MICROFLORA-MEDIATED IMMUNE REGULATION

IMPACT OF INTESTINAL MICROBIAL COMPOSITION ON THE REGULATION OF IMMUNOGLOBULIN E

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– ABSTRACT –

We are all born germ-free. Soon after birth, microbes colonize our body's surfaces, with the intestine housing the highest density of microbes on earth. Most of us remain blissfully unaware of this co-existence because inflammatory responses to the indigenous microbes are normally not triggered. Nonetheless, intestinal microbes are true educators of our immune system, which is exemplified by the immature immune system observed in germ-free animals. Accumulating evidence suggests that microbial exposure and/or composition impacts on immune regulation. As an example, isotype switch to immunoglobulin E (IgE) is normally very tightly regulated such that in healthy individuals and mice, serum levels are maintained at very low levels. In contrast, total serum IgE levels are elevated in germ-free mice, indicating that in the absence of microbes the regulatory pathway that maintains IgE at basal levels is disrupted. We hypothesize that in the absence of stimuli from the resident intestinal bacteria the immune system does not receive adequate educational signals. We showed that in germ-free mice class switch recombination (CSR) to IgE occurred at intestinal mucosal lymphoid sites a few weeks after birth. IgE levels then remained at elevated levels throughout life, even when intestinal bacteria were introduced after weaning. In the first part of this thesis, the mechanisms involved in this hygiene-induced IgE were investigateted. In a second part, the immunoregulatory role of commensal bacteria was extended to a model of autoimmunity.

Collectively these results demonstrate a new dimension of the impact of intestinal symbionts on the immune system: they dictate baseline immune system regulation. Elucidating the mechanisms whereby microbes induce immunoregulatory pathways may give insights into the increasing prevalence of allergic- and autoimmune diseases.

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– TABLE OF CONTENTS –

– LISTS OF FIGURES, TABLES AND APPENDICES –

– FIGURES –

– TABLES –

– APPENDICES –

– LIST OF ABBREVIATIONS –

ADA	Adenosine deaminase
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
APRIL	A proliferation inducing ligand
ASF	altered Schaedler flora
BAFF	B-cell activating factor
BALT	Bronchus-associated lymphoid tissue
BB-DP	Bio-breeding diabetes prone
BCMA	B cell maturation antigen
BCR	B cell receptor
BM	Bone marrow
BSA	Bovine serum albumin
CBA	Cytometric bead array
CD40L	CD40 ligand
cLP	Colon lamina propria
CpG ODN	CpG oligodeoxynucleotides
CSR	Class switch recombination
D	Diversity
d	day
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DSS	Dextran sulfate sodium
ELISA	enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter
FAE	Follicle associated epithelium
FCS	Fetal calf serum
GALT	Gut-associated lymphoid tissue
GLT	Germline transcript
GVHD	Graft-versus-host disease
h	hour
HEPA	high efficiency particulate air
HIES	Hyper-IgE syndrome
HIV	Human immunodeficiency virus
I	Intronic promoter
i.p	Intra-peritoneal
i.v	Intravenous
IBD	Inflammatory bowel disease
IEL	Intraepithelial lymphocyte
IgH	Immunoglubulin heavy chain
IgL	Immunoglobulin light chain
ILF	Isolated lymphoid follicle
IMDM	Iscove's Modified Dulbecco's Medium
J	Joining
kb	kilobase
KGF	Keratinocyte growth factor
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer cell
M cell	Microfold cell
mAb	Monoclonal antibody
MACS	Magnetic-assisted cell sorting
MALT	Mucosa-associated lymphoid tissue
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MLN	Mesenteric lymph node
MS	Multiple sclerosis
MyD88	Myeloid Differentiation Marker 88
NALT	Nasal-associated lymphoid tissue
NHEJ	Non-homologous end joining
NK	Natural killer cell
NOD	Non-obese diabetic
OVA	ovalbumin
p.c	Post-coitus
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
pLN	Peripheral lymph node
PP	Peyer's patch
PRR	Pattern recognition receptor
PSA	Polysaccharide A
PW	Peritoneal wash
qPCR	Quantitative real-time PCR
RAG	Recombination Activating gene
RALDH	Retinal dehydrogenases
rDNA	Ribosomal DNA
RORγt	Retinoic acid-related orphan receptor γt
RT	Room temperature
S	Switch
SD	Standard deviation
SFB	Segmented filamentous bacteria
SHM	Somatic hypermutation
SILT	Solitary intestinal lymphoid tissue
T1D	Type I diabetes
TACI	Transmembrane activator and calcium-modulator and cyclophilin ligand interactor

IPEX	Immune dysregulation, polyendocrinopathy, enteropathy, X-linked
TCR	T cell receptor
TRIF	TIR-domain-containing adapter-inducing interferon-β
Teff	T effector cell
Th	T helper cell
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	Tumor necrosis factor
Tr1	T regulatory type 1
Treg	T regulatory cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TSLP	Thymic stromal lymphopoietin
V	Variable
VSV	Vesicular stomatitis virus
w/v	Weight per volume
ZAP-70	Zeta-chain-associated protein kinase 70

– DECLARATION OF ACADEMIC ACHIEVEMENT –

My supervisor Kathy McCoy and myself designed the experiments. I performed and analyzed all the experiments presented in the first part. Markus Geuking and Kathy McCoy helped for the depletion of Peyer's patches and the adoptive T cell transfer experiments presented in Figures 7D and 12B. In the second part, I initiated the project, which was then pursued by Katia Safroneeva. The bacterial flow cytometry plots presented in Figure 24B and the histological grades of the islets presented in Figure C-D were performed by Katia Safroneeva.

Our animal technicians led by Jorum Kirundi provided animal care and assured the germ-free and gnotobiotic statuses of the mice. Surgical re-derivation to germ-free status from 2-cell stage embryos was performed by Kathy McCoy. Embryo collection and/or cryopreservation was performed by Kathy McCoy, Markus Geuking and myself.

1 Introduction

1.1 We are born germ-free

We are all born germ-free. With every newly born infant, a new microbial ecosystem is also formed. After birth, our intestines and other mucosal surfaces transit from complete sterility to become densely colonized, with the intestine eventually harboring the densest bacterial ecosystem known on earth (Macpherson et al., 2007). The microbes living with us outnumber our human cells by an order of magnitude (10^{13} eukaryotic animal cells compared to 10^{14} indigenous microbial cells). As such we can be redefined as a supraorganism, consisting of the cells of *Homo sapiens* and bacterial cells (Savage, 1977; Turnbaugh et al., 2007). Commensal (non-pathogenic) microbes colonize the skin and mucosal surfaces, with the densest concentration found in the colon (up to 10^{12}/ml of luminal contents) (Dethlefsen et al., 2007; Garrett et al., 2010). An estimate of up to 1000 different bacterial species inhabits the intestinal lumen (Eckburg et al., 2005; Gill et al., 2006; Qin et al., 2010; Suau et al., 1999; Turnbaugh et al., 2010). The majority of the intestinal bacteria is currently unculturable but with increased use of non-culture based techniques the high complexity of this microbial ecosystem begins to be appreciated (Handelsman, 2004). These techniques rely on genetic analysis of the gut microbial communities such as the sequences of 16S ribosomal (r)DNA, which are conserved but yet sufficiently varied amongst microorganisms (Turnbaugh et al., 2010). Using such metagenomic techniques, it has become evident that each individual appears to possess an idiosyncratic microbial fingerprint (Eckburg et al., 2005; Hayashi et al., 2002; Lay et al., 2005) that remains stable over months (Eckburg et al., 2005; Zoetendal et al., 1998). The factors contributing to this microbial uniqueness are a

matter of intense research and are critical towards understanding the impact of intestinal bacteria in health and disease. For example, it is known that diet directly influences microbial composition. Indeed it has been shown recently that similar microbial communities were observed in hosts with comparable diets both across mammalian species and within humans (Ley et al., 2008; Muegge et al., 2011). Moreover, bacterial composition was altered in a predictable manner following dietary changes, as demonstrated in gnotobiotic mice associated with a defined and limited number of microbial species (Faith et al., 2011; Turnbaugh et al., 2009).

The gut lumen is a highly specialized culture medium, which allows growth of only a small portion of bacterial species present on earth. Intestinal bacteria are classified in only eight-ten of the 55 bacterial divisions and of these, five are very rare. For comparison, the open ocean and the soil host 12 and 20 divisions, respectively (Bäckhed et al., 2005). Intriguingly gut bacteria seem to cluster into just three distinct and stable microbial combinations (called enterotypes) that are neither nation nor continent specific (Arumugam et al., 2011). Each enterotype is characterized by the variation in the levels of one of three bacterial genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3). It is possible that tight evolution between host and bacteria resulted in these three best-fitted enterotypes.

Commensal bacteria make essential contributions to the host while thriving in a safe niche, rich in nutrients provided by the ingested food. The intestinal microbes protect against invading mucosal pathogens by filling up microbial niches (Benson et al., 2009; Stecher et al., 2005), referred to as colonization resistance. Intestinal bacteria participate in metabolism by processing polysaccharides and synthesizing vitamin K (Hooper et al., 2002) and they impact post-natal intestinal development including

epithelial cell maturation (Hooper et al., 2001) and microvasculature angiogenesis (Stappenbeck et al., 2002). Despite this mutualistic relationship, hosting nearly 100 trillion of bacteria represents a persistent threat for the host. Sophisticated protection mechanisms at the bacterial-host interface insure a peaceful co-existance with indigenous microbes.

1.2 Homeostasis at the bacterial-host interface

Innate and adaptive arms of the immune system orchestrate refined immunity at the mucosal interface. While in a constant state of alertness against invading pathogens, no overt inflammation against innocuous commensal bacteria is normally elicited. Compromised bacterial-host equilibrium results in harmful consequences such as inflammatory bowel disease (IBD) (Elson et al., 2004). Unique mechanisms evolved within the mucosal immune system to live in an armed peace with our microbial symbionts. While the mucosal immune system assures that microbes are kept within the mucosal compartment, the systemic immune system remains mainly ignorant of their presence (Konrad et al., 2006; Macpherson and Harris, 2004). Therefore the mucosal immune system forms an entity separate from the classic systemic immune system.

1.2.1 Form follows function: anatomy of the gut-associated lymphoid tissues

The mucosal compartment is composed of various lymphoid tissues collectively referred to as the mucosa-associated lymphoid tissues (MALT) (Macpherson et al., 2008). Since no afferent lymphatics converge to the MALT, antigen is sampled directly from epithelial surfaces. The MALT is further subdivided into distinct functional areas, the NALT (nasal-associated lymphoid tissue), the BALT (bronchial associated lymphoid tissue) and gut associated lymphoid tissue (GALT) (Kiyono and Fukuyama, 2004; Ruddle and Akirav, 2009). In the GALT, distinct

lymphoid inductive and effector sites integrate stimuli from the gut lumen. Peyer's patches (PP), mesenteric lymph nodes (MLN) and isolated lymphoid follicles (ILF) are the main inductive sites whereas the lamina propria (LP) is the main effector site (Figure 1).

PP are found along the small intestine opposed to the line of attachment of the mesentery. They consist of lymphoid aggregates located underneath the follicle-associated epithelium (FAE), which is in direct contact with the gut lumen. Specialized cells devoid of microvilli, the M (microfold) cells, are situated in the FAE. M cells are the entry port for luminal antigens (Neutra et al., 2001). Sampled antigen is engulfed by dendritic cells (DC) either located underneath in the M cell pocket or in the underlying subepithelial dome. A fraction of the antigen-loaded DC migrate to the interfollicular T cell rich region, which surrounds the B cell follicles and germinal centers of the PP (Macpherson et al., 2008).

Cryptopatches and ILFs are small lymphoid aggregates scattered throughout the intestinal lamina propria collectively referred to as solitary intestinal lymphoid tissues (SILT) (Eberl and Sawa, 2010). After birth, hundreds of cryptopatches form between crypts (hence the name cryptopatch) and some of them mature to ILFs in a microbial-dependent manner (Bouskra et al., 2008; Kanamori et al., 1996). As such the intestinal lamina propria contains a fixed number of SILTs made up from a variable number of cryptopatches and ILFs. ILFs are large isolated B cell clusters organized into a germinal center (Hamada et al., 2002). Similar to PP, they are overlaid by M cells which deliver antigen to the DCs.

Antigen can also be sampled by a subset of DC expressing the fractalkine receptor CX_3CR1, which project dendrites across the epithelium into the gut lumen (Niess et al., 2005; Rescigno et al., 2001). These CX_3CR1-expressing DC are developmentally distinct from the migratory subset of

DC expressing the αE integrin CD103 (Jaensson et al., 2008; Schulz et al., 2009).

Collectively, all antigen-loaded DC can migrate to the MLN through the afferent lymphatics that drain to the MLN, which lies intercalated between two sheets of the peritoneal membrane. Lymphocytes residing in the MLN can enter the blood stream through the thoracic duct via the efferent lymphatics of the MLN. Recirculating mucosal lymphocytes eventually home back to the lamina propria, where they participate in frontline mucosal immunity.

1.2.2 Mechanisms to limit bacterial-epithelial interaction

A single epithelial layer separates the densely colonized intestinal lumen from the sterile body. Overlying the epithelial cells is a thick gel-forming layer, the mucus, produced by specialized epithelial cells, the goblet cells. Besides its role in physical compartmentalization, the epithelial layer secretes anti-microbial proteins belonging to the family of defensins, cathelicidins and C-type lectins. Together, the viscous mucus and these concentrated natural antibiotics limit bacteria from gaining close proximity to the epithelium (Johansson et al., 2008).

The lamina propria of the intestine contains 80% of the B cell-derived plasma cell pool, which produces an astonishing amount of IgA. Production of IgA exceeds all other isotypes combined (IgM, IgG, IgE) ranging from 40 to 60 mg/kg per day in humans (Fagarasan and Honjo, 2003). IgA is thought to limit bacterial association with the epithelium (Suzuki et al., 2004) and its penetration through the epithelium (Macpherson and Uhr, 2004). Following IgA induction in mucosal compartments including PP (Gearhart and Cebra, 1979), ILFs (Tsuji et al., 2008), MLN (Tezuka et al., 2011), and to a lesser extent the diffuse tissue of the lamina propria (Fagarasan et al., 2001), IgA is transcytosed across

the epithelium into the intestinal lumen. In neonates, prior to self-sustained IgA production, maternal IgA antibodies delivered by the transplacental route or through lactation maintain integrity at the bacterial-epithelial interface (Harris et al., 2006).

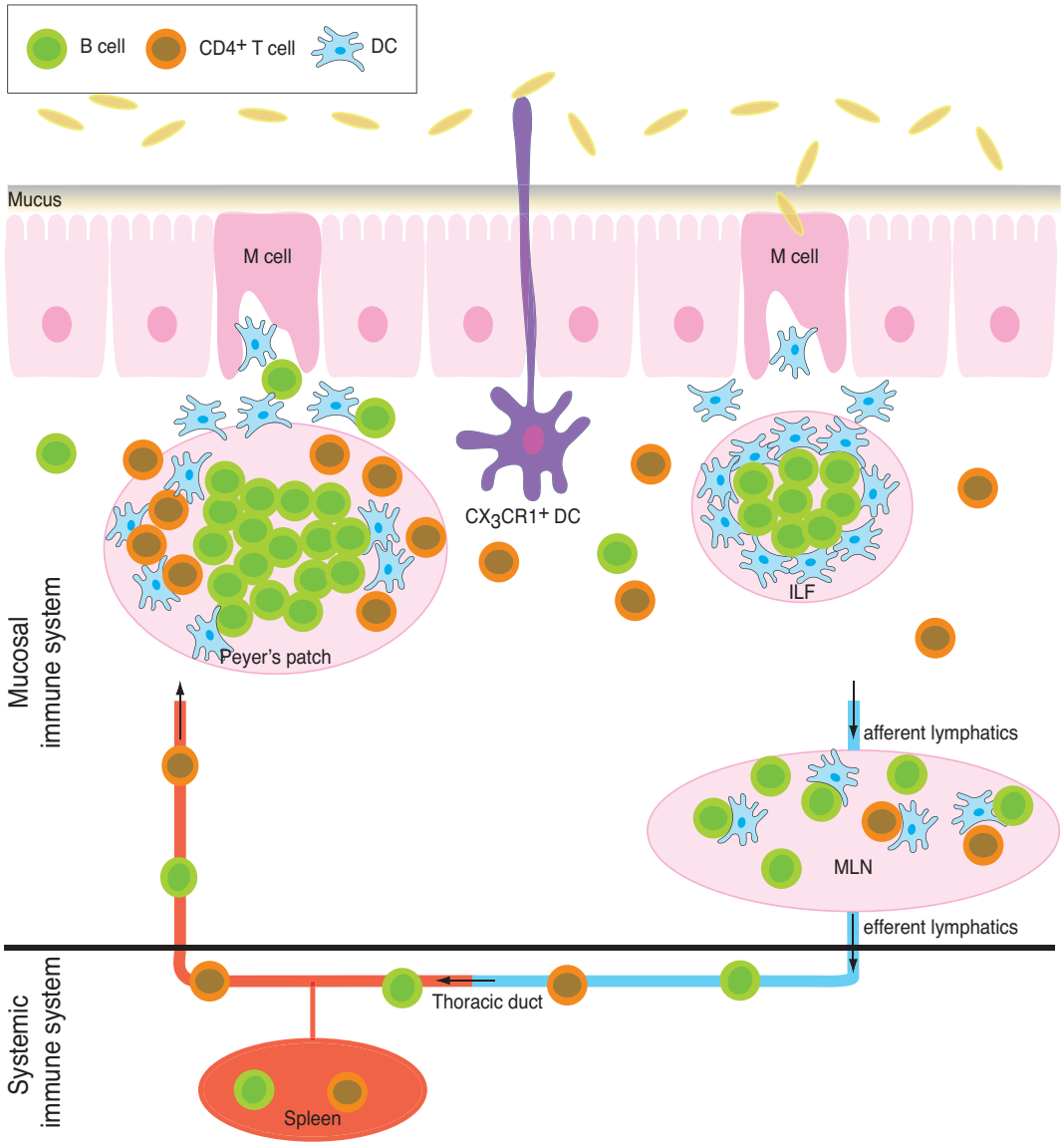


Figure 1. Compartmentalization of gut-associated lymphoid tissue. Schematic representation of the GALT illustrating the organized lymphoid structures. PP and ILFs are composed of specialized FAE containing M cells, a DC-rich subepithelial dome, and a B cell follicle with germinal centers. DC are strategically positioned underneath M cells, which allows for efficient uptake of sampled antigens. Alternatively, antigen can be sampled directly in the lumen by dendrites of specialized CX_3CR1^+ DC. Lymphocytes can

migrate to the MLN via the afferent lymphatics. From there they can enter the blood circulation via the thoracic duct and home back to the lamina propria. DC are shown in blue, B cells in green and T cells in orange. Lamina propria also contains large numbers of macrophages (not shown).

1.2.3 The immune system at the mucosal interface

Due to the tremendous number of bacterial cells living within the gut lumen, some bacterial cells will occasionally breach the epithelial layer. In the lamina propria a sophisticated dialogue between innate and adaptive immune cells ensures homeostasis while actively fighting bacterial breach of the epithelium.

Pattern recognition receptors (PRRs) are a family of germline-encoded receptors that recognize evolutionary conserved patterns, called pathogen-associated molecular patterns (PAMPs), displayed by microorganisms. The term PAMP suggests that these molecular patterns are restricted to pathogens, but the molecules that are referred to as PAMPs include lipopolysaccharide (LPS), peptidoglycan, and bacterial flagellin, which are also expressed by commensal bacteria. Therefore it has been proposed to change the name to microbe-associated molecular patterns (MAMPs) (Ausubel, 2005). PRRs expressed by innate immune cells such as DC, monocytes, macrophages, granulocytes, natural killer (NK) and epithelial cells ensure a rapid but non-specific response (Janeway and Medzhitov, 2002; Medzhitov, 2009). Macrophages are present in high numbers within the intestine and are found in very close proximity to the epithelial cells (Lee et al., 1985) where they engulf and kill bacteria (Smythies et al., 2005). Intestinal macrophages have distinctive features compared to monocytes in that they are able to phagocytose and kill bacteria but do so without secreting pro-inflammatory cytokines (Smith et al., 2011). Recently, a subset of intestinal NK cells expressing NKp46 and the transcription factor retinoic acid-related orphan receptor γt (RORγt) has been shown to secrete copious amounts of IL-22, a cytokine

contributing to epithelial cell homeostasis and antimicrobial peptide induction (Sanos et al., 2009; Satoh-Takayama et al., 2008). Similarly, $CD4^+$ lymphoid tissue inducer (LTi) cells also produce IL-22 and have been implicated in the innate immune response towards bacterial invasion (Sonnenberg et al., 2010). Intestinal epithelial lymphocytes (IELs) are innate immune leukocytes residing in the intestinal epithelium and participate in the maintenance of epithelial integrity. IELs expressing the $\gamma\partial$ T cell receptor (TCR) are critical players in the repair response following dextran sulphate sodium (DSS)-induced intestinal epithelial cell injury. Following injury, IELs express the intestinal epithelial cell mitogen keratinocyte growth factor (KGF) (Boismenu and Havran, 1994; Chen et al., 2002), heat shock proteins and cytoprotective factors (Ismail et al., 2009). Moreover, production of antibacterial factors by IELs limits bacterial penetration both during injury (Ismail et al., 2009) and at steady state (Ismail et al., 2011). In addition, the $\alpha\beta$TCR-expressing IELs may play a regulatory role in a model of colitis as shown by their protective effect upon adoptive transfer into lymphopenic hosts (Poussier et al., 2002). Collectively, these innate immune signals assure a first line of defense against a breach in the epithelial barrier.

In contrast to innate responses, somatically recombined B cell receptors (BCR) and TCR displayed on the adaptive B and T cells, respectively, provide a timely delayed but specific response (Cooper and Alder, 2006). Although the majority of resident bacteria are symbionts, some bacterial species (referred to as pathobionts) have the potential to become opportunistic pathogens when the immune system is weakened (Chow and Mazmanian, 2010). T helper (Th) cell subsets ensure homeostasis in the presence of both symbionts and pathobionts. Symbionts such as the eight bacterial species contained in the altered Schaedler flora (ASF), Clostridium species, or the polysaccharide A (PSA)-expressing

Bacteroides fragilis induce a T regulatory cell (Treg) differentiation program critical for the maintenance of intestinal homeostasis (Atarashi et al., 2010; Geuking et al., 2011; Round et al., 2011). On the other hand pathobionts, such as segmented filamentous bacteria (SFB), a spore-forming Clostridium located in very close contact with the epithelium of the intestine, are potent inducers of Th17 (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Other Th cell lineages such as Th1 and Treg cells are also induced by the presence of SFB although to a lesser extent than Th17 (Gaboriau-Routhiau et al., 2009). As expected, no Th17 cells and very few Treg cells are detected in the colonic lamina propria of germ-free mice (Atarashi et al., 2008; Geuking et al., 2011; Ivanov et al., 2008). Therefore intestinal DC promote non-inflammatory Th2 and Treg cell differentiation, which dampen inflammatory Th1 and Th17 responses (Cong et al., 2009; Rimoldi et al., 2005; Tsuji et al., 2009). These observations suggest that while Treg induced by symbionts are critical for intestinal homeostasis, Th17 induced by pathobionts are critical for combating invasive pathobionts.

1.2.4 MLN: the firewall at the mucosal-systemic interface

A fraction of invading bacteria are engulfed by resident intestinal DC that migrate to the MLN where they induce specific B and T cell responses, including protective IgA (Figure 1) (Macpherson and Uhr, 2004). Importantly, in immunocompetent hosts, live intestinal microbes do not migrate further than the MLN (Macpherson and Uhr, 2004). Both restricted phagocytic activity (Nagl et al., 2002) and rapid turnover of DC, contribute to the sequestration of intestinal microbes within the mucosal compartment. In contrast, microbial-induced B and T lymphocytes can enter the blood stream and home back to the lamina propria (GOWANS and KNIGHT, 1964; Husband and GOWANS, 1978). Therefore the MLN

are the firewall at the mucosal-systemic junction and explain the compartmentalization of the mucosal immune system. This is elegantly exemplified following MLN adenectomy, which results in infiltration of indigenous intestinal bacteria into the blood stream thereby eliciting systemic immunity (Macpherson and Uhr, 2004).

1.3 Maturation of the immune system by the microflora: lessons from germ-free animals

Infants are born sterile and progressively become colonized by microbial communities that will eventually converge towards a generic profile observed in adults. This transitional intestinal colonization follows a highly dynamic and random pattern characterized by a unique combination of bacterial species, which appear or disappear over time. It is thought that infants acquire a microflora through opportunistic colonization of the bacteria present in their environment, which would explain this observed "trial and error" colonization pattern (Kurokawa et al., 2007; Palmer et al., 2007; Stark and Lee, 1982; Tannock, 2007). Interestingly, the development of the microbial ecosystem occurs concomitantly with the maturation of the immune system.

Germ-free animals have been invaluable tools to experimentally address whether these two early-life events are causally linked (Smith et al., 2007a). Germ-free (axenic) animals are born and raised in flexible-film isolators maintained under positive pressure with high efficiency particulate air (HEPA)-filtered sterile air, which maintains an environment free of bacteria, archea, viruses, fungi and parasites. Exchange between sterile isolator and exterior is insured by a side port with a double door system, which is sterilized with a 2% peracetic acid mist prior to each opening. This allows import of thoroughly sterilized supplies, including food, water, cages, bedding, and experimental instruments. Initial re-derivation to germ-free status can be achieved by timed Caesarian section

whereby the pups are transferred to a germ-free foster mother. Another method routinely performed in our laboratory consists in the collection of two-cell stage embryos (which are always sterile) from a mother that underwent a timed hormone treatment. These embryos, either fresh or recovered from a cryopreserved stock, are surgically implanted in the oviducts of a germ-free pseudo-pregnant foster mother.

Deliberate and controlled experimental bacterial colonization of germ-free animals mimics the formation of a bacterial ecosystem at birth. Environmental bacteria quickly colonize an “empty” germ-free host, which confirms that the intestinal lumen is an excellent culture medium. Technically, colonization experiments are performed by simple co-housing of a germ-free mouse with a mouse harboring a bacterial flora or by oral gavage of bacteria. The power of such colonization experiments resides in the capability to experimentally manipulate the colonizing microflora and/or the genotype of the host. For example, germ-free mice can become mono-associated (colonized with one bacterial species), gnotobiotic (colonized with a limited number of defined bacterial species such as the ASF) or SPF (colonized with a non-defined microflora devoid of listed pathogens). Such landmark experiments demonstrated the profound impact of intestinal bacteria on the maturation of the immune system. The mucosal compartment undergoes a true immunologic development upon intestinal bacterial encounter. While few IgA-expressing B cells are detected in the lamina propria of germ-free mice, a massive B cell proliferation and induction of IgA follows bacterial encounter (Benveniste et al., 1971b; Hapfelmeier et al., 2010). Moreover, proliferation of B cells promotes an increase in the size and number of germinal centers in the PP (Shroff et al., 1995) and maturation of cryptopatches into ILFs (Tsuji et al., 2008). The numbers of intestinal lamina propria $CD4^+$ T cells (Macpherson and Harris, 2004) and IELs expressing the $\alpha\beta$TCR are increased (Bandeira et

al., 1990; Guy-Grand et al., 1991). Furthermore, genes involved in host-microbial interactions such as nutrient absorption, epithelial barrier strengthening, angiogenesis and xenobiotic metabolism are actively transcribed (Hooper et al., 2001).

Despite the compartmentalization of intestinal microbes within the mucosa, their impact on the immune system reaches beyond this physical boundary (Macpherson and Harris, 2004). Following colonization, the rate of B cell egress from the bone marrow (BM) is higher (unpublished observations from our laboratory) while total serum Igs increase (except IgE). In the spleen and peripheral lymph nodes (pLN) germinal centers gain cellularity through an unknown mechanism (Macpherson and Harris, 2004).

Therefore, indigenous microbes truly shape the immune system, which places them in the critical interface between health and disease.

1.4 Hygiene-mediated immune dysregulation

Based on the landmark epidemiological study of 17000 British children, hay fever has been shown to be less prevalent in children with older siblings (Strachan, 1989). Strachan, the author of this study which was published in 1989, coined the term “hygiene hypothesis” to explain this finding. The hygiene hypothesis argues that increased sanitation in industrialized countries led to decreased infections with common pathogens and a concomitant rise in allergic disorders (Strachan, 1989). In the last decades westernized countries undertook drastic measures to increase hygiene including water decontamination, food pasteurization and sterilization, uninterrupted cold chain, vaccination and wide-use of antibiotics. Affecting 10-15% of the western population, the prevalence of atopic allergic diseases has doubled over the last two decades and asthma is now described as an epidemic (Masoli et al., 2004; Prioult and

Nagler-Anderson, 2005). In 2002, the hygiene hypothesis was extended to autoimmunity. Jean-François Bach made the observation that, similarly to allergic disorders, the incidence of autoimmune diseases such as type 1 diabetes (T1D) (Harjutsalo et al., 2008), multiple sclerosis (MS) and IBD has also increased in industrialized and developing countries (Bach, 2002).

Th2-driven allergy and Th1-driven autoimmunity are immune-mediated diseases that must arise due to the lack of immune regulation. However, the mechanisms governing the strong epidemiologic link between hygiene and the increasing disease incidence are still a matter of debate. Nonetheless, a body of literature convincingly shows that environment plays a central role (Okada et al., 2010). As mentioned, the strong increase in immune dysregulation is exclusively observed in industrialized and developing countries where sanitation is high. Although diagnosis and health care infrastructures may be better in westernized countries, this sole explanation can't explain the dramatic rise noted in the last decades within countries. Genetic background certainly plays a role in these diseases and is clearly involved in the predisposition to disease in some individuals but genetics alone does not explain the rising incidence of disease. For example, T1D incidence is six times higher in Finland compared to the neighboring Russian Karelia despite the same genetic background (Kondrashova et al., 2005). Moreover, within one generation, children from migrants acquire the disease incidence prevailing in the host country, as shown in the case of T1D (Bodansky et al., 1992) and MS (Hammond et al., 2000; Leibowitz et al., 1973).

Since the publication of the hygiene hypothesis, different immunologic mechanisms have been proposed to explain the epidemiological data. At first this phenomenon was described only for allergic disorders and it was therefore postulated that a Th1/Th2 imbalance would explain the

prominent Th2 responses seen in allergy. It was argued that a reduction in early childhood infections inducing a Th1 response would favor the development of Th2 responses specific for innocuous environmental allergens (Holt, 1998). Alternatively, it was proposed that decreased microbial burden would delay the maturation of the immune system and therefore the Th2 polarization observed in newborns would not deviate to Th1 (Martinez and Holt, 1999). The Th1/Th2 paradigm was revisited with the finding that the prevalence of Th1-driven autoimmune diseases was also increasing. The counter-regulation model suggests that in the absence of immune regulatory mechanisms, such as IL-10 and TGF-β, inflammatory responses are not inhibited, which results in allergy and autoimmunity (Wills-Karp et al., 2001). The old friends hypothesis extends this view. In this model, immunoregulatory responses would be elicited by "old friends", which refer to the relatively harmless microorganisms that co-evolved with humans including helminths, saprophytic mycobacteria and lactobacilli (Rook et al., 2004). Indeed experimental helminth infections have been correlated with strong expansion of immunoregulatory cells including alternatively activated macrophages, Treg cells, and IL-10-producing regulatory B cells (Finney et al., 2007; Mangan et al., 2004; Reece et al., 2006). Indications that parasitic infections reduce the risk of immune dysregulation are provided by epidemiological studies (Cooper et al., 2003; van den Biggelaar et al., 2000) and by mouse models (Bashir et al., 2002). The balanced microflora hypothesis (Noverr and Huffnagle, 2005) proposes that a shift in the composition of indigenous microbes rather than pathogenic infections is at the origin of loss of immune regulation.

Because we are living in a peaceful mutualism with 100 trillions of intestinal microbes, it is tempting to believe that this lifelong relationship has a considerable impact on immune regulation. Accumulating evidence

indicates a shift in the composition of indigenous intestinal microbes in westernized countries (Walter and Ley, 2011). Changes in life style in industrialized countries such as antibiotic usage and dietary habits undoubtedly impact microbiota composition. Experimental evidence that changes in type and level of microbial stimulation can impact disease outcome, are mainly supported by animal models. The non-obese diabetic (NOD) mouse and the biobreeding diabetes-prone (BB-DP) rat develop a disease that shares many similarities with T1D and are therefore a good animal model for the study of T1D. Interestingly, the incidence of T1D in these animals was correlated to the hygiene conditions prevailing in the animal facility. The incidence of diabetes was lower in animals raised under conventional status compared to animals raised under SPF status (Bach, 2002; Like et al., 1991). A direct link between microbiota and T1D was shown in NOD mice deficient in the innate signaling molecule myeloid differentiation marker 88 (MyD88). While germ-free MyD88$^{-/-}$NOD mice had a high diabetes incidence, their SPF counterparts were protected from disease (Wen et al., 2008). The authors concluded that the results suggest that normal microbial stimuli protect from diabetes in a MyD88-independent manner (although alternative explanations exist that will be discussed later). In a model of ovalbumin (OVA)-induced asthma, an increased number of infiltrating lymphocytes and eosinophils with more pronounced secretion of Th2 cytokines was observed in airways of germ-free mice compared to SPF mice (Herbst et al., 2011). Similarly, in a model of peanut allergy, mice treated with antibiotics or deficient in toll-like receptor (TLR)4 underwent anaphylaxis, suggesting that bacterial-induced TLR4 signaling is critical in limiting inflammation (Bashir et al., 2004). In addition, the microflora may modulate oral tolerance. Following systemic OVA challenge, germ-free mice elicited greater Th2 responses including IL-4 production, and OVA-specific IgE and IgG_1 compared to mice

harboring microbes (Sudo et al., 1997). Clinical studies comparing the microflora from healthy controls and patients suffering from allergic- or autoimmune disease are in agreement with the animal models (Prioult and Nagler-Anderson, 2005; Sokol and Seksik, 2010). For instance, patients suffering from Crohn's disease had a reduced diversity in the microflora especially for the Firmicutes phylum (Frank et al., 2007; Manichanh et al., 2006). Administration of *Faecalibacterium prausnitzii* to mice, a species of Firmicutes that was markedly depleted in Crohn's disease patients, could ameliorate disease outcome in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis (Sokol et al., 2008). While some studies comparing the microbial composition of atopic allergic individuals to healthy individuals observed differences (Björkstén et al., 1999; Kalliomäki et al., 2001), others could not reproduce this finding (Adlerberth et al., 2007). Such comparative studies have to be interpreted with caution because dysbiosis could be a consequence rather than the cause of inflammatory disease. Nonetheless a vast literature ranging from animal models to clinical studies supports the concept that immune regulation can be influenced by the composition of intestinal microbes.

1.5 Immune dysregulation driven by partial T cell immunodeficiency

Partial T cell immunodeficiency, defined by the presence of T cells that are reduced in numbers or function, have been associated with immune dysregulation extending beyond an intrinsic T cell dysregulation and range from autoimmunity to elevated serum IgE (Liston et al., 2008). Interestingly, IgE antibodies are tightly regulated in healthy individuals such that basal serum IgE levels are maintained very low (100 ng/ml compared to 10 mg/ml for IgG; IgE accounts for <0.0001% of serum immunoglobulins) (Grimbacher et al., 2002; Sutton and Gould, 1993). IgE levels are typically increased up to one thousand fold in atopic allergic

diseases (Barberá et al., 1977) and parasitic infections (Durmaz et al., 1998). Therefore, elevated IgE titers in patients with partial T cell immunodeficiency reflect a dysfunctional immune network that can no longer inhibit isotype switch to IgE (Liston et al., 2008). In humans, markedly elevated levels of serum IgE are observed in at least eight primary immunodeficiencies: Adenosine deaminase (ADA) deficiency; atypical complete DiGeorge syndrome; hyper-IgE syndrome (HIES); immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome; Ommen syndrome; Wiskott-Aldrich syndrome and zeta-chain-associated protein kinase 70 (ZAP-70) deficiency (Appendix 1). In addition, elevated IgE levels and oligoclonal T cell expansion have been observed in BM-transplanted patients with acute graft versus host disease (GVHD) (Dietrich et al., 1992; Du et al., 2007; Geha et al., 1980). Furthermore, elevated IgE levels have been reported in patients infected with human immunodeficiency virus (HIV), which causes a decline of $CD4^+$ T cells over the course of the infection (Marone et al., 2001). Together these observations support the hypothesis whereby class switch recombination (CSR) to IgE is highly activated in immunocompromised patients with T cell anomalies. As listed in Appendix 2, genetically-modified mice with compromised T cell functions also display elevated IgE titers, thus arguing against parasite- or atopy-induced IgE.

In summary, IgE regulation is lost in the event of T cell signaling defects, suggesting that a complex immunological network maintains IgE titers low, as seen in normal healthy conditions (Geha et al., 2003). Therefore, in the absence of parasitic infections, elevated serum IgE levels is an accurate biomarker of immune dysregulation. Interestingly, we have measured elevated IgE levels in wild-type C57BL/6 germ-free mice. We have shown that this IgE is polyclonal and germline encoded with no evidence for somatic hypermutation (SHM), arguing against atopic allergic reaction

(McCoy et al., 2006). Collectively these observations indicate that B cells integrate signals that favor IgE induction in the absence of microbes.

1.6 B cell subsets

Most of the B cells in peripheral tissues are developed from B cell precursors in the adult BM and are known as B-2 cells. Follicular B cells (B-2 cells) are key players of adaptive immunity through their surface-expression and secretion of highly specific antibodies against non-self antigens. In contrast, some antibodies distinguish themselves by their innate-like properties, with polyreactivity towards self- and microbial antigens. These natural antibodies are mainly produced by the B-1 cells, which arise early in ontogeny, prior to B-2 cells, hence their terminology.

1.6.1 B-2 cells: follicular B cells and marginal zone B cells

B-2 cells represent the largest fraction of B cells and arise in the fetal liver and in the adult BM. They are comprised of follicular B cells (sometimes referred to as B-2 cells) and marginal zone B cells.

Follicular B cells recirculate and populate the follicles in secondary lymphoid organs (Pillai and Cariappa, 2009). B cell follicles are found neighboring T cell zones and this strategic organization allows activated follicular B cells and activated Th cells to interact at the interface between these two areas. Thus follicular B cells are particularly well adapted for T cell dependent responses to protein antigens. In addition, follicular B cells can also populate a niche in the BM in close proximity to the sinusoids. In this case they preferentially mount a T cell independent immune response to blood-borne pathogens (Cariappa et al., 2007; Cariappa et al., 2005).

Marginal zone B cells do not recirculate and reside in the outer white pulp of the spleen between the marginal sinus and the red pulp. Phenotypically marginal zone B cells are characterized by their high expression of CD21

and CD1d (whereas follicular B cells express intermediate levels of CD21 and no CD1d). Marginal zone B cells are early participators in immune responses to blood-borne pathogens as they trap and bind antigens with their semi-invariant immunoglobulins, which results in rapid proliferation and antibody secretion (Martin and Kearney, 2002). While follicular B cells have a limited life span of a few weeks, marginal zone B cells are self-renewing (Hao and Rajewsky, 2001).

1.6.2 B-1 cells, the main producers of natural antibodies

Natural antibodies are induced in the complete absence of foreign antigens by poorly understood regulatory mechanisms. Induction of natural antibodies is independent of microorganisms since germ-free animals produce antibodies (which are all natural antibodies) (Bos et al., 1989; Haury et al., 1997; Hooijkaas et al., 1984). B-1 cells distinguish themselves from the predominant B-2 cells by their ontogeny, selection and function. B-1 cells were first described in 1983 as B cells expressing the prototypic T cell marker CD5 (Hayakawa et al., 1983). However, CD5 expression can be present (B-1a) or absent (B-1b), which does not qualify it as a reliable marker for B-1 cell discrimination. Usually B-1 cells are defined by a combination of surface markers including $CD19^{+}CD23^{-}$ $CD43^{+}IgM^{+}CD5^{\pm}$. Therefore characterization of B-1 cells is challenging due to the phenotypic heterogeneity and scarcity outside of the pleural and peritoneal cavities. Although B-1 cells have been described in the majority of the cellular compartments, they are most prominent in pleural and peritoneal cavities (35-70% of B cells) (Baumgarth, 2010). The ontogeny of B-1 cells remains an enigma and two hypotheses attempt to explain it. The “lineage hypothesis” argues that B-1 cells and B-2 cells arise from different B cell lineages. In contrast the “induced differentiation hypothesis” proposes that both subsets arise from a common B cell precursor but that

different environmental cues lead to cell fate decision to B-1 or B-2 cells (Dorshkind and Montecino-Rodriguez, 2007). A B-1 cell precursor has recently been characterized in the fetal liver and BM and is the most convincing experimental evidence up-to-date for the lineage hypothesis (Montecino-Rodriguez et al., 2006). However, the two hypotheses are not mutually exclusive and it is possible that both development pathways contribute to the heterogeneous B-1 cell population. While self-reactive B-2 cells undergo apoptosis during germinal center reactions to prevent autoimmunity, B-1 cells are selected for self-reactivity (Hayakawa et al., 1999). Natural antibodies are crossreactive towards a wide array of antigens such as oxidized lipids (Chou et al., 2009), annexin IV, phosphatidylcholine (Kulik et al., 2009), phosphorylcholine and LPS (Baumgarth et al., 2005; Bouvet and Dighiero, 1998). Similar to embryonic B cells, gene segment usage in the variable region is more restricted than in B-2 cells (Tornberg and Holmberg, 1995). In contrast to the life-long *de novo* B-2 cell output from the BM, a process called “self-renewal” maintains a stable number of B-1 cells (Lalor et al., 1989). Under steady state conditions, dying cells are replaced by limited proliferation dependent on IL-5 (Moon et al., 2004) and IL-9 (Vink et al., 1999). Most of the natural antibodies are IgM but they can switch to any other isotype (Coutinho et al., 1995). In the lamina propria of the intestine up to half of the IgA antibodies are from B-1 origin (Kroese et al., 1989). It is known that germ-free mice colonized with intestinal microbes induce IgA in the intestine, but interestingly one study suggested that microbial-induced IgA^+ B cells are B-2 cells (Thurnheer et al., 2003). Thus, consistent with the efficient prenatal and neonatal development of B-1 cells, B-1-producing IgA cells may only be induced in the first few weeks of life in response to the colonizing intestinal bacteria.

1.6.3 Regulatory B cells

The newly described regulatory B cells have been shown to secrete copious amounts of IL-10, which reduces chronic intestinal inflammation (Mizoguchi et al., 2002) and T cell dependent contact hypersensitivity (Yanaba et al., 2008). The relationship to B-1 cells and B-2 cells is currently unclear. B regulatory cells express characteristic markers of B-1a cells (CD5) and marginal zone B cells (CD1d, CD21). Furthermore B-1 cells have also been shown to have the potential to secrete IL-10 (O'Garra et al., 1992).

Despite the challenging task to classify and distinguish marginal zone B cells, B-1 cells and B regulatory B cells, these B cells share a common characteristic in that they are innate-like as opposed to the adaptive follicular B-2 cells.

1.7 B cell diversity, affinity and functionality conferred by somatic genetic recombination

B cells possess the unique ability to undergo three distinct somatic genetic alterations, which together explain BCR functional- and antigenic diversity. The BCR is an immunoglobulin molecule formed by the assembly of two distinct chains encoded at separate alleles, a heavy chain and light chain. Immunoglobulin heavy (IgH) and immunoglobulin light (IgL) are each comprised of a variable region, which together form the variable domain of the antibody.

1.7.1 Antibody diversity

During B cell ontogeny in the fetal liver or BM a variable region in IgH and IgL is assembled in an antigen-independent fashion. This genetic alteration named V(D)J recombination is formed by the stochastic assembly of one variable (V), one diversity (D) and one joining (J) gene

segment in IgH and one V and one J segment in IgL. In humans there are 121 V (51 V_H, 40 $V\kappa_L$, 30 $V\lambda_L$), 27 D and 15 J (6 J_H, 5 $V\kappa_L$, 4 $V\lambda_L$) segments and in mice 221 V (134 V_H, 85 $V\kappa_L$, 2 $V\lambda_L$), 13 D and 11 J (4 J_H, 4 $V\kappa_L$, 3 $V\lambda_L$) segments. Thus more than 10^6 V(D)J combinatorial associations between IgH and IgL are possible. V(D)J recombination involves site-directed DNA excision and ligation depending on the recombination-activating gene (RAG)-1 and RAG-2 endonucleases (Oettinger et al., 1990; Schatz et al., 1989) and the nonhomologous end joining (NHEJ) machinery, respectively (Bassing et al., 2002; Jung and Alt, 2004). Besides random V(D)J joining and association of IgH and IgL, diversity of the variable region is further enhanced during ligation by junctional flexibility and nucleotide additions. Following successful V(D)J recombination, IgH and IgL combine as surface IgM and IgD on mature B cells. An estimated 5×10^6 mature B cells egress every day into the periphery to populate the secondary lymphoid organs such as the spleen and LN.

1.7.2 Antibody affinity

In the periphery antibody affinity is increased upon antigen encounter through another genetic modification called SHM. The B cell-intrinsic enzyme activation-induced cytidine deaminanse (AID) introduces a higher than normal rate of point mutations in the variable region of both IgH and IgV (Martin and Scharff, 2002; Papavasiliou and Schatz, 2002; Yélamos et al., 1995). This results in the selection and proliferation of B cell clones with high affinity towards antigen.

1.7.3 Antibody functionality

Antibody function can be altered to fit the antigenic stimulus received by the B cells during a process called class switch recombination (CSR).

CSR describes the somatic genetic alteration whereby an immunoglobulin changes its functional properties while retaining its antigen specificity (Honjo et al., 1981). Immunoglobulin function is determined by the constant region (δ, μ, γ, ε, α encoding IgD, IgM, IgG, IgE and IgA, respectively) whereas immunoglobulin specificity is determined by the variable domain, composed of V, D and J exons. Mechanistically, CSR is a deletion-recombination process (Figure 2). In the case of CSR to IgE, the Th2 cytokine IL-4 initiates transcription at an intronic (I) promoter Iε. Intronic promoters are located upstream of each switch (S) region with the exception of Cδ (Stavnezer, 1996). S regions are highly repetitive 1-12 kilobases (kb) sequences located upstream of each C_H gene. Transcription proceeds through Sε and Cε regions, which form a transcriptional entity. The resulting transcript is processed into an epsilon germ-line transcript (εGLT), which does not encode for a protein. εGLT (or any other GLT) is a hallmark of active CSR owing to its transient transcription prior to CSR (Muramatsu et al., 2000; Stavnezer, 1996). The functionality of sterile GLT has been a subject of intense research and it is thought that transcription of GLT aids DNA unwinding, which in turn facilitates access for the CSR excision/recombination machinery (Chaudhuri and Alt, 2004). AID is a critical player in this machinery and acts by converting cytidine to uridine (Rada et al., 2002), which induces DNA-repair pathways (Muramatsu et al., 2000; Revy et al., 2000). Ultimately these G-U mismatches result in DNA double strand breaks at the G-rich S regions. The intervening DNA sequences are looped out into an episomal circle (Kinoshita et al., 2001) and the two DNA ends are ligated together. This results in a new immunoglobulin isotype (IgG3, IgG1, IgG2b, IgG2a, IgE or IgA).

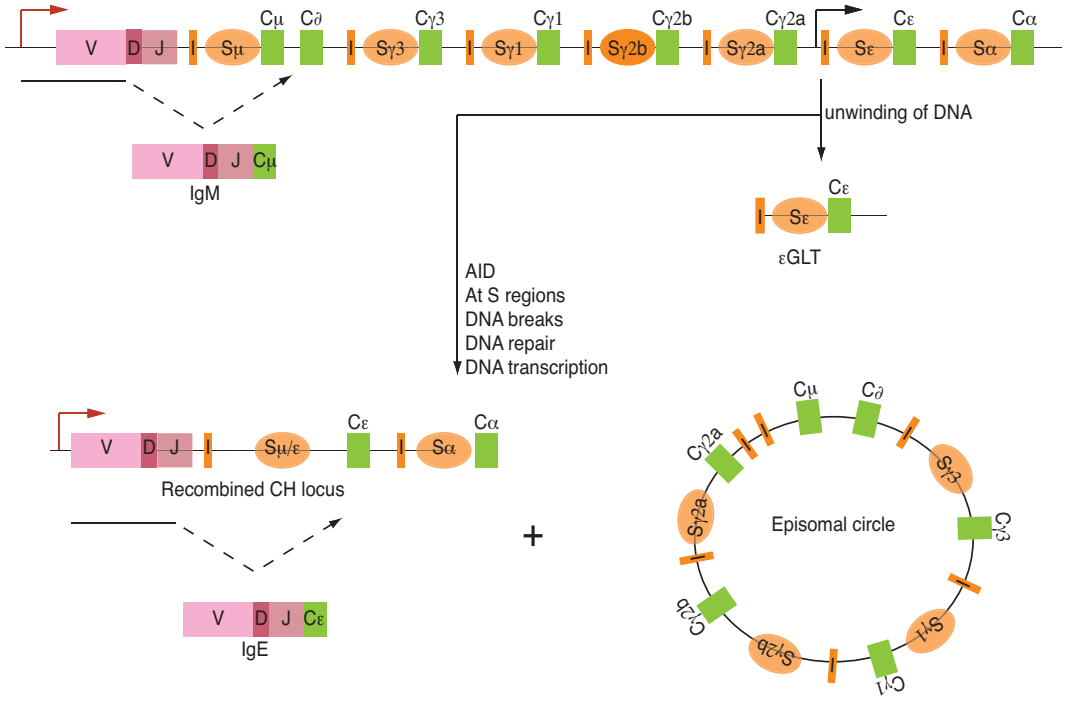


Figure 2. Class switch recombination at the immunoglobulin heavy-chain locus. The variable region is assembled from one V, one D and one J gene segments during the process of V(D)J recombination. Transcription from a promoter upstream of the variable region (red arrow) promotes synthesis of the μ heavy chain which, together with a light chain, forms the IgM molecule expressed on the surface of a mature B cell. Following antigen encounter, constant heavy chain isotype can be exchanged in a process called CSR. The constant region of the heavy chain is traded with a set of downstream constant-region genes (CSR to IgE is shown). The process is initiated with the transcription of the sterile εGLT (black arrow), which is thought to facilitate access of the excision/recombination machinery. The enzyme AID introduces DNA mutations at the Sμ and Sε regions, which initiates DNA-repair pathways that eventually result in DNA double strand breaks. This leads to the recombination of the DNA fragments at the Sμ and Sε regions and the looping out of the intervening DNA fragments in an episomal circle. Transcription upstream of the variable region (red arrow) leads to the synthesis of IgE antibody.

1.8 Effector functions of IgE

Th2 responses in general and IgE in particular are believed to have evolved for protection against extracellular multicellular parasites, such as helminths (Allen and Maizels, 2011). This beneficial response can turn harmful as seen in atopic allergic diseases such as allergic rhinitis, asthma and atopic eczema (Kay, 2001). IgE initiates multiple innate and adaptive

immune responses by binding to- and activating high or low affinity FcεR expressed on innate immune cells.

1.8.1 Protection against helminths

The association between helminth infection and elevated IgE levels was first reported in 1964 (OGILVIE, 1964). Nonetheless, the biological functions of IgE in parasite infections remain controversial. Interestingly, the majority of parasite-induced IgE is polyclonal and does not bind to parasite antigens, which may reflect the co-evolution between host and parasite (Dessaint et al., 1975). Polyclonal IgE may benefit the parasite to escape immune response and may benefit the host to reduce the risk of anaphylaxis (Pritchard, 1993). Whereas evidence of IgE-mediated immunity to helminths is clearly demonstrated in humans (Dunne et al., 1992) and sheep (Kooyman et al., 2000), it is more limited in mice, possibly due to the absence of high affinity IgE receptor on eosinophils in mice (de Andres et al., 1997). Nevertheless, experimental evidence supports a protective role of IgE in mice. *In vitro*, IgE has been shown to kill helminth larvae through antibody-dependent cellular cytotoxicity (Capron and Capron, 1994). Moreover, both eosinophils and IgE were required for vaccine-mediated protection against *Onchocerca volvulus* larvae (Abraham et al., 2004) and infection with *Trichinella spiralis* in IgE-deficient mice resulted in a delayed worm expulsion (Gurish et al., 2004). Furthermore, an epitope from *Nippostrongylus brasiliensis* has been shown to elicit specific IgE antibodies amongst the predominantly polyclonal IgE response. This purified helminth specific IgE could induce mast cell degranulation upon antigen crosslinking (Pochanke et al., 2007). In *Heligmosomoides polygyrus* infection, specific IgE was detected late after primary infection and accumulated following multiple rounds of infection as shown by clonal selection and affinity maturation (McCoy et

al., 2008). Collectively, these results suggest that IgE is critical for immunity against incoming larvae during concomitant or secondary infections. However it has to be borne in mind that multicellular parasites encompass a broad family of species differing by factors such as life cycle or infection site. Thus induction of parasite immunity may be strongly species- and site-dependent.

1.8.2 Type I mediated hypersensitivity

In 1902 Paul Portier and Charles Richet made the observation that a second injection of fluids isolated from jellyfish induced a harsh inflammatory response in dogs that showed no symptoms at the first injection (Cohen and Zelaya-Quesada, 2002). This was the discovery of anaphylaxis, which led to the Nobel Prize in physiology or medicine for Charles Richet in 1913. Anaphylaxis is currently referred to as a type I hypersensitivity reaction and is induced by antigens that elicit secretion of specific IgE. Mast cells and basophils express the high affinity receptor for IgE, FcεRI (Wedemeyer et al., 2000). Interaction between IgE and FcεRI is very stable (IgE-FcεRI: Ka = 10^9 M^{-1}, IgG-FcγRIII: Ka = 5×10^5 M^{-1}) which greatly delays IgE degradation ($t_{1/2}$ of IgE bound to FcεRI = 2 weeks, $t_{1/2}$ of serum IgE = 2 d). Exposure to an antigenic allergen in atopic individuals induces specific IgE antibodies, which bind to FcεRI on mast cells and basophils. Such sensitized cells can be activated within minutes following a secondary exposure to the same antigen. Antigen crosslinks specific IgE molecules bound to FcεRI, which triggers degranulation of preformed inflammatory mediators such as histamine. In addition, activation of mast cells and basophils induces *de novo* synthesis of inflammatory mediators such as prostaglandin D_2, leukotriene C_4 and proinflammatory cytokines (Gould et al., 2003). Altogether the release of inflammatory mediators results in inflammatory symptoms, which include enhanced local vascular

permeability, increased cutaneous blood flow, erythema and itching (Kay, 2001).

1.8.3 Prolonged mast cell survival by monomeric IgE

Binding of antigen-free (monomeric) IgE to FcεRI on mast cells promotes increased cell survival, a process mediated by autocrine cytokines (Asai et al., 2001; Kalesnikoff et al., 2001; Kitaura et al., 2003). Moreover, engagement of monomeric IgE with FcεRI amplifies surface expression of FcεRI on mast cells and basophils (Lantz et al., 1997; Yamaguchi et al., 1997). The physiological relevance of these findings remains to be determined. Yet the combination of increased mast cell survival (more inflammatory mediators) and surface expression of high affinity IgE receptor (more antigenic specificities) may be a mechanism to combat pathogens. Conversely in the context of allergies, this positive feedback mechanism may contribute to exacerbation of inflammatory disease.

1.9 Pathways of IgE CSR

Most antigens initiate Ig responses in a T-dependent process in lymphoid follicles containing germinal centers, which promote antibody diversification (CSR) and affinity maturation (SHM). This conventional T-dependent follicular pathway is also involved in IgE induction. However a vast literature shows that alternative pathways can induce IgE. In addition, recent advances in the Th2 field unfold exciting new views of IgE regulation in particular and initiation of Th2 responses in general.

1.9.1 The conventional T-dependent follicular pathway of IgE CSR

Antibodies initiated through the T-dependent pathway require five-seven days to reach the effector sites (Bendelac et al., 2001; Fagarasan and Honjo, 2000).

IgE is an immunoglobulin generated during Th2 responses, which are associated with the production of IL-4, IL-5, IL-9, IL-13 and IL-25 (Paul and Zhu, 2010). Since the discovery of Th1 and Th2 cells 25 years ago by Mosmann and Coffman (Mosmann et al., 1986), the cascade of events leading to induction of Th2 immunity remains unresolved. While classical Th1 differentiation relies on signaling through PRR expressed on DC, which promotes secretion of the Th1-polarizing cytokine IL-12 (Edwards et al., 2002), no such linear signaling pathway can explain the inception of Th2 responses. Nonetheless, *in vitro* studies have elucidated the conventional model of IgE induction in great detail and give insights into mechanisms operating *in vivo*. In this model, antigen, typically from an allergen or a parasite, is internalized by DC. Antigenic epitopes displayed on DC are presented to $CD4^+$ T cells via major histocompatibility complex (MHC) Class II cognate interactions. This leads to activation of $CD4^+$ T cells and DC, which upregulate the costimulatory molecules CD28 and CD40 Ligand (CD40L) on T cells and their respective ligands CD80/CD86 and CD40 on DC. Antigen-specific $CD4^+$ T cells and B cells undergo cognate interaction via B cell-expressed MHC Class II/peptide complexes leading to T cell derived IL-4 and IL-13 (Del Prete et al., 1988; Finkelman et al., 1988). Ultimately signals within B cells from IL-4 and CD40 synergize to induce transcription of AID and εGLT, which together initiate IgE CSR (Dedeoglu et al., 2004; Geha et al., 2003). Classical IgE responses are thus dependent on $CD4^+$ T cells and IL-4. However, numerous *in vitro* and *in vivo* experiments show alternative pathways for IgE CSR that can bypass one or more of these requirements.

1.9.2 Alternative pathways of IgE CSR

A large body of literature shows evidence of alternative pathways of IgE CSR. In different experimental contexts, IgE has been detected in the absence of one of the following parameters: $CD4^+$ T cells, IL-4, IL-4 and IL-13, MHC Class II molecules, or CD40 (Grunewald et al., 2001; Markowitz et al., 1993; Morawetz et al., 1996). Infection with a helminth could even bypass the need for mature IgM^+ B cells to induce IgE, as shown in the induction of IgE in μMT mice that lack IgM required for classical B cell development, which probably reflects an ancient compensatory mechanism that evolved to combat parasites (Perona-Wright et al., 2008). Our laboratory measured elevated levels of natural polyclonal IgE in mice deficient for CD4 or MHC Class II molecules or in athymic nude mice. In this model, CSR to IgE was independent of MHC Class II cognate help yet dependent on T cell derived IL-4. This may possibly represent a primitive pathway of IgE induction since lymphoid structure formation was dispensable (McCoy et al., 2006). Moreover, progression to IgE^+ plasma cells has been shown to have unique features in a model of immunization-induced hyper IgE in T/B monoclonal mice and in a model of infection with the helminth *Nippostrongylus brasiliensis* (Erazo et al., 2007). In these two models, T-dependent IgE antibodies underwent affinity maturation in the germinal center. However IgE^+ B cells could only be detected outside of the germinal centers after sequential isotype switch from IgG1.

1.9.3 CSR signals delivered by BAFF, APRIL or TSLP

The delayed induction (five-seven days) of antibodies via the follicular T-dependent pathway is not suited for rapid responses to harmful antigens (especially at mucosal interfaces). To compensate for this restriction, antibodies are induced through a faster T-independent pathway that

involves recognition of highly conserved microbial and viral products by low affinity BCRs and TLRs. Similarly to innate immune cells, B cells express TLRs (Lanzavecchia and Sallusto, 2007). Transcription of AID is induced upon TLR engagement, which leads to antibody secretion and B cell proliferation (He et al., 2004; Xu et al., 2008). Additionally, signaling through TLRs in innate immune cells including DC, monocytes, macrophages, granulocytes, germinal center follicular DC and epithelial cells (including intestinal epithelial cells) leads to the secretion of B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) that deliver CSR signals to B cells (He et al., 2007; Litinskiy et al., 2002). BAFF and APRIL are members of the tumor necrosis factor (TNF) family and bind to receptors expressed on B cells. Both engage two receptors expressed on B cells, transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) and B cell maturation protein (BCMA). In addition, BAFF engages a third receptor on B cells, BAFF-R. BAFF and/or APRIL can act in synergy with IL-4 to induce IgE CSR (Castigli et al., 2005). Importantly signaling pathways induced downstream of TLR and TACI cooperate in B cells as recently demonstrated by Cerutti and colleagues (He et al., 2010). MyD88, the prototypical molecule involved in relaying downstream TLR signals, was shown to bind to a highly conserved cytoplasmic domain of TACI. This interaction resulted in a signaling cascade reminiscent of the TLR pathway converging in the expression of AID and subsequent CSR.

Thymic stromal lymphopoietin (TSLP), a member of the IL-7 family, was discovered more than a decade ago and owes its name to its identification in conditioned supernatant of a mouse thymic stromal cell line as a B-cell growth factor (Friend et al., 1994). In humans T-independent induction of intestinal IgA_2 was shown to depend on epithelial-released TSLP, following microbial-induced TLR signals. Engagement of TSLP to TSLP-R

expressed on DC promoted the secretion of BAFF and APRIL, which led to B cell CSR to IgA_2 (He et al., 2007). It is tempting to think that TSLP is involved in IgE CSR because of the increasing amount of studies indicating the strong Th2-polarizing effect of TSLP. In models of allergic diseases, TSLP exacerbated inflammation (Zhang et al., 2009; Zhou et al., 2005) and in *Trichuris muris* helminth infection it enhanced protective Th2 responses (Liu et al., 2007). However, TSLP was dispensable for the induction of a protective anti-helminth Th2 response in mice infected with *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis* (Massacand et al., 2009). Moreover, TSLP produced by intestinal epithelial cells conditions lamina propria DC. Upon bacterial breach of the epithelial cell layer TSLP secretion was increased, which conditioned DC to induce Th1 polarization. In contrast under steady-state conditions, the concentration of TSLP favored a non-inflammatory Th2 response with DC secreting IL-6 and IL-10. Thus modulation of TSLP concentration may serve as an indicator of intestinal epithelial cell integrity (Rimoldi et al., 2005; Ziegler and Artis, 2010). Consistent with those results, genetic ablation of intrinsic intestinal epithelial cell TLR signaling via NF-κB lowered TSLP production and increased IL-12-producing DC. As a result, the development of protective Th2 responses was impaired after infection with *Trichuris muris* (Zaph et al., 2007). OX40L is mainly expressed on professional antigen presenting cells (APC) and its expression can be induced by various factors including signals through CD40, BCR, TLR as well as TSLP (Croft, 2010). Interestingly, DC conditioned with TSLP upregulate OX40L, which promotes Th2 polarization in the absence of IL-12 (Ito et al., 2005).

To summarize, rather than following a linear differentiation pathway, Th2 cells may arise from a complex network involving different and redundant cell types and cytokines. Furthermore, the site of induction (gut, lungs,

skin) and the type of antigen may determine the dominant path amongst this complex Th2 network.

1.10 Hypothesis and aims

I hypothesize that beyond the well-established role in maturation of the immune system, symbiotic microbes contribute to regulation of the immune system. In support of this, we observed elevated serum IgE levels in germ-free mice, which are devoid of microbes. Thus, absence of microbes compromises immune regulation, which is reflected by abnormally high levels of serum IgE in germ-free mice. We previously showed that this IgE repertoire was polyclonal and germ-line encoded with no evidence for somatic hypermutation. In the first part of this thesis I investigated the pathways leading to high IgE in germ-free mice and investigated the mechanisms leading to hygiene-mediated immune dysregulation.

In the second part of this thesis, I applied our hypothesis to a model of T1D using the diabetes-prone NOD mouse model. I investigated the development of diabetes after systemic challenge with the intestinal symbiont *E. cloacae.*

Defining the impact of normal indigenous microbes on the regulation of the immune system is relevant to understand the mechanistic basis of the increasing rates of atopic allergic diseases and autoimmune diseases.

2 Results I - Intestinal microbial composition contributes to IgE regulation

To gain insights into the impact of microbial composition on regulation of the immune system, mice were kept under three well-defined levels of hygiene: germ-free, gnotobiotic and SPF.

Germ-free mice are born and raised in a complete sterile environment within flexible film isolators and thus harbor no microorganisms on their skin or mucosal surfaces. Gnotobiotic mice are associated with a limited and defined microflora. In these experiments gnotobiotic mice were associated with the eight bacterial species of the altered Schaedler's flora consisting of four fastidious anaerobes and four aerotolerant bacterial species as listed in Table 1 (Dewhirst et al., 1999). Gnotobiotic ASF-colonized mice were maintained in a clean room within individually ventilated cages. SPF mice are devoid of a defined list of mouse pathogens but harbor a diversified and undefined flora, which widely varies across animal facilities. In these experiments SPF mice were purchased from Taconic or were bred and maintained in the SPF animal facility at the University of Bern.

2.1 Total serum IgE levels inversely correlate with the degree of intestinal microbial diversity

Induction of IgE is normally very tightly regulated such that in healthy individuals IgE levels remain below the detection limit of standard enzyme-linked immunosorbent assay (ELISA) assays (Sutton and Gould, 1993). The same is true for experimental mice housed under SPF conditions. To gain insights into the role of the microflora in maintaining IgE levels at basal level, serum IgE levels were measured in C57BL/6 mice kept under different levels of hygiene. As expected, mice harboring a diverse and

complex SPF flora had very low or undetectable levels of IgE (0.5 ± 1 ng/ml, n=17, range: 0.1-5, Figure 3). In contrast, a proportion of ASF-colonized gnotobiotic mice showed increased IgE in the serum (33/53, Figure 3). Even more striking was the observation that mice housed under germ-free conditions displayed even greater serum IgE levels (Figure 3). This observation was not a phenomenon isolated to a single animal facility as elevated IgE levels in germ-free and ASF-associated mice were confirmed in three independent facilities: the Labortierkunde at the University of Zürich (Zürich, Switzerland) (McCoy et al., 2006), the Axenic and Gnotobiotic Unit at McMaster University (Hamilton, Canada) (germ-free: 665 ± 1664 ng/ml, n=113, range: 0.1-14853; ASF: 20 ± 54 ng/ml, n=35, range: 0.1-311) and the Clean Mouse Facility of the University of Bern and the Inselspital (Bern, Switzerland) (germ-free: 578 ± 783 ng/ml, n=11, range: 0.1-2126; ASF: 27 ± 28 ng/ml, n=18, range: 0.1-99).

Antibodies found in germ-free animals are not triggered in response to conventional antigen-driven immune activation, thus all antibodies produced in germ-free animals are defined as natural antibodies (Manz et al., 2005). Collectively, these observations led us to conclude that in the absence or reduction of microbial stimuli, B cells preferentially produce natural antibodies of the IgE isotype.

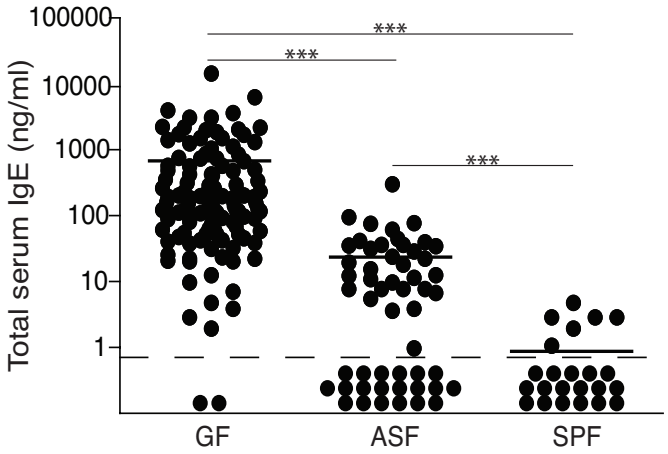


Figure 3. Serum IgE levels increase with hygiene. Total serum IgE was measured in adult C57BL/6 mice (>4 weeks of age) born and raised under different hygiene conditions as indicated: germ-free (GF), ASF-colonized (ASF), specific pathogen free (SPF). The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set. Statistical significance was determined with the unpaired two-tailed t-test (*** P<0.001).

2.2 Serum IgE levels in GF mice start to increase early in life

To evaluate if B cells switched to IgE during a precise time window or rather this was a stochastic process, we studied the kinetics of serum IgE appearance. Serum IgE levels were monitored in C57BL/6 germ-free mice starting from one week before weaning. Whereas IgE titers remained low in most mice until day 28 of age, they began to elevate after this time point (Figure 4A). It may be important to note that day 28 is also the time point of weaning of germ-free pups. As expected, no detectable IgE was measured in SPF mice (Figure 4B). This observation indicates that the elevated IgE levels measured in adult germ-free mice are the result of an early-life isotype switch to IgE and that the complete absence of bacterial stimuli during this time leads to a preferential isotype switch to IgE.

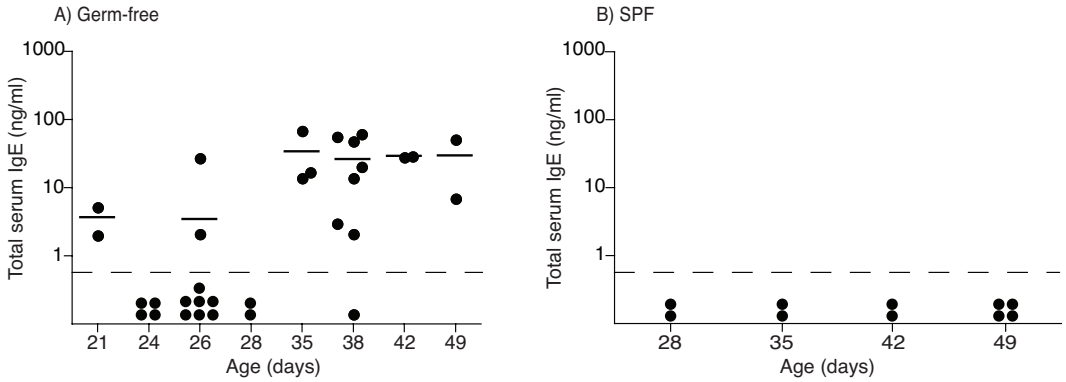


Figure 4. Isotype switch to IgE is induced very early in postnatal life of germ-free C57BL/6 mice. Serum IgE titers were measured at the indicated ages in (A) germ-free and (B) SPF mice. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set.

2.3 Increased natural IgE in germ-free mice is independent of maternal antibody transfer and dietary antigens

Maternal antibodies are passed to the offspring before birth by the transplacental route and after birth by breastfeeding (Hanson et al., 2003; Israel et al., 1995). Because IgE levels first became detectable in the serum at around day 28 of age, which is when the pups were weaned, we set out to investigate the contribution of maternal antibodies in preventing the early-life IgE switch. It is possible that the withdrawal of maternal antibodies at the time of weaning could remove an inhibitory factor or even trigger the hygiene-induced IgE switch. To investigate this we made use of the $J_H^{-/-}$ mouse strain (C57BL/6 background) that lacks all mature B cells (and therefore all antibodies) due to deletion of the J gene segments in the IgH chain locus (Chen et al., 1993). Germ-free female $J_H^{-/-}$ mice were bred with germ-free male C57BL/6 mice to generate pups heterozygous for the J_H lesion. Heterozygous $J_H^{+/-}$ offspring were therefore immunocompetent and could produce B cells and antibodies but were deprived of any maternal-derived antibodies because the mother was $J_H^{-/-}$ (Harris et al., 2006). As shown in Figure 5A, $J_H^{+/-}$ pups born to $J_H^{-/-}$ dams did not show any differences in the induction of IgE compared to wild-type mice. These

data suggest that maternal antibodies did not contribute to or inhibit hygiene-induced IgE in germ-free mice.

Not only does weaning correspond to the removal of maternal antibodies but it also coincides with the introduction of solid chow. Germ-free mice are fed an autoclaved rodent chow that contains dietary antigens and, despite its sterility, may contain significant amounts of dead bacteria and bacterial products such as LPS (Hrncir et al., 2008). To test the hypothesis that the increase in natural IgE may occur in response to food antigens, germ-free mice were fed an irradiated elemental diet. This diet consisted of extensively hydrolyzed proteins supplemented with fats, vitamins and minerals. To further reduce environmental antigens, baked sand was used as bedding. The initial "antigen-free" colony consisted of two C57BL/6 breeding pairs. Although C57BL/6 mice could be maintained on the elemental diet and had several litters of the expected litter size, the pups never survived past two-three days after birth. We suspected that the mortality of the pups might have been due to insufficient nutrients in the milk provided by the mothers fed an elemental diet. In an attempt to circumvent this we exchanged the inbred C57BL/6 females with outbred NIH-Swiss females as outbred mice are known to be more resilient. The resulting NIH-Swiss×C57BL/6 F1 pups survived through to adulthood and IgE levels were measured at different ages (Figure 5B). Whereas at early age (day 35) IgE was below detection limit, it increased rapidly onwards to reach very elevated levels (day 105: 1205 ± 464 ng/ml, n=4, range: 678-1699). We therefore concluded that antigens or bacterial products contained in the autoclaved chow were not required for the early IgE switch in germ-free mice.

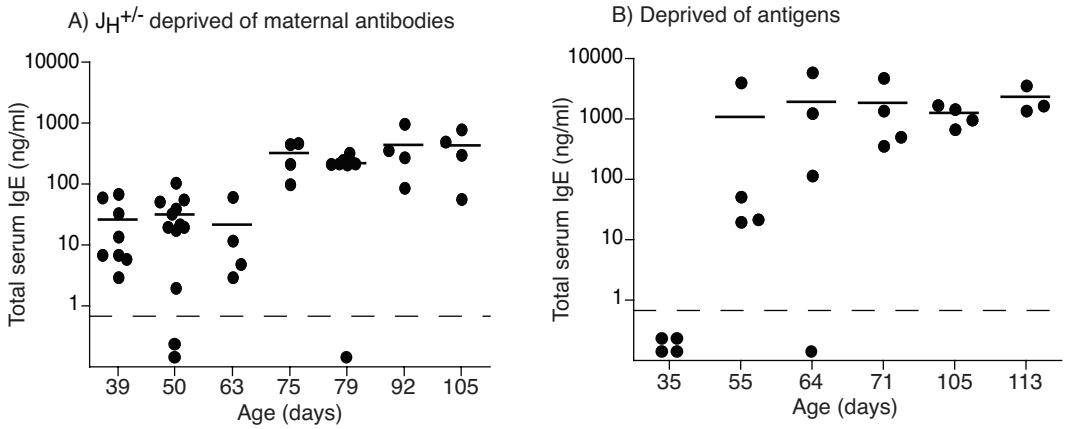


Figure 5. Elevated natural IgE in germ-free mice is independent of maternal antibodies and dietary antigens. Serum IgE titers were measured at the indicated ages in (A) heterozygous $J_H^{+/-}$ mice nursed by a $J_H^{-/-}$ mother and (B) F1 offspring from NIH-Swiss×C57BL/6 breeding pairs born and raised on an elemental diet. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set.

2.4 Elevated natural IgE levels in the serum of adult germ-free mice are not modulated by bacterial colonization

The observation that the absence of intestinal bacteria is associated with high levels of IgE led us to investigate whether colonization with a diverse SPF flora could modulate these levels. Adult (eight-nine week old) C57BL/6 germ-free mice were co-housed with a SPF "colonizer" mouse and total serum immunoglobulin levels (IgA, IgM, IgG_1, IgG_{2b}, IgG_{2c}, IgG_3, and IgE) were measured for the following eight weeks. While, as expected (Macpherson and Harris, 2004), serum concentrations of IgA, IgM, and IgG increased following colonization (Figure 6A), IgE concentrations were not markedly modulated (Figure 6B), remaining high or even increasing in two animals starting with low IgE levels. Mice that are born in a SPF environment do not develop high levels of IgE and yet colonization of an adult germ-free mouse with a SPF flora does not modulate the preexisting high levels of IgE over a period of eight weeks. We therefore suggest that bacterial colonization must happen during a critical time window early in life to prevent B cell switch to IgE.

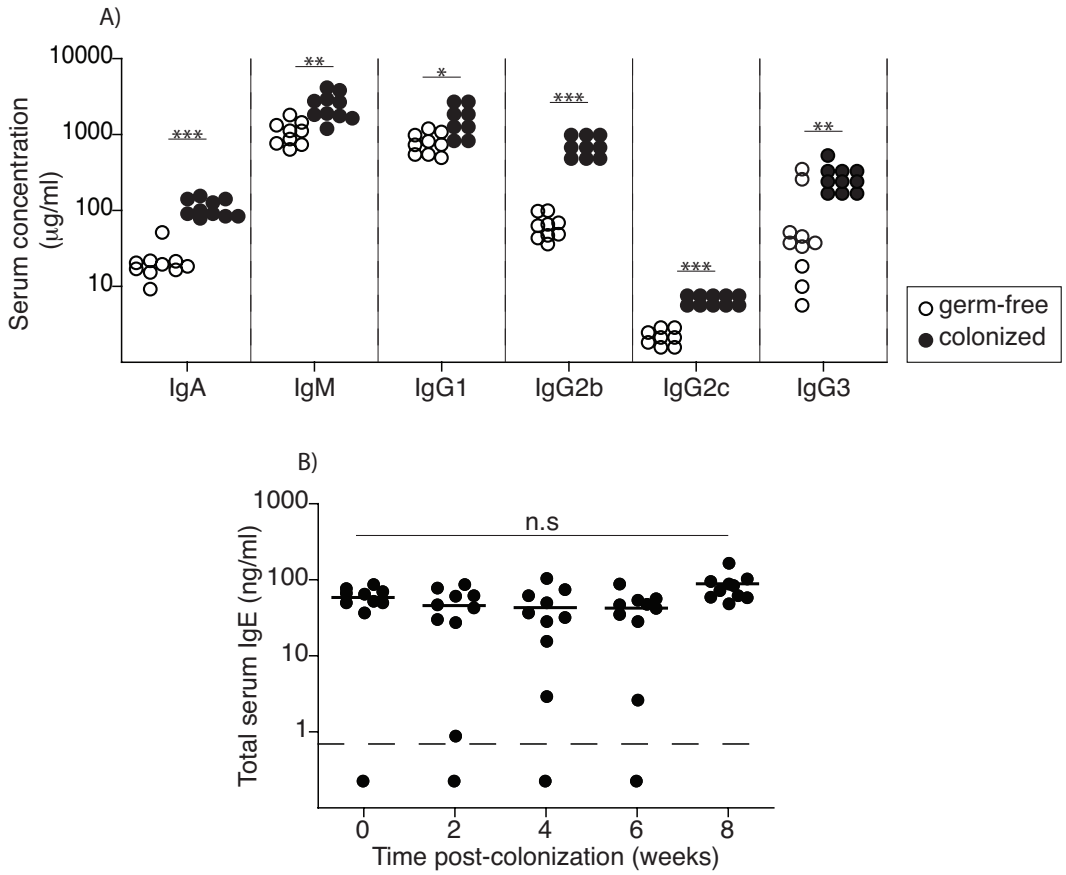


Figure 6. Colonization with intestinal microflora from a SPF mouse does not modulate preexisting elevated IgE levels. Adult (eight-nine week old) C57BL/6 germ-free mice were co-housed with a SPF colonizer mouse and total serum antibodies were measured. (A) Serum IgA, IgM, IgG_1, IgG_{2b}, IgG_{2c}, and IgG_3 concentrations at experiment start (o) and 8 weeks after colonization (●) and (B) longitudinal serum IgE concentrations at the indicated time points following colonization are shown. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set. Statistical significance was determined with the unpaired two-tailed t-test (n.s: not significant, *P<0.5, **P<0.01, *** P<0.001).

2.5 Isotype switch to IgE occurs in mucosal lymphoid sites

Class switch recombination to IgE is commonly associated with B cell activating antigens derived from parasites or allergens. In these circumstances B cells undergo IgE CSR at the site of antigen entry. In the present case, CSR to IgE occurs in the absence of antigens. We therefore set out to elucidate the lymphoid compartments in which B cells initiated IgE CSR in the absence of inhibitory microbial stimuli. Detection of sterile

εGLT is a hallmark for active CSR to IgE (Geha et al., 2003) as it is transcribed for a very limited time prior to active CSR. To this end, quantitative real-time PCR (qPCR) primers specific for εGLT were designed and validated on magnetic-assisted cell sorting (MACS)-enriched $CD19^+$ B cells cultured *in vitro* under conditions known to induce CSR to IgE. Specifically, $CD19^+$ B cells were cultured with IL-4 and anti-CD40 *in vitro* for 36 hours. As an additional positive control, B cells were also isolated from C57BL/6 mice infected for 14 days with the mouse helminth *Heligmosomoides polygyrus* (Figure 7A inset) as this parasite is known to induce a strong IgE response (Allen and Maizels, 2011). As a negative control for primer specificity, B cells were isolated from the spleen of $IgE^{-/-}$ mice. Corresponding with the absence of serum IgE, εGLT was not detected in B cells from spleen, BM, MLN, PP or peritoneal wash (PW) from SPF mice (Figure 7A inset). As IgE can be detected in the serum very early in post-natal life (Figure 4A) and B cell CSR must precede this, we determined the levels of εGLT in spleen, BM, pLN, MLN, PP and peritoneal wash (PW) of neonatal germ-free mice aged 7, 14, 21, 28, 35, 42, and 49 days-old. *εGLT* could be detected on day 35 (Figure 7A), consistent with the appearance of serum IgE (Figure 4A). Of note, *εGLT* expression was almost exclusively expressed in the mucosal-associated lymphoid tissues, the MLN and PP (Figure 7A). We thus conclude that during post-natal life the total absence of bacterial stimuli favors an environment in the MLN and PP where B cells preferentially undergo switch to IgE.

The presence of *εGLT* in both MLN and PP suggested that the microenvironment of both mucosal lymphoid sites in the absence of microbial stimulation promoted CSR to IgE. However, it is possible that the presence of *εGLT* in MLN represents B cells that have recently migrated there from PP. Therefore in order to determine if PP were the primary site

for induction of IgE we selectively depleted PP *in vivo*. Organogenesis of PP is dependent on IL-7 signaling and a single intravenous injection of anti-IL7Rα (CD127) on day 14.5-15.5 post-coitus (p.c) has been shown to result in an effective and specific ablation of PP in the progeny (Yoshida et al., 1999). We thus made use of this protocol under germ-free conditions and monitored serum IgE in the PP-deficient progeny (Figure 7B). The progeny of anti-IL7Rα-treated mothers did not develop PP (Figure 7C), and had very low or undetectable serum IgE levels (Figure 7D). These data suggest that PP may be critical for the initiation of natural IgE induction since MLN alone are not able to compensate for the absence of PP.

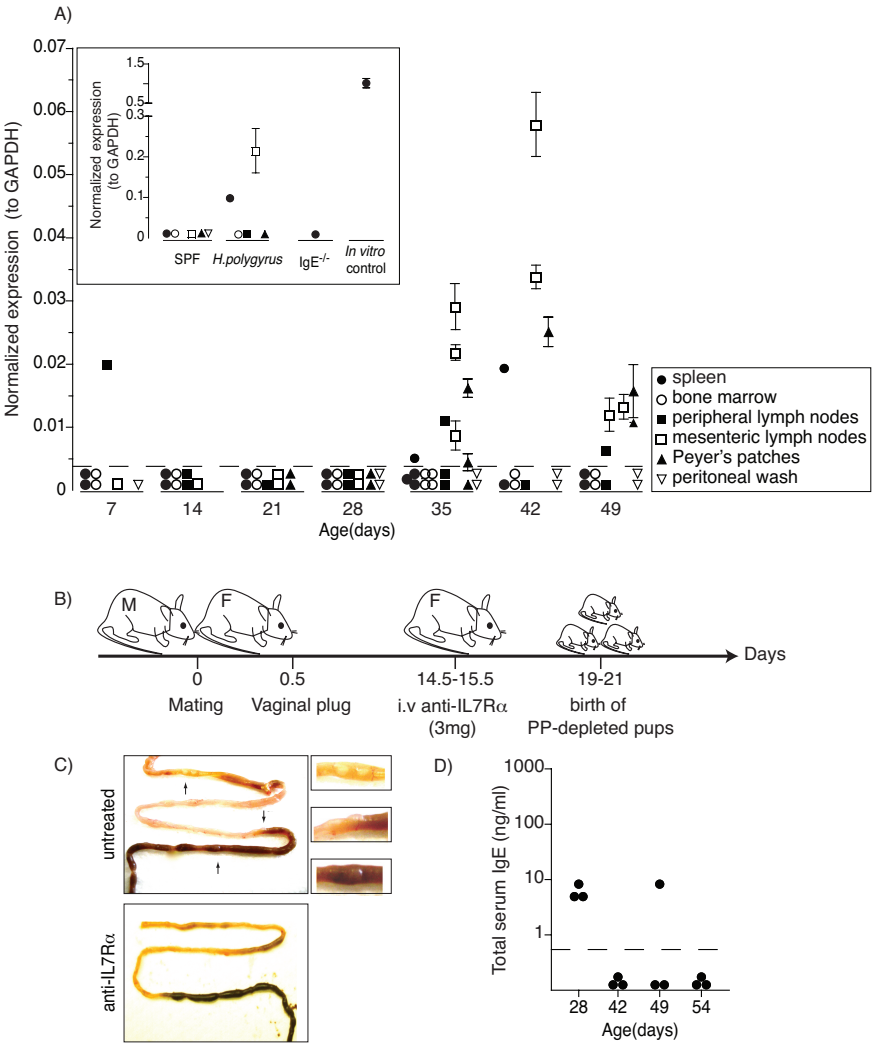


Figure 7. In the absence of intestinal bacteria *εGLT* is detected in the MLN and PP and natural IgE induction is dependent on PP formation. (A) Normalized expression of *εGLT* from B cells residing in different compartments of germ-free mice at weekly intervals starting from one week after birth. qPCR was performed on total RNA isolated from MACS-enriched $CD19^+$ cells from spleen, BM, pLN, MLN and PW or total cells from PP. Expression was normalized to *GAPDH* and expression level of *in vitro* control set to 1. The dotted line indicates the limit of detection and individual samples (individual mice or 2 pooled mice) are plotted as mean ± standard deviation (SD) of technical repeats. The inset indicates the normalized expression of the positive and negative controls used in this study. (B) Formation of PP was inhibited in germ-free C57BL/6 mice following blockade of IL7-Rα during ontogeny. (C) Depletion of PP was confirmed at experiment end. Shown are representative pictures of the small intestine of age-matched C57BL/6 germ-free mice either left untreated (top) or treated with anti-IL7Rα (bottom). Arrows indicate the location of PP, which are shown magnified in the insets. (D) Total serum IgE levels were measured at the indicated ages. The dotted line indicates the lower limit of detection (0.8 ng/ml) and each point indicates a single animal.

2.6 CSR to natural IgE is T cell dependent

Although isotype switch is usually dependent on T cells, mucosal B cells can undergo isotype switch in a T cell independent manner (Macpherson et al., 2000; Tezuka et al., 2011). T cell dependence for early natural IgE switch was thus investigated in germ-free mice using two different approaches. Firstly, total serum IgE levels were measured in TCR$\beta\delta$-deficient mice (C57BL/6 background) kept under germ-free or gnotobiotic ASF conditions. Whereas IgE levels are elevated in their wild-type counterparts (Figure 3), no detectable IgE was measured in germ-free (n=16) or ASF (n=8) adult T cell deficient mice (Figure 8A). In a second approach, germ-free C57BL/6 mice were depleted of $CD4^+$ T cells by injection of a monoclonal antibody (mAb) against CD4 (clone YTS191.1.2) beginning at 28 days of age when IgE levels are still below detection limit. Germ-free mice were intra-peritoneally (i.p) injected twice a week with 200 μg depleting antibody for a period of 68 days, which resulted in a rapid and effective loss of $CD4^+$ T cells (Figure 8B). While, as expected, untreated mice developed elevated natural IgE, anti-CD4-treated mice had IgE levels below the limit of detection (Figure 8C). Taken these results together, we can conclude that early switch to IgE is dependent on $CD4^+$ T cells.

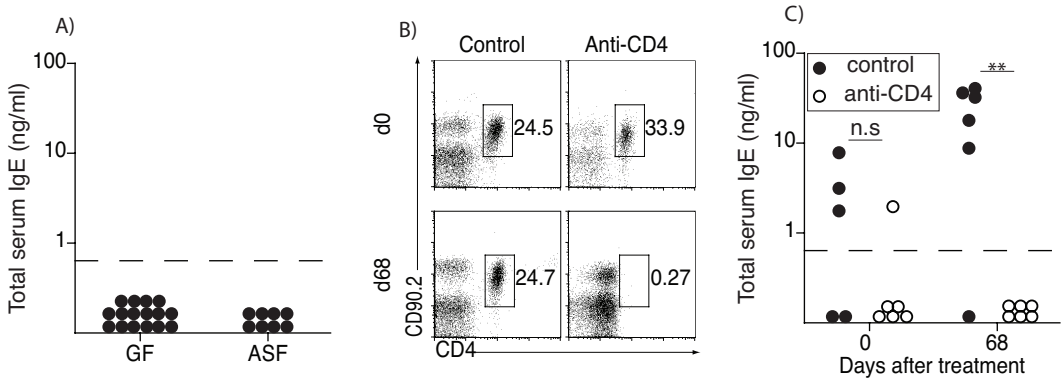


Figure 8. Natural IgE induction is T cell dependent. (A) Total serum IgE of adult TCR$\beta\delta^{-/-}$ mice housed under germ-free or ASF conditions was measured. (B-C) 28-day old germ-free C57BL/6 were depleted of $CD4^+$ T cells by bi-weekly injection of a CD4-depleting antibody (clone YTS191.1.2) for a period of 68 days. Depletion of $CD4^+$ T cells was assessed (B) by flow cytometry on blood and (C) total serum IgE was measured in control or antibody-treated mice at experiment start (d0) and end (d68). Representative flow cytometry plots are shown. Numbers beside outlined areas indicate the frequency of CD4+ T cells within the live lymphocyte gate. The dotted line indicates the lower limit of detection (0.8 ng/ml) and each point indicates a single animal. Statistical significance was determined with the unpaired two-tailed t-test (n.s: not significant, **P<0.01).

2.7 Towards elucidating the cytokines involved in early IgE CSR

During CSR the prevailing cytokines in the environment dictate B cell isotype fate decision. In response to defined cytokines, transcription through the CH gene that will encode the new isotype is initiated. This in turn leads to formation of GLT and the subsequent deletion-recombination process of CSR. In the case of CSR to IgE, IL-4 initiates transcription upstream of the Cε transcriptional unit (Stavnezer et al., 2008). Nonetheless accumulating evidence suggests that in certain circumstances CSR to IgE is dependent on other Th2 cytokines such as IL-13, TSLP or IL-10. Although not directly inducing transcription of the Cε locus, these cytokines can condition APCs such as DC, which in turn influence the initiation of CSR.

2.7.1 The classical Th2 cytokines, IL-4 and IL-13

The prototypical Th2 cytokines IL-4 and IL-13 are critical for IgE isotype switch in allergic diseases and helminth infection (Urban et al., 1995; Urban et al., 1998). Because high levels of IgE are observed in germ-free mice, it is possible that in the absence of intestinal bacteria a Th2 environment with high IL-4 and/or IL-13 dominates in the MLN and PP.

To determine whether *in vivo* IL-4 depletion could inhibit IgE CSR in germ-free mice, newly weaned (28-day old) germ-free mice were administered an IL-4 neutralizing mAb (clone 11B11) twice a week with 200 μg/injection. As expected, both the treated group and untreated group had undetectable IgE levels at the time of weaning (Figure 9A). However, after 34 days of treatment, IgE titers appeared to be reduced in the IL-4-depleted group as compared to control mice (Figure 9A). From this initial observation, we concluded that IL-4 seemed to be involved in hygiene-induced IgE but that other cytokines (such as IL-13) may also be involved. Alternatively, our treatment regimen was not sufficient to completely neutralize all IL-4, although this is extremely difficult to determine. In order to assess whether IL-13 may also be involved we assessed the concentration of key cytokines in the serum of germ-free and SPF mice. While IL-4 was below limit of detection in both groups (data not shown), IL-13 was significantly higher in the serum of germ-free (n=6) compared to SPF mice (n=6), indicative of more circulating IL-13-producing cells or higher production of IL-13 in the periphery (Figure 9B). In order to provide further information on the induction of these cytokines under germ-free conditions we determined gene expression levels of IL-4 and IL-13 by qPCR in germ-free mice and SPF mice aged 90- (n=8-11) or 138 (n=6-8) days of age. Gene expression of IL-4 and IL-13 was not increased in the MLN or spleen of germ-free mice compared to SPF (Figure 9C). Thus, in

adult germ-free mice with high IgE titers IL-4 and IL-13 gene expression were not upregulated.

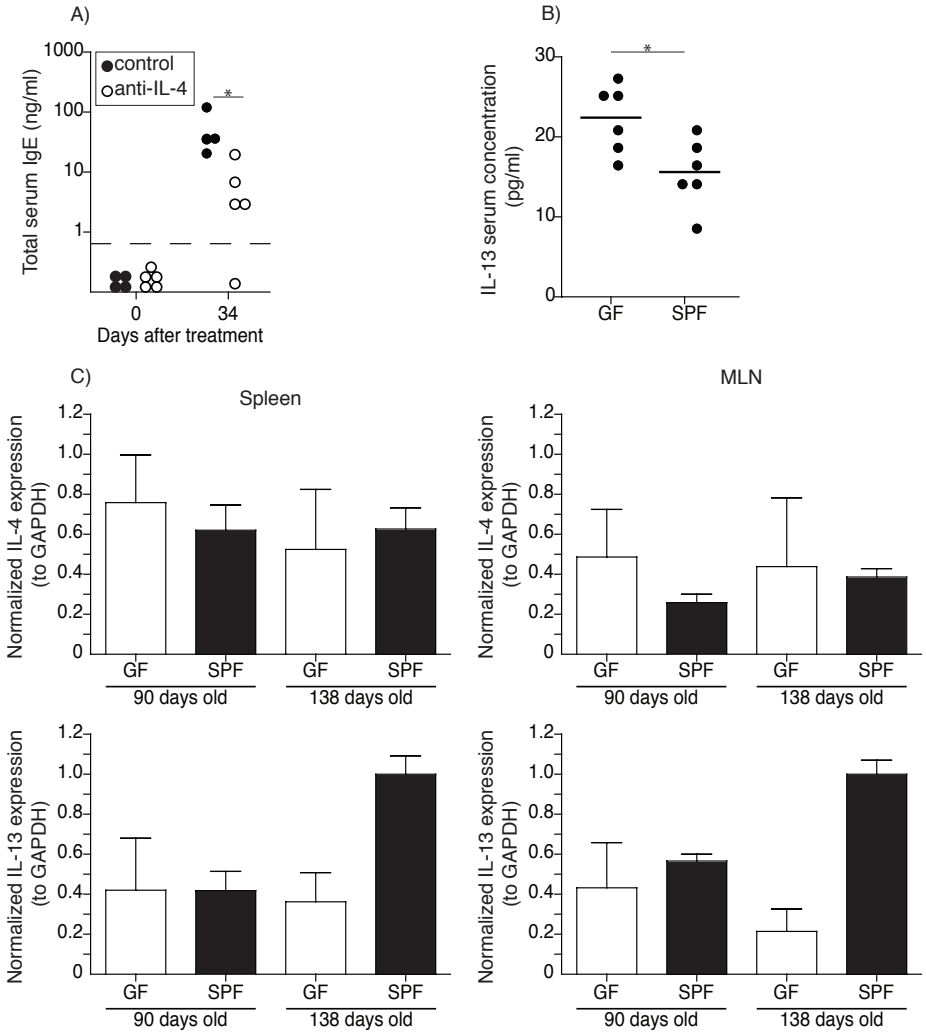


Figure 9. The role of IL-4 and IL-13 in natural IgE induction. (A) Newly weaned (28 days-old) C57BL/6 germ-free were i.p injected with an IL-4 neutralizing mAb (clone 11B11) twice a week with 200 μg/injection (anti-IL4) or left untreated (control) and IgE levels were monitored. The dotted line indicates the lower limit of detection (0.8 ng/ml). (B) IL-13 serum concentration was measured with CBA assay in C57BL/6 mice kept under GF or SPF conditions. Each point indicates a single animal and the horizontal line represents the mean of each data set. Statistical significance was determined with the unpaired two-tailed t-test (*P<0.05). (C) Normalized expression of IL-4 and IL-13. qPCR was performed on total RNA isolated from MACS-enriched $CD19^-$ cells from spleen (left column) or MLN (right column). Expression was normalized to *GAPDH* and the sample with highest expression level set to 1. GF expression was plotted as the mean of 8-11 (90 days-old) or 6-8 (138 days-old) individual animals ± SD and SPF expression as the mean ± SD of technical repeats

2.7.2 TSLP

Accumulating evidence has demonstrated that TSLP can play a key role in certain models of Th2 responses, such as allergic diseases and parasite infections (Ziegler and Artis, 2010). In addition, TSLP is constitutively expressed by IECs and thus believed to be a critical regulator of intestinal immune homeostasis by acting on the underlying DC (Rimoldi et al., 2005; Taylor et al., 2009). We thus wondered whether absence of microbes would alter TSLP expression and would explain the B cell switch to the Th2-isotype IgE. To investigate whether TSLP is involved in early IgE CSR in germ-free mice, TSLP was depleted using a specific anti-TSLP neutralizing mAb (clone 28F12). The contribution of TSLP was investigated in two groups of mice; the first group received anti-TSLP starting from the time of weaning when IgE levels were still low, while the second group received anti-TSLP as adults when IgE was already elevated. Anti-TSLP neutralizing mAb was injected i.p bi-weekly while the control groups were left untreated. Neutralization of TSLP at the time of weaning did not lead to lower IgE levels as compared to control mice (Figure 10A). In contrast, the initial increase in IgE was even more pronounced than in the untreated group (wk 2 and 4 following treatment, Figure 10A). On the other hand, in the adult group, IgE levels in control and anti-TSLP-treated mice were comparable (Figure 10A). These data therefore indicate that natural IgE induction in germ-free mice is not dependent on TSLP and its absence may even promote a more rapid onset of IgE switch. However, it could be argued that antibody-mediated depletion was ineffective in completely neutralizing TSLP *in vivo* and in particular neutralization in mucosal sites may be difficult to achieve. Therefore in order to confirm our observation, $TSLPR^{-/-}$ mice (C57BL/6 background) were rederived to germ-free status and IgE levels monitored over time. Germ-free TSLPR-deficient mice showed elevated IgE levels,

therefore verifying the results obtained through antibody depletion (Figure 10B). Furthermore, TSLPR-deficiency contributed to very high levels of IgE that continuously increased with age (mean d40: 11 ng/ml, mean d156: 31 000 ng/ml). These results suggest that natural IgE CSR is not only independent of TSLPR downstream signaling but may even be enhanced in its absence.

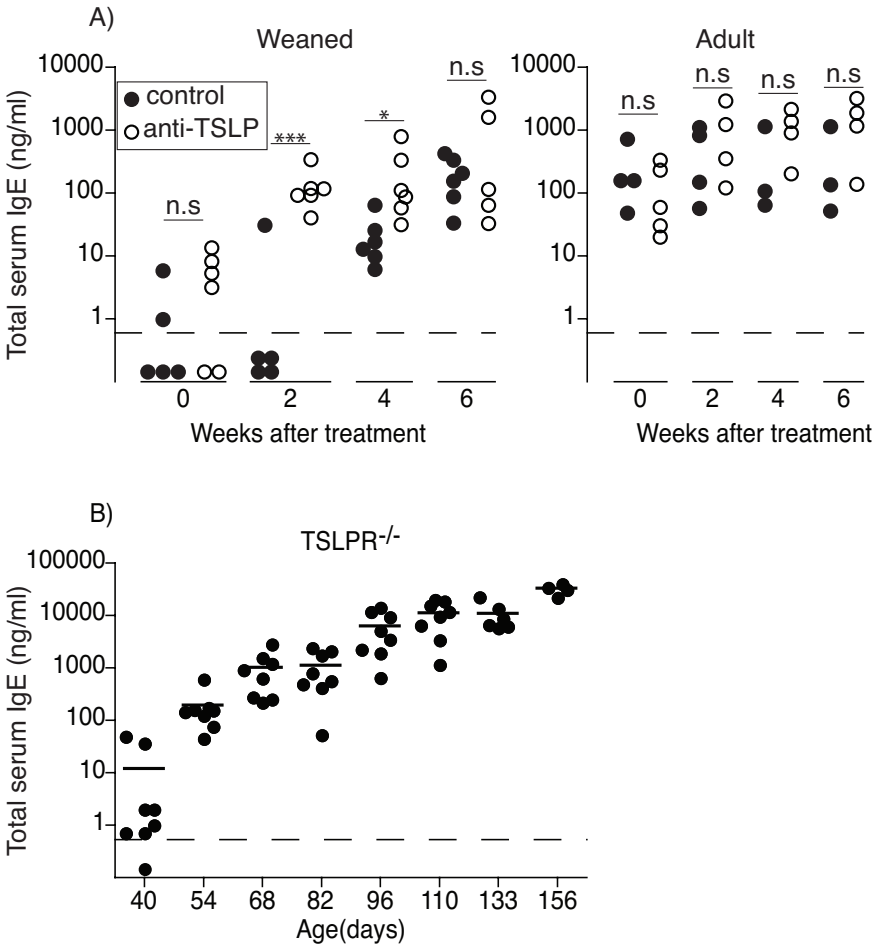


Figure 10. Natural IgE CSR is not dependent on TSLP. (A) Neutralization of TSLP by bi-weekly i.p injection of a mAb (clone 28F12) was performed from the time of weaning (28-day old) or in adult germ-free mice and total serum IgE was measured in untreated (control) and anti-TSLP-treated mice. Statistical significance was determined with the unpaired two-tailed t-test (n.s: not significant, *P<0.05, ***P<0.001). (B) Total serum IgE levels were monitored in germ-free TSLPR$^{-/-}$ at the indicated ages. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set.

2.7.3 Interleukin-10

IL-10 is a nonredundant anti-inflammatory cytokine that is highly expressed in the intestine (Kühn et al., 1993). Our research group has demonstrated that the establishment of a commensal mutualistic ASF microbiota is associated with a stark increase in regulatory T cells ($CD4^+FoxP3^+$) and IL-10 expression in the colon lamina propria (cLP) (Geuking et al., 2011). We thus wondered whether low IL-10 production in germ-free animals might explain the high IgE phenotype. The concentration of IL-10 in the serum was therefore measured by cytometric bead array (CBA) and a small but significant increase in serum IL-10 was observed in germ-free animals (n=6) compared to SPF (n=6) (Figure 11A). Therefore serum IL-10 levels were elevated, rather than decreased, in the absence of intestinal bacteria. However, since CSR to IgE in germ-free mice occurs in mucosal lymphoid sites (Figure 7), we determined expression levels of IL-10 RNA in the spleen and MLN from germ-free and SPF animals aged 90- and 138-days old. Although IL-10 expression was comparable in germ-free and ASF in the spleen, it was greatly reduced in germ-free compared to SPF mice in the MLN in both age groups (90- and 138-days old) (Figure 11B). Therefore, under germ-free conditions, gene expression of IL-10 was decreased in the same lymphoid organ that εGLT was increased (Figure 7). To test if this observation was functionally correlated, IL-10 signaling was abolished by injecting newly weaned (25-days old) germ-free mice with a blocking mAb against the IL-10R. Serum IgE levels increased shortly after weaning in both the isotype-treated control group (clone 36.61; IgG1, κ) and the anti-IL10R-treated group (clone IBI-2) (Figure 11C). Of note, at week 3 following treatment initiation, IgE levels were significantly decreased but yet elevated in the anti-IL10R-treated group compared to the isotype-treated control group. Nonetheless,

anti-IL10R treatment (500 μg i.p twice per week) could not prevent the high IgE phenotype observed in germ-free mice.

IL-10 is produced by several immune cells, such as $CD4^+$ regulatory T cells including the $FoxP3^+$ and T regulatory type 1 (Tr1) subsets (Roncarolo and Battaglia, 2007) and the recently identified IL-10-producing regulatory B cells (Mizoguchi et al., 2002; Yanaba et al., 2008; Yang et al., 2010). As mentioned above, we showed that bacterial colonization induces IL-10 RNA expression but the cellular source(s) remains unknown. To address this, we made use of the 10BiT IL-10 reporter strain (Maynard et al., 2007) and re-derived this strain to germ-free status. In this reporter strain *Il10* expression can be faithfully identified by surface Thy1.1 (CD90.1) staining because the first coding exon of *Il-10* has been replaced by Thy1.1. *Il-10* expression on $CD4^+$ T- and $CD19^+$ B cells was compared in germ-free and ASF-colonized (28 days) 10BiT mice. Following colonization, a striking increase in T cells expressing CD4 and Thy1.1 was measured in the BM (GF: 2.49%; ASF-colonized: 10.48%) and cLP (GF: 14.86%; ASF-colonized: 31.69%) while no major differences were detected in spleen, pLN, MLN, PP and PW (Figure 11D upper panel). Of note, IL-10 expression was upregulated in both $FoxP3^+$ and $FoxP3^-$ $CD4^+$ T cells following colonization implying that both Treg and Tr1 $CD4^+$ T cell regulatory subsets might contribute to bacterial-induced IL-10 expression. IL-10-expressing $CD19^+$ B cells were identified in cLP and starkly increased following colonization (GF: 7.69%; ASF-colonized: 27.5%) (Figure 11D bottom panel). A more moderate increase of IL-10 expressing B cells was observed in PW (GF: 3.97%; ASF-colonized: 6.83%) and in all other compartments (spleen, BM, pLN, MLN, PP) no noticeable differences were observed. Taken together, these results indicate that both $CD4^+$ T cells and $CD19^+$ B cells can be a source of

colonization-induced IL-10. Moreover, the highest increase in IL-10-secreting cells was found in cLP and BM.

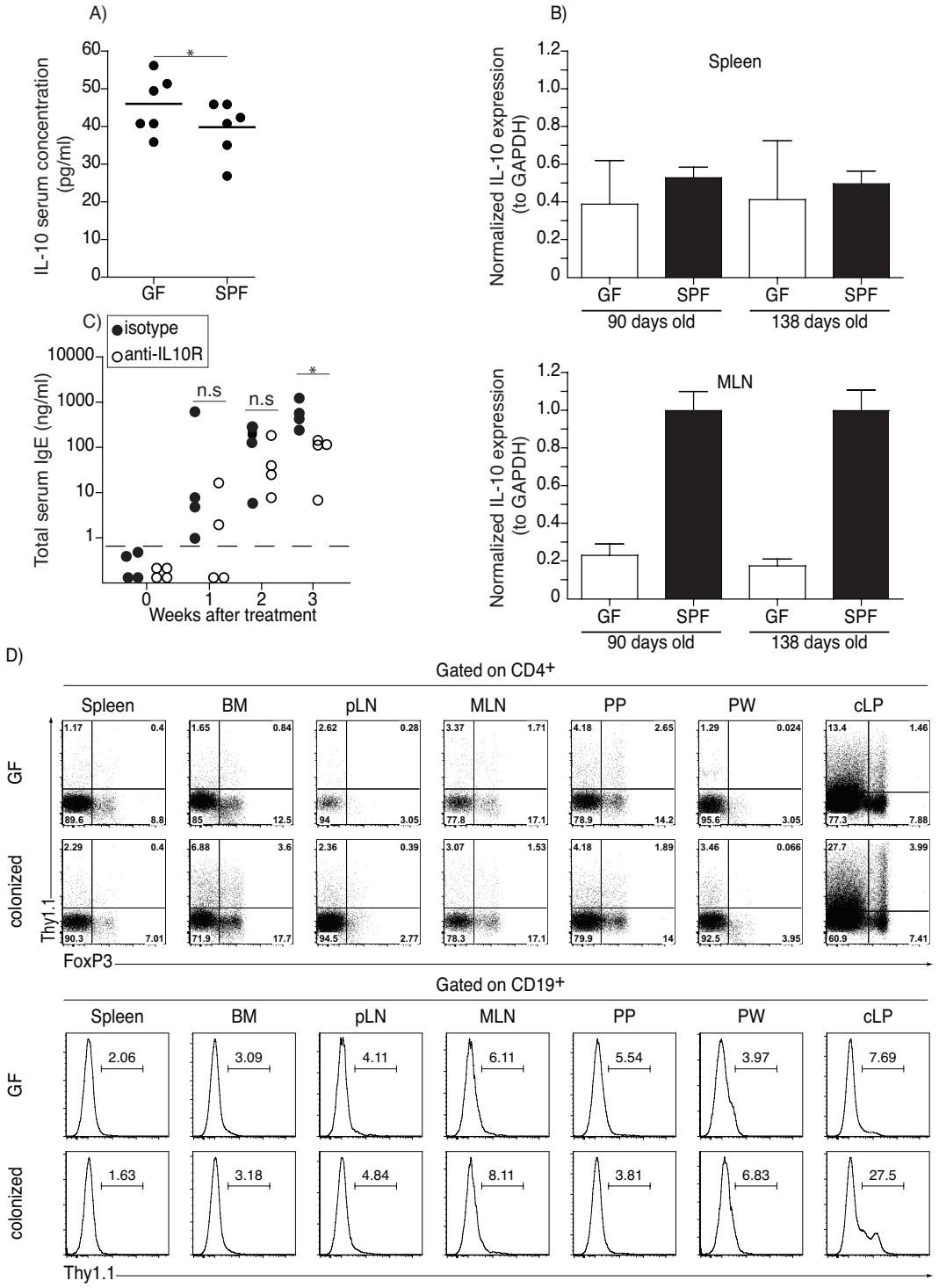

A)
IL-10 serum concentration (pg/ml)
GF
SPF
B)
Spleen
Normalized IL-10 expression (to GAPDH)
90 days old
138 days old
MLN
C)
isotype
anti-IL10R
Total serum IgE (ng/ml)
n.s
Weeks after treatment
D)
Gated on CD4+
Spleen
BM
pLN
MLN
PP
PW
cLP
colonized
Thy1.1
FoxP3
Gated on CD19+

Figure 11. IL-10 expression is altered in germ-free compared to bacterial-colonized mice. (A) IL-10 serum concentration was measured by CBA assay in C57BL/6 mice kept under GF or SPF conditions. Each point indicates a single animal and the horizontal line represents the mean of each data set. (B) Normalized expression of IL-10. qPCR was performed on total RNA isolated from MACS-enriched $CD19^-$ cells from spleen (top panel) or MLN (bottom panel). Expression was normalized to *GAPDH* and the sample with highest expression level set to 1. GF expression represents the mean of 8-11 (90 days-old) or 6-8 (138 days-old) individual animals ± SD and SPF expression represents the mean ± SD of technical repeats. (C) Newly weaned (25 days old) C57BL/6 germ-free were i.p injected with an anti-IL-10R blocking mAb (clone IBI-2) or isotype-matched control (clone 35.61) twice a week (500 μg/injection) and IgE levels were monitored. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal. (D) Lymphocytes from the indicated organs from germ-free and colonized (28 days) 10BiT mice were analyzed by flow cytometry. In the upper panel live lymphocytes were gated on $CD4^+$ cells and analyzed for IL-10 (Thy1.1) and Foxp3 expression. Numbers in quadrants represent cell frequencies. In the lower panel IL-10 (Thy1.1) expression on $CD19^+$ cells is shown. The marker indicates the percentage of $CD19^+$ cells expressing Thy1.1. Statistical significance was determined with the unpaired two-tailed t-test (n.s: not significant, *P<0.05).

2.8 Regulatory T cells may control early IgE induction

We have shown that bacterial colonization shapes $CD4^+$ T cells with increased IL-10 production especially in the cLP from both $FoxP3^+$ and $FoxP3^-$ subsets (Figure 11D). We therefore hypothesized that mucosal $CD4^+$ T cells from germ-free mice may be unable to inhibit IgE switch due to low numbers and/or functional defects. To test this, MACS-enriched T cell subsets from spleen or cLP from C57BL/6 ASF mice were adoptively transferred into 28-day old germ-free recipients and total serum IgE levels monitored. The following T cell subsets (shown in Figure 12A) were transferred into germ-free hosts: total splenic $CD4^+$ (n=4, 5×10^6), splenic $CD4^+CD25^-$ (n=4, 5×10^6), splenic $CD4^+CD25^+$ (n=4, 1×10^6), cLP $CD4^+CD25^-$ (n=3, 1×10^6), or cLP $CD4^+CD25^+$ (n=2, 2×10^5). As expected, IgE levels in untreated control germ-free mice increased at young age and at 49 days of age all the mice had detectable IgE (Figure 12B). Regardless of the subsets, increased $CD4^+$ T cell numbers did not prevent CSR to IgE (Figure 12B). Interestingly in mice transferred with splenic $CD4^+CD25^+$ or cLP $CD4^+CD25^+$ T cells CSR to IgE was delayed with IgE levels maintained under the limit of detection until days 42 (Figure 12B).

Whether bacterial exposed $CD4^+CD25^+$ T cells explain the observed delayed IgE induction is unknown and the experiment is currently being repeated as this thesis is written. In this experiment, Tregs from the cLP from germ-free (natural Tregs), ASF (induced Tregs) or Smarta1 mice (natural Tregs with irrelevant specificity) were adoptively transferred into 28-day old C57BL/6 germ-free hosts.

2.9 Natural IgE switch requires signaling through adaptor molecules MyD88 and/or TRIF

Despite the fact that germ-free mice are devoid of microbes, they are still exposed to antigens and microbial products contained in the autoclaved chow and bedding. For instance, germ-free mice are exposed to LPS that is contained in the chow (Hrncir et al., 2008). It is possible that exposure to microbial products is required for IgE CSR. The family of TLR relays signals to induce innate immunity after sensing an array of conserved motifs shared by many pathogens, called PAMPs (O'Neill and Bowie, 2007). Most TLRs signal through the adaptor molecule MyD88, with the exception of TLR3, which signals through the adaptor molecule TIR-domain-containing adapter-inducing interferon-β (TRIF). In addition, TLR4 can signal through either MyD88 or TRIF. However, MyD88 is not only involved in signaling in recognition of PAMPs but is also a downstream adaptor protein in IL-1, IL-18 and IL-33 signaling (Barksby et al., 2007; O'Neill and Bowie, 2007). Therefore the double knockout mouse $MyD88^{-/-}$ $TRIF^{-/-}$ is unable to signal through TLRs, IL-1R, IL-18R and IL-33R. IL-1 cytokines play an important role in immune regulation and inflammation (Dinarello, 2002) and IL-18 induces neutrophil migration and activation in addition to Th1 responses (Gracie et al., 2003). In contrast, IL-33 has been shown to be involved in Th2 polarization (Schmitz et al., 2005). We therefore rederived $MyD88^{-/-}TRIF^{-/-}$ (C57BL/6 background) mice to germ-free free status to evaluate if early natural IgE was dependent on MyD88

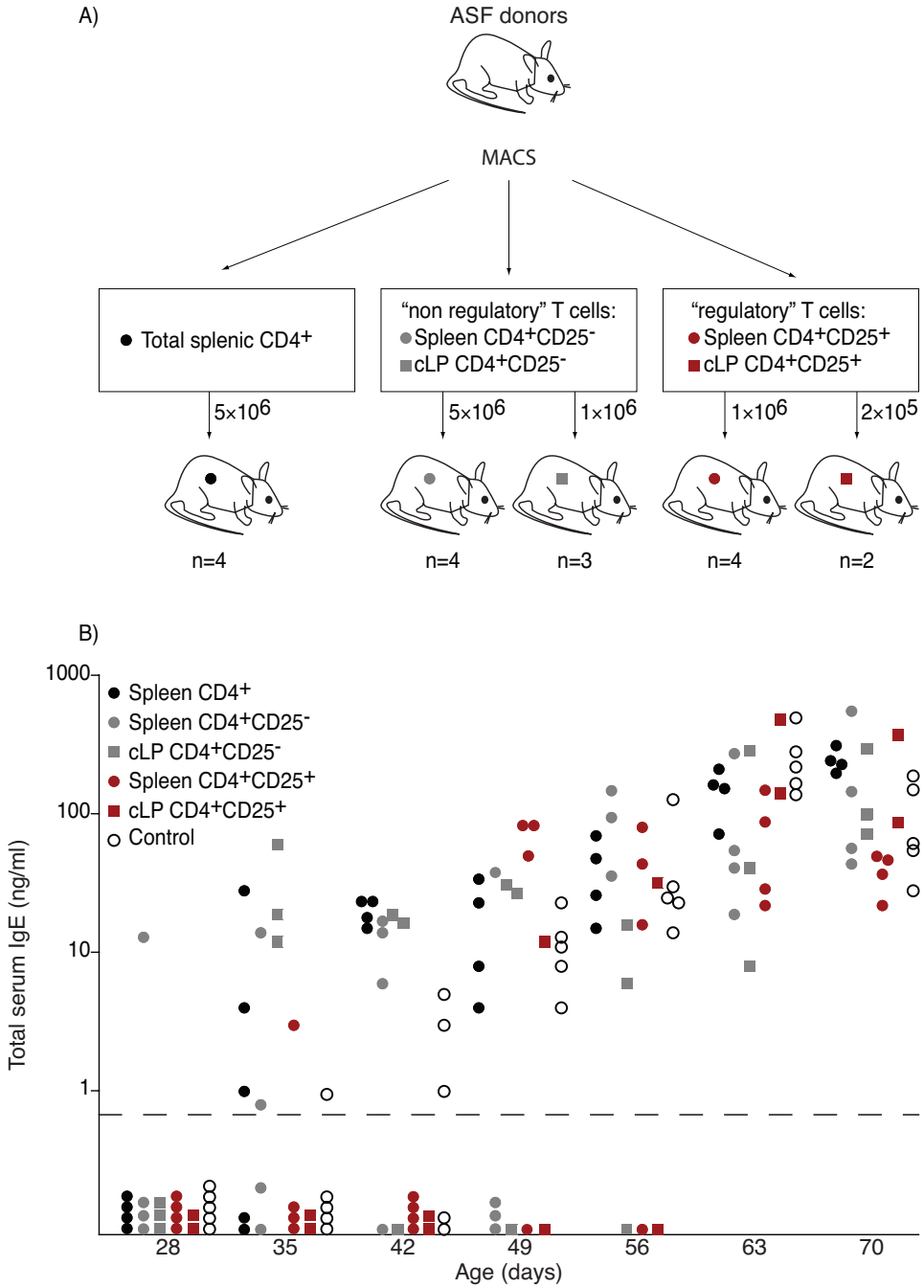


Figure 12. Adoptive transfer of regulatory CD4$^+$CD25$^+$ T cells from ASF mice may delay IgE induction in germ-free hosts. (A) Total splenic CD4$^+$ T cells, non-regulatory CD4$^+$CD25$^-$ T cells or regulatory CD4$^+$CD25$^+$ T cells from spleen or cLP from ASF donors were adoptively transferred into 28-day old germ-free C57BL/6 recipients as depicted. (B) Total serum IgE levels were measured in germ-free T cell recipients and controls at the indicated ages. The dotted line indicates the lower limit of detection (0.8 ng/ml) and each symbol a single animal.

and/or TRIF signaling. As shown in Figure 13, the deletion of MyD88 and TRIF adaptor proteins abrogated the elevated levels of natural IgE induction observed early in life. However at very old ages (>250 days old), IgE concentrations became elevated. From this we conclude that the early induction of natural IgE is dependent on MyD88 and/or TRIF signaling but at very old ages IgE is induced in a MyD88- and/or TRIF-independent manner.

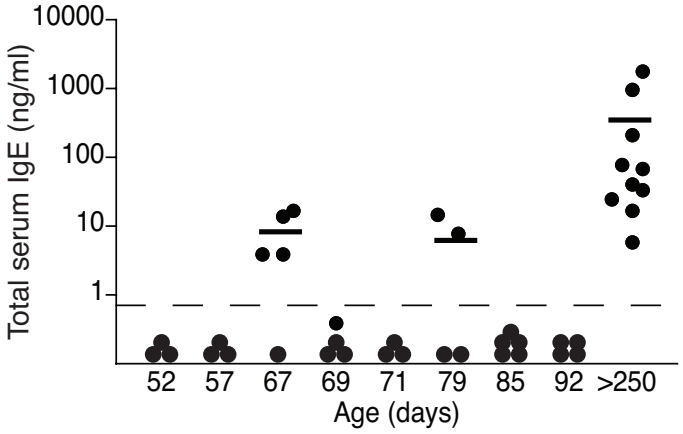


Figure 13. Early IgE is dependent on MyD88 and/or TRIF signaling. Total serum IgE titers of germ-free MyD88$^{-/-}$TRIF$^{-/-}$ (C57BL/6 background) were measured at the indicated ages. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set.

Elevated IgE levels in antigen-free mice (Figure 5B) suggest that microbial products in the food or bedding, which would signal through TLR and require MyD88, are not required for hygiene-induced IgE. This therefore suggests that signaling through IL-1R, IL-18R, or IL-33R may be crucial for hygiene-mediated IgE.

2.10 B-1a cell frequency in peritoneal cavities and expression of CD23 (FcεRII) on B cells are modulated with hygiene status

Microbial exposure is known to mature the immune system in many aspects (Macpherson and Harris, 2004). Yet a careful phenotypic cellular characterization (cell subsets frequencies, maturation and activation

status) between mice housed under different hygiene status has not yet been reported. This is of particular importance for understanding the mechanisms leading to the observed immune dysregulation.

In a first experiment we determined the proportions of B-1 cell populations in six-nine week old germ-free (n=2), ASF (n=2) and SPF (n=2) C57BL/6 mice. B-1 cells are the main source of natural antibodies and are key players in the maintenance of immune homeostasis and the defense against mucosal pathogens (Baumgarth, 2010). B-1 cells differ from the classical B-2 cells in that they are long-lived, self-renewing and polyspecific. Most of the B cells in neonates are thought to be of the B-1 subset (Hardy and Hayakawa, 1991). B-1 cells were identified by flow cytometry with a combination of surface markers having the phenotype $CD19^{+}CD3^{-}CD23^{-}IgM^{+}CD5^{\pm}$ with $CD5^{+}$ representing the B-1a cells and $CD5^{-}$ the B-1b cells. An example of the gating strategy is depicted in Figure 14A. When comparing the proportions of B cells expressing the B-1a cell marker CD5, no major differences were noticed between hygiene status in spleen, pLN, MLN and PP (Figure 14B). The same was observed for B cells negative for the CD5 surface molecule (Figure 14B). However in the PW, the compartment where the B-1 cells are most abundant (typically 35-70% of $CD19^{+}$ B cells compared to at the most 2% in other body compartments (Baumgarth, 2010)), CD5 was expressed on a larger proportion of B cells in SPF compared to germ-free and ASF mice (GF and ASF: 22.3 ± 2.5%; SPF: 44 ± 5.5%) (Figure 14B far right panel). Almost the entire CD5-expressing B cell population in the PW was negative for CD23 and thus confirmed to be B-1a cells (Figure 14C far right column). Therefore in the peritoneal cavities, SPF animals have a higher proportion of B-1a cells compared to GF and ASF animals.

We next analyzed the expression of CD23 on $CD19^{+}CD5^{+}$ and $CD19^{+}CD5^{-}$ B cells. Depicted in Figure 14C is the CD5-expressing subset

of B cells and we noted that the CD23-negative/low proportion was higher in the MLN and PP of SPF mice compared to GF and ASF mice (MLN in GF and ASF: 45.9 ± 5.7%; SPF: 87.3 ± 3.6% - PP in GF and ASF: 63.9 ± 4%; SPF: 88.8 ± 0.3%). The differences were not as obvious in the extra-mucosal sites (spleen, pLN, and PW). Accordingly the mean fluorescence intensity (MFI) of CD23 on $CD19^{+}CD5^{+}$ was reduced in the MLN and PP of SPF compared to GF and SPF mice (Figure 14E). Similarly, in the CD5-negative B cell subset, expression of surface CD23 was markedly reduced in SPF mice compared to GF and ASF mice and this observed across all analyzed cellular compartments (spleen, pLN, MLN, PP, PW) (Figure 14D). As expected, the MFI of CD23 on $CD19^{+}CD5^{-}$ was lower in SPF mice compared to GF and ASF in all lymphoid sites (Figure 14F). Importantly $CD19^{+}CD5^{-}CD23^{-}$ expressing cells outside of the peritoneal cavity can be B-1b cells, transitional-1 B cells developing into follicular B cells or marginal zone B cells from the spleen. In addition to the surface markers $CD19^{+}CD5^{-}CD23^{-}$, transitional-1 B cells can be identified with the AA4.1 marker (Cambier et al., 2007) and marginal zone B cells with the high expression of CD1d and CD21 (Pillai and Cariappa, 2009). Because we did not include these markers in this experiment we cannot conclude if the low CD23 expression on $CD19^{+}CD5^{-}$ cells could be explained by a selected reduced proportion in B cell subsets.

To conclude we observed a lower proportion of peritoneal B-1a cells in GF and ASF compared to SPF mice. Moreover CD23 expression on B cells may be regulated by microbial stimuli: in SPF mice, CD23 expression was markedly reduced in mucosal lymphoid tissues in $CD5^{+}$ B cells and in all lymphoid tissues in $CD5^{-}$ B cells.

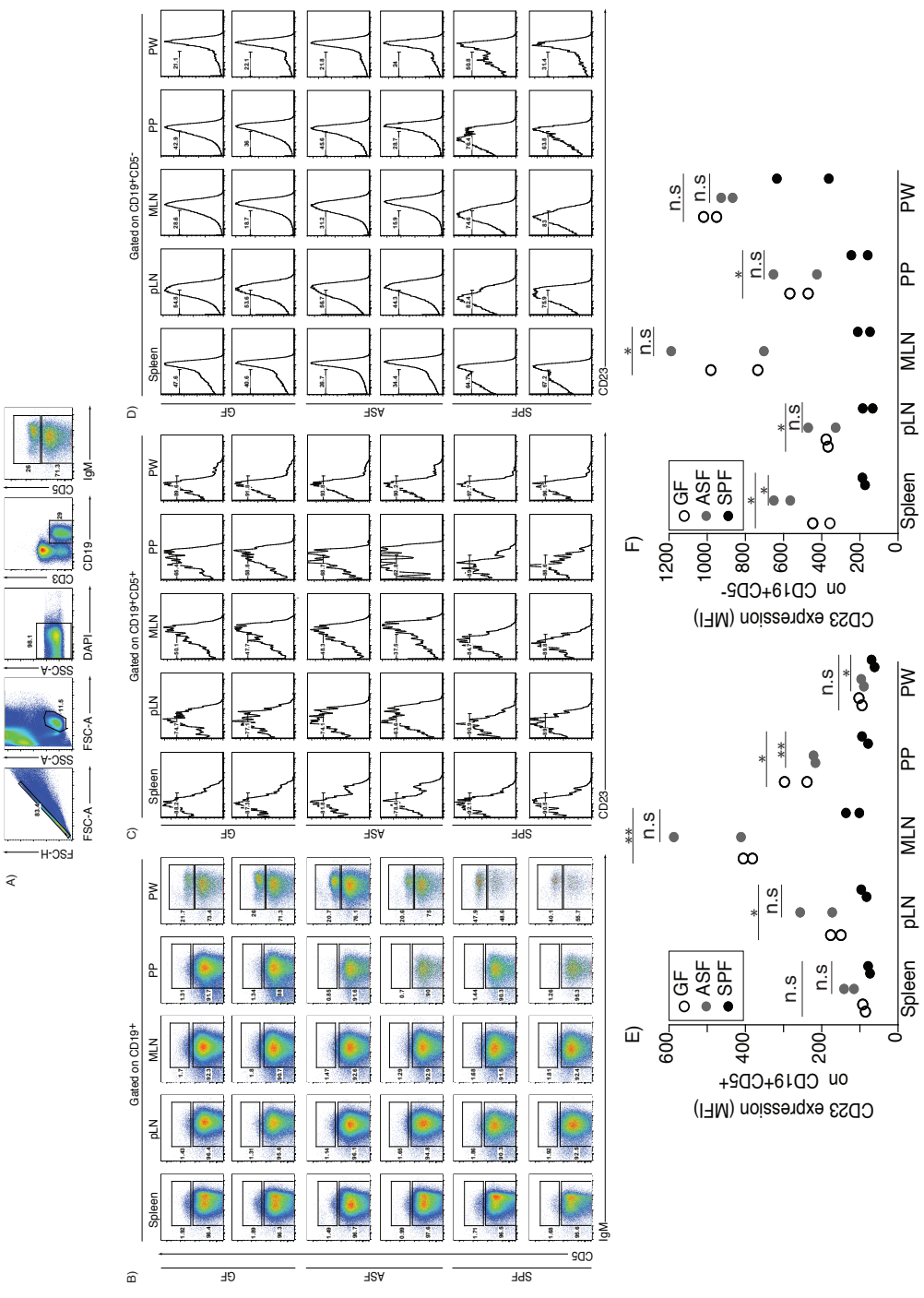

A)
FSC-H
FSC-A
SSC-A
FSC-A
SSC-A
DAPI
CD3
CD19
CD5
IgM
B)
Gated on CD19+
Spleen
pLN
MLN
PP
PW
GF
ASF
SPF
CD5
IgM
C)
Gated on CD19+CD5+
CD23
D)
Gated on CD19+CD5-
E)
CD23 expression (MFI)
on CD19+CD5+
GF
ASF
SPF
n.s
F)
CD23 expression (MFI)
on CD19+CD5-

Figure 14. Phenotypic characterization of $CD5^+$ and $CD5^-$ $CD19^+$ B cells in GF, ASF and SPF mice. Lymphocytes from the indicated compartments from GF, ASF, and SPF mice (as indicated) were analyzed by flow cytometry. The gating scheme used for all plots is shown in (A). Duplets were excluded by gating on single cells (FSC-A to FSC-H), lymphocytes were gated based on size and granularity (FSC-A to SSC-A) and dead cells were excluded by gating on the $DAPI^-$ population. The resulting live lymphocyte population was then subgated on the $CD3^-CD19^+$ B cell population and expression of IgM and CD5 shown in (B). The percentages of $CD5^+IgM^+$ and $CD5^-IgM^+$ B cells are indicated next to each gate. CD23 expression is shown on gated $CD19^+CD5^+$ (C) and $CD19^+CD5^-$ B cells (D). The markers indicate the percentage of $CD19^+IgM^+CD5^+$ or $CD19^+IgM^+CD5^-$ B cells that are negative for CD23 expression. Each plot represents a single animal. (E-F) MFI of CD23 on (E) $CD19^+CD5^+$ and (F) $CD19^+CD5^-$ cell subsets of germ-free (o), ASF (●) and SPF (●) animals, as indicated. Each point indicates a single animal. Statistical significance was determined with the unpaired two-tailed t-test (n.s: not significant, **P<0.01, *P<0.05).

2.11 B cell maturation and activation markers are not majorly altered by different hygiene statuses

To further phenotypically characterize B cells under different housing conditions (GF, ASF and SPF), we investigated the maturation and/or activation status of conventional bone-marrow-derived B2 cells in systemic and mucosal lymphoid tissues (spleen, pLN, MLN and PP) of six-nine week-old germ-free (n=2), ASF (n=2) and SPF (n=2) C57BL/6 mice. Expression of a panel of classic costimulatory markers (CD40, CD80, CD86), MHC Class II, and TACI and BAFF-R, the receptors for the maturation and survival factors BAFF and APRIL (MacLennan and Vinuesa, 2002) were analyzed. Of interest, it has been reported that TACI is not expressed in neonates (Kanswal et al., 2008) and is involved in a novel pathway leading to extrafollicular antibody switch (He et al., 2010). A representative gating strategy for all subsequent analysis of B cells is shown in Figure 15A. The staining was validated on $CD19^+$-sorted B cells cultured overnight in the presence of CpG oligodeoxynucleotides (CpG ODN) (1 μg/ml), a potent B cell activator known to upregulate costimulatory molecules, TACI and BAFF-R (Figure 15B). In addition, cells stained for B cell markers but not the activation markers were used to set the appropriate gate (Figure 15B).

As expected, MHC Class II and CD40 were constitutively expressed on B cells regardless of the compartment or hygiene status (Figure 16). Although the B7 family members CD80 and CD86 are normally upregulated on B cells upon activating signals (Hathcock et al., 1994), the frequency of B cells expressing these molecules was comparable between GF, ASF and SPF animals in all the analyzed cellular compartments (Figure 17). In these young naïve mice, TACI and BAFF-R were expressed at very low levels on B cells in all three experimental groups (Figure 18). Collectively, these results suggest that there are no major changes in activation and/or maturation status of B cells in spleen, pLN, MLN and PP in young naïve mice as determined by the frequency of B cells expressing CD80, CD86, TACI and BAFF-R.

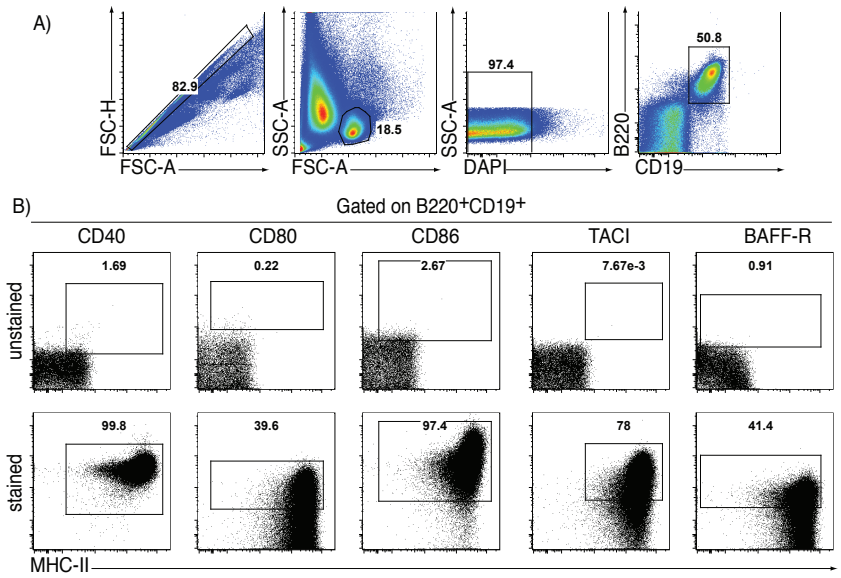


Figure 15. Gating strategy and validation of activation/maturation markers on CD19$^+$B220$^+$ B cells. (A) Gating scheme used to analyze activation/maturation markers on CD19$^+$B220$^+$ B cells. Duplets were excluded by gating on single cells (FSC-A to FSC-H), lymphocytes were gated based on size and granularity (FSC-A to SSC-A) and dead cells were excluded by gating on the DAPI$^-$ population. The resulting live lymphocyte population was then subgated on the CD19$^+$B220$^+$ B cell population. (B) MACS-enriched CD19$^+$ B cells were stimulated overnight with CpG ODN (1 μg/ml) to stimulate expression of MHC-II, CD40, CD80, CD86, TACI and BAFF-R and then analyzed by flow cytometry. Percentage of CD19$^+$B220$^+$ B cells expressing these surface markers is indicated next to each gate in unstained (top row) or stained (bottom row).

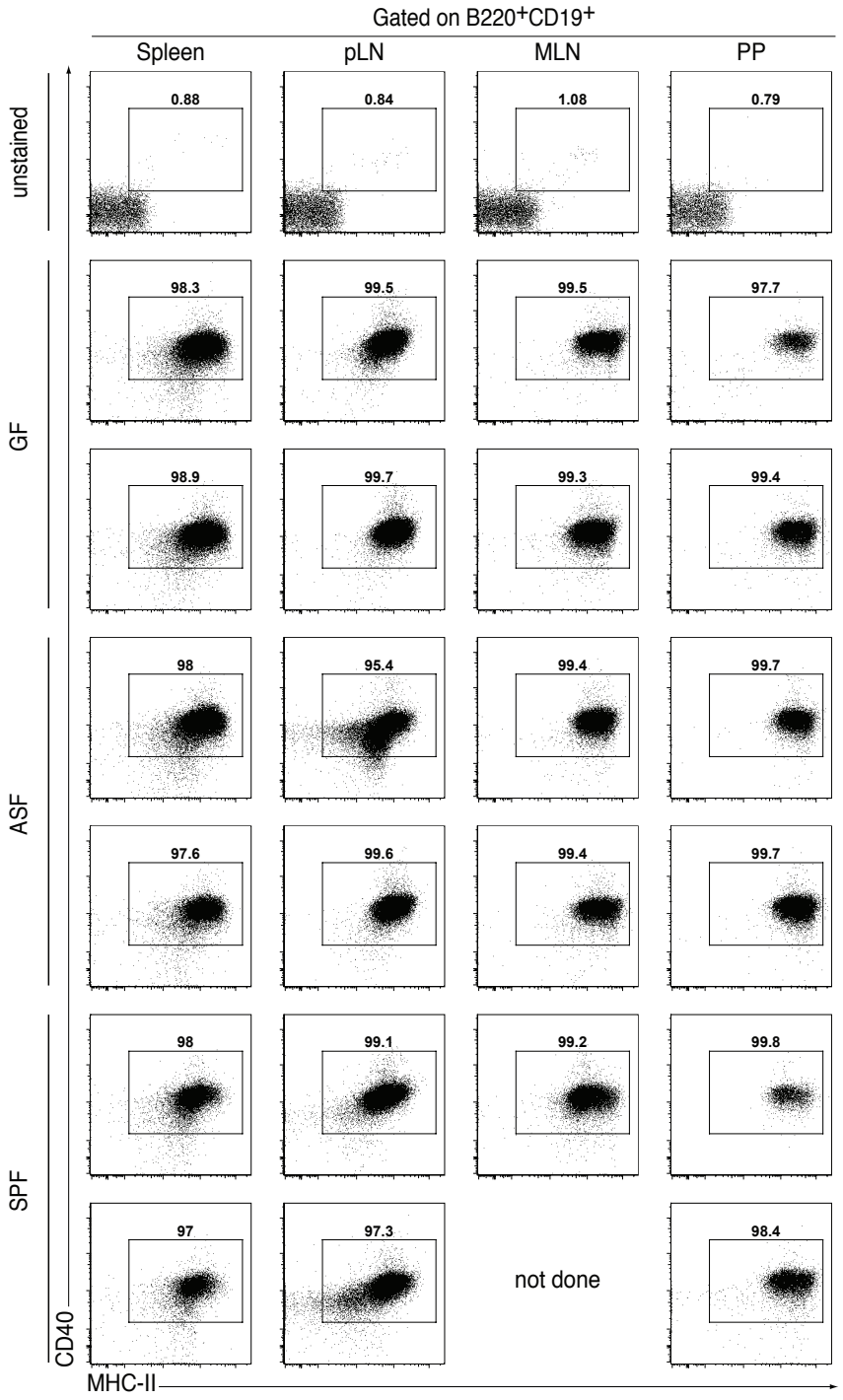


Figure 16. Phenotypic characterization of expression of CD40 and MHC-II on CD19$^+$B220$^+$ B cells in GF, ASF and SPF mice. Lymphocytes from the indicated organs from GF, ASF, and SPF mice (as indicated) were analyzed by flow cytometry. The gating scheme used for all plots is shown in **Figure 15**A. The percentage of MHC-II$^+$CD40$^+$ is indicated next to each gate. Each plot represents a single animal.

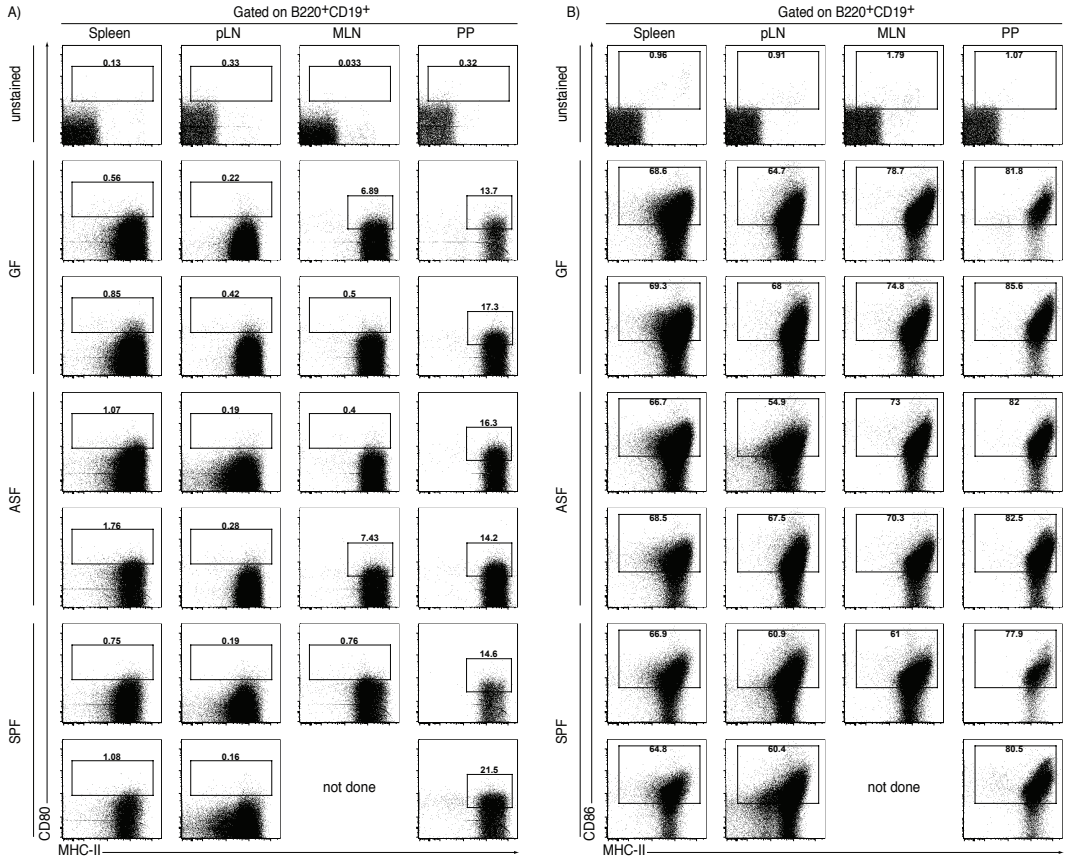


Figure 17. Phenotypic characterization of expression of CD80 and CD86 on CD19$^+$B220$^+$ B cells in GF, ASF and SPF mice. Lymphocytes from the indicated organs from GF, ASF, and SPF mice (as indicated) were analyzed by flow cytometry. The gating scheme used for all plots is shown in **Figure 15**A. The percentage of MHC-II$^+$CD80$^+$ and MHC-II$^+$CD86$^+$ is indicated next to each gate. Each plot represents a single animal.

2.12 Similar DC frequency and activation in spleen and pLN in GF, ASF and SPF animals

We next addressed whether the frequency and/or phenotype of DC were altered by different hygiene conditions. DC are professional APC that are critical in shaping B and T cell responses and are involved in classical IgE CSR (Geha et al., 2003). DC from spleen and pLN were characterized by flow cytometry in six-nine week-old germ-free (n=2), ASF (n=1) and SPF (n=2) C57BL/6 mice. DC were identified as CD11c$^+$F4/80$^-$ gated on live lymphocytes (Herbst et al., 2011) (Figure 19A-B). Different hygiene conditions did not contribute to gross differences in DC proportions in

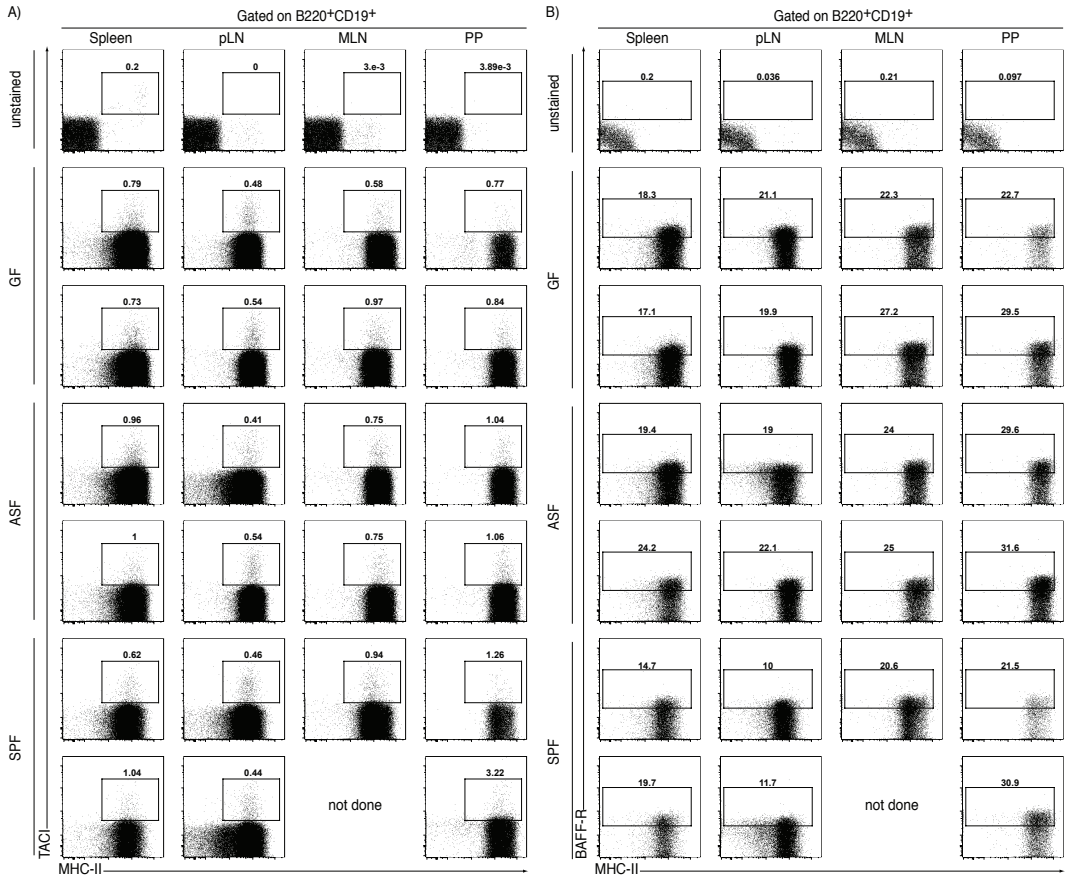


Figure 18. Phenotypic characterization of expression of TACI and BAFF-R on CD19$^+$B220$^+$ B cells in GF, ASF and SPF mice. Lymphocytes from the indicated organs from GF, ASF, and SPF mice (as indicated) were analyzed by flow cytometry. The gating scheme used for all plots is shown in **Figure 15**A. The percentage of MHC-II$^+$TACI$^+$ and MHC-II$^+$BAFF-R$^+$ is indicated next to each gate. Each plot represents a single animal.

either the spleen or pLN. Activation/maturation markers of DC were evaluated by analysis of surface expression of MHC Class II, CD40, CD86 and OX40-L expression. Under neutral conditions, engagement of OX40-L with OX40 expressed on naive CD4$^+$ T cells can drive Th2 polarization in the presence of IL-4 (Croft, 2010). We found no changes in expression of activation markers between GF, ASF and SPF in either spleen or pLN (Figure 19C). In summary, we conclude that bacterial diversity does not have a direct influence on the proportions and activation of DC in the peripheral organs spleen and pLN. However it is possible that bacterial

diversity shapes DC in the mucosal lymphoid tissues, which remains to be examined.

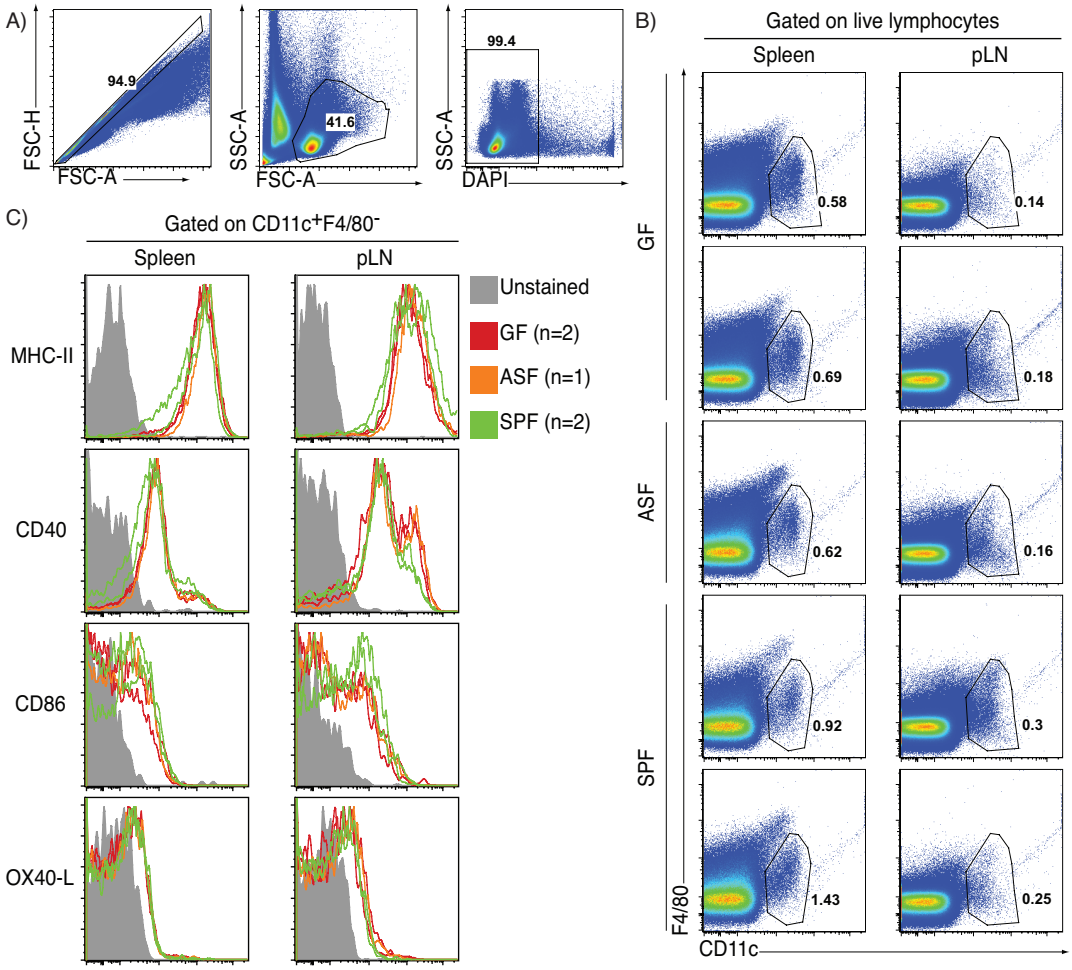


Figure 19. Phenotypic characterization of CD11c$^+$F4/80$^-$ DC in GF, ASF and SPF mice. Lymphocytes from the indicated organs from GF, ASF, and SPF mice (as indicated) were analyzed by flow cytometry. The gating scheme used for all plots is shown in (A). Duplets were excluded by gating on single cells (FSC-A to FSC-H), lymphocytes were gated based on size and granularity (FSC-A to SSC-A) and dead cells were excluded by gating on the DAPI$^-$ population. (B) The resulting live lymphocyte population was then subgated on the CD11c$^+$F4/80$^-$ DC population. The percentage of CD11c$^+$F4/80$^-$ is indicated next to each gate. (C) MHC-II, CD40, CD86 and OX40L expression is shown on gated CD11c$^+$F4/80$^-$. Each plot represents a single animal.

2.13 Natural IgE induction may be independent of TCR and BCR specificity

Given that limited TCR and BCR diversity has been correlated with immune dysregulation, we investigated the role of the specificity of T- and BCR in IgE induction (Liston et al., 2008; Milner et al., 2007a). Smarta1 (C57BL/6 background) mice express a transgenic TCR with >95% of $CD4^+$ T cells expressing a TCR specific for a Lymphocytic choriomeningitis virus (LCMV) glycoprotein (Oxenius et al., 1998) and therefore provide a good model of restricted TCR repertoire. Germ-free Smarta1 TCR transgenic mice expressing a transgenic TCR of irrelevant specificity developed high IgE levels, similar to C57BL/6 wild-type mice (Figure 20). This result suggests that IgE switch is not impaired with a restricted TCR repertoire diversity and since endogenous $CD4^+$ T cells represent a small proportion in Smarta1 mice, it is tempting to think that TCR specificity may be irrelevant for the induction of IgE.

VI-10 mice are heavy chain BCR knock-in mice expressing the vesicular stomatitis virus (VSV)-neutralizing BCR formed by the heavy chain transgene and endogenous light chains. The knocked-in heavy chain gene pairs with endogenous light chains, which can form BCRs with other specificities than VSV. Nevertheless BCR diversity is reduced in this mouse strain with 10-15% of total B cells expressing a BCR with high affinity for VSV (Hangartner et al., 2003). This restriction of BCR repertoire did not hamper IgE induction, as shown in Figure 20. Thus, despite a restricted BCR repertoire diversity, B cells still undergo CSR to IgE under germ-free conditions. To confirm that BCR specificity is irrelevant, VI-10 mice could be crossed to a transgenic mouse expressing a VSV-specific light chain, called YEN. More than 95% of B cells have been shown to be specific for VSV in VI-10×YEN mice (Hangartner et al., 2003).

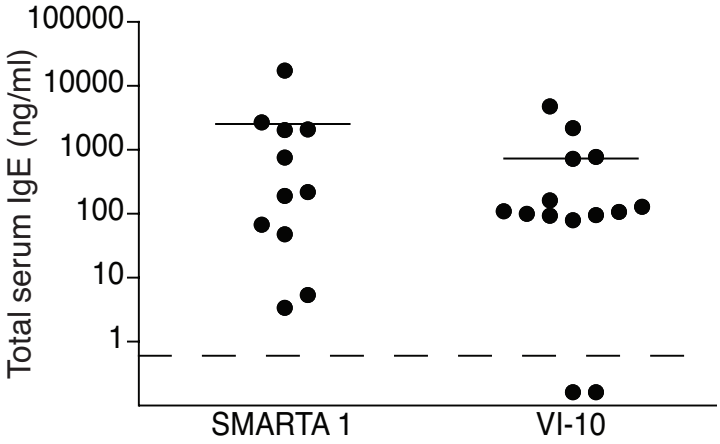


Figure 20. TCR transgenic Smarta1 and BCR knock-in VI-10 germ-free mice develop high IgE levels similar to C57BL/6 wild-type mice. Serum IgE levels were measured in germ-free adult Smarta1 and VI-10 mice. The dotted line indicates the lower limit of detection (0.8 ng/ml). Each point indicates a single animal and the horizontal line represents the mean of each data set.

2.14 Both the germ-free environment and the genetic background contribute to the induction of natural IgE

Up to this point, natural IgE induction in germ-free mice was studied in the inbred mouse strain C57BL/6. Because a tight interplay between environment and genes dictates the outcome of immune dysregulations, we determined IgE levels in germ-free mouse strains with different genetic backgrounds. Whereas, similarly to C57BL/6, BALB/c and NIH-Swiss had elevated IgE, NMRI and Swiss Webster mice had undetectable or very low IgE (Figure 21A). Given that IgE induction appears to have a genetic component, C57BL/6 with high IgE were paired with Swiss Webster with undetectable IgE and serum IgE of the progeny was evaluated (Figure 21B). As shown previously (Figure 21A), Swiss Webster mice were confirmed to have no IgE induction. In contrast, C57BL/6×Swiss Webster F1 progeny developed natural IgE, although this was not consistently observed in all animals. Taken together these observations are indicative of an influence of the genetic background in driving early IgE switch in germ-free mice.

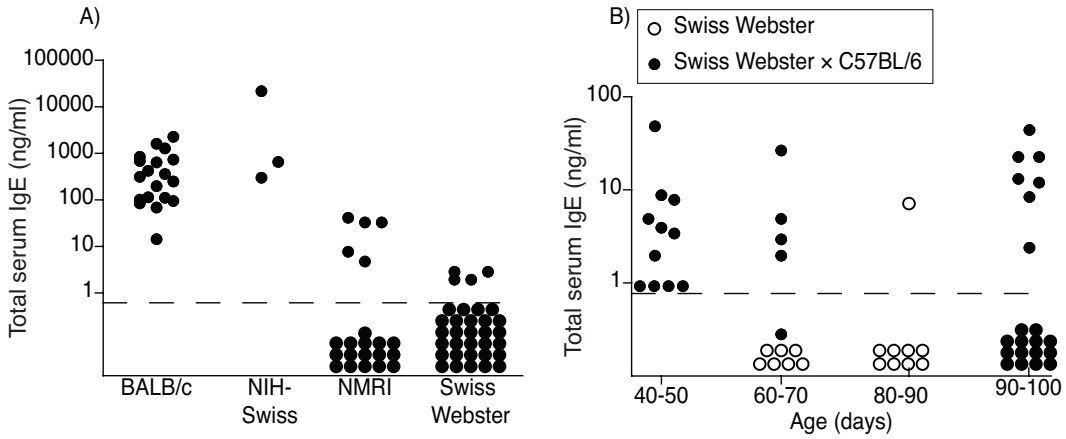


Figure 21. Genetic background is critical for induction of IgE in a germ-free environment. (A) Serum IgE levels were measured in the indicated adult mouse strains. BALB/c is an inbred strain whereas NIH-Swiss, NMRI and Swiss Webster are outbred strains. (B) C57BL/6 (high IgE) and Swiss Webster (undetectable IgE) were paired and IgE measured in the progeny at the indicated ages. The dotted line indicates the lower limit of detection (0.8 ng/ml) and each point indicates a single animal.

2.15 Summary

Soon after birth, microbes (such as the ones contained within the SPF flora) colonize the intestine and provide immunoregulatory stimuli that prevent CSR to IgE. In germ-free (and some ASF) C57BL/6 mice lacking such a threshold stimulus baseline IgE levels become abnormally elevated. Thus, beyond the well-known effects on the maturation of the immune system, the composition of indigenous intestinal bacteria molds baseline immune regulation. In germ-free C57BL/6 mice CSR to IgE was induced early in post-natal life at mucosal sites (PP and MLN) in a process that was independent of food-derived antigens and maternal antibodies. Natural IgE was T cell- and MyD88-TRIF-dependent but TSLP-independent. IL-4 may also be required, at least partially, in induction of hygiene-induced IgE. In addition, low concentrations of IL-10 at mucosal sites might favor CSR to IgE.

Investigating the complex crosstalk between indigenous microbial composition and immune regulation will be critical towards understanding the increasing rates of immune dysregulation in westernized countries.

3 Results II - Systemic exposure with the intestinal commensal *Enterobacter cloacae* modulates diabetes incidence

Insulin-dependent diabetes mellitus, also known as T1D, is an organ-specific autoimmune disease that involves specific and targeted destruction of insulin-producing β-cells in the pancreatic islets of Langerhans. It is thought to be a Th1-mediated disease that involves $CD8^+$ T cells and innate immune cells. The direct consequences of the disease are lymphocytic infiltrates in the pancreatic islets (insulitis) and resulting hyperglycemia but chronic lack of insulin can cause diabetes-associated complications in several organs (Atkinson and Maclaren, 1994; Castaño and Eisenbarth, 1990). Diabetes was lethal until the 1920s, when Banting and Best identified insulin as the pancreatic hormone maintaining blood glucose homeostasis (Banting et al., 1922). Diabetes is a multifactorial disease with both genetic and environmental factors regulating disease onset (Lehuen et al., 2010). Studies conducted in twins, families and animal models helped to elucidate genetic susceptibilities and identified a vast number of genetic loci contributing to disease, such as MHC Class II and insulin (Redondo et al., 2001). The role of environmental factors is exemplified by the observation that 90% of T1D patients have no affected relatives (Bingley et al., 1993) or the 40-60% concordance rate for diabetes onset in identical twins (Kyvik et al., 1995; Redondo et al., 2008). Furthermore, and in line with the hygiene hypothesis, the prevalence of T1D has dramatically increased in the last decades in industrialized countries (Bach, 2002).

Because this fast increasing rate of T1D can't be explained by genetic alteration, efforts to identify infectious agents that may precipitate or delay diabetes onset have been made. The NOD mouse (Adorini et al., 2002)

and the BB-DP rat (Allen and Braverman, 1996) spontaneously develop a disease reminiscent of T1D in humans, including the presence of islet antigen-specific CD4+ and $CD8^+$ T cells and genetic linkage to disease. Whereas in BB-DP rats there is no sex-difference in diabetes incidence, female NOD mice have a higher incidence of diabetes than males. Studies conducted in these diabetes-prone animals revealed that infectious agents such as viruses, bacteria or parasites, could either accelerate (Table 1) or delay (Table 2) onset of diabetes.

Table 1. Systemic viral infections in NOD mice associated with an acceleration of onset of diabetes

Infectious agent	References
Endogenous retrovirus	(Gaskins et al., 1992; Nakagawa et al., 1992; Suenaga and Yoon, 1988)
Coxsackievirus	(Horwitz et al., 1998; Serreze et al., 2000)
Rotavirus	(Graham et al., 2008)

As shown in Table 1, viruses can accelerate onset of T1D and mechanisms involved are thought to include molecular mimicry, induction of bystander damage of β-cells, release of sequestered antigens and alteration of the Treg:Teff cell ratio. In contrast, viruses have also been shown to have a protective role in T1D (Table 2). For example, rotavirus has been associated with protection (Graham et al., 2007) in young NOD mice, but with exacerbation when the mice are older (Graham et al., 2008). A similar dual role was reported for coxsackievirus (Horwitz et al., 1998; Serreze et al., 2000; Tracy et al., 2002). It is postulated that viral- induced protection from diabetes is associated with

Table 2. Systemic infectious agents in NOD mice associated with a delay or protection in onset of diabetes

Infectious agent	References
Bacteria	
Mycobacterium avium	(Brás and Aguas, 1996)
Salmonella typhimurium	(Zaccone et al., 2004)
Viruses	
Encephalomyocarditis virus-D	(Hermitte et al., 1990)
Coxsackievirus	(Filippi et al., 2009; Tracy et al., 2002)
Lactate dehydrogenase virus	(Takei et al., 1992)
LCMV	(Filippi et al., 2009; Oldstone, 1990)
Persistent murine hepatitis virus	(Wilberz et al., 1991)
Murine gammaherpesvirus	(Smith et al., 2007b)
Rotavirus	(Graham et al., 2007)
Helminths	
Schistosoma mansoni	(Cooke et al., 1999)
Trichinella spiralis	(Saunders et al., 2007)
Heligmosomoides polygyrus	(Saunders et al., 2007)
Litomosoides sigmodontis	(Hübner et al., 2009)

the induction of regulatory mechanisms, which control diabetogenic cells. Interestingly,pathogens such as bacteria and parasites are mostly preventive whereas viruses seem to have a dual role in the pathogenesis of diabetes.

Beyond the implications of the possible immunoregulatory roles of pathogens, indigenous microbiota have been shown to have a critical role in the progression towards diabetes. NOD mice deficient in MyD88 developed diabetes under germ-free conditions but were protected under SPF conditions, indicating that microbes protect from diabetes in a MyD88-independent manner in a poorly understood mechanism (Wen et al., 2008). The authors suggest that in the absence of MyD88, intestinal dysbiosis occurs, which may explain the protection observed under SPF conditions. However, work conducted in our laboratory showed that

C57BL/6 mice deficient in the TLR signaling molecules, MyD88 and TRIF, were systemically primed against intestinal microbes due to defective bacterial compartmentalization (Slack et al., 2009). Therefore, systemic priming, rather than dysbiosis, may explain the protective effects of MyD88 deficiency in SPF conditions. Moreover, it is well established that the hygiene status of NOD mice can explain differences in diabetes incidence across animal facilities with lowest incidence in conventional facilities (that may harbor pathogens) (Bach, 2002; Like et al., 1991; Wen et al., 2008). Collectively, these observations suggest that intestinal microbes can modulate diabetes incidence possibly by breaching systemic ignorance.

We thus hypothesized that systemic priming of microbes, even non-pathogenic such as commensal intestinal microbes, could delay onset of diabetes. To do so, we standardized the microflora in our colony of NOD mice by re-derivation to germ-free status and subsequent association to the ASF gnotobiotic microflora.

3.1 Systemic exposure to the commensal *Enterobacter cloacae* protects from onset of diabetes in young ASF-colonized NOD mice

Gnotobiotic NOD mice were devoid of *E. cloacae*, a commensal commonly present in SPF and conventional colonies. We therefore investigated the effects of repeated systemic exposure to *E. cloacae* in gnotobiotic NOD mice. To do so, 30-50 day-old female and male NOD mice were intravenously injected with either phosphate buffered saline (PBS) or 10^8 *E. cloacae* twice a week for a period of three weeks. As shown in Figure 22, the incidence of diabetes in female and male NOD mice was reduced upon systemic exposure to *E. cloacae*. Moreover diabetes incidence was robust over time as survival curves show the complied data collected over two years.

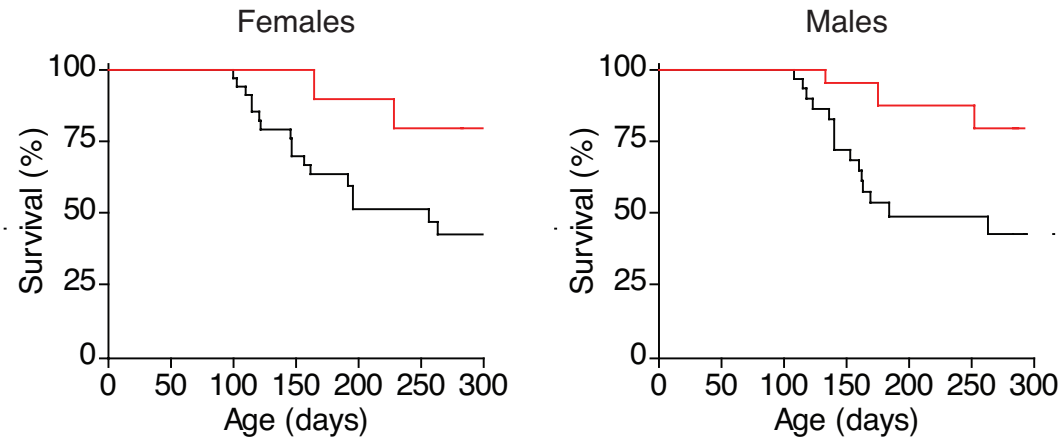


Figure 22. Systemic exposure with *E. cloacae* in gnotobiotic NOD mice protects from diabetes development. Female or male NOD mice (as indicated) were i.v injected twice a week for a period of three weeks with either PBS (black line; females n=35, males n=31) or 10^8 *E. cloacae* (red line; females n=10, males n=22). Blood glucose levels were monitored weekly and mice were considered diabetic when two successive blood glucose levels were >16 mmol/L.

3.2 Systemic exposure to the commensal *Enterobacter cloacae* partially protects from onset of diabetes in aged ASF-colonized NOD mice

Systemic administration of *E. cloacae* was correlated with strong protection from diabetes onset in NOD mice aged 30-50 days, when overt signs of disease are usually not observed in our colony. We thus investigated if there was a critical time window when systemic priming needed to occur to confer protection from diabetes. The cohort was expanded with female and male NOD mice aged 50-70, 70-100 and >100 days at the time of first injection. Systemic priming of older female mice was not as protective from onset of diabetes as seen in mice aged 30-50 days (Figure 23). Surprisingly however, priming of female NOD mice aged >100 days resulted in increased survival compared to mice aged 30-50 days. This may reflect a biased selection of naturally diabetes-resistant NOD mice because mice with overt diabetes would have been excluded from this experimental age group. Intriguingly, protection from diabetes was more robust in males compared to females even at older ages (Figure

23) suggesting that in this model bacterial immunoregulation may be sex-dependent.

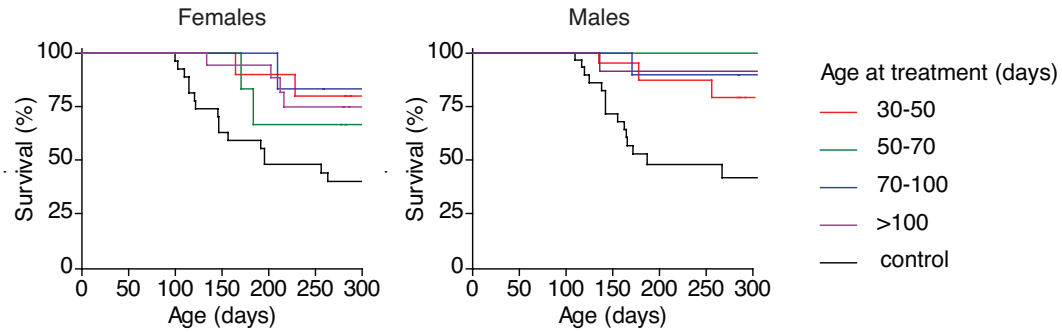


Figure 23. Systemic exposure with *E. cloacae* in older gnotobiotic NOD mice partially protects from diabetes development. Female or male NOD mice (as indicated) were i.v injected twice a week for a period of three weeks with either PBS (black line; females n=35, males n=31) or 10^8 *E. cloacae.* Mice primed with bacteria were either 30-50 (red line; females n=10, males n=22), 50-70 (green line; females n=9, males n=22), 70-100 (blue line; females n=13, males n=12) or >100 (purple line; females n=18, males n=12) days of age at the first injection. Blood glucose levels were monitored weekly and mice were considered diabetic when two successive blood glucose levels were >16 mmol/L.

3.3 Systemic exposure to *Enterobacter cloacae* breaks systemic ignorance

Intestinal microbes are tightly maintained within the mucosal compartment such that the systemic immune system remains mainly ignorant to them. Serum antibodies from immunocompetent mice born and raised SPF do not bind to indigenous intestinal microbes (Slack et al., 2009). We thus tested whether repeated systemic exposure to *E. cloacae* in NOD mice could break systemic ignorance and induce anti-bacterial antibodies in the serum. Binding of serum antibodies to *E. cloacae* was evaluated using live bacterial flow cytometry, a method developed and validated in our laboratory (Slack et al., 2009). Serum was collected one week following the last intravenous injection and binding of serum IgM and IgG2a to *E. cloacae* was compared between control and intravenous (i.v) primed NOD

mice (Figure 24A). As expected, control mice had no serum IgM or IgG2a binding to *E. cloacae.* In contrast, serum IgG2a and to a lesser extent IgM specifically bound to *E. cloacae* in i.v primed mice. To test if specific antibodies to bacteria were long-lived, serum was analyzed at 6 weeks and 12 weeks post-priming. All primed female and male NOD mice showed strong binding of serum IgG2b to *E. cloacae* even 12 weeks post-injection (Figure 24B). Moreover, antibodies did not bind to *Salmonella typhimurium,* a bacterium mice were previously not exposed to (Figure 24B). Therefore systemic exposure to bacteria induces a long-lived and specific antibody response.

3.4 Systemic exposure to *Enterobacter cloacae* protects from diabetes onset even in mice with insulitis

Progression to T1D is initiated with insulitis, a process whereby immune cells destroy the insulin-producing β-cells. Eventually this results in hyperglycemia recorded by blood glucose measurements above 16 mmol/L. Thus far, we have shown that systemic exposure to bacteria could protect from hyperglycemia. We next investigated the effects of bacterial priming on insulitis progression. Comparison of insulitis in untreated and systemically primed NOD mice was evaluated with immunohistochemistry of pancreatic sections from NOD mice. We first evaluated insulitis in NOD mice that were primed with *E. cloacae* at young age (30-50 days) and remained protected from diabetes. Sections from pancreata collected 21 weeks after systemic priming showed no lymphocytic infiltration in H&E (Figure 25A) and CD3 (Figure 25B) stains.

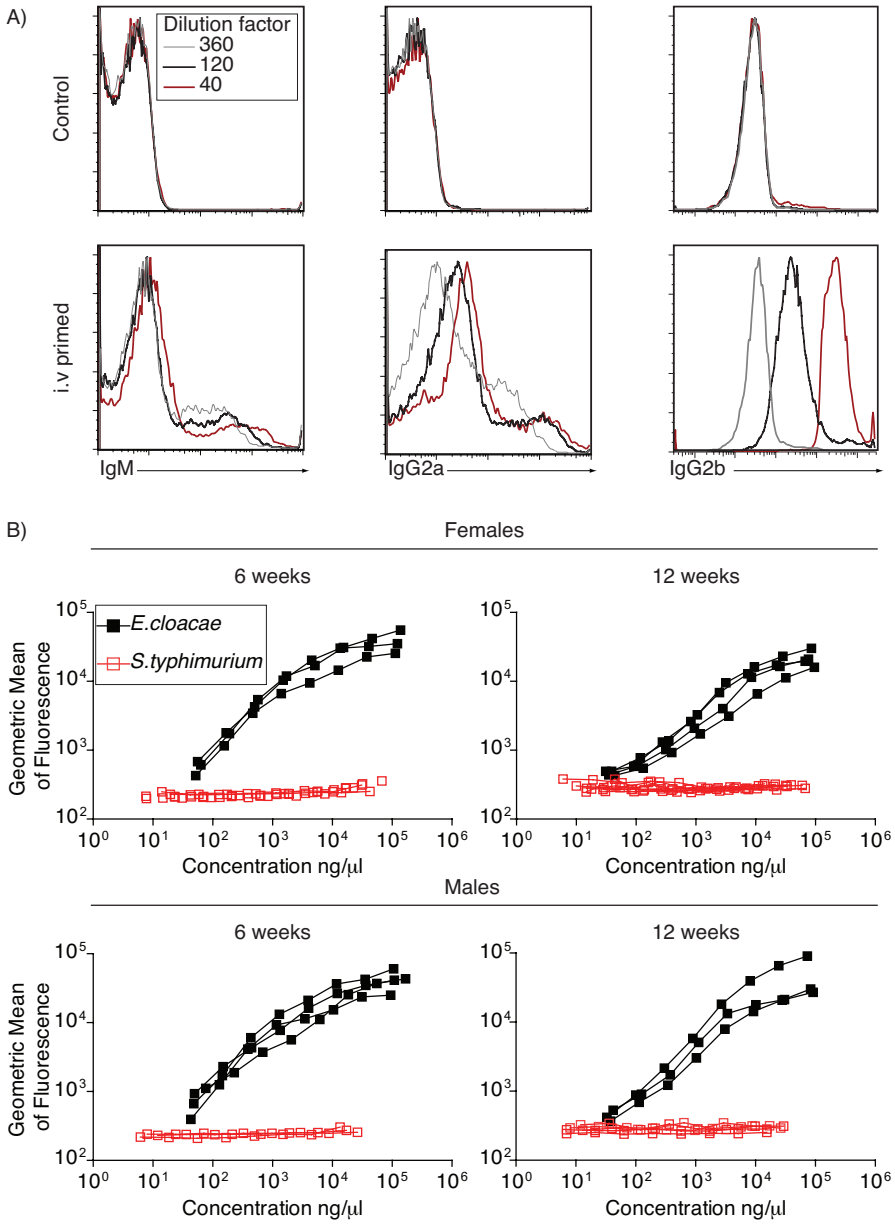
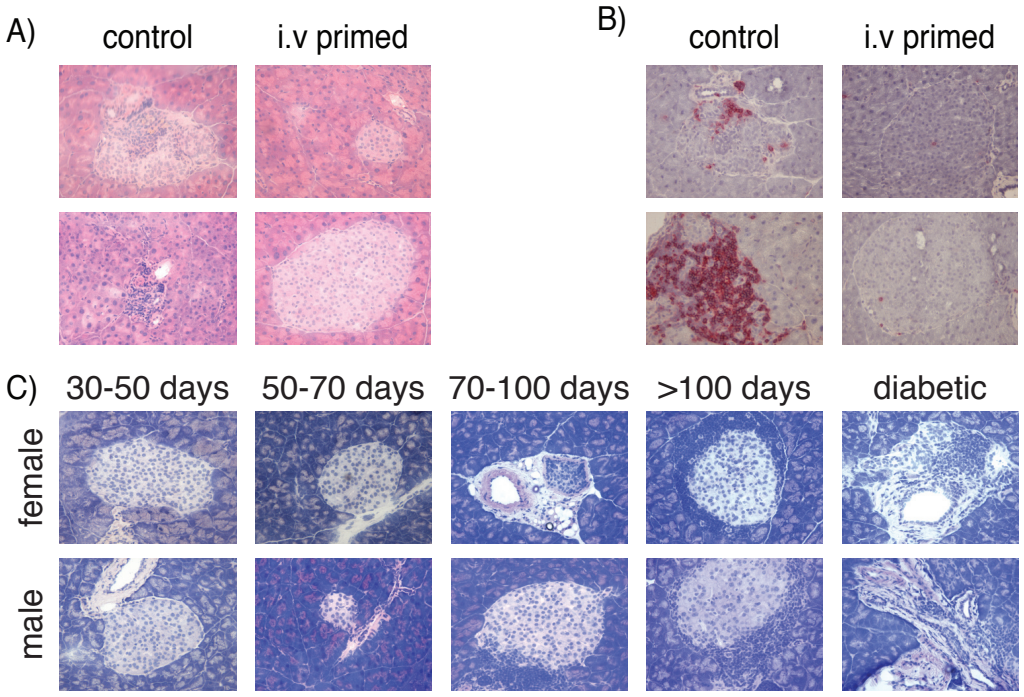


Figure 24. Systemic exposure with *E. cloacae* in gnotobiotic NOD mice induces specific serum antibodies. NOD mice were either left untreated or i.v injected with 10^8 *E. cloacae* twice a week for a period of three weeks. (A) Serum was collected one week following the last i.v injection, serially diluted (as indicated) and added to live *E. cloacae* prior to staining and detection of bound anti-mouse IgM, IgG2a and IgG2b by flow cytometry. Shown is one representative plot of fluorescence dilution out of 14 controls and out of 14 i.v primed female NOD mice. (B) Serum was collected 6 and 12 weeks following the last i.v injection and live *E. cloacae* (black) or *Salmonella typhimurium* (red) were added to serum as in (A) followed by detection of anti-mouse IgG2b. The mean fluorescence is plotted against total serum IgG2b as determined by ELISA. Each line represents one mouse.

As expected, untreated control counterparts developed hyperglycemia and hence showed lymphocytic infiltrates. Therefore systemic priming at a young age not only protected from hyperglycemia but also from insulitis. We next extended the analysis of insulitis on mice primed at older ages including 50-70, 70-100 and >100 days of age (Figure 25B). Pancreata were collected at 300 days of age or when mice became hyperglycemic and stained for H&E. Our previous observation that priming at young age (30-50 days) was correlated with protection form development of insulitis was reproduced in this experiment. However, the older the age at the time of systemic priming, the greater the insulitis, despite protection from hyperglycemia. Collectively these results suggest that systemic priming with *E. cloacae* can induce protection from diabetes even when inflammation in the islets was already initiated.



D)

	Degree of Insulitis (% of islets of each grade)									
	Grade 0		Grade 1		Grade 2		Grade 3		Grade 4	
Priming	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
70-100 d	26	25	10	18	8	42	5	1	51	14
>100 d	17	8	11	13	17	20	8	24	47	35
Control	2	9	17	20	30	19	7	17	44	35

Figure 25. Systemic exposure with *E. cloacae* in gnotobiotic NOD mice prevents insulitis in young mice or stops insulitis progression in older mice. NOD mice were i.v injected twice a week for a period of three weeks with 10^8 *E. cloacae* or left untreated. (A-B) Male NOD mice aged 30-50 days were primed or left untreated (as indicated), pancreata collected 21 weeks after and stained with H&E (A) or CD3 (B). Shown are representative sections from two controls and two i.v primed mice. (C-D) Female and male NOD mice were primed at the indicated ages. Pancreata were collected from all mice either when they became diabetic (controls) or at 300 days of age and stained with H&E (C). One representative section per age and sex is shown. (D) Average insulitis score from a minimum of 25 separate islets was determined by the Leiter scoring system.

3.5 Summary

Systemic exposure to *E. cloacae* starkly reduced diabetes incidence in ASF NOD mice even at older ages (>100 days old). In addition to protection of hyperglycemia, young ASF mice were protected from the development of insulitis and in older mice, insulitis progression was halted yet not reversed. Intravenous administration of *E. cloacae* induced specific antibodies in the serum, indicative of a breach in the systemic ignorance.

Understanding the underlying immune mechanisms whereby systemic exposure with a symbiont interferes with the development of diabetogenic responses will be instrumental towards elucidating the environment-diabetes crosstalk.

4 Discussion

It is well accepted that post-natal maturation of the immune system is largely dependent on microbes (Hooper and Macpherson, 2010). In this thesis I demonstrated a new dimension of the impact of intestinal symbionts on the immune system: they dictate baseline immune system regulation. I have shown this in experimental models with strictly defined microflora. In the first part I reported that the complete absence of symbionts in C57BL/6 mice led to abnormally elevated levels of baseline serum IgE, an immunoglobulin that is normally present at very low levels in a SPF environment. The presence of the restricted ASF microflora consisting of eight bacterial species dampened this effect although IgE levels remained considerably elevated in a proportion of mice. In a second part, the ability of exposure to commensal bacteria to impact on immune regulation was applied to a model of autoimmunity in the diabetes-prone NOD mouse. When NOD mice harboring the gnotobiotic ASF were systemically primed with the intestinal symbiont *E. cloacae*, disease incidence was drastically reduced.

4.1 Intestinal microbial composition imprints isotype fate decisions

IgE is mainly associated with atopic allergic diseases or parasite infections (Sutton and Gould, 1993) and thus is thought to have evolved to combat parasite infection without causing extensive self-damage (Allen and Maizels, 2011). We have previously demonstrated that IgE in germ-free mice were polyclonal and germ-line encoded with no evidence for somatic hypermutation, which strongly argues against atopy-mediated IgE (McCoy et al., 2006). We thus propose that an underlying immune dysregulation causes this striking phenotype. Indeed, immune dysregulation has been

associated with increased levels of IgE in patients (Appendix 1) and in mouse models (Appendix 2) with partial T cell immunodeficiency. Given that IgE switch is under strict control in normal healthy individuals and in mice harboring a SPF flora, elevated IgE in the absence of atopy and parasite infections is a reliable biomarker for underlying immune dysregulation. Understanding the interplay between intestinal bacterial composition and immune dysregulation is crucial in times when atopic allergic diseases and autoimmunity are dramatically rising in the westernized world (Okada et al., 2010). We thus aimed to elucidate the mechanism of elevated IgE in germ-free mice in order to identify which immune pathways are dysregulated in the absence of microbial signals.

A threshold of microbial stimuli has to be reached to potently inhibit IgE switch since the very limited ASF did not efficiently inhibit CSR to IgE. This supports the idea that the microbial makeup of each individual is at the health-disease interface (Prioult and Nagler-Anderson, 2005) and that germ-free mice are an excellent model to study immune dysregulation. Serum IgE titers were maintained at high levels even after colonization of adult germ-free mice with a SPF flora. One possible explanation for this observation is that IgE-producing long-lived plasma cells maintained serum levels high despite abrogation of active transcription of IgE. Detection of εGLT following colonization should clarify this. Another explanation is that bacterial colonization must happen during a critical time window at post-natal life beyond which IgE switch is no longer reversible. Yet another possibility is that the SPF flora used in this experiment (SPF mice kept in individually ventilated cages at the Central Animal Facility at McMaster University, Canada) was unable to provide the necessary inhibitory stimuli, despite the absence of elevated IgE in mice born in this environment. Indeed it has been reported that the SPF flora from the SPF mice kept at the Institute of Laboratory Animal Science (University of

Zurich, Switzerland) could reduce the high levels of IgE in germ-free mice (Herbst et al., 2011).

The period of weaning coincided with the initial rise in serum IgE, which reached a plateau and was maintained stable throughout adulthood. Of note this was neither dependent on the withdrawal of maternal antibodies nor on the transition to solid chow indicating that the time of IgE appearance coincides with post-natal immune development. Therefore we suggest that absence of microbes in post-natal life does not properly shape the immune network, which leads to this early rise in IgE possibly due to a lack of inhibitory signals. Interestingly, IgA is virtually absent in neonates or in germ-free mice and is detectable at three-four weeks following bacterial colonization (Benveniste et al., 1971a; Benveniste et al., 1971b; Moreau et al., 1978). Thus, at the time when SPF mice develop IgA antibodies, germ-free mice develop IgE antibodies. In addition to this timing coincidence, IgA and IgE share induction sites as εGLT was detected in the MLN and PP. Whether, similarly to IgA, IgE can also switch in the lamina propria, remains to be investigated. Interestingly, *in utero* depletion of PP abrogated induction of IgE. Providing that this finding can be repeated in an independent experiment (planned), this would imply that initiation of B cell CSR to IgE occurs in the PP whereupon B cells egress to the MLN. Alternatively, it is possible that the sampling of luminal antigens through the PP is important for the initiation of IgE switch. For example, PP "conditioned" DC may traffic to the MLN to initiate CSR. Paradoxically IgE switch in allergic conditions or parasite infections occurs at the site of antigen entry and in germ-free animals at the site of "no antigen". Thus microbes condition cells in the MLN and PP in such a way that they prevent spontaneous CSR to IgE and favor CSR to IgA. However, while IgA can be produced in a T cell-independent pathway, IgE required the presence of $CD4^+$ T cells. Whether $CD4^+$ T cells are involved

in MHC Class II cognate interactions or provide a favorable cytokine environment remains unclear. Moreover, early IgE switch was dependent on signaling through MyD88 and TRIF, which relay signals from most of the TLRs and IL-1R, IL-18R and IL-33R. At present we can only speculate as to which of these pathways is involved in the CSR to IgE. Reducing TLR ligands did not impair IgE induction as shown in mice fed an elemental diet, therefore we propose that TLR signaling may not be critical in this process. The MyD88 signaling cytokines IL-1, IL-18 and IL-33 belong to the IL-1 family of cytokines and have divergent functions. While IL-1 and IL-18 are mainly involved in proinflammatory Th1 responses, IL-33 has been implicated in Th2 responses. In 2005, IL-33 was discovered *in silico* as a new member of the IL-1 family and its exogenous administration led to strong Th2 polarization and high IgE (Schmitz et al., 2005). Moreover, it has been suggested that IL-33 is transiently expressed in the cecum upon infection with *Trichuris muris* (Humphreys et al., 2008). Thus it is tempting to hypothesize that IL-33 production by intestinal epithelial cells of germ-free mice is altered and thus contributes to IgE switch. To test this, IL-33$^{-/-}$ or IL-33R$^{-/-}$ (C57BL/6 background) will be re-derived to germ-free for future experiments.

In an effort to identify the immunological milieu prevailing in germ-free mice that conditions B cells to undergo CSR to IgE, B cells and DC from GF, ASF and SPF mice were compared. We found that B cell and DC activation was not majorly altered between hygiene statuses. However DC from mucosal lymphoid tissues were not analyzed and thus it remains possible that mucosal DC are shaped differently between hygiene conditions. Indeed it has been shown that signaling through TLR1 and TLR2 on DC was necessary and sufficient for the induction of functional intestinal DC with high expression of retinal dehydrogenases (RALDH) enzymes (Wang et al., 2011) critical for retinoic acid synthesis which

imprints gut-homing lymphocytes (Eksteen et al., 2009; Iwata et al., 2004; Mora et al., 2006). Likewise activation markers of B cells and DC from the lamina propria were not analyzed here. Notwithstanding this, it is striking how similar the analyzed activation markers on B cells and DC were across hygiene statuses. Despite similar activation, significant phenotypic differences were noted in B cells across hygiene environments. Notably, SPF mice had a remarkably higher proportion of peritoneal B-1a cells compared to germ-free and ASF. This is somewhat unexpected given that the majority of the B-1 cell pool develops during ontogeny and that B-1 cells have been found in increased numbers in newborns (Baumgarth, 2010; Hardy and Hayakawa, 1991). Thus, these findings imply that neonatal B-1 cell development is stimulated by microbial colonization. This is in agreement with the fact that B-1 cells contribute to IgA secretion in the lamina propria of the intestine (Kroese et al., 1989). Moreover, given that we did not detect εGLT in peritoneal B cells, this strongly suggests that natural IgE is produced by B-2 cells rather than peritoneal B-1 cells. Another striking phenotypic difference was the higher proportion of B cells not expressing CD23 (FcεRII) in SPF mice. Follicular B cells express CD23 while transitional-1 B-2, B-1 or splenic marginal zone B cells do not. Therefore this observation is difficult to interpret because the $CD23^-$ B cell subset represents a heterogonous B cell population. Including signature cell markers such as AA4.1 for transitional-1 B-2, CD43 for B-1 and CD21 and CD1d for marginal zone B cells will be instrumental to clarify this. Given the paucity of B-1 cells outside of the peritoneal cavities (up to 2% of $CD19^+$) and the location of marginal zone B cells restricted to the spleen, one can speculate that reduced transitional-1 B-2 cells in SPF mice explains such a significant shift in proportion (Baumgarth, 2010). Indeed bacterial colonization transiently induces increased proportions of transitional B cells in the spleen (R. McArthur and K. McCoy, unpublished

data). Similarly, a decreased $CD23^-$ B cell subset in SPF mice was observed in $CD5^+$ B cells although the effect was restricted to mucosal tissues (MLN and PP). In this case, reduced proportion of transitional-1 B-2 cells in SPF mice is not a satisfactory explanation since it is highly unlikely that the latter express CD5. Alternatively, one can speculate that microbial signals or the presence of serum IgE directly influence surface expression of CD23, which is the low affinity receptor for IgE. One study has suggested that capture of monomeric IgE by CD23 contributes to the reduction of free IgE molecules (Cheng et al., 2010).

Accumulating evidence indicated that symbionts induce Tregs in the lamina propria of the intestine, a process that ensures homeostasis. In our laboratory we have shown that Treg cells are induced in the cLP following 28 days-ASF colonization (Geuking et al., 2011). Atarashi et *al* (Atarashi et al., 2010) followed the kinetics of Treg appearance in the lamina propria of SPF mice, and noted a sharp increase at 28 days of age. From these results we can conclude that post-natal colonization and experimental colonization both induce Tregs in the lamina propria of the intestine at around four weeks after bacterial encounter. Interestingly this four-week period corresponds to the induction of IgA (bacterial colonization) or IgE (germ-free). Recently, Liston and coworkers (Tian et al., 2011) compared the outcome of Th cell subsets following acute quantitative loss of Treg cells using the *Foxp3*^{DTR} mice. Whereas loss of Treg cells did not influence Th17 and IL-10-producing subsets, Th1 and Th2 both increased. However the increase in Th2 was more pronounced (20 fold) than Th1 (5 fold), suggesting that Tregs actively silence Th2 responses. In patients with HIES, a partial T cell immunodeficiency associated with high levels of IgE (along with other pathologies), Treg induction was defective (Saito et al., 2011). HIES results from mutations in *signal transducer and activator of transcription 3* (*STAT3*) or *Tyrosine kinase 2* (*TYK2*) impairing the IL-10

signaling pathway in DC, which failed to become tolerogenic and thus could not induce Treg differentiation. Taken those results together it is tempting to believe that reduced numbers of Treg cells favors the spontaneous switch to IgE in germ-free mice. To test this hypothesis we adoptively transferred T cells from ASF mice into 28-day old germ-free hosts and monitored IgE levels. Transfer of $CD4^+$ T cells did not prevent high IgE but a possible delayed onset of IgE induction was observed after transfer of $CD4^+CD25^+$ thought to be Tregs. As this thesis is written, this experiment is currently being repeated.

CSR to IgE normally depends on the cytokine IL-4, which induces the transcription of εGLT. In addition, other Th2 cytokines are critical for the conditioning of the APC. We thus studied the cytokine milieu prevailing in germ-free mice to elucidate the pathway of early IgE switch.

IL-4 promoted IgE switch in germ-free mice as indicated by decreased serum IgE after treatment with an anti-IL-4 mAb. Because IgE was not completely abolished after treatment, it is uncertain if other cytokines are involved or if the *in vivo* depletion was not efficient. Expression of IL-4 and IL-13 mRNA was similar in adult germ-free and SPF mice. However, given that CSR to IgE is initiated at early age, comparing adult mice may not be a faithful indication of the cytokine environment involved in the process, although CSR to IgE was observed in adult germ-free mice (data not shown).

Judging from the differences in IL-10 production between germ-free mice and mice harboring a microbiota, it is likely that IL-10 contributes to the regulation of IgE synthesis. While IL-10 expression was lower in the MLN of adult germ-free compared to SPF mice, it was comparable in the spleen. Thus, low IL-10 expression was restricted to the site of IgE induction. Moreover, ASF colonization of 10Bit (C57BL/6 background) for 28 days induced increased IL-10 production in Treg and Tr1 $CD4^+$ T cells

and in B cells in the cLP (in addition to the BM for $CD4^+$ T cells). Serum concentration of IL-10 was unexpectedly increased in germ-free compared to SPF. However this may reflect different cellular compartmentalization with mucosal IL-10 produced by Treg and Tr1 $CD4^+$ T cells in addition to B cells and peripheral IL-10 by recirculating Th2 cells. However, abrogation of the IL-10 signaling pathway did not markedly enhance IgE induction. It is possible that anti-IL10R antibody did not reach mucosal sites. Alternatively, the absence of IL-10 in mucosal tissues is underlying IgE induction, and therefore blockade of any residual (likely systemic) IL-10 signaling would have no effect. *In vivo* administration of IL-10 would be an alternative way to test the function of IL-10.

TSLP has been implicated in Th2 (and IgE) induction in models of allergy and parasite infection (Ziegler and Artis, 2010) and luminal stimuli modulate secretion of TSLP from intestinal epithelial cells (Rimoldi et al., 2005). Thus TSLP was an attractive cytokine to investigate but I could clearly demonstrate that TSLP was not involved in early IgE switch. On the contrary, *in vivo* depletion of TSLP in young germ-free mice accelerated IgE induction and in germ-free TSLPR-deficient mice IgE levels increased to very elevated titers without reaching a plateau. In future experiments enhanced IgE production in the absence of TSLP signaling should be addressed by comparing IgE levels of germ-free homozygous $TSLPR^{-/-}$ and heterozygous $TSLPR^{+/-}$. Supporting this are preliminary data from our laboratory indicating an amplified reduction in cLP Treg in $TSLPR^{-/-}$ compared to C57BL/6 germ-free mice (M. Geuking, unpublished).

Elevated levels of IgE and loss of immune regulation have been correlated with restriction of the TCR repertoire. This has been shown in patients with partial T cell immunodeficiency (Appendix 1), with GVHD (Geha et al., 1980) or with HIV infection (Marone et al., 2001). These observations hold true in genetically modified mice (Appendix 2) or upon adoptive transfer of

a limited number of $CD4^+$ T cells (3×10^4) into lymphopenic hosts (Milner et al., 2007a; Milner et al., 2007b). It is possible that lack of or reduced microbial stimuli reduces the TCR repertoire but demonstrating this would require demanding techniques (Lin and Welsh, 1998; Pannetier et al., 1993). We therefore evaluated serum IgE levels in germ-free TCR transgenic Smarta1 mice (C57BL/6 background) with >95% of $CD4^+$ T cells expressing a TCR specific for an irrelevant glycoprotein from LCMV (Oxenius et al., 1998). The measured high levels of IgE imply that TCR specificity may be irrelevant for CSR to IgE. Complete absence of endogenous TCRs (for example $RAG1^{-/-}\times$Smarta1) should provide a definitive proof for this. Similarly to TCR specificity, it is possible that BCR specificity is irrelevant. High IgE levels were measured in VI-10 mice (C57BL/6 background) with 10-15% of total B cells expressing a BCR with high affinity for an irrelevant epitope of VSV (Hangartner et al., 2003). However, this finding needs to be confirmed in VI-10×YEN mice, which have a much higher proportion of B cells specific for VSV (>95%) (Hangartner et al., 2003).

In addition to environmental factors, genetic background plays a pivotal role in the predisposition to immune dysregulation (Paul and Zhu, 2010) and the same held true in the present model of hyper-IgE in germ-free C57BL/6 mice. While, similarly to C57BL/6, BALB/c and NIH-Swiss mice had high levels of IgE, most of the NMRI and Swiss Webster mice had low or undetectable levels of IgE. As expected the progeny of C57BL/6 and Swiss Webster had intermediate levels of IgE. Taken together these results suggest that genetic predisposition together with environmental factors (or reduced bacterial stimuli) contribute to loss of immune regulation.

The search for the pathway responsible for the induction of IgE has been proven to be intricate. This can be viewed as another example of the

operational redundancy involved in the inception of Th2 responses and IgE. Indeed, searching for one cell type and one cytokine type may be fruitless as it has been shown in many instances that Th2 responses result from a network of pathways (Paul and Zhu, 2010). Thus, in stark contrast to the linear pathway leading to Th1 responses, the Th2 pathway can be viewed as a neural network. Since the start of my PhD thesis in 2007, new Th2 cytokines and innate type 2 cells have been discovered, that altogether integrate in this “neural network”.

Long viewed as the barrier between gut lumen and the underlying lamina propria, intestinal epithelial cells truly acquired the status of innate immune cells. Intestinal epithelial cells express TLRs allowing for sensing of luminal stimuli, which are relayed and modulate cytokine expression. Notably intestinal epithelial cells secrete the Th2 cytokines IL-25, IL-33 and TSLP, which in turn can condition APC and subsets of the emerging innate type 2 cells (Saenz et al., 2008; Angkasekwinai et al., 2007). Natural helper cells were reported by Moro et *al*. (Moro et al., 2009) as clusters of cells found in the mesentery and in fatty deposits in the peritoneal cavity and surrounding the kidney. Although it is unknown how natural helper cells get activated, they express high levels of IL-4, IL-5, IL-6 and IL-13 and have been implicated in the proliferation of B-1 cells and IgA production. Nuocytes and innate type2 helper cells found in the spleen and MLN (in addition to the liver for innate type2 helper cells) express high levels of IL-5 and IL-13 following exogenous administration of IL-25 or IL-33 or helminth infection (Neill et al., 2010; Price et al., 2010). Multipotent progenitor type 2 (MPP^{type2}) cells are progenitor cells that can be differentiated into macrophages basophils and mast cells (Saenz et al., 2010). They were described in the MLN, PP and cecal patch and were activated upon exogenous IL-25 or helminth infection, producing high amounts of IL-4, IL-5 and IL-13. All innate type 2 cells (natural helper cells,

nuocytes, innate type2 helper cells, MPP^{type2}) have been implicated in anti-helminth responses. Whether such innate type 2 cells are integrating in the network of germless IgE induction is currently unknown. However, it is interesting that serum IL-13 levels were found to be slightly elevated in germ-free mice and all of these innate type 2 cells have been described to be high producers of IL-13.

A model proposing potential mechanisms for hygiene-induced IgE compared to bacterial-induced IgA is shown in Figure 26. Future studies will try to elucidate these proposed mechanisms in greater detail.

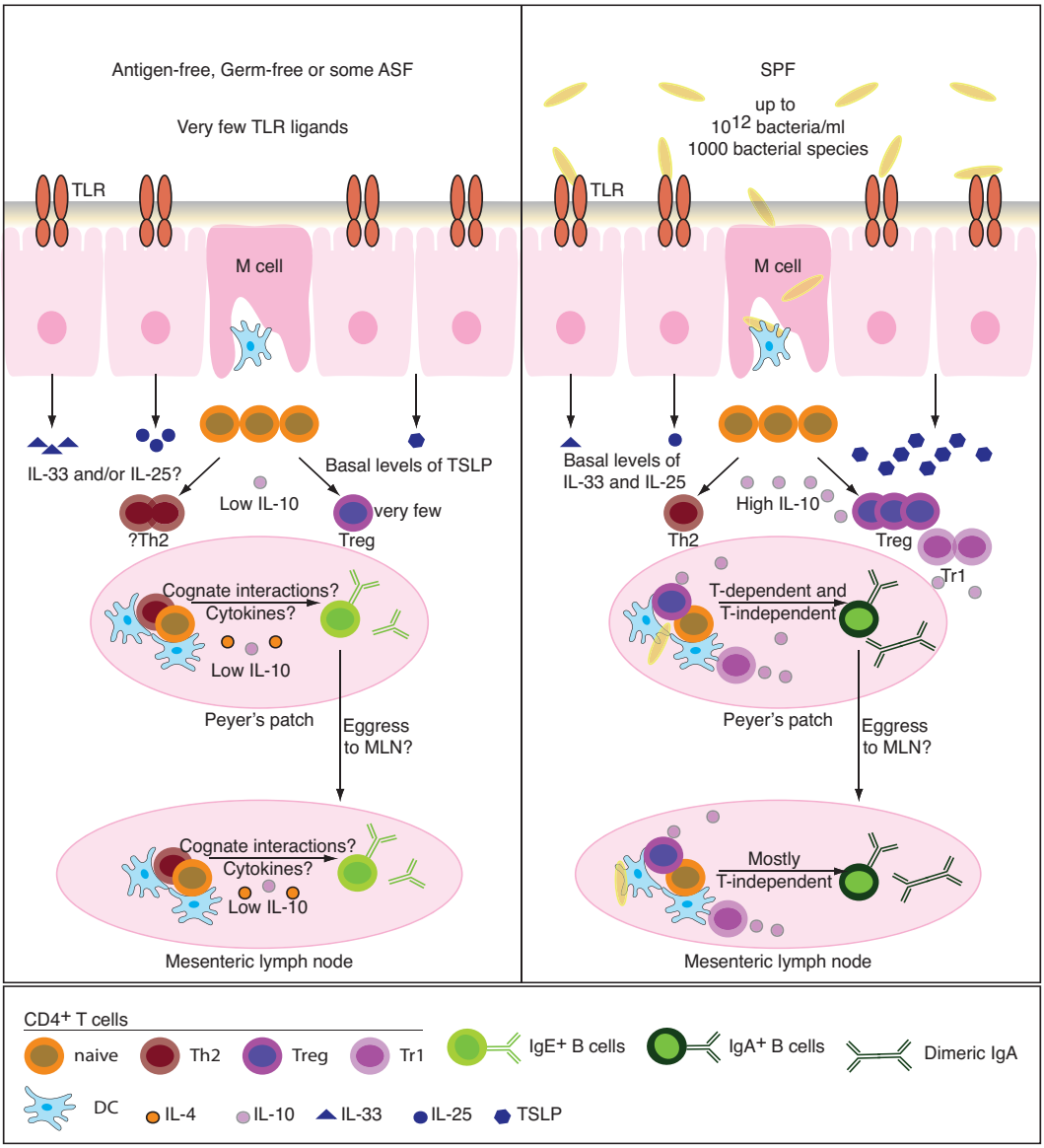


Figure 26. Comparison of hygiene-mediated IgE and bacterial-mediated IgA. In antigen-free, germ-free and some ASF C57BL/6 mice, luminal TLR ligands are reduced compared to SPF mice associated with a diverse microbiota. Consequently epithelial cells may alter the secretion of epithelial-derived cytokines. TSLP may be produced at lower levels, which may be unfavorable for a Treg differentiation program. The epithelial-derived Th2 cytokines IL-25 and IL-33 may be produced at higher levels, which would support a Th2 differentiation program (although Th2 cells have not been observed in the lamina propria of germ-free and ASF mice). The absence of inhibitory signals such as IL-10 (produced by Tregs, Tr1 and B cells in colonized mice) and Tregs and the presence of IL-4 may favor spontaneous CSR to IgE in the PP and the MLN. Alternatively induction of CSR to IgE may occur in the PP whereupon IgE^+ B cells would migrate to the MLN.

4.2 Systemic exposure to an intestinal symbiont reduces incidence of diabetes in gnotobiotic NOD mice

In agreement with the impact of intestinal microbes on immune regulation, evidence suggests that microbiota composition in diabetes-prone NOD mice modulates disease incidence (Bach, 2002; Like et al., 1991; Wen et al., 2008). In this thesis I demonstrated protection from diabetes in NOD mice following systemic exposure to an intestinal symbiont commonly found in SPF animals (*E. cloacae*). Importantly these experiments were carried out in gnotobiotic NOD mice indicating that the observed effects were independent from variability in microbiota.

Despite induction of insulitis, the protective effect was maintained in older mice (>100 days old). We suggest that systemic bacterial exposure induces an immunoregulatory pathway that halts insulitis progression, thus protecting from overt hyperglycemia.

Understanding the mechanisms underlying these observations is critical to elucidate the complex dialogue between genes, environmental factors and immune regulation. Notwithstanding the importance of genetic susceptibility (>50 genetic loci associated with increased risk for T1D (Todd, 2010)), environmental stimuli represent critical checkpoints to disease progression. In addition to the low concordance rate for T1D in genetically identical twins, the rate of disease progression is heterogeneous between susceptible individuals (Ziegler and Nepom, 2010). It is tempting to believe that immunoregulatory pathways explain such differences in disease progression at the population level.

It is undisputed that delivery into the blood stream of 10^8 live bacteria will induce vigorous innate immune responses. Our preliminary data indicate a stark increase in the proportion of blood monocytes and granulocytes (K. Safroneeva and K. McCoy, unpublished). In addition, systemic exposure with *E. cloacae* triggered the adaptive arm of immunity as I measured *E. cloacae*-specific serum antibodies, which did not crossreact to *Salmonella*

typhimurium. Typically, immunoglobulins with high affinity and specificity are generated five-seven days following antigen encounter in germinal center reactions, which involves an interplay between T cells, B cells and DC (Cerutti et al., 2011). Current knowledge indicates that disease progression in T1D requires both $CD4^+$ and $CD8^+$ T cells that engage β cell antigens displayed on APC including DC and B cells in the pancreatic LN (Santamaria, 2010). The mechanism whereby systemic *E. cloacae* interferes interacts with these diabetogenic cells is unknown. We can speculate that an immune reaction against *E. cloacae* can inhibit or deviate the autoreactive immune response. For example, it has been reported that formation of autoreactive cells is not inhibited since Treg function and numbers are decreased in NOD mice (Gregori et al., 2003; Sgouroudis et al., 2008; Yamanouchi et al., 2007). Whether systemic *E. cloacae* induces Treg remains to be tested as does the secretion of IL-10 or TGF-β cytokines. Alternatively it is possible that systemic priming induces a massive proliferation of *E. cloacae*-specific cells that deviate the diabetogenic response.

Immunocompetent SPF mice are normally ignorant to their microflora. However in the lifetime of an individual (which is not SPF and will experience infections), intestinal microbes can occasionally reach systemic sites and induce specific antibodies (C. Urbaniak, K. McCoy and A. Macpherson, submitted). Intestinal microbes can also access systemic sites in immunocompromised mice as seen in the TLR-defective $MyD88^{-/-}$ $TRIF^{-/-}$ mice, which induce specific serum antibodies against their indigenous microbes (Slack et al., 2009). Wen et *al* reported protection from diabetes in SPF $MyD88^{-/-}$NOD mice. This protective effect was conferred to the indigenous microbiota because germ-free $MyD88^{-/-}$NOD developed diabetes (Wen et al., 2008). Although not addressed in this publication, it is very likely that similarly to $MyD88^{-/-}TRIF^{-/-}$ mice, SPF

MyD88$^{-/-}$NOD mice fail to contain their indigenous microbes within the mucosal compartment. Therefore we suggest that the microbiota-induced protection reported by Wen et *al* and our observations involve similar immunoregulatory pathways.

Following this descriptive effect, future studies will concentrate on the immunoregulatory mechanisms triggered after a breach of systemic ignorance. These studies should be instrumental to reveal the immunological basis of microbial-induced protection from diabetes.

5 Materials and methods

5.1 Mouse strains, hygiene status and bacterial colonization

Mouse strains C57BL/6, TCR$\beta\delta^{-/-}$, TSLPR$^{-/-}$, Myd88$^{-/-}$TRIF$^{-/-}$, TgH(VI10), Smarta1 (all on a C57BL/6 background), BALB/c, NIH-Swiss, Swiss Webster, NMRI and NOD were re-derived to germ-free status from two cell embryos and bred and maintained in flexible-film isolators as described previously (Smith et al., 2007a) at the Farncombe Axenic and Gnotobiotic Unit, McMaster University, Canada or in the Clean Mouse Facility, University of Bern, Switzerland. The absence of bacteria in germ-free mice was routinely confirmed by culture of cecal contents under aerobic (Luria-Bertani Broth (LB) agar plates) and anaerobic (blood agar plates). In addition, DNA staining of cecal contents using Sytox green was used to confirm the absence of bacterial DNA in the cecal contents. The absence of parasites, bacteria and virus contamination was also independently confirmed every three months by shipping sentinel germ-free mice to Charles River Research Animal Diagnostic Services (MA, USA) or Biolytix AG (Witterswil, CH).

Gnotobiotic mice associated with the altered Schaedler's flora (Dewhirst et al., 1999) were originally obtained from germ-free mice co-housed with an ASF colonizer. The ASF consists of *Lactobacillus acidophilus* (ASF 360), *Lactobacillus murinus* (ASF 361), *Bacteroides distasonis,* (ASF 519), *Mucispirillum schaedleri* (ASF 457), *Eubacterium plexicaudatum (ASF 492),* a Fusiform-shaped bacterium (ASF 356) and two Clostridium species (ASF 500, ASF 502). The presence of these bacteria was confirmed by qPCR using the primers shown in Table 3.

Table 3. Primers for qPCR specific for the eight bacterial species composing the ASF.

Species	Primer sequences (5'-3')
ASF 356	Fwd: GATGCCTCCTAAGAACCGTATGC
	Rev: GCGGACGGGTGAGTAACGT
ASF 360	Fwd: AAGTCGAGCGAGCTGAACCA
	Rev: ATCCCAGACTTTAGGGCAGGTTA
ASF 361	Fwd: AGGTGGCTATGCTACCGCTTT
	Rev: CTCAGTTCGGCTACGCATCAT
ASF 457	Fwd: CGTTTGCAAGAATGAAACTCAAA
	Rev: CACAGCATTATCTCTAACGCCTT
ASF 492	Fwd: GGGACGACGTCAAATCATCAT
	Rev: GGCTTCGCTTCCCTCTGTCT
ASF 500	Fwd: TCGGACAACTGAAGGGAATCC
	Rev: CCAGTGCCATGCGACACA
ASF 502	Fwd: GAGGGAAGAGAGGGTGACTTAGC
	Rev: TCTTATGCGGTATTAGCAATCATTTC
ASF 519	Fwd: TTGCCGTTGAAACTGGTTGA
	Rev: GGAGTTCTGCGTGATATCTATGCA

For colonization experiments, germ-free C57BL/6 mice were exported from flexible-film isolators and co-housed with gnotobiotic mice associated with ASF or with SPF mice, as indicated. All animal experiments were carried out in accordance with the McMaster University animal utilization protocols and the Canadian Council on Animal Care (CCAC) guidelines or in accordance with Swiss federal regulations.

5.2 Germ-free mice fed an elemental diet

Germ-free breeding pairs (C57BL/6♂×NIH-Swiss♀) were housed on endotoxin-free sand (baked at 250°C for 30 mins). An antigen-free diet was provided by an irradiated (5M Rad Co-60 for 20 h) infant formula

consisting of extensively hydrolyzed proteins supplemented with fats, vitamins and minerals (Pregestimil, Enfamil).

5.3 Detection of serum antibodies and cytokines

Blood was collected in serum-separating tubes and total serum IgE, IgA, IgM, IgG1, IgG2a, IgG2c and IgG3 concentrations determined by ELISA. ELISA plates (Nunc) were coated overnight at 4°C with 2.5 μg/ml rat anti-mouse IgE (clone 6HD5, grown in-house) or with 1 μg/ml goat anti-mouse IgA, IgM, IgG1, IgG2a, IgG2c or IgG3 (Southern Biotechnologies) in 0.1 M $NaHCO_3$. Plates were washed with 0.05% Tween 20 in PBS and blocked for 2 h at room temperature (RT) with 5% (IgE) or 2.5% (all other isotypes) weight per volume (w/v) bovine serum albumin (BSA) in PBS. Blocking buffer was flicked off and serially diluted serum or standards, IgE (clone TIB-141) or IgA, IgM, IgG1, IgG2a, IgG2c and IgG3 (Zymed), were added and incubated for 2 h at RT. Bound IgE was detected with biotinylated anti-mouse IgE (clone RIE-4, grown in-house) at 2.5 μg/ml followed by horseradish peroxidase-conjugated streptdavidin (Biolegend) at 1 μg/ml, following plate-wash for each step. Bound IgA, IgM, IgG1, IgG2a, IgG2c and IgG3 were detected by horseradish peroxidase-conjugated anti-mouse IgA, IgM or IgG (Sigma). ABTS peroxidase solution (0.1 mg/ml 2,2'-azino-bis-[3-ethylbenzthiazidine-6-sulfonic acid], 0.05% H_20_2 in 0.1 M NaH_2PO_4) was used for colorimetric reaction. Reaction was read at 405 nm with microplate reader (Bio-Rad) and standard curves and antibody concentrations analyzed with Microplate Manager III software (Bio-Rad).

Cytokines in serum were detected with cytometric bead array reagents (BD Biosciences) according to the manufacturer's protocol, acquired on a fluorescence-activated cell sorter (FACS) Array (BD Biosciences) and analyzed with FCAP Array software (Soft Flow).

5.4 Quantitative real time PCR

For single cell suspensions, lymphoid tissues were homogenized and tibia and femur were crushed to obtain BM cells. PP were digested with Liberase C1 (Boehringer Mannheim) for 20 min at 37˚C prior to homogenization. For the detection of εGLT expression, B cells were enriched with CD19 magnetic beads (Miltenyi Biotec). The negative fraction ($CD19^-$) was collected for the detection of cytokine expression. Cells were resuspended into TRIzol reagent (Invitrogen Life Technologies) and RNA obtained according to the manufacturer's instructions. DNA impurities were removed using the DNA-free kit (Ambion). RNA quality and concentration were assessed with RNA 6000 Nano kit (Agilent) on a Bioanalyzer 2100 (Agilent). Synthesis of cDNA was performed using random hexamers and Superscript III RT (Invitrogen Life Technologies) according to the manufacturer's instructions. Amplification of cDNA by qPCR was performed using SYBR Green Supermix or SsoFast EvaGreen Supermix (Bio-Rad). Primer sequences were as follows: εGLT: 5'-GCC TGC ACA GGG GGC AGA AG-3' and 5'-ATG ACC CTG GGC TGC CTG GT-3', IL-4: 5'-TCA TCG GCA TTT TGA ACG AG-3' and 5'-CGT TTG GCA CAT CCA TCT CC-3', IL-10: 5'-TTT GAA TTC CCT GGG TGA GAA-3' and 5'-GGA GAA ATC GAT GAC AGC GC-3', IL-13: 5'-GAG CAA CAT CAC ACA AGA CCA GA-3' and 5'-GGC CAG GTC CAC ACT CCA TA-3', GAPDH: 5'-CAT CAA GAA GGT GGT GAA GC-3' and 5'-CCT GTT GCT GTA GCC GTA TT-3'. PCR and analysis were performed on an iQ5 or CFX384 (Bio-Rad) platform and software. Gene expression was calculated relative to GAPDH.

5.5 Flow cytometry

For single cell suspensions, lymphoid tissues were homogenized and tibia and femur were crushed to obtain BM cells. MLN and PP were digested in

Iscove's Modified Dulbecco's Medium (IMDM) (Gibco) containing 0.14 Wünsch units/ml of Liberase C1 (Boehringer Mannheim) for 20 min at 37˚C prior to homogenization. Collected cells were surface-stained for 20 min at 4°C with a combination of the following antibodies and corresponding clones: CD19-FITC (1D3), CD11c-PE (HL3), CD23-PE (B3B4), CD5-biotin (53-7.3) conjugated to streptavidin-APC, CD4-PerCP (RM4-5), B220-APC/Cy7 (RA36B2) purchased from BD PharMingen; CD90.2-APC (30-H12) purchased from Sunnybrook antibody core facility, Toronto; CD90.1-FITC (OX-7), TACI-PE (8F10), CD40-PE/Cy5 (3/23), CD3-PerCP/Cy5.5 (145-2C11), IgM-PE/Cy7 (RMM-1), CD86-PE/Cy7 (GL-1), BAFF-R-AlexaFluor647 (7H22-E16), OX40-L-AlexaFluor647 (RM134L), I-A/I-E-AlexaFluor700 (M5/114.15.2), CD4-PacificBlue (RM4-5), CD80-PacificBlue (16-10A1), CD86-PacificBlue (GL-1) purchased from Biolegend; F4/80-FITC (BM8), CD19-APC/Cy7 (6D5) purchased from eBioscience. For results shown in Figure 14-19**,** cells were acquired directly after the staining procedure and dead cells were excluded from analysis with the DNA-intercalating dye 4',6-diamidino-2-phenylindole (DAPI, 0.5 μg). Intracellular staining with Foxp3-AlexaFluor700 (FJK-16a, eBioscience) was used in combination with the Foxp3 staining kit (eBioscience) according to the manufacturer's instructions. For staining of blood, erythrocytes were lysed with BD FACS lysing solution (BD) prior to surface staining. Data were acquired on a FACSCalibur (BD) or an LSRII (BD) and analyzed with FlowJo software (TreeStar, Inc.).

5.6 *In vivo* mAb treatment

Germ-free C57BL/6 mice were i.p-treated twice a week (200 μg or 500 μg for anti-IL10R) with one of the following sterilely prepared mAbs grown in-house: anti-CD4 (clone YTS191.1.2), anti-IL-4 (clone 11B11), anti-TSLP (clone 28F12, hybridoma developed by Andrew Farr, University of

Washington, USA) or anti-IL10R (clone 1B1-2) and its isotype-matched control (35.61, IgG1, κ).

5.7 PP depletion

Germ-free C57BL/6 mice were bred and females were monitored each morning for the presence of vaginal plugs. The time of plug observation was counted as d0.5 of gestation. At d14.5-15.5 of gestation, a single i.v injection of anti-IL7Rα (3 mg, clone A7R34, BioxCell) was administered under axenic conditions in the laminar flow hood. Successful depletion of PP in the progeny was verified at the end of the experiment.

5.8 Adoptive T cell transfer

$CD4^+$, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were enriched from splenocytes or cLP lymphocytes with microbeads specific for CD4 or the regulatory T cell isolation kit (Miltenyi Biotec) in combination with an OctoMACS or autoMACS (Miltenyi Biotec) according to the manufacturer's instructions. Cells were resuspended in IMDM (Gibco), counted and i.v-transferred under axenic conditions in the laminar flow hood in germ-free recipients.

5.9 *In vitro* activation of B cells

B cells were enriched using CD19 microbeads (Miltenyi Biotec) in combination with an OctoMACS (Miltenyi Biotec), resuspended in IMDM supplemented with 10% fetal calf serum (FCS), 50 μM streptomycin, 50 U/ml penicillin, 2 mM L-Glutamine, and 50 μM β-Mercaptoethanol and added to a 24 well-plate ($1\text{-}2\times10^6$ cells/well in 1 ml). B cells were stimulated with 1 μg/ml type B CpG 1668 (PAGE-purified from Microsynth (Switzerland), 5'-TCCATGACGTTCCTGAATAAT-3', all bonds phosphorothioate) or with recombinant IL-4 (20 ng/ml, Sigma) and anti-

CD40 mAb (15 μg/ml, clone FGK45.5). Cells were placed in a humidified incubator at 37°C with 5% CO_2 for 24 h or 36 h.

5.10 cLP preparation

Colons from two mice per group were pooled to obtain sufficient numbers of cells. The excised colon was flushed to remove contents, opened longitudinally, washed thoroughly in Mg_2Cl_2- and $CaCl_2$-free DPBS (Gibco), and then cut into of 3-5 mm pieces that were incubated 4-5 times in 25 ml EDTA/HEPES/DPBS solution at 37°C for 20 min to remove the epithelial layer. Intestinal pieces were collagenase-digested for 40 min at 37°C in 25 ml IMDM containing 0.5 mg/ml collagenase type VIII (Sigma), 50 U DNaseI (Roche), and 0.01M HEPES (Gibco). The crude cell suspension was loaded onto a 30%/100% percoll (GE Healthcare) gradient and centrifuged at 680×g for 30 min at RT with acceleration and brake turned off. Cells were collected from the 30%/100% interphase.

5.11 Systemic priming with *Enterobacter cloacae* in NOD mice and blood glucose monitoring

A single colony from plated *E. cloacae* was inoculated in LB medium. Bacteria were grown overnight with shaking at 160 rpm, 37°C and harvested by centrifugation (15 min, 3500×g, 4°C). Bacteria were washed with sterile PBS and the concentration was determined with OD_{570} and adjusted for i.v injection. NOD mice were injected with 10^8 CFU twice per week for three weeks and subsequently monitored weekly for blood glucose with a glucometer (BD). Mice were considered diabetic and euthanized when two successive blood glucose levels were >16 mmol/L.

5.12 Bacterial flow cytometry

E. cloacae were grown as described in 5.11. The bacterial culture (1 ml) was harvested by centrifugation in a minifuge (3 min at 7000 rpm) and the bacterial pellet washed twice with sterile PBS. Serum was diluted in PBS/2%BSA (1:20), heat-inactivated (56°C for 30 min), centrifuged in a minifuge to remove bacterial-sized particles (5 min at 7000 rpm) and 3-fold serially diluted in 96 well plates for flow cytometry (BD). Bacteria were resuspended in sterile-filtered PBS/2%BSA/0.005%NaN_3, added to the serum (10^6 bacteria/well in 25 μl) and incubated for 30 min on ice. Bacteria were centrifuged (10 min at 4000 rpm), washed twice with PBS/2%BSA and stained for 30 mins at 4°C for serum antibody binding with mouse anti-IgM-APC (clone II-41), anti-IgG2a-FITC (clone R19-15), or anti-IgG2b-FITC (clone R12-3) from BD PharMingen. Bacteria were washed twice, resuspended in sterile-filtered 2% paraformaldehyde (PFA)/PBS and acquired on a FACSArray (BD) using FSC and SSC parameters in logarithmic mode. Data were analyzed using FlowJo software (TreeStar, Inc.).

5.13 Immunohistochemistry

Pancreas were embedded in Tissue-Tek OCT compound (Sakura Finetek), snap frozen in liquid nitrogen, and stored at -80°C. Cryosections (6 μm) were mounted on glass slides and air-dried for 2 h at RT. For H&E stains, sections were fixed in Wollman solution (95% EtOH, 5% Acetic Acid), prior to hematoxylin (Vector) and eosin (Sigma) staining and mounted with VectaMount (Vector). Islets were individually graded with the Leiter scoring system as follows: 0, no infiltration; 1, perivascular/periductular infiltrates with leukocytes touching islet perimeters but not penetrating; 2, leukocytic penetration of up to 25% of

islet mass; 3, leukocytic penetration of up to 75% of islet mass; 4, end stage insulitis with <20% of islet mass remaining.

For detection of peroxidase-labeled CD3, sections were fixed in acetone, prior to staining with purified rat anti-mouse CD3 (BD PharMingen), purified donkey anti-rat (Cedarlane) and donkey anti-goat labeled with horseradish peroxidase. 3-Amino-9-ethylcarbazole (AEC, Sigma) substrate was used for colorimetric reaction. Slides were mounted with glycerin-gelatin.

6 Appendices

Appendix 1: Partial T cell immunodeficiencies linked with high IgE in humans

Disease	Gene(s)	Immune dysregulation(s)	Symptoms	References
Atypical complete DiGeorge syndrome	~90% due to deletion of 22q11	- Reduced T cells numbers - Oligoclonal T cells	- Autoimmunity (juvenile rheumatoid arthritis, idiopathic thrombocytopaenia purpura)	(Chinen et al., 2003; Jawad et al., 2001; Markert et al., 2007; Sullivan et al., 1997)
Adenosine Deaminase (ADA)-deficiency	ADA	- Varying levels of T cell deficiency	- Autoimmunity (Idiopathic thrombocytopaenia purpura), - Asthma	(Hirschhorn et al., 1994; Hirschhorn et al., 1996; Shovlin et al., 1993)
Type I hyper-IgE syndrome (HIES)	Most cases dominant negative mutation of *Sat3*	- Decreased Th1 cytokines - Skewing towards Th2 - Deficiency of Th17 cells due to mutations in *Stat3* - Impaired signaling of IL-6 and IL-10 - Impaired generation of tolerogenic DCs and iTregs - Defective salivary activity (antimicrobial proteins)	- Abscesses, pneumonia, afebrile - Abnormalities of the connective tissue, skeleton and dentition, and coronary artery aneurysms - Abnormal susceptibility to a narrow spectrum of infections (*Staphylococcus aureus* and *Candida albicans)*	(Minegishi et al., 2007) (Holland et al., 2007) (Ma et al., 2008) (Grimbacher et al., 1999) (Ito et al., 2003) (Netea et al., 2005) (Saito et al., 2011) (Conti et al., 2011)
Type II hyper-IgE syndrome (HIES)	Largely unknown In one patient a null mutation in *Tyrosine kinase 2* (*Tyk2*)	- Reduced T cell proliferative response - Impaired Th1 - Enhanced Th2 - Defective signaling of IL-6, IL-10, IL-12, IL-23, IFNα and IFNβ	- Eosinophilia, eczema - Autoimmunity (antinuclear antibodies, autoimmune haemolytic anemia, vasculitis) - Severe viral infection, intracellular bacterial infection	(Minegishi and Karasuyama, 2007; Minegishi et al., 2006; Renner et al., 2004)
Immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX)	Mutation in *FoxP3*	- Lack $FoxP3^{+}CD25^{+}$ Tregs	- Elevated IgA levels - Severe eczema, eosinophilia and food allergy, enteropathy, endocrinopathy (diabetes or thyroid disease), and atopic dermatitis	(Hori et al., 2003) (Torgerson and Ochs, 2007) (Bennett and Ochs, 2001) (Wildin et al., 2001)

Disease	Gene(s)	Immune dysregulation(s)	Symptoms	References
Omenn syndrome	Mutations: *Rag1*, *Rag2*, *Artemis*, *Il7ra*, *Rmrp*, *Il2rg*, *Zap70* Deficiencies: ADA DNA ligase IV	- Oligoclonal TCR repertoire - Reduced T cell proliferation - Reduced Treg cells - Increased IL-4, IL-5 - Reduced IL-2 and IFNγ	- Decreased IgG, IgA and IgM - Eosinophilia, lymphadenopathy - hepatosplenomegaly, erythrodermia - Recurrent infections - Chronic diarrhea, failure to thrive, alopecia - Autoimmunity	(Grimbacher et al., 2002) (Roifman et al., 2006) (Giliani et al., 2006) (Shibata et al., 2007) (Santagata et al., 2000) (Aleman et al., 2001) (Marrella et al., 2008) (Turul et al., 2009) (Roifman et al., 2008) (Grunebaum et al., 2008)
Wiskott-Aldrich syndrome	*Was*	- Reduced $CD8^+$ T cells numbers - Reduced proliferative response of T cells - Actin cytoskeletal abnormalities and disturbed immune synapse stability - Function of Tregs impaired - Increased IL-4 (due to antigen overload?) - Defective Th1 response	- Low levels of IgM, normal levels of IgG, increased levels of IgA, IgD - Eosinophilia, eczema, thrombocytopenia with small platelets - Autoimmunity (autoimmune haemolytic anemia, juvenile rheumatoid arthritis, idiopathic thrombocytopaenia purpura) - Recurrent infections	(Derry et al., 1994; Dupuis-Girod et al., 2003; Kolluri et al., 1995; Schurman and Candotti, 2003; Sullivan et al., 1994) (Ochs and Thrasher, 2006) (Adriani et al., 2007; Humblet-Baron et al., 2007; Maillard et al., 2007; Marangoni et al., 2007) (Trifari et al., 2006)
ZAP-70 deficiency with compensation of SYK which partially restores TCR signaling	*Zap70*	- Absence of $CD8^+$ T cells - Reduced proliferative response of $CD4^+$ T cells		(Arpaia et al., 1994) (Chan et al., 1994; Elder et al., 1994) (Toyabe et al., 2001)

Appendix 2: Partial T cell immunodeficiencies linked with high IgE in mice

Mouse strain	Genetic modification	Immune dysregulation(s)	Symptoms	References
(Vav1-Cre)Itgb8$^{fl/fl}$	Conditional knockout of αvβ8 on leukocytes	- Increased effector/memory T cells - Failure to induce Treg cells - Serum IgA and IgG1 elevated	- IBD and age-related autoimmunity	(Travis et al., 2007)
B6 → aly/aly chimera	aly/aly point mutation in nuclear factor-κB-inducing kinase (Nik))	- absence of organized lymphoid structures due to impaired signaling through LTβR (	- elevated blood and skin eosinophilia, - airway hyperresponsiveness	(McCoy et al., 2006)
Bcl6$^{-/-}$	Knockout	- Lack robust germinal center responses - Absence of T follicular helper cells		(Ye et al., 1997) (Harris et al., 1999) (Nurieva et al., 2009) (Johnston et al., 2009)
CD4$^{-/-}$	Knockout	- Compromised MHCII-restricted T cell selection		(McCoy et al., 2006)
CD4cre-fur$^{f/f}$	Conditional knockout of furin on leukocytes	- Impaired function of Treg cells	- defective periperal tolerance - At 6 months of age, severe inflammatory disease	(Pesu et al., 2008)
CKA (CTLA4 knockout in Tregs)	Knockout	- Systemic lymphoproliferation	- Moribund at 7 weeks - Fatal T cell mediated autoimmune disease	(Wing et al., 2008)
Id2$^{-/-}$	Knockout	- Skewing to the Th2-cell phenotype - Effector T cells have higher apoptosis rates - Differentiation into memory T cells diminished		(Kusunoki et al., 2003) (Cannarile et al., 2006)
IRF-2$^{-/-}$	Knockout	- STAT6-independent increase of basophils and Th2 polarization		(Hida et al., 2005)
LAT^{Y136F}	Designed allele	- Impaired LAT signaling and T cell homeostasis - Th2 polarization - Defect in Foxp3^{+} Tregs	- Inflammation - Eosinophilia - High IgG1 - Disrupted central tolerance	(Aguado et al., 2002) (Sommers et al., 2002) (Koonpaew et al., 2006)
LAT$^{Y7/8/9F}$	Designed allele	- Elevated IgG1 - Accumulation of γδ CD4^{+} T cells in the spleen and lymph nodes with characteristics of αβ CD4^{+} T cells		(Nuñez-Cruz et al., 2003)
MHCII$^{-/-}$	Knockout	- Lack of thymic positive CD4^{+} T cell selection		(McCoy et al., 2006)

Mouse strain	Genetic modification	Immune dysregulation(s)	Symptoms	References
nu/nu	Single bp deletion in winged helix nude (*whn*)	- No $CD4/8^+$ T cells of thymic origin - Low $CD4^+$ and $CD8^+$ T cells in peripheral blood - Substantial populations of γδ IEL		(Ito et al., 1979) (McCoy et al., 2006)
$Rag1^{R972Q}$ (12% of wt activity)	Spontaneous mutant	- Elevated IgM, IgG2b and IgG2a - Dysregulated homeostatic proliferation of $CD4^+$ T cells - High Th2 cytokines	- Eosinophilia - Lymphopenic	(Khiong et al., 2007)
$Rag2^{R229Q}$ (150 fold reduction of recombinase activity)	Designed allele	- Elevated eosinophilis, - Reduced proportion of $Foxp3^+$ Tregs in thymus and spleen - Reduced thymic expression of *Aire*	- Inflammatory diarrhea - Alopecia	(Marrella et al., 2007)
Scurfy	Knockout (*FoxP3*)	- Deficient in $Foxp3^+$ Treg cells	- Lethal, lymphoproliferative autoimmune syndrome - Dermatitis with eosinophilic infiltration, and increased expression of Th2 cytokines	(Fontenot et al., 2003) (Torgerson and Ochs, 2007)
$SLP\text{-}76^{-/-}Cbl^{-/-}$	Knockout	- Elevated IgG1 - Multiorgan infiltration - Expansion of peripheral $CD4^+$ T cells, B cells and macrophages	- Splenomegaly - Lymphadenopathy - Lymphoid infiltrates of multiple parenchymal organs	(Chiang et al., 2004)
$TCR\alpha^{-/-}$	Knockout	- Increased IgG1	- Autoimmunity	(Wen et al., 1994)
$Wip^{-/-}$	Knockout	- Elevated IgM - T cells fail to proliferate - B cells show an enhanced proliferation		(Antón et al., 2002) (Curcio et al., 2007)
$Zap70^{mrd/mrt}$	ENU mutant	- Autoantibodies - Impaired Th function - Elevated IgG		(Siggs et al., 2007)

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