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Microbiota-induced tonic type I interferons instruct a transcriptional, epigenetic and metabolic program that defines the poised basal state of conventional dendritic cells

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Für meine Familie

Zu einem guten Ende gehört auch ein guter Beginn.

~ Konfuzius ~

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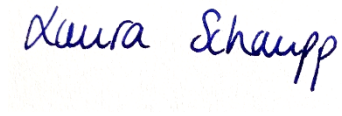

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1 Abstract

Border surfaces in the human body are colonized in high density by bacteria, fungi and viruses, commonly referred to as microbiota. It has becoming increasingly clear that, depending on environmental factors and host genetics, the microbiota has profound effects on shaping host physiology. The microbial impact is not only restricted, for example, to the support of nutrient uptake and epithelial tissue homeostasis, but also largely contributes to the education of the host's immune system.

In my dissertation, I present evidence that microbiota-derived signals are required to program splenic conventional dendritic cells (cDCs) during steady-state, so that they can immediately respond to pathogen encounter with firstly, the production of pro-inflammatory cytokines and secondly, the induction of the adaptive immune system by activating T cells.

The molecular mechanisms behind this microbiota-dependent instructive program cDCs are undergoing are not well understood though. In this thesis, type I interferons (IFNs) are identified as signaling molecules required for the instruction process of cDCs. These specialized cytokines are constantly produced in steady-state and are required to instruct a poised basal state in cDCs, rendering them highly responsive to pathogens. Specifically, plasmacytoid dendritic cells (pDCs) were found to be the main cellular source of constitutive, microbiota-controlled type I IFNs.

The implementation of genome-wide analyses of transcriptional (RNA-Sequencing) and epigenetic states (ChIP-Sequencing for H3K4me3 and H3K27me3) in cDCs from germ-free (GF) mice and from mice genetically deficient for the type I IFN receptor (*Ifnar1*$^{-/-}$), reveals that tonic type I IFN receptor signaling within cDCs instructs a specific epigenetic and metabolic basal state, that is indispensable for cDC functionality in pathogen defense.

However, such beneficial biological function comes with a trade-off. The microbiota-induced instruction process authorizes cDCs to prime CD8^{+} T cell responses against harmless peripheral antigens in steady-state, in case barriers of peripheral tolerance mechanisms are removed.

Collectively, I provide new insights into how the indigenous microbiota affects the immunological functions of cDCs residing in non-mucosal tissue on a transcriptional, epigenetic and metabolic level. This thesis contributes to a better understanding of the evolutionary trade-offs that come with the successful adaptation of vertebrates to their microbial environment.

2 Zusammenfassung

Sämtliche Zelloberflächen im menschlichen Körper sind von einer Vielzahl an Mikroben, wie Bakterien, Pilze und Viren, besiedelt, die in ihrer Gesamtheit als Mikrobiota bezeichnet wird. Abhängig von Umwelt- und genetischen Faktoren, nimmt die Mikrobiota einen wesentlichen Einfluss auf zahlreiche physiologische Funktionen ihres Wirts, worunter sowohl das angeborene als auch adaptive Immunsystem fällt.

Diese Arbeit zeigt, dass in der Milz lokalisierte konventionelle dendritische Zellen (cDCs) von der Mikrobiota kommende Signale im Steady-State benötigen, um sofort gegen eindringende Pathogene agieren zu können, indem sie pro-inflammatorische Zytokine ausschütten und T-Zellen des adaptiven Immunsystems aktivieren. Die molekularen Mechanismen, die diesem Mikrobiota-induzierten Instruktionsprogramm zugrunde liegen und welches cDCs durchlaufen, sind allerdings kaum entschlüsselt. In dieser Studie werden Typ I Interferone (IFN) als Schlüsselmoleküle identifiziert, die essentiell für den Instruktionsprozess von cDCs sind. Diese speziellen Zytokine werden permanent im Steady-State produziert und sind notwendig, um cDCs in eine Art Bereitschaftszustand zu versetzen, der sie dazu befähigt umgehend auf Pathogene reagieren zu können. Dabei konnten plasmazytoide dendritische Zellen (pDCs) als Hauptproduzenten von Mikrobiota-kontrolliertem, konstitutivem Typ I IFN identifiziert werden.

Die Analysen des Transkriptoms (RNA-Sequenzierung) und Epigenoms (H3K4me3 und H3K27me3) von cDCs aus keimfreien Mäusen und genetisch manipulierten Mäusen, die defizient für den IFN-I-Rezeptor (*Ifnar1*$^{-/-}$) sind, zeigen, dass der durch IFN-I induzierte Bereitschaftszustand in cDCs ebenfalls mit epigenetischen und metabolischen Veränderungen assoziiert ist und für ihre biologische Funktion in der Immunabwehr essentiell ist.

Allerdings geht mit dieser eigentlich sehr nützlichen Fähigkeit ein gewisser biologischer Konflikt einher. Denn der durch die Mikrobiota-induzierte Instruktionsprozess von cDCs befähigt diese im Steady-State CD8^{+} T-Zellen auch gegen harmlose Antigene zu aktivieren, wenn die Mechanismen der peripheren Toleranz aufgehoben werden.

Zusammenfassend liefert die vorliegende Arbeit neue Erkenntnisse darüber, wie die Mikrobiota die immunologische Funktion von in nicht-mukosalen Geweben residierenden cDCs auf transkriptioneller, epigenetischer und metabolischer Ebene beeinflusst. Sie trägt zu einem besseren Verständnis über der evolutionär bedingten biologischen Konflikte bei, die mit der erfolgreichen Adaption von Vertebraten an ihr mikrobielles Umfeld einhergehen.

3 Introduction

In higher vertebrates, protection against infectious microbes is mediated by the immune system that is able to discriminate between antigens originating from the external environment ("nonself") and antigens derived from within the body ("self"). While it is able to initiate a powerful response towards the former one, it remains tolerant towards the latter one in order to prevent the onset of autoimmune diseases. The host immune system is divided into two layers: innate and adaptive immunity that differ in cell composition and effector functions but yet communicate on various levels.

The innate immune system is indispensable for pathogen combat in the first hours post-infection as it promptly elicits a response. The key components of innate immunity are the epithelium and antimicrobial proteins, representing anatomical and chemical barriers, the complement system, and various innate immune cell populations, including macrophages, monocytes and conventional dendritic cells (cDCs), which are commonly referred to as mononuclear phagocytes (MPhs), plasmacytoid dendritic cells (pDCs), neutrophils, mast cells, natural killer (NK) and innate lymphoid cells (ILC). Most of those immune cells, like cDCs, macrophages and mast cells are constantly present in almost every tissue, patrolling as sentinels for invading pathogens (Branzk et al., 2018; Iwasaki and Medzhitov, 2004; Swiecki and Colonna, 2015). The components of innate immunity and their specific functions will be discussed in more detail in Chapter 3.2.

Adaptive immune responses occur later and are mediated by B and T lymphocytes. These immune cells bear one single antigen receptor, namely the B or T cell receptor (BCR/TCR), respectively. However, due to somatic recombination of germline-encoded receptor segments, they are able to recognize a vast number of distinct antigens, commonly known as diversity, and thus, permit a very specific reaction. To prevent reactivity towards "self" antigens, several tolerance mechanisms developed that generally include elimination of lymphocytes expressing receptors specific for "self" antigens and self-reactive lymphocytes, or suppressing their activity by other regulatory cells. Another feature of adaptive immunity is its generation of long-lived and antigen-specific memory cells during the primary immune responses. This immunologic memory function provides the basis for the immune system's capability to initiate a much faster and stronger response if the same antigen is encountered a second time (Litman et al., 2010).

The immune system is constantly exposed to microbes as border surfaces, such as the gastrointestinal, respiratory and genitourinary tract and the skin. These surfaces are colonized by a vast number of bacteria, fungi, viruses and other microbial or eukaryotic species,

collectively known as the microbiota. It is suggested that the microbiota has co-evolved with its host to maintain a symbiotic relationship, affecting multiple physiological processes within the host (Honda and Littman, 2012). The influence of the microbiota on the development, education and function of the host's immune system will be elucidated in the following chapters.

3.1 Microbiota – Host interactions

Border surfaces that are permanently in close contact with the commensal microbiota represent defined environments with specialized compartmentalization, cell types and native defense mechanisms. The microbiota's ability to shape mucosal immunity is well acknowledged. In addition, its influence on systemic innate and adaptive immune responses is becoming increasingly clear. Under optimal, physiological circumstances, the commensal microbiota, considered as nonpathogenic, exists in a mutualistic interaction with the host. However, distinct exogenous factors, e.g. diets or antibiotics, can affect the composition of the indigenous microbiota, which may favor the outgrowth of pathogens or opportunistic pathogens (Turnbaugh et al., 2009). Pathogens, such as *Mycobacterium tuberculosis* or the enteric pathogenic species of *Salmonella* and *Shigella*, are generally defined by their ability to cause disease in healthy hosts, while opportunistic pathogens, e.g. *Clostridium difficile* or *Staphylococcus epidermidis,* have a restricted infectious potential and require an impaired immune system or/and a particular microbiota composition to induce disease (Dekker and Frank, 2015; Hornef, 2015). Further, an increasing number of bacteria are classified as pathobionts. Although a consistent definition for this term has not been provided yet, pathobionts are originally considered as symbionts, but having the potential to initiate pathology by influencing the host's immune system when definite genetic and environmental factors are altered (Chow and Mazmanian, 2010; Jochum and Stecher, 2020). In previous studies, for instance, *Helicobacter hepaticus*, distinct *Escherichia coli* strains, *Enterococcus faecalis* and *Streptococcus pneumoniae* have been denoted as pathobionts (Ayres et al., 2012; Balish and Warner, 2002; Chow and Mazmanian, 2010; Leonard et al., 2020).

Given the close interaction of microbes with the host, an improved understanding of the complex microbiota-host interplay is pivotal, especially in the context of developing novel therapeutic strategies to either prevent or treat diseases associated with an altered microbiota.

3.1.1 Impact of the microbiota on host health

Microbial colonization begins during the passage through the maternal birth canal. The composition of the neonate's microbiota is highly similar to their mother's vaginal microbiota, characterized by a prevalence of *Lactobacillus*, *Prevotella*, or *Sneathia* spp. In contrast, infants delivered by cesarean section display a distinct microbial structure, mainly composed of *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp., bacteria usually found on the skin (Dominguez-Bello et al., 2010). The mechanisms how neonates adapt to their increasing exposure to the microbial environment is still poorly understood. However, maternal milk was shown to contain Immunoglobulin A (IgA), immune cells, cytokines and microbes that support microbiota constitution and shape the host's responses towards those microbes (Belkaid and Hand, 2014). The end of the breast-feeding period is associated with an alteration of the intestinal microbiota composition, as obligate anaerobes become more present, whereas the number of facultative anaerobes decreases (Honda and Littman, 2012). In addition, the virome, describing the entirety of all viruses and virus-derived components within the human body, becomes established during infancy, driven by the environment and maternal contact, and remains relatively stable in adults over time (Breitbart et al., 2008; Reyes et al., 2010; Virgin, 2014). It is assumed that with the age of three years the microbiota composition becomes nearly stable, nevertheless showing interindividual variation (Schloissnig et al., 2013; Yatsunenko et al., 2012). It is estimated that the human small intestinal harbors up to 10^3 to 10^9 bacterial cells/ml intestinal contents, the large intestine can even achieve a microbial density of 10^{11} to 10^{12} cells/ml intestinal contents, converting the colon into a nutritious and protecting environment for microbial growth, likely resembling a natural bioreactor (Berg, 1996; Ley et al., 2006).

The adult human gut microbiota is predominated by four phyla, among these more than 90 % belong to Firmicutes (including *Clostridium*, *Lactobacillus*, and *Bacillus*) and Bacteroidetes (including *Bacteroides* and *Prevotella*), whereas Proteobacteria (including *Escherichia*) and Actinobacteria (including *Bifidobacterium*) are less abundant (Eckburg et al., 2005). Extensive studies like the Human Microbiome Project or METAgenomics of the Human Intestinal Tract (MetaHit) have revealed though that at the lower taxonomic level a clearly higher variation between individuals exists. This is probably due to the fact that the composition of microbial communities is deeply affected by various lifestyle factors, such as nutrition, geographical location, intake of antibiotics or other medicines (Dethlefsen et al., 2008; Peterson et al., 2009; Qin et al., 2010; Turnbaugh et al., 2009). Host genetics were shown to be an additional factor in shaping the intestinal microbiota, mainly affecting the

bacterial and not so much the viral communities (Benson et al., 2010; Reyes et al., 2010). Despite these high interpersonal variances in the microbiota composition, a bacterial core gene set (~ 35 %) is shared across individuals, indicating that diverse microbial compositions may exhibit similar function (Burke et al., 2011; Qin et al., 2010). Additionally, a separation of the human population into three enterotypes is suggested, defined by altered levels of one of the following genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3) (Arumugam et al., 2011). However, the mechanisms by which the enterotypes influence the host's fitness remain to be elusive.

Over the past years, several studies have elucidated the numerous favorable effects of the commensal gut microbiota on host health and physiology, for instance, support in food digestion and nutrients uptake, intestinal development, education of the immune system and protection against invading pathogens (Bouskra et al., 2008; Koh and Bäckhed, 2020; Lathrop et al., 2011). It has been shown that some of those effects are mediated by microbiota-derived metabolites, such as short-chain fatty acids (SCFA), ATP, bile acids and tryptophan metabolites, functioning as communicators between the host and its microbiota (Koh and Bäckhed, 2020; Uchimura et al., 2018). Acetate, butyrate and propionate belong to the most studied SCFAs, which result from microbial fermentation of dietary fibers. They mediate their multifaceted effects on host health by either G-protein coupled receptor (GPCR) activation or inhibition of histone deacetylases (HDAC). For instance, butyrate has been shown to exert immunomodulatory functions through regulating IL-10 production by T cells and IL-18 secretion in the intestinal epithelium (Koh et al., 2016). Bile acids, which are generated from cholesterol in the liver, are released upon food intake to assist in lipid absorption and are known to signal through the nuclear farnesoid X receptor (FXR) and Takeda G protein-coupled receptor 5 (TGR5) (Maruyama et al., 2002; Sinal et al., 2000). Intestinal bacteria with bile salt hydrolase-activity further metabolize bile acids to prevent bile toxicity (Jones et al., 2008). However, if deconjugated bile acids enter the colon, they are converted into secondary bile acids, mostly lithocholic or deoxycholic acid, by specific bacteria, e.g. *Clostridium* (clusters XIVa and XI) and *Eubacterium*. Increased levels of deoxycholic acid have been reported to be associated with cancer and obesity in mice, indicating their profound ability to impact host metabolism (Yoshimoto et al., 2013). Tryptophan can be metabolized by the microbiota into different products that partially activate the aryl hydrocarbon receptor (AhR). Commonly, murine AhR activation is associated with maintenance of gut homeostasis and decreased inflammation (Cervantes-Barragan et al., 2017; Wlodarska et al., 2017). In a recent study, the tryptophan-derived microbial metabolite indole-3-aldehyde has been demonstrated

to have a protective role in a mouse model of autoimmune diabetes by inducing IL-22 production in pancreatic ILCs (Miani et al., 2018).

However, those beneficial effects of the microbiota on host physiology can turn into a detrimental relation, if the gut microbial composition changes in a way that growth of potential pathogenic bacteria is favored and/or the presence of commensal bacteria is diminished, a process also known as dysbiosis (Levy et al., 2017). Previous studies have shown that the imbalance of the intestinal microbiota is linked to several disorders, such as inflammatory bowel disease (IBD), obesity, type 2 diabetes, asthma, rheumatoid arthritis (RA) and celiac disease (Elinav et al., 2011; Gevers et al., 2014; Mazmanian et al., 2005; Qin et al., 2012; Turnbaugh et al., 2006). Animal models of obesity, either induced by high-fat diet or genetic manipulations, are associated with a dramatic alteration in the microbiota composition in which the number of *Firmicutes* is enriched, whereas the number of *Bacteroidetes* is decreased. The shift in the relative distribution of these indigenous phyla results in an enhanced capacity to resorb energy from ingested food (Ley et al., 2005). Interestingly, solely the transplantation of fecal microbiota from obese mice into healthy recipients induced the obese phenotype (Turnbaugh et al., 2006). Similar findings could be obtained by recent studies investigating the microbiota's impact on IBD development, a disease associated with a constant dysbiotic state that favors the outgrowth of *Proteobacteria* and *Actinobacteria*, thus promoting inflammation (Frank et al., 2007; Saleh and Elson, 2011). For instance, transmission of specific bacteria or the complete intestinal microbiota of IBD patients, that is generally of lower complexity compared to healthy individuals, into germ-free mice dramatically exacerbate intestinal inflammation in distinct colitis models (Britton et al., 2019; Frank et al., 2007; Palm et al., 2014).

Collectively, these examples illustrate the powerful influence of the microbiota on host health, as dysbiosis, induced by environmental or genetic factors, causes several diseases that are mostly associated with a disturbed immune reactivity. In order to develop new potentially microbiota-modulating therapies to prevent or treat such diseases, it is essential to understand the complex interplay between the microbiota and the host's immune system.

3.1.2 Microbiota as an immune modulator

Millions of years of co-evolution established under optimal circumstances a beneficial alliance between host and the microbiota that is based on constitutive sensing of commensals by the innate immune system via so called microbial-associated molecular patterns (MAMPs)

(Belkaid and Hand, 2014). Innate immune cells are equipped with a plethora of distinct pattern-recognition receptors (PRRs). Among these, mainly Toll-like receptors (TLRs) are used to recognize MAMPs, including bacterial cell wall components, like lipopolysaccharide (LPS) and peptidoglycan or flagellin. In addition, several studies have found that, depending on the anatomical location, sensing through TLRs could also mediate immunologic tolerance and inhibit inflammatory responses. Consequently, TLR deficiencies lead to an impaired development of gut and mucosal immune systems (O'Hara and Shanahan, 2006; Round et al., 2011).

The requirement for constitutive signaling of the microbiota to maintain the intestinal epithelial integrity and host-commensal-homeostasis, including innate and adaptive immunity, is demonstrated by numerous studies using germ-free (GF) mouse models that display several immune impairments (Bouskra et al., 2008; Hooper and Macpherson, 2010; Ichinohe et al., 2011; Rakoff-Nahoum et al., 2004). GF mice are born and housed in sterile, over-pressured flexible film isolators under strict axenic conditions. Together with gnotobiots, mice that are colonized with single bacterial strains or a more complex microbiota of known identity, they represent an important and versatile tool to investigate the impact of the microbiota on the host's immune system (Hapfelmeier et al., 2010; Smith et al., 2007). Administration of antibiotics is an additional, commonly used method to deplete specific bacterial taxa or the indigenous microflora. However, it is important to consider that microbial communities mainly exist in a complex interplay with each other. For instance, it has been shown that vancomycin treatment, an antibiotic that attacks gram-positive bacteria, leads also to a reduction of gram-negative bacteria of the phylum *Bacteroidetes* (Sekirov et al., 2008; Ubeda et al., 2010).

As a consequence, the constant sensing of commensal microbes is absent in GF mice, resulting in an impaired production of tissue repair factors, antimicrobial proteins and IgA, leading to disturbed barrier integrity and translocation of bacteria across the epithelial layer (Honda and Littman, 2012; Rakoff-Nahoum et al., 2004). Especially, microbiota-induced IgA production plays a crucial role in mediating protection of mucosal surfaces and support host-commensal mutualism (Clemente et al., 2012; Macpherson et al., 2011; Peterson et al., 2007).

GF mice also display an impaired development of intestinal lymphoid structures, such as Peyer's patches (PP), cryptopatches (CP) and isolated lymphoid follicles (ILFs), and also have smaller mesenteric lymph nodes (Bouskra et al., 2008; Round and Mazmanian, 2009). CPs are small cell clusters mainly composed of RORγt-expressing lymphoid tissue inducer (LTi)-like group 3 innate lymphoid (ILC3) cells and cDCs, that are located below small

intestinal crypts. With increasing microbial colonization B cells are recruited to CPs that mature subsequently into ILFs, representing organized B cell follicles (Eberl and Littman, 2004; Pabst et al., 2005). PP are nodule-like lymphoid clusters located in the wall of the small intestine that are composed of a subepithelial dome, B cell follicles and interfollicular T cell area (Hooper and Macpherson, 2010). M cells within the epithelium overlaying the PP or lamina propria cDCs constantly take up antigens from bacteria associated with mucosal surfaces. Antigen-carrying cDCs migrate to PP or mesenteric lymph nodes where they induce B cell differentiation into IgA-producing plasma cells. IgA^+ plasma cells then translocate through lymph and blood to the lamina propria and secrete IgA that is transcytosed across the epithelium. On the luminal site, IgA binds to bacteria, thus, minimizing the bacteria-epithelium contact and restricting bacterial infiltration into host tissue (Macpherson and Uhr, 2004). Additionally, ILC3s have been shown to support IgA production as they promote B cell activation by secreting ligands of the TNF superfamily, such as lymphotoxin (LT) $\alpha_1\beta_2$, $LT\alpha_3$, RANKL or BAFF (Kruglov et al., 2013; Tsuji et al., 2008). Importantly, microbial signals further increase ILC3-mediated IL-22 secretion which plays a crucial role in the production of AMPs in epithelial cells, such as RegIIIγ, and intestinal stem cell maintenance (Gronke and Diefenbach, 2015; Lindemans et al., 2015; Mukherjee and Hooper, 2015). Consequently, this positive effect of ILC3s on epithelium maintenance is diminished in GF mice (Sanos et al., 2009; Satoh-Takayama et al., 2008).

$CD4^+$ T cells belong to another group of immune cells that are frequently present in mucosa-associated tissues and whose functionality and numbers are greatly affected by the commensal microflora. In steady-state, forkhead box P3 (Foxp3)-expressing regulatory T cells (T_{reg}) and T helper type 17 (Th17) cells are the $CD4^+$ T cell subsets predominantly present in the intestinal lamina propria, specifically in the small intestine. $Foxp3^+$ T_{regs} play a crucial role in mediating immunological tolerance towards "self" antigens and suppressing hyperreactive immune cells, e.g. colitogenic effector T cells (Asseman et al., 2003; Sakaguchi et al., 2008). In antibiotics-treated or GF mice the numbers of $Foxp3^+$ T_{reg} cells residing in the colonic LP are significantly reduced (Atarashi et al., 2011). Interestingly, bacteria of the genus *Clostridium* were found to be potent inducers of T_{reg} differentiation in the colon (Atarashi et al., 2011). Colonization of GF mice with 46 known strains of *Clostridium* was sufficient to normalize frequencies and absolute numbers of colonic LP T_{regs} and even promoted high expression levels of the immune-suppressive molecules IL-10 and CTLA-4 (Atarashi et al., 2013). Moreover, the genus of *Clostridium* (clusters XIVa and IV) seems to be underrepresented in the microbial community in IBD patients compared to healthy

controls, indicating the beneficial ability of *Clostridium* to shape T_{reg} function in mediating prevention of harmful inflammation and autoimmunity (Frank et al., 2007). Th17 cells that are defined by their production of IL-17A, IL-17F, IL-21 and IL-22, are essential for epithelial homeostasis and host defense against invading microbes. In GF or antibiotics-treated mice, the numbers of intestinal LP Th17 cells, as well as IFNγ-producing Th1 cells, are considerably reduced (Gaboriau-Routhiau et al., 2009; Geuking et al., 2011; Ivanov et al., 2008). Interestingly, varying intestinal LP Th17 cell numbers were observed even in wildtype mice maintained in different SPF facilities. Those distinct ratios were ascribed to one single bacterium, namely segmented filamentous bacteria (SFB) (Ivanov et al., 2009). SFB are known to be potent inducers of IgA production and studies using mono-colonized GF mice have revealed that SFB also strongly promote intestinal Th17 cell accumulation and responses (Gaboriau-Routhiau et al., 2009; Talham et al., 1999). A recent study has demonstrated that the functionality of intestinal IFNγ-producing $CD8^+$ T cells is also regulated by the indigenous microbiota. In antibiotics-treated or GF mice, numbers and frequencies of intestinal $IFN\gamma^+$ $CD8^+$ T cells were greatly reduced. Re-colonization of GF mice with a consortium of 11 bacterial strains, isolated from the human fecal microbiota, restored this deficit in $IFN\gamma^+$ $CD8^+$ T cell accumulation, leading to an improved protection against *Listeria monocytogenes* infection and an enhanced anti-tumor immunity (Tanoue et al., 2019).

Importantly, the microbiota not only affect mucosal immunity, but also has a deep impact on shaping systemic immune responses. For instance, peritoneal macrophages from microbiota-depleted mice have been shown to display dysfunctional responses to type I and II IFNs and an impaired ability to restrict viral replication, rendering antibiotics-treated mice more susceptible to lymphocytic choriomeningitis virus (LCMV), murine cytomegalovirus (MCMV) or influenza virus infection (Abt et al., 2012; Ganal et al., 2012). In line with this, mononuclear phagocytes of GF mice residing in non-mucosal, lymphoid tissues were unable to upregulate pro-inflammatory cytokine and type I IFN production in response to microbial stimulation. Consequently, NK cell priming and antiviral immunity were markedly impaired (Ganal et al., 2012). Furthermore, neutrophils in the bone marrow required signaling of peptidoglycan via the PRR NOD1 to be fully functional. Peptidoglycan derived from the intestinal microbiota reached the neutrophils in the bone marrow via systemic circulation. Thereby, neutrophil function correlated with serum peptidoglycan concentrations (Clarke et al., 2010). Another study has demonstrated that the commensal microbiota regulates the activation of $CD4^+$ and $CD8^+$ T cells following respiratory influenza virus infection. Microbiota-derived signal were required to initiate an inflammasome-dependent migration of

lung cDCs to the draining lymph node where T cell priming occurred (Ichinohe et al., 2011). Additionally, the commensal microflora also affects the homeostasis of microglia, a specific cell type of brain-resident macrophages. Upon viral infection, GF mice display an impaired microglial immune response, which manifests itself in a reduced production of pro-inflammatory cytokines, such as TNF and IL-1β, and is accompanied by remarkable morphological changes. Further, it was demonstrated that those microbiota-driven effects were mediated by SCFAs (Erny et al., 2015).

Altogether, these studies highlight the multifaceted impact of the microbiota on host health and fitness, with a special focus on the education of the immune system at local mucosal sites, but also on a systemic level. Only in rare cases, an immune phenotype is caused by a single effector strain, such as the SFB-driven Th17 cell responses in the intestinal LP. Microbial communities mostly stay in a complex interaction with each other. The development of next-generation sequencing technologies and analytical approaches, such as the GF and gnotobiotic mouse models, improved our understanding of, not only, the complex host-microbiota interaction on a molecular level, but also highlighted the existing interdependency of distinct microbial populations that may need to be considered additionally. Those new insights provide a useful basis for future development of microbiota-modulating therapies to prevent or treat diseases.

3.2 Innate host response

The innate immune system represents the first line of defense upon pathogen encounter and it consists of distinct but fine-tuned defense mechanisms.

The first level of innate defense comprises epithelial surfaces that function as physical barriers preventing the entry of pathogens. This barrier function is further supported by the production of mucus, AMPs and IgA (Gallo and Hooper, 2012; Zasloff, 2002). Another defense mechanism relies on the complement system in which soluble proteins tag pathogens for phagocytosis.

The third level of innate defense is based on sensing of microbial invaders by specialized receptors localized on the surface of immune cells, which then orchestrate a powerful immune response. These inborn PRRs are germline-encoded and thus, do not rely on genetic recombination as it is the case for antigen receptors found on the cell surface of lymphocytes, the branch of adaptive immunity (Janeway and Medzhitov, 2002).

3.2.1 Antimicrobial proteins

Epithelial cells and phagocytes contribute with the production of bactericidal agents which comprise antimicrobial enzymes and various peptides (Lai and Gallo, 2009). Those antimicrobial peptides include defensins, cathelicidins and histatins that are secreted into the mucus layer at mucosal surfaces or released into tissues by phagocytes. Their mode of action is based on electrostatic interactions with bacterial membrane lipids, leading to membrane disruption (Zhang and Gallo, 2016). Another group of bactericidal peptides comprises lectins that mediate their antimicrobial function by binding carbohydrate structures in the bacterial cell wall component peptidoglycan. A prominent example is the C-type lectin RegIIIγ that is exclusively produced by Paneth cells of the intestinal epithelium (Lehotzky et al., 2010). In contrast, bactericidal enzymes, e.g. secretory phospholipase A_2 or lysozyme, digest bacterial cell walls by hydrolyzing phospholipids or by cleaving chemical bonds in the peptidoglycan structure, respectively (Zhang and Gallo, 2016).

3.2.2 Complement activation

The complement system represents a more specialized network with more than 30 distinct plasma proteins. Complement proteins, usually present as inactive zymogens, are activated in the presence of pathogens or by antibodies bound to pathogens and initiate a sequential cascade of proteolytic reactions resulting in the final activation of a C3 convertase (Janssen et al., 2005). This enzyme generates different peptides that in turn induce different effector pathways. For example, the anaphylatoxins C3a and C5a promote inflammation by recruiting phagocytes to the sites of infection. C3b functions as opsonin by binding covalently to the microbial surface and thus facilitating engulfment of microbes by phagocytic cells (Janssen et al., 2006). Another effector molecule is C5b which assemble with several terminal complement proteins (C6-9) into a membrane-attack complex (MAC) on the surface of pathogens, forming a pore within the cell wall that finally results in cell lysis (Serna et al., 2016). Since the activation of the complement system leads to strong inflammatory responses, it underlies strict regulatory mechanisms preventing the onset of autoimmune diseases, such as multiple sclerosis or asthma (Ingram et al., 2009; Wills-Karp, 2007).

3.2.3 Pathogen recognition receptor signaling

As pathogens also developed strategies to resist antimicrobials and to overcome complement activation, another level of innate immunity evolved that includes direct sensing of invading microbes by specific innate immune cells equipped with PRRs, leading to the induction of an effective immune response.

Numerous immune cells, e.g. macrophages, monocytes, cDCs and pDCs, but also non-immune cells, like epithelial cells, express various PRRs on their cell surface enabling them to sense structural components of invading microbes, so called pathogen-associated molecular patterns (PAMPs) (Janeway and Medzhitov, 2002). Until now, several PRR systems have been characterized. These include the membrane-bound Toll-like receptor (TLR) family, the cytoplasmatic sensors of NOD-like receptors (NLR) and RIG-I-like receptors (RLR), as well as the more recently described sensor modules stimulator of interferon genes (STING) and cyclic GMP-AMP (cGAMP) synthase (cGAS).

Although recognizing different PAMPs and initiating different intracellular signaling programs, the outcome of each PRR ligation is the production of pro-inflammatory and anti-viral cytokines in order to induce of a powerful immune response.

3.2.3.1 Toll- like receptor signaling

TLRs belong to the most ancient family of proteins that met the prerequisites of a PRR according to Janeway's prediction (Janeway, 1989). By now, 10 and 12 different *Tlr* genes were identified in humans and in mice, respectively. All receptors are transmembrane proteins and share a conserved protein structure composed of an N-terminal ectodomain containing leucine-rich repeats (LRR) and a C-terminal cytoplasmic area termed Toll-IL-1-receptor (TIR) domain (Medzhitov et al., 1997; Rock et al., 1998).

TLRs can be found at two distinct cellular sites: either they are located at the cell surface binding extracellular ligands (TLR-1, 2, 4, 5, 6, 11) or they are anchored in endosomal membranes sensing intracellular PAMPs (TLR-3, 7, 8, 9, 13). TLRs are able to recognize a broad spectrum of microbial molecular patterns that include bacterial components, e.g. flagellin (TLR-5), lipoproteins (TLR-1, 2, 6), lipopolysaccharides (LPS) (TLR-4), and motifs present in protozoan parasites like profilins (TLR-11) (Akira et al., 2006; Lee et al., 2014; Park et al., 2009). The endosomal TLRs detect virus-derived nucleotide structures, e.g. double- or single stranded ribonuclear acid (dsRNA or ssRNA) (TLR-3, 7, 8), bacterial ribosomal (r) RNA (TLR-13) or unmethylated DNA (TLR-9) (Ohto et al., 2018; Shibata et

al., 2016; Tanji et al., 2013). Ligand binding initiates a dimerization of the receptors allowing the cytoplasmic TIR domains to interact with specific adaptor molecules that are essential for TLR signaling. Mammalian TLRs employ four adaptors named myeloid differentiation primary response protein 88 (MyD88), MyD88-adapter-like protein (MAL), TIR domain-containing adapter inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM) in different combinations (Horng et al., 2001; Lin et al., 2010). The majority of TLRs exclusively use MyD88 as adaptor protein, whereas TLR-3 requires only TRIF for signaling. The TLR heterodimers TLR-2-1/2-6 interact with the combination of MyD88 and MAL. TLR-4 requires both adaptor pairings, MyD88/MAL and TRIF/TRAM (Bonham et al., 2014; Motshwene et al., 2009). Most notably, in dependency on which adaptor molecule will be activated by TLR ligation, different downstream signaling cascades will be initiated (Fig. 1).

The activation of MyD88 by TLRs located on the cell surface leads to the recruitment of IL-1-receptor associated kinases 1 and 4 (IRAK1/4) which act as a scaffold, facilitating the binding of the protein termed tumor necrosis factor receptor-associated factor 6 (TRAF6). Together they recruit another signaling complex composed of the TGF-β-activated kinase 1 (TAK1) binding proteins (TAB1/2) and the serine/threonine kinase TAK1 which activates several mitogen-activated protein kinases (MAPK) and the IκB kinase β (IKKβ) by phosphorylation. IKKβ in turn phosphorylates the inhibitor of κB (IκB) which releases the transcription factor nuclear factor κB (NF-κB) into the nucleus inducing transcription of genes encoding for pro-inflammatory cytokines like TNF, IL-1β, IL-6 and IL-12 (Emmerich et al., 2013; Tan and Kagan, 2019).

The TLRs that signal through TRIF/TRAM (TLR-3, 4) activate the E3 ubiquitin ligase TRAF3. This protein acts as scaffold and in turn recruits the kinases IKKε and TANK-binding kinase 1 (TBK1) which phosphorylate the transcription factor interferon regulatory factor 3 (IRF3). Activated, it then translocates into the nucleus initiating the transcription of type I interferon (IFN) genes, promoting antiviral defense. The ligation of the nucleic-acid sensing TLRs (TLR-7, 8, 9, 13) also leads to the production of type I IFNs, with the difference that gene transcription is induced by IRF7 which is previously activated by the IRAK/TRAF complex (Häcker et al., 2006; Kawai et al., 2004).

TLRs can activate the transcription of both NF-κB and IRF target genes, which enable them to promote either antibacterial or antiviral immune responses.

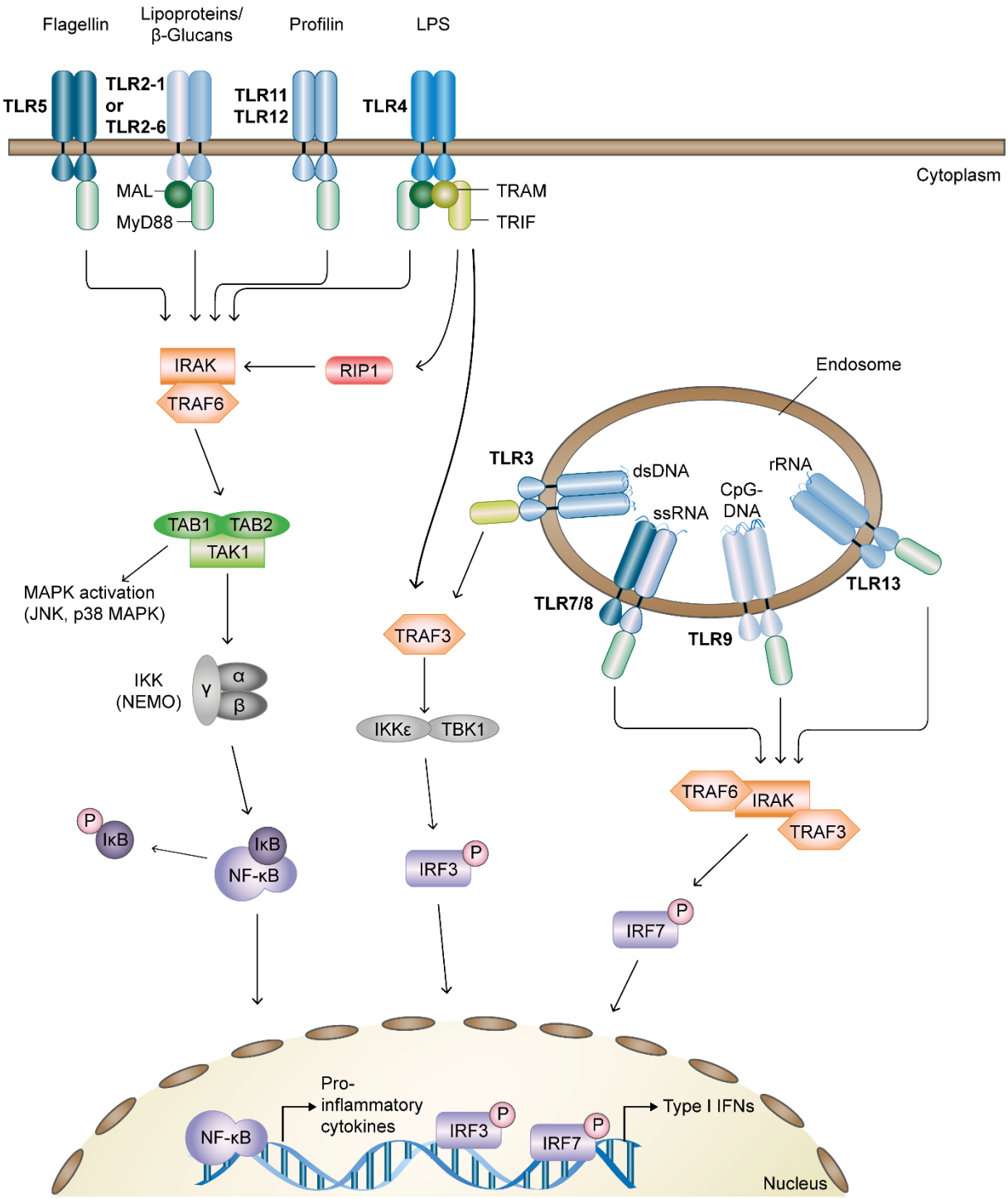

Figure 1: Toll-like receptors are an ancient family of pattern-recognition receptors.

TLR-5, 4, 11 and the heterodimers TLR-2/1 or 2/6 are localized on the cell surface recognizing extracellular PAMPs, e.g. LPS or flagellin, whereas the nucleic acid-sensing TLR-3, 7, 8, 9 and 13 are anchored in endosomal membranes. TLR signaling requires the adaptor proteins MAL, MyD88, TRAM and TRIF that, depending on each TLR, are associated in different constellations with the cytosolic TIR domains of the receptors. Ligation of TLRs induces their dimerization, thus activating the respective adaptor proteins. Signaling through MAL/MyD88 leads either to activation of pro-inflammatory cytokine gene expression via NF-κB and MAPK induction (surface TLRs) or to the production of type I interferons by IRF7 activation (endosomal TLRs). TRIF/TRAM associated TLRs induce the expression of type I interferon through IRF3 activation. IKK: IκB kinase; IκB: inhibitor of κB; IRAK: IL-1-receptor associated kinases; IRF: interferon regulatory factor; LPS: Lipopolysaccharide; MAPK: mitogen-activated protein kinases; NF-κB: factor nuclear factor κB; MyD88: myeloid

3.2.3.2 Nucleotide-binding oligomerization domain (NOD)-like receptor signaling

NLRs are cytosolic sensors that recognize not only PAMPs, but also host-derived molecules released from injured cells, e.g. ATP or hyaluronan, commonly referred to as damage/danger-associated molecular patterns (DAMPs) (Mariathasan et al., 2006; Yamasaki et al., 2009). The NLR family comprises numerous members sharing a similar domain organization, including a central nucleotide-binding NACHT domain enabling oligomerization and a C-terminal LRR region involved in ligand sensing. The N-termini of each NLR vary and thus different subfamilies can be classified (Lamkanfi and Dixit, 2014). In humans five of such subfamilies exist, and the members of the NLRC and NLRP subfamilies belong to the most prominent and best-characterized NLRs (Platnich and Muruve, 2019).

The nucleotide-binding oligomerization domain-containing proteins 1 and 2 (NOD1, 2) are considered as founding NLRs and exhibit one or two N-terminal caspase activation and recruitment domains (CARD), respectively, allowing for recognition of bacterial peptidoglycan structures (Strober et al., 2006). Specifically, NOD1 detects γ-glutamyl diaminopimelic acid (iE-DAP), whereas NOD2 recognizes muramyl dipeptides (MDP), but ligation of both NOD1 and 2 results in the activation of MAPK and NF-κB-dependent gene transcription of pro-inflammatory cytokines (Chamaillard et al., 2003; Girardin et al., 2003; Philpott et al., 2014).

The members of the NLRP family are characterized by an N-terminal pyrin domain and have been reported to signal through inflammasome formation. Inflammasomes are intracellular multiprotein complexes composed of the respective NLRP, the apoptosis-associated speck like protein (ASC) and the inactive protease pro-caspase 1 (Broz and Dixit, 2016; Lamkanfi and Dixit, 2014). Inflammasome activation initiates autocleavage of pro-caspase-1. The resulting active enzyme accomplishes the proteolytic processing of immature pro-inflammatory cytokines, specifically IL-1β and IL-18, into their active forms and induces the inflammatory cell death termed pyroptosis (Agostini et al., 2004; Bergsbaken et al., 2009; Sutterwala et al., 2006).

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differentiation primary response protein 88; MAL: MyD88-adapter-like protein; P: phosphorylation; TAK1: TGF-beta-activated kinase 1; TAB: TAK binding proteins; TBK1: TANK-binding kinase 1; TLR: Toll-like receptor; TRAM: TRIF-related adaptor molecule; TRAF: tumor necrosis factor receptor-associated factor; TRIF: TIR domain-containing adapter inducing IFN-β. Modified from (O'Neill et al., 2013; Takeuchi and Akira, 2010)

3.2.3.3 RIG-I-like receptor (RLR) signaling

RLRs are sensors of viral RNA that are localized in the cytoplasm and are expressed in most tissues and cell types (Rehwinkel and Gack, 2020). This protein family consists of three members: the retinoic acid-inducible gene 1 (RIG-I), the melanoma differentiation-associated protein 5 (MDA5) and the receptor termed laboratory of genetics and physiology 2 (LGP2) (Goubau et al., 2013; Loo and Gale, 2011). All three RLR share common structural features including a central DExH/D box helicase domain, involved in RNA binding, and a repressor domain (RD) located within the carboxy-terminal domain (CTD) (Saito et al., 2007; Yoneyama et al., 2004). RIG-I and MDA5 carry two additional CARDs enabling downstream signaling. LGP2 lacks such CARDs and therefore a rather regulatory role is attributed to this RLR (Yoneyama et al., 2005).

In steady-state, RIG-I and MDA5 are present in a closed conformation which is maintained by the close interaction between the CARDs and the helicase domains. RNA binding initiates conformational changes resulting in the release of the CARDs allowing for the recruitment of the E3 Ligases TRIM25 and Riplet (Cui et al., 2008; Kowalinski et al., 2011). These enzymes trigger the generation of a K63-linked polyubiquitin scaffold, either linked to the second CARD or as free polyubiquitin chains, facilitating the interaction of RIG-I and MDA5 with the downstream adaptor protein mitochondrial antiviral signaling protein (MAVS, also known as CARDIF, IPS-1 or VISA) via their CARDs (Fig. 2) (Gack et al., 2007; Zeng et al., 2010). MAVS is anchored in the outer membrane of mitochondria and upon interaction with RIG-I or MDA5 members of the TRAF family are recruited (Paz et al., 2011). Following this, IRF3 and IRF7 are activated via TBK1, resulting in the production of IFN-I, similar as described for TLR-3 signaling. In parallel, MAVS ligation can also be translated into NFκB activation that is mediated by the TNFR-associated death domain (TRADD) protein/Fas-associated death domain-containing protein (FADD) complex and IKK activity, leading to the induction of transcription of pro-inflammatory cytokine genes (Konno et al., 2009; Michallet et al., 2008; Paz et al., 2006). Although initiating the same downstream pathways, RIG-I and MDA5 differ in their ligand specificity. While RIG-I recognizes viral ssRNA through its unprocessed 5'-triphosphate end, from e.g. paramyxoviruses and flaviviruses, MDA5 preferentially binds dsRNA, as it is the case for picornaviruses (Hornung et al., 2006; Kato et al., 2006; Loo et al., 2008; Sumpter et al., 2005).

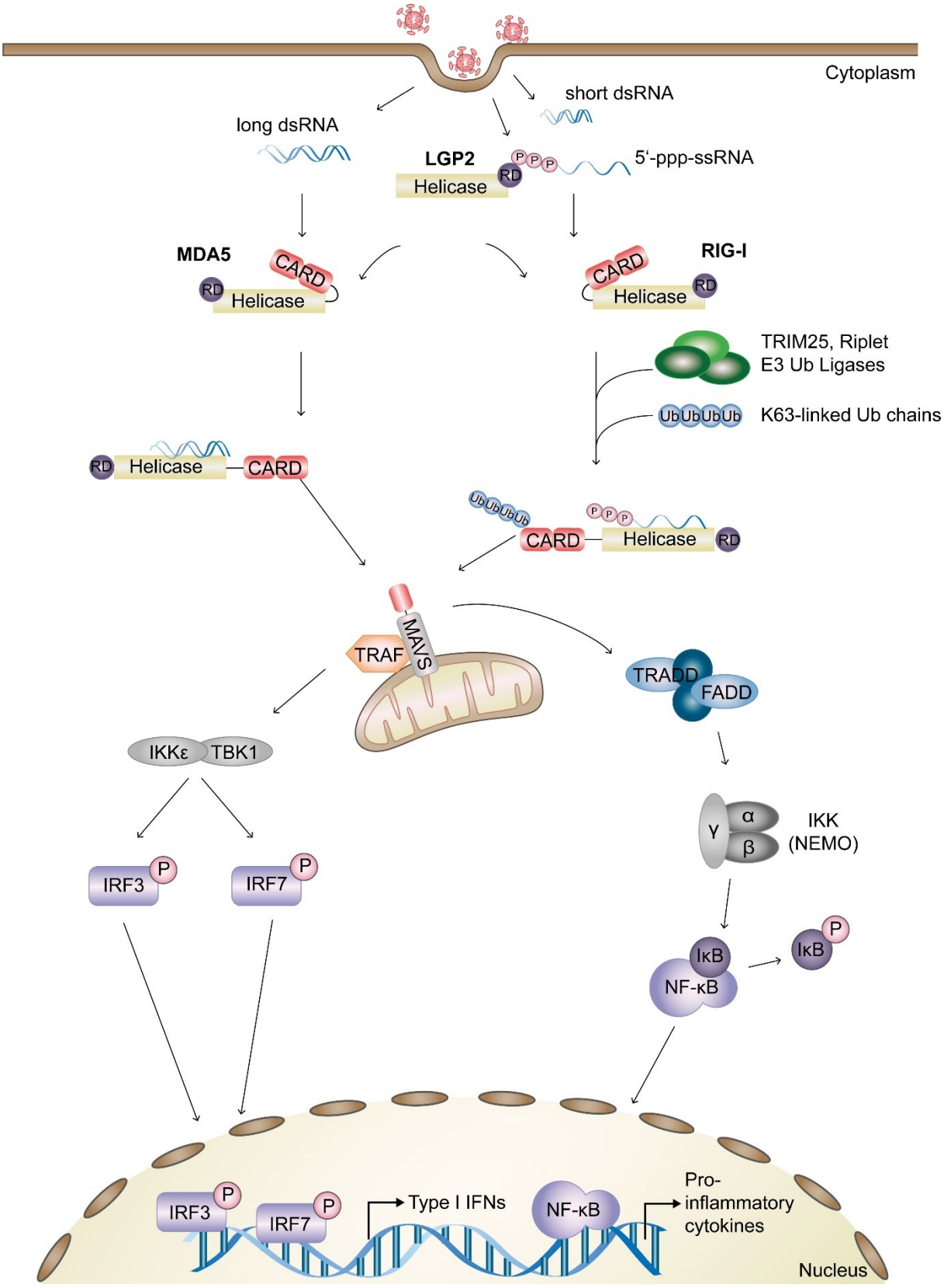

Figure 2: Viral encounters are recognized by the cytoplasmic sensors RIG-I, MDA5 and LGP2.

Viral RNA is recognized by the helicase domains of the sensor proteins. RNA binding induces structural rearrangements of the CARD domains of RIG-I and MDA5 leading to the recruitment of the E3 ligases TRIM25 and Riplet. These enzymes catalyze the generation of K63-linked polyubiquitin scaffolds that facilitate the interaction of RIG-I and MDA5 with the downstream adaptor protein MAVS localized in the mitochondria via their CARDs. MAVS signal transduction leads either to

3.2.3.4 cGAS-STING signaling

The family of microbial nucleic acid sensors also includes, besides the already described RLRs and TLRs (TLR-3, 7, 8, 9), the DNA-sensing enzyme cGAS and its downstream adaptor STING. In mammalian cells, cGAS is localized in the cytoplasm and possesses two DNA binding domains as well as a nucleotidyltransferase domain (Sun et al., 2013). cGAS recognizes dsDNA of different origins, including viral and bacterial DNA, but also host-derived DNA that, for instance, originates from damaged mitochondria or dead cells (Chen et al., 2016; Motwani et al., 2019).

DNA binding causes conformational changes that leads to the induction of enzymatic activity (Civril et al., 2013; Zhang et al., 2014). Activated cGAS catalyzes the generation of 2'3'-cGAMP from ATP and GTP, that then function as second messenger (Kranzusch et al., 2013; Wu et al., 2013). 2'3'-cGAMP binds to STING, an endoplasmic reticulum (ER)-associated adaptor, inducing conformational changes that result in the migration of STING from the ER into an ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus (Dobbs et al., 2015; Ishikawa and Barber, 2008; Saitoh et al., 2009). During this process, STING interacts with the kinases TBK1 and IKK. The former one activates the transcription factor IRF3 by phosphorylation, resulting in the induction of IFN-I production. The latter one regulates NF-κB-dependent transcription of pro-inflammatory genes. (Liu et al., 2015; Tanaka and Chen, 2012) (Fig. 3). STAT6 is another transcription factor reported to be activated by STING and TBK1, leading to the expression of specific chemokines genes, e.g. *Ccl2*, *Ccl20* and *Ccl26*, mainly participating in antiviral defense (Chen et al., 2011).

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gene expression of type I IFNs by IRF3/IRF7 activation or to the production of pro-inflammatory cytokines induced by activated NF-κB. CARD: caspase activation and recruitment domain; FADD: fibroblasts lacking Fas-associated death domain; IκB: inhibitor of κB; IKK: IκB kinase; IRF: interferon regulatory factor; LGP2: laboratory of genetics and physiology 2; MAVS: mitochondrial antiviral signaling protein; MDA5: melanoma differentiation-associated protein 5; NF-κB: nuclear factor κB; P: phosphorylation; RD: repressor domain; RIG-I: retinoic acid-inducible gene 1; TBK: TANK-binding kinase; TRADD: TNFR-associated death domain; TRAF: tumor necrosis factor receptor-associated factor; Ub: ubiquitin. Modified from (Takeuchi and Akira, 2010).

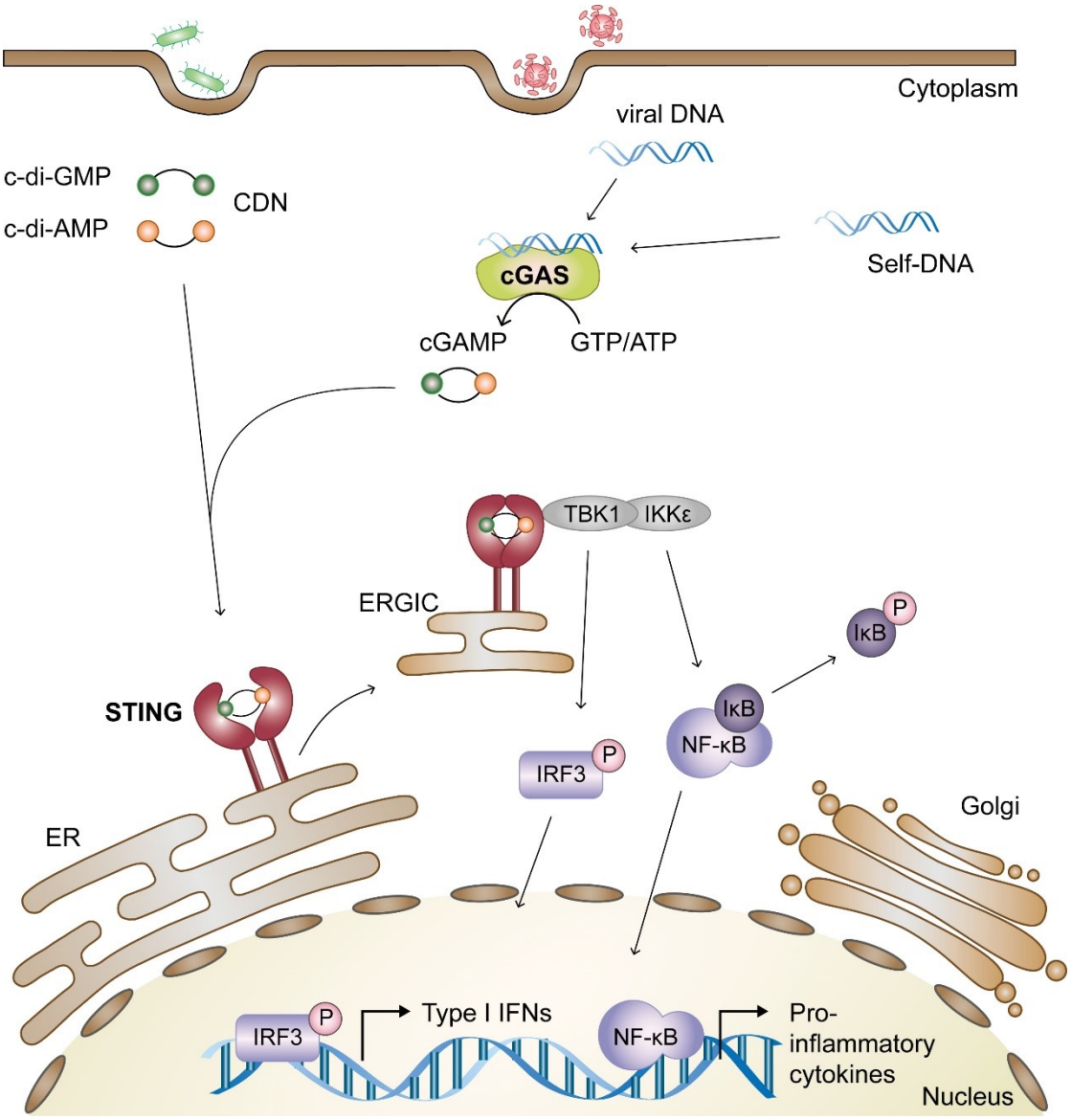

Figure 3: cGAS and STING are cytoplasmic dsDNA sensors.

Binding of DNA induces conformational changes leading to cGAS activation, followed by generation of 2'3'-cGAMP from ATP and GTP. 2'3'-cGAMP functions as second messenger activating the downstream adaptor and ER-associated protein STING. Upon ligation, STING migrates in the ERGIC to the Golgi apparatus thereby triggering the activation of the kinases TBK1 and IKKε that phosphorylate IRF3 and IκB, respectively. Activated IRF3 induces gene expression of type I IFNs, whereas NF-κB initiates transcription of pro-inflammatory cytokine genes. STING may also be activated directly by bacterial CDN. CDN: cyclic dinucleotides; cGAS: cyclic GMP-AMP (cGAMP) synthase; ER: endoplasmic reticulum; ERGIC: ER-Golgi intermediate compartment; IκB: inhibitor of κB; IKKε: IκB kinase ε; IRF: interferon regulatory factor; NF-κB: nuclear factor κB; P: phosphorylation; STING: stimulator of interferon genes. Modified from (Chen et al., 2016; Motwani et al., 2019).

STING is also activated directly by bacterial cyclic dinucleotides (CDN), including cyclic di-AMP and cyclic di-GMP, but studies reported a significantly higher affinity for the isomer 2'3'-cGAMP that precedes the activation of cGAS (Zhang et al., 2013).

3.3 Interferons are key players in the antiviral defense

Interferons (IFNs) are a specialized type of cytokines that are produced in high amounts upon viral infection. Hence, their naming stems from the observation made by Isaacs and Lindenmann in 1957: the ability to interfere with viral replication (Isaacs and Lindenmann, 1987). Since then, IFNs have been under comprehensive investigation, manifesting their roles as key players in the antiviral host defense. Firstly, IFNs activate the transcription of a specific gene set, termed interferon-stimulated genes (ISG), including various genes coding for proteins involved in the prevention of viral spread. Secondly, IFNs activate cDCs and macrophages and promote Natural Killer (NK) cells to kill virus-infected cells. Thirdly, IFNs promote chemokine production to attract lymphocytes and induce an elevated expression of MHC class I on the surface of nearly all cell types of the body, thus enhancing the recognition of infected cells by cytotoxic $CD8^+$ T cells (Ivashkiv and Donlin, 2014). Consequently, IFNs are potent inducers of innate and adaptive immune responses upon viral encounter.

3.3.1 Classification of interferons and signaling

The IFN family is subdivided into three classes, namely type I, II and III IFNs (Fig. 4). The murine type I IFN system is the family of IFNs described earliest. It comprises 14 IFNα subtypes, and the single gene products IFNβ, IFNε, IFNκ, IFNξ (Isaacs and Lindenmann, 1987; Lazear et al., 2019; Pestka et al., 2004). All members share structural homology enabling them to bind one common and ubiquitously expressed type I IFN receptor (IFNAR) that consists of the two subunits IFNAR1 and IFNAR2 (Kontsek, 1994; McNab et al., 2015; Müller et al., 1994). Despite the apparent redundancy among type I IFNs, it is suggested that they exert distinct functions due to dissimilarities in promoter sequences and biochemical properties (Schreiber, 2017). While IFNβ can be produced by most cells of the body, IFNα production is more restricted to specialized cell types, e.g. pDCs (Colonna et al., 2004). IFNγ represents the only type II IFN that is mainly produced by T cells, NK cells and ILC1. It signals through the heterodimeric IFNγ receptor (IFNGR), composed of the subunits IFNGR1 and IFNGR2 (Schoenborn and Wilson, 2007). The murine type III IFN family includes two single gene products IFNλ2 and IFNλ3, also known as IL-28A and IL-28B. These members have been described most recently and conduct their activity by binding to the IFNλ receptor (IFNLR) that consists of the IFNLR1 and the IL-10 receptor subunit-β (IL-10Rβ) (Kotenko and Durbin, 2017; Kotenko et al., 2003; Sheppard et al., 2003). The IL-10Rβ is broadly

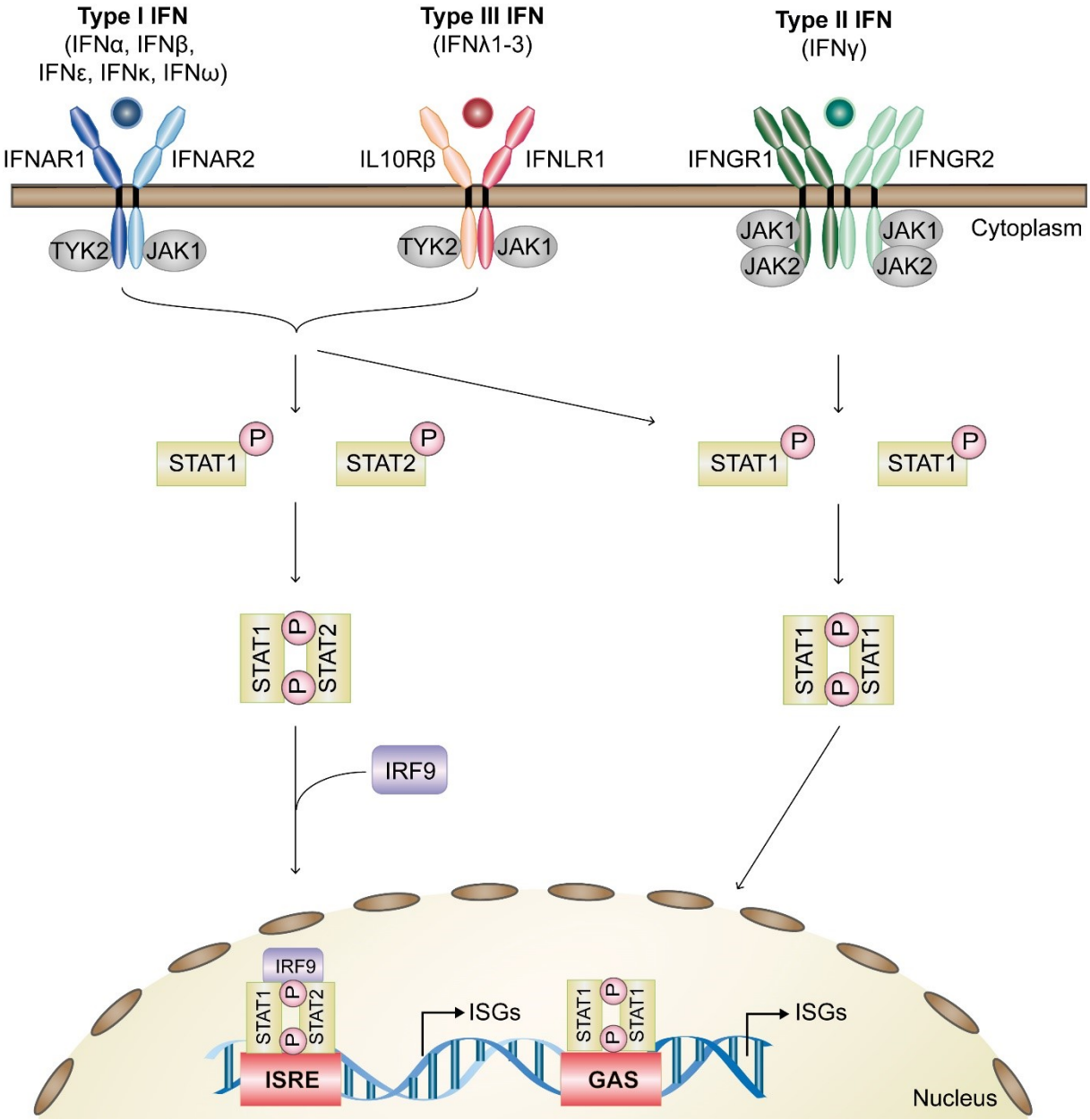

Figure 4: Canonical interferon signaling pathway.

The IFN receptors are heterodimeric proteins: the type I IFN receptor consists of IFNAR1 and IFNAR2, the type III IFN is built of IL10Rβ and IFNLR1 and the type II IFN receptor is composed of IFNGR1 and IFNGR2. The cytosolic domains of the type I and III receptors are associated with the kinases TYK2 and JAK1. Upon receptor engagement, they become activated and phosphorylate STAT1 and STAT2 initiating dimerization. Together with IRF9 they assemble into an active ISGF3 complex that binds ISRE motifs in the promoter regions of specific ISGs inducing their transcription. In contrast, the type II IFN receptor signals through JAK1 and JAK2 leading to the activation of only STAT1. Phosphorylated STAT1 proteins dimerize, translocate into the nucleus and bind only to GAS elements, thus initiating the expression of a distinct set of ISGs. GAS: IFNγ-activated site elements; IFN: interferon; IFNAR: IFNα receptor; IFNGR: IFNγ receptor; IFNLR: IFNγ receptor; IL10Rβ: IL-10 receptor subunit-β; IRF: interferon regulatory factor; ISG: interferon stimulated gene; ISRE: IFN-stimulated response elements; JAK: Janus kinase; STAT: signal transducer and activator; TYK: tyrosine kinase. Modified from (Borden et al., 2007; Ivashkiv and Donlin, 2014).

expressed, whereas the IFNLR1 has been reported to be predominantly expressed on epithelial cells from the gastrointestinal tract and the airways (Mahlakõiv et al., 2015; Ye et al., 2019).

Engagement of the type III receptors initiates a similar signaling cascade as it is described for the type I IFN receptor. The cytosolic domains of both receptors are associated with the protein kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) which are activated upon receptor ligation. Functionally active, both enzymes phosphorylate the transcription factors signal transducer and activator of transcription 1 and 2 (STAT1 and 2), followed by dimerization. Together with IRF9 they form a multimeric complex, called IFN-stimulated gene factor 3 (ISGF3), that translocates into the nucleus. There, it binds to specific DNA motifs contained in the promotor regions of several ISGs, generally termed IFN-stimulated response elements (ISRE), initiating their expression (Levy and Darnell, 2002; Stark and Darnell, 2012). However, type I IFNs can also signal through noncanonical pathways, including STAT1 homodimers that activate the transcription of a distinct set of ISGs that are characterized by IFN-γ-activated site (GAS) elements in their promotor regions. Additionally, type I IFNs can also operate through STATs that are typically involved in other cytokine-related signaling pathways, e.g. STAT3 or STAT4. Consequently, type I IFNs are able to induce the expression of a broad range of ISGs participating in the antiviral immune response (Ivashkiv and Donlin, 2014; Schoggins et al., 2011). IFNγ, on the other hand, only induces the expression of GAS-containing ISGs. Ligation of the IFNGR leads to the activation of JAK1 and 2, followed by STAT1 phosphorylation. Activated STAT1 proteins homodimerize, enter the nucleus and initiate ISG expression (Aaronson and Horvath, 2002).

3.3.2 Induction of type I interferons and their physiological functions

The production of type I IFNs is commonly initiated by the engagement of nucleic acid sensing PRRs, including the endosomal TLRs 3, 7, 8, 9, 13 and the cytosolic sensors RIG-I/MDA5 and cGAS/STING, as described in Chapter 3.2.2. Downstream signaling of those pathways rely on the activation of transcription factors belonging to the IRF protein family. Especially, IRF3 and IRF7 have essential roles in activating type I IFN gene expression. It is acknowledged that IRF3 primarily induces *Ifnb* and *Ifna4* gene expression that in turn activate the transcription of *Irf7*. Following this, in a positive feedback loop IRF7 initiates the expression of further *Ifna* genes, thus enhancing the type I IFN response (Honda et al., 2006; Tamura et al., 2008).

Type I IFNs then induce the expression of several hundred ISGs by signaling through IFNAR (Fig. 4) and thus promoting an "antiviral state" (Stetson and Medzhitov, 2006; van Boxel-Dezaire et al., 2006). This descriptive term mostly implies the antiviral function of proteins that are encoded by ISGs and the molecular basis of their activity could partially be unraveled over the past decades. In general, ISG products prevent viral transmission on different mechanistic levels, such as degradation of viral nucleic acids or inhibition of viral protein synthesis and virus replication (MacMicking, 2012; Schoggins et al., 2011). The families of OAS, MX, IFIT and IFITM proteins represent prominent ISG products. Oligoadenylate synthetases (OAS) are activated upon viral dsRNA sensing leading to the generation of 2'-5' oligoadenylates. In turn, these oligomers activate the endoribonuclease RNase L promoting the degradation of viral RNA (Hornung et al., 2014; Stark et al., 1998). Another well-known antiviral factor is the dsRNA-activated protein kinase (PKR) that phosphorylates the eukaryotic initiation factor 2α (eIF2α), resulting in the inhibition of protein translation and thus preventing viral replication (Kaufman, 1999; Pfaller et al., 2011). In contrast to OAS and PKR, that exhibit relatively unspecific antiviral property, myxoma resistant (MX) proteins have been reported to show activity more specifically against RNA viruses, such as influenza or vesicular stomatitis virus (VSV). It is assumed that MX proteins target nucleocapsid-like structures via their dynamin-like guanosine triphosphatase (GTPase) domain, but up until now it is only poorly understood how these proteins interfere exactly with virus transmission (Haller et al., 2015; Verhelst et al., 2013). IFN-induced proteins with tetratricoid repeats (IFIT) are another class of antiviral effectors that exert their function by restraining the translational machinery. IFITs bind to eIF3, thus preventing the interaction with eIF2 and finally the assembly into the ribosomal pre-initiation complex (Hyde et al., 2014; Terenzi et al., 2006). The IFN-induced transmembrane protein (IFITM) family interact in the early steps of virus infection. Although the molecular mechanisms are still unclear, IFITMs have been shown to be restrictors for influenza viruses and flaviviruses, such as Dengue or West Nile virus, by possibly inhibiting endocytosis and fusion of virus particles with host endosomes (Brass et al., 2009). Additionally, ISGs not only encode for specific sets of antiviral proteins as described above, but also proteins of the host viral detection systems or the IFN signaling pathway are ISG products themselves, such as RIG-I, MDA5, members of the IRF family, cGAS or STAT1, revealing the broad spectrum of ISG products (Schoggins et al., 2011). Transcriptome profiling studies revealed that, especially, the type I and III IFN signatures are largely overlapping. Commonly, type I IFNs are considered to be more potent than type III IFNs and the ISGs induced by type III IFNs represent a subset of those that are

induced by type I IFNs (Crotta et al., 2013; Marcello et al., 2006; Zhou et al., 2007). Nevertheless, IFN signaling can have diverse biological effects that probably result from differences in magnitude and frequency of signaling, and the cell types responding to type I *vs*. III IFNs (Lazear et al., 2019).

Besides the ability to induce the wide-ranging expression of ISGs, type I IFNs also have immunomodulatory effects on immune cells of the innate and adaptive immune system, thus mediating antiviral activities. For example, type I IFNs have been reported to promote surface expression of MHC and co-stimulatory molecules, e.g. CD80 and CD86, on cDCs as well as the migration of those cells into lymph nodes leading to an overall enhanced activation of $CD4^+$ and $CD8^+$ T cells (Ito et al., 2001; Le Bon et al., 2003). Further studies demonstrated the ability of type I IFNs to directly shape T cell responses. For instance, they promote survival and clonal expansion of $CD4^+$/$CD8^+$ T cells upon viral infection, induce differentiation into IFNγ-producing Th1 cells, positively influence $CD8^+$ T cell cytotoxicity and affect memory $CD8^+$ T cell function (Agarwal et al., 2009; Brinkmann et al., 1993; Havenar-Daughton et al., 2006; Thompson et al., 2006). NK cells represent another immune population whose antiviral functions are affected by type I IFNs. Generally, type I IFNs have been reported to boost NK cell survival and to modulate their cytolytic effector functions as well as their capacity to produce IFNγ (Lucas et al., 2007; Martinez et al., 2008). Investigation of the molecular mechanisms underlying those modulatory effects of type IFNs revealed that these are predominantly driven by differential regulation of the endogenous STAT protein levels as well as their activation (Miyagi et al., 2007; Tanabe et al., 2005).

Collectively, these examples illustrate the multifaceted effects of type I IFNs, manifesting their role as potent inducers of innate and adaptive immune responses upon viral entry. However, due to their high potency, dysregulated type I IFN responses can have severe impacts on host physiology, as they are linked to systemic inflammatory disorders with variable degrees of autoimmunity, that are commonly referred to as type I interferonopathies (Crow, 2011). A hallmark of all interferonopathies is a chronically upregulated IFN signature that is mainly caused by genetic, but also environmental factors. This novel group of diseases includes, for instance, the inflammatory disorders Aicardi-Goutières syndrome or STING-associated vasculopathy with infantile-onset, as well as autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Recent studies identified several gene mutations that result in aberrant type I IFN signaling causing disease onset by distinct mechanisms. These include, e.g. loss-of-function mutations in genes encoding for cytosolic DNases (*Trex1*), RNases (*Rnaseh2a-c*) and RNA-modifying enzymes (*Adar1*)

leading to increased levels of cytosolic DNA and DNA repair metabolites or unedited dsRNA, triggering type I IFN production through cGAS/STING and MDA5 signaling, respectively (Ablasser et al., 2014; Liddicoat et al., 2015; Mackenzie et al., 2016). Pathogenic type I IFN responses are also induced by gain-of-function mutations in the cytosolic DNA and RNA sensors, like STING and MDA5, leading to their constitutive activation (Konno et al., 2018; Rice et al., 2014). Finally, loss-of-function mutations in the genes of the negative regulators of IFNAR signaling ISG15 and ubiquitin-specific protease (USP18) cause enhanced type I IFN induction (Meuwissen et al., 2016; Zhang et al., 2015). Particular for SLE development, additional high-risk mutations have been described. For instance, these include deficiencies in complement proteins (C1q, C2 and C4), entailing an impaired clearance of cell debris, finally leading to an increased availability of nucleic-acid-containing products (Troedson et al., 2013; Truedsson et al., 2007). Furthermore, SLE-associated single-nucleotide polymorphisms were found in *Irf5* and *Irf7* genes, correlating with elevated type I IFN serum levels in lupus patients (Niewold et al., 2008).

Dysregulated type I IFN responses are also associated with diseases that are not necessarily determined by genetics, such as diabetes or Sjögren's syndrome (Banchereau and Pascual, 2006). Therefore, several regulatory mechanisms have evolved that tightly control for a balanced type I IFN response, thus preventing aberrant IFN signaling.

3.3.3 Various regulatory mechanisms control for a balanced type I interferon response

The cellular control mechanisms mainly rely on the modulation of the endogenous level and activation of signaling proteins by post-translational modifications (PTM) or on the epigenetic regulation of IFN-induced gene expression. Suppression of type I IFN signaling can be achieved by internalization of IFNAR. This process is primarily initiated by phosphorylation of a serine residue contained in a specific motif within the intracellular IFNAR1 domain, called degron sequence. Following this, E3 ubiquitin ligases are recruited leading to ubiquitination and receptor degradation. Studies reported that serine phosphorylation is mediated by the kinases TYK2, D2 or casein kinase 1α in a ligand-dependent manner (Bhattacharya et al., 2010; Zheng et al., 2011). Additionally, TLR stimulation and close interaction with immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors abolish IFNAR signaling through recruitment of protein kinases C (PKC), followed by the activation of the protein tyrosine phosphatase non-receptor type 11 (PTPN11) that in turn dephosphorylates signaling intermediates (Du et al., 2005; Huynh et al., 2012). Further on, type I IFNs simultaneously induce the production of inhibitory proteins,

e.g. suppressors of cytokine signaling (SOCSs) or ubiquitin carboxyl-terminal hydrolase 18 (USP18), functioning as a negative feedback loop to restrict the scope of the IFN response. SOCS1 has been reported to interact with TYK2, regulating its kinase activities or to bind to IFNAR1, interfering with STAT1 phosphorylation, whereas USP18 mediates the disassociation of JAK1 from IFNAR2 (Fenner et al., 2006; Piganis et al., 2011; Sarasin-Filipowicz et al., 2009). Attenuation of type I IFN signaling is also regulated by distinct PTMs of STAT proteins. For instance, either STAT1 is ubiquitinylated by different E3 ligases resulting in proteasome-mediated degradation or is tagged with small ubiquitin-related modifiers (SUMO). The protein inhibitors of activated STAT (PIAS1) represent one example of such enzymes that possess SUMO E3 ligase activity that finally leads to repression of ISG transcription (Liu et al., 2004; Tanaka et al., 2005).

Additionally, type I IFN responses are also regulated on a transcriptional level, mediated by repressors or epigenetic modifications. Previous studies described the transcription factor forkhead box protein O3 (FOXO3) as a negative regulator of ISG expression. Especially, FOXO3 binding motifs were identified in the *Irf7* gene locus, repressing basal ISG induction (Litvak et al., 2012). The SIN3 transcription regulator homolog A (SIN3A) represents another important factor controlling the transcriptional activity of ISGF3 during viral infections. SIN3A interacts with STAT3, causing its silencing by deacetylation and thus favoring ISGF3-induced ISG expression (Icardi et al., 2012). Additionally, in the quiescent state, ISG transcription is silenced by nucleosome barriers or repressive epigenetic marks, such as the histone modification H3 lysine 9 di-methylation (H3K9me2) that is mediated by the methyltransferase G9a. Studies reported cell-type specific H3K9me2 density occupancies that inversely correlated with the extent of the type I IFN response and ISG transcription (Fang et al., 2012). Further, induction of ISG expression by type I IFNs precedes removal of nucleosome barriers by remodeling complexes. The process of chromatin remodeling is accomplished by the activated ISGF3 complex that binds to ISG promoters and enhancer regions leading to the recruitment of nucleosome-remodeling enzymes as well as histone acetyltransferases (HATs). For instance, activated STAT1/2 interacts with the histone acetyltransferase KAT2A (also known GCN5), leading to acetylation of histone H3, a common permissive mark for transcription (Paulson et al., 2002). Bromodomain (BRD)-containing proteins, like BRD4, belong to a class of chromatin-associated proteins, which recognize acetylated H3 and promote transcriptional elongation through recruitment of the positive transcription elongation factor b (pTEFb) (Nicodeme et al., 2010; Patel et al., 2013). Further studies proposed that histone deacetylases (HDAC)

might also play a role in the transcriptional activation of ISGs. Applications of distinct HDAC inhibitors have revealed a decreased ISG expression in response to viral infections on a global level, suggesting a rather universal than specific mechanism for rapid ISG induction (Chang et al., 2004). Type I IFNs also enable ubiquitylation of histone H2B, a permissive mark, by the E3 ubiquitin ligase BRE1, thus promoting ISG expression that is, for instance, eluded by adenoviruses (Fonseca et al., 2012). Micro RNAs (miRNA) represent another control system that is involved in the regulation of type I IFN responses. These small non-coding RNAs (~ 22 nucleotides) exert their function as post-transcriptional regulators by mRNA silencing. Studies have demonstrated that, e.g. miR-29a promotes decreased IFNAR surface expression in thymic stromal cells, thus preventing thymus involution caused by hypersensitivity towards external type I IFN stimuli (Papadopoulou et al., 2011). miR-155 has been reported to broadly inhibit type I IFN signaling in activated $CD8^+$ T cells, thereby promoting an increased cell proliferation and a strengthened $CD8^+$ T cell response against bacterial or viral infections (Gracias et al., 2013). Furthermore, it has been reported that the stability of *Ifna* and *Ifnb* mRNA is controlled by specific regulatory elements found in the 3' or 5' untranslated region (UTR) of their own transcripts. Depending on binding of distinct RNA-binding proteins, either mRNA stabilization or decay is mediated (Khabar and Young, 2007). During infection with certain viruses, *Ifnb* transcript stability was maintained by protein kinase RNA-activated activity that restrained mRNA de-adenylation (Schulz et al., 2010).

In summary, these studies illustrate that a complex network has evolved on the cellular level that controls for an effective and at the same time balanced type I IFN response. This network is based on a tightly regulated interplay between control systems on distinct levels, including transcriptional (repressors, histone modifications, chromatin-remodeling enzymes) and post-translational regulation (miRNAs, PTMs), ensuring proper type I IFN signaling.

3.4 Conventional and plasmacytoid dendritic cells

Our body is constantly exposed to environmental, potentially harmful entities, including bacteria, viruses, fungi, parasites or toxins and thus, requires protection that is mediated by our immune system. Conventional dendritic cells (cDC) play a key role in the initiation of a powerful immune response, as they are specialized in pathogen sensing and activating T cells. Plasmacytoid dendritic cells (pDC) play a key role in antiviral defense, as they rapidly produce massive amounts of type I IFNs upon recognition of virus-infected cells. The

development of those two immune cell populations as well as their physiological functions will be discussed in detail in the following chapters.

3.4.1 Ontogeny of cDCs and pDCs

cDCs originate from myeloid progenitors in the bone marrow (BM) in dependency of fms-like tyrosine kinase 3 ligand (FLT3L) and mature in peripheral tissues either into cDC1 or cDC2 subsets that are defined by distinct transcriptional programs controlling for their differentiation, specific surface markers and their different ability to prime $CD8^+$ or $CD4^+$ T cells (Guilliams et al., 2014). Although the exact map of cDC and pDC hematopoiesis is still not complete, latest research using state-of-the-art technologies shed light on the transcriptional program underlying this complex differentiation process. Collectively, the data suggests that cDCs and pDCs share the FLT3L-driven development pathway until the common DC progenitor (CDP) stage, with an additional contribution of lymphoid progenitors to pDC development.

Imprinting of a cDC lineage signature could already be detected in early hematopoietic progenitors, such as the lymphoid-primed multipotent progenitor (LMPP) that developmentally branches into common lymphoid progenitors (CLP) and macrophage DC progenitors (MDP) (Naik et al., 2013) (Fig. 5). It is reported that cDC can also originate from CLPs, nevertheless myeloid progenitors are supposed to present the major contributors to cDC development (Karsunky et al., 2003; Manz et al., 2001). MDPs can give rise to either $Ly6C^+$/$Ly6C^-$ monocytes or differentiate into CDPs and subsequently into pre-DCs (Onai et al., 2007). In a study performed by Schlitzer et al., the authors demonstrated that the pool of pre-DCs is quite heterogeneous by identifying four different subsets that were defined by the expression of the surface markers Siglec-H and Ly6C (Schlitzer et al., 2015). Siglec-H^- $Ly6C^-$ and Siglec-H^- $Ly6C^+$ pre-DCs showed the highest level of ZBTB46, a transcription factor that is only expressed by cDCs and not pDCs, indicating a cDC1 and cDC2 commitment. This assumption was supported by clonal differentiation assays *in vitro* and adoptive cell transfers *in vivo* revealing that Siglec-H^- $Ly6C^-$ and Siglec-H^- $Ly6C^+$ pre-DCs exclusively gave rise to cDC1 or cDC2, respectively. In contrast, Siglec-H^+ $Ly6C^-$ and Siglec-H^+ $Ly6C^+$ pre-DCs were not subset-primed, and even retained pDC potential (Siglec-H^+ $Ly6C^-$). Conclusively, the imprinting of the cDC1 or cDC2 lineage with their specific transcriptional signature occurs in the BM during the transition from the CDP into the pre-DC stage. The cDC1/2-committed pre-DCs then migrate into peripheral lymphoid and non-lymphoid tissue, where

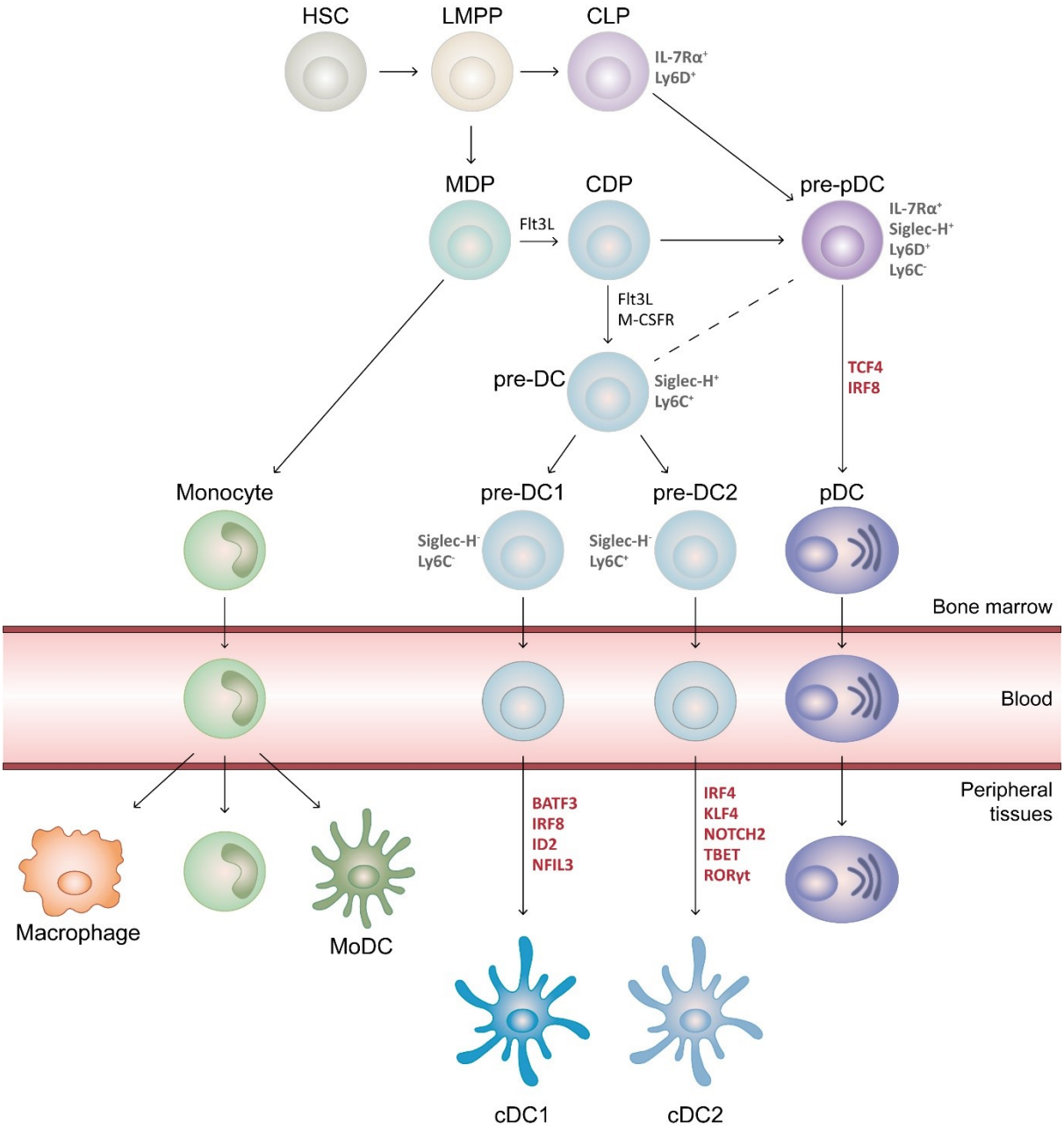

Figure 5: Ontogeny pathway of conventional and plasmacytoid dendritic cells.

cDC and pDC develop partially from common progenitors in the BM, whereby cDC1 and cDC2 complete their differentiation only in peripheral tissue, pDCs fully mature in the bone marrow instead. HSCs convert into LMPPs that either give rise to the lymphoid (CLP) or myeloid (MDP) lineage. MDPs are able to differentiate into monocytes, that may develop into tissue-resident macrophages or monocyte-derived (Mo) DCs, or give rise to CDP in a Flt3L-dependent manner. CDPs further differentiate into pre-DCs that, in dependency of their Siglec-H and Ly6C expression, are immediate precursors of cDC1, cDC2 or pDCs. IL-7Rα$^+$ Ly6D$^+$ lymphoid progenitors mainly give rise to pDC, whose commitment is generally dependent on the transcription factors TCF4 and IRF8. Differentiation of cDC1 is defined by BATF3, IRF8, ID2 and NFIL3, whereas cDC2 development is commonly dependent on IRF4, KLF4 and NOTCH2. A further division of the cDC2 subset is mediated by either TBET or RORγt expression. CDP: common DC progenitor; CLP; common lymphoid progenitor; HSC: hematopoietic stem cell; LMPP: lymphoid-primed multipotent progenitor; MDP: macrophage DC progenitor. Modified from (Dress et al., 2018; Merad et al., 2013).

they differentiate into mature cDC1 or cDC2 and gain their specific functionality, instructed by environmental cues (Guilliams et al., 2016; Schlitzer et al., 2015). It is well acknowledged that individual cell fate decisions are driven by the activity of specific transcription factors. Commitment into cDC1 is dependent on BATF3, IRF8, NFIL3 and ID2 (Grajales-Reyes et al., 2015; Hildner et al., 2008; Kashiwada et al., 2011; Murphy et al., 2016). Recently, PU.1 has also been reported to favor cDC1 differentiation by inducing the transcriptional regulator DC-SCRIPT that represses cDC2- and pDC-specific gene expression (Chopin et al., 2019). The cDC2 subset was found to be a more heterogeneous population with variable dependency on IRF4, KLF4 and NOTCH2 (Satpathy et al., 2013; Schlitzer et al., 2013; Tussiwand et al., 2015). A recent study suggested a further separation of the cDC2 subset into two subpopulations (cDC2A/B) classified by the exclusive expression of the transcription factors T-BET or RORγt. Interestingly, cDC2s acquire their expression only in peripheral tissues, most likely in response to environmental cues, elucidating an additional level of imprinting caused by tissue-specific factors (Brown et al., 2019).

pDCs have the potential to develop from myeloid or lymphoid progenitors and their maturation process is already completed within the BM. Schlitzer and colleagues reported that pDC signatures occur at the CDP stage, showing Siglec-H$^+$ Ly6C$^-$ pre-DCs to be pDC-committed (Schlitzer et al., 2015). In another recent study, the authors performed a more precise analysis of the CDP pool, revealing that only Ly6D$^+$ CD115$^-$ CPDs can give rise to pre-pDCs, whereas CD115$^+$ CPDs are committed to the cDC lineage (Dress et al., 2019). Although CDPs have the potential to develop into cDCs and pDCs, their contribution to the total pDC pool is relatively low compared to the quantitative numbers of pDCs versus cDCs *in vivo*. Indeed, by using *in vitro* clonal differentiation assays and *in vivo* cell transfers, Rodrigues et al. demonstrated that (FLT3$^+$ M-CSFR$^-$) IL-7Rα expressing lymphoid progenitors (LP) possess the major pDC differentiation potential that is five-to tenfold higher than those of CDPs or IL-7Rα$^-$ LPs. Further, they classified the IL-7Rα$^+$ LPs into three subpopulations based on their expression of Siglec-H and Ly6D, an early B cell differentiation marker, showing that Siglec-H$^+$ Ly6D$^+$ progenitors had exclusive pDC commitment. Transcriptome analyses proofed pDC-lineage specifications at this stage, prompting the authors to refer to the Siglec-H$^+$ Ly6D$^+$ progenitor as pre-pDC (Rodrigues et al., 2018).

The pDC transcriptional program is probably initiated by IRF8 expression, while following commitment of pDCs is highly dependent on the E protein transcription factor TCF4, also known as E2-2, that interacts with its cofactor MTG16 and BCL11A to induce

pDC development (Cisse et al., 2008; Ghosh et al., 2014; Ippolito et al., 2014; Upadhaya et al., 2018). TCF4 shows close homology to TCF3 and TCF12, the major regulators of lymphocyte development, and consequently, shares the ability to activate several genes in pDCs that are also activated by TCF3/12 in differentiating lymphocytes (Ghosh et al., 2014). Collectively, these studies pointed towards a close developmental relationship between pDCs and lymphocytes.

Moreover, bulk- and single-cell RNA sequencing approaches performed by Rodrigues et al. revealed an existing heterogeneity within the pDC population residing in the spleen and BM. They distinguish the smaller subset of pDC-like cells from conventional pDC only by their expression of ZBTB46 and CX_3CR1. Functionally, pDC-like cells, unlike pDCs, did not respond to CpG-B stimulation with IFNα production, but exhibited higher MHCII and CD86 upregulation when stimulated with CpG-A. Additionally, pDC-like cells were more efficient in antigen uptake and processing, leading to enhanced T cell priming. Transcriptionally, pDC-like cells are similar to conventional pDCs, but also display cDC characteristics. Conclusively, based on the transcriptional and functional profiles, one can speculate that pDC-like cells may develop from CDPs, while conventional pDCs may derive from IL-7Rα$^+$ Siglec-H$^+$ Ly6D$^+$ lymphoid progenitors (Manz, 2018; Rodrigues et al., 2018). Conversely, Dress and colleagues postulated that pDCs arise only from lymphoid progenitors and the so far suggested contribution of myeloid progenitors resulted only from contamination due to lacking resolution of flow cytometry gating strategies. Finally, they provoke the idea that pDCs might be a subpopulation of “innate, type I IFN-producing lymphoid cells” (Dress et al., 2019).

3.4.2 cDCs bridging innate and adaptive immune responses

The DC subsets can be distinguished not only due to their individual transcriptional signatures, but also by distinct phenotypic markers and functional specializations.

Besides the expression of the pan-cDC markers CD11c and MHCII, classical murine cDC1 markers are CD8α, XCR1, CD103, CLEC9A or CD24 (Durai and Murphy, 2016; Guilliams et al., 2016), whereas murine cDC2 are defined by CD4, CD11b and CD172α expression (Durai and Murphy, 2016; Guilliams et al., 2014). Nevertheless, it needs to be considered that the surface marker expression of the respective immune cell population varies between species and even between tissues within the same species, e.g. lymphoid *vs*. nonlymphoid tissue-residing cDCs.

cDCs are professional sentinels that steadily examine their environment for foreign molecules or signals of cell damage. These short-lived cells are equipped with a plethora of PRRs, e.g. TLRs, that enables them to efficiently detect those PAMPs or DAMPs, phagocytose antigens and present them via MHCI or MHCII molecules to $CD8^+$ and $CD4^+$ T cells, respectively (Dress et al., 2018; Kamath et al., 2002). The differentiation process of naïve T cells into effector T cells and their full activation requires three distinct cDC-mediated signals. T cell priming is initiated by the engagement of the TCR with peptide:MHC complexes displayed on the cell surface of cDCs (signal 1) (Heath and Carbone, 2009; Itano and Jenkins, 2003). In addition to antigen-induced signals, the concurrent expression of co-stimulatory molecules on the surface of activated cDCs is vital to promote T cell survival and expansion (signal 2). Well-characterized co-stimulatory molecules are members of the B7 family, such as CD80 and CD86, which are recognized by the T cell surface receptor CD28 (Schildberg et al., 2016). Finally, cytokines secreted by activated cDCs are necessary to induce the differentiation into distinct subsets of effector T cells (signal 3) (O'Shea and Paul, 2010).

Given the cDC's remarkable role in the activation of naïve T cells, it appears reasonable, that another special feature of cDCs is their contribution to central and peripheral tolerance, a mechanism that prevents reactivity of T lymphocytes to "self" or harmless, ubiquitous environmental antigens that would otherwise lead to deleterious auto-inflammatory responses. Central tolerance occurs during the maturation of T cells in the thymus by either eliminating autoreactive thymocytes through the process of negative selection or by initiating the development of T_{regs} that enter peripheral tissues (Goodnow et al., 2005). Peripheral tolerance is mainly based on the mechanisms of anergy (functional unresponsiveness), suppression of autoreactive lymphocytes by T_{regs} and deletion through activation-induced cell death. Anergy describes a process in which T lymphocytes become functionally inactivated (anergic) when they recognize antigens presented by cDCs in the absence of strong co-stimulatory molecules (signal 2) due to a blockade in TCR signaling or the engagement of inhibitory receptors, like CTLA-4 or PD-1 (Ishida et al., 1992; Okazaki et al., 2013; Tivol et al., 1995). In addition, T_{regs} possess the ability to suppress self-reactive lymphocytes. In case, a T_{reg} encounters its antigen in a peripheral tissue, it inhibits all autoreactive lymphocytes in close proximity, independent of their antigen specificity, by producing immunosuppressive cytokines, such as TGF-β1 and IL-10 (Vignali et al., 2008). Finally, the deletion of highly autoreactive T lymphocytes is mediated by apoptotic cell death (Griffith and Ferguson, 2011).

Thus, cDCs possess a bridging function in initiating and balancing innate and adaptive immune responses.

Among the cDC subsets, a variation in *Tlr* gene expression could be detected: cDC1 exclusively express TLR-3/11/12, whereas cDC2 mainly express TLR-1/2/5/6/7-9. TLR-4 expression was found to be present in both subsets that, consequently, allows them to sense and respond to diverse stimuli (Brown et al., 2019). cDC1 exert specialized function in cross-presenting exogenous antigens on MHCI molecules to $CD8^+$ cytotoxic T cells, mostly in response to viral and intracellular bacterial infections. cDC1 are capable of producing relatively high amounts of IL-12p70, a Th1 polarizing cytokine, and regulatory cytokines, like TGFβ or IL-10, elucidating their important role in mediating peripheral tolerance in steady-state while promoting immunity during infection (den Haan et al., 2000; Merad et al., 2013). In contrast, cDC2 present antigens on MHCII molecules to $CD4^+$ T cells and elicit Th2 and Th17 responses by producing the pro-inflammatory cytokines IL-4/5/13 or IL-23 upon activation (Dudziak et al., 2007; Williams et al., 2013). A recent study performed by Brown and colleagues support the observation of distinct functionalities of cDC1s and cDC2s by showing different expression patterns of chemokines and chemokine/cytokine receptors across both subsets. The group further refined the characterization of the cDC2 population and identified two subsets with specialized features: upon activation, cDC2Bs exhibit a higher production of pro-inflammatory cytokines, such as TNF or IL-6, show an increased expression of MHCII and co-stimulatory molecules and are more efficient in polarizing naïve T cells into IFNγ/IL-17A producing T cells compared to cDC2As. Hence, cDC2Bs are generally suggested to be involved in tissue inflammation, whereas cDC2As mainly play a role in tissue remodeling or repair (Brown et al., 2019).

3.4.3 pDCs are unique virus detectors

Murine pDCs are specified by intermediate CD11c, but high Siglec-H, mPDCA-1 (BST2, CD317) and B220 (CD45RA) expression. Within this population, some pDCs were also reported to express CD4, CD8α, CD9, CCR9, Ly49Q, Ly6C or Sca-1. Compared to cDCs, pDCs do not express high levels of MHCII at steady-state, but are able to upregulate its expression during activation. In addition, pDCs are also capable to upregulate co-stimulatory molecules, such as CD80 or CD86, upon activation (Merad et al., 2013).

pDCs reside and recirculate through lymphoid tissues, exhibiting a relatively short life span of only 1-2 weeks and are therefore constantly replenished from the BM (Geissmann et al., 2010; Zhan et al., 2016). Furthermore, pDCs are characterized by their strong expression

of the endosomal TLR-7 and 9, rendering them highly reactive towards viral or bacterial nucleic acids by a massive production of type I IFNs within 1-3 hours post-infection. Quiescent pDCs have been shown to express high levels of IRF7, a key inducer of type I IFN gene transcription that probably enables such rapid type I IFN secretion. Thus, pDCs were also designated as "natural interferon-producing cells" (Colonna et al., 2004; Swiecki and Colonna, 2015). Additionally, pDCs are able to produce type III IFNs, pro-inflammatory cytokines, like TNF, or chemokines, allowing for an overall rapid immune response against pathogens (Gilliet et al., 2008; Reizis et al., 2011). In the past years, the mechanisms of virus recognition by pDCs were under detailed investigations, revealing that pDCs rather recognize infected cells than the virus *per se*. This new "paradigm of virus recognition" could be observed for several distinct RNA viruses and was shown to be TLR-7-dependent and required physical contact between pDCs and the infected cell (Frenz et al., 2014; Takahashi et al., 2010). A recent study described this necessary pDC-infected cell-contact as an "interferogenic synapse" that is formed by integrin-ICAM-1 adhesion complexes and thus, enables viral RNA transfer and promotes antiviral responses (Assil et al., 2019). Altogether, pDCs seem to have acquired a unique capability to detect intracellular PAMPs, closely resembling other sentinels, like NK or cytotoxic T cells (Reizis, 2019). Interestingly, pDCs also exhibit a potential role in modulating adaptive immune responses upon viral infection. For instance, pDC-deficient mice showed impaired survival of cytotoxic T cells and inefficient $CD4^+$/$CD8^+$ T cell priming after VSV or LCMV infection, respectively (Cervantes-Barragan et al., 2012; Swiecki et al., 2010). The involvement of pDCs in T cell priming may partially be arbitrated through a close interaction with specifically $XCR1^+$ cDC1, resulting in an enhanced $CD8^+$ T cell cross-priming (Brewitz et al., 2017). Additionally, pDCs have been reported to capture and present peripheral antigens to developing thymocytes, thus contributing to central tolerance (Hadeiba et al., 2012).

4 Aims and Significance

Over the past years, various studies have highlighted the importance of the microbiota's multifaceted role in shaping host physiology. The substantial progress in the development and application of cutting-edge technologies has provided an extended knowledge about potentially beneficial or detrimental host-microbiota interactions. However, we are still at the beginning to understand to which extent the microbiota in general or only single members and their metabolites influence the host's fitness and how these effects are mediated and transduced within the relevant cell populations on a molecular level. Investigating the impacts of the microbiota on the host's immune system has become a major focus of microbiome research, as GF mice display several immune defects. For instance, GF mice show an impaired T cell differentiation and accumulation in the intestine, a reduced anti-microbial peptide production and a decreased tumor rejection ability due to an impaired function of tumor-infiltrating myeloid cells during cancer therapy (Cash et al., 2006; Iida et al., 2013; Ivanov et al., 2008). Furthermore, GF mice were also more susceptible to virus infections that resulted from an incapability of MPhs to respond with cytokine and chemokine production. Expression of those genes was not initiated because chromatin barriers could not be properly removed (Ganal et al., 2012). Interestingly, this impaired responsiveness also affects MPhs residing in non-mucosal sites, which are not in close contact to commensals, such as in spleen, lymph nodes or brain (Abt et al., 2012; Erny et al., 2015; Ganal et al., 2012). Conclusively, it appears that microbiota-derived cues are required during steady-state to calibrate MPhs for an effective immune defense against pathogens. This state of MPhs is referred to as their poised basal state.

cDCs represent a subset of MPhs that possess specialized functions in initiating innate as well as adaptive immunity upon pathogen entry and simultaneously mediating tolerance towards "self" and harmless, environmental antigens. However, little is known about the signals and molecular networks instructing the poised basal state of systemically distributed cDCs, but might be of relevance regarding the pathophysiology and treatment of autoimmune or inflammatory diseases. In view of this fact, the specific objective of this study was to investigate thoroughly the impact of the commensal microbiota on the basal state of splenic cDCs on a molecular level.

First, I aimed to investigate if the unresponsiveness of splenic cDCs to microbial stimulation observed in GF mice was restorable. For this purpose, GF mice were mono-

colonized with one single strain of *Escherichia coli* (JM83) or with a more complex microbiota, consisting of 12 different bacterial strains (sDMDMm2), and the cDC's ability to respond to distinct microbial stimuli was analyzed. Further, I set out to interrogate if the calibration process of cDCs might also rely on tonic sensing of microbes though PRRs, since it is well acknowledged, that the constant sensing of the commensal microbiota through PRRs plays an important role in the education of the host's immune system. To address this aim, mice that were genetically deficient for the adaptor proteins MyD88, TRIF and CARDIF, critically involved in the TLR and RIG-I sensing pathway, were employed and cDC function after direct activation with a CD40 antibody was examined.

To obtain insights into the molecular mechanisms underlying this cDC calibration process, genome-wide profiles of cDCs from GF and specific pathogen-free (SPF) control mice were compared, showing a reduced type I IFN signature in the former one, indicating that microbiota-controlled type I IFNs instruct the poised basal state of cDCs.

However, the cellular source producing tonic type I IFNs remained unknown until this point. Thus, distinct splenic and intestinal leukocyte populations were sort-purified and *Ifnb1* transcript levels were determined. This is the first study showing that splenic and intestinal pDCs were the main producers of tonic type I IFNs. Constitutive, pDC-dependent type I IFN production was induced by the commensal microbiota and required tonic PRR signaling.

In a next step, I aimed to understand how a permanent type I IFN signaling affects the functionality of cDCs and what happens on a molecular level, when cDCs are deprived of type I IFN signals. Therefore, I investigated both cDC subsets, cDC1s and cDC2s, from mice deficient for the type I IFN receptor (*Ifnar1*$^{-/-}$) on a transcriptomic level (RNA-sequencing) and compared those to the cDC subsets from GF and SPF control mice. Additionally, epigenomic profiling (ChIP-sequencing for H3K4me3 and H3K27me3) of cDCs from SPF, GF and *Ifnar1*$^{-/-}$ mice was performed.

Following this, a series of further experiments were performed in order to link tonic type I IFN signaling to cDC function. To exclude, that an altered microbiota composition in *Ifnar1*$^{-/-}$ mice might cause the cDC's incapability to upregulate cytokine production upon activation, cDC responses to microbial stimulation in co-housed *Ifnar1*$^{+/+}$, *Ifnar1*$^{+/-}$ and *Ifnar1*$^{-/-}$ littermates were examined. Further, mice with a cDC-specific IFNAR1 deletion (*Ifnar1*$^{\Delta cDC}$) were generated and cytokine production of cDCs after microbial stimulation was analyzed with the aim to provide evidence that cDCs require direct tonic type I IFN signaling to become fully functional. Additionally, to proof that the deprivation of tonic type I IFNs is

caused by the GF status, GF *Ifnar1*$^{-/-}$ mice were generated and the ability of cDCs to induce an inflammatory response after microbial stimulation was compared to those of GF and *Ifnar1*$^{-/-}$ (SPF) mice.

Furthermore, epigenomic and transcriptomic profiling revealed that genes associated with the mitochondrial respiratory chain showed a significant higher density of the repressive mark H3K27me3 and consequently, were highly downregulated in cDCs from GF and *Ifnar1*$^{-/-}$ mice compared to SPF cDCs. Thus, I aimed to obtain more insight into the metabolic activity of mitochondria in cDCs. Subsequently, the mitochondrial membrane potential and the mitochondrial mass were investigated using distinct probes, whose uptake into mitochondria is dependent or independent, respectively, on the membrane potential. Additionally, the physical appearance of mitochondria in cDCs from GF and *Ifnar1*$^{-/-}$ and control mice was examined by transmission electron microscopy. Lastly, live mitochondrial respiration in freshly isolated splenic cDCs was conducted using extracellular flux analysis.

Altogether, this study provides a new and essential aspect in the activation process of cDCs, as cDCs require experiencing a specific instruction program at steady-state, which is critically regulated by microbiota-induced tonic type I IFNs. Consequently, cDCs deprived of tonic type I IFN signaling (GF and *Ifnar1*$^{-/-}$ mice) were unable to initiate a powerful immune response towards microbial stimulation. This study profoundly elucidated the molecular mechanisms underlying this calibration process of the cDC basal state that is based on a specific transcriptional, epigenetic and metabolic program, but at the same time, admits also unwanted priming of T cells. Conclusively, these findings may give a cause of thought for developing therapies for treatment of autoimmune or inflammatory diseases that are associated with an enhanced type I IFN gene expression signature, such as SLE, Sjögren's syndrome or type I diabetes, by controlling type I IFN production through modulation of the microbiota composition. Further, the efficacies of checkpoint inhibitors for anti-cancer treatments have been reported to be microbiota-dependent (Iida et al., 2013; Sivan et al., 2015; Vétizou et al., 2015). Thus, it would be intriguing to speculate if the microbiota-controlled calibration of cDC function may be of relevance for responsiveness to immunotherapies.

5 Research articles

Schaupp L*, Muth S*, Rogell L*, Kofoed-Branzk M, Melchior F, Lienenklaus S, Ganal-Vonarburg SC, Klein M, Guendel F, Hain T, Schütze K, Grundmann U, Schmitt V, Dorsch M, Spanier J, Larsen P-K, Schwanz T, Jäckel S, Reinhardt C, Bopp T, Danckwardt S, Mahnke K, Heinz GA, Mashreghi M-F, Durek P, Kalinke U, Kretz O, Huber TB, Weiss S, Wilhelm C, Macpherson AJ, Schild H, Diefenbach A and Probst HC

Microbiota-Induced Type I Interferons Instruct a Poised Basal State of Dendritic Cells

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* These authors contributed equally.

Contribution statement

This research article represents a comprehensive collaborative work, resulting in a co-first authorship consisting of me, S. Muth and L. Rogell. This article contains data that I mainly generated during my work as Ph.D. student and the content will be profoundly discussed in this doctoral thesis. Hereinafter, I disclose my contributions to the individual data sets: I planned, performed and analyzed the experiments shown in Fig. 1 A-C/E-F; Fig. 2 I-J, Fig. 5 C-F; S1 A-B/F-I; S2 A; S5 E-L. Furthermore, I contributed to Fig. 4, S4 and Fig. 6 D by performing the preparation, sorting and processing of splenic cDC1s and cDC2s from *Ifnar1*$^{+/+}$ (SPF), *Ifnar1*$^{+/+}$ (GF) and *Ifnar1*$^{-/-}$ (SPF) mice, following RNA purification for subsequent sequencing analyses. I contributed to Fig. 7 F-G and H-I, S7 C-E by assisting in experiment performance and conducting the preparation, sorting and processing of splenic cDCs from *Ifnar1*$^{+/+}$ (SPF), *Ifnar1*$^{+/+}$ (GF) and *Ifnar1*$^{-/-}$ (SPF) mice for subsequent transmission electron microscopy analyses, respectively.

6 Results

The data that I generated in my PhD project and that will be presented in the following Chapter has been, in large parts, published in the research article "Microbiota-Induced Type I Interferons Instruct a Poised Basal State of Dendritic Cells" (Schaupp et al., 2020), as mentioned in Chapter 5. Specifically, Figures 6 A-E; 7 A-F; 8 A-E; 9 A-D; 10-11; 12 B-D, F, H, K-L and 13 A, D, G-N are identical or very similar to those found in the original publication. In certain paragraphs, I will also refer to data from this paper that was not exclusively generated by myself, but my co-authors. Although I did not generate this data, it will be helpful to substantiate core findings and put them into perspective.

6.1 cDCs from GF mice are unresponsive to microbial stimulation

Conventional dendritic cells (cDCs) are highly efficient sentinels, constantly patrolling their environment for foreign molecules or signals of cell damage. Upon detection of, for example, PAMPs from invading pathogens, cDCs react promptly with the production of inflammatory cytokines, e.g. TNF, IL-6, IL-12 etc., thereby orchestrating an innate immune response (Merad et al., 2013). In the following experiments, we used the cDC's capacity to produce cytokines after stimulation as an indicator of their functionality.

In previous studies, cDCs from microbiota-depleted mice have been reported to display an impaired immune response upon encounter of viruses, which, for instance, manifested in a reduced pro-inflammatory cytokine production (Abt et al., 2012; Ganal et al., 2012). In extension to this data, we investigated the capacity to produce inflammatory cytokines of both splenic cDC subsets, cDC1s and cDC2s, from specific-pathogen-free (SPF) and germ-free (GF) mice after direct stimulation with an agonistic α-CD40 antibody by flow cytometry (Rolink et al., 1996). CD40 is broadly expressed on the cell surface of antigen-presenting cells, such as cDCs, and ligation of CD40 induces the activation of multiple pathways, including MAPK, STAT3 and NFκB, that finally leads to inflammatory cytokine gene expression (Eliopoulos et al., 2000; Grewal and Flavell, 1998). Interestingly, both cDC subsets, cDC1s and cDC2s, from GF mice were largely unresponsive to direct stimulation with α-CD40 antibody, as they displayed significantly reduced production of TNF (Fig. 6 A-C). To interrogate whether this functional incompetence in producing inflammatory cytokines was restricted to splenic cDCs, we measured serum cytokine concentration and splenic type I IFN transcript levels in α-CD40-stimulated SPF and GF mice by ELISA or qRT-PCR, respectively. Importantly, GF mice exhibited low serum TNF and type I IFN levels (Fig. 6 D-

E), as well as a significant reduction in *Ifna4* and *Ifnb1* transcripts in splenic tissue (Fig. 6 F-G) after α-CD40 challenge. Altogether, these data indicate that splenic cDCs, including cDC1s and cDC2s, from GF mice are highly unresponsive in terms of cytokine production,

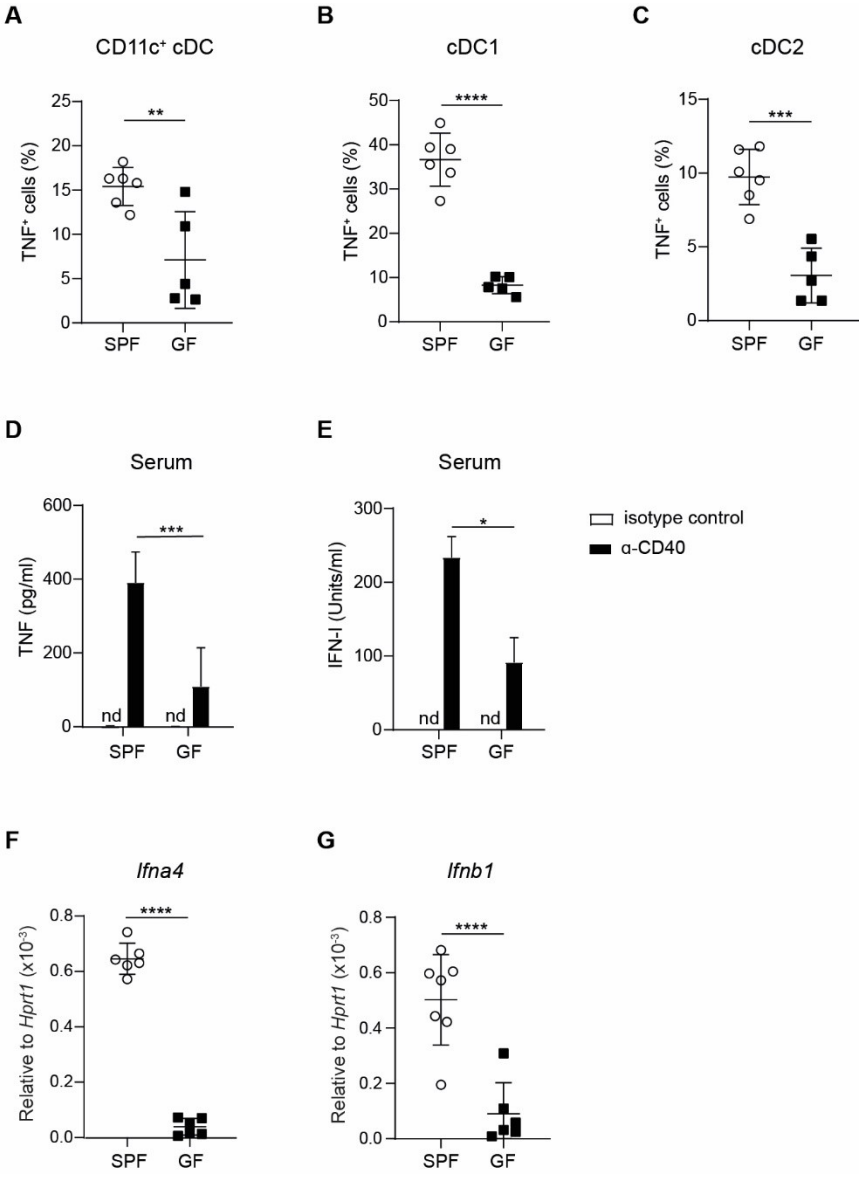


Figure 6: The commensal microbiota instructs cDCs for cytokine responses upon activation.

(A-C) SPF and GF mice were injected i.p. with an α-CD40 antibody and TNF production of all splenic CD11c$^+$ cDCs (A) and the subsets cDC1 (B) and cDC2 (C) was analyzed by flow cytometry.

(D-E) Serum TNF and type I IFN concentrations from the indicated mice in (A-C) were determined by ELISA or VSV bioassay, respectively. (F-G) Splenic tissue from α-CD40-stimulated mice shown in (A-C) was collected for RNA extraction. *Ifna4* and *Ifnb1* transcript levels were subsequently determined by qRT-PCR.

Data originates from experiments shown in Fig. 8 and was split up for a better overview. Data are representative of two independent experiments. Horizontal bars represent mean ± SD (A-G, n = 5-7). Statistical significance was determined with Student's t test, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; nd, not detectable.

even after direct stimulation. However, this unresponsiveness is not necessarily restricted to splenic cDCs.

Initiating adaptive immunity by priming T cells is another elementally function of activated cDCs. To analyze cDC-induced T cell priming we used a specific transgenic mouse model, termed DIETER, in which the expression of a class I MHC-restricted $CD8^+$ T cell epitope specifically on cDCs is induced by tamoxifen administration (Probst et al., 2003). Additionally, when peripheral tolerance mechanisms are eliminated by antibody-mediated $CD4^+$ T cell depletion, this model allows for analyzing $CD8^+$ T cell priming by cDCs at steady-state (Muth et al., 2012). To explore, if microbiota-derived signals are also necessary for the cDC-induced T cell priming, those mice were treated with antibiotics (ABX) to deplete the indigenous microflora. Indeed, cDCs from ABX-treated mice were unable to induce $CD8^+$ T cell expansion (data not shown; Figure 1 G of Schaupp et al., 2020).

Collectively, these findings indicate that signals of the commensal microflora are required to instruct cDCs at steady-state, hereinafter referred to as the cDC basal state, allowing for a powerful cytokine immune response upon activation. However, the cDC basal state also authorizes the activation of T cells against harmless antigens, that is normally prevented by the mechanisms of peripheral tolerance.

6.2 cDC function can be restored by re-colonization of GF mice with a model microbiota

So far, the data show that the commensal microbiota is required to instruct the cDC basal state, enabling a powerful cytokine immune response upon activation. Consequently, cDCs from GF mice exhibit a considerably impaired capacity in producing inflammatory cytokines after stimulation. Thus, we wondered if re-colonization of GF mice with consortia of distinct bacterial strains would restore the functional incompetence of splenic cDCs to respond towards microbial stimuli. To address this question, we monocolonized GF mice with one single strain of *Escherichia coli* (JM83) or with a more complex microbiota, consisting of 12 different bacterial strains representing the five major bacterial phyla of the intestinal microbiota (sDMDMm2) (Hapfelmeier et al., 2010; Uchimura et al., 2016). Mice colonized

with JM83 or sDMDMm2 as well as GF and SPF mice were challenged with LPS or poly(I:C). The capacity of cDCs to respond to those stimuli was analyzed by flow cytometry (Fig. 7 A, D).

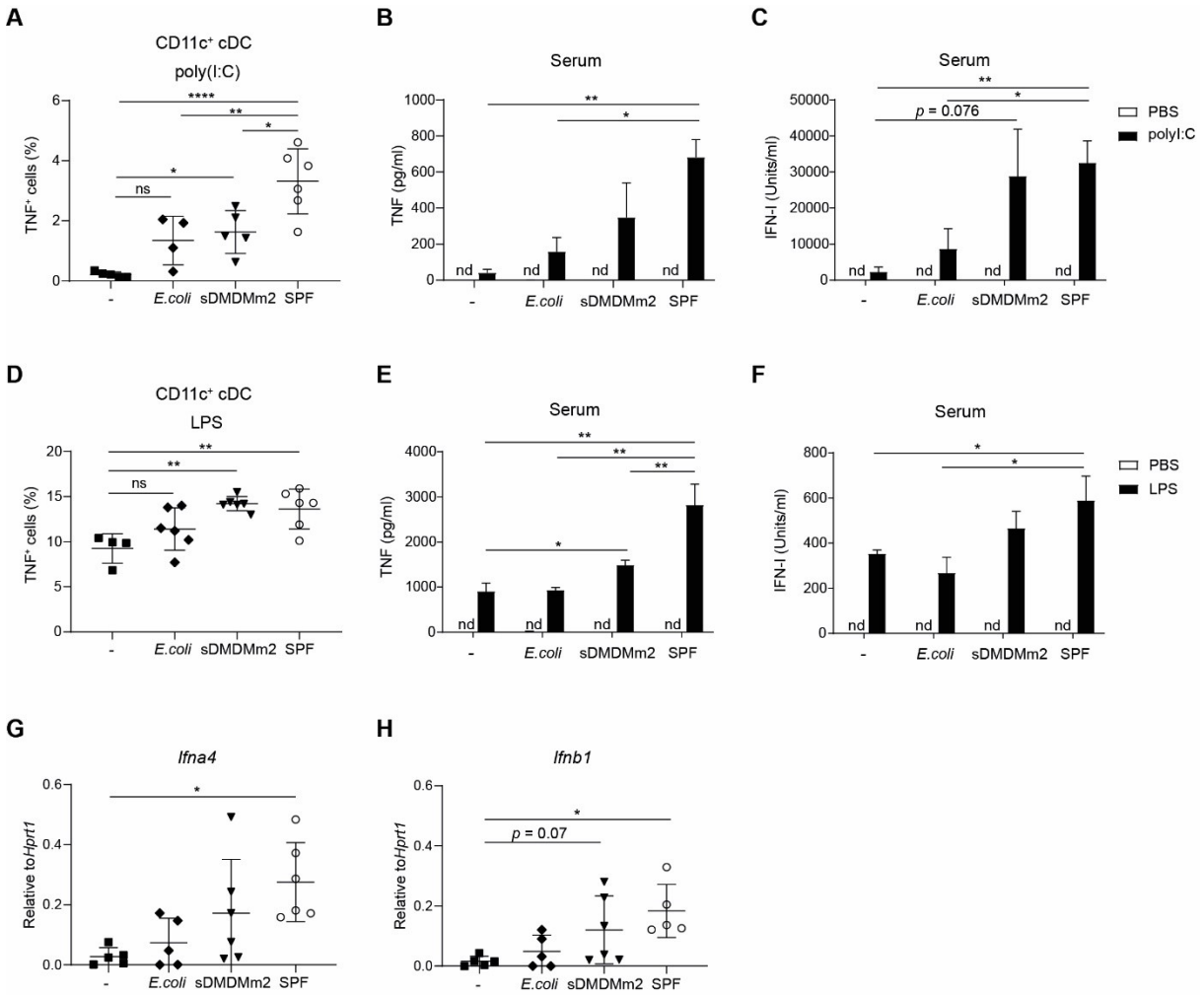


Figure 7: Re-colonization of GF mice with the model microbiota sDMDMm2 restored cDC function.

(A-H) GF mice (-) were re-colonized with the *E. coli* strain JM83 or with a complex microbiota (sDMDMm2 or SPF). (A, D) Mice were injected i.p. with poly(I:C) (A) or LPS (D) and TNF production of splenic cDCs was analyzed by flow cytometry. (B-C, E-F) Serum TNF and type I IFN concentrations were determined after poly(I:C) (B-C) or LPS (E-F) stimulation by ELISA or VSV bioassay, respectively.

(G, H) Splenic tissue was collected after poly(I:C) stimulation for RNA extraction. *Ifna4* and *Ifnb1* transcript level were determined by qRT-PCR.

Data are representative of two independent experiments. Horizontal bars represent mean ± SD (A-H, n = 4-6). Statistical significance was determined with one-way ANOVA with Bonferroni's multiple comparisons corrections, *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001; ns, not significant; nd, not detectable.

cDCs from mice monocolonized with *E. coli* JM83 showed a slight increase in their TNF production after stimulation with LPS or poly(I:C) compared to GF mice (Fig. 7 A, D). But only re-colonization of GF mice with the more complex microbiota sDMDMm2 fully reconstituted the cDC's capacity to produce TNF in response to LPS, and significantly increased their ability to respond towards poly(I:C) (Fig. 7 A, D). Additionally, to investigate the responsiveness of the systemic mononuclear phagocyte system, serum cytokine and type I IFN transcript levels in splenic tissue were determined by ELISA or qRT-PCR, respectively. Similar to the results obtained by flow cytometry analysis, only mice re-colonized with the more complex microbiota sDMDMm2 exhibited type I IFN serum and *Ifna4* and *Ifnb1* transcript levels nearly comparable to SPF controls (Fig. 7 C, F, G, H). TNF serum levels in sDMDMm2-colonized mice were increased compared to GF mice, but did not fully reach the capacity of systemic TNF production of SPF mice.

Collectively, the results indicate that re-colonization of GF mice with a complex model microbiota can in principle restore cDC responsiveness towards microbial stimulation. Further, it appears that the cDC's capacity of cytokine production was progressively increased dependent on microbiota complexity. Similar results were obtained when analyzing systemic TNF and type I IFN serum as well as *Ifna4* and *Ifnb1* transcript levels, suggesting that probably the entire mononuclear phagocyte system relied on receiving signals from the indigenous microbiota in order to respond with a powerful inflammatory cytokine production towards microbial stimuli. Although, it cannot be excluded that microbiota components other than bacteria may contribute to the calibration of the cDC basal state, the data indicate that the complexity level of the indigenous microbiota probably determines the magnitude of the cDC's ability to produce inflammatory cytokines in response to microbial stimulation.

6.3 PRR signaling at steady-state is required to instruct a poised basal state of cDCs

Up to here, the data suggests that microbiota-controlled signals are indispensable for the instruction of the cDC basal state at steady-state. Because it is well known, that the constant sensing of the commensal microbiota through PRRs plays an important role in the education of the host's immune system, we set out to interrogate if the calibration process of cDCs might also rely on tonic sensing of microbes though PRRs. To verify this, we made use of

mice that were genetically deficient for the adaptors MyD88 ("My"), TRIF ("Tr") and CARDIF ("Ca"). MyD88 and TRIF are adaptor proteins critically involved in signaling of all TLRs (Fig. 1), while CARDIF is involved in RIG-I/MDA5-mediated sensing of viral ssRNA and dsRNA (Fig. 2). We analyzed the ability of cDCs derived from mice deficient for one or multiple adaptor proteins to produce TNF after α-CD40 stimulation, which activates cDCs independent of those adaptor proteins, by flow cytometry (Fig. 8 A-C).

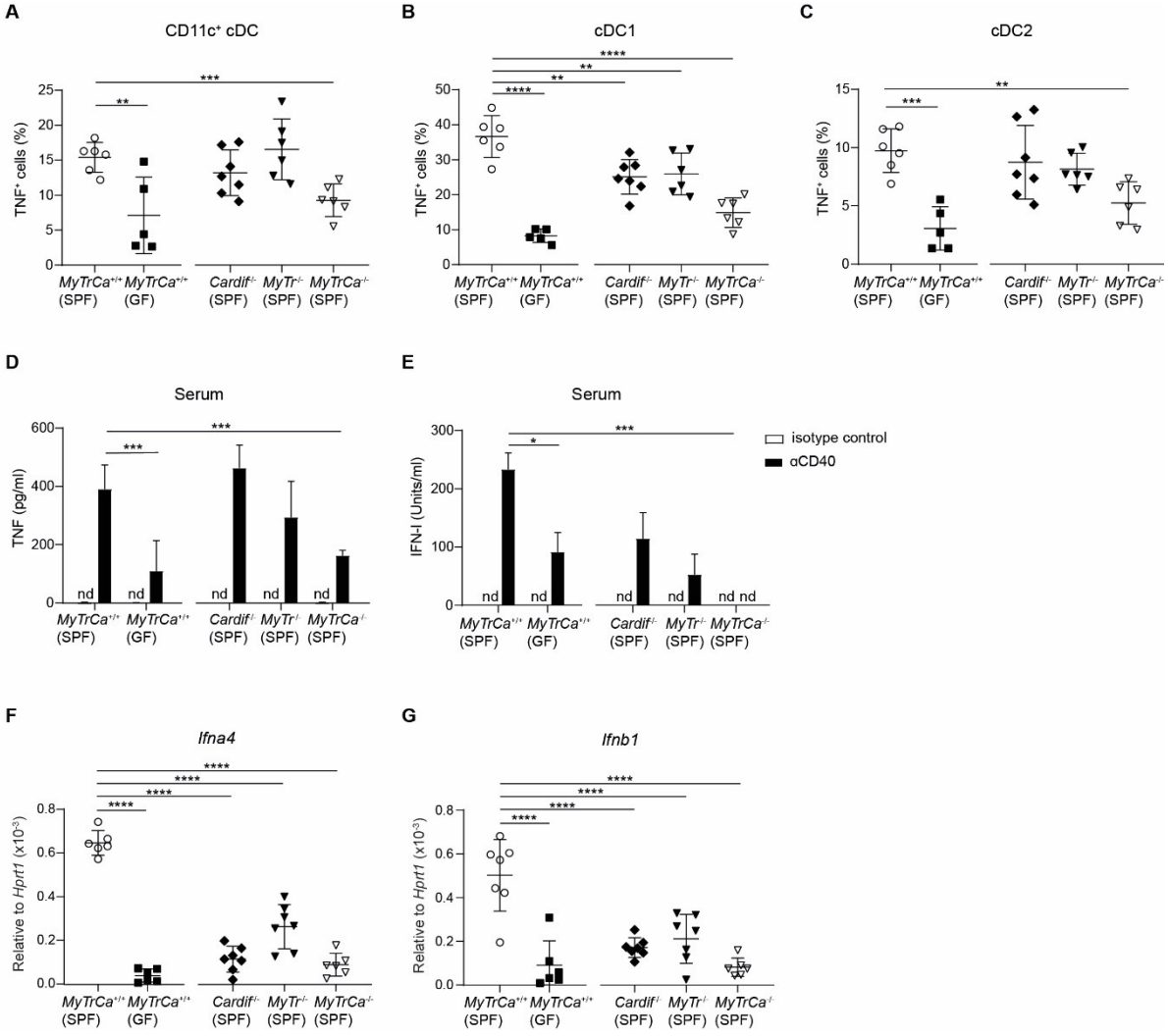


Figure 8: Instruction of the cDC basal state requires tonic PRR signaling.

(A-C) Mice of the indicated strains were injected i.p. with a CD40 antibody and TNF production of all splenic $CD11c^+$ cDCs (A) and the subsets cDC1 (B) and cDC2 (C) was analyzed by flow cytometry.

(D-E) Serum TNF and type I IFN concentrations from the indicated mice in (A-C) were determined by ELISA or VSV bioassay, respectively.

cDCs from *MyTr*$^{-/-}$ and *Cardif*$^{-/-}$ mice displayed no significant decrease in TNF production, although cDC1s seemed to be affected, as they produced slightly less TNF when compared to SPF controls (Fig. 8 A-C). Interestingly, both cDC subsets, cDC1s and cDC2s, from mice deficient for all three adaptors (*MyTrCa*$^{-/-}$) showed a robust impairment in TNF production after stimulation, that was nearly comparable to that of cDCs from GF mice (Fig. 8 A-C). Similar, the measurement of serum TNF concentrations nearly reflected the above findings also on a systemic level, showing *MyTrCa*$^{-/-}$ mice to have the most severe defect in the initiation of cytokine production after direct CD40 stimulation (Fig. 8 D). Interestingly, serum type I IFN levels were decreased in all deficient mice when compared to SPF controls (Fig. 8 E). In line with this, the qRT-PCR data revealed a significant reduced *Ifna4* and *Ifnb1* gene expression in splenic tissue of all three PRR-deficient mouse models in response to α-CD40 stimulation (Fig. 8 F-G).

Recapitulating, these findings indicate that sensing of microbes at steady-state through PRRs is required to program the cDC basal state. Further it seems, that the ability of cDCs to induce production of inflammatory cytokines or a type I IFN response probably relies on distinct tonic PRR signaling. The cDC's capacity to upregulate cytokine production, e.g. TNF, required redundant PRR signaling through one of the available pathways, MyD88/TRIF or CARDIF, whereas the induction of type I IFN gene expression was seemingly dependent on concomitant signaling of all PRR systems.

(F-G) Splenic tissue from α-CD40-stimulated mice shown in (A-C) was collected for RNA extraction. *Ifna4* and *Ifnb1* transcript levels were subsequently determined by qRT-PCR.

Data are representative of two independent experiments. Horizontal bars represent mean ± SD (A-G, n = 5-7). Statistical significance was determined with one-way ANOVA with Bonferroni's multiple comparisons corrections, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001; nd, not detectable.

6.4 Commensal microbiota controls tonic type I interferon production by pDCs

So far, the data indicate that the calibration of the cDC basal state, which allows for a powerful cytokine immune response upon activation, strongly depends on microbiota-controlled signals involving tonic PRR signaling. However, the molecular mechanisms underlying this cDC calibration process at steady-state were less clear and the microbiota-controlled signaling molecules involved in this process were unknown as well.

To obtain insights into the molecular circuits, we performed RNA-sequencing analyses of splenic I-A/I-E^+ $CD11c^+$ cDCs isolated from SPF and GF mice (data not shown; Figure 2 A and S2 B-D of Schaupp et al., 2020). The results indicate that type I IFNs act as the microbiota-controlled calibrator of the cDC instruction process, since ISGs as well as STAT1-target genes were markedly downregulated in cDCs from GF mice when compared to SPF control mice.

Further, we aimed to identify the cellular source of tonic type I IFNs, which remained unknown until this point. Basal type I IFN production is notoriously difficult to analyze because they are produced in such low amounts. We tried to determine steady-state type I IFN protein levels in lysates of sorted cells by using the most sensitive, commercially available ELISA-based kits (Mouse IFN beta ELISA Kit, High Sensitivity, PBL assay science; U-Plex Interferon Combo 1 mouse, MSD). However, both assays failed to detect basal IFN concentration (data not shown). Therefore, qRT-PCR analysis and the usage of IFN-β luciferase reporter mice are currently the only available methods that reliably detect basal type I IFN levels.

To this end, distinct splenic cell populations were sorted and *Ifnb1* transcript levels were determined via qRT-PCR (Fig. 9 A, B). Clearly, pDCs showed the highest steady-state *Ifnb1* gene expression amongst all analyzed populations, although $Ly6C^{hi}$ Monocytes/MPh exhibited also a slightly increased expression of *Ifnb1* (Fig. 9 B). Neither cDCs nor other indicated cell population displayed any detectable tonic type I IFN expression (Fig. 9 B). Those qRT-PCR results could also be recapitulated in a comparable experimental set-up using IFN-β luciferase reporter mice, revealing that pDCs contributed with over 60 % to the total steady-state IFN-β signal (data not shown; Figure 2 G-H of Schaupp et al., 2020). Most noticeably, *Ifnb1* transcript levels in splenic pDCs from GF mice were significantly decreased compared to pDCs from SPF mice (Fig. 9 C), whereas the *Ifnb1* expression in splenic $Ly6C^{hi}$ monocytes/MPh was not altered between GF and SPF mice (Fig. 9 D). Interestingly, a similar *Ifnb1* expression pattern was obtained for distinct gut mucosal-associated cell populations.

Again, intestinal pDCs exhibited at steady-state the highest *Ifnb1* transcript levels when compared to the residual cell populations (Fig. 9 E). Certainly, intestinal Ly6C^{hi} and Ly6C^{lo} monocytes/MPh showed also elevated *Ifnb1* gene expression. Neither intestinal cDCs nor other cell populations displayed any detectable tonic type I IFN expression (Fig. 9 E). Similar to the findings above, *Ifnb1* expression in intestinal pDCs from GF mice was significantly reduced compared to pDCs from SPF mice (Fig. 9 F). In contrast, *Ifnb1* transcript levels in both Ly6C^{hi} and Ly6C^{lo} monocyte/MPh populations were not different between GF and SPF mice (Fig. 9 G-H).

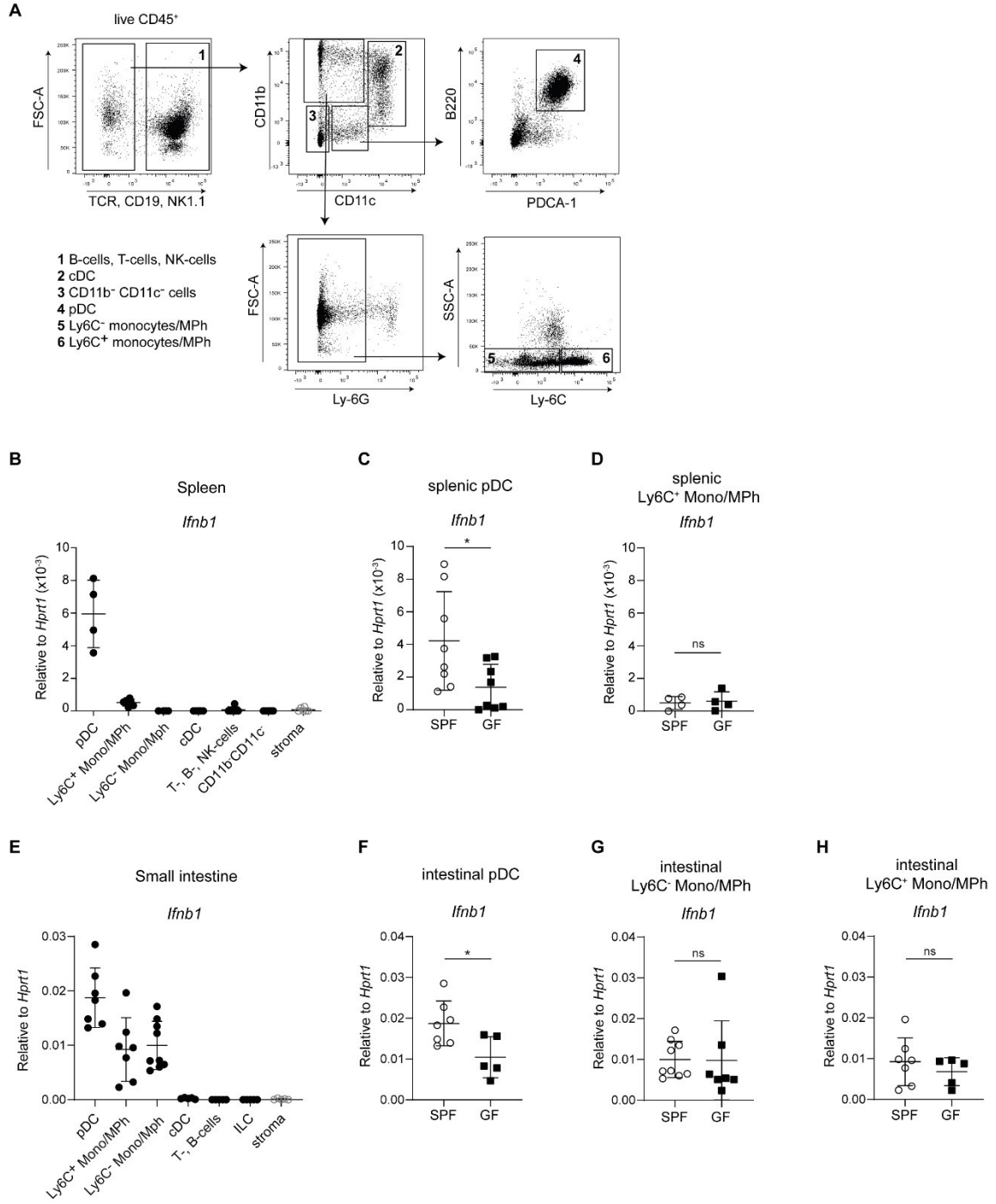


Figure 9: pDCs are the main producers of microbiota-regulated tonic type I IFNs.

(A) Gating strategy for flow cytometry-based cell sorting of leukocyte populations presented in (B-H). Stroma encompass all live CD45$^-$ cells; intestinal T/B-cells were identified as live, CD45$^+$ Lin1$^+$ Lin2$^-$ cells; intestinal ILCs were defined as live, CD45$^+$ Lin1+2$^-$ CD127$^+$ cells. Lin1: TCRβ, CD3, CD5, CD19, B220; Lin2: CD11c, CD11b, Gr-1, F4/80. (B-D) Splenic cell populations from SPF mice (B), pDCs and Ly6C^{hi} Mono/MPh from SPF and GF mice (C, D) were sorted as shown in (A) and *Ifnb1* transcript levels were determined by using qRT-PCR.

(E-H) Indicated lamina propria leukocyte populations from SPF mice (E), pDCs and Ly6C^{hi} or Ly6C^{lo} Mono/MPh from SPF and GF mice (F-H) were sort-purified and *Ifnb1* transcript levels were determined by using qRT-PCR.

Data are representative of two independent experiments. Horizontal bars represent mean ± SD (B+D, n = 4; C, n = 8; E = 7-8; F-H, n = 5-9). Statistical significance was determined with Student's t test, *p ≤ 0.05, **p ≤ 0.01; ns, not significant

In summary, pDCs, systemically distributed or localized in mucosal tissue, were identified as the main producers of tonic type I IFNs. Furthermore, compared to Ly6C^{hi} and Ly6C^{lo} monocytes/MPh, pDCs were the only cell population whose steady-state *Ifnb1* gene expression was microbiota-dependent.

6.5 Microbiota-induced type I IFNs control the cDC basal state through a core transcriptional program

Until now, the data indicate that microbiota-controlled tonic type I IFNs, produced by pDCs, act as instructor molecules of the cDC basal state. To obtain profound insights into the transcriptional circuits based on this cDC calibration process, we extended our initial genome-wide profiling of splenic cDCs by comparing full transcriptomes of the cDC subsets, cDC1 and cDC2, isolated from SPF and GF mice, and from mice genetically deficient for the type I IFN receptor (*Ifnar1*$^{-/-}$) (Fig. 10 and 11). Pearson correlation analysis confirmed previous findings that cDC1s and cDC2s transcriptomes are substantially different (Fig. 10 B) (Merad et al., 2013). We first focused on the analysis of cDC2s, as they represent the largest splenic cDC subset. Principal component analysis (PCA) clearly separates the transcriptomes of GF and *Ifnar1*$^{-/-}$ cDC2s from control SPF cDC2 in PC2 (Fig. 10 C). In line with the transcriptomic data from all CD11c^{+} cDCs, canonical ISGs were found to be consistently downregulated in cDC2s from GF and *Ifnar1*$^{-/-}$ mice (Fig. 10 D). In the next step, we performed Gene Ontology (GO) term analysis of GO terms, pre-defined to contain gene sets from 10 to 1000 genes categorized as biological processes using the database for annotation, visualization and integrated discovery (DAVID) (Huang da et al., 2009). As outcome, we obtained 43 GO terms that were enriched at a false discovery rate (FDR) < 0.05 in SPF cDC2s compared to GF and *Ifnar1*$^{-/-}$ cDC2s. Subsequently, an enrichment map was created, in which the GO terms were clustered according to gene overlap (Fig. 10 E). Even though, the edge cutoff, which corresponds to a similarity score between distinct gene sets, was set to 1, the clusters remained deeply interconnected and enriched quite similar immunological processes. These include two major clusters containing functional annotations for “Innate immune response” and “Response to virus” and two smaller clusters encompassing gene sets related to type I IFN signaling and function (Fig. 10 E). The observed consistency in the enrichment of the those GO terms is reflected by the fact that more than 35 % (81/222) of the genes are consistently downregulated in GF and *Ifnar1*$^{-/-}$ cDC2s and

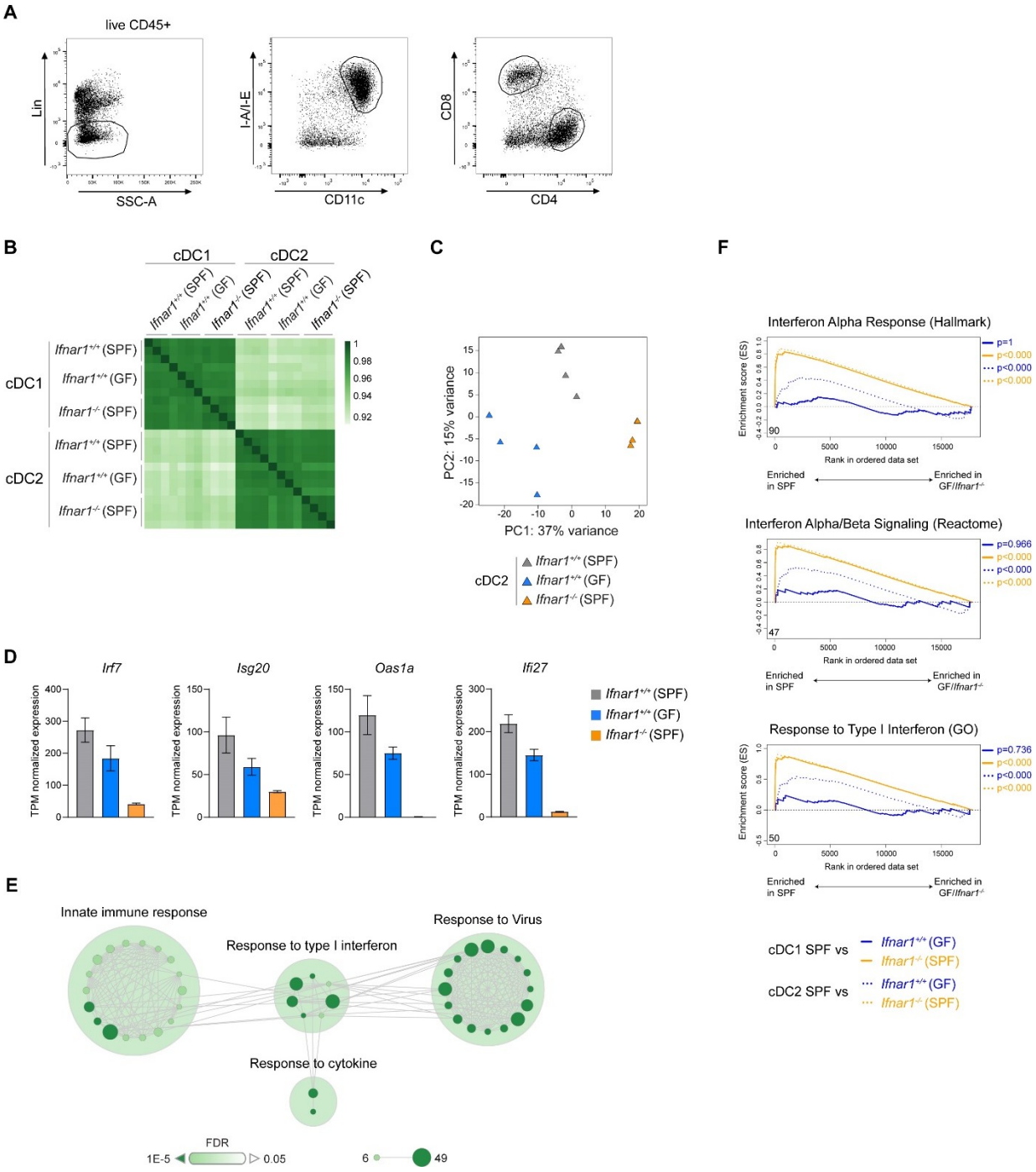


Figure 10: Microbiota and type I IFNs control a similar transcriptional regulome in cDC1s and cDC2s.

(A) Gating strategy for flow cytometry-based sorting of splenic cDC1 and cDC2 for subsequent RNA-sequencing. After duplicates exclusion, cells were gated as DAPI$^-$ CD45$^+$ Lin$^-$ I-A/I-E^{hi} CD11c^{hi} and finally separated by CD8 or CD4 expression into the cDC1 or cDC2 subset, respectively. The lineage cocktail contained CD3, CD19, B220, F4/80, Gr-1.

RNA-sequencing analyses of splenic cDC1 and cDC2 isolated from unstimulated GF, *Ifnar1*$^{-/-}$ and control SPF mice.

(B) Heatmap of Pearson correlation coefficients. Only genes with a non-zero value (normalized read counts) for all replicates in at least one group of cDC1s or cDC2s were included (~ 15,000 genes). Correlation coefficients were calculated using $\log_2$(normalized read counts +1).

almost 20 % (47/222) of these genes are represented in more than 5 distinct GO terms. Hence, the majority of genes consistently downregulated in GF and *Ifnar1*$^{-/-}$ cDC2s are part of the same immunological processes, namely type I IFN signaling and function.

In addition, gene set enrichment analysis (GSEA) was conducted, which required no previous definition of gene sets (Subramanian et al., 2005). Again, in cDC2s from GF and *Ifnar1*$^{-/-}$ mice selected type I IFN-related gene sets were negatively correlated (Fig. 10 F). In the GSEA, this negative correlation in type I IFN-associated gene sets could also be observed for the comparison of SPF *vs*. *Ifnar1*$^{-/-}$ cDC1s (Fig. 10 F).

However, the consistent downregulation of type I IFN-controlled genes in GF cDC1s was not equally apparent, but we found a clear negative correlation of related gene sets, such as “Interferon signaling”, “Interferon Gamma Response” and Interferon Gamma Signaling” (Fig. 11 A). Indeed, the type I IFN- and IFN-γ-related gene sets share many of the same genes and consequently, it cannot be strictly discriminated between type I and type II IFN-controlled genes (Fig. 11 B). Despite this gene overlap, we were still wondering why we obtained a clearer enrichment of interferon-related genes in cDC2s, but not in cDC1s. This observation could partially be explained by the fact, that most genes in those gene sets were expressed at much lower levels in SPF cDC1s and therefore, simply cannot be markedly downregulated (Fig. 11 C).

(C) Principal component analysis on variance-stabilized transformed (vst) counts from splenic cDC2s from the indicated mouse strains.

(D) Expression level of selected ISGs in cDC2s from the indicated mouse strains.

(E) Enrichment map of significantly enriched GO terms classified as biological processes through DAVID (FDR < 0.05).

(F) Enrichment score of selected gene sets for the comparisons SPF *vs*. GF (blue lines) and SPF *vs*. *Ifnar1*$^{-/-}$ (orange lines) for cDC1s (full lines) and cDC2s (dotted lines). Numbers in the lower left corner denote the number of human genes within the respective gene sets. p indicates the nominal p-value from the GSEA analysis.

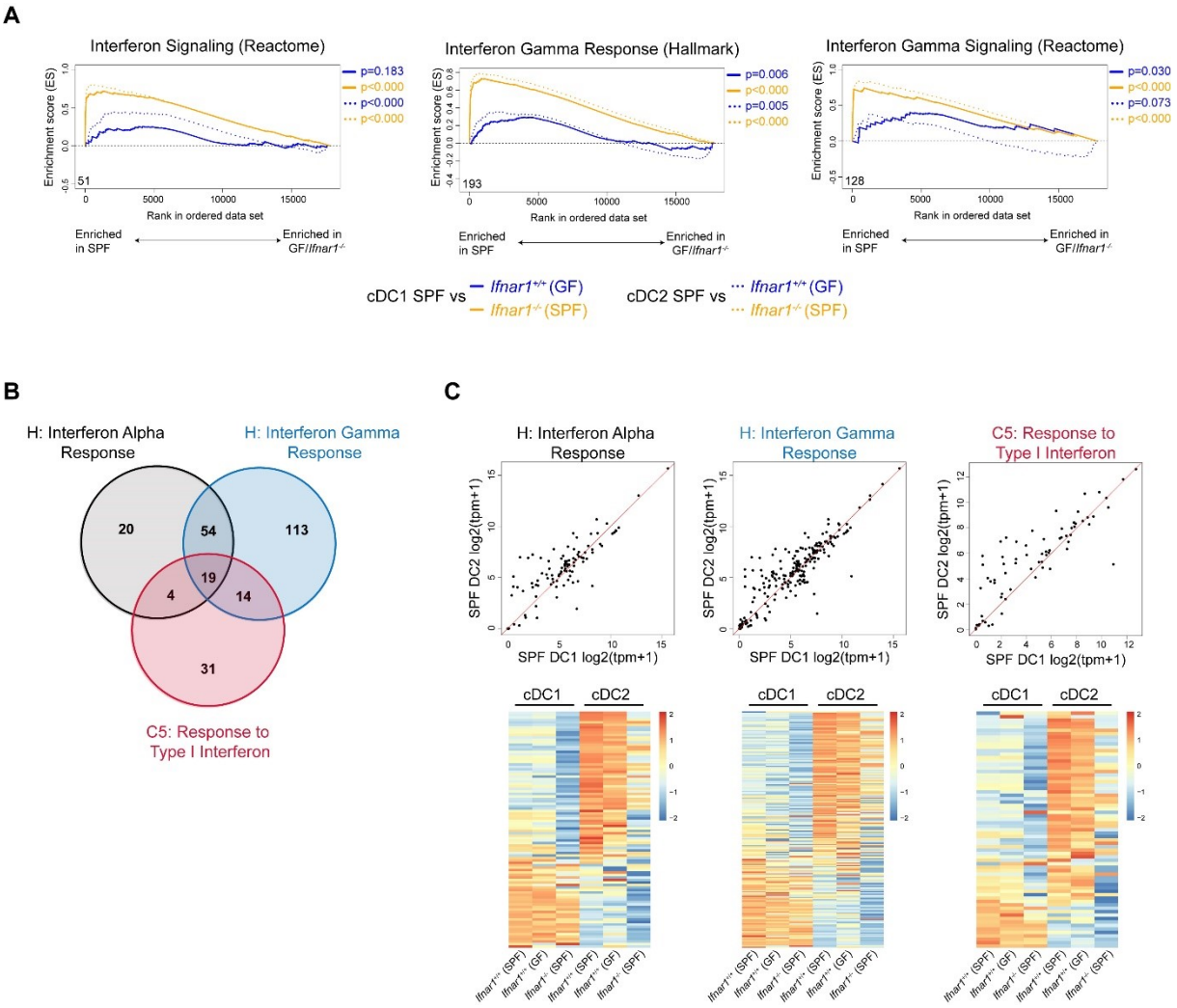


Figure 11: Transcriptional regulome in cDC1s and cDC2s from GF and *Ifnar1*$^{-/-}$ mice.

(A) Enrichment score of selected gene sets for the comparisons SPF *vs*. GF (blue lines) and SPF *vs*. *Ifnar1*$^{-/-}$ (orange lines) for cDC1s (full lines) and cDC2s (dotted lines). Numbers in the lower left corner denote the number of human genes within the respective gene sets. p indicates the nominal p-value from the GSEA analysis.

(B) Venn Diagram showing the overlap of the human genes present in the indicated GSEA gene sets.

(C) Dotplots displaying the expression (log_2 TPM+1) of all corresponding mouse genes present in the indicated GSEA gene set in SPF cDC1 *vs*. SPF cDC2. Red lines indicate equal expression. Heatmaps show the row-scaled transformed expression (log_2 TPM+1) for the same mouse genes across all groups of cDC1 and cDC2.

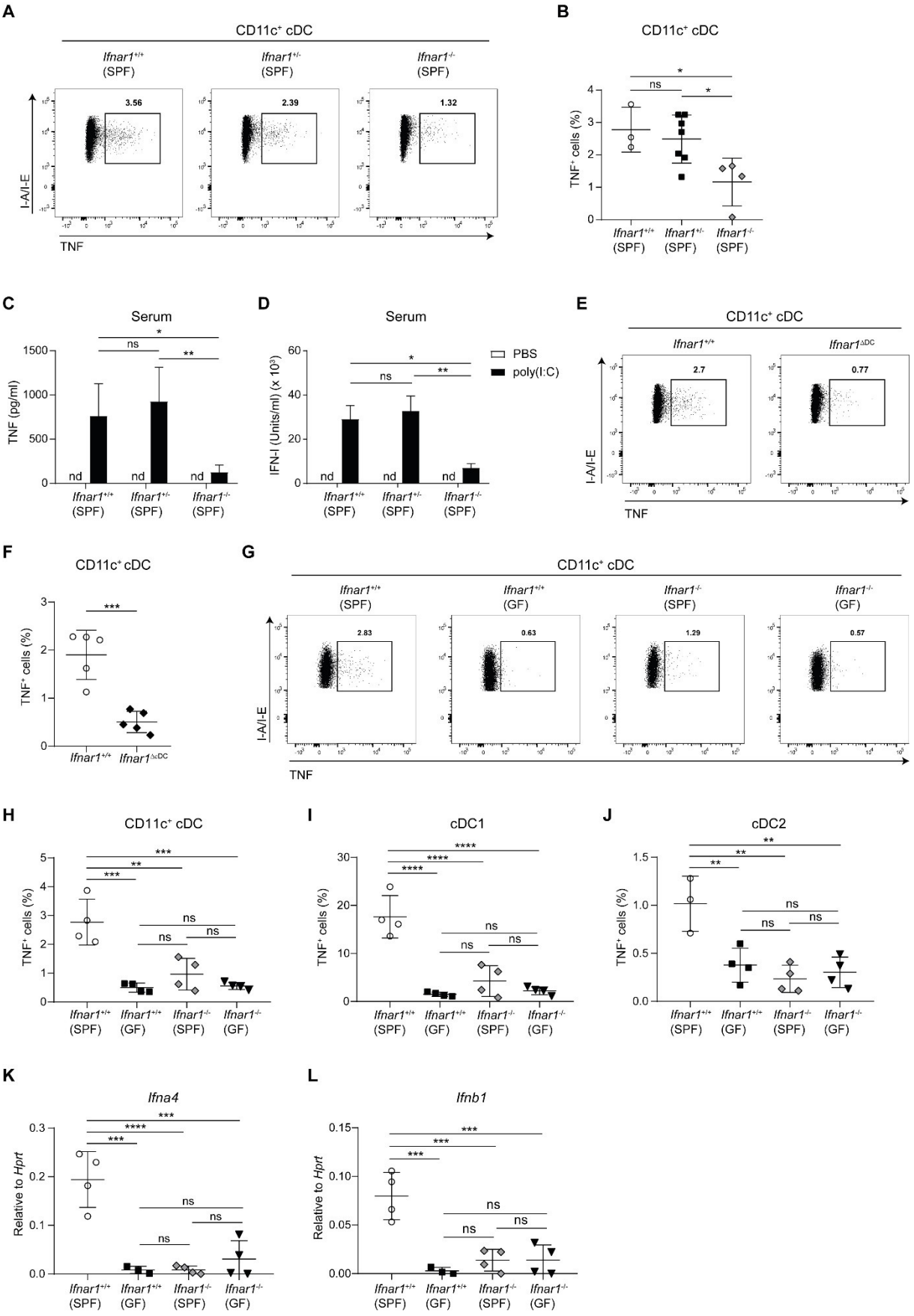
Collectively, the genome-wide transcriptional profiling of the unstimulated splenic cDC subsets, cDC1 and cDC2, revealed that the most downregulated gene sets in cDC1s and cDC2s from GF and *Ifnar1*$^{-/-}$ mice are those related to type I IFN signaling. Thus, these findings further support the essential role of microbiota-induced tonic type I IFN signaling in the instruction process of the cDC basal state.

6.6 Tonic type I IFNs prime cDCs for subsequent responses to microbial stimulation

Altogether, the data suggest that the commensal microbiota regulates the production of tonic type I IFNs by pDCs, which in turn are required to instruct the cDC basal state through a specific transcriptional program. To profoundly validate these findings, a series of further experiments were performed in order to link tonic type I IFN signaling to cDC function.

In a first approach, splenic I-A/I-E^{+} CD11c^{+} cDCs isolated from SPF, GF and *Ifnar1*$^{-/-}$ mice were stimulated with LPS and transcriptional profiling was conducted (data not shown; Figure 5 A-B, S5 A-C of Schaupp et al., 2020). The RNA-sequencing analyses showed an overall incompetence of GF and *Ifnar1*$^{-/-}$ cDCs to upregulate mainly type I IFN- and NF-κB-target genes upon microbial stimulation when compared to SPF cDCs. Furthermore, the capability of cDCs to induce an inflammatory response was clearly dependent on tonic type I IFNs, as cDCs deprived of type II (*Ifngr*$^{-/-}$) or type III (*Ifnlr1*$^{-/-}$) IFN signaling showed no impaired TNF production after microbial stimulation (data not shown; Figure S5 E of Schaupp et al., 2020).

To further exclude, that an altered microbiota composition in *Ifnar1*$^{-/-}$ mice might cause the evident incapability of cDCs to upregulate inflammatory cytokine production upon activation, cDC responses to poly(I:C) stimulation in co-housed *Ifnar1*$^{+/+}$, *Ifnar1*$^{+/-}$ and *Ifnar1*$^{-/-}$ littermates were investigated by flow cytometry (Fig. 12 A-B). The capacity of cDCs from *Ifnar1*$^{+/+}$ and *Ifnar1*$^{+/-}$ mice to upregulate TNF production was nearly comparable, whereas cDCs from co-housed, homozygous *Ifnar1*$^{-/-}$ mice displayed a significant decrease in TNF production in response to poly(I:C) (Fig. 12 B). The measurement of serum TNF and type I IFN concentrations in those mice reflects the results obtained by flow cytometry analysis (Fig. 12 C- D). Systemic TNF and type I IFN levels in *Ifnar1*$^{+/+}$ and *Ifnar1*$^{+/-}$ mice were not much different. In contrast, *Ifnar1*$^{-/-}$ mice exhibited significantly lower serum TNF and type I IFN concentrations after poly(I:C) stimulation (Fig. 12 C-D). Thus, the composition of the indigenous microbiota in *Ifnar1*$^{-/-}$ mice is not the causative factor to explain the observed functional differences between cDCs from *Ifnar1*$^{-/-}$ mice and control mice.


A
CD11c+ cDC
Ifnar1+/+ (SPF)
Ifnar1+/- (SPF)
Ifnar1-/- (SPF)
3.56
2.39
1.32
I-A/I-E
TNF
B
CD11c+ cDC
TNF+ cells (%)
ns
*
*
C
Serum
TNF (pg/ml)
nd
D
Serum
IFN-I (Units/ml) (x 10^3)
PBS
poly(I:C)
E
CD11c+ cDC
Ifnar1+/+
Ifnar1ΔDC
2.7
0.77
F
CD11c+ cDC
Ifnar1+/+
Ifnar1ΔcDC
G
CD11c+ cDC
Ifnar1+/+ (SPF)
Ifnar1+/+ (GF)
Ifnar1-/- (SPF)
Ifnar1-/- (GF)
2.83
0.63
1.29
0.57
H
CD11c+ cDC
I
cDC1
J
cDC2
K
Ifna4
Relative to Hprt
L
Ifnb1

Although this data provides the link of tonic type I IFN signals to a poised cDC basal state, it did not directly proof that type I IFNs act specifically on cDCs due to global IFNAR1 deficiency in *Ifnar1*$^{-/-}$ mice. To address this aspect, *Cd11c* (*Itgax*)-cre mice were crossed to *Ifnar1*$^{fl/fl}$ mice, generating a cDC-specific IFNAR1 deletion (*Ifnar1*$^{\Delta cDC}$) and the cDC response to poly(I:C) stimulation was investigated (Fig. 12 E-F). Flow cytometry analysis revealed that TNF production of splenic cDCs in *Ifnar1*$^{\Delta cDC}$ mice was significantly reduced compared to control mice (Fig. 12 F). This observation indicates that cDCs required indeed direct tonic type I IFN signaling, rendering them responsive towards microbial stimuli.

Additionally, to proof that the deprivation of tonic type I IFNs is caused by the GF status, we generated GF *Ifnar1*$^{-/-}$ mice and compared the cDC's ability to induce an inflammatory response after poly(I:C) stimulation to those of GF and *Ifnar1*$^{-/-}$ (SPF) mice (Fig. 12 G-J). Flow cytometry analysis clearly shows that all CD11c^{+} cDCs, including both subsets cDC1s and cDC2s, from GF, *Ifnar1*$^{-/-}$ (SPF) and *Ifnar1*$^{-/-}$ (GF) mice displayed a significantly decreased TNF production when compared to cDCs from SPF control mice (Fig. 12 H-J).

Figure 12: cDCs deprived of tonic type I IFN signaling cannot be activated upon microbial stimulation.

(A-B) Flow cytometry analyses of TNF production by splenic cDCs from co-housed *Ifnar1*$^{+/+}$, *Ifnar1*$^{+/-}$ and *Ifnar1*$^{-/-}$ littermates after poly(I:C) stimulation. Pre-gating contains duplicates exclusion, CD45^{+} Lin (CD3, CD19, B220, F4/80, Gr-1)$^{-}$ I-A/I-E^{hi} CD11c^{hi}. Representative dot plots (A) with corresponding quantitative analysis (B). (C-D) Serum TNF and type I IFN concentrations from mice shown in (A-B) were determined after poly(I:C) stimulation by ELISA or VSV bioassay, respectively.

(E-F) Flow cytometry analyses of TNF production by splenic cDCs from controls (*Ifnar1*$^{+/+}$) and from mice carrying a cDC-specific IFNAR1 deletion (*Ifnar1*$^{\Delta cDC}$) after poly(I:C) stimulation. Pre-gating contains duplicates exclusion, CD45^{+} Lin (CD3, CD19, B220, F4/80, Gr-1)$^{-}$ I-A/I-E^{hi} CD11c^{hi}. Representative dot plots (E) with corresponding quantitative analysis (F).

(G-J) Flow cytometry analyses of TNF production by splenic cDCs (H), cDC1s (I) and cDC2s (J) from SPF, GF, *Ifnar1*$^{-/-}$ and GF *Ifnar1*$^{-/-}$ mice after poly(I:C) stimulation. Pre-gating includes duplicates exclusion, CD45^{+} Lin (CD3, CD19, B220, F4/80, Gr-1)$^{-}$ I-A/I-E^{hi} CD11c^{hi}. The cDC subsets were further distinguished by CD8 (cDC1) or CD4 expression (cDC2). Representative dot plots (G) with corresponding quantitative analysis (H-J).

(K-L) Splenic tissue from poly(I:C)-stimulated mice shown in (G-J) was collected for RNA extraction. *Ifna4* (K) and *Ifnb1* (L) transcript levels were subsequently determined by qRT-PCR.

Data are representative of two independent experiments. Horizontal bars represent mean ± SD (A-D, n = 3-6; E-F, n = 5; G-L, n = 4). Statistical significance was determined with Student's t test (F) or with one-way ANOVA with Bonferroni's multiple comparisons corrections (B-D, H-L), *p ≤ 0.05, **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001; ns, not significant; nd,not detectable.

Further, *Ifna4* and *Ifnb1*transcript levels in splenic tissue of those mice were determined by qRT-PCR (Fig. 12 K-L). Similar to the findings above, GF, *Ifnar1*$^{-/-}$ (SPF) and *Ifnar1*$^{-/-}$ (GF) mice failed to upregulate type I IFN gene expression in response to poly(I:C) stimulation when compared to SPF mice. Interestingly, cDCs from *Ifnar1*$^{-/-}$ (GF) mice showed no further reduction in TNF production and systemic type I IFN expression when compared to cDCs isolated from GF and *Ifnar1*$^{-/-}$ (SPF) mice, suggesting that the majority of signals lacking in GF and in *Ifnar1*$^{-/-}$ (SPF) mice are rather linked than acting separately.

Collectively, the results demonstrate that cDCs require direct tonic type I IFN signaling to keep them in a poised state, ensuring a proper immune response upon microbial stimulation. Moreover, our data indicate the lack of the indigenous microbiota to be the causing factor for the deprivation of tonic type I IFN signals. Consequently, our data show that tonic type I IFN signaling and the calibration of the cDC basal state are in causal relation.

6.7 Microbiota-induced type I IFNs instruct a specific metabolic basal state of cDCs

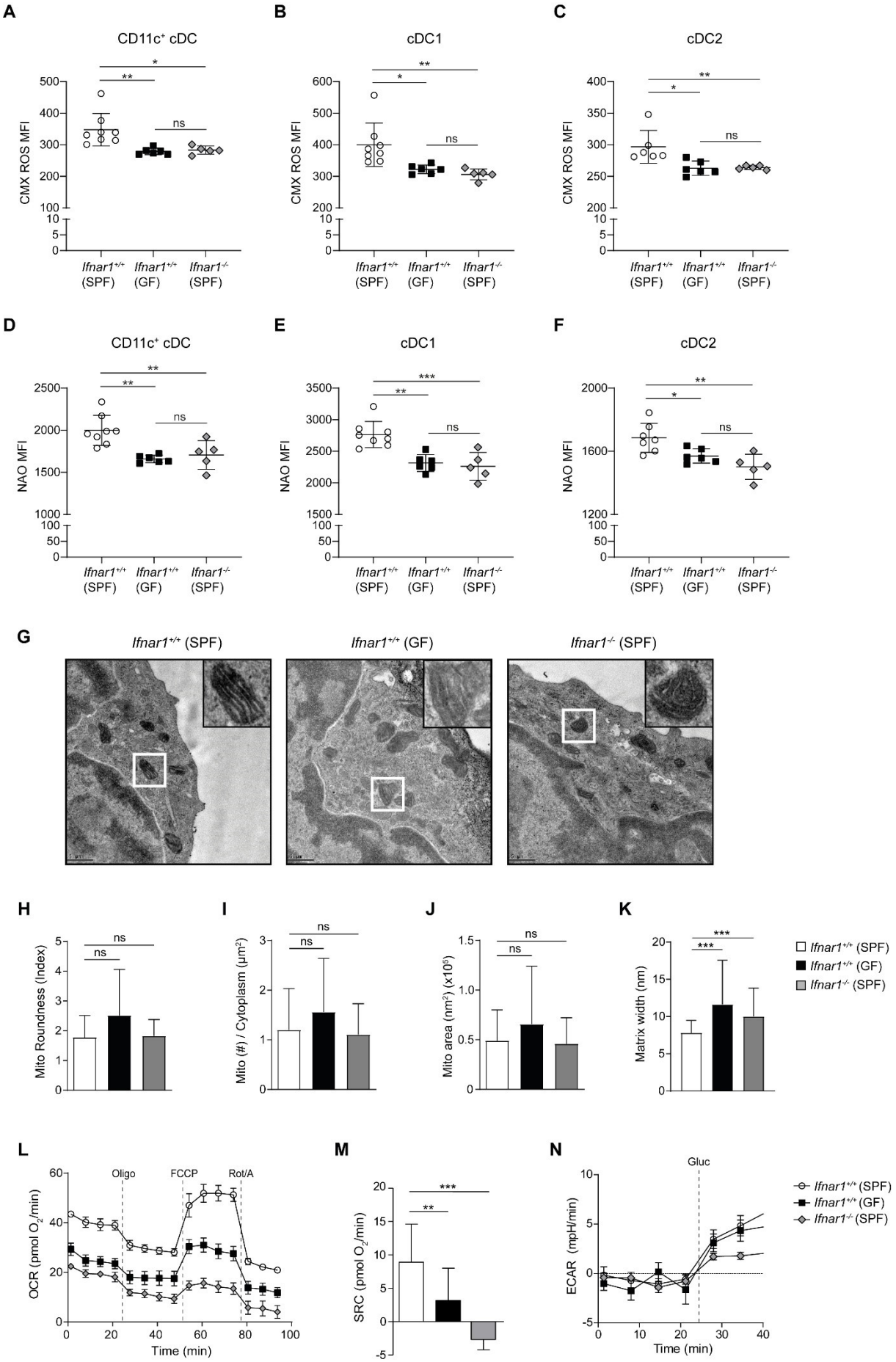
GSEA analysis of our initial RNA-sequencing data of all I-A/I-E^{+} CD11c^{+} splenic cDCs revealed not only a markedly reduction in ISG expression in cDCs from GF and *Ifnar1*$^{-/-}$ mice, but also a consistent downregulation of genes associated with mitochondrial function, including genes of the respiratory chain (data not shown; Figure 3 F of Schaupp et al., 2020). Interestingly, ChIP-sequencing directed against H3K27m3, a dominant mark of transcriptionally inactive genes, supported RNA-sequencing results, as primarily genes involved in metabolic processes were enriched with H3K27me3 in GF and *Ifnar1*$^{-/-}$ cDCs (data not shown; Figure 7 E of Schaupp et al., 2020). The oxidative phosphorylation pathway appeared to have the highest H3K27me3 occupancy, suggesting that cDCs from GF and *Ifnar1*$^{-/-}$ mice might possess dysfunctional mitochondria. To directly investigate mitochondrial function in cDCs from GF and *Ifnar1*$^{-/-}$ mice and to obtain more insight into the metabolic basal state of cDCs, a series of further experiments was performed.

In a first approach, the mitochondrial membrane potential was investigated using the MitoTracker Red CMX Ros, whose uptake is directly proportional to the mitochondrial membrane potential (Pendergrass et al., 2004). All CD11c^{+} cDCs and the subsets, cDC1s and cDC2s, from GF and *Ifnar1*$^{-/-}$ mice displayed a significant decrease in MitoTracker uptake at

stead-state when compared to SPF controls (Fig. 13 A-C). Further, total mitochondrial mass and inner membrane surface was analyzed using nonyl acridine orange (NAO), which is incorporated into mitochondria independent of their membrane potential (Maftah et al., 1989). Compared to SPF controls, cDCs, including both subsets cDC1s and cDC2s, from GF and *Ifnar1*$^{-/-}$ mice showed a reduced NAO integration (Fig. 13 D-F). Together, these findings further corroborate impaired mitochondrial function in cDCs from GF and *Ifnar1*$^{-/-}$ mice, as those cells displayed an overall reduced mitochondrial mass and a weaker membrane potential.

In a second approach, the physical appearance of mitochondria in cDCs from SPF, GF and *Ifnar1*$^{-/-}$ mice was investigated by using transmission electron microscopy (TEM) (Fig. 13 G). Several distinct parameters were determined, including mitochondria number, size and form. However, none of these parameters were significantly different between SPF, GF and *Ifnar1*$^{-/-}$ cDCs (Fig. 13 H-J). Interestingly, the matrix width of the mitochondria was significantly increased in cDCs from GF and *Ifnar1*$^{-/-}$ mice compared to cDCs from SPF controls (Fig. 13 K). The enlarged matrix width is accompanied by looser cristae and a wider inner membrane space that has previously been linked to decreased electron transport efficacy and impaired mitochondrial function in T cells (Klein Geltink et al., 2017).

In a third approach, we set the focus on investigating live metabolic function in freshly isolated splenic cDCs from SPF, GF and *Ifnar1*$^{-/-}$ mice using extracellular flux analysis (Fig. 13 L-N). First, mitochondrial oxidative phosphorylation was analyzed by measuring the oxygen consumption rate (OCR) (Fig. 13 L). Clearly, GF and *Ifnar1*$^{-/-}$ cDCs had a much lower basal respiration compared to SPF controls. Injection of the electron uncoupler FCCP forces the cell to its maximum respiratory capacity. cDCs from GF and *Ifnar1*$^{-/-}$ mice were distinctly unable to augment maximal respiration to the same extent as SPF cDCs. Consequently, the spare respiratory capacity, defined as the difference between maximal and basal respiration, was greatly reduced in cDCs from GF and *Ifnar1*$^{-/-}$ mice compared to SPF controls (Fig. 13 M).


A
CD11c+ cDC
CMX ROS MFI
B
cDC1
C
cDC2
D
CD11c+ cDC
NAO MFI
E
cDC1
F
cDC2
Ifnar1+/+ (SPF)
Ifnar1+/+ (GF)
Ifnar1-/- (SPF)
ns
G
Ifnar1+/+ (SPF)
Ifnar1+/+ (GF)
Ifnar1-/- (SPF)
H
Mito Roundness (Index)
I
Mito (#) / Cytoplasm (µm2)
J
Mito area (nm2) (x10e5)
K
Matrix width (nm)
L
OCR (pmol O2/min)
Oligo
FCCP
Rot/A
Time (min)
M
SRC (pmol O2/min)
N
ECAR (mpH/min)
Gluc
Time (min)

Secondly, the glycolytic activity of cDCs was determined by measuring the extracellular acidification rate (ECAR), which was not markedly different between SPF cDCs and cDCs from GF and *Ifnar1*$^{-/-}$ mice (Fig. 13 N).

Taken together, the data point towards that cDCs from GF and *Ifnar1*$^{-/-}$ mice have an impaired mitochondrial function, as the membrane potential, total mitochondria mass and oxidative phosphorylation were found to be significantly reduced in those. Consequently, microbiota-controlled tonic type I IFNs instruct also a specific basal metabolic state of cDCs, that primary relies on functional mitochondrial respiration.

Figure 13: Microbiota-induced tonic type I IFNs regulate the metabolic basal state of cDCs.

(A-C) Mitochondrial membrane potential in splenic cDCs, cDC1s and cDC2s from the indicated mouse strains was analyzed by flow cytometry using the MitoTracker Red CMX Ros. Data represent mean fluorescence intensity (MFI).

(D-F) Total mitochondrial mass in splenic cDCs, cDC1s and cDC2s from the indicated mouse strains was analyzed by flow cytometry using nonyl acridine orange (NAO). Data represent mean fluorescence intensity (MFI).

(G-K) Mitochondria of splenic cDCs isolated from the indicated mouse strains were analyzed using transmission electron microscopy. White squares marked regions electronically magnified in the inlays with a magnification of x60,000 (G). The number (#) per area cytoplasm (H), size (I), 'Roundness Index' (J), calculated as the longitudinal diameter divided by the transverse diameter, and the matrix width (K) of mitochondria were determined.

(L-N) Primary splenic cDCs were sorted from the indicated mouse strains. Mitochondrial respiration, denoted as oxygen consumptions rate (OCR) (pmol O_2/min) (L) and glycolytic activity, displayed as extracellular acidification rate (ECAR) (mpH/min) (N), during steady-state were measured using an extracellular flux analyzer (Agilent Seahorse); Oligo: Oligomycin; Rot/A: Rotenone/Antimycin. The spare respiratory capacity, related to (L) was calculated as the difference between the maximal and the basal respiration (M).

Data are representative of three independent experiments. Horizontal bars represent mean ± SD (A-F, n = 5-8; G-K, n = 3; L-N, n = 5). Statistical significance was determined with one-way ANOVA with Bonferroni's multiple comparisons corrections (A-C), *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ns, not significant.

7 Discussion

7.1 cDC unresponsiveness to microbial stimulation in GF mice can be restored through re-colonization with a defined microbiota

cDCs are equipped with a plethora of distinct PRRs rendering them highly efficient in sensing PAMPs and DAMPs. Engagement of PRRs leads to cDC activation resulting in pro-inflammatory cytokine secretion and priming of T cells, thereby representing the central mechanism that links innate immune recognition to antigen-specific adaptive immunity (Janeway and Medzhitov, 2002). My work now shows that cDCs need to undergo a microbiota-regulated "instruction" process at steady-state that is required for fulfilling these central functions. Previous studies performed in the group of Dr. Diefenbach and others demonstrated that, in the absence of the indigenous microbiota, cDCs and other MPh subsets cannot initiate a proper cytokine response upon stimulation, leading to higher susceptibility to some virus infections and reduced tumor rejection (Abt et al., 2012; Ganal et al., 2012; Iida et al., 2013; Kane et al., 2011). This incompetence is not restricted to splenic cDCs because also low serum cytokine and type I interferon transcript levels could be detected in GF mice treated i.v. with anti-CD40 (Fig. 6 D-G). In line with previous studies, this observation may point towards a general unresponsiveness of the MPh system to microbial stimulation under germ-free conditions (Abt et al., 2012; Ichinohe et al., 2011; Winkler et al., 2020). Most likely, cDCs residing in other lymphoid and non-lymphoid tissue, monocytes and macrophages, that represent a further cellular source of pro-inflammatory cytokine production upon activation, may also be affected (Abt et al., 2012; Davies et al., 2013; Erny et al., 2015; Hume, 2008). Erny and colleagues found microbiota-derived SCFAs, which mediate their effect partially through HDAC inhibition, to be important modulators of microglia function in the brain. However, we could not observe a restoration of cDC function from GF mice, when treated with HDAC inhibitors (Ganal et al., 2012).

Further research is required to explore to which extent the functionality of the individual, tissue-specific MPh subsets is regulated by the commensal microbiota and to unravel the molecular mechanisms underlying these processes.

cDCs play a central role in initiating not only innate, but also adaptive immune responses, when they become activated through PRR engagement. Efficient cDC-mediated T cell priming relies on simultaneous encounter of three signals presented by cDCs (antigen presentation, expression of co-stimulatory molecules and cytokine secretion) as described in

Chapter 3.4.2. Otherwise, mechanisms of peripheral tolerance will be induced, leading to T cell unresponsiveness. We have shown that cDCs deprived of tonic type I interferons (GF and *Ifnar1*$^{-/-}$) are unable to prime CD8^{+} T cells at steady-state or after activation (data not shown; Figure 1 D and 6 E). Recently, it has been described that splenic cDCs from *Ifnar1*$^{-/-}$ mice possess a reduced ability to present antigens (Zietara et al., 2009). However, we could not observe any difference in antigen presentation capacity between *Ifnar1*$^{-/-}$ and control cDCs (data not shown; Figure S6 E of Schaupp et al., 2020). Furthermore, expression of genes associated with T cell activation, such as co-stimulatory molecules or NF-κB regulators, was not significantly altered in cDCs from GF and *Ifnar1*$^{-/-}$ mice when compared to SPF mice (data not shown; Figure 6 D of Schaupp et al., 2020). Thus, the reduced CD8^{+} T cell priming by antigen-presenting cDCs deprived from tonic type I interferon signaling cannot be easily explained by a general reduction in "signal 1" or "signal 2", but rather demonstrate the fundamental requirement of cDCs to become "instructed" by microbiota-controlled tonic type I interferons. However, our data also demonstrates that the microbiota-induced instruction of the cDC basal state authorizes cDCs to prime T cells against harmless antigens, demanding the intervention by peripheral tolerance mechanisms, illustrating an example of an evolutionary successful adaption of vertebrates to their microbial environment.

Our data adds to recent insights showing that the function of myeloid cells is not entirely repetitious but depends on previous "experience". For example, macrophages that were previously stimulated by microbial PAMPs, such as fungal or bacterial cell wall components, on already instructed cDCs or MPhs show increased responses upon secondary stimulation (i.e., "trained immunity" or "innate immune memory"). This process is associated with epigenetic and metabolic reprogramming, enabling a stronger immune response upon re-challenge with those microbial ligands when compared to "untrained" cells (Cheng et al., 2014; Netea et al., 2016; Novakovic et al., 2016; Quintin et al., 2012).

It should be pointed out that cDC "instruction" which is mediated by the microbiota and "trained immunity" are considered two different processes, although they may share similar regulatory mechanisms, including chromatin remodeling and metabolic reprograming. The instruction process that cDCs need to undergo during steady-state in order to fulfill their future functions as key coordinators of innate and adaptive immunity is a continuous and likely tunable process that relies on a microbiota-regulated tonic IFN-I signal acting on cDCs and initiating a specific transcriptional, epigenetic and metabolic basal state.

In the current study, we showed that cDCs from adult GF mice were highly unresponsive towards microbial stimuli. cDCs from young SPF mice (3 or 4 weeks old) displayed a similarly impaired pro-inflammatory response upon LPS stimulation when compared to adult SPF mice (data not shown; Figure S1 C-D of Schaupp et al., 2020). Monocolonization of adult GF mice with the Gram-negative bacterium *E. coli* JM83 led to increased TNF production of cDC in response to LPS and poly(I:C) (Fig. 7). But only re-colonization with a more complex model microbiota (referred to as sDMDMm2), consisting of 12 defined bacterial species that represent five major prokaryote phyla of the gut microbiota community (Uchimura et al., 2016), restored cDC responsiveness to microbial stimulation, indicating that the complexity of the indigenous microbiota may determine the amplitude of the cDC immune response (Fig. 7). In vertebrates, microbial colonization begins at birth and in mice microbiota diversity becomes established within the first weeks of life (Gensollen et al., 2016). Several studies have demonstrated that during the neonatal period (from birth to weaning) the microbiota has a deep impact on shaping the host immune system with long-term effects on immune cell function and immunopathology (Gensollen et al., 2016; Thorburn et al., 2015). Furthermore, distinct studies have shown that perturbation of this "window of opportunity" can have severe consequences for the host. For instance, GF mice display an impaired development of intestinal lymphoid structures and reduced intestinal T_{reg} cell numbers (Al Nabhani et al., 2019; An et al., 2014). These defects are considered to be only transient though and could be overcome by microbial re-colonization later in life (Al Nabhani and Eberl, 2020). However, previous studies have shown that in some cases the immune deficiencies cannot be reverted by re-colonization and thus, persist throughout life. For example, it has been demonstrated that antibiotics treatment during the neonatal period but not later, or colonization of GF mice only after weaning led to an enhanced susceptibility to IBD and allergy in the gut and the lung in adulthood (Bashir et al., 2004; Olszak et al., 2012; Russell et al., 2012).

Although it cannot be ruled out that the imprinting by the microbiota early in life may be also important for the cDC instruction process, this study showed that cDC unresponsiveness in GF mice towards microbial stimuli was not an irreversible effect, as cDC function could be restored by re-colonization of adult GF mice with the complex model microbiota sDMDMm2. Most interestingly, these findings collectively suggest that the magnitude of the cDC's ability to induce an immune response to microbial stimulation may be determined in steady-state by the complexity of the indigenous microbiota composition.

7.2 Signaling of commensal microbiota through PRRs at steady-state is required to instruct a functional basal state of cDCs

Host-commensal mutualism is sustained by constant sensing of the microflora by innate immune cells through PPRs. The permanent sensing of MAMPs at steady-state is not only crucial for maintaining an intact epithelial barrier, including production and secretion of IgA, mucus, and antimicrobial proteins, but also for mediating immunological tolerance and thus, preventing deleterious auto-immune and inflammatory responses (Belkaid and Hand, 2014; Honda and Littman, 2012; Rakoff-Nahoum et al., 2004). Since constant microbial recognition is essential for the immune system's education, we wondered if the cDC "instruction" process would also require sensing of the commensal microbiota trough PRRs. To address this question, mice genetically deficient for the adaptor proteins MyD88, TRIF and CARDIF were employed. MyD88 and TRIF are essentially involved in the TLR system that senses a broad spectrum of microbial ligands, including bacterial, fungal and viral derived molecules (Fig. 1) (Horng et al., 2001; Lin et al., 2010). CARDIF is part of the RIG-I/MDA5 signaling pathway, representing professional sensors of viral ssRNA and dsRNA (Fig. 2) (Gack et al., 2007; Zeng et al., 2010). An agonistic anti-CD40 antibody was chosen as stimulus that activates cDCs independent of these PRR adaptors. CD40 is broadly expressed on the cell surface of antigen-presenting cells, such as cDC, macrophages and monocytes, and acts as co-stimulatory molecule in T cell-mediated immune responses. Ligation of CD40 induces the activation of multiple pathways, including MAPK, STAT3 and NF-κB, that finally leads to the initiation of pro-inflammatory cytokine gene expression (Eliopoulos et al., 2000; Grewal and Flavell, 1998).

cDCs from mice genetically deficient for all three adaptor proteins (*MyTrCa*$^{-/-}$) were largely unresponsive to anti-CD40 challenge, nearly comparable to GF mice (Fig. 8 A-C). The incapability of the MPh system to elicit an efficient immune response could also be observed systemically, as *MyTrCa*$^{-/-}$ mice showed decreased serum TNF and IFN-I levels, as well as a reduced expression of type I IFNs compared to controls (Fig. 8 D-G). Interestingly, the ability to induce an inflammatory cytokine or a type I IFN response required possibly distinct tonic PRR signaling, as systemic type IFN I expression and serum levels were also reduced in *MyTr*$^{-/-}$ and *Cardif*$^{-/-}$ mice, while TNF production was not impaired to the same extent. These findings indicate that programming of the cDC's ability to induce the production of inflammatory cytokines after activation requires redundant tonic PRR signaling through one of the modules, either MyD88/TRIF or CARDIF. However, the promotion of the

"antiviral state" by upregulating type I interferon production was apparently dependent on tonic signaling of PRR modules, specifically involved in detecting viral-derived molecules, as it is the case for CARDIF and certain TLRs, such as TLR-3, 7 and 9.

From this point on, it was still unclear which type of microbiota-induced signal is required for the instruction of the cDC basal state. Therefore, we decided to perform genome-wide RNA-sequencing of cDC isolated from GF and SPF control mice in order to obtain insights into the microbiota-regulated transcriptional circuits. Strikingly, ISGs and STAT1-target genes were robustly downregulated in GF cDCs (data not shown; Figure 5 A-B and S5 A-C of Schaupp et al., 2020). This observation was consistent with previous studies showing a reduced ISG transcription in microglia and peritoneal macrophages from GF mice, underlining again that the lack of the indigenous microbiota and consequently the missing microbiota-induced signals may entail a general functional impairment of the MPh system (Abt et al., 2012; Erny et al., 2015). STAT1 is the major regulator of canonical type I IFN signaling and we found also a significant decreased pool of phosphorylated STAT1 at steady-state in cDCs from GF mice when compared to SPF mice (data not shown; Figure 2 C of Schaupp et al., 2020).

Collectively, these data suggest that steady-state type I IFNs are the putative microbiota-controlled signal required for cDC calibration at steady-state. Since mixed BM chimera experiments revealed that cDCs themselves did not require direct tonic PRR signaling for their steady-state calibration (data not shown; Figure 1 I of Schaupp et al., 2020), we hypothesized that the cell population producing steady-state type I IFNs senses MAMPs through PRRs, thereby linking the microbiota to type I IFN production.

7.3 Microbiota controls tonic type I IFN production by pDCs

It is well acknowledged that type I IFNs are produced in low levels at steady-state and are critically involved in several processes of immune homeostasis, including resistance to viruses, hematopoietic stem cell maintenance, NK cell and macrophage function (Bocci, 1985; Essers et al., 2009; Gough et al., 2012; Lucas et al., 2007; McFarlane et al., 2017; Taniguchi and Takaoka, 2001). Importantly, several studies have shown that the production of type I IFNs in response to a microbial stimulus is regulated by the commensal microbiota

(Abt et al., 2012; Ganal et al., 2012; Kawashima et al., 2013; Stefan et al., 2020). However, no previous study has yet addressed the question in which manner and range microbiota-controlled tonic type I IFNs impact on cDC function. In general, GF mice displayed a reduced type I IFN production at steady-state compared to SPF mice (data not shown; Figure 1 D-E of Schaupp et al., 2020). Systemically distributed as well as mucosa-associated pDCs were found to be the major source of microbiota-regulated basal type I IFN production (Fig. 9 B, E). *Ifnb1* transcript level of intestinal pDCs seemed to be elevated compared to splenic pDCs. These data must be interpreted with caution though, as these are independent experiments. Analysis of tonic type I IFN production using IFN-β luciferase reporter mice revealed that tissues in close contact to the commensal microbiota did not preferably show higher type I IFN concentrations than peripheral tissues (data not shown; Figure 2 F of Schaupp et al., 2020). In order to validate those findings also on a transcript level, it is recommended to isolate and analyze pDCs from distinct tissues from the same mouse. Interestingly, a recent study reported that colonic cDCs, specifically the cDC2 subset, were a major source of microbiota-controlled constitutive IFN-β that supported natural resistance to virus infection (Stefan et al., 2020). However, the investigators did not analyze colonic pDCs and excluded a contribution of this cell population to tonic type IFN production, although a previous study by the same group showed a microbiota-dependent correlation between number of colonic pDCs and a type I IFN gene signature (Geva-Zatorsky et al., 2017).

In our study, we provided clear evidence that splenic and intestinal pDCs are the major producers of microbiota-regulated constitutive IFN-β production (Fig. 9 B, E). However, it is still unclear where pDCs encounter the relevant type I IFN-inducing microbiota-derived signals. One possibility is that pDCs receive microbial signals at mucosal sites and subsequently circulate through the lymph or blood system into peripheral tissues where they calibrate the cDC basal state by tonic type I IFN release. Previous studies have shown that the gut microbiota composition influenced the number of intestinal pDCs, however pDC function in terms of type I IFN production has not been analyzed (Fujiwara et al., 2008; Manfredo Vieira et al., 2018). Recently, a correlation between frequencies of pDCs, residing in the lamina propria of the small and large intestine, and a systemic type I IFN gene signature was reported, that was mainly provoked by distinct species of the genus *Bacteroides* (Geva-Zatorsky et al., 2017). Furthermore, it has been reported that various immune cell populations are able to migrate from intestinal sites into lymphatics and traffic between various lymphoid tissues (Morton et al., 2014). It is possible that intestinal pDCs also release tonic type I IFNs

into systemic circulation. But since we observed that even splenic pDCs produced high amounts of tonic type I IFNs, it is rather imaginable that "primed" pDCs may migrate from the gut into peripheral tissues releasing type I IFNs. In another conceivable scenario, microbiota-derived molecules may pass the epithelial barrier at mucosal sites entering systemic circulation. pDCs possibly sense those molecules in the blood, peripheral tissue or even in the bone marrow, as it was demonstrated for neutrophils that required peptidoglycan signaling in the bone marrow to become fully functional (Clarke et al., 2010). Another study reported that distinct gut-colonizing commensal bacteria are even capable to delocalize to mLN and systemic lymphoid tissues, such as spleen or peripheral lymph nodes, potentially involved in priming of pDCs within secondary lymphoid organs (Geva-Zatorsky et al., 2017). Further investigation is necessary to clarify which of the aforementioned models is correct.

Our data also revealed that steady-state type I IFN production by pDCs required tonic sensing of microbiota-derived molecules through MyD88, TRIF and CARDIF-dependent PRR modules (Fig. 8). Since differences in the MPh systems's ability to induce either cytokine or type I IFN production could be observed and probably rely on distinct tonic PRR signaling, it would be interesting to investigate in more detail whether specific microbial molecules may induce a higher tonic type I IFN expression by pDCs than others, for instance bacterial ligands such as LPS or CpG DNA, *vs*. viral molecules, like ssRNA or dsRNA. It is well known that pDCs express high levels of TLR-7 and -9, rendering them highly efficient in sensing bacterial and viral nucleic acids with subsequent extensive production of type I IFNs within a few hours post-infection (Colonna et al., 2004). According to the Immunological Genome Project Database, splenic pDCs express also increased levels of *Tlr12*, *Mavs* and *Sting1* (Heng and Painter, 2008). Thus, it would be intriguing to analyze if engagement of such particular PRR pathways generally contribute to basal type I IFN production and secondly, if, dependent on which PRR pathway will be activated, differences in the magnitude of tonic type I IFN production by pDCs, and following cDC function, might be observed.

Furthermore, it could be possible that microbe-derived metabolites augment basal type I IFN production. For instance, indole metabolites, resulting from tryptophan catabolism by the intestinal microflora, have been reported to induce type I IFN signaling in gut epithelial cells mediating a protective role by decreasing epithelial damage and pathological inflammation in a murine graft-versus-host disease model (Swimm et al., 2018). Desaminotyrosine (DAT) is another metabolite produced by the commensal microbiota whose beneficial effect on type I

IFN activity has been described in the context of influenza infection. DAT amplified type I IFN signaling and lung phagocytes subsequently mediate its protective effect (Steed et al., 2017). However, a contribution of SCFAs to tonic type I IFN production by pDCs seems unlikely. SCFAs partially mediate their effect by inhibiting HDACs that has been previously linked to anti-inflammatory responses in immune cells (Chen et al., 2012). Specifically, the application of HDAC inhibitors to activated cDCs and pDCs led to the suppression of pro-inflammatory cytokine as well as type I IFN production (Arbez et al., 2014; Frikeche et al., 2012). Nevertheless, further analyses are required to unravel a possible role of microbe-derived metabolites in influencing tonic type I IFN production by pDCs, and the subsequent calibration of the cDC basal state.

7.4 Microbiota-induced type I IFNs calibrate cDC function through a specific transcriptional, epigenetic and metabolic program

In the present study, the deciphering of the molecular circuits underlying the instruction of the cDC basal state revealed that this process is strongly dependent on microbiota-induced tonic type I IFN signaling, as the most consistently downregulated genes in GF and *Ifnar1*$^{-/-}$ cDCs, including both subsets cDC1s and cDC2s, were related to type I IFN signaling and function (Fig. 10 and 11). In line with this, similar findings could be obtained in genome-wide profiling studies of peritoneal macrophages isolated from antibiotics-treated mice (Abt et al., 2012). Thus, a decreased ISG signature, at least in specific MPh subsets, can possibly be considered as a hallmark of the germ-free or microbiota-depleted status.

Further, our study provides a functional link between tonic type I IFNs and cDC function. Firstly, the deprivation of specifically type I IFNs led to cDC unresponsiveness towards microbial stimulation and secondly, the lack of tonic type I IFNs was clearly attributed to the absence of the indigenous microbiota (Fig. 12 A-C and 13). In particular, cDCs required direct sensing of tonic type I IFNs through IFNAR, since cDCs with a conditional deletion of IFNAR using a *Cd11c*-cre (*Ifnar1*$^{\Delta cDC}$) exhibited a significant impaired immune response after poly(I:C) stimulation (Fig. 12 D). The *Cd11*-cre mediates efficient deletion of floxed alleles in splenic cDCs, while cDC precursors in the BM and pDCs are only marginally affected (Caton et al., 2007). The observation that cDCs required tonic type I IFN signals at their mature stage for functional priming mainly precludes an

indispensable effect of tonic type I IFNs on cDC precursors. cDCs fully differentiate into mature cDC1 or cDC2 in peripheral tissues and gain their specific functionality likely in response to distinct environmental cues (Brown et al., 2019; Guilliams et al., 2016). Thus, it can be suggested that the calibration of cDCs by microbiota-induced tonic type I IFNs might be part of the cDC final maturation process, considering tonic type I IFNs as possible environmental factors generally instructing mature cDCs to become fully functional.

In a previous study, the commensal microbiota has been demonstrated to affect chromatin states in cDCs. Ganal and colleagues reported that activated cDCs from GF mice were unable to initiate transcription of *Ifnb1* or inflammatory cytokine genes, such as *Tnf* and *Il6*, due to chromatin barriers preventing binding of transcription factors to the respective promoter sites (Ganal et al., 2012). In line with this, it has been demonstrated that the initiation of *Ifnb1* transcription required nucleosome remodeling mediated by the SWI/SNF-complex, an indication for their tight regulation as type I interferon-induced transcription factors, IRFs and STATs, are capable to promote nucleosome remodeling by themselves (Agalioti et al., 2000; Ramirez-Carrozzi et al., 2009; Smale, 2010). Here, we showed that the indigenous microbiota also influenced chromatin states in unstimulated cDCs, underlining the microbiota's role as important factor regulating selective gene transcription (data not shown; Figure 7 A-E and S7 A of Schaupp et al., 2020). The instruction process of the cDC basal state was associated with a specific epigenetic signature, showing a reduced occupancy of the active chromatin mark H3K4me3, especially at the promoter sites of ISGs in cDCs from type I IFN-deprived mice (GF and *Ifnar1*$^{-/-}$). This observation is consistent with our transcriptomic data, revealing an overall reduced steady-state ISG expression in GF and *Ifnar1*$^{-/-}$ cDCs. Interestingly, in cDCs from GF and *Ifnar1*$^{-/-}$ mice, genes implicated in metabolic processes, such as glycolysis and the mitochondrial respiratory chain or TCA cycle, were found to have a significant higher density of the repressive mark H3K27me3. In line with our transcriptional analyses, genes specifically involved in the mitochondrial respiratory electron transport chain showed the highest downregulation in cDCs from GF and *Ifnar1*$^{-/-}$ mice (data not shown; Figure 3 F of Schaupp et al., 2020). The electron transport chain is localized in the inner mitochondrial membrane and provides the cell's main energy in form of ATP through the process of oxidative phosphorylation (Pfeiffer et al., 2001).

During the past years, immunometabolism has been recognized as a central factor influencing various immune cell functions (O'Neill et al., 2016). Several studies highlighted

that cellular metabolism not only serves as a provider of energy maintaining cell survival, but also heavily affects individual cell fate decision and function in response to environmental cues, such as hypoxia, nutrients, cytokines or DAMPs, commonly referred to as metabolic reprogramming (Jung et al., 2019; O'Neill and Pearce, 2016). For instance, the metabolic profiles of distinct macrophage populations had been linked to distinct functions. Classical, LPS-activated macrophages (M1), in presence or absence of IFN-γ, upregulate aerobic glycolysis (Warburg effect) allowing for a rapid pro-inflammatory response (Galván-Peña and O'Neill, 2014). An increased consumption of glucose during activation could also be observed for B cells, effector T cells and NK cells (Donnelly et al., 2014; Doughty et al., 2006; Frauwirth et al., 2002). In contrast, macrophages activated by IL-4 during helminth infection (M2), enhance their oxidative metabolism by consuming various carbon sources, like fatty acids, glutamine and glucose, permitting a better energy supply for prolonged defense functions (Jha et al., 2015; Vats et al., 2006). Likewise, cDCs are undergoing different metabolic states during their maturation and activation. For instance, it has been shown that cDCs rapidly increase aerobic glycolysis during activation with TLR ligands (Jantsch et al., 2008; Krawczyk et al., 2010). This was further linked to an upregulation of fatty acid synthesis allowing for ER and Golgi expansion in order to adopt to a secretory state (Everts et al., 2014). In contrast, the cDC quiescent state mainly relies on mitochondrial oxidative phosphorylation which is additionally fueled by catabolism of proteins and triacylglycerols (Krawczyk et al., 2010). Further, cDC differentiation from monocytes is followed by enhanced mitochondrial biogenesis, primary regulated by the peroxisome proliferator-activated receptor-γ co-activator 1α (PGC1α). Additionally, the importance of oxidative phosphorylation during this process was elucidated, as treatment with rotenone, an inhibitor of complex I of the mitochondrial respiratory chain, led to blockade of cDC differentiation (Del Prete et al., 2008; Zaccagnino et al., 2012). However, the signals inducing and regulating the cDC's metabolic activity *in vivo* at steady-state remained unclear. Our data showed, that microbiota-controlled tonic type I IFNs instruct the cDC's metabolic adaption to mitochondrial respiration at steady-state (Fig. 13 L, M). Consequently, un-instructed, tonic type I IFN signaling-deprived cDCs (GF and *Ifnar1*$^{-/-}$), displayed an impaired mitochondrial respiration and thus, a decreased energy generation that finally did not permit the induction of an appropriate immune response even after direct cDC activation. Interestingly, the modulating effects of type I IFN on cellular metabolism has also been described for pDCs in the context of activation. CpG-induced type I IFNs boosted fatty acid oxidation and

mitochondrial oxidative phosphorylation that were critical for pDC activation (Wu et al., 2016).

However, further investigation will be required to obtain insights into the metabolic priming of cDC function by microbiota-induced tonic type I IFNs on a molecular level. In addition, it would be interesting to characterize the metabolic demands of pDCs at steady-state and to explore whether the commensal microbiota may also be involved in the regulation of pDC metabolism.

In summary, my work profoundly shed light on the molecular mechanisms underlying the calibration process that systemically distributed cDCs need to undergo in steady-state to become fully functional. This process is characterized by a specific transcriptional, epigenetic and metabolic program that is induced specifically by microbiota- and pDC-dependent tonic type I IFNs (Fig. 14). Furthermore, we found that tonic PRR signaling is involved in constitutive, pDC-mediated type I IFN production. Subsequently, tonic IFNAR signaling induced chromatin remodeling in cDCs, which allowed for constitutive ISG expression and maintaining mitochondrial respiration activity to ensure cDC's energy demands. Altogether, this study showed that microbiota-induced type I IFNs instruct a poised basal state of cDCs, enabling them to initiate a powerful immune response towards microbial stimulation, including inflammatory cytokine production and T cell activation.

Consequently, un-instructed, type I IFN-deprived cDCs from GF and *Ifnar1*$^{-/-}$ mice exhibited a markedly decreased type I IFN signature and mitochondrial respiration activity, that finally did not permit for an efficient immune response upon microbial stimulation.

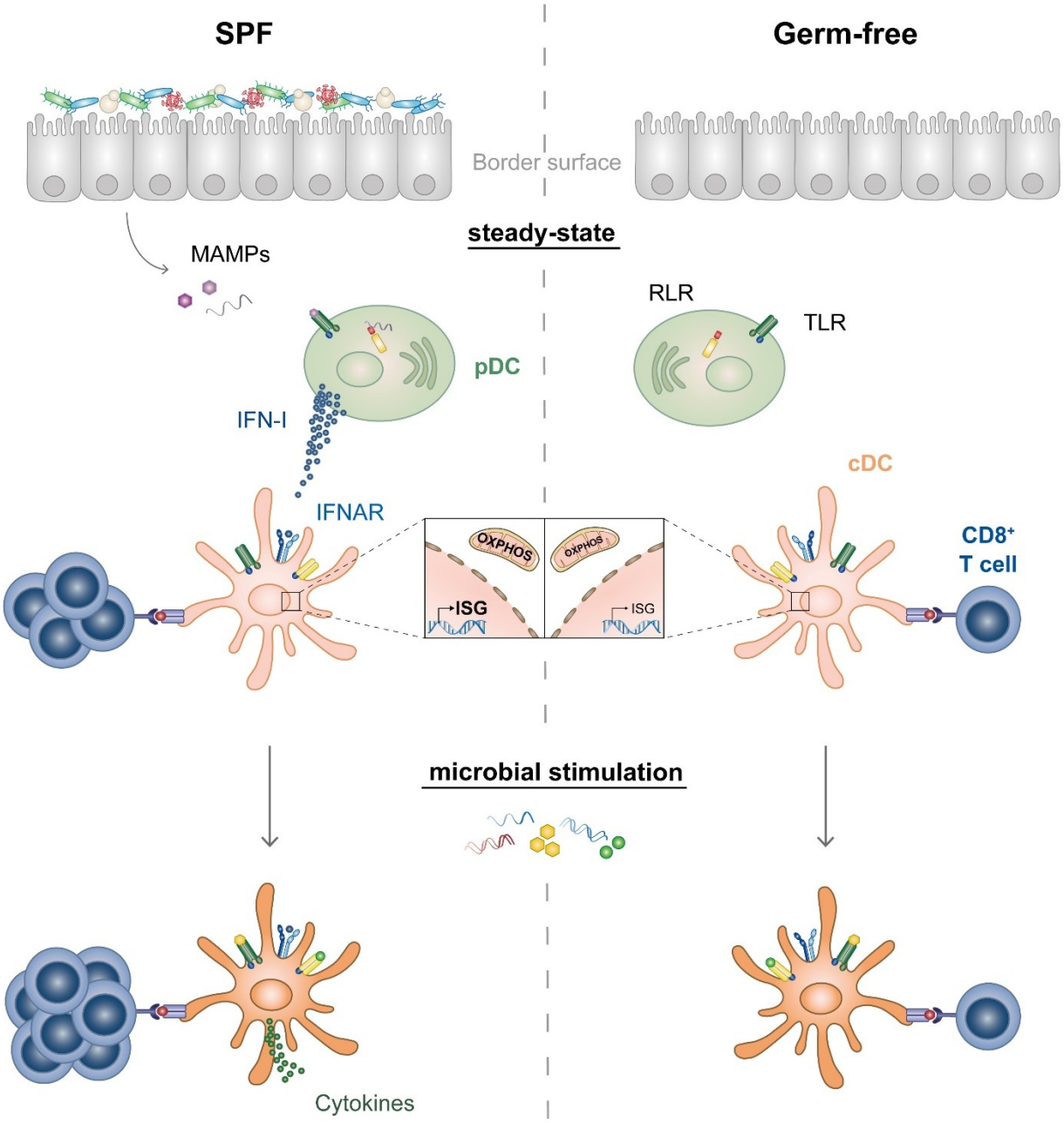


Figure 14: Model of the instruction process of the cDC poised basal state by microbiota-induced tonic type I IFNs. Microbiota-derived molecules induce tonic type I interferon production in pDCs through engagement of pattern recognition receptors. In turn, tonic type I interferon signaling in cDC instructs a poised basal state that relies on constitutive ISG expression and active mitochondrial respiration, rendering cDCs highly responsive towards microbial stimulation, including inflammatory cytokine production and T cell priming. In comparison, cDCs lacking tonic type I interferon signaling (GF and *Ifnar1*$^{-/-}$ mice) have a reduced basal ISG expression and a low mitochondrial respiration activity that does not enable a strong immune response upon pathogen encounter. IFNAR: type I interferon (IFN-I) receptor; ISG: interferon stimulated gene; MAMPs: microbial-associated molecular patterns; OXPHOS: oxidative phosphorylation; RLR: RIG-I-like receptor; TLR: Toll-like receptor.

Despite the fact, that our study provided comprehensive data elucidating how the commensal microbiota impacts on cDC function at steady-state on a molecular level, new questions have emerged that would be of value to address in future investigations. For instance, it has yet to be determined where and which type of microbiota-derived signals pDCs need to receive that finally induce tonic type I IFN production. Related to this, it would also be compelling to identify specific members of the commensal microbiota that preferably induce tonic type I IFN production in pDCs and consequently affect cDC function. Following analyses of the microbiota-mediated imprints on the transcriptome, epigenome and metabolome of pDCs at steady-state would complete the current model of the microbiota-regulated instruction process of the cDC poised basal state (Fig. 14). Since several autoimmune and inflammatory diseases are associated with an elevated type I IFN signature, in a long-term perspective, the results of these investigations may provide a basis for the development of new therapies by regulating pDC-dependent type I IFN production through modulation of the indigenous microbiota.

7.5 Concluding remarks: Microbiota-modulating strategies as novel therapy approaches to treat diseases associated with a dysregulated type I IFN signature?

My study showed that the commensal microbiota markedly shapes cDC function by regulating pDC-dependent tonic type I IFN production. Type I IFNs play a central role in antiviral immunity, but are also critically involved in multiple immune-related, homeostatic control processes, as discussed in Chapter 7.3 (Bocci, 1985; Essers et al., 2009; Gough et al., 2012; Lucas et al., 2007; McFarlane et al., 2017; Taniguchi and Takaoka, 2001). Due to their multifaceted and potent immunomodulatory abilities, dysregulated type I IFN responses have a severe impact on host physiology, as they have been shown to be implicated in several inflammatory and autoimmune diseases, including the Aicardi-Goutières syndrome or systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (Crow, 2011). This group of diseases is commonly designated as type I interferonopathies, as those disorders are characterized by chronically upregulated systemic type I IFN levels and ISG expression, primarily caused by genetic, but also environmental factors (Crow, 2014; Deng and Tsao, 2010).

Since the present study clearly demonstrated that pDC-dependent tonic type I IFN levels are regulated by the indigenous microbiota, it might be compelling to bring this new aspect into clinical relevance by addressing the question whether microbiota-modulating strategies might be a promising approach to treat or even prevent disorders associated with a dysregulated type I IFN signature.

SLE has become a major objective of current research by the nature of its complexity and its yet unpredictable disease progression. This, still incurable, autoimmune disease which predominantly affects young women is accompanied by variable clinical manifestations, including weariness, rashes, photosensitivity, arthritis and the more severe symptoms such as lupus nephritis, neurological complications and atherosclerosis (Crow, 2009; Faurschou et al., 2006; Yee et al., 2015). Many of those indications are caused through plasmablast-mediated formation and deposition of autoantibodies against nucleic acids (antinuclear antibodies) and nucleic acid-binding proteins (Arbuckle et al., 2003; Kaul et al., 2016; Sanz and Lee, 2010). These immune complexes potently activate nucleic acid-sensing endosomal TLRs, particularly TLR-7 and -9, inducing a constant type I IFN production, finally leading to inflammation and tissue damage (Lövgren et al., 2004). However, previous studies suggested an additional contribution of TLR-independent pathways to SLE pathogenesis, involving the nucleic acid sensors RIG-I, MDA5 and cGAS, (Gao et al., 2015; Oliveira et al., 2014).

pDCs were identified as the major mediators of an enhanced type I IFN signature in SLE (Rönnblom and Eloranta, 2013). Their importance in SLE development was further substantiated by the observation that pDC depletion in lupus-prone mice ameliorated disease progression (Baccala et al., 2013). In addition, other cell types, including macrophages, neutrophils and non-hematopoietic cells have been suggested to amplify pathological type I IFN signaling (Bennett et al., 2003; Gall et al., 2012; Lee et al., 2008).

It is well established that genetic factors contribute to SLE development, as already described in Chapter 3.3.2. However, the observation that genetically identical twins can exhibit distinct severities of the disease, heavily suggests an additional and essential role of environmental factors in lupus etiology (Hewagama and Richardson, 2009; Sullivan, 2000). The exposition to distinct environments with higher hygiene standards in developed countries and thus, a different microbiome status, possibly contributed to the distinct patterns of SLE incidence (Bach, 2002; Somers and Richardson, 2014). Indeed, several studies provided increasing evidence for a potential link between the microbiota and SLE development. In a cohort of female SLE patients, a decreasing *Firmicutes*/*Bacteroidetes* ratio correlated with

disease severity, even during remission phase (Hevia et al., 2014). Similarly, in lupus-prone mice an increased abundance of *Bacteroidetes* could be correlated with a more severe phenotype (Johnson et al., 2015). Furthermore, the intestinal microbiota composition of SLE patients seemed to be generally less diverse compared to healthy controls (Azzouz et al., 2019; van der Meulen et al., 2019). In successive studies, distinct gut pathobionts were identified to be causative for several disease manifestations. For instance, a study by Manfredo Vieira *et al*. showed that the translocation of the gut pathobiont *Enterococcus gallinarum* to the liver causes autoimmunity in lupus-prone mice by inducing antinuclear antibody formation and a systemic type I IFN signature. This bacterium could also be found in liver biopsies from a small group of SLE patients, suggesting a potential role of *E. gallinarum* in human autoimmune pathogenesis (Manfredo Vieira et al., 2018). In another study, the investigators could link the outgrowth of the gut commensal *Rumincococcus gnavus*, a member of the *Lachnospiraceae* family, to the development of lupus nephritis in SLE patients (Azzouz et al., 2019). Recently, Zegarra-Ruiz *et al*. described a major role of *Lactobacillus* species, specifically *L. reuteri*, in driving TLR7-and pDC-dependent systemic autoimmunity in lupus-prone mice. Additionally, the researchers demonstrated that a resistant starch-rich diet ameliorated lupus-related symptoms by SCFA-mediated inhibition of *L. reuteri* outgrowth (Zegarra-Ruiz et al., 2019).

Together, these studies support the view of an important contribution of the commensal microbiota to SLE pathogenesis, but also coincidently highlight that distinct pathobionts are able to affect disease development, although they may share several pathological mechanisms, such as translocation from the intestine into other organs or tissues and induction of global ISG expression. Additionally, it should be considered that distinct mouse models were applied in those studies, pointing towards that individual genetic and microbial constitutions may contribute to different SLE manifestations, which likely reflects distinct SLE phenotypes reported for humans (Banchereau et al., 2016). Against this backdrop, the study by Zegarra-Ruiz *et al*. demonstrated, though, that a diet-based influence of the indigenous microbiota can decrease the systemic type I IFN signature in lupus-prone mice by specifically preventing the outgrowth of the autoimmunity-causing pathobiont *L. reuteri* (Zegarra-Ruiz et al., 2019). However, a limitation of this study is the lack of data on pDC-specific contribution to global type I IFN gene expression and production, although the researchers provided evidence of a reduced systemic type I IFN gene signature, accompanied with normalized pDC numbers in lymphoid tissues.

Therefore, further investigations are required aiming to identify additional, potential pathobionts associated with an elevated type I IFN signature manifested in SLE, and also other interferonopathies, to understand their interaction with the host and other microbial members on a mechanistic level and lastly, to evaluate whether and how pDC-driven pathological type I IFN signaling can be regulated through manipulation of the indigenous microflora. Current therapies of SLE patients mainly rely on immunosuppressive medication (Thong and Olsen, 2017). Since this treatment leads long-term to a higher infection rate in those patients, a therapy based on microbiota modulation, achieved e.g. through a special diet or fecal transplantation of healthy donors into SLE patients, may represent a more efficient and less risky approach (Tektonidou et al., 2015). Additionally, host-dependent genetic and environmental factors, potentially affecting indigenous microbiota composition, should be considered in therapy development, that will possibly amount to personalized diets adapted for the patient's individual microflora constitution (Kau et al., 2011).

8 Materials and Methods

8.1 Mouse strains

Experiments were performed with age- (8 to 10 weeks old) and sex- (males and females) matched mice in accordance with local animal care and use committees. Germ-free mice C57BL/6 mice were housed in sterile, over-pressured, flexible film isolators under strict axenic or gnotobiotic conditions. Germ-free status was routinely monitored by culture-dependent and independent methods. Experiments were carried out with germ-free mice from facilities in Mainz, Bern and Berlin with comparable results.

MOUSE STRAIN	REFERENCE
C57BL/6J (SPF)	Janvier SAS, France
Myd88$^{-/-}$ (SPF)	Adachi et al., 1998
Trif$^{-/-}$ (SPF)	Hoebe et al., 2003
Cardif$^{-/-}$ (SPF)	Michallet et al., 2008
MyTrCa$^{-/-}$(SPF)	Spanier et al., 2014
Ifnar1$^{-/-}$ (SPF)	Muller et al., 1994
Cd11c(Itgax)-Cre (SPF)	Caton et al., 2007
Ifnar1$^{fl/fl}$ (SPF)	Prinz et al., 2008
C57BL/6 (Germ Free)	Andrew MacPherson (University of Bern), Christoph Reinhardt (Mainz University Medical Center) and Rainer Jumpertz-von Schwartzenberg (Charité University Medical Center, Berlin)
C57BL/6 (*E. coli* JM83)	Hapfelmaier et al., 2010, Andrew MacPherson (University of Bern)
C57BL/6 (sDMDMm2)	Uchimura et al., 2016, Andrew MacPherson (University of Bern)
Ifnar1$^{-/-}$ (Germ Free)	Muller et al., 1994, Andrew MacPherson (University of Bern)

8.2 Cell lines

CELL LINE	REFERENCE
L-929	ATCC, CCL-1

8.3 Antibodies

ANTIBODY	CLONE	MANUFACTURER
Anti-mouse CD16/CD32	2.4G2	self-purified
Anti-mouse CD19	MB19-1	eBioscience
Anti-mouse CD3	145-2C11	eBioscience
Anti-mouse CD5	53-7.3	eBioscience
Anti-human/mouse B220	RA3-6B2	eBioscience
Anti-mouse TCR-β	H57-597	Biolegend
Anti-mouse NK1.1	PK136	eBioscience
Anti-mouse CD127 (IL-7Rα)	A7R34	Biolegend
Anti-mouse Gr-1	RB6-8C5	eBioscience
Anti-mouse Ly-6C	HK1.4	eBioscience
Anti-mouse Ly-6G	1A8	Biolegend
Anti-mouse F4/80	BM8	eBioscience
Anti-mouse CD11c	N418	Biolegend
Anti-mouse CD11b	M1/70	eBioscience
Anti-mouse MHC-II (I-A/I-E)	M5/114.15.2	eBioscience
Anti-mouse CD4	RM4-5	eBioscience
Anti-mouse CD8	53-6.7	BD Bioscience
Anti-mouse CD45	30-F11	BD Bioscience
Anti-mouse Siglec H	eBio440c	eBioscience
Anti-mouse CD317	eBio129c	eBioscience
Anti-mouse TNF	MP6-XT22	Biolegend
InVivoMAb anti-mouse CD40	FGK4.5/FGK45	Bio X Cell
InVivoMAb rat IgG2a isotype control	clone 2A3	Bio X Cell

8.4 Critical Commercial Kits

KIT	MANUFACTURER
Seahorse XFe96 FluxPak mini	Agilent
miRNeasy Micro Kit	Qiagen
HS RNA 15nt Kit	Agilent
Smart-Seq v4 mRNA Ultra Low Input RNA Kit	Clontech
HS NGS Fragment Kit (1-6000bp)	Agilent
Qubit dsDNA HS Assay Kit	Invitrogen
Nextera XT library preparation kit	Illumina
NextSeq® 500/550 High Output Kit v2 (150 cycles)	Illumina
NEBNext Ultra RNA Library Prep Kit for Illumina	New England Biolabs
TURBO DNA-free Kit	Invitrogen
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems
SYBR™ Green PCR Master Mix	Applied Biosystems
Mouse TNF alpha ELISA	Invitrogen
Streptavidin Microbeads	Miltenyi

8.5 Chemicals, buffers and media

8.5.1 Chemicals

CHEMICAL	MANUFACTURER
LPS from *E. coli* O111:B4	Sigma Aldrich
Polyinosinic-polycytidylic acid (poly(I:C)), HMW	InvivoGen
Collagenase D	Roche
DNase I	Sigma Aldrich
Dispase	Corning
Recombinant Mouse IFN-β1	PBL
Brefeldin A	Sigma Aldrich
Trizol	Invitrogen
Chloroform	Sigma Aldrich

CHEMICAL	MANUFACTURER
2-Propanol	Sigma Aldrich
Glutaraldehyde (EM Grade)	Sigma Aldrich
DAPI (4',6-Diamidino-2-phenylindole dihydrochloride)	Sigma Aldrich
Mitotracker Red CMX Ros	Invitrogen
Nonyl acridine orange	Invitrogen
Thiazolyl blue tetrazolium bromide	Sigma Aldrich
Saponin	Sigma Aldrich
Ethylenediaminetetraacetic acid, pH 8.0	EurX, Molecular Biology Products
Percoll	Sigma Aldrich
HEPES (1M)	Gibco
Bovine Serum Albumin (protease, fatty acid and essentially globulin free)	Sigma Aldrich
Fetal Bovine Serum	Pan-Biotech
Hank's Balanced Salt Solution (HBSS), with and without phenol red	Sigma Aldrich
RPMI 1640 medium	Gibco
DMEM, high glucose medium	Gibco
Seahorse XF RPMI medium, pH 7.4	Agilent
FCCP (Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone)	Sigma Aldrich
Oligomycin A	Sigma Aldrich
Rotenone	Sigma Aldrich
Antimycin A	Sigma Aldrich
Ammonium chloride	Sigma Aldrich
Potassium bicarbonate	Sigma Aldrich
HEPES (1 M)	Gibco
MEM non-essential amino acid solution (100x)	Gibco
L-glutamine	Sigma Aldrich
Penicillin/Streptamycin	Gibco
Gentamicin	Lonza BioWhittaker
β-mercaptoethanol	Sigma Aldrich

8.5.2 Buffers and media

FACS BUFFER	
CHEMICAL	FINAL CONCENTRATION
PBS	1x
BSA	0.5 % (w/v)
EDTA, pH 8.0	2 mM

MACS BUFFER	
CHEMICAL	FINAL CONCENTRATION
PBS	1x
EDTA, pH 8.0	2 mM

SAPONIN BUFFER	
CHEMICAL	FINAL CONCENTRATION
PBS	1x
BSA	0.5 % (w/v)
EDTA, pH 8.0	2 mM
Saponin	0.5 % (w/v)

RED CELL LYSIS BUFFER	
CHEMICAL	FINAL CONCENTRATION
NH_4Cl	150 mM
$KHCO_3$	10 mM
EDTA, pH 8.0	100 µM

D10$^+$ MEDIUM	
CHEMICAL	FINAL CONCENTRATION
DMEM medium	-
FBS	10 % (v/v)
MS	1x

40X MEDIUM SUPPLEMENT (MS)	
CHEMICAL	FINAL CONCENTRATION
L-glutamine	8 mg/ml

Penicillin	2000 U/ml
Streptomycin	2000 µg/ml
Gentamycin	400 µg/ml
β-mercaptoethanol	0.014 % (v/v)

DISSOCIATION MEDIUM	
CHEMICAL	FINAL CONCENTRATION
HBSS (with phenol red)	-
EDTA, pH 8.0	5 mM
HEPES	10 mM

DIGESTION MIX	
CHEMICAL	FINAL CONCENTRATION
HBSS (without phenol red)	-
FBS	2.5 % (v/v)
Collagenase D	0.5 mg/ml
DNase I	0.5 mg/ml
Dispase	0.5 U/ml

8.6 Preparation of splenic cDC and pDC

Spleens were removed, injected with 3 ml digestion mix composed of 1 mg/ml collagenase D (Sigma-Aldrich) and 0.2 mg/ml DNase I (Sigma- Aldrich) in RPMI medium. Spleens were homogenized and incubated at 37 °C, 5 % CO_2 for 15 min. Subsequently, the cell suspension was filtered through a 70 µm cell strainer and washed with FACS buffer. Erythrocytes were removed through osmotic lysis by incubating the pelleted cells with 1 ml of red cell lysis buffer (RCLB) for 2 min at RT or with a Percoll density gradient (20 % / 80 %).

8.7 Preparation of lamina propria leukocytes

Lamina propria cells from the small intestine were isolated as described in (Gronke et al., 2017). In brief, small intestines were removed and were cleaned from mesenteric fat, followed by the excision of Peyer's Patches. Small intestines were cut open longitudinally and were washed rigorously in PBS to remove remaining feces. Epithelial cells were dislodged by

incubating the small intestines twice in 5 ml dissociation medium at 37 °C, 120 rpm for 15 min. Afterwards, the guts were cut into pieces using a scalpel and were digested in 3 rounds for 20 min each at 37 °C, 120 rpm in 5 ml digestion mix. After each digestion step, cells were filtered through a 70 µm cell strainer and were collected in $D10^+$ medium. Cells were sedimented at 2000 rpm for 10 min, 4 °C and a 40/80 % Percoll density gradient centrifugation was performed (2500 rpm, RT, w/o break). Cells located in the interphase were collected and washed with FACS buffer.

8.8 *In vivo* stimulation of splenic myeloid cells

For *in vivo* stimulation, 50 µg ultrapure LPS from *E. coli* O111:B4 (Sigma-Aldrich), 100 µg poly(I:C) (HMW, InvivoGen) or 75 µg anti-CD40 (clone FGK4.5, BioXCell) were diluted in 200 µl PBS and mice were injected intraperitoneally (i.p.).

8.9 Recolonization of germ-free mice

8.9.1 *E. coli* JM83 monocolonization

Escherichia coli JM83 was cultured overnight in LB medium at 37 °C, shaking at 200 rpm (Hapfelmeier et al., 2010). To prepare gavage solutions, bacteria were centrifuged for 10 min at 4000 xg and washed twice with sterile PBS. A dose of 1010 CFU was resuspended in 500 µl of sterile PBS and administered to germ-free mice by oral gavage. Mice were kept for 4 weeks in isolators in order to allow for stable colonization before analysis.

8.9.2 sDMDMm2 colonization

Mice colonized with the stable Defined Moderately Diverse Mouse microbiota 2 (sDMDMm2) were born to sDMDMm2 colonized dams and raised under this defined colonization status (Uchimura et al., 2016).

8.10 Flow cytometric analysis

Staining of single cell suspensions was performed in 96 V-bottom well plates and up to 5 x 10^6 cells per well were stained in a volume of 100 µl. In order to block F_c-receptors cells were incubated on ice for 20 min with anti-CD16/CD32 (clone 2.4G2, self-purified). Cells were washed with FACS buffer (500 xg, 4 °C, 5 min), followed by staining with

fluorochrome-coupled antibodies for 30 min at 4 °C in FACS buffer. Cells were washed twice with FACS buffer (500 xg, 4 °C, 5 min). Samples were analyzed on a FACS Canto II or a Fortessa X20 Cytometer (Becton Dickinson, Mountain View, CA) and data were analyzed with FlowJo Analysis Software (Tree Star Inc, Ashland, OR).

For intracellular cytokine stainings, cells were fixed and permeabilized after surface staining with BD Cytofix/Cytoperm on ice for 30 min. Cells were washed twice with Saponin buffer (600 xg, 4 °C, 5 min) and stained intracellularly for the respective cytokines in Saponin buffer for 2 h. Cells were washed twice with Saponin buffer and were resuspended in FACS buffer. Sample acquisition and data analysis were performed as described above.

8.11 Cell sorting and Magnet-activated cell sorting (MACS)

For sorting of splenic cDCs and pDCs, splenocyte single cell suspension was prepared as described above. $CD11c^+$ cells were pre-enriched using an anti-CD11c Biotin-coupled antibody in combination with Streptavidin magnetic microbeads (Miltenyi) according to the manufacturer's instructions. Before labelling, F_c receptors were blocked by incubating the cells on ice for 20 min with anti-CD16/CD32 (clone 2.4G2, self-purified), followed by one washing step with MACS buffer (300 xg, 4 °C, 10 min). For cell separation LS columns were used.

Cells were stained with the respective fluorophore-coupled antibodies for 30 min at 4 °C and washed with FACS buffer (300 xg, 4 °C, 10 min). DAPI was added to the cell suspension right before sorting in order to exclude dead cells. Cell sorting was performed on an ARIA II or an ARIA Fusion cell sorter.

The purification of various splenic or lamina propria leukocyte populations, including stromal cells, was performed as described in chapter 8.6 using a Percoll density gradient (20/ 80 %) and chapter 8.7, respectively. Cells were stained with the respective fluorophore-coupled antibodies for 30 min at 4 °C and washed with FACS buffer (300 xg, 4 °C, 10 min). DAPI was added to the cell suspension right before sorting in order to exclude dead cells. Cell sorting was performed on an ARIA II or an ARIA Fusion cell sorter (Becton Dickinson) based on a published strategy to analyze the splenic myeloid compartment in mice (Rose et al., 2012).

8.12 Determination of serum cytokine levels

TNF was measured by ELISA (Invitrogen) according to the manufacturer's instructions. IFN-I concentrations were determined by VSV bioassay (Ganal et al., 2012).

8.13 Mitochondrial stainings

Bulk splenocytes were incubated with either 5 µM Nonyl Acridine Orange (Thermo Fischer, Invitrogen) or 100 nM Mitotracker Red CMX Ros (Thermo Fischer, Invitrogen) for 15 min at 37 °C, 5 % CO_2 in serum-free RPMI 1640 medium. Cells were subsequently washed with FACS buffer (500 xg, 4 °C, 5 min) and stained with fluorophore-coupled antibodies, following an incubation period with a live/dead dye before they were analyzed by flow cytometry.

8.14 Transmission Electron microscopy

Sort-purified splenic cDCs were fixed on glass cover slips in 4% paraformaldehyde and 1% glutaraldehyde (EM Grade, Sigma-Aldrich) in 0.1 M phosphate buffer, pH 7.4 (PB). Following fixation, samples were washed in PB and post fixed in 1 % osmium tetroxide. After extensive washing in 0.1 M PB, samples were contrasted using 1 % uranyl acetate in 70% ethanol for 1 h, dehydrated in an ascending ethanol series, followed by propylen oxide and finally embedded in epoxy resin (Durcupan, Roth, Germany). Ultrathin sections were cut using an UC6 Leica ultramicrotome and imaged using a Philips CM100 Transmisson Electron Microscope (TEM). Quantitative image analysis was performed using Olympus ITEM software.

8.15 Extracellular flux analysis

For real-time measurement of OCR and ECAR, sort-purified splenic cDC were rested for 3 h until adherent to a XFe96 microplate. Media was exchanged with un-supplemented XF Assay Medium and analyzed with an XFe96 Extracellular Flux Analyzer (Agilent Seahorse). Four consecutive measurements were obtained under basal conditions followed by the addition of 10 mM glucose, 1 µM oligomycin, which inhibits the mitochondrial ATP synthase, 1.5 µM
Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), which uncouples ATP synthesis from oxygen consumption and a combination of 100 nM rotenone plus 1 µM

antimycin A, which inhibit the electron transport chain by blocking complex I and III, respectively. All chemicals used for these assays were purchased from Sigma-Aldrich. The SRC is calculated as the difference between basal OCR and the maximal OCR after the addition of FCCP.

8.16 Quantification of mRNA expression

Cells were resuspended in Trizol reagent (Invitrogen) and RNA was extracted according to the manufacturer's instructions. For DNA removal, the RNA was treated with the TURBO DNA-free kit (Invitrogen). The RNA concentration was measured using a Qubit 2.0 Fluorimeter (Invitrogen) or a NanoDrop spectrophotometer (Thermo Fisher Scientific). Subsequently, the isolated RNA amounts were normalized and the same amount of RNA was used in every reaction (up to 2 µg total RNA per reaction). RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative Real-Time PCR was performed in 384 well plates and run on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Primers (Sigma-Aldrich) were used at a final concentration of 400 nM in SYBR Green PCR Master Mix (Applied Biosystems). All reactions were performed in triplicates and gene expression was normalized to the expression of the house keeping gene hypoxanthine-guanine phosphoribosyl transferase 1 (*Hprt1*).

8.16.1 Primers

GENE	**FORWARD PRIMER SEQUENCE (5'→3')**	**REVERSE PRIMER SEQUENCE (5'→3')**
Ifna4	AGTGACCAGCATCTACAAGACC	CTGTCAAGGCCCTCTTGTTCC
Ifnb1	GTACGTCTCCTGGATGAACTCC	CCACGTCAATCTTTCCTCTTGC

8.17 RNA-sequencing analysis of splenic cDC1s and cDC2s

Total RNA was prepared from sort-purified splenic cDC1s and cDC2s (for each sample 3 individual mice were pooled) using Trizol reagent (Invitrogen) in combination with the miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. For RNA quality validation, samples were analyzed using the Fragment Analyzer System and the HS RNA 15nt Kit (Agilent). Only RNA samples with an RNA Quality Number ≥ 9 were selected for sequencing. For poly-A-dependent cDNA synthesis and a first amplification step, 10 ng of

total RNA was used as input in the Smart-Seq v4 mRNA Ultra Low Input RNA Kit (Clontech) and processed according to the manufacturer's instructions. After quality control (HS NGS Fragment Kit (1-6000bp), Agilent) and concentration measurement (Qubit dsDNA HS Assay Kit, Invitrogen), 1 ng of the purified cDNA was used for tagmentation and library completion with the Nextera XT library preparation kit (Illumina). In the following, 2x75nt paired-end sequencing was performed on a NextSeq500/550.

Sequenced reads for cDC1 and cDC2 samples were mapped to the RefSeq mouse transcriptome (mm10) using Salmon (v.0.14.1) with validation enabled and quantified at the gene level (Patro et al., 2017). Statistical analysis (Likelihood ratio tests) were performed using DESeq2 (v.1.24.0) controlling for gender when necessary (Love et al., 2014). Gene ontology using DAVID (6.8) was performed for GO terms classified as biological processes (Huang da et al., 2009). An enrichment map was created in Cytoscape (v3.7.2) using all significantly enriched GO terms (FDR < 0.05) with a gene set size between 10-1000 (Shannon et al., 2003). Overlap was used as metric and the cutoff was set to 1. Clustering was performed using AutoAnnotate (v1.3.2) with overlap used as edge weight values (Kucera et al., 2016). Normalized gene counts from DESeq2 were used as expression dataset for GSEA analysis (v4.0.1 with MSigDB v7.0) (Subramanian et al., 2005) and the Chip platform for conversion of mouse to human annotations was derived from MGI vertebrate homology reports. Permutation was set to gene_set and gene set size limits was set to 10-1000.

8.18 Quantification and Statistical Analysis

Statistical parameters including the exact value of n, the definition of center, dispersion and precision measures (mean ± SD) and statistical significance are reported in the figures and the corresponding figure legends. Data was judged statistically significant when $p \leq 0.05$ by two-tailed Student's t test or One-way Anova with Bonferroni's multiple comparison corrections. In figures, asterisks denote statistical significance as calculated by Student's t test or One-way Anova with Bonferroni's multiple comparison corrections, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$. Statistical analyses were performed in GraphPad Prism version 8.

9 List of abbreviations

-	-negative
+	-positive
ABX	antibiotics treated
AMP	antimicrobial proteins or adenosine monophosphate
APC	antigen-presenting cell
ATP	adenosine triphosphate
BCR	B cell receptor
BRD4	bromodomain-containing protein 4
BSA	bovine serum albumin
C2TA	MHC class 2 transcription activator
CARD	caspase recruitment domain
cDC	conventional dendritic cell
cDNA	complementary DNA
CFU	colony forming units
CMX Ros	mitotracker Red
CDN	cyclic dinucleotides
CDP	common dendritic cell progenitor
cGAMP	cyclic guanosine monophosphate–adenosine monophosphate
cGas	cyclic GMP-AMP synthase
ChIP	chromatin immunoprecipitation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CP	cryptopatch
CpG	cytosine-guanine dinulceotides
CTD	carboxy-terminal domain
CTLA-4	cytotoxic T-lymphocyte protein 4
DAMPs	danger-associated molecular patterns
DAT	desaminotyrosine
DAVID	database for annotation, visualization and integrated discovery
DMEM	Dulbecco’s modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA

ECAR	extracellular acidification rate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
FACS	fluorescence-activated cell sorting
FADD	FAS-associated death domain protein
FBS	fetal bovine serum
FCCP	Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone
FDR	false discovery rate
FOXO3	forkhead box protein O3
GAS	gamma interferon activation site elements
GF	germ-free
GMP	guanosine monophosphate
GO	gene ontology
GSEA	gene set enrichment analysis
GTP	guanosine triphosphate
H3K9me2	histone 3 lysine 9 di-methylation (repressive mark)
H3K4me3	histone 3 lysine 4 tri-methylation (permissive mark)
H3K27me3	histone 3 lysine 27 tri-methylation (repressive mark)
HAT	histone acetyltransferase
HDAC	histone deacetylases
HET-E	incompatibility locus protein from Podospora anserina
hi	high
HSC	hematopoietic stem cell
IBD	inflammatory bowel disease
iE-DAP	γ-glutamyl diaminopimelic acid
Ifna4	interferon α4 gene
Ifnb1	interferon β1 gene
IFN	interferon
IFNAR	interferon α/β receptor (type I IFN)
IFNGR	interferon γ receptor (type II IFN)
IFNLR	interferon λ receptor (type III IFN)
IκB	inhibitor of NF-κB
IKK	inhibitor of NF-κB kinase
IL	interleukin

ILC	innate lymphoid cell
ILF	isolated lymphoid follicle
i.p.	intraperitoneal
IRAK	interleukin-1 receptor-associated kinase
IRF	interferon regulatory factor
ISG	interferon stimulated gene
ISGF3	IFN-stimulated gene factor 3, complex consists of activated STAT1/2 dimers and IRF9
ISRE	interferon stimulated response element
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
JNK	c-Jun N-terminal kinases
KAT2A	lysine acetyltransferase 2A, also known as GCN5
LCMV	lymphocytic choriomeningitis virus
LGP2	laboratory of genetics and physiology 2
LPS	lipopolysaccharide
lo	low
MAC	membrane-attack complex
MACS	magnetic-activated cell sorting
MAL	MyD88-adapter-like protein, also known as TIRAP
MAMPs	microbial-associated molecular patterns
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral-signaling protein, also known as CARDIF, IPS-1 or VISA
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation-associated protein 5
MDP	macrophage - dendritic cell progenitor or muramyl dipeptides
MHC-I/II	class I/II major histocompatibility complex
mLN	mesenteric lymph nodes
MLP	myeloid-lymphoid progenitor
MPh	mononuclear phagocyte
MPP	multipotent progenitors
MTT	thiazolyl blue tetrazolium bromide
MyD88	myeloid differentiation primary response protein 88

NAO	nonyl acridine orange
NACHT	domain present in NAIP, C2TA, HET-E, and TP-1
NAIP	neuronal apoptosis inhibitor protein
NF-κB	nuclear factor kappa B
NK	natural killer
NLR	NOD-like receptor
NOD1/2	nucleotide-binding oligomerization domain 1/2
OCR	oxygen consumption rate
Oxphos	oxidative phosphorylation
P	phosphate
PAMPs	pathogen-associated molecular patterns
PB	phosphate buffer
PBS	phosphate-buffered saline
PCA	principal component analysis
pDC	plasmacytoid dendritic cell
PD-1	programmed cell death protein 1
PI3K	phosphatidylinositol-3-kinase
PIAS1	protein inhibitors of activated STAT
PKC	protein kinase C
pLN	peripheral lymph nodes
poly(I:C)	polyinosinic-polycytidylic acid
PP	Peyer's Patch
PGC1α	peroxisome proliferator- activated receptor-γ co-activator 1α
PRR	pattern recognition receptor
PSM	phenol-soluble modulins
pTEFb	positive transcription elongation factor b
PTM	post-translational modification
PTPN11	protein tyrosine phosphatase non-receptor type 11
RA	rheumatoid arthritis
RD	repressor domain
RIG-I	retinoic acid-inducible gene 1
RIP1	receptor (TNFRSF)-interacting serine-threonine kinase 1
RLCB	red cell lysis buffer
RLR	RIG-I-like receptor

RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SCFA	short-chain fatty acid
sDMDMm2	stable defined moderately diverse mouse microbiota 2
Seq	sequencing
SFB	segmented filamentous bacteria
SIN3A	SIN3 transcription regulator homolog A
SLE	systemic lupus erythematosus
SOCS	suppressors of cytokine signaling
SPF	specific pathogen-free
SRC	spare respiratory capacity
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
STING	stimulator of interferon genes protein
SUMO	small ubiquitin-related modifiers
TAB	TGF-beta-activated kinase 1 (MAP3K7) binding protein
TAK	TGF-beta-activated kinase 1
TBK1	TANK-binding kinase 1
TCA	tricarboxylic acid cycle
TCR	T cell receptor
TGF-β	transforming growth factor β
TIRAP	toll-interleukin 1 receptor (TIR) domain-containing adaptor protein
TLR	Toll-like receptor
TMAO	trimethylamine N-oxide
TNF	tumor necrosis factor
TP-1	telomerase-associated protein 1
TPM	transcripts per million
TRADD	tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAF	tumor necrosis factor receptor–associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter molecule 1, also known as TICAM-1

TRIM25	E3 ubiquitin/ISG15 ligase
TYK2	non-receptor tyrosine-protein kinase
Ub	ubiquitin
USP18	ubiquitin carboxyl-terminal hydrolase 18
VSV	vascular stomatitis virus
w/o	without
WT	wildtype

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11 Acknowledgements