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The interaction between invariant Natural Killer T cells and the mucosal microbiota

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Summary

The surface of mammalian bodies is colonized by a multitude of microbial organisms, which under normal conditions support the host and are considered beneficial commensals. This requires, however, that the composition of the commensal microbiota is tightly controlled and regulated. The host immune system plays an important role in the maintenance of this microbiota composition. Here we focus on the contribution of one particular immune cell type, invariant Natural Killer T (*i*NKT) cells, in this process. The *i*NKT cells are a unique subset of T cells characterized by two main features. First, they express an invariant T-cell receptor that recognizes glycolipid antigens presented by CD1d, a non-polymorphic major histocompatibility complex class I-like molecule. Second, *i*NKT cells develop as effector/memory cells and swiftly exert effector functions, like cytokine production and cytotoxicity, after activation. We outline the influence that the mucosal microbiota can have on *i*NKT cells, and how *i*NKT cells contribute to the maintenance of the microbiota composition.

Keywords: microbiota; mucosal immunology; invariant Natural Killer T cells.

Introduction

The bodies of animals are exposed to the environment on the skin and the mucosal surfaces, which have to reconcile two often conflicting aims: to support the exchange of the host with the environment and to protect the host from pathogenic colonization and invasion. Whereas the skin can afford a thick, multilayered barrier, the functional characteristics of the mucosal surfaces, mainly the airways, and the gastrointestinal and urogenital tracts, require at times a single-cell layer of separation between the host and the environment. These surfaces are colonized by a diverse mixture of microbial organisms, consisting of bacteria, fungi and viruses. Each of the mucosal surfaces hosts a unique microbiome, often with additional niches depending on the particularities of the location. Importantly, the interaction between the host and the microbiota has coevolved in such a manner that it is, under normal conditions, mutually beneficial, and the commensal microbiota contributes to the health of the host, not only at the mucosa, but also systemically. Furthermore, the composition of the commensal microbiota is not random, but is maintained by a complex, location-specific and bi-directional communication between the host and its commensals.[1–3] The immune system aims to protect the host against pathogens, and therefore, it is not surprising that it plays an important role in maintaining the truce with the commensal microbiota (Box 1). To this end, the mucosal immune system, composed of cells of haematopoietic origin, interacts closely with various tissue-resident cell types, such as epithelial cells, microfold cells, and Paneth cells (Box 1).

Invariant Natural Killer T (*i*NKT) cells are a unique subset of T lymphocytes that phenotypically and functionally resemble Natural Killer cells as well as memory T cells.[4–9] They are characterized by the expression of a canonical Vα14 to Jα18 T-cell receptor (TCR) rearrangement (Vα14*i*) in mice and an orthologous Vα24-Jα18 TCR chain (Vα24*i*) in humans. *i*NKT cells recognize glycolipids, especially glycosphingolipid structures, presented by CD1d, a non-polymorphic homologue of the major histocompatibility complex class I antigen-presenting molecules. The first and best-studied TCR agonist for mouse and human *i*NKT cells is α-galactosylceramide

Abbreviations: GF, germ-free; HDE, house dust extracts; IEL, intraepithelial lymphocyte; *i*NKT cells, invariant Natural Killer T cells; RF, restricted flora; TCR, T-cell receptor; αGalCer, α-galactosyl-ceramide

Box 1 Means of interaction between the immune system and the commensal microbiota

The commensal microbiota influences the health of the host in many ways, and the host actively shapes the composition of the commensal microbiota. This mutual interaction relies on various means of communication and influence. We outline here shortly those means pertaining the immune system and give examples for invariant Natural Killer T (*i*NKT) cells were possible.

(A) Means of the microbiota to influence the host immune system:

Three main ways have been described by which the commensal microbiota impacts the host immune system.

(1) Antigens

Microbially derived molecules are detected by the adaptive immune system when they can act as antigens by binding either to the B-cell receptor (BCR) of B cells (direct binding) or the T-cell receptor (TCR) of T cells (after processing by antigen-presenting cells (APCs) and presentation on major histocompatibility complex type molecules). This is the most direct way by which the microbiota can activate the adaptive immune system. The organisms known to carry specific antigens for *i*NKT cell are discussed in the text and in Table 1. As *i*NKT cells have some degree of auto-reactivity,[121] there exists another antigen-dependent method for *i*NKT cell activation. The microbially derived signal might alter the expression of self-antigens presented by CD1d on APCs[97,98] or change the expression levels of CD1d itself.[97,122]

(2) PAMPs

Microbially derived molecules are sensed similar to pathogen-associated molecular patterns (PAMPs) by the innate immune system through pattern-recognition receptors (PRRs). For example, the polysaccharide A from *Bacteroides fragilis* and the exopolysaccharide from *Bifidobacterium longum* both can expand regulatory T (Treg) cells *in vivo* by inducing interleukin-10 (IL-10) production in APCs.[123,124] In some cases, receptors for IgA can facilitate the uptake of bacteria.[125] Cytokines produced by PAMP-activated APCs can also stimulate cytokine production by *i*NKT cells (see text and Figure 1).

(3) Metabolites

Microbially derived molecules that influence metabolic and immunological processes in the host. Short-chain fatty acids (SCFAs), like acetate, propionate, butyrate; biogenic amines, like taurine and histamine; tryptophan catabolites, are all known to modulate metabolic processes in APCs and thereby the host immune response.[123,124] However, we are not aware of data that link these metabolites to *i*NKT cells.

(B) Means of the host immune system to influence the microbiota

The host immune system can impact the composition of the commensal microbiota in several ways:

(1) Anti-microbial peptides

Many anti-microbial peptides are produced directly by immune cells or are induced by them in, for example, epithelial cells via messenger molecules like cytokines. With regard to *i*NKT cells, it was shown that *i*NKT cells, via the production of interferon-γ, can regulate the production of anti-microbial peptides by Paneth cells.[68]

(2) Secretory IgA molecules

The majority of the antibodies produced in mammals are of the secretory IgA (sIgA) type that are transported across the endothelial and epithelial barriers of the mucosal surfaces. For example, around three-quarters of the commensal bacteria in the mouse gastrointestinal tract are coated with sIgA, which is essential for the maintenance of the bacterial homeostasis in the intestine.[125] CD1d-deficient mice show changes in their IgA-repertoire compared with wild-type mice, but it was suggested that this is not due to a direct interaction with *i*NKT cells, but rather a consequence of the altered microbial flora in the $CD1d^{-/-}$ mice.[46] However, it was reported that human *i*NKT cells could stimulate IgA and IgG secretion by B cells *in vitro* even in the absence of exogenous *i*NKT cell antigens.[126]

(3) Mucus production and glycosylation

The glycosylation of the epithelial cells and the production of mucus by goblet cells and enterocytes are important physical defence mechanisms of the mucosal surfaces.[127,128] For example, the glycosylation of intestinal epithelial cells can be regulated by dendritic cells and innate lymphoid cells (ILC3s). With regard to *i*NKT cells, it was suggested that *i*NKT cell-derived interleukin-13 (IL-13) regulates goblet cell homeostasis.[129]

(4) CD1d-retrograte signalling

Interaction of the invariant T-cell receptor (*i*TCR) of *i*NKT cells with CD1d not only can activate *i*NKT cells but can also influence the CD1d-expressing cells due to CD1d-retrograde signalling. This has been reported for APC (leading to IL-12 production);[130,131] epithelial cells (either IL-12 and IL-15,[94] or IL-10[132,133]); cancer cell lines (IL-12);[134] and ILC3s (IL-22).[135] In the context of the mucosa, pro-inflammatory responses, IL-22 by ILC3s,[135] and anti-inflammatory responses, IL-10 by intestinal epithelial cells,[132,133] have been reported for such CD1d-retrograte signalling.

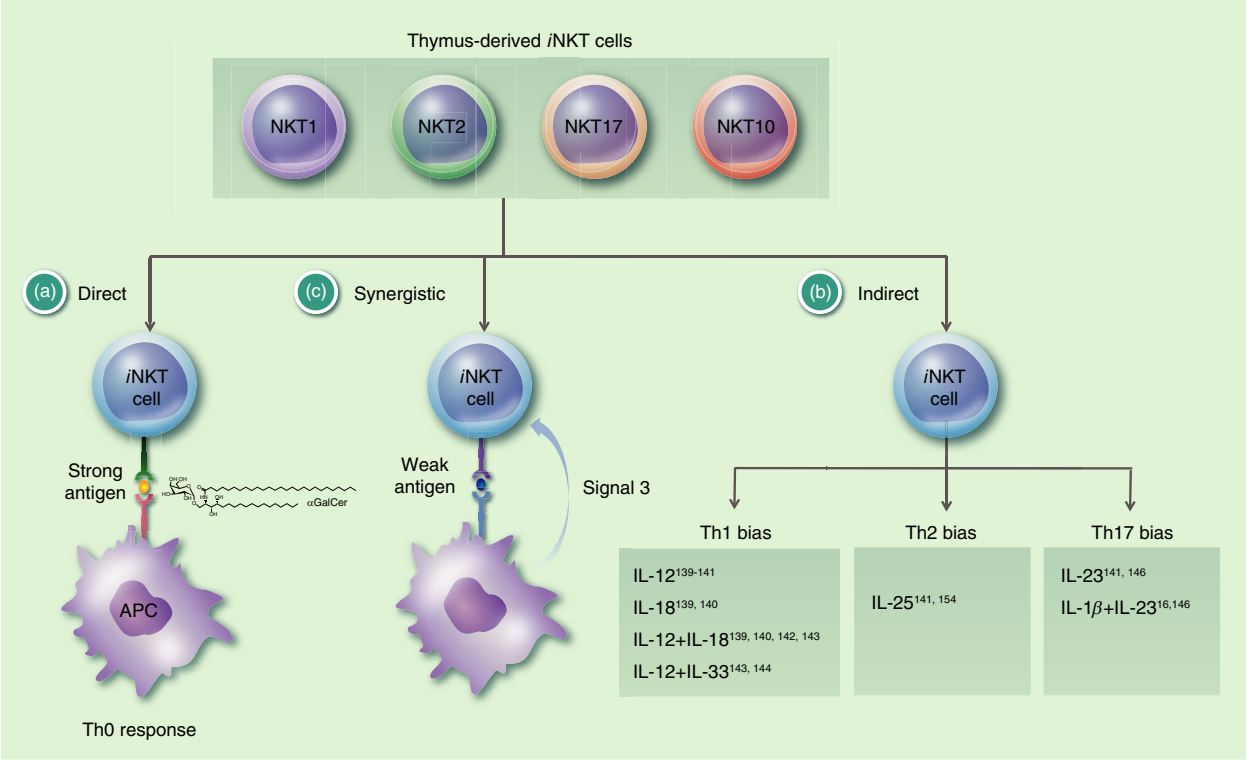

Figure 1. The route of invariant Natural Killer T (*i*NKT) cell activation impacts their effector function. For *i*NKT cells in unchallenged control mice, i.e. thymus-derived *i*NKT cells, three routes of activation have been described. (a) In the direct or antigen-dependent activation, a strong antigen, usually of foreign origin, binds to CD1d and activates *i*NKT cells via their semi-invariant T-cell receptor (TCR). αGalCer is depicted as an example. This direct activation leads to the production of both T helper type 1 (Th1) cytokines, like interferon-γ (IFN-γ) and tumour necrosis factor, and Th2 cytokines, like interleukin-4 (IL-4) and IL-13, by the *i*NKT cells, which is sometimes referred to as a Th0 response. (b) The indirect or antigen-independent activation does not require an engagement of TCR, but rather is achieved by the exposure of the *i*NKT cells to several pro-inflammatory cytokines alone or in combination. Some cytokines could induce a preferential production of particular cytokines by the *i*NKT cells, leading to a Th1-bias (more IFN-γ and/or less IL-4), Th2-bias (more IL-4, IL-13 and/or less IFN-γ), or Th17-bias (more IL-17A). The available data indicate a preferential activation of NKT17 cells in the case of the Th17-bias. However, in the case of the Th1- and Th2-biases it seems more likely that it is the result of a modulation of the *i*NKT cell cytokine response. (c) In the synergistic pathway the stimulation of *i*NKT cells depends both on the TCR and on cytokine receptors. In these cases, the stimulation provided by a weak antigen (signal 1) and sub-optimal cytokine concentrations (signal 3), which are both too weak on their own to drive *i*NKT cell activation, can act together to achieve the stimulation of the *i*NKT cells. The antigens bound to CD1d could be of self or foreign origin. In the direct and synergistic pathways, the signal can also be amplified by the up-regulation of the expression levels of CD1d[97,122] and/or self-antigens.[97,98] Additionally, signal two, i.e. co-stimulation/inhibition, can modulate *i*NKT cell responses.[25]

(αGalCer), a bacteria-derived glycolipid chemical optimized to yield its exceptionally strong agonistic potential. The *i*NKT cells develop as effector/memory cells and, following TCR stimulation, they rapidly produce copious amounts of various cytokines and display strong cytotoxicity.[10] Due to their cytokine production *i*NKT cells can impact a wide variety of different chronic and acute immune processes, ranging from responses to pathogens and tumours, to autoimmune responses.

Furthermore, *i*NKT cells are heterogeneous and based on significant biases in cytokine production and the expression of particular transcription factors, several subsets have been described. Some, like NKT1,[11] NKT2,[11] NKT10[12,13] and NKT17[14–18] cells, develop in the thymus, while others, such as NKT_{FH}[19,20] and FoxP3$^+$ *i*NKT[21] cells seem to arise or, like NKT10 cells,[12,22,23] greatly expand after immunization. For some of these subsets a preferential distribution to various organs has been described.[12,24]

The TCR-mediated activation of *i*NKT cells is called the direct or antigen-dependent activation.[4–9] Similar to memory T cells, *i*NKT cells can also be activated in a TCR-independent manner by cytokines alone. This indirect or antigen-independent activation can be induced by several pro-inflammatory cytokines alone or in combination. For some cytokine combinations a preferential activation of a particular *i*NKT cell subset has been suggested (Fig. 1). However, the direct and indirect activation pathways are not exclusive as cytokines can augment CD1d-dependent activation too. This synergistic pathway for *i*NKT cell activation seems particularly critical for stimulation with weak

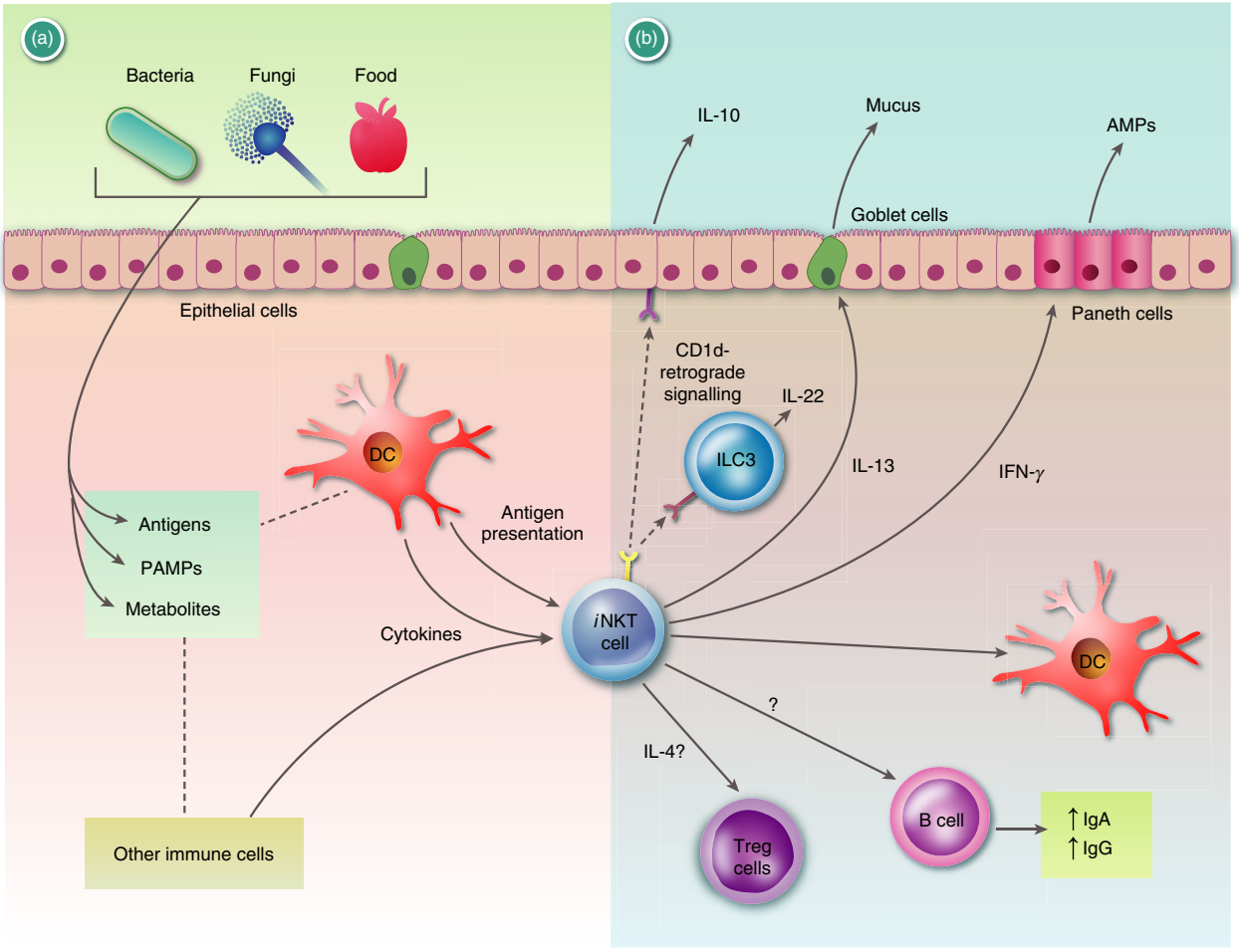

Figure 2. Graphic summary of the functions of invariant Natural Killer T (*i*NKT) cells at mucosal surfaces: (a) *i*NKT cells at mucosal surfaces can respond to many signals. Bacterium-, fungus-, and food-derived products can influence the tissue-resident cells of the mucosa and the local immune cells, via antigens, pathogen-associated molecular patterns (PAMPs), and metabolites (Box 1). These can alter the frequency and function of various cells (not depicted). Antigens for *i*NKT cells can be either self-antigens or antigens derived from the microbiota (Table 1). Some of the bacterially derived glycolipids binding to CD1d can also be inactive, competitive inhibitors. Furthermore, *i*NKT can respond to local cytokines either directly or synergistically with CD1d-bound antigens. (b) Invariant NKT cells are known to influence the mucosal microenvironment and the microbial composition via (1) cytokines that they produce and (2) direct cell–cell contact. (1) Invariant NKT cell derived interferon-γ (IFN-γ) or interleukin-13 (IL-13) has been shown to activate Paneth cells or goblet cells to increase production of anti-microbial peptides (AMPs) or mucus, respectively. Furthermore, *i*NKT cells have been shown to boost IgA and IgG production by B cells. Finally, CD1d$^{-/-}$ mice had a lower frequency of CD304^{+} regulatory T (Treg) cells in the mesenteric lymph nodes and *i*NKT cell-derived IL-4 was implicated.[46] (2) Binding of *i*NKT cells to CD1d can induce, via a CD1d-retrograde signal, IL-10 or IL-22 production by epithelial cells or innate lymphoid cells (ILC3s), respectively, which both support mucosal integrity. Please note that the figure does not attempt to represent a particular mucosal surface, but summarizes available data derived from various tissues and sides. For additional references we refer to the main text and Box 1. AMPs, anti-microbial peptides; ECs, epithelial cells.

antigens, which, importantly, can be either exogenous (foreign) or host-derived (self) antigens. Furthermore, surface co-stimulatory and co-inhibitory molecules have been suggested to modulate *i*NKT cell responses.[25]

As *i*NKT cells are positioned in peripheral tissues and display effector functions rapidly after activation, they are part of the first line of defence of the immune system and therefore play an important role at the mucosal surfaces as well. In this review we will outline our current knowledge on the interplay between *i*NKT cells and the mucosal microbiota, with a special emphasis on the respiratory and gastrointestinal tracts (Fig. 2).

*i*NKT cells and the microbiota in the gastrointestinal tract

The gastrointestinal tract, reaching from the mouth to the rectum, is usually divided into the upper

Table 1. Mucosal commensals and pathogens with known invariant Natural Killer T (*i*NKT) cell antigens

Organism	Phylum	Pathogenicity	Antigen	References
Bacteria				
Mycobacterium tuberculosis	*Actinobacteria*	Pathogen	Phosphatidylinositol mannoside (PIM)	78
Saccharopolyspora	*Actinobacteria*	Environmental, opportunistic pathogen	M-AcM-MAG	63
Rothia dentocariosa	*Actinobacteria*	Commensal, opportunistic pathogen	M-AcM-MAG	63
Arthrobacter	*Actinobacteria*	Commensal, opportunistic pathogen	M-AcM-MAG	63
Bacteroides fragilis	*Bacteriodetes*	Commensal, opportunistic pathogen	αGalCer(Bf)	59
Prevotella copri	*Bacteroidetes*	Commensal	αGalCer (~ 100 × lower concentration than *B. fragilis*)	61
Bacteroides vulgatus	*Bacteriodetes*	Commensal, opportunistic pathogen	αGalCer (~ 100 × lower concentration than *B. fragilis*)	61
Streptococcus pneumoniae and Group B streptococcus	*Firmicutes*	Commensal, opportunistic pathogens	SPN-Glc-DAG SPN-Gal-Glc-DAG	62
Lactobacillus casei	*Firmicutes*	Commensal	Glc-DAG	62
Sphingomonas paucimobilis	*Proteobacteria*	Commensal, opportunistic pathogen	α-glucuronosyl ceramide (GSL-1/ aGlcUCer)	50,51
Sphingomonas yanoikuyae	*Proteobacteria*	Environmental, commensal, opportunistic pathogen	α-galacturonosyl-ceramides	50
Ehrlichia muris	*Proteobacteria*	Pathogen in rodents, but not in humans	Antigen not defined	49
Helicobacter pylori	*Proteobacteria*	Commensal, opportunistic pathogen	Cholestoryl-α-glucosides, especially monoacyl α-CPG	30,56
Sphingomonas wittichii	*Proteobacteria*	No pathogenicity reported	α-galacturonosyl-ceramides	52
Borrelia burgdorferi	*Spirochaetae*	Pathogen	BbGL-II (1,2-di-*O*-acyl- 3-*O*-α-D-galactopyranosyl-*sn*-glycerol, 6)	52
Fungi				
Aspergillus fumigatus and *Aspergillus niger* (latter with lower antigenic content)	*Ascomycota*	Opportunistic pathogen	Asperamide B	136
Candida albicans	*Ascomycota*	Commensal, opportunistic pathogen	ChAcMan	63
Protozoa				
Entamoeba histolytica	*Amoebozoa*	Opportunistic pathogen (often asymptomatic)	lipopeptidophosphoglycan (EhLPPG)	137
Leishmania donovani	*Euglenozoa*	Opportunistic pathogen (often asymptomatic)	Lipophosphoglycan (LPG)	138

gastrointestinal tract (pharynx, oesophagus, stomach), the small intestine (duodenum, jejunum, ileum), and the large intestine (caecum, colon, rectum). Its main function is to extract and absorb nutrients from the food via its large surface of approximately 200 m^2, made up largely of small intestinal villi and microvilli. This digestive process is supported by the commensal microbiota of the intestine. The gastrointestinal tract harbours diverse sets of bacteria, fungi, Archaea, and viruses with varying densities, although the vast majority of the microbiome are bacteria. *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria* are the dominant bacterial phyla in the human intestine, with estimates suggesting over 1000 distinct species.[26] The bacterial density increases along the gastrointestinal tract, spanning from 10^3 to 10^4 bacteria/ml at the beginning of the small intestine up to 10^{11} bacteria/ml in the colon.[27] Besides digestion, the composition of the gut microbiota can influence many aspects of human health, including neural, gastrointestinal, metabolic and skeletal systems, as well as the immune system.[1–3] Holding such large numbers of bacteria, separated from the body by only one cell layer, at bay requires several defence mechanisms, with the immune system in a prominent position. The closest are the intraepithelial lymphocytes (IELs), which are sited between the mucosal epithelial cells. There are approximately 10–15 IELs for

every 100 epithelial cells in the small intestine with fewer cells in the large intestine.[28] Over 90% of these IELs are T cells with the large majority expressing CD8α. Furthermore, basically all IEL T cells express an effector/memory phenotype, bear a TCR with limited diversity, and many have unusual developmental requirements unique to this location.[28] In contrast, the lamina propria lymphocytes, which are scattered throughout the lamina propria underneath the epithelial layer, are more diverse in their cellular composition and resemble more those of other lymphatic organs.[29]

*i*NKT cells can be detected in the mouse intestine at frequencies only slightly lower than in other peripheral organs, like the spleen and lymph nodes (stomach,[30] small intestine,[31–33] large intestine[33–37]). Approximately 65% (BALB/c mice) to 85% (C57BL/6 mice) of intestinal *i*NKT cells are NKT1 cells, with the rest largely being NKT2 cells, and no differences in this distribution were noted for IELs versus lamina propria lymphocytes.[24] The distribution of intestinal *i*NKT cells correlated indirectly with the bacterial density, i.e. more *i*NKT cells in the small intestine than the large intestine, and the proximity of the bacteria, i.e. more *i*NKT cells in the lamina propria lymphocytes than in the IELs.[33] Invariant NKT cells can also be found in the human intestine, however, less is known about their distribution.[38–40]

Although *i*NKT cells do not require the commensal microbiota to develop an activated/memory phenotype and the ability to produce cytokines,[33,35,41–43] the microbiota nonetheless impacts their functionality. Indeed, *i*NKT cells from germ-free mice (GF) differed in their TCR Vβ7 frequency and expressed lower levels of activation markers[33] compared with the control specific pathogen-free mice. Furthermore, these *i*NKT cells were hyporesponsive and performed weaker effector functions (cytokine production, cytotoxicity) after antigenic stimulation.[33] Reconstitution of the GF animals with bacteria that contain *i*NKT cell antigens (*Sphingobium yanoikuyae*), but not with antigen-negative bacteria (*Escherichia coli*), could fully establish phenotypic and functional maturity of the *i*NKT cells.[33] Interestingly, the microbiota affected the distribution of *i*NKT cells as well. Whereas the frequency of *i*NKT cells in GF animals was lower in spleen and liver,[35,42] it was increased in the small and large intestines[33] and the colon.[35,37] This distribution was established within the first 5 weeks of life, as reconstitution of the GF mice with a normal microbiota at a later time did not change the frequency of *i*NKT cells.[35] Importantly, the increased frequency of *i*NKT cells in the intestine of GF mice has also functional consequences for intestinal immune responses. Oxazolone-induced colitis is a mouse model of ulcerative colitis in which *i*NKT cells are known to be pathogenic.[44] As a result of the higher numbers of effector *i*NKT cells in the mucosa at the onset of the disease, these GF animals were also more sensitive to oxazolone-induced colitis.[35] CXCL16 production by epithelial cells was implicated in the recruitment of *i*NKT cells to the intestine;[35] however, as the authors themselves published conflicting data,[37] the role of CXCL16 appears unclear. Data similar to the GF mice were obtained with mice with a highly restricted intestinal flora (RF mice), which is devoid of *Sphingobium* but enriched for *Firmicutes*.[42] The frequency of *i*NKT cells in RF mice was reduced in the spleen and liver, they displayed an altered TCR Vβ7 frequency, lower expression of activation markers, and produced smaller amounts of cytokines following activation with αGalCer.[33,42] Moreover, in the RF mice the intra-gastric administration of *S. yanoikuyae* bacteria increased the frequency of spleen and liver *i*NKT cells and their expression of activation markers within a day.[42] The data in the GF and RF animals demonstrate that it is not the microbiota *per se* that affects *i*NKT cells, but the composition of the microbiota and the presence of particular *i*NKT cell antigens. When we compared control specific pathogen-free mice obtained from different vendors, which are known to differ in their gut flora,[45] we again noticed differences in the frequency, Vβ7-usage, and tumour necrosis factor production of *i*NKT cells.[33] These differences were abolished by co-housing the offspring, which served to equalize the microbial flora.[33]

Several lines of data support the idea that specific *i*NKT cell antigens can be provided by the intestinal microbiota. First, the effects observed in the GF animals were independent of interleukin-12 or MyD88.[33,35] Second, using Nur77 expression as an indication for TCR-mediated activation, a CD1d-dependent stimulation of *i*NKT cells in the intestine was shown in the presence of the intestinal microbiota.[46] Third, intra-gastric administration of GF mice with a mixture of heat-killed bacteria leads to the expression of CD1d–antigen complexes, detected with the antibody clone L363,[47] on liver dendritic cells.[48] Finally, and most directly, *i*NKT cell antigens have been discovered in several bacteria present in the intestinal microbiota.

Besides the above mentioned α-Proteobacteria *Sphingobium yanoikuyae*,[49,50] *i*NKT cell antigens have been described in the closely related *Sphingomonas paucimobilis*,[49–51] *Sphingomonas wittichii*,[52] *Sphingomonas capsulata*,[49] *Novosphingobium aromaticivorans*,[53] and *Ehrichia muris*.[49] The Proteobacterium *Heliobacter pylori* is a common colonizer of the stomach, present in about half of the world population,[54] that can cause gastric ulcers. About 25% of *H. pylori*'s lipids are cholesteryl α-glucosides[55] that contain several related antigens for *i*NKT cells.[30,56] The intestinal microbiota in humans and mice is dominated by members of the phyla *Firmicutes* and *Bacteriodetes* (human,[57] mouse[58]). For one common member of these phyla, *Bacteroides fragilis*, an α-galactosylceramide antigen (α-GalCer(Bf)) reminiscent of αGalCer has been described that could stimulate human and

mouse *i*NKT cells.[59] Surprisingly, a subsequent study described a *B. fragilis*-derived α-galactosylceramide (GSL-Bf717) that could bind CD1d but was not stimulatory.[37] This suggests that some commensal-derived glycolipids can act as competitive inhibitors for other stimulatory *i*NKT cell antigens. Another example for this was the inhibition of αGalCer-induced *i*NKT cell activation *in vitro* by a lipid extract of *Bifidobacterium infantis*.[60] In addition to *B. fragilis*, *Prevotella copri* and *Bacteriodes vulgatus,* two other commensals belonging to the *Bacteriodetes* phylum, express *i*NKT cell antigens, although at approximately 100-fold lower concentrations than *B. fragilis*.[61] Within the *Firmicutes* phylum so far only the commensal strain *Lactobacillus casei* has been described to express an *i*NKT cell antigen.[62] Furthermore, a few other reports reported *i*NKT cell antigens from commensal bacteria, without clearly defining the source[48] or the exact structure of the antigen (e.g. *B. infantis*[60]). Besides bacteria, an *i*NKT cell antigen (ChAcMan) has been reported for the fungus *Candida albicans*, a gut commensal and opportunistic pathogen.[63] Finally, *i*NKT cell antigens have been described for several mucosal pathogens (Table 1). In summary, in the last decade *i*NKT cell antigens were discovered in many bacteria, fungi and protozoa, indicating that such antigens are widely distributed in the environment.[64]

Given the impact of the intestinal microbiota on *i*NKT cells it might not be too surprising that the elimination of the gut flora with antibiotics likewise influences *i*NKT cell responses. After 2 weeks of antibiotic treatment, the frequency of *i*NKT cells increased in the colon of wild-type C57BL/6 mice.[65] Interestingly, this increase disappeared within 1–2 weeks following the bacterial reconstitution.[65] Changes were also noted beyond the intestine. For example, the *i*NKT cell frequency in liver increased following antibiotic-mediated commensal depletion.[66] Furthermore, for several models the changes observed after the depletion of the commensal microbiota by antibiotics were dependent on *i*NKT cells: a delayed liver regeneration after partial hepatectomy,[66] an amelioration of experimental autoimmune encephalomyelitis,[67] but also an augmented concanavalin A-induced liver injury.[48]

Importantly, the interaction between the commensal microbiota and *i*NKT cells is mutual, as *i*NKT cells can influence the composition of the intestinal microflora. CD1d-deficient mice were found to host an altered gut microbiota,[46,68,69] which was pro-inflammatory upon transfer into wild-type animals.[69] Furthermore, CD1d-deficient mice were more susceptible to intestinal colonization by pathogenic bacteria as well.[68] Whereas in control mice the intestinal bacteria were largely separated from the intestinal epithelial cells by a mucus layer, this layer was impaired in the CD1d-deficient animals, leading to a direct contact of the bacteria and the epithelial cells.[46] The impact of *i*NKT cells on the intestinal microbiota was stronger in the small than the large intestine,[46,69] in line with the higher frequency of *i*NKT cells in the small intestine.[33] This might also explain why an analysis of faeces from CD1d-deficient pigs did not reveal any differences in the bacterial composition.[70] The changes in the intestinal microbiota seen in the CD1d-deficient animals could be replicated in mice where CD1d was selectively missing on $CD11c^+$ cells.[46] In contrast, in mice with a CD11c-specific deletion of CD1d, the separation between the intestinal bacteria and the epithelial cells was intact, as in the control animals.[46] This demonstrates that *i*NKT cells influence the intestinal microbiota in at least two independent ways involving $CD11c^+$ cells, presumably dendritic cells, and other $CD1d^+$ cells in the intestine. Finally, the intestinal microbiota could be influenced by antigen-specific activation of *i*NKT cells with αGalCer. On the one hand, oral challenge of control mice with αGalCer led to an increase in *Bacteriodetes* and *Proteobacteria* but a decrease in *Firmicutes* species.[46] On the other hand, injection of αGalCer into GF mice delayed their reconstitution with bacteria given orally.[68] However, information is still limited on how *i*NKT cells could achieve this influence on the microbiota (Box 1).

*i*NKT cells and the microbiota in the respiratory tract

The mammalian respiratory system is generally divided into the upper and lower respiratory tract. The upper respiratory tract is composed of the nose, nasal cavity and sinuses, pharynx (throat) and larynx (voice box) and is the first mucosal site where the body encounters airborne microorganisms. The lower respiratory tract includes the conducting airways (trachea, bronchi, bronchioles) and the alveoli, in which the gas exchange occurs. The upper respiratory tract retains larger particles; however, particles smaller than 1–3 μm, such as microorganisms, pollen and smoke, can pass to the lower respiratory tract. There, particles or microorganisms can be trapped in the mucous that is secreted by submucosal mucous glands and lines the lower airways. The rhythmic pulse-like movements by the cilia of the epithelial cells then transport the mucus and its trapped particles to the upper respiratory tract where they are eliminated either via the digestive tract or through the sneeze and cough reflexes. Furthermore, this mucus and the liquid layer on the airway surfaces of the lower respiratory tracts contain various antimicrobial peptides and antigen-specific secretory IgA to protect the lung.[71,72] Despite these mechanical and chemical defence mechanisms, the lower airways are not sterile, but host a unique commensal microbiota.

The airways are colonized immediately after birth and a stable commensal microbiota develops within the first years of life.[73] This commensal microbiota contributes to lung development and function, and to host defence; a

disordered microbiota (dysbiosis) is a common feature of chronic lung inflammations, like asthma.[71,73] The core microbiota of the healthy lung contains five bacteria phyla: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Fusobacteria* and *Actinobacteria*.[71,73]

*i*NKT cells are more frequent in the lung than in the secondary lymphatics,[24] with a strong influx from the blood following lung inflammation.[74] There is strong evidence for a major role for *i*NKT cells in regulating immune and inflammatory responses in the respiratory system. They are important contributors for most asthma models and have been implicated in the pathogenesis of human asthma.[75,76] Furthermore, *i*NKT cells are important for a protective immune response in the lung to many infectious agents.[77] It is clear now that several of these airway pathogens contain *i*NKT cell antigens.

Besides the *Sphingobium* bacteria mentioned above, *i*NKT cell antigens have been described for *Mycobacterium tuberculosis*, the causative bacteria of tuberculosis,[78] *Streptococcus pneumoniae*, the leading cause of community-acquired pneumonia,[62] group B streptococcus,[62] *Saccharopolyspora* spp., the leading cause for Farmer's lung,[63] and the fungus *Aspergillus fumigatus*.[79] In contrast to *M. tuberculosis*, which is considered an obligate pathogen, the other organisms on this list are opportunistic pathogens as they can be found in the human microbiota to varying extents and are usually asymptomatic in healthy individuals. Nonetheless, the presence of particular commensals or the composition of the lung microbiota can probably impact inflammatory responses. For example, patients with poorly controlled asthma have a higher bacterial count in the lung, and some of the taxa (e.g. *Sphingobium*) are known to bear *i*NKT cell antigens.[80]

Additionally, several of the organisms mentioned above can readily be detected in the environment, like *Sphingobium* spp., *Saccharopolyspora* spp., or *Aspergillus fumigatus*, indicating that the inhaled air is another source for *i*NKT cell antigens.[64] Indeed, the majority of sterile house dust extracts (HDEs) that we tested contained antigens for mouse and human *i*NKT cells.[81] HDEs are simple aqueous preparations from house dust that provide a relatively complete sampling of the environment without the need for *a priori* assumptions imposed by the nature of the purification.[82] The HDEs displayed adjuvant-like properties in an *i*NKT-cell-dependent allergen-induced mouse asthma model.[81] When different HDEs were tested, we noted a large variability in the antigenic strength as well as in the chemical nature, indicating that different HDEs could contain several distinct *i*NKT cell antigens.[81] Furthermore, as our experiments indicated that dust mites are probably not the source of the antigenic activity found in HDE,[81] we consider bacteria as the most likely source of these environmental *i*NKT cell antigens.[64] However, it should be noted that some preliminary data suggest that plants and their pollen might contain *i*NKT cell antigens as well.[83–85] Altogether, recent findings indicate that antigens for *i*NKT cells are almost ubiquitous indoors and in the environment. As they will reach the airways with the inhaled air, it seems likely that *i*NKT cell antigens are present under normal conditions in the lung at low levels and could under certain conditions contribute to airway inflammation.

Interestingly, however, the age at the time of the antigen exposure seems to be important as well. Whereas the *i*NKT cell antigens in the HDEs greatly augmented symptoms in an allergen-induced asthma model in adult mice,[81] they seemed to have an opposite effect early in life. The 'Urban Environment and Childhood Asthma' (URECA) project is a longitudinal study that follows infants born to parents with asthma from birth through to age 14–16 years.[86] As part of the URECA study we correlated the *i*NKT cell–antigen content of the house dust from children when they were 3 months old with the clinical symptoms of those children at age 3–7 years.[87] Our data indicate that infants growing up in homes containing more *i*NKT cell antigens were less likely to develop asthma.[87] A child's house that is rich in antigens for *i*NKT cells probably reflects an environment with increased microbe exposure. According to the 'hygiene hypothesis', increased microbial exposure in the first years of life protects children from asthma.[88,89] In line with this hypothesis are also the data on lung *i*NKT cells in GF mice. Such GF mice had a higher frequency of *i*NKT cells in the lung than specific pathogen-free control mice.[35] Similar to the intestine, this frequency remained high in the lung if the mice were not colonized with bacteria within the first 3 weeks of life.[35] Due to this increased frequency of effector *i*NKT cells in the lung, such mice were more susceptible to allergen-induced asthma as well.[35] Similarly, the deliberate activation of *i*NKT cells in 2-week-old mice with an *H. pylori*-derived antigen or the T helper type 1-biasing antigen C-GalCer protected mice against asthma 6 weeks later.[56] Surprisingly, a *Sphingobium*-derived antigen was not able to induce this protection.[56] These data demonstrate that the influence of the commensal microbiota on *i*NKT cells can have important roles in the regulation of the immune response in the airways and suggest that this impact is influenced by the age of the individual. In contrast to the intestine, no information is currently available as to whether *i*NKT cells can influence the lung microbiota or can regulate the production of anti-microbial peptides in the lung under steady-state conditions.

*i*NKT cells and microbiota in other mucosal tissues

Much less is known about the role of *i*NKT cells at other mucosal surfaces and of their interaction there with the local commensals than for the intestine or the lung.

Although, the microbial composition of the urogenital tract differs from the intestine, several of the bacteria known to bear *i*NKT cell antigens can be found in the urogenital tract of healthy individuals.[90,91] *i*NKT cells are functional in the bladder, as αGalCer activation was shown to support bacterial clearance in urinary tract infections.[92,93] Furthermore, urethral epithelial cells were shown to be receptive to retrograde signalling downstream of CD1d.[94] Therefore, it seems likely that *i*NKT cells play a role in the protection of the mucosal surface of the urogenital tract as well.

Concluding remarks

It is becoming increasingly clear how intricate and far-reaching the interaction between the host and its commensal microbiota is, where each individual player seems to have its own role at maintaining and fostering this mutually beneficial relationship. These data also establish a mutual interaction between the commensal microbiota and *i*NKT cells, where the microbiota is required for *i*NKT cells to gain function, and where *i*NKT cells can influence the composition of the mucosal flora. Moreover, they suggest that *i*NKT cells play an important role in supporting the health and homeostasis of the mucosal tissues by acting as an earlier sensor for tissue damage and bacterial translocation.

The microbiota is a major source for *i*NKT cell antigens. These include bacteria, fungi, protozoa and metazoa that either colonize the mucosa (true or facultative commensals, opportunistic pathogens), or temporarily present themselves at the mucosa following ingestion or inhalation (commensals, pathogens, environmental antigens) (Table 1). Besides microbes, environmental antigens[64] could be derived from food components too, like milk lipids,[95,96] and from airborne pollen,[83] either directly or after processing by the commensals. Furthermore, microbe-induced changes in the presentation of host-derived self-lipids or the expression levels of CD1d itself could conceivably modulate mucosal *i*NKT cell responses as well.[97,98] Finally, although the GF data indicated that pathogen-associated molecular pattern-induced signals are not necessary for full *i*NKT cell maturation,[33,35] it is likely that such signals are involved in the regulation of *i*NKT cell functions in the mucosa (Box 1). Such indirect signals could explain the long-lasting effects on *i*NKT cell frequency and functionality following virus infection.[56,99] In this context, it is notable that *i*NKT cells can also be activated by bacterially derived superantigens.[100,101]

Moreover, anything that changes the composition of the commensal microbiota could also potentially impact *i*NKT cells. Host-derived variables, like gender and genetics, are known to impact the microbiota.[102,103] The list is longer for environmental variables, inducing the diet (e.g. fibre content, antibiotics/probiotics), housing (e.g. urban/rural, pets), and the presence of chronic infections (e.g. helminths) or other 'pathobionts'.[104–107] Consequently, the microbiota is dynamic.[108]

An intriguing finding is the far-reaching impact that the microbiota can have on the host. On the one hand, changes in one mucosal surface can lead to changes in other mucosal sides. For example, using αGalCer as adjuvant for an sublingual vaccination led to increases in antigen-specific antibody levels also in vaginal washes.[109] Furthermore, changes in the microbiota of one mucosal tissue can impact the frequency and function of immune cells of other mucosal tissues.[110] On the other hand, the impact of the microbiota is not limited to the mucosal surfaces and can influence apparently any part of the body, including seemingly remote areas, such as the brain.[111] With regard to *i*NKT cells, the above-mentioned systemic effects of antibiotic treatment and the role of *i*NKT cells are relevant in sepsis,[112] which is often caused by translocated intestinal bacteria.[113] Furthermore, one member of the commensal *Sphingobium* species (*Novosphingobium aromaticivorans*) has been linked to *i*NKT cell-dependent autoimmune responses against the bile duct in mice[53] and humans.[114,115]

However, many open questions remain with regard to the details of the mutual regulation of *i*NKT cells and the commensal microbiota. For many of the observed influences the mechanistic understanding is still rudimentary, and many new microbial mediators will probably be discovered, adding to the complexity. It seems likely that different commensals provide at times complementary or opposing influences, as reported for example for *B. fragilis*.[37,59] Furthermore, the response of *i*NKT cells towards microbial-derived signals can be pro-inflammatory or anti-inflammatory and the decisive factors governing this outcome are largely unclear. Whereas the nature of the antigen-presenting cell probably plays a role,[116] the potential involvement of different *i*NKT cell subsets is currently unexplored. Finally, much needs to be learned about the mechanisms of the systemic impact on *i*NKT cells and the extent to which the microbiota impacts *i*NKT cell functions all over the body.

Invariant NKT cells are of great therapeutic potential as the lock-and-key principle of CD1d/*i*TCR is basically shared by every human being. Consequently, *i*NKT cell antigens are already in clinical trials for cancer therapy and for several vaccination approaches,[117,118] and we expect many new applications, in particular for mucosal vaccinations, in the near future. The data reviewed here also suggest that *i*NKT cells could be a promising therapeutic target to address microbial dysbiosis, which is linked to many mucosal diseases.[119,120] Furthermore, the finding that neonatal changes can have life-long impacts on the frequency of mucosal *i*NKT cells is intriguing, as it suggests an option for preventive approaches to treat, for example, asthma.

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The authors declare that they have no competing interests.

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