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Gut Microbiota-Derived Unconventional T Cell Ligands: Contribution to Host Immune Modulation

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Abstract

Besides the prototypic innate and adaptive pathways, immune responses by innate-like lymphocytes have gained significant attention due to their unique roles. Among innate-like lymphocytes, unconventional T cells such as NKT cells and mucosal-associated invariant T (MAIT) cells recognize small nonpeptide molecules of specific chemical classes. Endogenous or microbial ligands are loaded to MHC class I–like molecule CD1d or MR1, and inducing immediate effector T cell and ligand structure is one of the key determinants of NKT/MAIT cell functions. Unconventional T cells are in close, constant contact with symbiotic microbes at the mucosal layer, and CD1d/MR1 can accommodate diverse metabolites produced by gut microbiota. There is a strong interest to identify novel immunoactive molecules of endobiotic (symbiont-produced) origin as new NKT/MAIT cell ligands, as well as new cognate Ags for previously uncharacterized unconventional T cell subsets. Further studies will open an possibility to explore basic biology as well as therapeutic potential.

INTRODUCTION

Mammalian hosts have evolved a sophisticated system in response to various types of potentially pathogenic foreign molecules. The two main pillars of defense provide orthogonal and synergistic protection. First, innate immune cells provide immediate recognition of pathogen-associated molecular patterns, which are recognized by pattern-recognition receptors (PRRs). Induction of diverse downstream signaling pathways enables direct killing and phagocytosis of pathogens, as well as further recruitment of effector immune cells. At the same time, structural information is relayed to the adaptive arm of immunity. Foreign molecules are internalized and processed by APCs, and epitopes (mostly processed peptide) are presented by MHC proteins, which are recognized by the

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specific TCR with high affinity (1). Cognate recognition of the peptide Ag activates T cells, developing specific and lasting cellular and humoral responses.

Inclusive versus exclusive mechanisms of immunogenic ligand recognition

The primary requirement in immune surveillance is to distinguish foreign versus self. In order to generate the “non-self list” (Table I), the host uses two distinct mechanisms. For most innate leukocytes, multiple types of PRRs such as TLRs, Nod-like receptors, C-type lectin receptors, and RIG-I–like receptors have evolved to cover diverse foreign molecules over chemical and spatial spectra, from polar viral nucleic acids to sticky bacterial lipoproteins, located extracellularly or intracellularly (2). A host can have a long list of molecules (or narrow classes of molecules with similar chemical and structural properties) imprinted to innate leukocytes, and the usual suspects can be rapidly taken care of. This “inclusive” surveillance mechanism has several advantages. In many cases, PRR ligands are non-self, ubiquitous, and essential molecules for pathogens, such as microbial structural components (LPSs as prototypic TLR4 ligand or peptidoglycans as TLR2 agonist), or bacterial or viral-specific nucleic acids (TLR3/7/9 ligands) (3-5). Therefore, even relatively stringent structural requirements of an individual PRR can cover a wide range of foreign molecules. Furthermore, preparing a large number of cells with the capability to fight against the most common pathogens would be the best preparation for immediate responses. Nonetheless, activation by structural imprinting allows certain pathogens to develop biochemical pathways to modify the structures of PRR ligands, just enough to evade recognition but still maintain their own function. Such adaptations can greatly compromise the efficiency of immune responses, and it is clear that hosts cannot win such a genetic “arms race” against bugs.

As a complement to efficient but incomplete PRR-type recognition, mammalian hosts have developed foreign ligand recognition using almost the opposite strategy. MHC class I and II molecules can load processed peptides with unrestricted structural limitations. Therefore, structural changes in foreign molecules (e.g., a mutation in peptide sequence) do not matter, as long as the T cell with the receptor recognizing the mutated peptide exists. Instead, the host must avoid unnecessary and detrimental immune responses caused by self-antigen; removing the self-peptide responding T cell by negative selection is a key step (6). This “exclusive” type Ag recognition mechanism (removing autoreactive immune cells) also pairs well with immune memory and rapid reactivation, as maintaining previously activated lymphocytes especially designated for repeated challenge is a key to retaining protection with limited resource. At the same time, in order that adaptive responses can function properly, innate immunity as the first line of defense is critical, especially when the host encounters “novel” pathogens.

Out of dichotomy: innate-like lymphocytes.—The aforementioned mechanistic differences in foreign ligand recognition and potentiation of effector cells are some of the primary distinctions between innate and adaptive pathways. Alternatively, we mention that recognition of foreign ligands and activation of host immune responses are two distinct events and therefore could be dividable. Indeed, applying exclusive-type Ag recognition to innate cells (maintaining a significant number of each and every Ag-specific clone)

is too costly; however, if the TCR variability is small enough, MHC-TCR–mediated Ag recognition can also be paired with the innate-type, immediate effector functions. Therefore, similar to innate leukocytes, subsets of T cells recognize ligands with limited chemical and structural diversity. Because “recognition of variable peptide Ag by variable TCR” has been widely accepted as the defining characteristic of “conventional” T cells, these cells with limited TCR diversity are usually described as “unconventional” (7).

In addition to unconventional T cells, there are also leukocytes that do not follow the cell ontogeny of myeloid (innate)/lymphoid (adaptive) classification. Unlike typical effector lymphocytes, some tissue-resident cells of common lymphoid progenitor origin express neither T nor B cell receptors but show innate-like phenotypes (8). These innate lymphoid cells, along with the above-described unconventional T cells, constitute a major component of innate-like lymphocytes. Accumulating studies emphasize the importance of innate-like lymphocytes in the peripheral immune system, especially at the mucosal surface (9-17).

Bridging innate and adaptive immunities: unconventional T cells as fast-acting coordinators of immune responses at mucosal surfaces

Mucosal surfaces (the oro-gastrointestinal tract, lung, and vaginal cavity, to name a few) are where microbes interact directly with the host at the highest density and diversity. As they are actively involved in absorbing nutrients and oxygen, mucosal surfaces present fewer physical barriers. Therefore, more sophisticated immune mechanisms are required to keep the niche in balance. Unconventional T cells are capable of rapid cytokine release and robust effector function upon encountering cognate Ags, even in the absence of the peripheral immune synapse (18-20). These unique functions can fill the gap between innate and adaptive arms, where the physical barrier can easily be compromised by large numbers of external pathogens. Unconventional T cells are also important for the host defense early in life (when adaptive immunity has not fully developed), as they develop in specific tissue during development, preceding conventional effector T cells (10, 21, 22).

UNCONVENTIONAL T CELLS WITH KNOWN CLASS OF NONPEPTIDE LIGANDS

Unconventional T cells encompass multiple subsets of TCR-expressing lymphocytes, restricted by oligomorphic MHC class I (or MHC class I–like) molecules, such as CD1, MR1, Qa-1/2, or H2M3 (23). Among those, we primarily discuss NKT cells and MAIT cells in this review, focusing on their capability to recognize nonpeptide ligands (24-26).

Among MHC class Ib molecules, CD1 and MR1 evolved earlier (“old”) (27) and differ from MHC class II and MHC class Ia/“young” Ib molecules in two ways. First, CD1 and MR1 are essentially monomorphic and more selective in ligand binding, recognizing hydrophobic glycolipids (CD1d) or vitamin B metabolites (MR1) (28, 29). Second, TCRs that recognize these CD1/MR1 complexes are much less variant (often described as “semi-invariant”) (26, 30, 31). Both NKT cells and MAIT cells are preprogrammed with an effector phenotype during thymic differentiation, by recognition of self- or microbial-driven

Ags and subsequent expression of the transcription factor promyelocytic leukemia zinc finger protein (PLZF) (32-34).

NKT cells

Definition.—NKT cells are classified into two major subpopulations: type I NKT and type II NKT cells. Type I NKT cells express invariant αβTCRs comprising an invariant α-chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans) coupled to a limited set of β-chains (Vβ8, Vβ7, and Vβ2 in mice, Vβ11 in humans) (35, 36). In mice, type I NKT cells account for 1–3% of total T cells in most tissues and 30–40% of total T cells in the liver. In contrast, although human type I NKT cells account for only <0.5% of T cells in circulating blood and liver, they are abundant in omentum (37). Type II NKT cells express a more diverse TCR repertoire than do type I NKT cells, recognizing structurally related but different ligands such as sulfatide (38), which is a β-linked self-glycolipid and mainly presents in neuronal tissue. In this review, we focus on type I NKT cells.

Development.—In the thymus, NKT cells are positively selected by cortical double-positive thymocytes presenting endogenous glycolipid Ags loaded onto CD1d. PLZF is a critical transcription factor for NKT cell development and differentiation into effector subsets of NKT cells (32, 39). There are three functional subsets of NKT cells in the thymus. Analogous to conventional CD4 cell subsets, they express distinct transcription factors and effector cytokines and are designated NKT1, NKT2, and NKT17. NKT1 cells are $PLZF^{lo}T\text{-}bet^{+}$ and produce IFN-γ in response to stimulation. NKT2 cells are $PLZF^{hi}GATA\text{-}3^{+}$ and produce IL-4 both in the steady state and after stimulation. NKT17 cells are $PLZF^{int}ROR\gamma t^{+}$ and produce IL-17 after stimulation (40, 41). In addition to three NKT subsets developed in the thymus, other subpopulations of NKT cells have been reported. IL-10–producing regulatory NKT10 cells are greatly enriched in adipose tissue and they also can be induced by repeated and strong stimulation with α-galactosylceramide (α-GalCer). NKT10 cells express very low levels of PLZF, and do not express Foxp3, which is a master transcription factor of regulatory T cells. However, they express high levels of E4BP4, which induces IL-10 transcription (42). Follicular helper NKT cells (NKTfhs) are another subset of NKT cells, which are generated after immunization with α-GalCer–conjugated Ags. NKTfhs express Bcl6, PD-1, CXCR5, and ICOS, which are classical markers of follicular helper T cells. NKTfhs can be localized in germinal centers and provide help to B cells to promote Ab affinity maturation (43, 44).

CD1d ligands: initial discoveries.—NKT invariant TCRs can bind to a variety of lipid Ags complexed with CD1d, including ceramide-based glycolipids such as glycosphingolipids, microbial lipids, and endogenous self-lipids (45) (Table II). The prototypical ceramide-based glycolipid NKT cell Ag is an α-GalCer, and among α-GalCer classes, KRN7000 is the most studied synthetic α-GalCer. The KRN7000 structure was originally derived from the marine sponge *Agelas mauritianus* (46) and is known for its antitumor effects in mice (47, 48). CD1d-bound KRN7000 can be recognized by the NKT TCR, which leads to stimulation of type I NKT cells and massive production of various inflammatory cytokines such as IFN-γ, TNF-α, IL-4, IL-5, and IL-13. As KRN7000 nonspecifically induces a wide range of immune responses, there has been efforts to

develop synthetic analogs of KRN7000 inducing more specific types of immune responses. Furthermore, it has been shown that α-GalCer derivatives with truncated sphingosine chains preferentially induce IL-4 production, which indicates distinct cytokine responses by structural variants of NKT cell ligands (49, 50). One of the representative structural analogs of KRN7000 is OCH with C8 sphingosine, which mainly induces production of Th2 type cytokines such as IL-4 rather than IFN-γ. Along with chemical modification of naturally derived structures, chemical screening–based approaches also identified a nonlipid ligand (51), which is loaded to CD1d and activates a non-NKT unconventional T cell population.

NKT agonists of potentially pathogenic origins.—Microbial glycolipids derived from bacteria and viruses can be recognized by NKT cells and stimulate NKT cells to produce effector molecules such as cytokines and chemokines for host protection against exposure to microorganisms. A variety of α-linked ceramide-based glycolipids are produced exclusively by microorganisms and antigenic glycolipid Ags: α-glucuronosylceramides and α-galacturonosylceramides from *Sphingomonas* spp. (52, 53), α-galactosyldiacylglycerols from *Borrelia burgdorferi* (54), and α-glucosyldiacylglycerols from *Streptococcus pneumoniae* (55). There have been many reports of a more susceptible phenotype of mice lacking NKT cells against pathogenic injection, which suggests critical roles of appropriate NKT cell activation for host protection. NKT cells participate in immune responses clearing pathogens by producing inflammatory cytokines including IFN-γ, TNF-α, and IL-17 and interacting with other types of immune cells such as macrophages, neutrophils, and cytotoxic T cells.

Contribution to host immunity.—NKT cells are present in most nonlymphoid and lymphoid tissues, including liver, lungs, intestines, adipose tissue, spleen, lymph nodes, and bone marrow. NKT cells can be stimulated directly (by CD1d ligands) or indirectly (mediated by cytokines, such as IL-12 and IL-18) and hence can be involved in diverse bacterial and viral challenges. Dysregulation of NKT cells is associated with various human immune diseases such as infectious diseases, inflammatory bowel disease, cancer, metabolic disorders, asthma, and liver disease (56-62). Nonetheless, the impact of NKT cells to individual diseases is context-dependent: contrary to its expected immunostimulatory or proinflammatory functions, the presence of NKT cells can also protect the host from inflammatory responses in some diseases (63-65).

MAIT cells

Definition.—MAIT cells are abundant in humans, accounting for ~40% of liver T cells and ~10% of intestinal T cells, although they make up <1% of T cells in mouse tissues (66-68). MR1 is highly conserved between humans and mice, sharing >90% sequence homology at the protein level (69). For the recognition of MR1-presented Ags, MAIT cells express a semi-invariant TCR that consists of Vα19–Jα33 coupled with the Vβ8 or Vβ6 chain in mice and Vα7.2–Jα33 paired with the Vβ2 or Vβ13 chain in humans.

Development.—MAIT cells develop in the thymus after birth. After MAIT cells leave the thymus, they can gradually mature and expand in the periphery. MAIT cells are present in several peripheral tissues, including intestines, lung, liver, adipose tissue, and spleen (70).

Similar to other unconventional T cells, MAIT cells have effector and memory phenotypes that respond rapidly to Ag exposure. Activated TCRs by cognate Ags induce MAIT cells to produce effector molecules, including inflammatory cytokines (IFN-α, TNF-α, IL-17, and IL-22) and cytotoxic molecules (granzyme B and perforin), and upregulate chemokine receptors (CCR5, CCR6, and CXCR6), which finally allow migration of MAIT cells to the target tissues. MAIT cells can protect the host against pathogenic infection and may be involved in the control of noninfectious diseases such as autoimmune, allergic, and inflammatory disorders. Similar to NKT cells, MAIT cells can be also positively selected by Ags loaded onto MR1 of double-positive thymocytes and require PLZF expression for their development (34, 71). There are two distinct populations of MAIT cells in mice. $PLZF^{int}ROR\gamma t^{+}$ MAIT cells are the most abundant and produce IL-17, which resembles NKT17 and can be pathogenic in some diseases such as arthritis, inflammatory bowel disease, and multiple sclerosis. In contrast, $PLZF^{lo}T\text{-}bet^{+}$ MAIT cells are less abundant and can produce IFN-γ, which also resembles the phenotypes of NKT1 cells and conventional Th1 cells (72).

MR1 ligands.—The Ag-binding pocket of MR1 is relatively smaller than that of conventional MHCs or CD1s. Nevertheless, the MR1 binding pocket can accommodate a variety of molecule sizes, from 280 up to 1200 Da (73). The most representative MAIT cell ligands are metabolites derived from riboflavin (vitamin B_2) and folic acid (vitamin B_9) (Table III). 5-OP-RU (5-(2-oxopropylideneamino)-6-D-ribitylaminouracil) and 5-OERU (5-(2-oxoethylideneamino)-6-D-ribitylaminouracil) are derived from vitamin B_2 and are stimulatory ligands for MAIT cells (74). Bacteria and fungi have a de novo biosynthesis pathway for vitamin B_2, although mammals must acquire vitamin B_2 from the diet. Microorganisms synthesizing vitamin B_2 can stimulate MAIT cells. In contrast, 6-FP (6-formylpterin) derived from vitamin B_9 does not activate NKT cells, although it can be loaded onto the Ag-binding pocket of MR1 (29). The development of 5-OP-RU–loaded MR1 tetramers enabled the specific detection of MAIT cells and definition of MAIT cell developmental stages. However, studies of MAIT cell ligands other than vitamin B metabolites have been limited. In addition to microorganism-derived MAIT cell ligands, chemical library screening has enabled researchers to determine that small compounds such as drugs, drug metabolites, and drug-like molecules can be loaded onto MR1 and presented to MAIT cells (75, 76). Some of those compounds upregulate MR1 surface expression and compete with 5-OP-RU for MR1 binding. Although most identified ligands were not agonistic for MAIT cells, several drugs or drug metabolites, including diclofenac, 5-OH-diclofenac, and 5-formyl-salicylic acid, activated MAIT TCRs. Collectively, these findings show the versatility of the MR1 binding pocket.

Contribution to host immunity.—MAIT cells are widely distributed in multiple mammalian organs primarily at skin and mucosal tissue (77, 78). MAIT cells are reported to exert immunostimulatory actions against bacterial pathogens such as *Mycobacterium tuberculosis* (79) and *Escherichia coli* (80), in response to their MR1 ligand–producing property (81). MAIT cells also respond to multiple types of viral infection (82), including SARS-CoV-2 (83), via the TCR-independent IL-18 signaling pathway (84), similar to ligand-independent activation of NKT cells via IL-12 signaling. MAIT cells, both resident

and those recruited from circulation, contribute to the recovery after lung infections (79) as well as wound healing (85). MAIT cells are involved in not only host protection against acute infection but also immune-mediated chronic diseases such as inflammatory bowel diseases (86-88), asthma (89, 90), rheumatoid arthritis (90, 91), and obesity/type 2 diabetes (92, 93), playing either a protective or deleterious role in the context of each disease.

ROLE OF SYMBIONT-DERIVED LIGANDS IN NKT AND MAIT CELL DEVELOPMENT AND EFFECTOR FUNCTIONS

Host mucosal tissues constantly interact with symbiotic microbes, which critically regulate local and systemic immune development. Germ-free (GF) animals, which have not been exposed to live bacteria or fungi from birth, are physiologically and pathologically significantly different from animals naturally colonized with microbes (conventional, or specific pathogen-free (SPF) animals, in the laboratory setting). In most cases, the gut immune system of GF mice is considered immature in innate as well as adaptive components of the immune system: 1) smaller and underdeveloped GALT such as Peyer's patches; 2) fewer conventional T cells and plasma cells in the lamina propria; as well as 3) blunted secretion of antimicrobial peptides, complements, and IgA to the gut lumen (94). Systematic underdevelopment results in insufficient immune responses against invasive pathogens; hence, GF hosts are more prone to infection.

Conversely, education of the host immune system by symbionts is critical for homeostatic control of immunity and responses to inflammatory stimuli. For example, it is well known that TLRs are necessary to maintain epithelial homeostasis and regulation of inflammatory responses (95). Considering the vast chemical diversity of symbiont-derived metabolites, it is clear that the contribution of microbiota to immune development is specific not only to host cell type but also to ligand structure. Such effects have a particularly strong prominent impact on mucosal tissue–resident immune cells, which are exposed to hundreds if not thousands of bacterial species during the life-time of the host.

Regulation of early development by symbiotic microbiota and microbiota-derived ligands

Both NKT cells and MAIT cells develop at the gut lamina propria shortly after birth. Nonetheless, they show distinctive developmental differences in response to microbial colonization. Colonic NKT cells start proliferation shortly after birth, and CD1d is necessary for (both thymic and peripheral) development (96, 97). Contradicting the accepted dogma that symbiotic bacteria induce immune activation, NKT cells are more abundant in GF mice than in SPF mice. In fact, in accordance with the elevated number in the colon, GF mice are more prone to NKT cell–mediated (oxazolone) colitis. CD1d Ab treatment of GF mice can suppress colonic NKT cells, confirming that CD1d-mediated signaling is critical for NKT cell development (98). These findings imply that in the absence of gut microbes, murine colonic NKT cells are constitutively active in early life, potentially involving endogenous CD1d ligands functioning as NKT cell agonists. In this regard, several CD1d-associated lipids (phospholipids and glycosphingolipids) of endogenous origin have been identified (38, 99-102) (Table IV), although the contribution of each species to in vivo NKT cell development and regulation remains obscure.

When mice are conventionally colonized, colonic NKT cells are less proliferative, which results as a lower number and frequency in the adult stage. Cohousing of GF mice with SPF mice (conventionalization) at birth can normalize the NKT cell number. Of note, introduction of gut microbiota can regulate colonic NKT cells, but only in early life. Conventionalization at 14 d after birth, when tissue NKT cell numbers are already significantly higher, cannot normalize colonic NKT cells in adult animals. This is likely to reflect the distinct phenotype of tissue-resident NKT cells, as conventionalized GF mice at the adult stage can normalize circulating NKT cell phenotypes, at least to some degree (103).

These results strongly suggest the presence of symbiont-derived factors (possibly CD1d ligands) that regulate NKT proliferation in the mucosal tissue. Although specific ligands originating from conventional mouse microbiota remain to be confirmed, the regulatory actions of microbiota can be recapitulated by monoassociation with *Bacteroides fragilis* (104), as well as oral administration of purified α-galactosylceramides from *B. fragilis*. Similar to conventionalization, such interventions work only when the bacteria or purified lipid is given immediately after birth, confirming that CD1d-mediated regulation of NKT cell takes place in an age-specific manner.

Host immune regulation by gut microbiota-derived unconventional T cell ligands

The gut microbiota and microbiota-derived molecules significantly influence many aspects of host physiology. The lipid Ags produced by microbiota may function as NKT cell ligands (22). It has been demonstrated that colonization of GF mice with human gut bacteria *B. fragilis* was sufficient to normalize elevated levels of colonic NKT cells to the level of SPF mice (104). Interestingly, it has been determined that *B. fragilis* produces unique α-GalCers, which are loaded onto the CD1d molecule and recognized by NKT TCRs. Elevated colonic NKT cell levels of GF mice were normalized by administration of unique α-GalCers originated from *B. fragilis* (BfaGCs) during early developmental stages, and the susceptibility of GF mice to experimental colitis was also ameliorated by BfaGC treatment (105). Using chemically synthesized structural variants of BfaGCs, we show that branching on the sphinganine chain is critical to stimulate NKT cells, which also emphasizes the importance of the structure–activity relationship of NKT cell ligands.

Unlike NKT cells, commensal microbial Ags act as positive regulators of MAIT cell development. GF mice have very few MAIT cells in their colonic lamina propria, both during development as well as adults. Conventionalization of adult GF mice can increase thymic and peripheral MAIT cells in as little as 2 wk (106). Furthermore, it was also shown that colonization of GF mice with vitamin B_2–synthesizing gut commensal bacteria is required for the development of MAIT cells homing to the skin tissue (11). These results show the plasticity of MAIT cells in response to gut bacterial ligands, at local as well as at systemic levels. In addition, whether removal or dysbiosis of gut microbiota can change the maintenance or immune phenotypes of MAIT cells is an area of active investigation (11, 26, 34,106).

Cellular and molecular determinants of unconventional T cell–mediated immune responses

Since the first structural and functional characterization of agelasphins (47), exploiting the immunostimulatory action of NKT cells using strongly agonistic CD1d ligands has been a major interest of the field. Harnessing the antitumor activity of NKT cells in cancer therapy (48) has been one of the major clinical focuses. Nonetheless, little success has been achieved due to inconsistent responses and anergy in NKT cells (107). Activation of NKT cells with strong agonists causes internalization of NKT TCRs as well as exhaustion; thus, they become nonresponsive to repeated exposure. In this regard, recent studies of agonistic CD1d ligands as a vaccine adjuvant have been more successful (108), probably because one-time activation of NKT cells at immunization is sufficient to achieve necessary immunity.

In parallel, regulation of host immunity and protection from inflammatory diseases by CD1d ligands is also well appreciated. Of note, immunoprotective functions of NKT cells activated by synthetic agonists have been reported in different biological contexts. For example, the strongly immunostimulatory CD1d ligand KRN7000 can protect the host from oxazolone colitis. This is potentially by activating Th1 and suppressing Th2-type responses, the primary inflammatory pathway of the disease. Similarly, Th2-skewed CD1d ligand OCH can provide protection from murine experimental autoimmune encephalomyelitis (49).

Ligand structure is not the only decisive factor for NKT cell-mediated immune responses. The type of APCs can also direct the effector functions of NKT cells. Unlike APCs of hematopoietic origin, which generally induce a strong Th1 signal to NKT cells with KRN7000, other CD1d-expressing cell types such as colonic epithelial cells (109) or adipocytes (110) induce IL-10 production upon Ag stimulation. Of note, depending on the tissue type, IL-10 can be directly produced by NKT cells (42) or by APCs (109), adding further complexity.

Finally, several structure–activity relationship studies have implied that NKT cell agonistic α-GalCers can have multiple dimensions of immune activation, in addition to the Th1/Th2 dichotomy. BfaGCs have chain length and shape (terminal branching pattern) distinct from those of KRN7000 or OCH (as shown in Table II). When synthetic BfaGCs are given in vivo and splenic NKT cells are collected (hematopoietic APCs and NKT cells are expected to be major interacting cell types), the transcriptomic profile of NKT cell is significantly different from prototypic Th1/Th2 ligands (105).

As shown in NKT cells, whether the tissue specificity of MR1-expressing APCs can direct the effector function of MAIT cells warrants further investigation. Regarding the structure–activity relationship of MR1 ligands and MAIT function, a novel class of synthetic ligands that can modulate MAIT cell functions were identified by in silico screening, and their MR1-MAIT–dependent actions were confirmed with synthetic molecules (111). These novel ligands, which are chemically distinct from previously known CD1d/MR1 ligands, nonetheless still function via unconventional TCRs, are also an area of interest.

Impact of environmental (dietary/xenobiotic)factors on the structure and function of gut symbiont-derived, unconventional T cell ligands

Dietary factors as well as xenobiotics are the most prominent source of metabolites for gut microbiota. Symbionts use both small and large molecules that the host cannot break down (“indigestible”) or does not completely absorb (“spillover”). These metabolites can be further processed to extract energy (such as fiber fermentation), producing secondary metabolites (short-chain fatty acids), or can be incorporated as a building block of key components for the microbes (endobiotic metabolites).

As an example of the latter case, we recently elucidated the incorporation of host dietary branched-chain amino acid to the branched-chain fatty acid and branched-chain sphinganine of BfaGCs (105). Of note, this branching in the lipid structure serves as a key functional moiety for NKT cell activity. Blocking incorporation of branched-chain amino acid by genetic manipulation of *B. fragilis* deprives its gut NKT cell modulating function by colonization.

In parallel, methylglyoxal (MGO) is a key chemical necessary for the conversion of MAIT cell ligand. Microbial 5-amino-6-(D-ribitylamino)uracil (5-A-RU) is conjugated with endogenous MGO to form 5-OP-RU. Exogenous MGO can induce production of 5-OP-RU in vitro, which enhances activation of MAIT cells (112). Whether supplementation of MGO (rich in some functional foods) can achieve similar impact in vivo awaits further investigation. These reports collectively propose direct contributions of dietary components to the structures and functions of unconventional T cell ligands, providing molecular-level evidence of host–microbiota–environment interplay (Fig. 1).

CURRENT GAP IN KNOWLEDGE AND FUTURE PROSPECTS (“ON THE HORIZON”)

Seminal works on ligand structures and actions of NKT cells and MAIT cells have provided critical knowledge on unconventional T cell functions and regulation. Nonetheless, several missing pieces remain to be elucidated.

Contribution of gut microbiota to the unconventional T cell ligand pool

Identification of endogenous ligands of CD1d (113) has raised two major questions. In addition to the biological aspect that NKT cells can be regulated not only by foreign but also by self-lipids, diverse structures of endogenous ligands indicate that CD1d can accommodate several classes of complex lipids of a polar headgroup with two long acyl chains. Similarly, MR1 can also respond to small molecules of several different chemical classes. The gut microbiota is constantly present in the gut lumen and produces lipid molecules of large structural variety (114). Therefore, contribution of symbiotic gut microbial molecules to the NKT/MAIT cell activity can be easily recognized. Large-scale, metagenomic analysis of gut microbiota dramatically expanded the capability to identify potential gene products commonly generated by symbionts. Still, a large portion of the symbiotic microbiota metagenome remains unannotated. As a complementary strategy, recent advances in discovery metabolomics platforms may offer opportunities to identify

novel molecules that originated from microbiota (115). These novel metabolites may function as ligands of CD1d/MR1, or even further, as ligands of previously uncharacterized unconventional T cell subsets.

Functional delineation of ligand structure–activity relationship

Unlike typical ligand–membrane receptor interactions (as shown in G protein–coupled receptors or nuclear receptors), epitope/T cell recognition requires preceding ligand-presentation molecule complex formation, and the tertiary complex is the key structure for T cell activation. In this juncture, there are several points that can determine the activity of unconventional T cell ligands: 1) intracellular/extracellular loading or replacement mechanisms of the ligand to the presentation molecules (CD1d/MR1), 2) affinity (association/dissociation kinetics) of presentation molecule–ligand complexes, 3) recognition of the ligand-presentation molecule complex by specific TCRs, and 4) ligand-specific downstream signaling. Although each of these questions has been (at least partially) addressed, the structure–activity relationship of novel ligands remains to be determined. In addition, considering the nature of NKT/MAIT cells as early amplifiers in the scene, the in vivo milieu where NKT/MAIT cells interact neighboring immune cells is also a key determinant of the outcome.

Therapeutic application and human relevance

As discussed, recent investigations of the functional diversity of small-molecule ligands suggest a novel route to harness unconventional T cell activity, by customizing biogenic lead molecules to specific therapeutic targets with desired functions. Although the effector function of unconventional T cells is very context-dependent and most ligands identified thus far originated from gut bacteria, it does not limit the potential that novel ligands can target unconventional T cell populations as well as immune diseases out of the gastrointestinal tract. Deciphering the crosstalk between tissue-specific APCs and T cells is a complex and fascinating field of study. Furthermore, wide ligand-binding capability of CD1d and MR1 opens possibility for mining novel ligands and elucidating previously uncharacterized unconventional T cells restricted by those molecules.

One caveat of interpreting animal model results is that NKT/MAIT cell profiles in tissues and organs can be significantly different between humans and mice, and even among different mouse strains. Experimental findings from one murine model may not always be applicable to human diseases. One possible way to address this is to use ‘humanized’ system, as shown in the case of transgenic mice with humanized type I NKT cells (116, 117).

CONCLUSIONS

Further studies of the ligands on molecular structures and abundance (metabolomics), symbiont-originated biosynthesis (microbiology), as well as host actions (immunology) will contribute to dissect the molecular mechanisms of unconventional T cell ligands. Well-designed interdisciplinary investigation will open an exciting possibility to explore basic biology as well as therapeutic potential.

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Abbreviations used in this article:

BfaGC	α-GalCers originated from *Bacteroides fragilis*
α-GalCer	α-galactosylceramide
GF	germ-free
MAIT	mucosal-associated invariant T
MGO	methylglyoxal
NKTfh	follicular helper NKT cell
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
PLZF	promyelocytic leukemia zinc finger protein
PRR	pattern-recognition receptor

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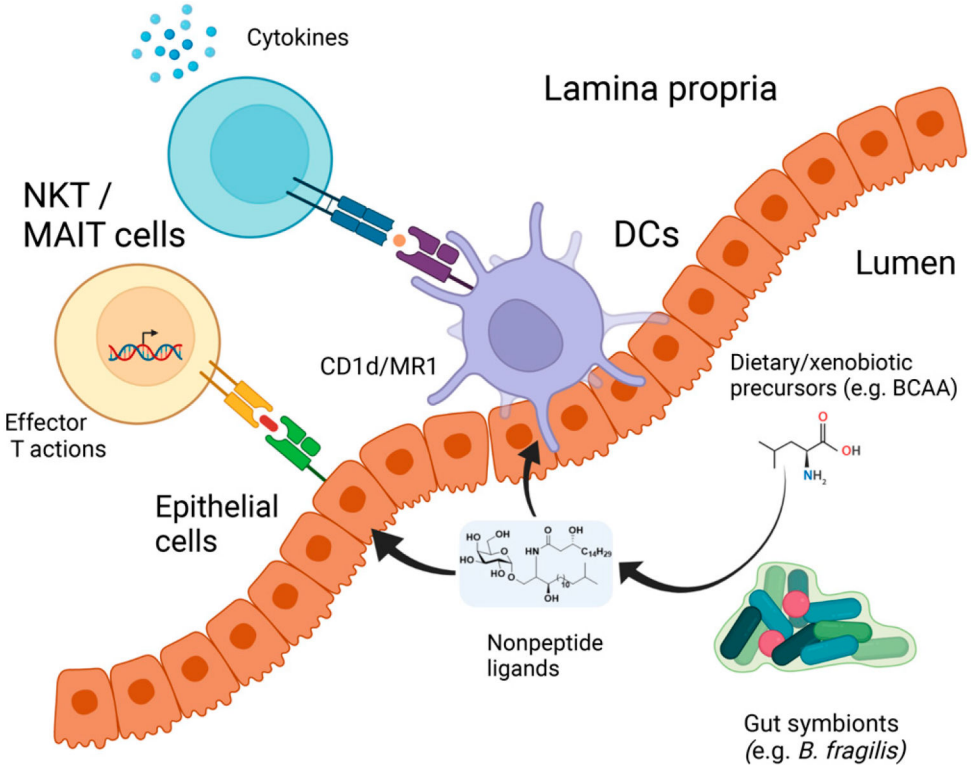


FIGURE 1.

Host–microbiota–environment crosstalk of unconventional T cell ligand production and recognition.

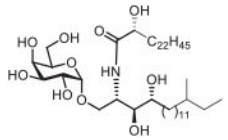
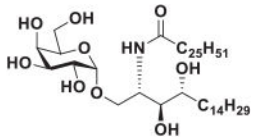
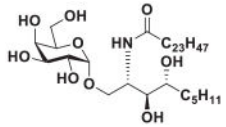
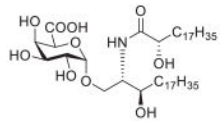
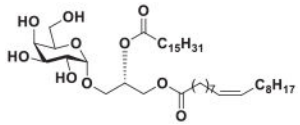
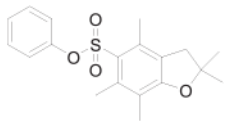
TABLE I.

Comparison of inclusive- versus exclusive-type recognition of foreign epitopes

Mechanisms	Inclusive	Exclusive
Concept of epitope recognition	Recognize specific foreign structures	Catch all, but exclude self
Ligand characteristics	Specific chemical class	Variable (peptide)
Presentation molecule variability	Monomorphic or oligomorphic	Polymorphic
Effector cell receptor variability	Limited	Variable
How to ensure specificity	Affinity of ligand–receptor interaction	Removal of autoreactive effector subset
Prototypic cell types	Innate immune cells (macrophages, dendritic cells) Unconventional T cells	Conventional T cells

TABLE II.

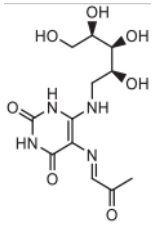
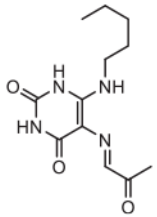
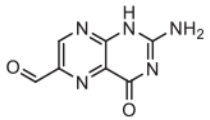
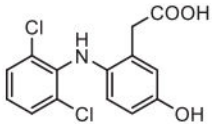
Structures and characteristics of representative microbial and synthetic CD1d ligands

Name	Chemical Structure	Category	Class	Activity	Reference
Agelasphin-11		Algal (*Agelas mauritanius*)	α-GalCer	Stimulatory	(47)
KRN7000		Agelasphine analog	α-GalCer	Stimulatory (Th1/Th2)	(48)
OCH		Agelasphine analog	α-GalCer	Stimulatory (Th2)	(50)
GSL-1		Microbial (*Sphingomonas* spp.)	α-GalACer	Weak agonist	(52)
BbGL-2		Microbial (*Borrelia burdoferei*)	α-GalDAG	Weak agonist	(54)
PPBF		Synthetic (chemical library)	Nonlipidic	Agonist of non-NKT, CD1d-restricted T cells	(51)

α-GalDAG, α-galactosyldiacylglycerol; PPBF, phenyl-pentamethyldihydrobenzofuran sulfonate.

TABLE III.

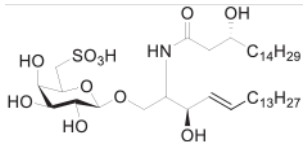
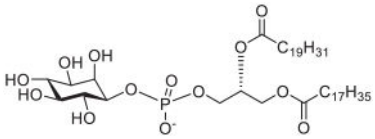
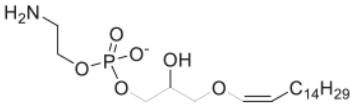
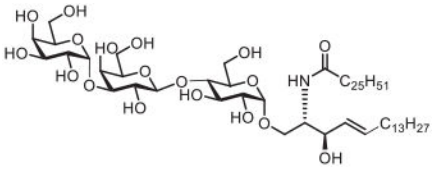
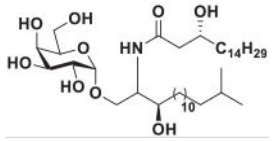
Structures and characteristics of representative MR1 ligands

Name	Chemical Structure	Origin	Class	Activity	Reference
5-OP-RU		Microbial	Vitamin B_2 metabolite	Stimulatory	(74)
6-Alkylamino-5-OP-RU		Microbial analog	Vitamin B_2 metabolite	Antagonistic	(76)
6-FP		Microbial	Vitamin B_9 metabolite	Antagonistic	(29
5-OH-diclofenac		Chemical library-derived	NSAID metabolite	Stimulatory	(75)

6-FP, 6-formylpterin; NSAID, nonsteroidal anti-inflammatory drug.

TABLE IV.

Endogenous (host-originated) and endobiotic (symbiont-originated) CD1d ligands

Name	Representative Chemical Structure	Category	Class	Activity	Reference
3′-Sulfo-galactosyl ceramide (“sulfatide”)		Endogenous ligand	β-Anomeric sulfatide	Type II NKT agonist	(38)
PI		Endogenous ligand	Glycerophospholipid	Agonist for specific NKT cell subset	(99)
p-LysoPE		Endogenous ligand	Lysophospholipid (ether-linked)	Agonist for thymic/peripheral NKT cells	(100)
iGb3		Endogenous ligand	Isogloboside	Weak agonist	(101)
BfaGC (shown as SB2217)		Endobiotic ligand (symbiotic *B. fragilis)*	α-GalCer	Immunomodulatory	(102)

iGb3, isoglobotrihexosylceramide; PI, phosphatidylinositol.