Nonclassical T Cells and Their Antigens in Tuberculosis

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T cells that recognize nonpeptidic antigens, and thereby are identified as nonclassical, represent important yet poorly characterized effectors of the immune response. They are present in large numbers in circulating blood and tissues and are as abundant as T cells recognizing peptide antigens. Nonclassical T cells exert multiple functions including immunoregulation, tumor control, and protection against infections. They recognize complexes of nonpeptidic antigens such as lipid and glycolipid molecules, vitamin B₂ precursors, and phosphorylated metabolites of the mevalonate pathway. Each of these antigens is presented by antigen-presenting molecules other than major histocompatibility complex (MHC), including CD1, MHC class I-related molecule 1 (MR1), and butyrophilin 3A1 (BTN3A1) molecules. Here, we discuss how nonclassical T cells participate in the recognition of mycobacterial antigens and in the mycobacterial-specific immune response.

The recognition by T lymphocytes of antigenic complexes formed by antigens and antigen-presenting molecules is a hallmark of the adaptive immune system. A wide range of exogenous and endogenous antigenic molecules reflecting a variety of chemical structures have been identified and are represented by proteins, lipids, phosphorylated metabolites, or vitamin B₂ pathway metabolites. The role of classical T cells, which recognize peptidic antigens associated with MHC-encoded molecules, during *Mycobacterium tuberculosis* (MTB) infection is well documented and studied for a long time (Cooper 2009). On the contrary, nonclassical T cells recognizing nonpeptidic antigens associ-

ated with non-MHC antigen-presenting molecules have been described more recently. They have also been identified as important contributors to host defense against infections, although many aspects of their physiological role still remain to be discovered. Based on the restriction molecule, antigen specificity, and T-cell receptor (TCR) structure, non-MHC-restricted T cells can be grouped into three categories: lipid- and glycolipid-specific CD1-restricted T cells, mucosal-associated invariant T (MAIT) MR1-restricted cells, and TCR $\gamma\delta$ BTN3A1-restricted T cells. In this article, we review critical issues of the basic immunobiology of non-MHC-restricted T cells related to recognition of

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mycobacterial antigens and discuss their roles during MTB infection.

LIPID-SPECIFIC CD1-RESTRICTED T CELLS

Lipid-specific T cells recognize lipid antigens as complexes formed with the CD1 antigen-presenting molecules. The human CD1a, CD1b, CD1c, and CD1d proteins bind and present lipid antigens (De Libero and Mori 2006), whereas CD1e behaves as a lipid chaperone, which participates in lipid antigen presentation without interacting with the TCR (Garcia-Alles et al. 2011). The CD1–lipid antigen complexes are mostly formed within antigen-presenting cells (APCs) in compartments where CD1 molecules recycle. Each CD1 molecule has unique modes of intracellular trafficking and thus can intersect with lipids, which also have different trafficking modes within APCs. Several studies have shown how CD1 structure, trafficking, and loading influence lipid antigen presentation, thereby directly contributing to lipid immunogenicity during immune responses (De Libero and Mori 2012). CD1-presented antigens are represented by amphipathic molecules characterized by a hydrophobic moiety, instrumental for binding within the CD1 pockets, and by a polar part, which in most of the cases directly interacts with the TCR.

Structure, Expression, and Intracellular Trafficking of CD1 Molecules

During mycobacterial infection the activation of lipid-specific T cells occurs upon the generation of stable CD1-lipid antigen complexes, which are formed within APCs. The immunogenicity of mycobacterial lipids is influenced by the type of cell that internalizes the lipid antigen and by the intersection of the lipid antigen with individual CD1 isoforms that recycle in different endosomal compartments.

All CD1 molecules show general structural features similar to MHC class I molecules, including two α helices in the distal $\alpha 1$ and $\alpha 2$ domains and a $\alpha 3$ domain that noncovalently associates with β_2 microglobulin. The antigen-binding groove of CD1 molecules allows inser-

tion of the hydrophobic moieties of lipid antigens, whereas the antigenic polar moieties usually remain outside the groove and, along with CD1 key amino acids, interact with the TCRs (Moody et al. 2005; Rossjohn et al. 2012). Among different CD1 molecules, lipid-binding pockets vary in number, shape, and total volume. Together, these structural properties allow selective binding of lipid antigens differing in the number and length of alkyl chains. An important difference between CD1 and MHC molecules is a very limited sequence diversity and thus almost absence of functional polymorphism (Han et al. 1999; Porcelli and Modlin 1999). Two examples of *CD1* gene polymorphisms were described that influence the response to lipid antigens. The first example concerns CD1d in congenic mice in which CD1d alleles influence development and presentation of endogenous and exogenous ligands to CD1d-restricted T cells (Zimmer et al. 2009). The second example applies to CD1e in humans. *CD1e* gene is the most polymorphic gene of the CD1 family, with six allelic variants reported so far. CD1e is necessary for processing of mycobacterial hexamannosylated phosphatidylinositol mannosides (PIM₆) (de La Salle et al. 2005) and controls the generation and persistence of complexes formed by different lipid antigens and all other CD1 molecules (Facciotti et al. 2011). The CD1e allele 4 is unable to participate in the presentation of PIM₆ to CD1b-restricted specific T cells (Tourne et al. 2008), suggesting that homozygous individuals might display altered immune responses to microbial glycolipid antigens.

A different mechanism controlling the presentation of lipid antigens in the population is represented by regulation of *CD1* gene expression. Indeed, a unique SNP in the 5' untranslated region of the human *CD1a* gene is associated with altered expression of CD1a and antigen presentation (Seshadri et al. 2013).

The immunological relevance of lipid-specific recognition during mycobacterial infections is also determined by the constitutive or inducible expression of CD1a, b, and c isoforms on unique cell types, indicating distinct roles during immune responses in tissues (Porcelli

1995; Dougan et al. 2007). CD1d expression is distributed on almost all types of hematopoietic cells (Brossay et al. 1997; Roark et al. 1998) and also on some cells of nonhematopoietic origin, including epithelial cells and adipocytes. Whether CD1d-expressing epithelial cells present mycobacterial antigens at the foci of infection remains to be investigated.

The third important feature of CD1 isoforms is their intracellular trafficking route that determines the encounter with different mycobacterial lipids. CD1 molecules use different trafficking pathways, mainly governed by motifs present in their cytoplasmic tails, which interact with various adaptor protein complexes. Although CD1b, CD1c, and CD1d molecules bind to the adaptor protein 2 and recycle through late endosomes where lipid antigens are loaded (Barral and Brenner 2007), CD1a localizes in membrane rafts and recycles through early endosomes (Barral et al. 2008; Sloma et al. 2008). Mouse CD1d and human CD1b also interact with the adaptor protein 3, responsible for lysosomal trafficking. Lysosomal route is instrumental for binding complex mycobacterial lipids, which require processing and low pH environment for CD1b loading.

Mycobacterial Lipid Antigens Presented by CD1 Molecules

Because of their abundance in mycobacterial cell envelope, several mycobacterial lipids are antigenic (Fig. 1; Table 1) (De Libero et al. 2009). Among all CD1 molecules, CD1b binds lipids of different size ranging from C12 to C80 (Beckman et al. 1994; Batuwangala et al. 2004; Guiard et al. 2009; Layre et al. 2009; Garcia-Alles et al. 2011). CD1b-restricted MTB-derived mycolic acid (MA) having α -branched β -hydroxy long chain fatty acid was the first lipid antigen found to stimulate CD1-specific T cells (Beckman et al. 1994). Glucose monomycolate (GMM), an MA with a single glucose molecule, is another CD1b-restricted immunogenic lipid, which is synthesized by pathogenic MTB upon use of glucose derived from its host (Moody et al. 2000a). Recently, a conserved T-cell population expressing TCR α chain with limited junctional

variability and with biased selection of TCR β chains was detected in the blood of some MTB-infected individuals. This type of conserved TCR has high affinity to mycolyl lipid-CD1b complexes and might be an example of “public” TCR expanding in response to mycobacterial antigen (Van Rhijn et al. 2013).

Glycerol monomycolate (GroMM), a MA with a glycerol, stimulates CD1-restricted lipid-specific T lymphocytes from TB patients (Layre et al. 2009). Chemical characterization of this antigen showed that it exists as two stereoisomers, one synthetic isomer being more stimulatory than the other. The hydroxyl groups of glycerol and mycolic acid length are critical for triggering T-cell responses. GroMM is presented by MTB-infected dendritic cells (DCs), showing that it is available for presentation during natural infection. Evidence from ex vivo experiments that GroMM stimulates T cells from vaccinated or latently infected donors but not cells from patients with active TB suggests that GroMM-specific T cells are primed during infection and their detection correlates with lack of clinical active disease (Layre et al. 2009). Other CD1b-restricted immunogenic mycobacterial lipids are glycosylphosphatidylinositols. They include phosphatidylinositol mannosides (PIMs) with varying numbers of mannose residues (Sieling et al. 1995; de La Salle et al. 2005), and further extensions of glycosylations like lipomannan (LM) (Ernst et al. 1998) and lipoarabinomannan (LAM) (Sieling et al. 1995). Of particular interest, diacylsulfoglycolipid (Ac₂SGL), found exclusively in the cell envelope of virulent MTB, is a potent antigen that stimulates CD1b- (Gilleron et al. 2004; Layre et al. 2009) and CD1c-restricted immune responses in TB (Lepore et al. 2014). The length of the linear acyl chain at position 2, the stereochemistry of the methyl-branched fatty acid chain at position 3, and the number of C-methyl-branched acyl groups influence antigenicity. A direct correlation was observed between the T-cell stimulatory capacities of the analogs and the number of methyl-branched groups, suggesting that the most abundant sulfoglycolipid form naturally produced by MTB is the most stimulatory one

Figure 1. Structure of mycobacterial lipid antigens. The structures of representative mycobacterial lipid antigens are illustrated. Diacylsulfoglycolipid is illustrated in the natural form found in MTB. DPG, diphosphatidylglycerol; GMM, glucose monomycolate; GroMM, glycerol monomycolate; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM₂, phosphatidylglucosylmonomycolate.

Table 1. Mycobacterial lipid antigens with CD1 restriction

Antigen	CD1	Reference(s)
Dideoxymycobactin	CD1a	Moody et al. 2004; Kasmar et al. 2013
Diacylsulfoglycolipid	CD1b	Gilleron et al. 2004
Glucose monomycolate	CD1b	Sieling et al. 1995
Glycerol monomycolate	CD1b	Layre et al. 2009
Mycolic acid	CD1b	Beckman et al. 1994
LAM	CD1b	Sieling et al. 1995
LM	CD1b	Sieling et al. 1995
PIMs	CD1b	Sieling et al. 1995
Mannosyl- β -1-phosphomycoketide	CD1c	Moody et al. 2000a; Matsunaga et al. 2004; Ly et al. 2013
PIM	CD1d	Fischer et al. 2004
Phosphoglycerolipids: PG, DPG, PI	CD1d	Tatituri et al. 2013

LAM, lipoarabinomannan; LM, lipomannan; PIMs, phosphatidylinositol mannosides; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PI, phosphatidylinositol.

for T cells (Gau et al. 2013; Geerdink et al. 2013).

A unique mode of binding lipid antigens is that of CD1a. This CD1 isoform shows a quite large and open F' pocket and a deep A' pocket, which bind and present mycobacterial dideoxymycobactin, a single chain lipopeptide precursor in the biosynthesis of mycobactin (Moody et al. 2004; Zajonc et al. 2005; Madigan et al. 2012). Mycobactin being essential in mycobacterial iron acquisition and survival of MTB in vivo (De Voss et al. 2000; Siegrist et al. 2009), the existence of T lymphocytes targeting cells presenting this type of antigen might represent an important protective mechanism. Another mycobacterial lipid antigen, mannosyl- β -1-phosphomycoketide (MPM) is stimulatory in association with CD1c and its tail is composed of a single saturated alkane with methyl branches on every fourth carbon (Moody et al. 2000b; Matsunaga et al. 2004). CD1c-restricted T cells recognizing synthetic mycoketide expand in the blood of TB patients as compared with unin-

fected controls (Moody et al. 2000b). Furthermore, an organism-wide survey has identified C32-phosphomycoketide (PM) from MTB as another CD1c-restricted lipid antigen (Ly et al. 2013).

Mycobacterial antigens that stimulate CD1d-restricted T cells, which are not invariant natural killer (NK) T cells, have been identified (Tatituri et al. 2013). These lipid antigens comprised several phospholipids, such as phosphatidylglycerol, diphosphatidylglycerol (or cardiolipin), and phosphatidylinositol. The response to these antigens required antigen internalization and engagement of the TCR with the CD1d-lipid complex. Two important aspects of this type of antigen specificity remain to be clarified: (i) the role of these responses in patients with mycobacterial infections, because only mouse hybridoma cells were studied, and (ii) the potential reactivity with self-phospholipids that show identical structures to the bacterial ones.

Functions of CD1-Restricted T Cells during Mycobacterial Infection

The absence of group 1 CD1 genes in mice and rats and the limited availability of reliable tools to directly visualize ex vivo antigen-specific CD1-restricted T cells has severely hampered the progress in the study of lipid-specific T cells. However, in vivo and ex vivo data on the presentation of mycobacterial lipids in humans and other animal species have provided direct evidence of the function of CD1-specific T cells during mycobacterial infections. These studies showed that group 1 CD1-restricted T cells have effector functions typical of T helper 1 (Th1), T helper 2 (Th2), and T helper 17 (Th17) cells, and that these functions increase during infection with MTB, suggesting their role in protection (Cohen et al. 2009). Experiments conducted with guinea pigs, which have an extended family of CD1 molecules, showed development of CD1b- and CD1c-restricted proliferative and cytolytic T-cell responses on immunization either by *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) or with partially purified lipid extracts of MTB (Hiromatsu et al. 2002; Watanabe et al. 2006). MTB-lipid-immunized guinea

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pigs had smaller lung lesions on challenge with MTB than vehicle-only-immunized animals (Dascher et al. 2003). In the cattle, which have *cd1a* and *cd1b* genes, a CD1b-restricted T-cell response could be detected after immunization with GMM (Nguyen et al. 2009). Studies conducted in TB patients suggested that CD1-restricted T cells are not infrequent and represent an important fraction of circulating T cells. We have shown the presence of cytolytic Ac₂SGL-specific CD1b-restricted T cells in latent TB individuals and active TB patients, but not in healthy donors (Gilleron et al. 2004). Ex vivo analysis of peripheral T cells has shown a CD1b-restricted response against GroMM in latent TB and BCG-vaccinated individuals, but not in active TB patients or healthy donors (Layre et al. 2009). In contrast, GMM was recognized ex vivo by T cells from latent TB individuals only (Ulrichs et al. 2003). More recently, using GMM-loaded CD1b tetramers a small CD4⁺ CD1b-restricted population was detected in TB patients (Kasmar et al. 2011). MA-specific CD1b-restricted T cells have been detected in the periphery and lungs of TB patients (Montamat-Sicotte et al. 2011). These T cells produced IFN- γ and IL-2, showed effector and central memory phenotypes and were absent in BCG-vaccinated individuals. Upon anti-TB therapy, MA-specific T cells contracted, became undetectable at 6 months post-initiation of treatment and exhibited recall expansion on antigen reencounter in vitro long after successful treatment, indicating persistence of lipid-specific memory T cells. The role of CD1-restricted responses in in vivo immune response was further strengthened by demonstration of the expansion of phosphomycoketide-specific CD1c-restricted T cells (Moody et al. 2000b) and detection of DDM-specific CD1a-restricted T cells (Kasmar et al. 2013) in TB patients. The use of CD1 tetramers loaded with different lipid antigens has revealed that the number of circulating CD1-restricted T cells is in the same range of peptide-specific T cells, at least for tested CD1-antigen complexes. It remains to be investigated whether CD1-restricted T cells accumulate within tissues in which lipid-specific responses occur (i.e., skin, lung, gut, and liver),

which might represent niches of preferential accumulation.

CD1d-restricted iNKT cells acquire an effector/memory phenotype and rapidly secrete large amounts of Th1 and Th2 cytokines on primary stimulation (Bendelac et al. 2007). iNKT cells contain preformed IFN- γ mRNA in their cytoplasm, which promotes immediate secretion of this cytokine on TCR stimulation at levels 200 times higher than conventional MHC-restricted CD4⁺ T cells (Stetson et al. 2003). This secretion of cytokines is controlled by the strength of TCR signal received during antigen recognition (Wang et al. 2008), which depends on the number of CD1 complexes as well as their persistence on the surface of APCs. In addition, human NKT cells express granzysin (Gansert et al. 2003) that participates in killing intracellular bacteria resident within target cells. The effector capacities of iNKT cells contribute to the activation of other cells including DCs, NK cells, and CD4⁺ and CD8⁺ T cells (Hermans et al. 2003), and thus iNKT cells can be considered as regulatory cells of adaptive immune responses. This function is important during mycobacterial infection for the expansion of CD8⁺ T cells that participate in protective immunity (Behar et al. 1999). Further, iNKT cells express a variety of chemokine receptors that may drive their traffic to inflamed tissues (Thomas et al. 2003). Intriguingly, iNKT cells display poor expression of the lymphoid homing receptors, CCR7 and CXCR5, suggesting that their function is mostly exerted in peripheral tissues rather than in peripheral lymphoid organs. Studies performed in mice infected with live mycobacteria or injected with purified MTB lipids provided evidence for the expansion, recruitment, and activation of iNKT cells (Ryll et al. 2001; Dieli et al. 2003) and suggested the importance of iNKT cells in the formation of granulomas (Gilleron et al. 2001). The importance of iNKT cells in anti-mycobacterial protective immunity has been further shown in experimental models using knockout animals with deletions in *cd1d* or TCR *Ja18* genes (Chen et al. 1997; Sousa et al. 2000; Kawakami et al. 2002; Sugawara et al. 2002). The role of iNKT cells in controlling

MTB infection in humans is indirectly suggested by phenotypic observations showing variations as compared with healthy individuals (Snyder-Cappione et al. 2007; Im et al. 2008; Montoya et al. 2008). Thus, clear evidence of the role of iNKT cells in protection against TB is still formally lacking.

Modulation of CD1 Expression and Evasion of CD1-Mediated Detection by MTB

MTB has evolved a series of strategies to modulate the expression of CD1 molecules and to prevent CD1-restricted responses. MTB infection of human monocytes inhibits the expression of CD1 molecules and induces the generation of CD1a[−], CD1b[−], and CD1c[−] DCs in the presence of IFN- α (Mariotti et al. 2002, 2004). This is attributed to cell wall-associated α -glucans of MTB that through CR3 activate the p38 MAPK pathway, and in turn the ATF-2 transcription factor (Gagliardi et al. 2007), leading to the inhibition of *cd1* gene transcription. MTB-infected monocytes differentiate into macrophage-like host cells and have reduced capacities to stimulate CD1-restricted T cells, thus representing an immunoprivileged niche for mycobacterial persistence. Other studies performed with CD1-expressing and mature DCs showed down-regulation of *cd1* gene transcription by MTB (Stenger et al. 1998), which was ascribed to engagement of Toll-like receptor 2 (TLR2) (Roura-Mir et al. 2005). Another important mechanism with potential effects on CD1-restricted lipid presentation is the inhibition of apoptosis of infected cells by MTB. As apoptosis is required for release of mycobacterial lipids and subsequent cross-presentation by CD1-expressing APCs (Schaible et al. 2003), its inhibition may also have indirect inhibitory effects on presentation of mycobacterial lipid antigens. The evolution of these evasion mechanisms suggests a protective role of CD1-restricted T-cell responses during MTB infection. Despite these in vitro compelling data, the physiological significance of CD1 protein down-regulation in vivo during mycobacterial infections remains to be investigated.

MAIT CELLS

MAIT cells are innate-like T lymphocytes present at high frequency in human blood (Martin et al. 2009). They were originally found enriched in the mucosal lamina propria, from where their name was derived (Treiner et al. 2003). Recently they also have been found to be a major population of liver-resident T cells (Dusseaux et al. 2011; Tang et al. 2013). MAIT cells express an evolutionarily conserved TCR- α chain characterized by the semi-invariant rearrangement V α 7.2-J α 33 in human and V α 19-J α 33 in mice paired with a limited set of V β chains, mainly V β 2 and V β 13 in humans and V β 6 and V β 8 in mice (Porcelli et al. 1993; Tilloy et al. 1999; Treiner et al. 2003; Kawachi et al. 2006; Goldfinch et al. 2010).

Antigens Stimulating MAIT Cells

MAIT cells recognize pterin-containing compounds generated during the synthesis of riboflavin (vitamin B₂) by bacteria and yeasts (Kjer-Nielsen et al. 2012) (Fig. 2A) and presented by nonpolymorphic MR1, which is the restriction element for these T lymphocytes (Treiner et al. 2003; Huang et al. 2005). MAIT cells react to APCs infected with a broad array of microbes able to synthesize riboflavin, whereas they are not stimulated by microbes that lack this capacity (Kjer-Nielsen et al. 2012). The responsiveness to a wide group of microorganisms suggests an important function of MAIT cells in antimicrobial immunity (Gold et al. 2010; Le Bourhis et al. 2010). Furthermore, MAIT cells are absent in the periphery of germ-free mice and their peripheral expansion depends on bacterial colonization (Treiner et al. 2005), thus further suggesting that they participate in the control of infections.

Structural studies have shown how the pterin-containing metabolites are inserted within the antigen-binding pocket of MR1 (Lopez-Sagaseta et al. 2013; Patel et al. 2013). The pocket is very large and delimited by hydrophilic and hydrophobic amino acids. Furthermore, the cocrystallized antigens showed a minimal occupancy of the groove, suggesting that additional

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Figure 2. Structure of antigenic metabolites. (A) Structures of three different ribityl lumazines (RL), which bind MR1 and stimulate MAIT cells. (B) Structures of isopentenyl pyrophosphate (IPP) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) metabolite antigens, which stimulate Vγ9-Vδ2 T cells.

large hydrophobic or hydrophilic molecules might also bind to MR1. There is no evidence so far that MR1 molecules can present other antigens to non-MAIT T cells.

Role of MR1-Restricted T Cells in Mycobacterial Infections

Mycobacteria, including MTB, *Mycobacterium smegmatis*, *Mycobacterium abscessus*, and BCG are potent stimulators of human and mouse MAIT cells (Gold et al. 2010; Le Bourhis et al. 2010; Chua et al. 2012). A recent work showed that MAIT cells are reduced in blood of pulmonary TB patients compared with individuals with latent TB and healthy controls (Gold et al. 2010). In this study the percentage of MTB-reactive T-cell clones restricted to MR1 was higher in healthy donors and LTB individuals compared with patients with active disease. These studies were performed by analyzing the ex vivo frequency of MAIT cells measuring TNF-α release in response to MTB-infected epithelial cells. The reduction of MR1-restricted T cells in blood of patients with active TB was possibly attributed to migration of MAIT cells into the lung, the natural site of MTB infection. The same study showed that a small percentage of

lung-infiltrating T cells expressing the TCR Vα7.2 from two donors released TNF-α in response to MTB-infected APCs in an MR1-dependent manner. As MTB-infected primary lung epithelial cells stimulate MAIT cells, they might be involved in the activation of MAIT cells in the lung (Gold et al. 2010). Another study described a reduction of MAIT cells in blood of patients with pulmonary bacterial infections, including TB, compared with healthy individuals (Le Bourhis et al. 2010). Here, MAIT cells were identified ex vivo by flow cytometry according to the coexpression of their specific markers TCR Vα7.2 and CD161. The drawback of this alternative approach is the known down-modulation of CD161 marker following antigen recognition, which limits the number of cells identified as true MAIT. However, it is not biased by the intrinsic differential capacity of individual T cells to adapt and grow in vitro following cloning. Despite the intrinsic limitations of used techniques, both studies confirmed that the MAIT cell compartment is impaired in blood of TB patients (Gold et al. 2010; Le Bourhis et al. 2010). As MAIT cells disappear from circulating blood on infection, they might accumulate at the site of infection. If this is the case, these findings raise the question of whether

MAIT cells accumulating in the lung are protective in the context of TB infection.

Effector Functions of MAIT Cells

Upon recognition of bacteria, including MTB, MAIT cells released large amounts of IFN- γ and TNF- α (Dusseaux et al. 2011; Chua et al. 2012; Gold et al. 2013), which are crucial cytokines for an effective immune response against MTB (reviewed in O'Garra et al. 2013). Furthermore, they killed in vitro APCs infected with *Escherichia coli* and BCG (Chua et al. 2012; Gold et al. 2013), thus contributing to control of intracellular infection and to limiting of pathogen spreading. Indications of a putative protective function of MAIT cells are also being investigated using MR1-deficient mice (MR1^{-/-}), which lack MAIT cells. These mice mounted a less efficient immune response at early time points than wild-type (WT) mice following intraperitoneal infection with *Klebsiella pneumoniae* (Georgel et al. 2011). It is of note that, in the same study, no differences between WT and MR1^{-/-} mice were observed when the animals were infected with *E. coli*, *Shigella dysenteriae*, and *Yersinia enterocolitica* (Georgel et al. 2011), although these bacteria can stimulate MAIT cells (Kjer-Nielsen et al. 2012). A transient impaired ability to clear aerosol-administered BCG was also described in mice lacking MAIT cells, as highlighted by the higher bacterial counts in the lung of MR1^{-/-} animals compared with WTones (Chua et al. 2012). In keeping with this, MR1-deficient V α 19-J α 33 transgenic mice, intravenously injected with *M. abscessus*, showed an increased bacterial burden in the spleen compared with their MR1-sufficient counterpart (Le Bourhis et al. 2010). In another study, purified human MAIT cells were reprogrammed into induced pluripotent stem cells and redifferentiated back in vitro into MAIT cells (re-MAIT) (Wakao et al. 2013). Re-MAIT showed MAIT-like phenotype and properties in vitro and, when adoptively transferred into immune-compromised NOD/SCID mice challenged with *M. abscessus*, elicited a protective function via a granulysin-dependent mechanism (Wakao et al. 2013). In all these

studies the protective activity of MAIT cells was noticed only at the early stage of infection, in line with the innate-like properties of these T lymphocytes. Nevertheless, a more recent work showed that MAIT cells can also elicit long-term bacterial control in a mouse model of pulmonary infection with the live vaccine strain of *Francisella tularensis* (Meierovics et al. 2013). The investigators found that CD4⁺ and CD8⁺ T-cell-depleted WT mice were resistant to a sublethal dose of *F. tularensis*, whereas MR1-deficient animals devoid of CD4⁺ and CD8⁺ T cells succumbed to the infection. These data represented, to our knowledge, the first report of an effective long-lasting antimicrobial role of MAIT cells in vivo. Importantly, the same study showed that MAIT cells accumulated in the lung of *F. tularensis*-challenged mice and induced a prompt recruitment of IFN- γ -producing CD4⁺ and CD8⁺ T cells to the infected site. Indeed MR1-deficient animals showed no MAIT accumulation in the lung and a significant delay in the recruitment of adaptive conventional T cells, resulting in a lower efficiency in the clearing of the bacteria (Meierovics et al. 2013). Such characteristics resemble those of CD1d-restricted iNKT cells, which have been defined as a “bridge” between innate and adaptive immunity (reviewed in Brennan et al. 2013).

It is still unclear whether the accumulation of MAIT cells in inflamed tissues resulted from the migration of circulating MAIT cells or from the expansion of the tissue-resident MAIT cell pool. In some instances, human MAIT cells do not proliferate in vitro after antigen stimulation (Dusseaux et al. 2011; Cosgrove et al. 2013), which instead induces an apoptotic program in these T lymphocytes (Cosgrove et al. 2013). However, in other laboratories MAIT cells have been successfully expanded and cloned (Gold et al. 2010; Lepore et al. 2014), showing that they survive in vitro when provided with the right culture conditions. Furthermore, they express high levels of tissue homing markers and chemokine receptors and display a marked tropism for solid organs (Kawachi et al. 2006; Dusseaux et al. 2011; Gold et al. 2013; Tang et al. 2013; Wakao et al. 2013). According to these findings, tissue-resident MAIT cells might be crucial as

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the first line of defense against invading pathogens. The prompt reactivity of “tissue-patrolling” MAIT cells to microbes might be important in containing the initial phase of local infections, further sustained by the fast influx of MAIT cells from blood, driven by chemotactic signals. The inflammatory environment resulting from MAIT cell activation would be important for the rapid recruitment of the effectors of the adaptive immunity. Further studies will be needed to challenge this model and to better characterize the potential protective activity of MAIT cells in TB. The definition of their role in the orchestrated mucosal immune response in the lung, their ability to modulate and regulate the function of other immune cells, such as macrophages and neutrophils, important in the early immune response against MTB (O’Garra et al. 2013), and the molecular mechanisms governing their antibacterial capacity remain unsolved and debated issues.

GAMMA-DELTA T CELLS

T cells expressing the gamma-delta ($\gamma\delta$) TCR represent the third population of nonclassical T cells in humans. The main characteristic of human $\gamma\delta$ T cells is their expression of unique TCR heterodimers. Three main populations of $\gamma\delta$ T cells are detected: The most abundant population in circulating blood and secondary lymphoid organs expresses the V δ 2 chain almost always paired with the V γ 9 chain. These TCR heterodimers are disulphide linked as they use the C γ 1 constant region and are polyclonal. Junctional diversity is present in the CDR3 regions of both the TCR γ and δ chains. The second main population expresses the V δ 1 chain, which is paired with different V γ chains, mostly belonging to the V γ I family (V γ 2, 3, 4, 5, and 8). This population of $\gamma\delta$ T cells is polyclonal and the TCR is not disulphide linked, as in most of the cases the C γ 2 constant region is used. The V δ 1-expressing cells are abundant in the lamina propria of the gut and in the intra-epithelial spaces. Noteworthy, these cells are also found in the lung and in the skin. The third population of $\gamma\delta$ T cells expresses the V δ 3

chain, which usually pairs with V γ chains of the V γ I family.

Antigen Recognition by $\gamma\delta$ Cells

Although we know a lot about TCR gene use, tissue localization, and effector functions of $\gamma\delta$ T cells, we remain quite unaware of the antigens that stimulate human $\gamma\delta$ T cells. In addition, we have only partial information on the function of this T-cell population in TB.

The population expressing the TCR V γ 9-V δ 2 heterodimer recognizes phosphorylated metabolites, such as isopentenyl pyrophosphate (IPP) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which are produced in the mevalonate and methyl erythritol pathways, respectively (Fig. 2B). IPP is made by eukaryotic and prokaryotic cells, whereas HMBPP is made only by some microorganisms, including MTB. IPP and HMBPP are very small molecules (245 and 262 Da, respectively) and require appropriate antigen presentation. Recently, it was reported that Butyrophilin 3A1 (BTN3A1) is required for activation of V γ 9-V δ 2 cells (Harly et al. 2012) and that this molecule is involved in the presentation of both phosphorylated metabolites to the TCR V γ 9-V δ 2 (Vavassori et al. 2013). Surface plasmon resonance and surface-enhanced Raman scattering showed a direct interaction of the TCR V γ 9-V δ 2 with BTN3A1, and the presence of IPP increased the binding (Vavassori et al. 2013). Studies performed with site-directed mutagenesis of TCR chains indicated that the TCR becomes activated only when several amino acids present in the CDR regions of both the TCR γ and δ chains interact with the antigen-presenting molecule (Wang et al. 2010).

Interestingly, BCG-infected cells stimulated only a subset of V γ 9-V δ 2 cells, and only a fraction of V γ 9-V δ 2 cells expanded in vitro with synthetic HMBPP was reactive to infected cells (Spencer et al. 2008). Whether the differential response results from recognition of additional mycobacterial antigens or reflects differences in TCR avidity or variable expression of co-stimulatory/inhibitory molecules remains to be investigated.

The antigens stimulating $\gamma\delta$ cells expressing non-V γ 9 chains and non-V δ 2 chains remain poorly characterized. These cells can be activated during CMV infections, and one clone expressing a V δ 5-V γ 4 TCR was activated by the endothelial protein C receptor (Willcox et al. 2012). V δ 1-expressing cells were also found to interact with the bacterial phycoerythrin protein in a manner resembling antigen–antibody recognition, without requirements for antigen processing or presentation by dedicated presenting molecules (Zeng et al. 2012).

A small number of non-V δ 2 cells recognize glycolipids presented by CD1c and CD1d molecules (Faure et al. 1990; Spada et al. 2000; Bai et al. 2012). The structure of the TCR $\gamma\delta$ -CD1-lipid complex showed a MHC-like recognition (Luoma et al. 2013; Uldrich et al. 2013), disclosing the enormous plasticity of antigen recognition by TCR $\gamma\delta$. So far, there is no evidence of recognition of mycobacterial lipid antigens by $\gamma\delta$ cells. Recent studies reported the identification of two mycobacterial proteins, namely of 1-deoxy-D-xylulose 5-phosphate synthase 2 and Rv2272 protein, which activated $\gamma\delta$ T cells from pulmonary TB patients (Xi et al. 2013). Responding $\gamma\delta$ cells secreted IFN- γ and monocyte chemoattractant protein 1 implicating an important role of these cells in protection and stimulation of monocyte chemotaxis toward the site of infection.

Role of $\gamma\delta$ Cells in TB

In TB patients many changes in $\gamma\delta$ cell populations have been detected for many years. However, molecular mechanisms and implications in protection and disease progression remain poorly characterized. Only in the recent past have novel mechanisms begun to be carefully investigated. In patients with active TB it was observed a decrease of circulating V γ 9V δ 2 cells and in particular of central memory cells and of effector memory and terminally differentiated cells, which exert immediate effector functions (Meraviglia et al. 2010). These changes reversed after antimycobacterial therapy, suggesting an indirect correlation with antigen burden. In severe TB patients, an increase of $\gamma\delta$ cells with

enhanced interleukin-10 production was also described (Pinheiro et al. 2012), indicating changes associated with disease progression. This plasticity in T-cell function was confirmed in another study conducted in acute TB patients, in which a progressive reduction of the cytolytic activity of $\alpha\beta$ cells was described. However, in the same patients, $\gamma\delta$ cells remained perforin⁺ and capable of killing intracellular bacteria (De La Barrera et al. 2003).

The implication of $\gamma\delta$ cells in the immune response within MTB-infected lungs is supported by a series of investigations. V γ 9V δ 2 cells accumulate within lung granulomas (Huang et al. 2012), and in a macaque model of BCG and MTB infection they rapidly expanded upon second exposure to mycobacteria (Shen et al. 2002). This behavior resembles that of adaptive immunity cells and suggests that V γ 9V δ 2 cells may contribute to adaptive immunity to mycobacterial infections. In pleural effusions of TB patients, two populations of $\gamma\delta$ cells were identified as TCR^{low} V δ 2⁺ and TCR^{high} V δ 1⁺ (Yokobori et al. 2009). The V δ 2 cells were CD45RA^{+/−}CCR7⁺CXCR3⁺, released large amounts of IFN- γ , and expressed the CD107a marker, indicating a status of activated cells within the tissue.

Finally, in a mouse model of lung infection with MTB, $\gamma\delta$ cells were instrumental to activate DCs in the lung and facilitate subsequent priming of lung CD8 cytotoxic T cells (Caccamo et al. 2006), thus representing important protagonists of the early response during infection.

CONCLUDING REMARKS

All together these studies depict nonclassical T cells as major players of the immune response during MTB infection. The capacity of these cells to recognize unique nonpeptidic antigens, together with their memory phenotype and effector capacities, represent good indications that they participate in protective mechanisms, alongside with MHC-restricted and peptide-specific T cells. Therefore, mycobacterial lipid antigens stimulating protective CD1-restricted T cells may be considered as candidate constituents of subunit anti-TB vaccines.

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A second important feature of other main populations of nonclassical T cells (MAIT and $\gamma\delta$ cells) is their recognition of small metabolites produced by most bacteria and their activation following TCR interaction with dedicated presenting molecules, such as MR1 and BTN3A1, which are both nonpolymorphic and almost ubiquitously expressed. These characteristics indicate a common strategy of tissue-resident nonclassical T-cell activation during early infection, which prompts immediate release of proinflammatory cytokines. This feature has not been exploited in animal models of TB and might represent a novel approach to facilitate early protection and efficient priming of adaptive T cells during MTB infection.

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