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Protective immune responses of major V γ 2V δ 2 T-cell subset in *M. tuberculosis* infection

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Abstract

Recent observation that prenyl pyrophosphates bind the Ig superfamily protein butyrophilin 3A1 (BTN3A1) suggests that modifying BTN3A1 activates major $\gamma\delta$ T-cell subset, V γ 2V δ 2 T cells. Studies also show that microbial phosphoantigen HMBPP is required for expansion, pulmonary response, effector functions and memory polarization of V γ 2V δ 2 T cells during infections. Broad repertoires of cytokines involve expansion, recall-like expansion and effector functions of V γ 2V δ 2 T cells after Mtb infection or vaccination. Finally, mechanistic studies in nonhuman primate TB model demonstrate early expansion and differentiation of V γ 2V δ 2 T cells during Mtb infection can increase immune resistance to TB in macaques, with a potential mechanism of early/sustained IFN- γ production and CTL killing.

Introduction

Dominant V γ 2V δ 2 (also V γ 9V δ 2) T-cell subset constitutes 65–90% of total circulating human $\gamma\delta$ T cells. This subset remain a sole $\gamma\delta$ T-cell population recognizing isoprenoid metabolites such as isopentenyl pyrophosphate (IPP) and (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP), which are usually referred to as phosphoantigens [1,2]. Isoprenoid metabolites are produced *via* two major pathways: the classical mevalonate pathway, or the alternative, non-mevalonate pathway. IPP is an intermediate metabolite found in both pathways, whereas HMBPP is only produced in the non-mevalonate pathway by some selected microbes including *Mycobacterium tuberculosis*, *M. bovis* BCG, *Listeria monocytogenes* and certain species of malaria parasites [1,2]. Phosphoantigen-specific V γ 2V δ 2 T-cell subset exist only in humans or nonhuman primates(NHP), not other species. It is clear that IPP or HMBPP activation of V γ 2V δ 2T cells is independent of MHC or CD1 molecules [3]. However, it has not been demonstrated until recently that the Ig superfamily protein butyrophilin (BTN)3A1 is required for prenyl pyrophosphate stimulation or activation. It has been shown that the extracellular BTN3A1 IgV domain binds prenyl pyrophosphates, resulting in the proposal that the V γ 2V δ 2 TCRs detect the complex [4**]. Nevertheless, other reports suggest that the intracellular B30.2 domain of BTN3A1 indeed binds prenyl pyrophosphates, leading to a change in the extracellular BTN3A1 dimer that is recognized by V γ 2V δ 2 TCRs [5**,6**]. It is likely that prenyl pyrophosphates-mediated

alteration or modification of BTN3A1 induces activation of V γ 2V δ 2 T cells [7]. These findings will further enhance our understanding of specific immune responses of V γ 2V δ 2 T cells in infections or cancers. This article reviews recent progress regarding how V γ 2V δ 2 T cells mount immune responses to microbial phosphoantigen HMBPP during host–pathogen interaction in infections. Importantly, *in vivo* manipulation of protective V γ 2V δ 2 T-cell responses will also be discussed in non-human primate TB model.

Phosphoantigen HMBPP is required for expansion, pulmonary trafficking, effector functions and memory polarization of V γ 2V δ 2 T cells during microbial infections

V γ 2V δ 2 T cells in humans and nonhuman primates (NHP) can remarkably expand during acute infections/ reinfections with HMBPP-producing *M. tuberculosis* or other pathogens [8,9]. In NHP models of TB, BCG, Listerial or orthopoxvirus re-infection, V γ 2V δ 2 T cells can mount remarkable recall expansion in systemic or lung compartments [8,10,11]. Such recall-like expansion resembles an adaptive immune response after re-infection or Mtb infection of BCG-vaccinated macaques as the secondary expansion displays a memory-like pattern of much faster, greater magnitudes and longer period, with selected clonotypic $\gamma\delta$ TCR sequences readily being seen in the re-expanded V γ 2V δ 2 T cells [8,10,11]. However, whether HMBPP, IPP, cytokine(s) or TLR/NKG2A ligands each contributes to the *in vivo* expansion needs to be addressed. This is not a trivial question as all these elements have been shown to somehow activate V γ 2V δ 2 T cells in cultures [9,12,13]. Recently, relative importance of HMBPP, IPP and others for *in vivo* expansion of V γ 2V δ 2 T cells has been evaluated in comparative NHP studies of HMBPP/IPP-coproducing parental *Listeria actA prfA** and HMBPP-deficient IPP-producing *Listeria actA gcpE prfA** mutant [14**]. HMBPP-deficient IPP-producing mutant shows comparable *in vivo* infectivity [14**]. Interestingly, infection of macaques with HMBPP-deficient IPP-producing mutant elicits much lower magnitudes of expansion and recall-like expansion of V γ 2V δ 2 T cells compared to parental HMBPP/IPP-coproducing strain (Figure 1). In addition, the mutant infection leads to marked decreases in trafficking and accumulation of V γ 2V δ 2 T cells in pulmonary compartment. The deficiency of HMBPP also remarkably influences memory polarization and differentiation of effector functions for cytokine production and for inhibiting intracellular bacteria [14**] (Figure 1). These findings may also apply to the setting of *M. tuberculosis* infection, whereas HMBPP-deficient Mtb cannot be evaluated due to a loss of decent Mtb replication after genetic knock-out of *gcpE* gene encoding enzymes for HMBPP production. Nevertheless, the results illustrate dominant roles of HMBPP during infections of primates with HMBPP-producing pathogens.

Broad repertoires of cytokines involve expansion, recall-like expansion and effector functions of V γ 2V δ 2 T cells after Mtb infection or vaccination

Although phosphoantigen HMBPP produced by Mtb or other pathogens is crucial for stimulating activation and expansion of V γ 2V δ 2 T cells, little is known about what cytokine(s) facilitates expansion or recall-like expansion of HMBPP-stimulated V γ 2V δ 2 T

cells during infections. It is noteworthy that, unlike CD4 T helper cells, V γ 2V δ 2 T cells activated by HMBPP alone without cytokine(s) co-signaling only produce little or no IL-2 required for clonal expansion of themselves. In this context, expansion of activated V γ 2V δ 2 T cells in cultures appears to depend largely on certain T-cell growth cytokines such as IL-2, IL-15, or IL-21 [8,15,16], whereas IL-17-producing V γ 2V δ 2 T cells could be induced in culture by Th17 cocktailed cytokines [17,18]. *In vivo* NHP studies have showed that only co-treatment of macaques with phosphoantigen HMBPP plus IL2, but not IL2 alone or HMBPP alone, could induce major expansion of V γ 2V δ 2 T cells in macaques [19,20]. However, primary active infection of NHP with Mtb or BCG did not appear to produce high levels of IL-2/IL-15 or IL-21 [21,22] at the time expansion of V γ 2V δ 2 T cells was clearly seen [8]. Instead, high levels of IL-22 and IL-23 were found coincident with major expansion of V γ 2V δ 2 T cells during Mtb or BCG infection of NHP [8,21,22]. This *in vivo* correlate prompts a hypothesis or possibility that Th17-related cytokines involve expansion or recall-like responses of V γ 2V δ 2 T cells after infections. Now new NHP studies showed that IL-17A/IL-17F, IL-22 or IL-23 could expand phosphoantigen HMBPP-stimulated V γ 2V δ 2 T cells from BCG-vaccinated macaques but hardly from naïve animals [23**] (Figure 2). IL-23 induced greater expansion than other Th17-related cytokines [23**]. Consistently, Mtb infection of macaques also enhanced the ability to IL-17/IL-22 or IL-23 to expand HMBPP-stimulated V γ 2V δ 2 T cells (Figure 2).

Interestingly, comparative studies showed that while IL-2 could remarkably expand HMBPP-activated V γ 2V δ 2 T cells in both naïve and Mtb/BCG-infected macaques, IL-23 favored recall-like of the activated $\gamma\delta$ T cells after infection or vaccination [23**] (Figure 2). The reason for such a difference is currently not known. IL-2 expansion of HMBPP-activated V γ 2V δ 2 T cells likely requires the signaling of the JAK1-JAK3/LCK/SYK pathway, whereas IL-23 appears to expand and differentiate Th17 cells *via* JAK3-TYK2/STAT3 signaling. However, it is likely that IL-2 and IL-23 each could distinctly act in concert with the HMBPP/BTN3A1-mediated TCR signal to differentiate into similar effector functions. In fact, V γ 2V δ 2 T cells expanded by HMBPP/IL-2 administration were shown to produce anti-TB cytokines IFN- γ , TNF- α , and perforin [24,25]. Similarly, IL-23 expansion of HMBPP-activated V γ 2V δ 2 T cells from macaques infected with Mtb, BCG or *Listeria* could differentiate effector function of these $\gamma\delta$ T cells for producing IL-17, IL-22, and IFN- γ [23**] (Figure 2). Furthermore, HMBPP/IL-23-induced proliferation of V γ 2V δ 2 T cells appeared to require APC contact and involve the conventional and novel protein kinase C (PKC) signaling pathways [23**]. Findings suggest that Th17-related cytokines can contribute to recall-like expansion of Ag-specific $\gamma\delta$ T cells after infection.

Notably, the production of the anti-TB cytokine IFN- γ by V γ 2V δ 2 T cells may be the intrinsic immune feature of HMBPP activation of V γ 2V δ 2 TCR during Mtb infection. Large amounts of IFN- γ can be produced after HMBPP-stimulated V γ 2V δ 2 T cells are expanded by IL-2 [25,26], variant IL-4 [27] and IL-23 [23**]. IFN- γ can be also produced by HMBPP-alone activation of 'mature' V γ 2V δ 2 T effector cells from naturally Mtb infected macaques [28]. More importantly, IFN- γ endogenously produced by V γ 2V δ 2 T effector cells appears to have multi-regulatory roles. Blockade experiments using neutralizing anti-IFN- γ and others showed that the endogenous IFN- γ could promote expansion of V γ 2V δ 2 T cells after co-activation by HMBPP/IL-2 or HMBPP/IL-23 [23**]. In addition, endogenous

IFN- γ was also required for the ability of V γ 2V δ 2 T effector cells to antagonize Foxp3+ Treg function [29**] and down-regulate over-active IL-22 production by $\alpha\beta$ T cells in TB [28]. This intrinsic production of IFN- γ by V γ 2V δ 2 T cells may contribute to anti-TB immune responses and regulate TB lesions in Mtb infection.

Protective responses and mechanisms of V γ 2V δ 2 T cells in Mtb infection

Despite that $\gamma\delta$ T cells have been discovered for 30 years, protective roles of human V γ 2V δ 2 T cells in TB and other infections remains unknown. Recently, in-depth mechanistic studies have been conducted in NHP TB model to determine whether early expansion and differentiation of V γ 2V δ 2 T cells during Mtb infection can increase immune resistance to TB in macaques. cHMBPP (HMBPP isoform) plus IL-2 administration reproducibly induced major expansion of phosphoantigen-specific V γ 2V δ 2 T cells during Mtb infection, while these expanded $\gamma\delta$ T cells could rapidly traffic to and accumulate in the pulmonary compartment [30] (Figure 3). Expanded V γ 2V δ 2 T cells were able to differentiate into multifunctional effector subpopulations capable of producing anti-TB cytokines IFN- γ , perforin and granulysin, and even co-producing perforin/granulysin in the lung tissue [30] (Figure 3). Importantly, early expansion and differentiation of phosphoantigen-specific V γ 2V δ 2 T cells significantly reduced Mtb burdens and attenuated TB lesions in the lung tissues compared to saline/BSA or IL-2 controls (Figure 3). These results provide first *in vivo* evidence implicating that early expansion and differentiation of V γ 2V δ 2 T effector cells during Mtb infection can increase immune resistance to TB.

Mechanistic experiments showed that, in addition to early/sustained production of anti-TB cytokine IFN- γ , perforin/granulysin-producing V γ 2V δ 2 T cells could limit intracellular Mtb growth, whereas macaque granulysin had Mtb-bactericidal effect, and inhibited intracellular Mtb in presence of perforin [30] (Figure 3). Consistently, human studies showed that potent *in vitro* stimulation of human V γ 2V δ 2 T cells allowed these highly-activated $\gamma\delta$ T cells to inhibit Mtb growth and that neutralization of perforin or granulysin by antibodies could reduce the anti-Mtb effector function [16,31–33]. The CTL killing mechanism seen in NHP and humans is supported further by the striking data derived from a paralleled NHP study, in which *in vivo* expanded V γ 2V δ 2 T cells during intracellular Listerial infection were able to directly kill infected target cells and inhibit intracellular bacterial replication in a perforin-dependent fashion even in the absence of *in vitro* phosphoantigen stimulation [34]. This indeed represents the first experimental evidence that *in vivo* expanded V γ 2V δ 2 T effector cells during infection can mediate direct lysis of infected target cells and inhibition of intracellular pathogen without the prior *in vitro* Ag stimulation. Of note, another novel mechanistic pathway whereby V γ 2V δ 2 T effector cells inhibit intracellular mycobacteria has recently been discovered. It has been shown that granzyme A produced in V γ 2V δ 2 T cells or purified granzyme A alone could inhibit intracellular mycobacteria, but such inhibition requires action of TNF- α in mycobacteria-infected monocytes. This mechanism involving granzyme A-mediated triggering of TNF- α production by monocytes was independent of autophagy, apoptosis, nitric oxide production, type I interferons, Fas/FasL and perforin [35**].

Another potential protective mechanism for V γ 2V δ 2 T cells may help to enhance the host immune system. Virtually, cHMBPP/IL-2 expansion and differentiation of V γ 2V δ 2 T cells led to enhanced pulmonary Th1 responses of Mtb protein Ag-specific $\alpha\beta$ T cells as IFN- γ -producing PPD-specific CD4 and CD8 T cells in airway were much higher after cHMBPP/IL-2 administration compared to controls [30]. Such immune enhancement was linked to the ability of V γ 2V δ 2 T effector cells to produce IL-12, a crucial cytokine required for Th1 polarization [30]. The enhanced anti-TB Th1 response appear to be in line with the recent interesting observations seen in NHP and humans. *In vitro* activation of human $\gamma\delta$ T cells could stimulate DC maturation, improve B cell production of antibodies and facilitate development of CD4 T helper cells [36,37,26]. Consistently, NHP studies showed that HMBPP/IL-2 expansion of V γ 2V δ 2 T cells during chronic SHIV infection, while reducing viral antigenic loads, could boost and sustain HIV Env-specific Ab titers for >170 days [24]. The peak expansion of V γ 2V δ 2 T cells displaying memory phenotypes also led to increased effector function of CD4 and CD8 $\alpha\beta$ T cells producing anti-TB cytokines IFN- γ , TNF- α [24]. Such enhancements do not appear to result from residual effects of IL-2 in the HMBPP/IL-2 regimen. In fact, IL-2 administration during mycobacterial infection of NHP indeed induced predominant expansion of CD4+CD25+Foxp3+ Treg and inhibited mycobacterium-specific Th1 immune responses [29**]. In contrast, combined HMBPP and IL-2 administration, not IL-2 alone, actually down-regulated IL-2-induced increases in Foxp3+ Treg cells and reversed IL-2/Foxp3+ Treg-mediated suppression of Th1 responses in mycobacterium-infected macaques [29**].

In parallel to the novel observation that V γ 2V δ 2 T effector cells have protective roles in TB, early expansion of V γ 2V δ 2 T effector cells have also been found to mediate protection against pneumonic plague lesions in the macaque model of acutely fatal *Yersinia pestis* infection [38]. It was shown that HMBPP/IL-2 administration, but not controls, after lethal-dose *Y. pestis* infection expanded lung parenchymal V γ 2V δ 2 T cells and led to the attenuation of inhalation plague lesions in lungs [38]. The protection correlated with FGF-7, a homeostatic mediator against tissue damages [39], although a precise mechanism is unknown.

Summary and outlook

Microbial phosphoantigen HMBPP is required for expansion, pulmonary response, effector functions and memory polarization of V γ 2V δ 2 T cells during infections, involving broad repertoires of cytokines for these effector functions of V γ 2V δ 2 T cells. *In vivo* manipulation of V γ 2V δ 2 T cells during Mtb infection can increase immune resistance to TB in macaques. The novel findings in the settings of experimental TB and plaque start to shed light on protective immune responses of HMBPP-specific human dominant $\gamma\delta$ T-cell subset in Mtb infection (Figure 4).

To define the principle that V γ 2V δ 2 T cells are protective against Mtb or other infections, adoptive transfer of V γ 2V δ 2 T cells or specific depletion of these $\gamma\delta$ T-cell subset may be one of the future proof-of-concept studies in NHP. This would require advanced development of technical aspects. On the other hand, the unique features of fast reaction/expansion and multi-effector functions for V γ 2V δ 2 T cells will help to guide rational

experimental designs including $\gamma\delta$ T cells for development of new vaccine or host-directed therapy against TB.

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Figure 1.

(a) Mean absolute numbers of V γ 2V δ 2 T cells at various time points following *Listeria* exposures (arrows). Macaque group shown in open symbols were challenged with parental *Listeria actA prfA** strain; those shown in solid symbols infected with the *gcpE* mutant (deleted of *gcpE* gene encoding enzyme for production of HMBPP). (b) Absolute numbers of phosphoantigen-specific IFN- γ + TNF- α +V γ 2+ T cells (double positives), TNF- α +V γ 2+ T cells or IFN- γ +V γ 2+ T cells in blood (μ L). Absolute numbers were calculated by first subtracting cytokine+ V γ 2+CD3+ T cell percentage from unstimulated panel from cytokine + V γ 2+CD3+ T cell percentage in the HMBPP stimulated panel, and then multiplying by total CD3+ T cells derived from CBC counts. (c) V γ 2V δ 2 T cells from macaques infected with HMBPP-deficient IPP-producing *Listeria actA gcpE prfA** mutant showed a reduced capability to inhibit intracellular listerial growth.

Figure 2.

(a) BCG vaccination enhances the ability of Th17-related cytokines, but not IL-2, to expand HMBPP-stimulated V γ 2V δ 2 T cells. **(b)** Primary Mtb infection of macaques promoted the ability of IL-23 or IL-17/IL-22 to expand HMBPP-stimulated V γ 2V δ 2 T cells. **(c)** HMBPP/IL-23-expanded V γ 2V δ 2 T cells from macaques infected with Mtb had effector function producing IL-17, IL-22, IL-2 and IFN- γ . Shown are mean percentage numbers of V γ 2V δ 2 T cells producing respective cytokines in intracellular cytokine staining and flow analysis.

Figure 3.

(a) Increases in V γ 2V δ 2 T cells in airway (BAL fluid) after picostim (cHMBPP)/IL-2 treatment of Mtb-infected macaques. (b) Median CFU counts of bacilli in lung tissue homogenates of different lung lobes collected at the end point (at day 65) from cHMBPP/IL-2-treated and control groups. (c) Median gross pathology scores for cHMBPP/IL-2-treated and control groups. Note that great scores denote severe TB. (d) Mean numbers of IFN- γ -producing V γ 2V δ 2T cells in airway of different groups. (e) Mean numbers of perforin-producing V γ 2V δ 2T cells in airway. Data in both e and d are derived from intracellular cytokine staining after HMBPP stimulation. (f) Perforin/granzyme-producing V γ 2V δ 2T cells inhibit intracellular Mtb in monocyte-derived macrophages.

Figure 4. Summary figure implicating protective roles of HMBPP-specific V γ 2V δ 2 T cells in Mtb infection.