Review

The Plasticity of γδ T Cells: Innate Immunity, Antigen Presentation and New Immunotherapy

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Several signals influence dendritic cell (DC) functions and consequent the immune responses to infectious pathogens. Our recent findings provide a new model of intervention on DCs implicating human γδ T cell stimuli. Vγ9Vδ2 T cells represent the major subset of circulating human γδ T cells and can be activated by non-peptidic molecules derived from different microorganisms or abnormal metabolic routes. With activated-Vγ9Vδ2 T cell co-culture, immature DCs acquire features of mature DCs, such as increasing the migratory activity, up-regulating the chemokine receptors, and triggering the Th1 immune response. Similar to the NK-derived signals, DC activation is mediated by soluble factors as well as cell-to-cell contact. Many non-peptidic molecules including nitrogen-containing bisphosphonates and pyrophosphomonoester drugs, can stimulate the activity of Vγ9Vδ2 T cells *in vitro* and *in vivo*. The relatively low *in vivo* toxicity of many of these drugs makes possible novel vaccine and immune-based strategies against infectious diseases. *Cellular & Molecular Immunology*. 2008;5(3):161-170.

Key Words: T cell, dendritic cell, innate immunity, immunotherapy

Introduction

Although most of the immune cells can be classified as innate or adaptive cells, the current concept of a strict dependent relationship between these two elements has changed the point of view on the regulation of the immune system. Indeed, during the host reactions, both adaptive and innate immune responses cooperate in the host's protection and the control of damages (1). During the lifetime of an individual, adaptive immunity develops as a response to particular infections conferring specific protective status. In contrast, innate immunity is common to an entire species, and it develops in a non-specific manner and does not confer long-lasting immunity to pathogens. However, the infection by a microbial pathogen often occurs in periphery whereas naïve lymphocytes are confined to lymphoid organs. In this context, γδ T cells play an important role together with other innate cells like natural killer cells. γδ T cells form an independent population of circulating lymphocytes with peculiar functions and distribution that have been an enigma now starting to be resolved. Recently, evidence shows a new impact of this T cell subset on antigen presentation through a cross talk with dendritic cells (DCs).

Although several elements have been shown to link innate and adaptive immune responses, a crucial and well-established role is played by DCs, considering the real bridge between local inflammatory response and systemic immunity (2, 3). DCs are the unique cells with the high capacity to export antigens to central immune tissues through dynamic processes, which can be influenced by different stimuli. During infections, DCs interacting with microbes or their products undergo to a complex cell transformation called "maturation" and referring to an intricate differentiation process whereby DCs respond rapidly to an environmental insult and become capable of eliciting the adaptive immune response. DCs directly sense pathogens *via* several kinds of receptors, acquire the capacity to migrate to lymphoid areas and trigger the specific immune response. Innate immune cells that participate in the early response against infecting microbes interact with DCs inducing their maturation and influencing their capacity to trigger the specific immune response. γδ T cells are able to sense pathogens and induce DC maturation, functional activation, DC migration and antigen presentation (4). In this review, we will summarize the recent findings on the plasticity of γδ T cells, including their roles in innate immunity, antigen presentation, regulation of DC functions and their novel application usage in new immunotherapy.

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γδ T cells and innate immunity

γδ T cells have been considered as cells of immuno-surveillance playing a significant role in the innate immunity against pathogens and tumors since their discovery. Like αβ T cells, γδ T cells carry antigen receptors that vary in the physical properties of their ligand-binding sites (5). Indeed, γδ TCRs have a great potential of diversity at their putative ligand-binding sites as well as αβ T and B cells. γδ T cells constitute an entire system of functionally specialized subsets that have been implicated in the innate responses in infectious diseases, in the regulation of immune responses, including cell recruitment and activation, and in tissue repair (6).

Murine γδ T cells are the first lineage of T lymphocytes appearing in the thymus, and later, predominating in epithelia. Vγ3Vδ1 is preponderant in the epidermis, Vγ2Vδ5/6 in the lung, Vγ4Vδ1 in the uterus and vagina, and Vγ5Vδ2/4/5/6 in the intestinal epithelium (7). Circulating murine γδ T cells mainly express the Vγ1Vδ5/6 or Vγ2Vδ5 TCR chains. Structures recognized by murine γδ TCR include I-E^k, heat shock protein 65 (HSP65), T10/22, HSV-gI and stressed epithelial cells (8-10). Based on TCR recombination, different settings of γδ T cells may be now classified. In normal mice, the first is represented by epidermal γδ T cells, which are permanent within the epidermis and constitute a large network of lymphocytes (11, 12). Although the ligand, able to activate this invariant subset, is not known at present, some evidence shows a reasonable self-molecule as candidate (9). Another population of γδ T cells in mice is present in a small number in some tissues including the reproductive tract and the lung (13). During pregnancy or infections, these cells increase and form a large but transient population (14). Even these cells are thought to recognize a self-molecule induced by inflammatory conditions. The third population of mouse γδ T cells resides in circulating tissues forming a small population subset compared to that of αβ T cells and B cells.

In the human fetal thymus, the first γδ T cells to emerge use the Vδ1 chain paired with different Vγ chains, and these preferentially enrich epithelial tissues as the intestine (15). Thus, although such Vδ1 T cells constitute only a minor proportion of human blood, they are a large population of the human intraepithelial cells and have been found to enrich various human epithelial tumors and lymphomas (16). Vδ1 T cells recognize stressed cells *via* presentation of self-lipids by CD1 and/or stress-induced molecules through the NKG2D receptor. In contrast, Vγ9Vδ2 T cells are the major subset of the adult peripheral blood of humans, ranging from 80/90% of γδ T cell pool. They typically recognize phosphor-monoester molecules synthesized in the mevalonate and DOXP metabolic pathway (Figure 1) (17).

The role of γδ T cells differs in different kinds of infection and compartments. For instance, the normal mouse lung contains 10^5 γδ T cells, and after intratracheal infection with *Cryptococcus neoformans,* γδ T cell number increases in 3-6 days. The depletion of pulmonary γδ T cells in the lung decreases the time of clearance, suggesting that γδ T cells down-modulating the Th1 inflammatory response may play a regulatory role during the infection (18). In humans, several studies have reported *in vitro* reactivity of both human Vδ2 and Vδ1 against normal cells infected with viruses, parasites and bacteria as well as tumor cells (19). A direct implication of these cells *in vivo* diseases is well accepted. However, distinct mechanisms in the recognizing and functions of Vδ2 and Vδ1 T cells have been demonstrated, also according to different infections and environments. In human infections, responses of Vγ9Vδ2 T cells to *Mycobacterium tuberculosis* (MTB) were described as early 1989 (20). Later, a range of studies described a marked expansion of this subset in the blood of tuberculosis (TB) patients and also with a range of other infections as leprosy, malaria, salmonella, *Streptococcus pneumoniae,* etc. The response to this variety of infectious agents is the direct result of the recognition of shared non-peptide compounds such as isopentenyl pyrophosphate (IPP), other intermediates of mevalonate pathway, and alkylamines the non-phosphate compounds found in plants and bacteria. The so-called phosphoantigens were identified as potent stimulators of Vγ9Vδ2 T cell functions (21). Although Vγ9Vδ2 T cells predominate in mycobacterial infections, Vδ1 T cells are preferentially expanded in HIV patients and in immunocompromised subjects undergoing CMV reactivation (22).

In viral infections, Vγ9Vδ2 T cells were found as anergic cells, but recently the role of human γδ T cells has been revised. The identity of specific viral antigens recognized by this subset of T cells, remains unknown. The metabolic pathway leading to T cell activation in bacterial infections, has not been demonstrated in viruses and there is not evidence for the presence of phosphoantigens of viral origin. Clearly, γδ T cells, during viral infections, could probably recognize metabolites of altered cellular pathway rather than molecules of viral origin. Moreover, virus exposed γδ T cells can be rapidly activated by type I interferons α and β, a phenomenon that is likely to contribute to the effective antiviral response. The antiviral role of γδ T cells seems to correlate with the production of IFN-γ by distinct γδ T cell subsets (22). With respect to transformed cells, the range of tumor cell lines recognized by Vγ9Vδ2 T cells is now extended to either haematopoietic or solid tumors (23). Both subsets of human γδ T cells are able to recognize and destroy tumor cells through different mechanisms.

The activation properties of γδ T cells in human

Most Vγ9Vδ2 T cells react against the same related set of non-peptidic, phosphorilated antigens recognized in a TCR-dependent manner (21). These compounds derive from the mevalonate pathway (MVA) that is essential for mammalian cells in the sterol synthesis, cell growth and membrane integrity. Other human γδ T cell antigens were only recently described. In this class, aminobisphosphonate

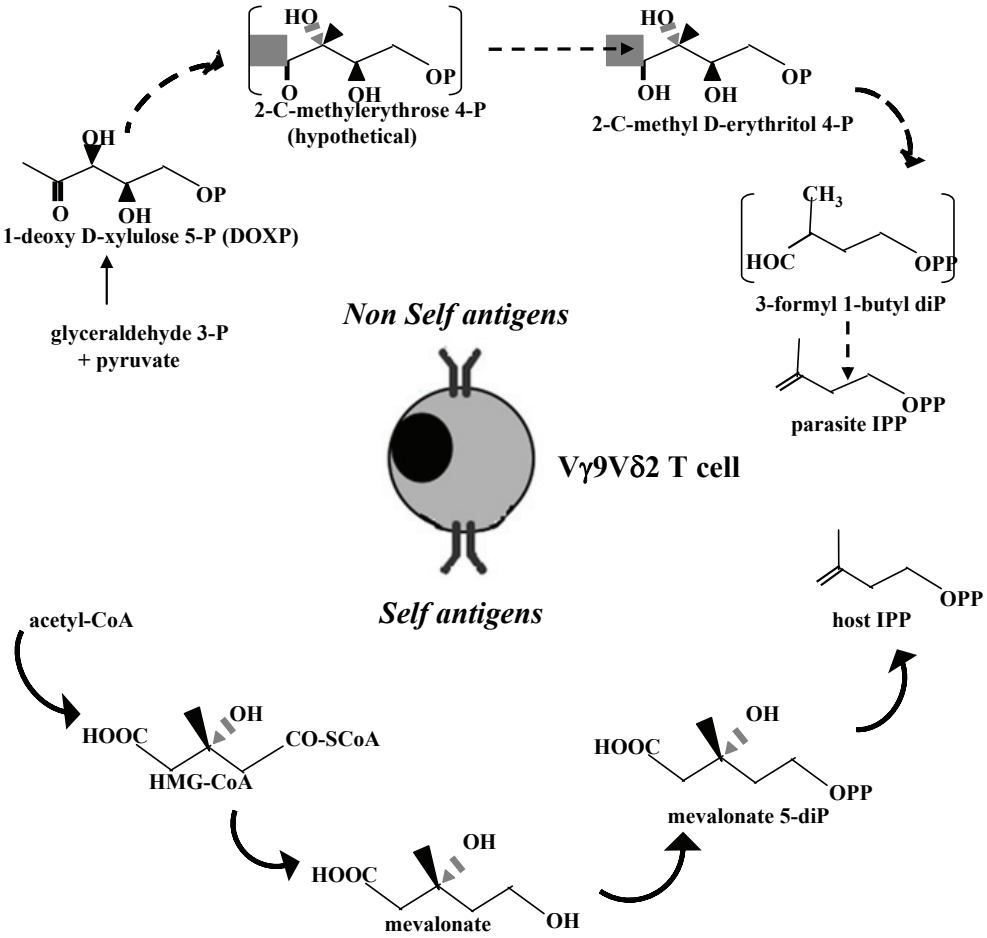

Figure 1. Phosphoantigen production. Self- and non-self-phosphoantigen production in the host cell. In parasites phosphoantigens derive from "1-deoxy-D-xylulose-5-phosphate pathway", and in host cells the production of phosphoantigens is due to the MVA pahway.

(ABP) compounds also stimulate Vγ9Vδ2 T cells through their ability to inhibit farnesyl pyrophosphate synthase, an enzyme acting downstream of IPP synthesis along MVA pathway, promoting intracellular accumulation of IPP. Finally, the alkylamines remain the debated class of γδ T cell antigens. A recent study strongly suggests that like ABPs, alkylamines promote intracellular accumulation of Vγ9Vδ2 agonists derived from MVA pathway, presumably through the same enzymatic way (23-25). Although phosphoantigen mediated activation of Vγ9Vδ2 T cells clearly requires the expression of TCR, how precisely this occurs remains unclear to date. The cell-cell contact required for the activation implicates that phosphoantigens induce either the structural modification of TCR or that surface molecules undefined to date present them.

Human γδ T cells express frequently activating or inhibitory NKR. NKG2D seems to be the major contributing receptor to Vγ9Vδ2 T cell activation. This receptor is a C-type lectin directed against MICA and MICB molecules (26, 27). They also express CD94/NKG2A that strongly inhibits the killing of MHC class I by Vγ9Vδ2 T cells, and CD94/NKG2C or the killer cell immunoglobulin-like receptors (28). NKR expression has been further correlated with the acquisition of memory markers in classical or non-classical T cells and may reflect the distinct functional/memory status in γδ T cell population. Although immunological memory is a hallmark of adaptive immune response, Vγ9Vδ2 T cells seem to develop some features of memory cells. The ability of Vγ9Vδ2 T cells to develop immunological memory remains debated. Studies on monkeys suggested that phosphoantigen-specific Vγ9Vδ2 T cells expanded during a primary tuberculosis vaccination, showing an accelerated response after a secondary challenge (29). However, owing to the IL-2 dependency of primate Vγ9Vδ2 T cells, these results have reflected the occurrence of memory T helper responses and this could not be taken as a formal demonstration of Vγ9Vδ2 T cell memory. The ubiquitous nature of exogenous and endogenous phosphoantigens for Vγ9Vδ2 T cells also suggests that the development of memory state may be quite different from that of conventional cells, which are programmed to respond to foreign peptide antigens. However, after antigen exposure,

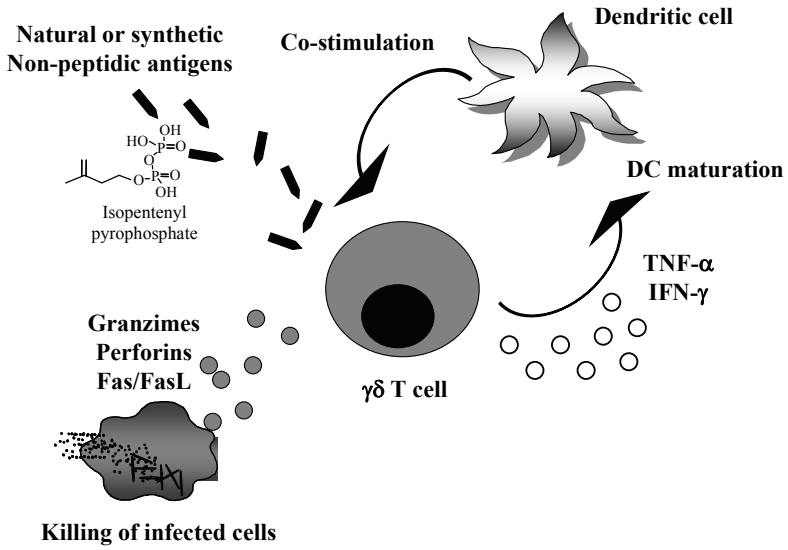

Figure 2. Human γδ T cell functions. Antigen activation of γδ T cells by phosphoantigens leads to the acquisition of effector functions such cytokine production, cytotoxic activity against tumor or infected cells and the capacity to interfere in DC functions.

Vγ9Vδ2 T cells undergo the same change of CD8 T cells. Basing on the expression of CD45RA and CD27 molecules on their surface, it is possible to distinguish 4 subsets of Vγ9Vδ2 T cells as naïve, central memory, effector memory and terminal differentiated effector cells. Vγ9Vδ2 T cells acquire CD45RO expression like early memory CD8 T cells, and are termed central memory Vγ9Vδ2 T cells. They lose CD27 and CD28 expression and re-express CD45RA becoming effector memory cells. Approximately 90% of Vγ9Vδ2 T cells in the adult have a memory phenotype (30). The central memory Vγ9Vδ2 T cell subset may represent an antigen-primed population trafficking to the lymph nodes, generating a new wave of effector cell. On the other hand, effector memory Vγ9Vδ2 T cells represent a readily available pool of antigen-primed Vγ9Vδ2 T cells entering the peripheral tissues, where they can eventually further differentiate into $CD45RA^{+}CD27^{-}$ cells, produce cytokines and exert cytotoxicity contributing to the containment of invading microbial pathogens. After their exposure to foreign infectious agents or dying and metabolic stressed cells, Vγ9Vδ2 T cells go to the rapid production of chemokines and Th1 cytokines as IFN-γ. They may stimulate other components of innate and adaptive immune system and exert cytotoxic activities against infected cells through the expression of perforin and granzymes (Figure 2).

Another pathway recognized by Vγ9Vδ2 T cells has been recently shown. A complex formed between apolipoprotein A1 and ATP synthase, a mitochondrial enzyme that is translocated on the surface of normal hepatocytes and some tumor cell lines, is able to activate Vγ9Vδ2 T cells in a TCR dependent fashion (31).

In the plethora of activation signals of human γδ T cells an important pathway is represented by Toll like receptors (TLR). TLR have emerged as central regulators of innate immunity being receptors specifically sensing molecular patterns of microbes, leading to immediate cellular responses through the activation of transcription factors, notably NF-kβ, AP-1 and IRF (32). Although certain TLRs are expressed on myeloid cells, several reports have shown the functional expression on B and T cells. It has been reported that TLR ligands including TLR3 ligand poly (I:C) and TLR9 ligand CpG enhance the activation of γδ T cells *in vitro via* promoting type I interferon production in myeloid and plasmacytoid DCs respectively (33). More recently, it has been shown that highly purified γδ T cells expressed more TLR3 mRNA than αβ T cells, thus opening the possibility that γδ T cells might respond directly to TLR3 ligands in the absence of APCs (34). Taken together, these results confirm that γδ T cells may play a crucial role in innate immune response and studies on the different signals activating or enhancing their functions may help to improve new strategies in immunotherapy (Figure 3).

γδ T cells amplify DC functions and antigen presentation

Continuous cross-talk between γδ T cells and myeloid cells is evident in histological studies, *in vitro* culture experiments and in animal models, indicating that this particular subset plays a functional role as an integral component of the innate and adaptive immune system. The first evidence of an influence exerted by γδ T cells on DC system comes from Ismaili et al., showing that human γδ T cells activated *in vitro* by phosphoantigens can maturate of monocyte-derived DCs (35). DCs increase the expression of HLA-DR, CD86, and CD83 on their surface significantly. In particular, this effect seems to be related to TNF-α secreted by γδ T cells. Human

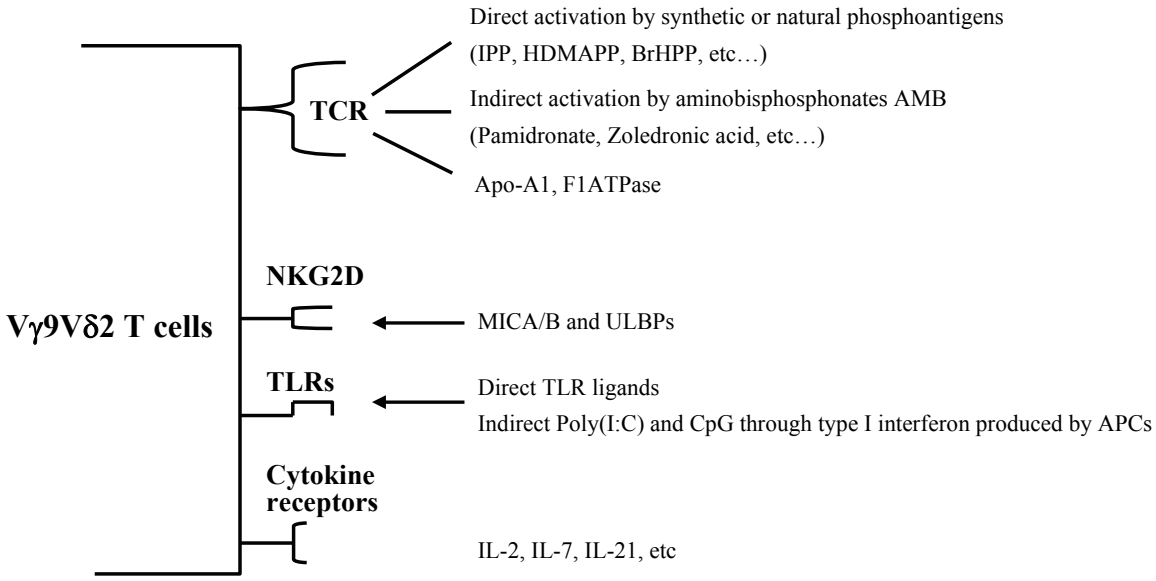

Figure 3. Human Vγ9Vδ2 T cell activation pathways. Activation of Vγ9Vδ2 T cells may be achieved through different stimulatory as TCR-dependent activation and co-stimulatory signals as cytokines, MICA/B or TLR ligands.

γδ T cells are known to produce high amounts of TNF-α and IFN-γ in response to phosphoantigen stimulation. IFN-γ produced by γδ T cells seems to play an important role in the induction of IL-12 by DCs. This DC profile is then responsible for the induction of a high production of IFN-γ by alloreactive T cells, when DCs were cultured with naïve αβ T cells. These findings established a new link between innate and adaptive immunity for γδ T cells that are rapidly activated in course of several infections providing a primary response.

The involvement of γδ T cells in the DC function is not limited to simple maturation induced by cytokines produced upon activation. The interaction between these compartments of the immune system is clearly more important and intriguing. In humans, Vγ9Vδ2 T cells represent the 70% of γδ T cell pool in peripheral blood, but their percentage can be increased in few days after infection and expands to 50% of total T cells. The highly restricted repertoire of their TCR is certainly the major feature of this subset that distinguishes them from conventional αβ T cells. The importance of Vγ9Vδ2 T cells in the systemic immune response is supported by the fact that these cells express high levels of CCR7 for the lymph node homing, where they can interact with DCs. The interaction between DCs and Vγ9Vδ2 T cells has been intensively studied and reciprocal activating interaction were reported by Conti et al. firstly in 2006 (36). In this study, it has been described that co-culture with γδ T cells activated by two classes of phosphoantigens, pamidronate (PAM) and IPP, leads to remarkable phenotypical and functional changes in DCs characterized by a marked up-regulation of CD86 and MHC class I molecules as well as the acquisition of functional activity typical of mature DCs. The modalities through which DC activation was achieved are completely different for the two antigens. In fact, PAM-activated γδ T cells required a physical contact with imDCs to induce full activation, while IPP activated γδ T cells do not require it. However, in both cases, the cytokines released during co-culture, mainly TNF-α and IFN-γ, played a central role in this process. Pamidronate belongs to a new class of synthetic compounds, the aminobisphosphonates, originally developed as therapeutic drugs for osteoporosis, and now used for cancer therapy (37). Bisphosphonates inhibit bone resorption by selective adsorption to mineral surfaces and subsequent internalization by bone-resorbing osteoclasts, where they interfere with various biochemical processes. The simpler, non-nitrogen-containing bisphosphonates (e.g., clodronate and etidronate) can be metabolically incorporated into non-hydrolysable analogues of adenosine triphosphate (ATP), inhibiting ATP-dependent intracellular enzymes. In contrast, the more potent nitrogen-containing bisphophonates (e.g., pamidronate, alendronate, risedronate, ibandronate, and zoledronate) inhibit a key enzyme, farnesyl pyrophosphate synthase, in the mevalonate pathway, thereby preventing the biosynthesis of isoprenoid compounds, which are essential for the post-translational modification of small guanosine triphosphate (GTP)-binding proteins (which are also GTPases), such as Rab, Rho, and Rac. The inhibition of protein prenylation and the function of these key regulatory proteins explain the loss of osteoclast activity (38). These drugs activate human γδ T cells leading to their cytotoxic activity toward a wide spectrum of tumors (39). Bisphophonates cause an accumulation of mavalonate pathway metabolites as IPP activating the TCR of human γδ T cells. In contrast to IPP, PAM induced activation and clustering of γδ T cells is strictly dependent on the presence of antigen presenting cells (40).

According to this, it has been observed that co-culture of PAM activated γδ T cells and imDCs leads to a marked up-regulation of CD25 and CD69 as well as cytokine production by γδ T cells, which is not observed when they are stimulated with PAM in the absence of DCs. Moreover, the evidence that this reciprocal interaction requires cell-cell contact is provided by the role played by CD86 on DCs. In fact, the effect is partially diminished by blocking CD86, suggesting that other DC molecules are likely involved in DC dependent PAM recognition of γδ T cells. In this regard, LFA-1/ICAM-1 seems to be involved in another model, as the interaction between γδ T cells and PAM presenting tumor cells (41). Furthermore, a difference between imDCs and mDCs in the potentiation of Vγ9Vδ2 T cell responses has been found and reported recently. In particular, imDCs show a strong capacity to potentiate cytokine production when cultured with γδ T cells respect to mDCs probably due to the different capacity to up-regulate and/or present Vγ9Vδ2 T cell antigens. Although it is not possible to precisely assess imDC or mDC ability to respond to pharmacological agents or agonists which promote the accumulation of Vγ9Vδ2, a correlation between pamidronate cell responsiveness and pinocytic activity has been previously reported (42). Recently, a more recent and potent aminobisphosphonate, zoledronic acid, has been shown to be a potent inducer of Vγ9Vδ2 T cell activation. In particular, DCs stimulated for 24 hours can induce a vigourous expansion of central memory and effector memory γδ T cells displaying anti-tumor effect. Antigen-specific MHC restricted immune responses, mediated by conventional αβ T cells, were improved by the concurrent γδ T cell activation (43). In summary, these results improve adoptive cell therapy and vaccine interventions against tumor and infections.

Human γδ T cells acquire themselves antigen presentation features

A first indication that human γδ T cells may indeed have APC functions was shown using tonsillar γδ T cells (48). By means of flow cytometric analysis, tonsillar γδ T cells could be separated into two populations based on MHC II expression. MHC II^- cells were in a resting state, whereas those belonging to the MHC II^+ subset were pre-activated. Of note, MHC II^+ γδ T cells expressed many cell surface proteins normally associated to APCs, including the co-stimulatory molecules such as CD80/CD86, the integrin ligand CD54 and co-stimulatory receptor CD40 (49). The appearance of these molecules on γδ T cells has been found be related to their activation. The subsequent study on freshly isolated γδ T cells from peripheral blood confimed that resting γδ T cells lack APC markers, whereas short-term IPP-activated Vγ9Vδ2 T cells express antigen presenting molecules at levels similar to those seen in stimulated monocyte-derived dendritic cells. This phenotype of γδ T cells has also been shown to correlate to the APC functions. In fact, using autologous CD4 T cells as responder cells, it has been demonstrated that activated γδ T cells were formidable antigen processing cells and APCs (49). γδ T-APCs compared favourably with mature DCs in terms of uptake and processing of soluble protein antigens and induction of CD4 T cell proliferation. In addition, they seem many fold more effective APCs than activated αβ T cells or monocytes. This unexpected result opens new ways in the immunotherapy based on γδ T cells and future investigations of γδ T-APCs will need to concentrate on the molecular mechanisms and factors contributing to the *in vitro* generation of this novel type of APCs. However, the physiological relevance remains to be assessed in an *in vivo* model.

Human γδ T cells and DCs during mycobacterial infections

However, in the infection environment, it is easy to hypothesize that a lot of stimuli influence the activation state of DCs and γδ T cells and particularly microbial products. In this context, we have shown that γδ T cells, activated by IPP, could play a complementary role in DC activation during the maturation induced by bacterial products. In fact, DCs and γδ T cells activated by LPS and IPP respectively show an enhanced status of activation and cytokine production, indicating that the singular activation by microbial molecules is not exhausted, but it may be increased in the presence of other cell types (44). The combination of IPP with either imDC or mDC induced a strong activation of γδ T cells, indicating an important role of DC in the boosting of antigen specific response mediated by γδ T cells. Similarly, using the bacillus Calmette-Guerin as model to test these effects in the live mycobacterial infection, we have shown that the co-culture of BCGs infected DC with human phosphoantigen activated γδ T cells, increases the expression of important co-stimulatory molecules, such as CD25, CD40, CD80 or CD86 on DCs. Moreover, this phenotype is associated to an increase of pro-inflammatory cytokine production such as TNF-α, showing a stronger capacity to elicit a local inflammatory immune response. In particular, TNF-α in synergism with IFN-γ triggers anti-mycobacterial mechanisms in macrophages. In contrast, we did not find differences in IL-10/IL-12 production by BCG infected DCs after exposure to activated γδ T cells, but we observed that activated γδ T cell co-culture induces a significant production of IL-15 by DCs (45). IL-15 is a cytokine that has an activity similar to IL-2, playing important functions in lymphocyte differentiation, homeostasis and expanding CD8 memory T cells. Furthermore, IL-15 is able to enhance the IFN-γ production by CD4 and CD8 T cells and warrants the protective immunity to mycobacteria (46). In this context, infected DCs, purified after activated γδ T cell co-culture, stimulate naïve CD4 T cells to produce higher levels IFN-γ than naïve DCs infected with BCG or those uncultured with γδ T cells. Indeed, the production of TNF-α as well as the polarization

of naïve CD4 T cells towards Th1 immunity was significantly increased in our *in vitro* model. This result, as well as the IL-15 production by infected DCs, reveals a relevant role of both cells in the early immunization against MTB and suggests new possible approaches in the TB vaccination strategies.

On the other hand, a new result has been found in our investigations analyzing the effect of DCs infected by BCG on Vγ9Vδ2 T cells. Although, it is still unclear what the role of DCs play in the differentiation of Vγ9Vδ2 T cells following the infection, we demonstrated that BCG infection of human monocyte-derived DCs leads to a rapid and strong activation of co-cultured Vγ9Vδ2 T cells without any further stimulations. After few days, they expand and become a functional competent cytotoxic subset, even if phenotypically immature, being central memory cells and not displaying lymph node homing receptors. In contrast, the Vγ9Vδ2 T cell population derived from BCG-infected DC may be distinguished in two subsets on the basis of perforin expression: perforin^{low} or perforin^{high} Vγ9Vδ2 T cells. They show cytotoxic activity against infected target cells or naïve tumor cell line (47).

The high proliferation of γδ T cells sustained by BCG-infected DCs without exogenous cytokine stimulation leads us to hypothesize that the infected DCs can sustain the local inflammatory response and amplify the signals to themselves. Furthermore, it has been shown that IPP-expanded Vγ9Vδ2 T cells do not arrest the growth of intracellular mycobacteria, whereas those expanded with BCG inhibit intracellular mycobacterial growth. Interestingly, they kill freshly infected monocytes, preserving the viability of co-cultured BCG-infected DCs. Thus, Vγ9Vδ2 T cells could play a role in the homeostasis of the immune response during bacterial infection: they could control the spread of infection, by killing recruited infected monocytes, and preserve the antigen presentation process exerted by *in situ* DCs. We therefore conclude that during bacterial infections, DCs stimulate and expand a Vγ9Vδ2 T cell population, which can functionally recognize infected target cells and complement the DC editing preserving the antigen presentation capability.

Activation of Vγ9Vδ2 T cells by synthetic phosphoantigens in vivo

γδ T cells have antimicrobial as well as anti-tumor activity through the production of pro-inflammatory cytokines, chemokines and cytotoxic molecules such as perforins. This suggests their involvement in the control of infections and tumors *in vivo*, and could be considered as target for new intriguing therapeutic approaches. Moreover, the capacity of γδ T cells to interfere in DC functions would allow their use in specific immunotherapy. Although other non-classical lymphocytes may support DC maturation and contribute to the antigen presentation, γδ T cells in humans represent an easy model to amplify the DC system. Given that the different classes of pharmacological agents are used in therapies for different diseases, the possibility to make new vaccines or adjuvants based on these compounds is very close. A variety of natural and synthetic non-peptidic antigens have been demonstrated to activate γδ T cells such as IPP, DMAPP, GGPP including N-Bps. At present two approaches have been shown exiting results.

There is good evidence that tumors can naturally be controlled by the immune system (50), and most immunotherapy strategies aim to induce adaptive immune response of B and MHC restricted αβ T cells, particularly CD8 T cells. Despite to advances in this area, the actual vaccine based strategies give rare durable responses and active immunotherapy is not yet an established modality. Tumor immunoevasion mechanisms are common and include the down-regulation of tumor-associated antigens, MHC, and co-stimulatory molecules. By contrast αβ T cells, γδ T cells are not MHC restricted and show less dependence on co-stimulatory molecules such as CD28. Moreover, γδ T cells are involved in the resistance of cutaneous carcinogenesis in mice, and display potent cytotoxicity against various human tumor cell lines *in vitro*. Indeed, human Vγ9Vδ2 T cells expanded *in vitro* and transferred to immunodeficient mice, xenografted with tumor cells, showed efficacy against B cell lymphoma, melanoma, and renal carcinoma (51). Building on this, in patients with multiple mieloma or with low-grade non-Hodgkin lymphoma, occurrences of acute phase reaction to intravenously injection of PAM were attributed to the systemic activation of γδ T cells (52), and this provoked the deliberate treatment of lymphoma patients with PAM and IL-2. Promising results were achieved after the patients were prescreened for substantively response to pamidronate and IL-2 of γδ T cells *in vitro*. By several criteria, zoledronate is more potent and efficacious than PAM. Previous studies in patients with breast and prostate tumors showed that zoledronate induced *in vivo* activation of peripheral γδ T cells into more potent cytotoxic and IFN-γ producing cells. Recently, a phase I clinical trial in metastatic HRPC has been conducted by Dieli F, et al. to determine the safety, feasibility, and response induced by Vγ9Vδ2 T cells *in vivo*, using zoledronate alone or in combination with low-doses of IL-2 (53). Results showed that six out of nine patients treated with zoledronate and IL-2 and two out of nine patients treated with zoledronate alone developed a transient flu-like syndrome that was easily controlled, suggesting the absence of any toxicity in this treatment. Six out of nine patients treated with zoledronate and IL-2 showed a strong increase of γδ T cells, across the treatment period, and favorable clinical responses, with a partial remission of cancer. The encouraging prospect that the activation of peripheral blood Vγ9Vδ2 T cells can be efficacious against solid tumors could be explained by the double role played by these cells; activated γδ T cells can infiltrate tumor sites and display cytotoxic activity against tumor cells or they help other cells as DCs to trigger an adequate specific CD8 T cell immune response.

Different interesting results have been shown in animal models to improve the actual vaccination against TB. As

known, although the vaccination with BCG protects children against disseminated TB, it is now clear that it does not protect efficiently against pulmonary disease. Therefore, the ever-increasing incidence of TB worldwide urges to improve this vaccine. It is widely accepted that one of the best immunological predictors of protective and long-lasting immunity to TB is a high frequency of MTB-specific IFN-γ-secreting cells (ISCs) in the peripheral blood (54). A quantitatively sizeable population of effector T cells able to release IFN-γ seems to promote the protective bioactivity of infected macrophages. Therefore, most of current TB vaccine candidates and injection regimens aim to increase the frequency of these MTB-specific ISC. These candidates comprise recombinant BCG, attenuated MTB, modified vaccinia virus, naked DNA, and subunit combinations of either MTB protein antigens or recombinant fusion proteins (55). It exists now a consensus on the ability of heterologous prime-boosts regimens to induce high titres of MTB-specific ISC. The priming with an optimized "starter" such as BCG or improved BCG could likely induce a broad diversity of memory cells. The further boost with antigens common to the priming would expand and differentiate into effector memory, MTB-specific ISC. The immunodominant protein antigens from MTB include members of the "proline-proline-glutamic acid family" proteins (Mtb39a-e), Mtb9.9, TB10.4, so-called "6-kDa early secretory antigenic target" (ESAT-6), and mycolyl transferase complex Ag85A, B, C (55). Despite their good specificity, these purified antigens were weakly immunogenic when injected alone and needed to be combined either to other antigens (hybrid proteins) or to adjuvants. Hybrids of the most promising proteic antigens, namely Mtb72F and H-1 have been generated: they corresponded to fusions Mtb39 to Mtb32 and ESAT-6 to mycolyl transferase complex antigen 85B (Ag85B), respectively. Hybrid H-1 is highly specific of MTB and induces a detectable ISC population but its immunogenicity was quite low, even after several boosts. Therefore, H-1 was combined with adjuvants such as Lipovac or IC31.

Non-human primates γδ T cells like those from rhesus macaques present a TCR with similarity of 90% to the human Vγ9Vδ2 TCR sequence, and the same pattern of specificity for phosphoantigens (56). Therefore, these animals represent a model suited to investigate the role of phosphoantigen-induced γδ T cell responses in immunity to TB. A pioneering analysis of rhesus infected with MTB demonstrated that rhesus γδ T lymphocytes mounted memory responses to mycobacteria. This adaptive response correlated with a faster γδ T cell expansion in the secondary respect to primary exposure to mycobacteria, and was associated with a reduced bacteremia and protection against fatal TB. Furthermore, two studies have independently shown that blood γδ T cells from several monkey species could be monitored using mAb reagents for human T cells. These studies confirmed that phosphoantigen-induced proliferation of naïve, central memory $CD27^+$ and effector memory $CD27^-$ γδ T cells require IL-2 *in vivo* (56, 57). Since macaque γδ T cells seem to react as human Vγ9Vδ2 T cells during BCG vaccination or TB infection and to phosphoantigen stimulation, it has been assessed *in vivo* the bioactivity of a synthetic phosphoantigen combined to a subunit vaccine candidate for TB.

Since TB mainly alters cytokine production and cytotoxic activity but not proliferation of human γδ T cells, this study focused on effector functions in defence against TB: secretion of Th1 cytokines, most notably IFN-γ, and perforin (58). In this paper, we reported an efficient immunogenicity against MTB antigens in naïve cynomolgus after a prime-boost with the hybrid H-1 solubilized in Lipovac adjuvant with or without the synthetic phosphoantigen Picostim. Although the IC31 adjuvant was selected for clinical trial of the H-1 subunit vaccine, in this work we preferred to use the adjuvant Lipovac for its lower bioactivity to detect any additional adjuvant effect of phosphoantigens. However, we found that Picostim, a new generation of synthetic phosphoantigens, induced immediate cytokine production by γδ T cells (IL-2, IL-6, IFN-γ and TNF-α), but their subsequent anergy up to 4 months after the initial administration. This phenomenon could be related to the TCR down-modulation/regulation or apoptosis induced cell death (59). However, this early γδ response translates into differential induction of recall response eliciting the H-1-specific αβ T cell responses, which essentially comprised recall of cytotoxic αβ T lymphocytes specific for Ag85B and few ISC αβ T lymphocytes in both groups of animals. So this study demonstrated that a prime-boost regimen with the H-1/phosphoantigen combination added a primary wave of adaptive immune responses from phospho-antigen-specific γδ T cells to the secondary wave of H-1-specific αβ T cells. In summary, non-human primates vaccinated with phosphoantigens associated to a subunit of anti-tuberculosis vaccine, mount a differential immune response by αβ or γδ T cells, where boosts anergized γδ T cells but promoted αβ recall responses. Finally, these models of usage of phosphoantigens against tumors and infections can help to design subunit combinations promoting memory by both classes of lymphocytes to improve the actual immunotherapy.

Concluding remarks and future directions

γδ T cells appear to combine properties of both adaptive and innate immunities. The identification of unusual compounds that are recognized by human γδ T cells but not by αβ T cells has recently stimulated great interest in the development of γδ T cell based therapies. In contrast to other potential effector cells, it is possible to envisage combined *in vivo* activation and adoptive cell therapy with *ex vivo* expanded γδ T cells, because several drugs as ABPs and synthetic phosphoantigens are licensed for clinical application and in clinical trials respectively. Moreover, the knowledge of different signals as TLR or NKG2D receptors may help to improve and optimize the cell therapy.

Recent advances on their multipotent functions, not only to the innate immune response, but to DC and antigen

presentation system, increase the interest in a possible usage in clinical treatments. The facility to use these compounds to stimulate a cytotoxic response against tumors as well as amplify the antigen presentation of soluble specific peptides through DCs, represents a new possibility in the approaches based on immune cells. Furthermore, the intriguing capacity of γδ T cells to naturally respond to particular infections, such as TB, and the new advances of their capacity to acquire antigen presenting feature themselves, put these cells in a central place mainly in those pathologies where the classical presentation of antigens is compromised. However, γδ T cells are part of the multicellular immune system that is tightly regulated by multiple pathways and cells including the regulatory cells. Therefore, this point of view should be considered. Moreover, the sustained stimulation of γδ T cells by non-peptide antigens could lead to the anergy of these cells and the lack of an important compartment of innate system. However, we still know very little about the nature of γδ T cell antigens, their precise recognition mechanism and their therapeutic relevance. Also the mechanism regarding the DC/γδ T cells cross talk, is still not clear, for instance, the receptors and ligands involved in this interaction, the molecular factors as well as and the possibility to verify this interaction in a model *in vivo*. Future studies should also address the possible advantage of combining γδ T cell therapy with conventional therapy or other therapeutical approaches.

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