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Self/non-self discrimination by human γδ T cells: simple solutions for a complex issue?

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Acknowledgements
We thank Innate Pharma (Marseille, France) for kindly providing phospho-antigens; Brigitte Gicquel and Nathalie Winter (Institut Pasteur, Paris) for kindly providing *Mycobacteria* strains; and Emmanuel Donnadieu (Institut Cochin, Paris) for expert work in videomicroscopy. The authors are supported in their research by grants from INSERM, from the Commission of the European Union Programme 6FP (LSH-CT-2003-503367) TB-VAC, from ANR (projects #A05118GS and PRIB/017), and from ARC (#3662).

Immunological Reviews 2007
Vol. 215: 123–135

Immunological Reviews
0105-2896

Summary: Although γδ T cells express clonally distributed T-cell receptors (TCRs), a hallmark of adaptive immunity, they are classically considered as innate-like effectors, owing to the high frequency of preactivated γδ T cells, with restricted antigen recognition repertoire in particular tissue locations. Actually, such features are shared only by a fraction of γδ T-cell subsets located in the skin and reproductive organ mucosa in rodents or in peripheral blood in humans. By contrast, other γδ subsets, e.g. those found in rodent and human spleen, show diverse antigenic reactivity patterns and mixed naive/memory phenotypes. Thus, γδ T cells are made of both 'primitive' subsets endowed with innate-like properties and 'evolved' subsets able to mount anamnestic responses like conventional major histocompatibility complex-restricted αβ T cells. In this article, we show that human γδ T cells, although heterogeneous, do share recurrent innate features that distinguish them from mainstream αβ T cells. In particular, most of them are activated on TCR- or natural killer receptor-mediated recognition of a restricted set of conserved yet poorly defined endogenous stress determinants. This rather simple recognition mechanism allows human γδ T cells to discriminate healthy cells from altered cells and to exert a variety of immunostimulatory or regulatory functions. The recent availability of synthetic γδ T-cell agonists mimicking these natural stress-induced ligands have fostered development of immunotherapeutic strategies, with broad indications against infectious and tumor diseases, which are briefly reviewed here.

Keywords: *T-cell receptor, T lymphocyte, innate immunity, immunotherapy*

Introduction

The highly restricted T-cell receptor (TCR) V region repertoire of γδ T cells is certainly one of the most salient features that distinguish these lymphocytes from conventional major histocompatibility complex (MHC)-restricted αβ T cells. In humans, γδ T cells use three main Vδ and at most six Vγ region genes to make their TCRs (1, 2). The actual peripheral γδ TCR combinatorial diversity is even more limited because the TCR V region repertoire of human γδ T cells, as in rodents, is highly skewed in particular tissue locations (3, 4). Indeed, most peripheral blood γδ T cells express Vδ2 TCR chains paired with Vγ9 chains, whereas in other tissues, γδ T cells express TCRs

primarily made of Vδ1 or Vδ3 regions paired with a diverse array of Vγ regions. Vδ2 and non-Vδ2 subsets differ in several aspects. Most Vδ2 cells display a memory phenotype acquired during perinatal life, whereas non-Vδ2 cells are mainly naive in young adults (5). Moreover, most Vγ9Vδ2 T cells from peripheral blood lymphocytes (PBLs) react against the same related set of non-peptidic, phosphorylated compounds (3), whereas non-Vδ2 cells seem to recognize heterogeneous yet ill-defined antigens presumably unrelated to Vδ2 agonists. In this article, we review recent studies, providing new insights into the fine mode of activation of Vγ9Vδ2 T cells by their non-peptidic agonists and into the biological significance of this recognition process. We underline the similarities between the activation contexts of Vδ2 and non-Vδ2 subsets and show how recognition of a restricted set of stress-induced natural killer receptor (NKR) and TCR ligands allows these cells to express immunoprotective and regulatory functions complementary to those ensured by conventional αβ T lymphocytes.

Target cell recognition by human γδ T cells

At least three kinds of receptor–ligand interactions contribute to γδ T-cell activation by their target cells: adhesion molecules, TCRs, and NKRs. The adhesion receptors playing a key role in T-target cell interactions, such as leukocyte-function-associated antigen-1/intercellular adhesion molecule-1, are shared in most instances by both γδ (6, 7) and αβ T cells and are not discussed further.

γδ TCR stimuli: Vγ9Vδ2 T cells

Vγ9Vδ2 T cells recognize in a TCR-dependent fashion a restricted set of phosphorylated compounds referred to as 'phospho-antigens', which are produced through the isoprenoid biosynthetic pathway (reviewed in 3, 8–10). The most potent agonists, like hydroxy-dimethyl-allyl-pyrophosphate (HDMAPP), are intermediates of the so-called '1-deoxy-D-xylulose-5-phosphate pathway', restricted to plant cells and some microorganisms. Intermediates of the mevalonate (MVA) pathway used by mammalian cells and some bacteria, such as isopentenyl pyrophosphate (IPP), can also activate Vγ9Vδ2 T cells, although at concentrations of 10- to 100 000-fold higher than those for microbial agonists. Because IPP production is strongly upregulated in tumor cells (11), recognition of isoprenoid pathway intermediates by Vγ9Vδ2 lymphocytes allows discrimination not only between infected and non-infected cells (through detection of highly potent microbial phospho-antigens) but also between resting and transformed cells (through detection of overexpressed IPP). Two additional classes of compounds, namely aminobisphosphonates (ABPs) and alkylamines, have been shown to activate Vγ9Vδ2 T cells. Through their ability to inhibit farnesyl pyrophosphate synthase (FPPS) (12), an enzyme acting downstream of IPP synthesis along the MVA pathway, ABPs promote intracellular accumulation of IPP. Such a mechanism most likely underlies the Vγ9Vδ2-stimulating activity of ABPs (11, 13). The mode of action of alkylamines has remained debated until recently. It was initially assumed that these compounds, like phospho-antigens, were directly recognized by Vγ9Vδ2 T cells (14). However, owing to the non-phosphorylated nature of alkylamines, such a hypothesis was difficult to reconcile, with studies showing a key contribution of pyrophosphate moieties to Vγ9Vδ2 agonist activity of phospho-antigens. A recent study strongly suggests that like ABPs, alkylamines promote intracellular accumulation of Vγ9Vδ2 agonists derived from the MVA pathway, presumably through inhibition of FPPS (15). Therefore, it appears that in all instances, Vγ9Vδ2 T cells are stimulated by phosphorylated intermediates of the isoprenoid biosynthetic pathway (*Fig. 1A*).

How precisely the Vγ9Vδ2 T cells are activated by phospho-antigens remains unclear. Phospho-antigen-induced activation of T cells requires expression of a Vγ9Vδ2 TCR, as indicated by antibody blocking and gene transfer approaches (16, 17). Moreover, phospho-antigens induce within seconds a burst of respiratory activity (18) and within minutes a strong Ca^{2+} signaling by Vγ9Vδ2 T cells (E. Scotet, M.-C. Devilder, M. Bonneville, unpublished data), suggesting a very swift engagement of TCRs on incubation with phospho-antigens. However, all attempts to show cognate interactions between recombinant, soluble Vγ9Vδ2 TCRs and phospho-antigens have failed so far. Because respiratory burst and Ca^{2+} signaling events are observed in pelleted Vγ9Vδ2 cells but not when they are maintained in suspension, phospho-antigens may not be recognized *per se* but instead may be recognized in association with a yet undefined surface presenting receptor (PR). It is also possible that phospho-antigens, whose Vγ9Vδ2 agonist activity closely correlates with their chemical reactivity (reviewed in 8), induce conformational or chemical modification of a surface receptor(s), which would be recognized in turn by Vγ9Vδ2 TCRs (*Fig. 1B*). Several groups have tried to get evidence on binding between phospho-antigens and their putative PRs through analysis of target cell susceptibility to Vγ9Vδ2 T-cell lysis after preincubation with phospho-antigens. Although previous studies failed to show any activation of Vγ9Vδ2 cells by target cells pretreated with semipurified microbial agonists or IPP (19), efficient Vγ9Vδ2 sensitization of target cells preincubated with the most potent agonists, like

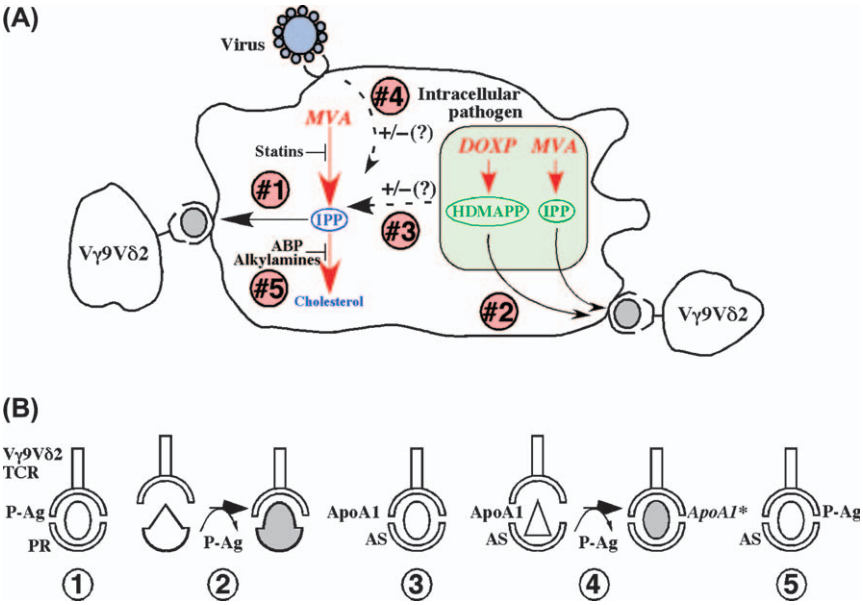

***Fig. 1.* Activation of Vγ9Vδ2 T cells by phospho-antigens.** (A). Discrimination between normal versus altered self by Vγ9Vδ2 T cells through recognition of isoprenoid pathway intermediates. Vγ9Vδ2 T cells are activated either by weak phosphorylated agonists (such as IPP) produced through the MVA pathway found in mammalian cells or by strong agonists (such as HDMAPP) produced through the 1-deoxy-D-xylulose-5-phosphate pathway found in some microorganisms. Upregulation of IPP production in activated/transformed cells (pathway 1) and/or of HDMAPP production in cells infected by intracellular bacteria (pathway 2) allows respective recognition of tumor and infected cells by Vγ9Vδ2 T cells. Bacteria and/or viruses may also modulate endogenous IPP production (pathways 3 and 4, respectively) and subsequent Vγ9Vδ2 activation by infected cells. Finally, pharmacological inhibitors of the MVA pathway acting upstream (like statins) or downstream of IPP synthesis (like ABP or alkylamines), respectively, decrease or enhance treated cell recognition by Vγ9Vδ2 T cells (pathway 5). (?) refers to pathway not yet formally proven. (B). Possible mechanisms of TCR-mediated activation of Vγ9Vδ2 T cells by their phosphorylated and tumor antigen. 1: Recognition of a complex formed by P-Ag and a putative PR. 2: Recognition of a surface molecule modified by phospho-antigen. 3: Recognition of a complex made of apoA1 and AS. 4: Recognition of AS loaded with a chemically modified apoA1 (possibly mediated by phospho-antigen). 5: Recognition of P-Ag in the context of AS. # indicates the pathway number. P-Ag, phospho-antigen. * refers to chemically modified ApoA1.

HDMAPP, was more recently reported (20). In our experience, the latter results are most likely explained by insufficient washing of HDMAPP, which acts at picomolar concentrations, rather than by stable binding of phospho-antigens to their PRs (M.-C. Devilder, E. Scotet, M. Bonneville, unpublished data). In line with these results, several recent observations suggest that phospho-antigen-induced activation is a very transient process. First, phospho-antigen-stimulated γδ T cells exhibit 'spiky' Ca^{2+} fluxes, unlike antigen-stimulated αβ T cells, which show an early and sustained Ca^{2+} signaling (*Fig. 2A*). Moreover, the phosphorylation kinetics of protein tyrosine kinases linked to the TCR signaling cascade are quite delayed in phospho-antigen-activated γδ T cells compared with the peptide-stimulated αβ T cells (*Fig. 2B*). These results suggest serial but highly transient engagements of TCRs by phospho-antigen–PR complexes, possibly explained by formation of very labile phospho-antigen–PR complexes and/or low-affinity interactions between TCR and phospho-antigen–PR complexes.

In addition to phospho-antigens, Vγ9Vδ2 T cells were recently shown to recognize a complex formed between apolipoprotein (apo)A1and adenosine triphosphate synthase (AS), an enzymatic complex normally found in the inner mitochondrial membrane that is translocated to the cell surface of some tumor cells (21). Activation of Vγ9Vδ2 T cells by apoA1 is reminiscent of apoH recognition by some murine γδ hybridomas (22). As the oxidized forms of apoA1 have been detected in particular inflammatory contexts linked to atherosclerosis (reviewed in 23), the above results could reflect an implication of γδ T cells in lipid metabolism and immune control of some cardiovascular pathological processes. The apoA1 also contributes to innate defense against some parasitic infections (24) and may represent a stress-associated cue recognized by γδ T cells. However, although apoA1 enhances tumor cell recognition by Vγ9Vδ2 T cells, it is neither necessary nor sufficient for this process because the purified apoA1 does not readily activate Vγ9Vδ2 T cells, unlike beads coated with purified AS alone. Given the ability of AS to bind phosphorylated compounds, this receptor could be a logical candidate for phospho-antigen presentation (*Fig. 1B*), although this association remains to be proven in cell-free systems. The mechanisms

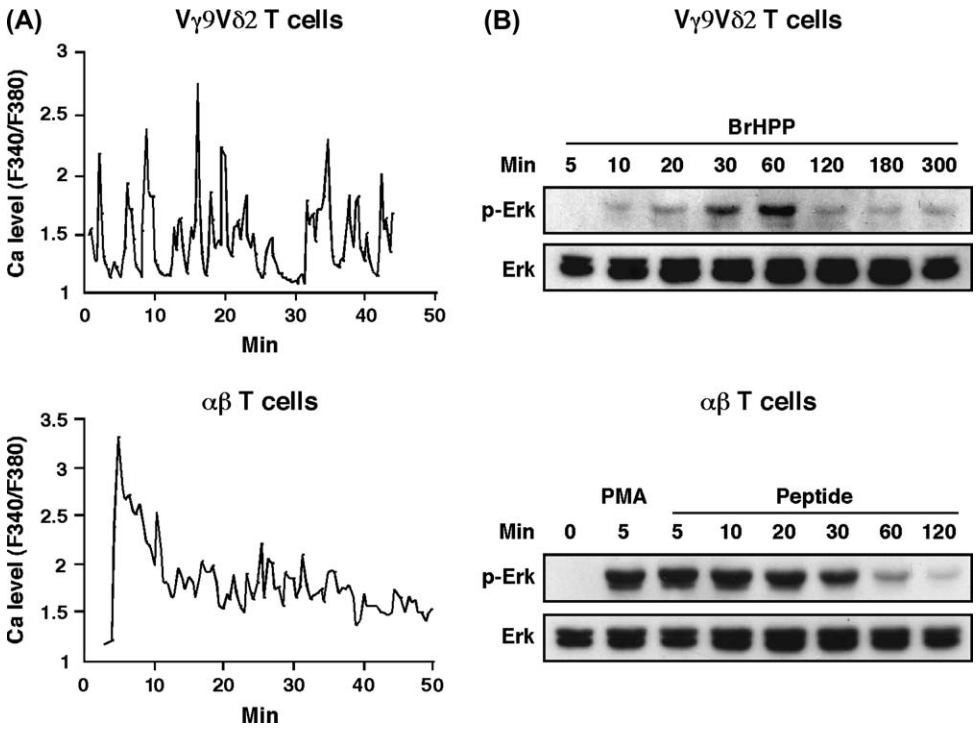

Fig. 2. Vγ9Vδ2 and αβ T lymphocytes exhibit distinct signaling profiles following antigenic stimulation. (A) Characteristics of T-cell Ca^{2+} responses following antigenic activation. Fura-2-AM-loaded T cells (Vγ9Vδ2 clone G42 and αβ clone A4.5) were co-incubated with DCs in the presence of either soluble phospho-antigen (BrHPP, 3 μM) or HLA-A*0201-Epstein–Barr virus peptide (BMLF1/GLCTLVAML, 10 μM) respectively. Single-cell Ca^{2+} levels were analyzed by collecting 510-nm emissions (340/380-nm excitation) for the indicated times (Min). (B). Kinetics of extracellular signal-regulated kinase (Erk) activation in human Vγ9Vδ2 and αβ T cells. T-cell clone GR72 (Vγ9Vδ2) and clone A4.5 (αβ) were incubated in the presence of BrHPP (3 μM) and BMLF1 peptide (20 μM) respectively for the indicated times (Min). Total cellular proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and showed by western blot using an anti-p42/44 Erk antibody (recognizing phosphorylated and active forms of Erk-1 and Erk-2) (*p-Erk*). Normalization was performed by stripping the antibody and subsequent incubation with anti-Erk antibody (recognizing total p42/44 Erk) (*Erk*). PMA was used at 0.1 μM as a positive control. PMA, Phorbol 12-myristate 13-acetate.

of surface translocation of AS are still unclear. Such a process could result from fusion events between the plasma membrane and the inner membrane of disrupted mitochondria that are generated during apoptosis. AS recognition could therefore allow Vγ9Vδ2 T cells to sense stressed and/or pre-apoptotic cells. In light of a study suggesting strong upregulation of mycobacterium-derived AS subunits in infected macrophages (25) and owing to the extensive phylogenetic conservation of AS from bacteria to mammalian cells, AS may also represent, like microbial phospho-antigen, a pathogen-associated molecular pattern, permitting detection of infected cells by Vγ9Vδ2 effectors. AS has also been found on the surface of the endothelial cells (26, 27). Therefore, its targeting by Vγ9Vδ2 T cells could reflect a possible implication of this γδ subset in the regulation of angiogenesis and anti-tumor immunity.

γδ TCR stimuli: non-Vδ2 subsets

The extensive structural diversity of Vδ1 and Vδ3 TCRs and the existence of Vδ1 clones reactive against MHC, MHC-like, or non-MHC molecules (*Table 1*) suggest recognition of a highly diverse and heterogeneous set of antigens by non-Vδ2 cells. However, several important issues remain unsettled. Cognate interactions between non-Vδ2 TCRs and any of the above antigens have not been shown yet, and they have been indirectly supported by gene transfer experiments in a few cases only [e.g. for CD1c-specific clones (28)]. MHC class-I-chain-related gene A (MICA) has also been proposed as an important tumor antigen recognized by Vδ1 T cells, owing to TCR-dependent recognition of MICA-positive tumor cells by Vδ1-lymphocytes infiltrating colon carcinomas (29–31). However, the very low affinity of MICA–Vδ1 TCR interactions estimated by surface plasmon resonance analyses raises doubts about the functional relevance of MICA or MHC class-I-chain-related gene B (MICB) recognition by Vδ1 TCRs (32). Moreover, the frequency of Vδ1 or Vδ3 clones directed against any of the above antigens has not been assessed but is presumably low, at least with respect to CD1 molecules. Hence, the antigens recognized by non-Vδ2 T cells remain in most instances unknown.

Table 1. **Marked reactivity of human γδ T cells toward self-stimuli upregulated in various physiopathological contexts**

γδ subset	Physiopathological context	Antigen origin	Antigen structure	References
Vδ1	Tumors	Self	MICA/MICB	Groh *et al.* (29,30)
		Self	?	Halary *et al.* (37)
	Infections	Self	CD1c (+ self ?)	Spada *et al.* (28)
		Self	?	Poccia *et al.* (33)
		Self	?	Halary *et al.* (37)
	Alloreactivity	Non-Self	HLA-DR	Flament *et al.* (85)
Vδ2	Tumor cells	Self	IPP	Gober *et al.* (11)
		Self	AS/Apo A1	Scotet *et al.* (21)
	Viral infections	Self	?	Poccia *et al.* (33)
	Bacterial infections	Self	IPP/HDMAPP/TubAg	Bonneville and Fournie (3), Espinosa *et al.* (8), Poupot and Fournie (9), Bonneville and Scotet (10)
		Non-Self (?)	?	
Vδ3	Viral infections, tumors	Self	?	Halary *et al.* (37)
Vδ5	Viral infections, tumors	Self	?	Halary *et al.* (37)

? refers to undefied antigen or not yet formally proven mechanism.

Non-Vδ2 γδ T cells are expanded in various infectious contexts involving intracellular bacteria (*Mycobacteria*, *Listeria*, *Borrelia*, etc.) and viruses [HIV, cytomegalovirus (CMV)] (reviewed in 10, 33). In most instances, the stimuli that trigger Vδ1 expansion are not derived from pathogens but instead correspond to endogenous gene products presumably upregulated on infection. For instance, most Vδ1 T-cell clones expanded after coculture with autologous dendritic cells (DCs) loaded with microbial lipids derived from Gram-negative bacteria (*Escherichia coli*, *Salmonella typhimurium*) actually recognize endogenous DC-derived stimuli upregulated by interleukin (IL)-12 (34). Both Vδ1 and Vδ3 subsets are dramatically expanded in immunosuppressed patients undergoing CMV reactivation, and a large fraction of clonally expanded Vδ1 and Vδ3 cells from these patients recognizes in a TCR-dependent fashion CMV-infected fibroblasts *in vitro* (35, 36). A recent study indicates that the putative Vδ1/Vδ3 antigens recognized by CMV-reactive subsets are not virally encoded but instead correspond to endogenous stress-induced ligands possibly shared by CMV-infected cells and several colon tumors (37). Importantly, the frequency of Vδ1 PBLs reactive against these CMV-induced antigens might be quite high, even in healthy donors who have not undergone CMV reactivation (38). The antigen recognized by non-Vδ2 T cells expanded in the above infectious contexts have not been characterized, but the fact that Vδ1 T-cell responses are not blocked by monoclonal antibody directed against known classical or non-classical MHC molecules suggests recognition of a new class of conserved stress-induced antigens.

NKR signals

Human γδ T cells frequently express activating and/or inhibitory NKRs (iNKRs) that can fine-tune their activation threshold (reviewed in 39, 40) (*Fig.* 3). NKG2D, an activating C-type lectin receptor directed against MICA/MICB and UL16-binding protein (ULBP) molecules, seems to be a major costimulator of both Vδ2 and non-Vδ2 γδ T cells (41). Indeed, NKG2D is recruited within the Vγ9Vδ2 immunological synapse (42) and enhances recognition by Vγ9Vδ2 T cells of *Mycobacteria*-infected DCs and various MICA/MICB$^+$ or ULBP$^+$ hemopoietic and non-hemopoietic tumors (7, 43). NKG2D has also been involved in Vδ1 T-cell-mediated recognition of infected DCs and epithelial and hemopoietic tumors (29, 34, 44). NKG2D is upregulated by inflammatory cytokines (e.g. IL-15) (45), and NKG2D ligands are induced after a physical or genotoxic stress and/or along infection by intracellular pathogens. Therefore, NKG2D is a key stress sensor that strongly enhances recognition of altered or infected self by human γδ T cells.

The γδ T cells also express several other activating and iNKRs belonging to either the C-type lectin (e.g. CD94/NKG2A, CD94/NKG2C) or the immunoglobulin [e.g. killer cell immunoglobulin-like receptor or immunoglobulin-like transcript 2 (ILT2)] superfamilies. Although ILT2 is frequently found on Vδ2 subset and presumably on non-Vδ2 subset (C. Benezech, R. Breathnach, personal communication), CD94/NKG2A is expressed by the majority of Vδ2 cells but not by Vδ1 cells (46). NKR expression has been correlated with acquisition of effector memory markers within both mainstream MHC-restricted αβ T cells and non-classical, CD1d-restricted, invariant natural killer T (iNKT) cells. Thus, the different NKR

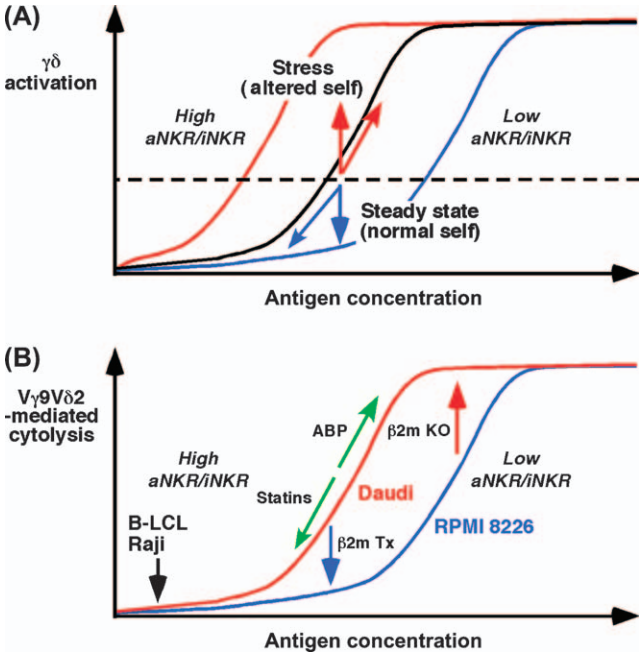

***Fig. 3.* Modulation of γδ T-cell activation by TCR and NKR signals.** (A). The amount of antigen required to activate γδ effector/proliferative responses (activation threshold represented as a dotted line) is controlled by inhibitory and activating signals delivered by engaged NKRs expressed by γδ T cells. It is decreased at high aNKR/iNKR signaling ratios (red antigen response curve) or increased at low aNKR/iNKR ratios (blue antigen response curve). In the steady state, low expression of γδ TCR antigen and aNKR stimuli and/or high expression of MHC class I/class Ib that engage ILT2 and CD94/NKG2A iNKR prevents self-recognition by γδ T cells (blue arrows). Stress (infection/cell transformation) may lead to increased γδ antigen and/or aNKR ligand expression and decreased MHC class I/class Ib expression (red arrows), resulting in γδ T-cell activation. (B). Balance between TCR and NKR signals in tumor B-cell lines showing different susceptibility to Vγ9Vδ2 T-cell-mediated lysis. Vγ9Vδ2 T cells efficiently lyse the Daudi Burkitt's lymphoma and the RPMI 8226 myeloma but not the Raji Burkitt's lymphoma nor B-LCL. Daudi cells do not express MHC class I and class Ib on their surface because of the lack of functional β2m, and are thus unable to deliver iNKR signals to ILT2/NKG2A+Vγ9Vδ2 T cells. Transfection of functional β2m (β2m Tx) in Daudi cells strongly inhibit their lysis by Vγ9Vδ2 T cells, presumably through iNKR engagement (blue arrow). Daudi lysis can also be modulated by inhibiting or increasing endogenous IPP levels using pharmacological inhibitors of the MVA pathway (green arrows). RPMI 8226 cells are as efficiently lysed by Vγ9Vδ2 than Daudi cells, although they express 'normal' levels of MHC class I/class Ib molecules. Because Daudi and RPMI 8226 cells express similar levels of NKG2D ligands, this finding suggests that the latter cells express higher endogenous levels of phospho-antigen than the former cells. Knockdown of β2m (β2m KO) expression in RPMI 8226 cells prevents iNKR engagement on Vγ9Vδ2 effectors and strongly enhances target cell lysis (red arrow). Finally, Vγ9Vδ2-mediated lysis of B-LCL or Raji cells is not enhanced by MHC class I/class Ib knockdown, consistent with very low expression of Vγ9Vδ2 antigen. aNKR, activating natural killer receptor; β2m, β2 microglobulin; B-LCL, B-lymphoblastoid cell lines.

phenotypes of Vδ2 versus Vδ1 cells may merely reflect distinct functional/memory status of these γδ subsets (5). On a functional standpoint, both ILT2 and CD94/NKG2A receptors strongly inhibit killing of MHC class I^+ or class Ib^+ target cells by Vγ9Vδ2 T cells (47-49). As the partial downmodulation of MHC class I and class Ib molecules, which frequently occurs along cell transformation, prevents engagement of ILT2 and inhibitory CD94/iNKG2A receptors, these iNKRs may ensure efficient discrimination of transformed versus normal cells by Vγ9Vδ2 T lymphocytes (48, 49) (*Fig.* 3).

Factors contributing to human γδ T-cell activation and homeostasis

Cytokines

Purified Vγ9Vδ2 cells are unable to proliferate after *in vitro* antigen stimulation, unless supplemented with IL-2 or incubated with stimulated conventional T helper (Th) cells (50). Similarly, strong *in vivo* expansion of Vγ9Vδ2 PBLs is observed in primates injected with both Vγ9Vδ2 synthetic agonists and IL-2 but not in individuals receiving Vγ9Vδ2 agonists alone (51, 52). Terminal differentiation of most Vγ9Vδ2 PBLs into effector or effector/memory T cells probably explains their poor IL-2 responses. In this regard, most IL-2-producing Vγ9Vδ2 PBLs display a typical $CD27^{bright}CD11a^{dull}$ phenotype shared with conventional αβ naive T cells (5). IL-15 can also restore the *in vitro* proliferative responses of antigen-stimulated Vγ9Vδ2 T cells to a similar extent as IL-2 (A. Thedrez, E. Scotet, M. Bonneville, unpublished data). As for conventional memory T cells, IL-15 seems to be an important homeostatic cytokine for peripheral Vγ9Vδ2 PBLs, at least *in vitro* (53). Several other growth factors, such as IL-7 and IL-21, may also contribute, although to a lesser extent, to proliferation of this γδ subset (A. Thedrez, E. Scotet, M. Bonneville, unpublished data). Soluble factors contributing to non-Vδ2 T-cell activation remain ill defined as in most instances, the fine antigen specificity of Vδ1 and Vδ3 subsets has not been characterized. Moreover, in-depth *ex vivo* functional analysis of these subsets has been hampered by their low frequency in readily accessible samples such as peripheral blood.

Enhancement of γδ T-cell responses by DCs

In line with their effector/memory phenotype, *ex vivo* Vγ9Vδ2 T cells can be readily activated by specific agonists in the absence of any professional antigen-presenting cells (APCs). However, Vγ9Vδ2 cytokine responses but not cytolytic activity are significantly enhanced when these cells are stimulated in the presence of DCs (54). Quite unexpectedly, immature DCs (iDCs) are much better cytokine-response enhancers than mature DCs (mDCs). The factors responsible for this iDC-mediated potentiating effect remain unknown. They are presumably

membrane bound, as suggested by *Transwell* experiments, but are distinct from several costimulators known to be strongly expressed on iDCs, such as CD2, LIGHT (homologous to lymphotoxins, inducible expression, competes with herpes simplex virus glycoprotein D for herpes virus entry mediator, a receptor expressed on T lymphocytes), or APRIL (a proliferation-inducing ligand) (M.-C. Devilder, S. M, E. Scotet, and M. Bonneville, unpublished data). Importantly, iDC-mediated potentiation of cytokine responses is also observed on antigen-stimulated MHC class-I-restricted $CD8^+$ T-cell clones and on *ex vivo* $CD4^+$ T cells stimulated by superantigen, which indicates that it is a general property of *ex vivo* memory T cells (M.-C. Devilder, E. Scotet, M. Bonneville, unpublished data). These *in vitro* findings, whose physiological relevance is still unclear, are clearly in line with previous reports implicating DCs in both the *in vivo* maintenance of conventional memory T cells (55) and optimal *in vivo* activation of glycolipid-specific memory natural killer T cells (56, 57).

Human γδ T-cell physiological roles

γδ T-cell-mediated priming of adaptive and innate immune effectors

Both Vδ1 and Vδ2 γδ T cells can activate iDCs *in vitro* through various mechanisms (*Fig.* 4). Activation of iDCs into IL-12-producing mDCs mediated by CD1c-restricted γδ T cells is a two-step process initiated by CD40 and tumor necrosis factor-α (TNFα) engagement and completed through an interferon (IFN)-γ/IL-12-positive feedback loop (58). IL-12-dependent upregulation of endogenous DC-derived Vδ1 ligands by some intracellular bacteria may also trigger IFNγ release by CD1-independent Vδ1 cells and presumably subsequent iDC activation (34). Moreover, a recent study indicates that Vδ1 synovial fluid lymphocytes from patients with Lyme arthritis can promote activation of *Borrelia*-infected iDCs in a Fas/Fas-ligand-dependent fashion (59).

Unlike CD1c-specific Vδ1 cells, Vδ2 T cells do not directly recognize iDCs, unless incubated with Vγ9Vδ2 agonists or infected by Vγ9Vδ2-stimulating pathogens. Antigen-stimulated Vγ9Vδ2 cells can induce rapid and complete iDC activation into IL-12-producing cells (54). As for Vδ1 or iNKT cells, this process involves CD40/CD40 ligand, TNFα, and IFNγ (60). The iDC-mediated potentiation of Th1 cytokine production by γδ and, more generally, by memory T cells may further enhance their own activation and the subsequent priming of proinflammatory adaptive immune responses. Accordingly, both iNKT cells and murine γδ T cells were shown to induce priming of conventional Th1 responses (61–63) in rodent models and, in the former case, in humans as well (64). Natural memory T cells, such as iNKT or Vγ9Vδ2 lymphocytes, could be major players in such a priming process, owing to their high frequency in pre-immune individuals and their reactivity against broadly distributed conserved antigens. Conventional memory αβ T cells directed against common environmental agents (like influenza, Epstein–Barr virus, CMV, or *Mycobacteria*) could also significantly contribute to such a phenomenon because these cells represent up to several percent of the peripheral lymphoid pool in primed individuals. The iDC-mediated potentiation of memory-T-cell cytokine responses and subsequent priming of naive immune responses, which remains to be formally shown for both human γδ and conventional T cells, could be key in situations where T-cell-stimulating pathogens are unable to induce full iDC activation, as is the case for *Mycobacteria* or Toll-like receptor-deficient α-proteobacteria infections.

γδ T cells as effectors of protective immunity against pathogens and tumors

Numerous studies have reported *in vitro* reactivity of both Vδ2 and non-Vδ2 γδ T-cell subsets against a broad range of tumor cell lines and normal cells infected by a variety of viruses, parasites, and bacteria [reviewed by Bonneville and Fournie (3), Poccia *et al.* (33), Dechanet *et al.* (65) and Ferrarini *et al.* (66)]. With respect to transformed cells, the range of cell lines recognized by Vγ9Vδ2 T cells, initially thought to be primarily restricted to hemopoietic tumors (47), was recently extended to several solid tumors, such as renal and colon carcinomas (7, 43). Both Vδ2 and non-Vδ2 subsets are able to directly kill target cells *in vitro*, express bactericidal molecules such as granulysin, and express proinflammatory cytokines involved in clearance of tumor and infected cells (3, 28, 67, 68). Therefore, a direct implication of both subsets in anti-tumor and anti-infectious protective immunity is highly likely. However, Vδ2 and non-Vδ2 subsets may exert distinct functions according to the type of tumor or infectious agent and/or the tissue environment. For instance, although Vδ2 T cells predominate within the mycobacterial lesions in patients with tuberculosis, Vδ1 cells are preferentially expanded in HIV-infected patients with systemic *Mycobacterium avium* complex infections (69). Major Vδ1/Vδ3 expansions have been reported in HIV-infected patients and in immunosuppressed patients undergoing CMV reactivation [reviewed by Poccia *et al.* (33) and Dechanet *et al.*], whereas Vδ2 cells are mostly anergic in such situations. In a tumor context, the frequency of Vδ2 cells within lymphocytes infiltrating solid tumors is generally low, even within Vγ9Vδ2-susceptible tumors such as renal and colon

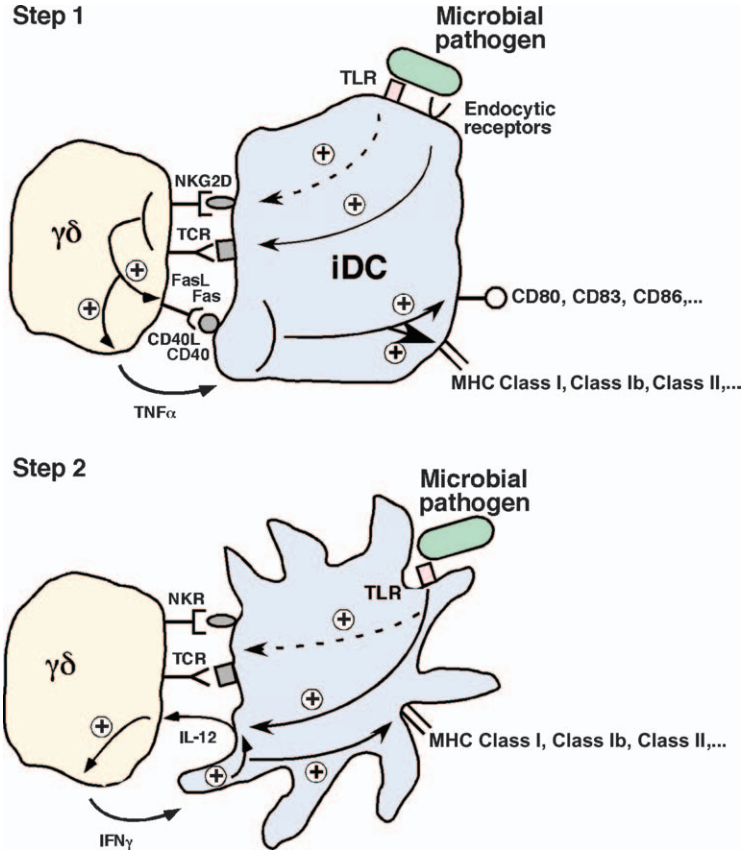

***Fig. 4.* Cross talk between γδ T cells and DCs.** The γδ T cells are activated by DCs through recognition of either microbial antigen (e.g. phospho-antigen specific for Vδ2 cells) or self-antigen upregulated on viral or microbial infection (as is presumably the case for CMV-specific Vδ1 cells or virus-induced Vδ2 cells). Microbial or viral pathogens may also induce, presumably through Toll-like receptor engagement and expression of stress-associated ligands recognized by NKG2D, which can then enhance activation of NKG2D$^+$ γδ T cells. Both TCR- and NKR-induced signals trigger expression of T-cell-derived molecules belonging to the TNF/TNFR family (CD40 ligand, Fas ligand, TNFα). These molecules in turn may upregulate expression of costimulatory (CD80, CD83, CD86) and MHC class I/class II molecules on iDCs after engagement of their respective counter receptors (step 1). Although CD40 ligand/CD40 signaling plays an important role in iDC maturation induced by Vδ2 cells, Fas ligand/Fas signaling may significantly contribute to iDC activation induced by some Vδ1 cells (e.g. specific for *Borrelia burgdorferi*). Soluble factors such as TNFα can also contribute to DC maturation mediated by antigen-stimulated Vδ2 cells and CD1c-specific Vδ1 cells. Complete maturation of DCs into IL-12p70-producing cells (step 2) requires both Toll-like receptor engagements by PAMPs and T-cell-derived IFNγ. IL-12 production can be readily induced by high concentrations of PAMPs alone (e.g. microgram amounts of lipopolysaccharide) or by low PAMP concentrations (e.g. nanogram amounts of lipopolysaccharide) together with T-cell-derived maturation signals such as IFNγ. IL-12 upregulates IFNγ production, which in turn upregulates IL-12 through a positive feedback loop. IL-12 may also upregulate γδ TCR/NKR ligands, as suggested by analysis of some Vδ1 subsets expanded *in vitro* after incubation with DCs treated with Gram-negative bacterial extracts. Finally, the ability of iDCs to potentiate both TNFα and IFNγ production by γδ T cells may further enhance T-cell-induced iDC activation. PAMPs, pathogen-associated molecular patterns.

carcinomas (7, 43). By contrast, Vδ1 cells are quite frequent within T cells infiltrating solid tumors (reviewed in 70). Similarly, Vδ1 cells but not Vδ2 cells are present within non-inflamed skin, consistent with a specific role played by the former subset in skin immunosurveillance (71).

In line with the above-mentioned observations, *in vivo* studies in severe combined immunodeficient disease (SCID)/hu models indicate that although both Vδ2 and Vδ1 cells can clear human melanoma cells in grafted mice with SCID when injected within the tumor, only Vδ1 cells can clear tumor cells when injected systemically (72). These distinct behaviors of Vδ2 versus Vδ1 cells could be explained by expression of a different set of homing receptors on these γδ subsets (71, 73). However, such a functional dichotomy between Vδ1 and Vδ2 cells may not apply for all solid tumors, as suggested by enhanced elimination of melanoma (74) or renal carcinoma cells by Vδ2 cells in very similar SCID/hu models (Y. Morel, personal communication). In mice with SCID/huPBL, Vγ9Vδ2 PBLs activated *in vivo* can migrate to and clear human kidney tumors grafted subcutaneously. These seemingly discrepant results could be explained by upregulation of skin-homing receptors on activated Vγ9Vδ2 T cells and/or by different levels of

tumor-induced inflammation in the above models, leading in only some cases to *in situ* migration of Vγ9Vδ2 cells, which have a strong tropism for inflammatory sites. In an infectious context, Bukowski and colleagues (75) have reported efficient clearance of intraperitoneal bacterial infections in SCID mice following adoptive transfer of Vγ9Vδ2 PBLs, in accordance with their *in vitro* anti-bacterial effector properties (see above).

γδ T cells as professional APCs

As suggested by a recent study (76), Vγ9Vδ2 T cells can acquire within few hours after antigenic activation several attributes of APCs, such as expression of costimulatory molecules (e.g. CD40), which allow them to efficiently stimulate naive T cells. These are clearly new and quite exciting observations, whose overall physiological relevance remains to be assessed in *ad hoc in vivo* models.

In vivo/in vitro manipulation of human γδ T cells for immunotherapeutic purposes

Ongoing Vγ9Vδ2 T-cell-based immunotherapies

Broad reactivity of Vγ9Vδ2 PBLs toward a broad range of tumors and recent availability of several synthetic clinical-grade Vγ9Vδ2 agonists or pharmacological inhibitors able to trigger selective Vγ9Vδ2 expansion and enhance Vγ9Vδ2 recognition of tumor cells have fostered development of several new immunotherapeutic approaches targeting this γδ subset. Toxicity and efficacy of adoptively transferred Vγ9Vδ2 lymphocytes after *in vitro* expansion are currently assessed in patients with renal carcinoma through several phase I and phase I/II clinical trials (77). Although it is too early to draw conclusions about the therapeutic efficacy of such protocols, these trials have shown that it is possible to expand under GMP conditions up to 8 billion T cells highly enriched for Vγ9Vδ2 lymphocytes (*Fig. 5A*), which can be injected into patients without overt toxicity. Nevertheless, *in vitro* proliferative responses of Vγ9Vδ2 PBLs from patients with cancer here turned out to be significantly lower than those obtained with their healthy-donor-derived counterparts. This phenomenon could be accounted for by chronic Vγ9Vδ2 stimulation, tumor-induced specific or systemic anergy induction, or iatrogenic effects. Irrespective of the mechanisms involved, this decreased proliferative activity is an important drawback that may significantly hamper development of Vγ9Vδ2 T-cell-based adoptive immunotherapy. This problem could be circumvented by administration of allogeneic Vγ9Vδ2 T cells derived from healthy donors because this γδ subset is not alloreactive and has not been involved so far in graft-versus-host (GVH) reactions.

Several active immunotherapy trials aim at directly activating Vγ9Vδ2 T cells *in vivo*, using either synthetic GMP-grade phospho-antigen or ABP (78, 79). As mentioned, these compounds do not induce Vγ9Vδ2 expansion *in vivo* in primates, unless co-injected with IL-2 (51, 52). In monkeys treated with both γδ T-cell agonists and IL-2, the frequency of Vγ9Vδ2 PBLs increases dramatically between days 5 and 8 but rapidly goes down to basal levels within 10–15 days. Moreover, the magnitude of Vγ9Vδ2 PBL expansions rapidly decreases after repeated treatments with γδ T-cell agonists and IL-2 (*Fig. 5B*). This outcome could reflect an exhaustion of the naive compartment and/or terminal maturation of Vγ9Vδ2 T cells in treated monkeys. However, *ex vivo* phenotypic studies did not evidence long-term modification of the effector/memory phenotype of Vδ2 PBLs from treated monkeys. Repeated phospho-antigen treatments may also lead to depletion of a high-affinity Vγ9Vδ2 subset, as suggested by preliminary *in vitro* analyses of primate PBL responses to phospho-antigen (J.-J. Fournié, unpublished data). In humans, ABPs can induce dramatic expansion of Vγ9Vδ2 PBLs associated with an acute-phase response in a small fraction of treated patients, indicating that γδ T cells can proliferate in an autocrine fashion on antigenic stimulation *in vivo*. However, in line with primate studies, most ABP- or phospho-antigen-treated patients do not show any *in vivo* amplification of their Vγ9Vδ2 PBLs, unless they are co-treated with IL-2. Importantly, stable responses or partial tumor remission has been observed in several patients with multiple myeloma receiving ABP and IL-2 (79, 80). Confirmation of these encouraging yet very preliminary results may come from several ongoing phase I or phase I/II trials, which are based on repeated administration of synthetic phospho-antigen and IL-2 in patients carrying either hemopoietic or solid tumors.

Optimization of γδ immunotherapeutic approaches

Researchers designing γδ T-cell-based immunotherapies are still facing several problems that currently preclude careful assessment of the therapeutic potential of these protocols. Current optimization strategies in this field (*Fig. 5C*) aim to (i) improve long-term maintenance of adoptively transferred γδ T cells through administration of homeostatic cytokines (such as IL-15) or induction of lymphodepletion prior to adoptive cell transfer; (ii) combine ABP treatment, which increases tumor cell susceptibility to Vγ9Vδ2 lysis, with adoptive transfer of Vγ9Vδ2 T cells; (iii) use allogeneic Vγ9Vδ2 T cells, which are presumably unable to induce GVH owing to their Th dependency and their lack of alloreactivity; (iv) improve effector functions of adoptively transferred cells during their *in vitro* expansion, e.g. through addition of IL-15 (which may

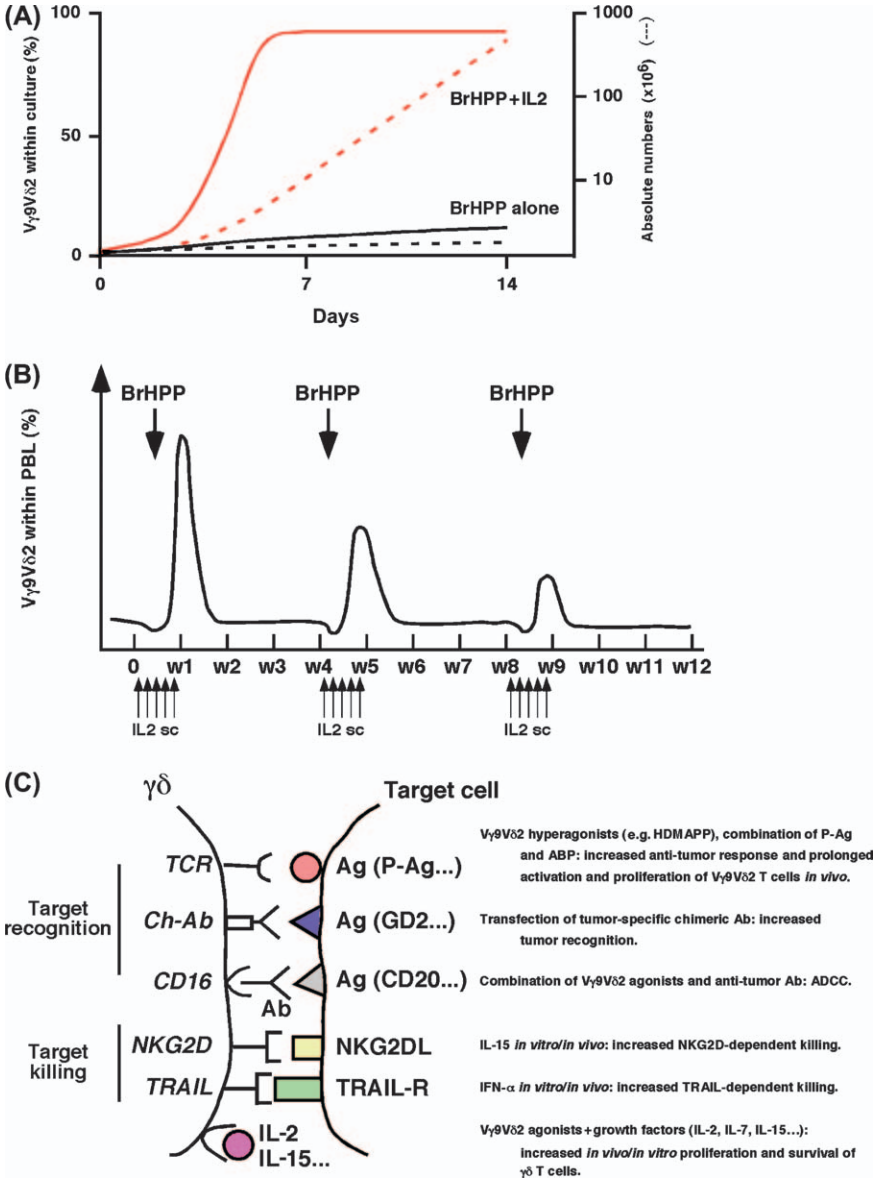

***Fig. 5. In vivo/in vitro* stimulation of Vγ9Vδ2 T cells for immunotherapeutic purposes.** (A). Selective *in vitro* expansion of Vγ9Vδ2 T cells within PBLs cultured with synthetic P-Ag (BrHPP/Phosphostim®, Innate Pharma SA, Marseilles, France) and recombinant IL-2. Growth curves deduced from representative data obtained with *in vitro*-stimulated PBLs from healthy donors (A. Thedrez, E. Scotet, M. Bonneville, unpublished data). (B). *In vivo* expansion of Vγ9Vδ2 PBLs in Cynomolgus monkeys treated with Phosphostim® and IL-2 sc. Percentage and absolute numbers of Vγ9Vδ2 PBLs peak at 7–10 days after each treatment but go down to basal levels after 2 weeks. Moreover, the intensity of Vγ9Vδ2 *in vivo* expansion rapidly decreases after repeated treatments, suggesting γδ T-cell exhaustion/anergy [typical response pattern drawn from Sicard *et al.*(52)]. A similar phenomenon is observed in patients receiving multiple treatments with Vγ9Vδ2 agonists and IL-2 (P-Ag or ABP) (J. Bennouna, personal communication). (C). Possible ways to improve γδ T-cell-based immunotherapeutic protocols. Ab, antibody; IL-2 sc, IL-2 subcutaneously; P-Ag, phospho-antigen; TRAIL, TNF-related apoptosis-inducing ligand.

increase cytolytic properties and anti-tumor reactivity of γδ T cells through upregulation of NKG2D signaling), IL-4 (which may allow acquisition of anti-inflammatory and/or B-helper functions for some infectious and/or allergic indications), or IFNα (which may increase TNF-related, apoptosis-inducing, ligand-dependent killing of tumor cells); and (v) transduce Vγ9Vδ2 T cells with tumor-specific TCRs (e.g. specific for leukemia-specific minor histocompatibility antigen) (81) or chimeric tumor-specific immunoglobulin receptors (e.g. specific for gangliosides or CD19) (82).

The γδ T cells can improve monoclonal-antibody-mediated tumor clearance *in vitro* and *in vivo* [e.g. anti-GD2 (82)], presumably through antibody-dependent, cell-mediated cytotoxicity. In this respect, a significant fraction of Vγ9Vδ2

T cells expresses intermediate levels of CD16, which is preferentially upregulated within the effector/recently activated (the so-called 'TEMRA') T-cell subset (83, 84). This provides a strong rationale for combination therapies using tumor-specific monoclonal antibodies and either adoptively transferred γδ T cells or γδ T-cell agonists. *In vivo* Vγ9Vδ2 T-cell responses following treatment with ABP or phosphoantigen may be improved through replacement of IL-2 by IL-15 or IL-7, which contribute to peripheral maintenance of memory γδ T cells and may prevent exhaustion of the naive compartment, respectively. Administration of strong Vγ9Vδ2 agonists, like HDMAPP, can induce prolonged *in vivo* expansion of Vγ9Vδ2 T cells in primates, which may last up to several weeks. Such new generation compounds represent promising means to improve the pharmacodynamics of γδ T-cell responses in patients with cancer and possibly their anti-tumor efficacy.

Conclusions

Studies performed during the past 5 years have brought important new information regarding the specificity, activation modalities, and *in vivo* function of human γδ T cells. However, we still know very little about the nature of human γδ antigens, their precise recognition mechanisms, and their therapeutic potential. It is clear that the major efforts recently put into large-scale production of clinical-grade γδ T-cell agonists and the design and optimization of immunotherapeutic protocols targeting γδ T cells will certainly help address several of these challenging issues. Another major challenge will be to define the right therapeutic window for immunotherapeutic treatments, including γδ T-cell-based ones and the proper way to combine these approaches with new therapies targeting tumor cells and/or angiogenesis.

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