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Abstract

T lymphocytes expressing the γδ-type of T cell receptors for antigens contribute to all aspects of immune responses, including defenses against viruses, bacteria, parasites and tumors, allergy and autoimmunity. Multiple subsets have been individualized in humans as well as in mice and they appear to recognize in a TCR-dependent manner antigens as diverse as small non-peptidic molecules, soluble or membrane-anchored polypeptides and molecules related to MHC antigens on cell surfaces, implying diverse modes of antigen recognition. We review here the γδ TCR ligands which have been identified along the years and their characteristics, with emphasis on a few systems which have been extensively studied such as human γδ T cells responding to phosphoantigens or murine γδ T cells activated by allogeneic MHC antigens. We discuss a speculative model of antigen recognition involving simultaneous TCR recognition of MHC-like and non-MHC ligands which could fit with most available data and shares many similarities with the classical model of MHC-restricted antigen recognition for peptides or lipids by T cells subsets with αβ-type TCRs.

MESH Keywords Animals ; Antigens ; metabolism ; Humans ; Immunity, Innate ; Ligands ; Models, Immunological ; Receptors, Antigen, T-Cell, gamma-delta ; metabolism ; Signal Transduction ; T-Lymphocytes ; immunology

Author Keywords Gamma delta lymphocytes ; T cell receptor ; Innate immunity ; Antigen recognition ; Major Histocompatibility complex

Introduction

On the basis of the T cell antigen receptor (TCR) chains which they express and use to recognize antigens, human and murine T lymphocytes comprise two subtypes: T cells using an α/β TCR heterodimer (αβ T cells) and those using a γ/δ heterodimer (γδ T cells), which associate to a common CD3 signal transduction module (Hayday 2000 ; Kabelitz and Wesch 2003 ; Pennington et al. 2005). The majority of αβ T cells in blood and secondary lymphoid organs express clonotypic TCR chains with a strong diversity originating from usage of a large number of Vα and Vβ genes, as well as extensive junctional diversity allowing the recognition of a large array of antigenic peptides in complex with polymorphic presenting MHC class I or class II molecules. Besides these “classical” T cells, NKT cells are present in small numbers in blood, liver and spleen and recognize non-polymorphic CD1d, an MHC-class I-like antigen (MHC-Ib) which associates with lipids. Among these, a subset of invariant NKT cells (iNKT) use a precise combination of Vα and Vβ genes and carry very limited junctional diversity, having in particular no N-region insertions in the third complementarity-determining region (CDR3) of their TCR. They may recognize a limited set of antigenic endogenous lipids. Other NKT cells use a limited set of V genes and an extensive junctional diversity to recognize exogenous bacterial lipids presented by CD1d (Godfrey et al. 2004 ; Godfrey et al. 2010). Mucosal-associated invariant T cells (MAIT) are abundant in blood and are also found in epithelial tissues. They are in fact semi-invariant as although they also use a defined combination of Vα/Jα and use preferentially a limited set of Vβ genes, these can combine with multiple Vβ genes carrying some N-region junctional diversity (Treiner et al. 2005). This limited diversity is probably used to recognize uncharacterized antigens from pathogens in combination with MR1, another MHC-Ib antigen (Le Bourhis et al. 2010). All these αβ T cell populations are present in both humans and mice and their biology appears closely homologous.

In contrast and although they are also present in both species, there is accumulating evidence that human and murine γδ T cells have substantially diverged. Subpopulations of γδ T cells are recognized in both systems. In the murine system, most of these are characterized by their predominant localization in defined epithelial tissues and have characteristics of NKT or MAIT cells by their expression of preferential Vγ and Vδ TCR region genes. Vγ5Vδ1 cells and Vγ6Vδ1 cells colonize the skin (DETC) and the reproductive tract epithelium (r-IELs), respectively, early during the fetal life and do not express junctional diversity. Other γδ populations appear later and use particular Vγ genes in correlation with their homing to preferential epithelial tissues which continues after birth (intestinal, lung and other mucosal γδ IELs). These Vγ genes are used in combination with multiple Vδ genes and the diversity of their repertoire is further increased by an extensive junctional diversity (Allison and Raulet 1990). In humans, and especially in adults, one γδ subset expressing Vγ9 and Vδ2 TCR regions starts to expand early after birth and is particularly abundant in adult blood an peripheral lymphoid tissues, although it is also present in epithelia (usually ~70% of γδ cells). These Vγ9Vδ2 cells, also called Vγ2Vδ2, carry extensive junctional diversity in CDR3 regions but share a similar antigenic reactivity although they may display diverse effector functions (Nedellec et al. 2010). A specific tissue distribution for other γδ cell populations in humans as a function of V gene expression is not as clear as in mice. Although γδ T cells expressing Vδ1 regions predominantly reside in epithelial tissues (and in large proportion in the intestine), they are diverse and may express different Vγ regions (Holtmeier 2003).

It thus appears that there are for γδ cells as well as for αβ cells multiple populations which may differ significantly in their biological properties. It seems likely that invariant DETC in the mouse recognize a tissue-specific and non-polymorphic endogenous ligand to perform homeostatic functions in the skin (Jameson and Havran 2007). Other populations may have completely different roles from the recognition of endogenous stress signals in epithelia to the recognition of pathogen-derived antigens so that a single antigen recognition mechanism may not apply to all γδ cell populations (Born et al. 2010). In mice as well as in humans, the total γδ TCR repertoire is characterized by a limited number of available Vγ and Vδ genes, and preferential usage of a few V segments in preferential Vγ/Vδ combinations. In contrast, there is a potential for extensive junctional diversity in particular in CDR3δ which results from usage of multiple Dδ gene segments in tandem, so that most of the TCR diversity is confined to CDR3 regions (Hayday 2000). This potential diversity is not used by the murine invariant γδ populations and how it is used by other populations is still not really understood. The limited V region repertoire probably reflects the specialization of γδ cells for the recognition of a limited number of conserved ligands expressed in selected tissues during stress or infection.

Activation of γδ cells may be achieved through multiple non-TCR activating receptors, among which TLRs (Martin et al. 2009 ; Mokuno et al. 2000 ; Pietschmann et al. 2009), CD16, CD226 (Gertner-Dardenne et al. 2009 ; Toutirais et al. 2009), NKRs (Das et al. 2001 ; Fisch et al. 2000 ; Halary et al. 1999 ; Lafarge et al. 2005), CD28 (Sperling et al. 1993) and NKG2D. These receptors are thought to be co-stimulators rather than providers of a primary stimulus (Nedellec et al. 2010). Focusing on TCR activation, the list of antigens which have been found to activate γδ T cells in a TCR-dependent manner include polypeptides, non-peptidic molecules and an increasing list of MHC-like structures so that no unifying concept about γδ T cell reactivity has yet emerged. The necessity for antigen presentation in the case of γδ T cells has been questioned as some soluble antigens can activate γδ cells in the absence of antigen presenting cells (Born and O'Brien 2009). The understanding of γδ cell biology is further complicated by the wide expression of NK receptors for MHC and MHC-like molecules (NKR) by murine and human γδ T cells and these may act in conjunction with the TCR to modulate its activity.

We will review here the different putative γδ TCR ligands that have been found recently and in the past and discuss the data which support their role in TCR ligation. Through a more detailed description of a few murine and human models which have been extensively studied, we will discuss how the recognition of many diverse structures may be integrated in a common model of antigen recognition involving simultaneously MHC-like and non-MHC-like ligands and sharing many features of classical antigen restriction. For Vγ/δ gene nomenclature used here, refer to (Kabelitz and Wesch 2003).

γδ TCR ligands belonging to the MHC family

Besides few exceptions, γδ T cells do not recognize antigens restricted by MHC complex class I and class II molecules as αβ T cells do. Spits *et al.* reported isolation of cytotoxic human γδ T cell clones which proliferated against JY EBV-B-cells and were inhibited by anti-MHC-I antibody and anti-β2m. The fine specificity of these clones was however not reported (Spits et al. 1989). MHC-Ia and Ib molecules interact with NKRs and modulate antigen recognition on many γδ T cell populations. This has led to the concept that these T cells recognize antigens while sensing the context of their expression on target cells (Bonneville and Fournie 2005). Nevertheless, although γδ cells recognizing MHC-like molecules were initially thought to be exceptions, the list of MHC-Ib molecules which seem to interact directly with γδ TCRs has recently grown and the concept that MHC molecules are essentially targets for NKRs deserves to be revisited.

CD1

CD1 is a non polymorphic MHC-Ib protein associated with β2-microglobulin (β2m). It is expressed on immature dendritic cells, can present phospholipids and comprises four isoforms in humans (CD1a, b, c, d) and one in mice (CD1d) (Vincent et al. 2003). Human γδ T cell clones activated by the recognition of CD1c have been isolated from peripheral blood (Faure et al. 1990 ; Leslie et al. 2002 ; Spada et al. 2000). In the study by Faure *et al.* , CD1c reactivity was found in one γδ T cell clone out of 43 obtained from peripheral blood. In the study by Spada *et al.* , these were raised by repeated stimulation with dendritic cells and mycobacterial extracts, although these extracts were not required for reactivation. Due to the ability of CD1c to present lipids, it was assumed that these clones were recognizing endogenous lipids bound to CD1c. γδ TCR transfection in TCR-deficient J.RT3-T3.5 cells together with the usage of CD1c-transfected target HeLa or C1R cells have confirmed the specific recognition of CD1c in a TCR-dependent manner. All four clones analyzed expressed Vδ1 associated with a different Vγ chain (Vγ1.2, Vγ1.3, Vγ1.4, Vγ2) with no recognizable homology in the CDR3γ or δ regions.

Human T cell clones recognizing the lipid antigen phosphatidyl-ethanolamine have been isolated from normal blood and were enriched in the blood and nasal mucosa of patients allergic to pollen lipids. These were all found to be strictly antigen-specific and restricted by CD1d (Russano et al. 2006 ; Russano et al. 2007) when tested on HeLa cells transfected with various CD1 isoforms. Some expressed Vδ2 whereas the majority expressed Vδ1. Most of them were also $CD4^+$ as opposed to most human γδ cells. Although their reactivity appears very similar to that of NKT cells, the involvement of the TCR in this reactivity has not been formally proven. In the same line, Cui *et al.* (Cui et al. 2005 ; Cui et al. 2009)have reported that human γδ T cells from peripheral blood could be activated by

lipid-A, a component of LPS, loaded on monocyte-derived dendritic cells or CD1-transfected C1R cells. The response appeared to involve specifically CD1b or CD1c (not CD1a or CD1d), and could be blocked by anti-TCRγδ, anti-lipid-A and anti-CD1b/c antibodies. Nevertheless, the direct involvement of the TCR in lipid-A recognition is not ascertained and lipid-A/CD1-mediated stimulation may represent a co-stimulus for γδ T cells since anti-TLR-4 antibodies also inhibited γδ cell reactivity. In addition, Vδ2$^+$ as well as Vδ1$^+$ cells seemed to be responding and anti-TCR antibodies used for γδ-cell preparation may have had a role in activation.

MHC class-I-related chain A/B (MICA/B)

MICA/B are polymorphic β2m-linked MHC-Ib molecules constitutively expressed in intestinal epithelial cells and many tumors. They are inducible by stress on multiple cell types. Both are ligands for the activating receptor NKG2D expressed on NK cells, some cytolytic CD8$^+$ T cells and subsets of γδ cells (Champsaur and Lanier 2010). Groh *et al.* have obtained MICA/MICB-reactive γδ T cell clones from human intestinal mucosae and found that these reacted specifically against MICA-transfected C1R targets but not against C1R transfected with another NKG2D ligand (ULBP1). All 5 clones described expressed Vδ1 associated with different Vγ chains (Vγ1.3, 1.4, 1.5 or 1.8), again with no obvious homology in CDR3γ/δ besides a common usage of Jδ1, suggesting that CDR3 regions do not contribute significantly to MIC reactivity (Groh et al. 1998). The usage of MICA tetramers indicated that these specifically stained MICA-reactive clones (Wu et al. 2002). Strikingly, some Vδ1/Vγ1.4 clones were non-reactive to MICA and did not stain with MICA tetramers, suggesting either a role for CDR3 or the involvement of co-receptors or co-activators besides the TCR. As γδ T cells frequently express the NKG2D receptor which also binds to MIC proteins, this may have been involved. TCR-dependence of this recognition was however analyzed using TCR transfer in HPB-ALL T cells which lack NKG2D expression. Although these cells could not be functionally tested for antigen reactivity, MICA tetramers specifically stained cells transfected with reactive TCRs. Strikingly again, although the contribution of gamma chains did not appear previously, transfection of mixed combinations of Vγ and Vδ chains from responder clones produced TCRs which did not stain with the tetramers.

MICA reactivity was also described for γδ clones infiltrating ovarian carcinomas. These clones were expanded using recombinant α1α 2 MICA protein constructs and expressed Vδ1 associated with Vγ2, Vγ3 or Vγ4 (Zhao et al. 2006). Vδ1$^+$ iIELs also react against MICA/B from multiple primate species despite considerable variation or alterations in the structure of α-helical regions of theses α1α2 domains (Steinle et al. 1998). Soluble recombinant single-chain TCR constructs corresponding to these TCRs as well as mixed combinations of Vγ and Vδ chains were subsequently tested for binding on HeLa cells which expressed MICA. Authors show a strong dependence on Vδ1 chains for soluble TCR binding to HeLa cells with little dependence on the associated γ- chain. In both studies, TCR-MICA interactions could be inhibited by antibodies recognizing the α1 or α2 domains of MICA.

UL16-binding protein family (ULBP/RAET1)

This family of MHC-Ib antigens comprises six known functional members in humans (Eagle et al. 2009 ; Eagle and Trowsdale 2007 ; Samarakoon et al. 2009). They are non-polymorphic, do not associate with β2m and mice also express genes homologous to the human ones. Members of this family of NKG2D ligands are frequently expressed in tumors, virally-infected cells and selective normal tissues, and are inducible by retinoic acid and cellular stress. They can be linked to the plasma membrane through a trans-membrane domain or a GPI-anchor. Unlike MIC proteins, they lack the α3 MHC-I domain (Champsaur and Lanier 2010). Their expression is modulated following CMV infection through binding to UL16 viral protein and through miRNAs (Stern-Ginossar et al. 2007 ; Stern-Ginossar and Mandelboim 2009). In normal tissues, ULBP4 expression is restricted to skin and small intestine (Bacon et al. 2004 ; Chalupny et al. 2003 ; Conejo-Garcia et al. 2003 ; Eagle et al. 2006 ; Radosavljevic et al. 2002). γδ T cells infiltrating colonic and ovarian tumors could be stimulated and amplified with immobilized recombinant ULBP4, and the majority of these expressed Vδ2 although Vδ1 cells were also detected in cultures (Kong et al. 2009). The paring of Vδ2 with Vγ9 in these tumor infiltrating lymphocytes (TIL) was not documented. Sequencing of Vδ2 chains revealed considerable diversity in CDR3 regions although most were associated with Jδ1. Using a Vγ9Vδ2 TCR-Fc recombinant soluble construct carrying the Vδ2 TIL sequence and a syntheticVγ9-Jγ1.2 (also called JP) γ-chain, the authors have shown that this could stain specifically EL4 cells transfected with ULBP4. It also stained Daudi lymphoma cells which are classical targets for Vγ9Vδ2 cells, although it remains to be shown that this is due to ligation of ULBP4 on these cells. Reactivity of Vγ9Vδ2 cells against EL4-ULBP4 could be inhibited by both anti-TCR and anti-NKG2D antibodies, indicating that both receptors were involved in ULBP4 recognition.

ULBP1 has been shown to be determinant in the recognition of hematopoietic tumors by Vγ9Vδ2 lymphocytes expanded from peripheral blood. Nevertheless, in Lança's study (Lanca et al. 2010), this appeared to be mediated essentially through ULBP1 interaction with NKG2D rather than with the TCR and was not hampered by anti-TCR antibodies. Similarly, although ULBP3 expression on B-CLL is determinant for their recognition by some Vδ1 cells, there is no evidence in favor or against an involvement of the TCR in their recognition of ULBP3 (Poggi et al. 2004).

HLA-E and Qa-1

Barkonyi *et al.* reported that Vγ9Vδ2 cells expanded from peripheral blood recognized preferentially and made conjugates with choriocarcinoma targets (JAR) transfected with HLA-G/E whereas Vδ1 cells did not. There is no evidence for an involvement of the TCR in HLA-E/G recognition (Barakonyi et al. 2002). Nevertheless, in the mouse, the activation of some γδ cells were reported to be dependent on the recognition of Qa-1, the murine counterpart of HLA-E. These included a Vγ2/Vδ6.1 hybridoma (DGT3) recognizing Glu-Tyr polymer + Qa-1b (Vidovic and Dembic 1991 ; Vidovic et al. 1989), and, more recently, a population of $CD8^+$ iIEL expressing Vγ 4 and expanding in the course of salmonella infections (Davies et al. 2004). Although Qa-1 can activate CD94/NKG2D family of receptors on γδ T cells, these receptors were found absent on this particular γδ subset. The presence of these cells in the intestine is strongly dependent on TAP and Qa-1. Although not a definitive proof, this strong dependency on Qa-1 for development suggests an involvement of the TCR in Qa-1 recognition. Nevertheless, since Qa-1 expression is dependent on endogenous peptides for expression and may be altered by TAP deficiency, TAP sensitivity of Vγ4 T cell development does not imply that they recognize peptides in a Qa-1-restricted manner (Kambayashi et al. 2004).

T10/T22

Since the initial description that some murine γδ T cells were stimulated by MHC antigens encoded in the T region of the MHC (Bluestone et al. 1988 ; Bonneville et al. 1989), T cell clones recognizing the two closely homologous MHC-Ib antigens T10 and T22 have been isolated and extensively studied (reviewed in (Chien and Konigshofer 2007 ; Meyer et al. 2010)). These molecules are not ubiquitously expressed and cannot present peptides due to an alteration of their α1/α2 domains similar to that of MICA (Wingren et al. 2000). KN6 and G8 γδ T cell clones can be directly activated by immobilized T10 or T22 molecules in complexes with β2m, independently of peptides. T22 tetramers can stain 0.2 to 2% of splenic γδ T cells in normal mice and most are $V\gamma1^+$ or $V\gamma4^+$ (Crowley et al. 2000). They also stain few $V\gamma7^+$ cells in the intestine. Although T22-specific clones may also use different Vγ regions, it was found that there is a strict requirement for the expression of a CDR3δ region bearing the mostly Dδ2-encoded motif (S)EGYEL, whereas surrounding N, P and Jδ-encoded diversity had little influence on antigen recognition. The crystallographic structure of the G8 TCR in complex with T22 has been obtained and confirmed the involvement of the CDR3δ loop in the contact with T22. Strikingly however, it was found that G8 binding to T22 differed considerably from that of αβ TCRs with peptide-MHC complexes, in that the two complexes were bound at an angle and CDR loops other than CDR3δ did not seem to contribute significantly to this binding (Adams et al. 2005). Thus, the G8 TCR does not recognize its target MHC antigen as do αβ TCRs which must align with the MHC molecule and use all six CDR regions to make contacts with the MHC-peptide complex.

This unexpected interaction suggests that the G8 TCR might interact simultaneously with molecules surrounding the T22 complex on stimulatory cells, an interaction which may involve other CDR regions and could influence fine specificity. The T22 molecule would thus be only part of the physiological TCR ligand. T22 tetramers can stain efficiently cells expressing the relevant TCRs and accordingly T10 or T22 recombinant complexes can stimulate G8 cells in experimental settings (Chien and Konigshofer 2007). This may be explained by an inherent high affinity of the (S)EGYEL CDR3δ motif for T10/T22, multivalence of the tetramers and the high amounts of antigen used experimentally for stimulation. This interaction may however not be sufficient in more physiological conditions. This hypothesis would lead to reconsider the interpretation of findings relating to G8-TCR T cell development in mice lacking T10/T22 and will be discussed further below.

MHC-class II molecules

A human γδ T cell clone (Vγ9Vδ2) with dual specificity has been reported to recognize mycobacterial antigens in a non-MHC restricted fashion and a tetanus toxoid (TT) peptide in the contect of an MHC-II molecule (DRw53) (Holoshitz et al. 1989). Kozbor *et al.* reported the isolation of TT-specific γδ T cell clones from a TT-hyper-immune individual. These responded specifically to TT in the presence of autologous APCs, were $CD8^+$, could be inhibited by anti-DR antibodies and appeared DR4-restricted (Kozbor et al. 1989). The frequency of such apparent MHC restriction to protein antigens is unknown. Moreover, although the reactivity was strikingly similar to that of MHC-II restricted αβ T cells, evidence that TT was recognized in the form of classical peptide-MHC complexes was not reported. MHC-II-alloreactive murine γδ clones have been obtained (Matis et al. 1989). LKB5 (specific for IE alleles) and LKD1 (specific for IA^d) have been isolated and LKB5 has been extensively studied (see (Chien et al. 1996 ; Chien and Konigshofer 2007) for a complete review on this system). Both clones were found to bind MHC independently of peptide and peptide-loading mechanisms and could be stimulated by purified peptide-stabilized MHC-II complexes as well as MHC-II-transfected CHO cells. Moreover, they share identical Vγ and Vδ sequences except in CDR3 regions (Matis et al. 1989 ; Meyer et al. 2010). The scanning of IE mutations which affected TCR recognition have revealed that the γδ TCR epitopes did not involve amino acid locations usually involved in MHC-II recognition by αβ TCRs but instead a functional polymorphic epitope near β67/70. Recognition was also affected by a residue at one end of the peptide groove (α79) possibly affecting the glycosylation state of the molecule on the α82 residue (Schild et al. 1994). From these data, the interaction between LKB5 TCR and IE must differ fundamentally from that of αβ TCR and IE. Nevertheless IE^k +peptide tetramer complexes could stain relevant IE^k -restricted αβ T cell clones but did not stain LKB5, possibly because of a particularly low affinity of the

TCR for the MHC ligand (Chien and Konigshofer 2007). We suggest the possibility that the LKB5 TCR co-recognizes an unknown membrane ligand associated with IE outside of the peptide binding groove resulting in a low affinity for IE alone.

γδ TCR ligands non related to MHC

Many of these putative antigens have been reviewed recently (Born and O'Brien 2009 ; Konigshofer and Chien 2006). For convenience, non-MHC ligands for Vγ9Vδ2 cells are described in separate sections.

Herpes simplex virus 1-glycoprotein I (HSV-gI)

HSV-gI (US7 gene product) is thought to be involved in viral assembly and intracellular routing in neurones. In HSV-infected mice, Bluestone and coworkers have found that γδ T cells baring Vγ1.2 and Vδ8 expend in infected mice and react in vitro specifically to gI (Johnson et al. 1992). T cell stimulation could be obtained with purified protein in the absence of APCs, did not require glycosylation and was conformation-dependent (Sciammas and Bluestone 1998). gI could also be recognized on the surface of gI-transfected fibroblasts, and cells expressing gI mutants incapable to reach the cell surface were not recognized. Recognition did not require MHC-I, MHC-II or TAP, excluding conventional recognition of antigenic peptides (Sciammas et al. 1994). gI was thus recognized either as a conformed soluble or membrane-anchored protein and specialized APCs were not required. HSV-reactive γδ cells have also been found in humans (Bukowski et al. 1994). These belonged to the Vγ9Vδ2 subset and probably recognized an endogenous, virus-induced ligand instead of a viral protein as they were equally reactive with T cell blasts infected with HSV and the unrelated vaccinia virus.

Lipids

The possible involvement of lipids in conjunction with CD1 isoforms in the stimulation of γδ cells has been mentioned above in the CD1 section. γδ T cells responsive to glycerophospholipids in an unusual manner have also been described (Born et al. 2003). Indeed, some murine γδ T cell hydridomas derived from non-immunized mice were found to respond in a TCR-dependent manner to cardiolipin and, although more weakly, to the related phospholipids phosphatidyl glycerol, phosphatidic acid and cytididine diphosphate-phosphatidyl glycerol, whereas phophatidyl inositol, phosphatidyl-serine and phosphatidyl choline were not stimulatory. In addition to particular head groups, the acyl chains were also important for antigenic activity as deacylated lipids did not stimulate. Responsive γδ cells all expressed murine Vγ1 and two characterized Vδ chains belonged to the Vδ6 family. Stimulation did not require APCs. TCR transfer experiments have confirmed the strict TCR dependence of lipid recognition. However, these hybidomas as well as the TCR tranfectants were characterized by a strong, also TCR-dependent, self reactivity (lymphokine secretion in absence of added lipid) as well as by their dependence on a serum component which could be replaced by β2-glycoprotein-1 (β2-GP1). This protein (also called apolipoprotein H) can be a component of circulating lipoproteins and was independently shown to bind to cell surfaces, in particular apoptotic cells, and to undergo conformation and orientation changes upon lipid binding (Wang et al. 2002 ; Wang et al. 2000). As pathogenic human anti-phospholipid autoantibodies have been shown to recognize cardiolipin-β2-GP1 complexes, it is quite possible that the murine hybridomas recognize similar complexes through their TCR. Nevertheless, one cannot exclude that another component present on the T cell or hybridoma surface is co-recognized and is responsible for the self-reactivity of their TCR.

Heat shock proteins (HSP)

In humans as well as in mice, γδ T cells expand in response to mycobacterial infections. Earlier works have shown that murine γδ T cells could be stimulated with HSP65 from mycobacterial extracts and similarly with recombinant proteins including the close relative endogenous mammalian HSP60 (Kabelitz et al. 1990 ; Kobayashi et al. 1994). Reactive murine cells expressed Vγ1 in association with several Vδ2 genes, predominantly Vγ6, and represented 10–20% of unselected splenic or lymph node γδ T cells (Kobayashi et al. 1994 ; O' Brien et al. 1992). As for lipid-reactive γδ cells, HSP-reactive T cell clones and hybridomas were found to have significant autoreactivity. Autoreactivity as well as HSP60 reactivity could be conferred to non-reactive cells by relevant γδ TCR transfer experiments. A minimal 7aa FGLQLEL conserved peptide ($HSP60^{181–190}$) was also found efficient with an essential contributions of amino cid positions 181 and 183 (Fu et al. 1994). APCs and antigen processing were not required for HSP recognition and recognition was not restricted by MHC-I or MHC-II classical antigens. This establishes a direct involvement of the TCR in HSP60 reactivity. A cognate interaction of the TCR with the stimulating peptide is not ascertained and HSP peptides could possibly alter the expression or the conformation of another TCR ligand expressed on the surface of hybridomas and responsible for their inherent autoreactivity.

Endogenous HSP70 family proteins were also found to stimulate murine Vδ8 cells (Kishi et al. 2001). In humans, Vγ9Vδ2 T cells which react against mycobacterial antigens were initially found to be stimulated by HSP60 homologs (Haregewoin et al. 1989) and an endogenous protein of this family was found to be expressed on the surface of Daudi lymphoma cells which can activate cells expressing a Vγ9Vδ2 TCR after TCR tranfer (Bukowski et al. 1998 ; Fisch et al. 1990 ; Kaur et al. 1993). Although anti-HSP antibodies could stain cells which were not Vγ9Vδ2 targets, they could however block recognition of Vγ9Vδ2-targeted tumors (Selin et al. 1992). Since these earlier studies, potent non-peptidic phosphoantigens rather than HSPs have been found to be the essential stimulatory components of mycobacterial extracts for human Vγ9Vδ2 cells and no TCR transfer experiment have demonstrated that human γδ TCRs have a direct role

in HSP recognition. Expansion of Vγ9Vδ2 cells have been reported using HSP60 as well as HSP70 proteins (Chauhan et al. 2007 ; Wadia et al. 2005 ; Zhang et al. 2005).

Although they are essentially cytosolic, secreted through exosomes (Gupta and Knowlton 2007 ; Lancaster and Febbraio 2005) and/or transported to the cell surface by mechanisms which are not well defined, HSPs were found expressed on normal, transformed or apoptotic cells and their surface expression can be up-regulated by various stimuli, in particular TLR stimulation, bacterial and parasitic infections (Hirsh et al. 2006 ; Hirsh and Junger 2008). Endogenous or exogenous HSPs and HSP peptides can bind to cell surfaces via multiple receptors including CD91, may provide a potent stimulus for some γδ T cell subpopulations and this is not be limited to HSP60 family (Binder et al. 2004 ; Habich et al. 2002 ; MacAry et al. 2004). Zhang *et al.* have reported the stimulation of Vγ9Vδ2 cells by EBV-transformed LCL expressing HSP70 and this was abrogated by silencing HSP70 expression in target cells (Zhang et al. 2005). HSP70 expression in lung neutrophils may also be involved in their killing by γδ cells in a murine model of sepsis (Hirsh et al. 2006). In many of these systems, there is no substantial evidence that HSP recognition involves the TCR. HSP70 was found to bind to TLR2 and TLR4 (Asea 2008). More generally, HSPs may signal through TLRs, directly or indirectly through binding peptides or hydrophobic ligands, including LPS (Tsan and Gao 2009 ; Zhao et al. 2007). Nevertheless, HSPs could also be non-specific ligands for the TCR or target antigens and affect specific TCR-antigen cognate interaction.

Insulin peptide

Insulin provided in aerosol has been reported to induce γδ cells with regulatory activity in autoimmune diabetic NOD mice (Harrison et al. 1996). In addition to insulin peptide-specific αβ T cells, γδ T cell hybridomas responding to a diabetogenic insulin B:9-23 epitope could be derived from NOD mice with or without peptide immunization. Peptide-reactive γδ hybridomas were diverse in terms of Vγ and Vδ gene usage. Unlike αβ-T cell hybridomas which responded to the same epitope, reactive γδ hybridomas did not require APCs and peptide mutations altered differently the response of αβ and γδ hybridomas. Simultaneous transfer of the γ and δ chains from a responsive hybridoma to αβTCR-deficient cells could confer peptide reactivity although unlike the original hybridoma their response was amplified by provision of APCs. Finally, B:9-23-specific γδ cells responded to the processed peptide but not to native insulin (Zhang et al. 2010).

Glu-Tyr polymer

The biological relevance of Glu-Tyr polymer is not clear although it may mimic natural proteins from diverse pathogens. A reassessment of the reactivity of Glu-Tyr polymer-reactive γδ cells revealed a similar frequency of these cells in β2m-deficient mice, questioning the role of Qa-1 in polymer recognition (Cady et al. 2000). Unlike the DGT3 clone (see above HLE/Qa-1 section), new γδ hybridomas recognized Glu-Tyr polymer in the absence of APCs and did not require Qa-1. They expressed mainly Vγ1 in association with various possible Vδ genes. In one case, reactivity could be transferred through γ and δ TCR chain expression, did not require APCs and was maximal with the Glu^{50} Tyr^{50} polypeptide.

6-kDa early secreted antigenic target protein (ESAT-6)

ESAT-6 from *M. tuberculosis* is one member of 23 related proteins secreted by the pathogen which constitute determinant virulence factors and are thought to play a role in pathogen escape from phagolysosomes. This family of proteins carries potent immunogenic epitopes in particular for $CD4^+$ αβ T lymphocytes. ESAT-6 binds to and can disrupt phospholipid membranes, an interaction which can induce conformational changes in the protein (Meher et al. 2006). The protein can be post-transcriptionally modified by cleavage or acetylation and makes heterodimers with another family member, culture filtrate component-10 (CFP10) (Brodin et al. 2004 ; Renshaw et al. 2005). A number of reports link γδ T cell responses to the recognition of ESAT-6 following stimulation with mycobacterial extracts. When screening PPD components for stimulatory activity on bovine T cells, Rhodes *et al.* found that purified ESAT-6 could recapitulate the effects of PPD stimulation on WC1 (+) bovine γδ cells (Welsh et al. 2002). Reindeer γδ cells proliferate in response to ESAT-6 (Waters et al. 2006). Bovine γδ cells were also found to respond, although more weakly, to the pyrophosphate antigens (Welsh et al. 2002) and thus some of the stimulatory activity of ESAT-6 on γδ cells could be due to co-purified phosphoantigens (see below), although in the latter study recombinant ESAT-6 was used. Li and Wu have observed a similar activation of human peripheral blood Vγ9Vδ2 T cells with ESAT-6 which increased their CD69 and CD25 expression, produced IFNγ, underwent cell division, and stimulations were increased by CD28 co-stimulation (Li and Wu 2008). Although Vγ and Vδ expression was not determined, the responding cells are assumed to belong to the Vγ9Vδ2 subset which is responsive to mycobacterial extracts. Nevertheless, Casetti *et al.* could not confirm the responsiveness of human Vδ1 or Vδ2 subsets to ESAT-6 and the co-immunization of cynomolgus monkeys with an ESAT-6 fusion protein and a phosphoantigen did not significantly modulate the *in vivo* phosphoantigen response (Casetti et al. 2008 ; Cendron et al. 2007). Discrepancies between studies may be due to differences in ESAT-6 antigen preparations which may contain uncharacterized components, including phosphoantigens, or on the conformation of the protein. The properties of ESAT-6 as a γδ TCR antigen have thus to be ascertained notably because TLR-2 can also be expressed on γδ T cells (Martin et al. 2009 ; Mokuno et al. 2000 ; Pietschmann et al. 2009), and because ESAT-6 was found to bind TLR-2 (Pathak et al. 2007).

$V_{\gamma}9V_{\delta}2$ T cell antigens

Human Vγ9Vδ2 T cells have been the focus of recent reviews (Bonneville and Fournie 2005 ; Nedellec et al. 2010). Their response to antigens does not require prior immunization and may result in a dramatic polyclonal amplification, leading to the concept that they recognize frequent pathogen- or tumor-associated determinants, and participate to the innate line of immune defense (reviewed in (Kabelitz and Wesch 2003)). They are activated by multiple tumor cells types *in vitro* , infiltrate human tumors *in vivo* and are thought to play a role in tumor immunity (Tanaka 2006). They also proliferate in the course of bacterial and parasitic infections and mediate protective responses against these pathogens (Morita et al. 1999). It is clear that the TCR is only one of the receptors which can promote their activation in these different contexts and that TLRs, CD16, NKG2D and NKRs, either of the lectin or immunoglobulin super-families tightly determine or regulate their activity. Nevertheless, multiple proteins and non-peptidic antigens have been described as potential TCR stimuli and the diversity of these ligands excludes a common mechanism of action. Some of them may however contribute in different ways to the mechanism of TCR-mediated antigen recognition. Highly specific stimulators comprise the non peptidic substances phosphoantigens, aminobisphosphonates, alkylamines. Candidate proteins involved in their activation include apolipoprotein A-I, F1-ATPase, ULBP proteins and the above mentioned HSPs and ESAT-6.

Phosphoantigens and related non-peptidic compounds

Phosphoantigens were first isolated as phosphatase-sensitive and protease-resistant stimulatory components of mycobacterial extracts (Constant et al. 1994 ; Pfeffer et al. 1990 ; Schoel et al. 1994 ; Tanaka et al. 1995). The main antigen characterized is hydroxymethyl butenyl pyrophosphate (HMBPP, also called HDMAPP), a metabolite produced in many bacteria and apicomplexan parasites through the methyl-erythritol phosphate biochemical pathway (MEP, also called DOXP pathway) leading to isopentenyl pyrophosphate (IPP), a precursor for isoprenoid and steroid synthesis (Eberl et al. 2003 ; Hintz et al. 2001 ; Jomaa et al. 1999 ; Poupot and Fournie 2004). Together with HMBPP, nucleotide derivatives have been isolated from bacterial extracts and can also activate specifically Vγ9Vδ2 T cells (Constant et al. 1994 ; Poquet et al. 1996a ; Poquet et al. 1996b). The direct role of phosphoantigens in T cell stimulation is confirmed by the fact that multiple synthetic molecules with stimulatory activity have been produced, and this has confirmed the general requirement for a pyrophosphate or triphosphate nucleotide moiety linked through the terminal phosphate to a short alkyl chain, usually of five carbons (Espinosa et al. 2001 ; Morita et al. 2001 ; Zhang et al. 2006). Few molecules qualified as phosphoantigens do not follow this structure, in particular monoethyl phosphate, 2,3-diphosphoglyceric acid, β-D-ribosyl phosphate, xylose-1-phosphate and glycerol-3 phosphoric acid which require high micro or millimolar concentrations for stimulation (Burk et al. 1997 ; Burk et al. 1995 ; Morita et al. 1999 ; Tanaka et al. 1994). As the the ability of pyrophosphate antigens has been linked to the ability of the phosphodiester bond to be cleavable (Belmant et al. 1999 ; Belmant et al. 2000), is should be ascertained that a similar mechanism accounts fort the stimulatory activity of all these phosphorylated compounds. Replacement of the phosphoester bond linking the alkyl chain to its proximal phosphate by a phosphatase-resistant phosphonate bond however leads to compounds with increased activity (Zgani et al. 2004). Several phosphoantigens carry one unsaturation on the alkyl chain and their stereochemical conformation was found determinant for activity (Boedec et al. 2008). Although active at concentrations far above that of the bacterial HMBPP (μM versus nM range), IPP and its isomer DMAPP are phosphoantigens universally produced by bacterial and eukaryotic cells through the mevalonate pathway of isoprenoid synthesis (Poupot and Fournie 2004). Experimental manipulation of the mevalonate pathway in human tumor cells affects their stimulatory activity. Daudi Burkitt lymphoma cells are natural activators and targets for cytotoxic Vγ9Vδ2 and their stimulatory activity is abrogated by statins such as lovastatin and mevastatin, which block hydroxymethylglutaryl-CoA reductase (HMGR), an enzyme of the mevalonate pathway upstream of IPP/DMAPP synthesis (Gober et al. 2003 ; Thompson et al. 2002). Conversely, non-stimulatory cells become stimulant following accumulation of these compounds resulting from inhibiting farnesyl pyrophosphate synthase (FPP), the enzyme which consumes IPP for downstream isoprenoid synthesis. *E. coli* , a bacterium which cannot make HDMAPP because it does not carry the MEP pathway, can induce HMGR activation through dephosphorylation and alters the mevalonate metabolism in infected macrophages resulting in their stimulatory activity for Vγ9Vδ2 cells (Kistowska et al. 2008). T cell reactivity against pyrophosphate antigens can be transferred by Vγ9Vδ2 TCR expression in TCR-deficient cells and this confers reactivity to soluble antigens as well as against stimulatory tumors, demonstrating a direct involvement of the TCR in these responses (Bukowski et al. 1995).

Alkylamins (AA) and aminobisphosphonates (ABP) are two other classes of compounds which were found to have stimulatory activity for Vγ9Vδ2 cells. AAs are small aminated alkyl molecules from plants and microorganisms (Bukowski et al. 1999 ; Kamath et al. 2003). ABPs such as pamidronate and zoledronate are synthetic drugs used for treating bone resorption. Although their structure is reminiscent of that of phosphoantigens, they have different requirements for activity (Thompson et al. 2010). Whereas phosphoantigens of the pyrophosphate group can directly activate γδ T cells in the absence of APCs, alkylamines and aminobisphosphonates have an absolute requirement for APCs of human or primate origin, although many non-professional APCs are efficient for presentation (Green et al. 2004 ; Kato et al. 2003 ; Miyagawa et al. 2001b). It is now clear that ABPs act indirectly as inhibitors of FPP thus inducing phosphoantigen accumulation in APCs which subsequently become stimulatory (Li et al. 2009 ; Rogers 2003). A similar property has been ascribed to AAs (Thompson et al. 2006). In addition to IPP, ABP stimulation of cells induces accumulation of triphosphoric acid 1-adenosin-5-yl ester 3- (3-methylbut-3-enyl) ester, an ATP derivative of isopentenyl (ApppI) (Monkkonen et al. 2006 ; Monkkonen et al. 2007 ; Rogers et al. 1996 ; Rogers et al. 1994). This product can be detected in Daudi cells as well as in cells treated with pamidronate (Monkkonen et al. 2007 ; Vantourout et al. 2009). Strikingly, we could not stimulate directly directly γδ T cells with synthetic ApppI which required to be

pulsed on APCs. Its activity was not abrogated by statins and thus it did not appear to act as a modulator of the mevalonate pathway (Vantourout et al. 2009). It is not clear whether other nucleotidic phosphoantigens have a similar requirement for APCs, as their activity was generally measured in the presence of APCs (Morita et al. 2001).

Although pyrophosphate antigens can directly activate purified Vγ9Vδ2 cells, early studies uncovered a minimal requirement for autologous T cell-T cell contact (Lang et al. 1995 ; Morita et al. 1995). Although this might reveal the involvement of co-stimulatory or adhesion molecules, there is accumulating evidence that phosphoantigens are displayed on cellular membranes for T cell recognition. Vγ 9Vδ2 reactivity with tumor surfaces supports this hypothesis. Sarikonda *et al.* could crosslink photoreactive phosphoantigens on cell surfaces and these were stimulatory in the absence of MHC-I, MHC-II or CD1 antigens (Sarikonda et al. 2008). In another study, Wei *et al.* have used macaque Vγ9Vδ2 TCR tetramer to reveal specific ligands on HMBPP-pulsed cells of monocytes, T or B cells of human or primate origin as well as mycobacteria-infected human monocytes. These tetramers did not bind mouse, rat or pig cells. In the latter case, recognition was abrogated by trypsin, suggesting that the phosphoantigen was recognized in a protein context (Wei et al. 2008).

Apolipoprotein A-I and F1-ATPase

As Daudi Burkitt lymphoma cells are killed almost constantly by Vγ9Vδ2 cells, we have produced anti-Daudi monoclonal antibodies and selected one which did not react with non-stimulatory Raji Burkitt lymphoma cells and had modulatory activity on Daudi cell killing by Vγ9Vδ2 cells. This antibody, M5, could decrease cytotoxicity by 30% and was found to recognize apolipoprotein A-I (ApoA-I) adsorbed on the surface of Daudi cells (Scotet et al. 2005). Strikingly, positive M5 antibody staining of hematopoietic tumors correlated with low MHC-I expression (unpublished data). ApoA-I addition in killing assays performed in the absence of serum revealed that although the cytolytic activity of several γδ T cell clones was increased by apoA-I, this was not always required. A possible effect of MHC-I interaction with inhibitory NKRs could have accounted for the observed correlation between M5 positivity (low MHC-I) and sensitivity to γδ killing. Nevertheless, purified apoA-I but not apoA-II, was found to bind specifically to TCR Vγ9Vδ2 tetramers by surface plasmon resonance and was thus a possible TCR ligand. Subsequently, known apoA-I receptors were screened for there presence on Vγ9Vδ2- targeted tumor lines. The ecto-F_1 -ATPase is one such receptor expressed on hepatocytes (Martinez et al. 2003). As for M5 staining, the detection of the α and or β chains of the mitochondrial ATP synthase/F_1 -ATPase on tumor lines correlated, although not perfectly, with sensitivity to Vγ9Vδ2 killing. A purified F_1 -ATPase bovine complex could also bind specifically to Vγ9Vδ2 TCR tetramers in surface plasmon resonance and ELISA assays, and this binding was increased by apoA-I, suggesting that apoA-I is an optional ligand which can stabilize F_1 -ATPase-TCR interaction. When immobilized on polystyrene beads, the F_1 -ATPase complex could be used to stimulate T cells from peripheral blood and induced detectable lymphokine production in the Vγ9Vδ2 subset (Scotet et al. 2005).

Ecto-F_1 -ATPase (also called cell surface ATP synthase) is thought to be similar to the ATP synthase located in the inner membrane of mitochondria. This complex uses the energy of the electrochemical proton gradient generated by the respiratory chain between the intermembrane space and the mitochondrial matrix to catalyze ATP synthesis from ADP and inorganic phosphate while translocating protons across the inner mitochondrial membrane. In the absence of a proper proton gradient, the complex can hydrolyze ATP into ADP, a process which is normally regulated in mitochondria by the soluble polypeptide IF1 (inhibitor of F_1). The mitochondrial complex comprises an extramembrane F_1 domain (α3β3) which binds nucleotides and performs ATP synthesis/hydrolysis, and an intramembrane domain which functions as a rotor (c12 ring) and is the proton channel. The two domains are connected by a peripheral stalk of subunits (a, e, f, g, A6L, b, F6, d and OSCP) and a mobile central stalk (γδε) which is attached to the rotor and produces conformational changes in the F_1 domain during rotation of the c-ring associated with proton translocation (Devenish et al. 2008). The α and β chains can be detected on cell surfaces of many cell types which orientates the complex with the catalytic F_1 -domain on the outside (Champagne et al. 2006). F_1 and other components of this complex have been detected on rat cell membranes (b, g and OSCP) (Mangiullo et al. 2008), on osteosarcoma cells (d and OSCP) (Yonally and Capaldi 2006), or in membrane lipid raft or caveolae-enriched extracts (Bae et al. 2004 ; Bini et al. 2003 ; Kim et al. 2004 ; Sprenger et al. 2004 ; von Haller et al. 2001 ; Yu et al. 2005). F_1 -ATPase-expressing cells include endothelial cells, hepatocytes, neurones, adipocytes (reviewed in. (Champagne et al. 2006)). This probably underscores the real tissue expression as detection of the complex on cell surfaces has proven difficult on tissue sections in particular due to the sensitivity of epitopes to fixation procedures. The strong mitochondrial expression hampers detection of the membrane complex by fluorescence techniques. F_1 epitopes are also removed by trypsinization of cells (unpublished observations). There is increasing evidence that the membrane complex is enzymatically active although there are discrepancies on the reaction it performs. ATP synthesis (Burrell et al. 2005 ; Kim et al. 2004 ; Mangiullo et al. 2008 ; Moser et al. 2001)as well as ATP hydrolysis (Fabre et al. 2006 ; Mangiullo et al. 2008 ; Martinez et al. 2003 ; Radojkovic et al. 2009 ; Yegutkin et al. 2001) have been reported and may depend on the extracellular environment of the cell as well as on its metabolic status. The metabolic activity of ecto-F_1 -ATPase has been shown to modulate lipoprotein internalization by hepatocytes, to regulate endothelial and keratinocyte proliferation and apoptosis, and to regulate intestinal lipid absorption. In most instances, these functions were uncovered through evidence that this complex acts as a receptor for specific regulatory polypeptides: apoA-I, enterostatin, angiostatin, EMAPII and the regulatory subunits IF1 and F6 which can be found as circulating serum proteins (Champagne et al. 2006). How F_1 -ATPase reaches the cell surface is unknown and presumably involves an intracellular vesicular pathway. A possible involvement of F_1 -ATPase in intracellular vesicular trafficking is further supported by its association with phagosomes (Garin et al. 2001 ; Kovarova et

al. 2002 ; Li et al. 2003). The α-subunit may be glycosylated on the surface of murine cells suggesting that the complex may transit through the endoplasmic reticulum or Golgi apparatus (Schmidt et al. 2008).

Strikingly we could immunoprecipitate F_1 -ATPase components from the membrane extracts of cells where it was not detected by immunofluorescence, such as Raji or Jurkat cells. We assumed that F_1 -ATPase was indeed expressed on Raji and many other tumor lines but was not detected due to a masking of antibody epitopes by surrounding membrane components. MHC-I antigens are in large part involved in this interaction as stimulation of their expression leads to lower detection of F_1 -ATPase, whereas their disruption through β2m removal by acid treatment leads to higher detection. MHC-I and F_1 -ATPase can be co-precipitated and colocalize partially in membrane patch-like domains on HepG2 cells (Vantourout et al. 2008). These findings indicate a close association of MHC-I antigens and F_1 -ATPase on the cell membrane. ApoA-I binding (detected with the M5 antibody) as well as the binding of most anti-F_1 -ATPase antibodies is likely to occur on an area of the F_1 surface which is alternatively occupied by an interaction with MHC-I, presumably close to the plasma membrane.

Possible role of F1-ATPase in phosphoantigen presentation

The presence of F_1 -ATPase in intracellular non-mitochondrial compartments and the modulator properties of chloroquine on phosphoantigen presentation (Rojas et al. 2002) are compatible with an involvement of this complex in some form of phosphoantigen antigen processing and presentation analogous to the mechanisms used for peptides and lipids. More investigations are required to determine if this is truly the case. Nevertheless, F_1 -ATPase is probably one of the determinant factors involved in phosphoantigen recognition by Vγ9Vδ2 cells. Although IPP does not induce a calcium flux in cells which do not make contacts with other cells, contact with beads coated with purified bovine F_1 -ATPase in the presence of IPP induces a week and delayed calcium signal in individual cells indicating that F_1 -ATPase partly replaces the required interaction with autologous cell membranes. In accordance with this, the ability of contacting cells to induce a calcium flux in response to IPP correlated with their expression of F_1 -ATPase. In addition, cells which did not promote calcium flux were equally unable to promote Vγ9Vδ2 T cell responses after treatment with aminobisphosphonates and this correlated with undetectable F_1 -ATPase on their surface. These data are in favor of a strict requirement for F_1 -ATPase in phosphoantigen responses (Mookerjee-Basu et al. 2010).

Pichler *et al.* have shown that some some αβ T cells specific for hapten drugs in an MHC-II restricted fashion did not require stable binding of the drug to the MHC molecule although a TCR-dependent recognition of both the drug and the MHC were required. Accordingly, sulfametoxazole (SMX) cannot be pulsed on APCs but, when in solution, SMX can activate drug-specific T cells provided fixed APC with the relevant MHC are available for contact (Pichler et al. 2006 ; Schnyder et al. 1997 ; Zanni et al. 1998). A similar situation seems to occur with IPP: although some pyrophosphate antigens can be pulsed on APCs, this is usually regarded as poorly efficient, in particular when weak antigens are concerned, and the response to pyrophosphates requires contact with APCs, partially replaceable by beads carrying F_1 -ATPase (Mookerjee-Basu et al. 2010).

Purified F_1 -ATPases of multiple origins can bind multiple nucleotide analogs on three catalytic sites and additional non-catalytic sites of unknown function. It is thus not surprising that bovine F_1 -ATPase immobilized on polystyrene beads stably binds ApppI. Such complexes were found to induce TCR aggregation on Vγ9Vδ2 cells although F_1 -ATPase alone did not. We have observed however that they were unable to promote lymphokine secretion and expression of cytolytic activity marker CD107 unless a nucleotide pyrophosphatase (NPP) cleaving ApppI into IPP + AMP was added in the assay (Mookerjee-Basu et al. 2010).

There are conflicting reports on the ability of pyrophosphate antigens to induce TCR modulation: in the study by Lafont et al. (Lafont et al. 2001), γδ T cell stimulation with IPP was unable to induce TCR down-modulation, whereas Sireci *et al.* (Sireci et al. 2001)found modulation with weak agonists including IPP but not with a stronger agonist BrHPP. Pyrophosphate antigens are probably too small to crosslink TCRs and it seems thus likely that TCR cross-linking and internalization results from the recognition of a putative presentation molecule (O'Brien et al. 2007). The efficiency of cross-linking and modulation may require a physical link between the phosphoantigen and the presentation molecule and this link may depend on chemistry of the phosphoantigen and experimental procedures. As opposed to the recognition of soluble phosphoantigens, the recognition of antigenic molecules on tumors, on ABP-treated or on ApppI-treated cells invariably induces TCR aggregation and down-modulation. In these cases, antigen-loaded cells can be washed and remain stimulatory, suggesting that these situations lead to efficient antigen presentation through an endogenous pathway (APB, tumors), or through an exogenous route (ApppI and possibly phosphate antigens). ApppI can be captured by APCs (tumor cells or dendritic cells) and is efficiently presented to γδ cell. In our hands this process was more efficient for ApppI than for IPP on a molar concentration basis. Although it is not clear whether this is due to better stability, more efficient capture by the cell or to a better efficiency of the presentation mechanism, this suggests that nucleotide derivatives could be a form of storage, transport and/or presentation for phosphoantigens produced intracellularly. Once ApppI was loaded on APCs, the stimulatory activity was similar to that of APB-treated cells or stimulatory tumors since it was resistant to extensive washing of stimulatory cells and did not require exogenous pyrophosphatase activity as opposed to ApppI loaded on purified F_1 -ATPase.

A definitive proof that F_1-ATPase is a presentation molecule for phosphoantigens will require demonstration of its physical interaction with endogenously produced antigens and demonstration of an association of both components on the cell surface in physiological conditions. Another degree of complexity in the mechanism of phosphoantigen recognition is suspected from the observation that hydrolysis of ApppI is required for full T cell activation by F_1-ATPase/ApppI complexes, although this hydrolysis is not necessary for recognition and TCR aggregation. This may indicate that ApppI/F_1 are not physiological ligands. In support of this possibility, Vγ9Vδ2 TCR tetramers from macaques (Wei et al. 2008) can stain surface ligands on cells loaded with HMBPP, suggesting that HMBPP itself is efficiently presented. Additionally, cells which are stimulatory either naturally or following pulsing with ABP or ApppI do no require exogenous NPP activity. Nevertheless, an alternative possibility is that HMBPP, like IPP, can be converted into a nucleotide derivative by cells and presented as such in complex with the presentation molecule for recognition. In support, IPP does not make stable stimulatory complexes with F_1-ATPase in vitro. A requirement for such complexes for initial recognition does not preclude the necessity of a hydrolysis step for full TCR activation, as is observed with ApppI/F_1 complexes. The known hydrolytic activity of F_1-ATPase cannot perform this hydrolysis as it would release isopentenyl monophosphate which is not a stimulus for Vγ9Vδ2 cells. Although it is not excluded that the F_1-ATPase complex has unknown activities, such hydrolysis could be performed by one of the multiple ecto-nucleotide pyrophosphatases present on APC surfaces (Bollen et al. 2000 ; Goding et al. 2003 ; Yegutkin 2008).

Finally, it is possible that F_1-ATPase is one among several possible presentation molecules for phosphoantigens. Spencer *et al.* have found that the T cells which were responsive to mycobacterial antigens presented by mycobacteria-infected macrophages, presumably through en endogenous route, were only a subset of the Vγ9Vδ2 which were expanded with pyrophosphate antigens loaded on dendritic cells, suggesting an involvement of different antigen presentation molecules or processing pathways (Spencer et al. 2008).

Vγ9Vδ2 TCR epitopes involved in ligand recognition

γδ cells responding to mycobacteria and purified phosphate antigens all express Vγ9 and Vδ2 and TCR transfer experiments with mixed TCR chains from responder and non-responder γδ clones have demonstrated the contribution of both Vγ9 and Vδ2 chains. Although responder clones carry an extreme diversity in the CDR3γ and δ regions, most of them express Jγ1.2 and mutational analysis have demonstrated the tight contribution of two lysine residues encoded by Jγ1.2 (K^{108}-K^{109}). TCR transfer experiment with TCRs mutated in other CDR regions highlighted the contribution of a frequent N-region neutral or hydrophobic residue in position 97 of the δ chain (5′end of CDR3δ), and an arginine residue in CDR2δ (R51) (Bukowski et al. 1998 ; Miyagawa et al. 2001a ; Morita et al. 2001 ; Yamashita et al. 2003). The three lysine residues and R^{51} are thought to provide an anchor for negatively charged phosphates in the antigen and the existence of a possible binding pocket for phosphoantigens involving these residues was substantiated by the crystal structure of a Vγ9Vδ2 TCR (Allison et al. 2001 ; Morita et al. 2001). Recently, a more extensive analysis of mutations which influence reactivity to phosphoantigens revealed that the area covered by critical mutations was much larger than the pocket initially defined, involved all six CDR regions and was largely germ-line encoded. Since this area exceeds the size of pyrophosphate antigens, Wang *et al.* conclude that these new critical residues likely contact a presentation molecule whereas pyrophosphate recognition mainly involvesCDR3γδ (Wang et al. 2010).

Xu *et al.* have shown that a peptide derived from the CDR3δ sequence of a $Vδ2^+$ lymphocyte infiltrating an ovarian tumor, either alone or grafted onto an IgG, could be used to detect a surface ligand on epithelial tumors as well as in protein extracts from selected tissues (Xu et al. 2007). This CDR3δ peptide displayed selectivity for certain tumors. Probing of a random peptide library with this peptide allowed the identification of sequences homologous to possible ligands, two of which HSP60 and MSH-2 were also detected with monoclonal antibodies on the cell surface of epithelial tumors (Chen et al. 2008). The implication of these candidate proteins as TCR targets has to be further explored, as well as the relationship between $Vδ2^+$ ovarian and colon carcinoma infiltrating cells and the Vγ9Vδ2 T cell population which reacts to phosphoantigens.

Other possible protein ligands for γδ TCRS

Recently, the *Skint1* gene was identified by a genetic approach as a member of a multigene family which determines the presence of V γ5/Vδ1 DETCs in the skin of mice (Boyden et al. 2008). There is multiple evidence that DETCs recognize an endogenous ligand expressed in murine skin upon infections or stress (Jameson and Havran 2007). The *Skint1* gene encodes a protein of the immunoglobulin superfamily. Its expression is confined to murine thymus and epidermis and thus it could possibly be a TCR ligand determining thymic differentiation and tissue homing of DETC cells. Evidence for surface expression of the protein on murine cells which do not overexpress the gene artificially is still lacking but surface expression could be driven by contact with pathogens as suggested for H-2M3 or MR1 which are ligands for of αβ TCRs (Gulden et al. 1996 ; Le Bourhis et al. 2010 ; Lenz et al. 1996). Nevertheless, Skint may not be directly involved in TCR-mediated antigen recognition and an alternative possibility is that it is somehow involved in TCR ligand expression. Skint proteins share significant homology with proteins of the butyrophilin (BTN) family which are expressed in humans as well as in mice. In humans, the butyrophilin family comprises seven members some of which are expressed on the cell surface and have a wide tissue distribution (Rhodes et al. 2001). BTN products have also been shown to affect TCR-dependent stimulation of CD4 and CD8 αβ T cells (Smith et al. 2010) but it is not known whether this is also true human γδ T cells.

Non-Vδ2 γδ T cells expand in the course of CMV infection or reactivation in transplanted patients (Dechanet et al. 1999 ; Pitard et al. 2008). CMV-specific γδ T cell clones cross- react with determinants expressed on tumoral epithelial cells suggesting recognition of an endogenous ligand induced by CMV or tumoral processes (Halary et al. 2005). Similar expansions are observed in the course of CMV infection *in utero* (Vermijlen et al. 2010). In this latter study, γδ T cells bearing a public TCR consisting of Vδ1 paired with Vγ8 were strikingly enriched in infected newborns. These TCRs were mostly germline-encoded as they had no P/N nucleotide additions in CDR3 and carried very similar CDR3 γ and δ sequences. This may be explained by the immaturity of the immune system. This population of cells is undetectable in uninfected newborns and is a unique example of γδ T cell which can mount a vigourous oligoclonal response to a viral infection.

For completeness, one should mention the streptococcal superantigens SEA and SEB which have been shown to activate human Vδ1 (SEB) and Vδ2 (SEA) T cells, respectively, and do so by cross-linking the TCR γ-chain and MHC-II molecules from APCs in much the same way they bridge selected Vβ regions with MHC-II molecules to activate αβ-T cells (Fikri et al. 2001 ; Loh et al. 1994 ; Maeurer et al. 1995 ; Ramesh et al. 1995). Superantigens represent an alternative way to activate γδ as well as αβ T cells through bypassing their fine antigen specificity (Rust and Koning 1993).

MHC-dependent antigen recognition and the "docking partner hypothesis"

Based on the analysis of CDR3 length distributions, Chien and collaborators have shown that the antigen-binding region of γδ TCRs more closely resembles that of immunoglobulins than that of αβ TCRs (Chien and Konigshofer 2007 ; Rock et al. 1994). The discovery of non-peptidic antigens and peptidic ones which did not require classical processing comforted the hypothesis that γδ TCRs can recognize soluble antigens. Nevertheless, in most instances if not all, antigen recognition is potentiated by APCs and multiple evidence that antigens are preferentially recognized on cell surfaces have already been presented. In most instances, antigen recognition by γδ TCRs appears to be dependent on germ line-encoded TCR elements. Born et al. thus proposed that γδ TCRs have evolved to recognize conserved antigenic structures and that the particularly high potential variability of CDR sequences serves to provide TCR adaptability to diverse cell surface molecules which constitute the context in which these antigens are expressed.

From this review, it appears that candidate ligands for γδ cells fall in three categories: MHC-like molecules, unrelated polypeptides and, in the case of Vγ9Vδ2 cells, small non-peptidic molecules. T cell activation by such diverse structures cannot involve similar TCR-ligand interactions. Although the example of superantigens (and also mitogens) shows that TCR activation can be achieved through multiple alternative interactions with MHC molecules, data in the Vγ9Vδ2 system do not fit well with this situation. The specificity of Vγ 9Vδ2 cells for targeted tumors has been shown to be determined through the recognition of phosphoantigens produced endogenously, F_1 -ATPase expressed at the cell surface, and MHC-Ib molecules. As described above, there is molecular evidence for TCR interaction with each of these structures although the TCR interaction with phosphoantigens still relies on indirect data. The involvement of molecules of the MHC family is ambiguous as they also participate to T cell activation through NKRs. The case of MICA recognition by human Vδ1 cells is striking as this antigen can bind the NKD2D receptor as well as the TCR. A similar situation is observed with Vδ2 TCRs which possibly bind ULBP4. To the diverse antigenic context proposed by Born et al, we thus suggest the variant hypothesis that antigens appear in a relatively defined context and our view is that above observations converge to a preferential involvement of MHC-related molecules recognized simultaneously with antigenic ligand.

The model of peptide-MHC recognition by αβ T cells indicates that MHC molecules perform different essential functions: a) they provide an anchor for antigens on cell surfaces, the antigen binding cleft; b) they insure that these antigens are efficiently targeted by TCRs by providing a recognizable framework for recognition. This is usually called self-MHC restriction and is achieved through thymic positive selection; c) they are involved in specific presentation routes for exogenous or endogenous antigens which ensure that the antigens are recognized on defined APCs and initiate an appropriate immune response. This model is valid for αβ TCRs which recognize polymorphic MHC-I or MHC-II molecules and those recognizing non-polymorphic CD1 together with lipids. How does it apply to γδ -TCR antigen recognition? There is no evidence that homologous binding clefts in MHC-related γδ TCR ligands provide anchors for antigens since they either do not have a proper binding cleft (ULBPs, MICA/B, T10/T22) or are recognized independently of peptides (IE). In the case of γδ cells recognizing CD1, the role of lipids may similarly have no role in CD1-dependent recognition by γδ TCRs, although they may be required for proper surface expression of complexes. Available data fit with a general model based on a simultaneous recognition of MHC molecules and ligands which are tightly associated outside the peptide groove. The complex would be recognized by the TCR through CDR regions although other framework TCR regions may also be involved. This model is supported by the following observations.

Based on available crystallographic data, the G8 TCR in complex with T22 uses almost exclusively CDR3δ to contact the MHC ligand, with a minor contribution of CDR3γ, none from other CDRs and with some docking flexibility. The difference in G8 TCR docking on T10 is extreme as compared with peptide/MHC restricted TCRs. Differences in docking on MHC ligand, as compared to αβ-TCRs, is also reported for the TCRs of NKT cells on CD1, revealing that homologous MHC molecules can be ligated by different TCRs without

using conserved docking sites and alignment (Borg et al. 2007 ; Gadola et al. 2006 ; Godfrey et al. 2010 ; Kjer-Nielsen et al. 2006 ; Zajonc et al. 2008). The crystallographic analysis of unbound Vγ9Vδ2 TCR also uncovered a considerable difference between αβ and γd TCRs architecture, that is an acute Vγ/Cγ interdomain angle of 42°C observed in the G115 TCR (Allison et al. 2001). Although it is not yet reported that other γδ TCRs have a similar tilted V domain, these structural features suggest that docking of γδ TCRs on their ligands obeys to constraints which are different from those of other TCRs. CDR3δ peptides can be used to detect ligands on the surface of target cells in an apparently specific manner supporting a relative autonomy for CDR3δ in ligand binding. Finally, as already mentioned, CDR3δ length distribution is uniquely long and is not constrained as other CDR regions in αβ and γδ TCRs (Chien and Konigshofer 2007 ; Rock et al. 1994). As CDR3δ makes the essential contribution to the binding to T10, it seems plausible that other CDR regions are available for docking on tightly associated partners.

Some of the γδ TCR ligands are membrane-anchored proteins which may represent such docking partners: the viral HSV-gI protein for Vδ8 murine TCRs and the F_1-ATPase for human Vδ2 cells. Whereas association with MHC-like proteins is not documented for gI, F_1-ATPase appears to associate with classical MHC-I molecules. We find plausible that it can also interact with ULBP proteins which would then be co-recognized with F_1-ATPase. The presence or absence of an α3 domain in the MHC-Ib molecule would then be determinant for defining the docking area on the [MHC/docking partner] complex, so that classical MHC-I antigens might not allow proper docking as opposed to ULBPs. In the case of F_1-ATPase which is a self ubiquitous ligand, antigenicity could be conferred by associated phosphoantigens, whereas HSV-gI would be antigenic by itself. An inherent and somewhat autonomous affinity of different regions of the γδ TCRs for MHC-like proteins and their partners would explain the physical associations observed in vitro between soluble TCRs and ULBP, MICA, T10/T22 on one side and F_1-ATPase or gI on the other.

There are multiple lines of evidence that thymic positive selection occurs for murine Vγ5/Vδ1 DETC and related epidermic T cells (Lewis et al. 2006 ; Mallick-Wood et al. 1998 ; Xiong et al. 2004) although the selecting ligand is not yet identified. Mice which are deficient for MHC-II or MHC-I have normal numbers of γδ cells indicating that classical MHC expression is not generally required for their development (Bigby et al. 1993 ; Correa et al. 1992). Nevertheless, in the case of murine γδ T cells reacting against the β 2m-associated MHC-Ib T10/T22, it is intriguing that thymic antigen expression does not determine thymic selection of cells carrying relevant TCRs and G8 TCR-transgenic mice bred on $\beta 2m^0$ background have increased numbers of fully mature antigen-reactive γδ cells (Schweighoffer and Fowlkes 1996). The recent work by Jensen *et al.* demonstrates that thymic expression on T10/T22 influences a functional switch in lymphokine secretion ability of the mature γδ progeny and the straightforward conclusion was that these cells could have a unique thymic selection program and do not need positive selection (Jensen and Chien 2009). Thymic MHC-I/T10 expression appears to affect the phenotype of T10 tetramer-positive mature T cells, leading to higher expression of CD122 and lower HSA expression, indicative that they have encountered antigen in the thymus whereas cells developing in the absence of T10 are naive (Jensen and Chien 2009 ; Shibata et al. 2008). An alternative explanation would be that a binding partner of T10/T22 promotes thymic selection whereas T10/T22 are dispensable or replaceable by another MHC-like, β2m-independent ligand for positive selection whereas T10/T22 would only influence TCR binding affinity.

The recognition of a combination of MHC-Ib molecules and a docking partner implies that each partner could contribute to the antigenicity of the complex. The T10/T22 system suggests at first sight that antigenicity is conferred by the polymorphic T10. This may however not be the case: although the original $T10^b$-reactive clone G8 does not react with $T10^d$, G8 transgenic mice carrying the $T10^d$ haplotype have reduced numbers of γδ thymocytes and Schweigorfer and Fowlkes provide evidence that in these mice the transgenic thymocytes undergo thymic deletion and have thus made cognate interactions with $T10^d$ (Schweighoffer and Fowlkes 1996). Although we cannot exclude that $T10^d$ induces anergy in mature cells, an alternative possibility is that $T10^d$ cannot pair with the selecting partner of $T10^b$, altering thymic selection. The thymic partner of $T10^b$ would possibly be replaced by another which induces thymic deletion of G8 cells. The non-reactivity of G8 cells towards $T10^d$ would imply that this alternative partner is not expressed by APCs used for specificity assays. The existence of multiple tissue-specific binding partners would also explain why γδ cells use different V genes for T22 recognition within different tissues while retaining the common CDR3δ WSEGYEL motif (Shin et al. 2005). If this is the case, the antigenicity would be conferred by a combination of T10 with its partner rather that T10 itself and the MHC would confer only part of the γδ-TCR recognizable framework.

Soluble molecules identified as possible γδ TCR ligands may play different roles in antigen recognition. Phosphoantigens could confer antigenicity to F_1-ATPase which could provide the anchoring function for the antigen. Similarly, phospholipids such as cardiolipin could confer antigenicity to some unknown conserved ligand. Hydrophobic proteins such as HSPs and apolipoproteins (apoA-I, apoH) could participate in TCR-ligand complexes by their ability to bind TCRs, MHC or partners through less specific interactions, and could facilitate or stabilize interactions. Their role may be more significant in the case of low affinity TCR-ligand interactions.

The possible recognition of NKG2D ligands by both the TCR and NKG2D raises the question of a possible co-ligand function for NKG2D. However, structural data on NKG2D/ligand complexes do not leave much room for a possible simultaneous interaction of both receptors on the same molecule (Radaev and Sun 2003). Together with other NKRs, NKG2D might rather signal in separate complexes

and allow γδ cells to sense the cellular context of the antigenic stimulus. This would be essential in the case of endogenous and ubiquitous antigens such as IPP/ApppI in tumor cells. These putative autoantigens are however weak stimulators of Vγ9/Vδ2 cells and probably become significant only when they are strongly over-expressed or when the inhibitory signals by NKRs are decreased. They may as well provide a basal TCR signal licensing the γδ T cell for a response to other receptors activated by stress or pathogen-associated stimuli.

Conclusion

The hypothetical [MHC + docking partner] recognition hypothesis is not much different from classical MHC-restriction for peptide-MHC recognition, and differs only by the nature of the ligand, the mode of ligand binding to the MHC, and the mode of docking of the TCR on the combined ligand. If many of the possible MHC-Ib molecules possibly involved are non-polymorphic, MICA proteins display high polymorphism concentrated on the outer edge of the residual cleft as well as in the α3 domain which may influence interactions with surrounding membrane proteins and putative ligands. Together with the emerging view that γδ T cells recognize antigens on cell surfaces, the points discussed here seem to indicate that antigen recognition by γδ T cells shares most of the characteristics of peptide recognition by αβ TCRs. Nevertheless it is likely that most of the antigenic ligands for γδ T cells have not been fully identified and if a unifying model for antigen recognition by γδ cells is proposed here, experimental evidence are still scarce and our view of antigen diversity is still unclear.

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List of abbreviations

AA : alkylamin
ABP : aminobisphosphonate
apoA-I : apolipoprotein A-I
ApppI : triphosphoric acid 1-adenosin-5′-yl ester 3- (3-methylbut-3-enyl) ester
β2m : β2-microglobulin
BTN : butyrophilin family
BrHPP : bromohydrin pyrophosphate
CDR : complementarity determining region of the TCR
DMAPP : dimethylallyl pyrophosphate
EMAP II : endothelial monocyte-activating polypeptide II
ESAT-6 : early secreted antigenic target protein 6
HMBPP : (E)-1-hydroxy-2-methylbut-2-enyl 4-diphosphate
HMGR : 3-hydroxy-3-methyl-glutaryl-CoA reductase
HSP : heat shock protein
HSV : herpes simplex virus
IEL : intraepithelial lymphocytes
IF1 : inhibitor of F_1 -ATPase
F_1 -ATPase : F_1 adenosine triphosphatase
FPP : farnesyl pyrophosphate synthase
IPP : isopentenyl pyrophosphate
MAIT : Mucosal-associated invariant T cells
MEP : 2-C-methyl-D-erythritol-4-phosphate
MICA/B : MHC class-I-related chain A/B
NKR : Natural killer receptor
PPD : partially purified tuberculin derivative
SEA/B : staphylococcal enterotoxin A/B
SMX : sulfamethoxazole
TAP : transporter associated with antigen processing
TIL : tumor infiltrating lymphocyte
TT : tetanus toxoid
ULBP/RAET1 : unique long 16 (UL16)-binding protein/retinoic acid early transcript 1

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