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MULTI-AUTHOR REVIEW

Searching for "signal 2": costimulation requirements of $\gamma\delta$ T cells

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Abstract T cell activation requires the integration of signals that arise from various types of receptors. Although TCR triggering is a necessary condition, it is often not sufficient to induce full T-cell activation, as reflected in cell proliferation and cytokine secretion. This has been firmly demonstrated for conventional $\alpha\beta$ T cells, for which a large panel of costimulatory receptors has been identified. By contrast, the area remains more obscure for unconventional, innate-like $\gamma\delta$ T cells, as the literature has been scarce and at times contradictory. Here we review the current state of the art on the costimulatory requirements of $\gamma\delta$ T cell activation. We highlight the roles of members of the immunoglobulin (like CD28 or JAML) or tumour necrosis factor receptor (like CD27) superfamilies of coreceptors, but also of more atypical costimulatory molecules, such as NKG2D or CD46. Finally, we identify various areas where our knowledge is still markedly insufficient, hoping to provoke future research on $\gamma\delta$ T cell costimulation.

Keywords $\gamma\delta$ T cells · T cell activation · Costimulation · CD27 · CD28 · NKG2D

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T cell costimulation in brief

T cells use their signature TCR to recognise antigens and initiate cellular immune responses whose magnitude depends on the integrated activation of a series of signalling pathways. These must ultimately lead to critical changes in gene expression, such as induction of pro-survival and cell cycle genes that control T cell expansion, or cytokine genes that orchestrate T cell effector function. Thus, in a nutshell, T cell activation typically requires signal transduction via the MAPK/ Erk and PI3 K/ Akt pathways, which relay information to Fos/Jun and NF-kB/ NFAT transcription factors, respectively, and these control the expression of genes like IL-2, cyclins and Bcl2 family members (reviewed in [1]).

While the TCR makes a key contribution to the activation of these molecular pathways, extensive research on $\alpha\beta$ T cell responses has convincingly established that TCR signalling alone is not sufficient to release $\alpha\beta$ T cells from their quiescent state. In fact, TCR$\alpha\beta$ stimulation ("signal 1") in the absence of additional activating signals results in a non-responsive state (also called "anergy") that is refractory to restimulation (reviewed in [1]). To avoid "anergy" and induce $\alpha\beta$ T cell activation, co-ligation of other receptors, which provide "signal 2" (or costimulation), is usually required.

Much of what we know about the importance of "signal 2" has come from studies on the CD28 coreceptor. CD28 signalling has been shown to produce both qualitative and quantitative changes leading to lower activation threshold and enhanced T cell activity. This is critically reflected in the impaired immune responses mounted by CD28-deficient mice against a variety of infectious agents (reviewed in [2]).

The list of costimulatory receptors that, like CD28, affect T cell activation, division, survival and cytokine

secretion, has been growing steadily over the past 20 years. Typical costimulatory receptors are type I transmembrane proteins that can be divided into two groups, based on their structural characteristics: Immunoglobulin (Ig) or tumour necrosis factor receptor (TNFR) superfamilies. Ig superfamily members are characterised by a variable Ig-like extracellular domain and a short cytoplasmic tail, whereas TNFR family members present extracellular domains rich in six cysteine repeats (which form disulphide bridges), and posess a more complex cytoplasmic tail (reviewed in [3]).

Ig superfamily costimulatory receptors are also collectively denominated the CD28 family. Although CD28 was the first costimulatory receptor to be identified, other Ig superfamily members were later shown to share structural and functional characteristics with CD28. These include CD2, CTLA-4, ICOS and PD-1, among others (reviewed in [4]). On the other hand, within the TNFR superfamily we find the costimulatory 4-1BB (CD137), OX40 (CD134), CD27 and HVEM, among others (reviewed in [5]).

These two main types of costimulatory receptors use distinct modes of intracellular signalling: whereas the CD28 family members associate directly with protein kinases (such as PI3 K), TNFR superfamily coreceptors require the adaptor proteins TRAF (TNFR-associated factor), namely TRAF2 and TRAF5, to link to downstream signalling events (Fig. 1). Of note, whereas many non-costimulatory members of the TNFR superfamily contain cytoplasmic death domains, the costimulatory members lack death domains, and instead contain TRAF-binding motifs (reviewed in [3]).

While the molecular mechanisms of costimulatory signal transduction are likely to be conserved, this review will focus on the particular functional outcomes of coreceptor triggering on the various subsets of human and murine $\gamma\delta$ T cells [6]. In humans, the major (>60%) subset of peripheral blood $\gamma\delta$ T cells expresses a Vγ9Vδ2 TCR, which enables the cells to (uniquely) respond to non-peptidic prenyl pyrophosphates ("phosphoantigens") [7, 8]. Importantly, the presence of antigen presenting cells (APC) can enhance Vγ9Vδ2 T cell responses [9], suggesting that accessory molecules are also involved. The most promising costimulatory receptors thus far characterised on human and/or murine $\gamma\delta$ T cells will be discussed in this review.

Ig superfamily coreceptors in $\gamma\delta$ T cell activation

The complex role of CD28

CD28 ligation by its ligands B7.1 (CD80) or B7.2 (CD86) (Fig. 2) is known to promote proliferation, survival and cytokine production of $CD4^+$ and $CD8^+$ T cells. Thus, CD28 costimulation increases IL-2 transcription [10] and

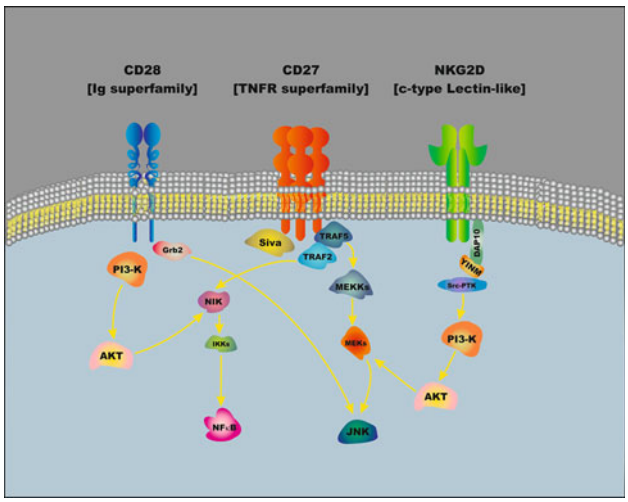

Fig. 1 T cell costimulatory receptors activate distinct signalling pathways. Schematic of the first layer of signalling events downstream of three classes of costimulatory receptors: Immunoglobulin (CD28), tumour necrosis factor receptor (CD27) and c-type lectin-like (NKG2D). CD28 and NKG2D activate PI3 K/Akt either directly (CD28) or via Src-PTK (NKG2D). CD27 activates the MEKK/JNK and IKK/NFκB pathways through the adaptors TRAF2 and TRAF5. Signal transduction downstream of JNK, NFκB and Akt will then modify gene expression in the nucleus (not represented)

mRNA stability [11], and it enhances the expression of anti-apoptotic Bcl-x_L [12]. As a result, $\alpha\beta$ T cell responses are very frequently impaired when CD28 signaling is impeded in vitro (using blocking reagents) or in vivo (using CD28-deficient mice) (reviewed in [2]).

Taking into account the key role of CD28 in $\alpha\beta$ T cell activation, it is maybe surprising that its relevance for $\gamma\delta$ T cell responses remains controversial. This possibly stems from some discrepancies among reports mostly published in the 1990s and from the complex pattern of expression of CD28 in $\gamma\delta$ T cell populations. Thus, whereas $\alpha\beta$ T cells constitutively express high levels of CD28, resting murine $\gamma\delta$ splenocytes [13] and various intraepithelial lymphocytes (IEL) subsets [14–17], as well as ruminant $\gamma\delta$ T cells [18], were reported to mostly lack CD28. However, CD28 levels were shown to increase significantly upon activation of mouse [13] or avian [19] $\gamma\delta$ T cells. This pattern contrasts with that observed with human peripheral blood $\gamma\delta$ T cells: CD28 is expressed by 40–60% of freshly isolated cells [20–22], but very few (<10%) activated cells [22]. It is therefore difficult to predict the role of CD28 in $\gamma\delta$ T cell physiology based on such atypical expression patterns.

Nonetheless, a series of functional assays performed by various groups in the 1990s suggested an active role for CD28 in $\gamma\delta$ T cell activation. Thus, anti-CD28 agonist antibodies enhanced human $\gamma\delta$ T cell proliferation [20], whereas blocking antibodies inhibited it significantly [21]. Moreover, the generation of TCR$\gamma\delta$ transgenic mouse lines

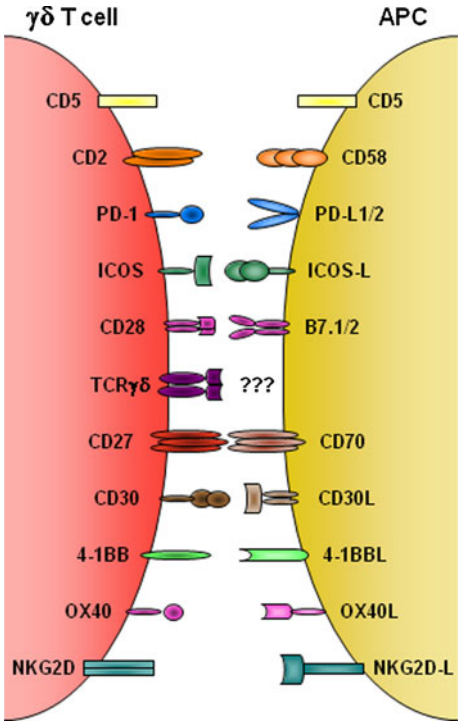

Fig. 2 Costimulatory receptors on γδ T cells and corresponding ligands on antigen-presenting cells. Schematic of the main costimulatory receptors known to be expressed on human and/or mouse γδ T cells, which are discussed in this review (CD46 and JAML are not represented). With exceptions of the inhibitory molecules PD-1 and CD5, the coreceptors provide positive signals that enhance (TCR-driven) γδ T cell proliferation and/or cytokine production. This is contingent on receptor binding to cognate ligands expressed on a variety of possible "antigen presenting cells" (APC), such as dendritic cells, epithelial cells or activated lymphocytes

allowed the examination of CD28 function in molecularly well-defined models. Namely, proliferation of G8 transgenic γδ splenocytes and IELs was reduced upon treatment with an antagonistic fusion protein (CTLA-4Ig), but augmented by CD28 agonistic antibodies or exogenous IL-2 [13]. This has also been reproduced more recently using polyclonal "wild-type" splenocytes [17]. In another study, Tak Mak and collaborators showed that the alloreactivity of Vγ2$^+$ transgenic lymph node cells was also severely impaired in the CD28-deficient background [23].

Although these reports suggested that CD28 costimulation promotes the proliferation of peripheral γδ T cells, it is important to note that other biological processes appear to be CD28-independent. Namely, the development and activation (alloreactivity) of murine Vγ2$^+$ transgenic thymocytes was normal in the genetic absence of CD28 [23]. Moreover, TNF-α was shown to be produced by Vγ9Vδ2 T cells that mostly lacked CD28 [22].

An important limitation of the studies aimed at investigating the role of CD28 in γδ T cell activation is that functional responses, particularly to infectious agents, were not evaluated in CD28-deficient mice. While this was justifiable in the 1990s, when most of the studies reviewed here were performed, they could easily have been revisited more recently, as has been done for the TNFR superfamily receptor CD27 (see ahead).

A contribution by CD2 at the immunological synapse

CD2 is an Ig superfamily receptor that has been implicated in T cell activation for over 2 decades. CD2 is expressed on all T and NK lymphocytes, and also on a subset of human DCs [24]. CD2 is known to contribute to T and NK cell activation through its interaction with LFA-3 (CD58) expressed on antigen-presenting cells (APC) (Fig. 2). CD2-CD58 ligation optimises T cell recognition through stabilisation of cell-cell contact, "anchoring" cell membranes at a distance suitable for TCR-ligand interaction, while also inducing T cell polarisation [25]. CD2 signaling occurs in microdomains formed by actin-dependent coalescence of signaling molecules shared with TCR cascades, such as TCRξ, Lck or LAT [26]. Moreover, CD2 and TCR appear to synergise in recruiting and activating phospholipase Cγ1 (PLCγ1), a key component of the calcium mobilisation pathway, at the immunological synapse [27]. Specifically on γδ T cells, CD2 has been shown to be enriched at the cell surface contact point with tumour target cells [28].

The role of CD2 in γδ T cell activation was first addressed by Pawelec et al. [29] and by Kabelitz and coworkers [30]. These two studies showed that distinct panels of agonist antibodies against CD2 promoted human γδ T cell proliferation [29, 30] and IL-2 secretion [30]. Interestingly, they also demonstrated that γδ T cell activation was induced by a single anti-CD2 mAb (directed against the sheep erythrocyte-binding epitope, T11.1), by contrast to αβ T cells that required simultaneous engagement of a second mAb directed against a different (T11.2 or T11.3) CD2 epitope [29, 30].

Conversely, antagonist antibodies to CD2 were shown to inhibit γδ T cell proliferation [21]. Wang and Malkovksy reported that blockade of CD2 or CD58 led to reduced proliferation and impaired TNF-α and IL-2 secretion by Vγ9Vδ2 T cells, without affecting their cytotoxic activity [9]. Moreover, the impact of CD2/CD58 blockade on cytokine production could be reversed by adding exogenous IL-2 [9]. The interplay between CD2 signals and another key cytokine, IL-12, was addressed by Lopez et al. [31]. The authors showed that agonistic CD2 mAb and IL-12 acted synergistically to promote human γδ T cell expansion in vitro. Thus, while CD2 signals conferred resistance to activation-induced cell death, the presence of IL-12 was required for active cell proliferation [31].

More recently, Brenner and collaborators examined the role of CD2 in the activation of the Vδ1 subset of human γδ

T cells [32], which is enriched at epithelial surfaces [6]. By blocking CD2 or CD58 in co-cultures between Vδ1 T cells and DCs, they obtained a significant, dose-dependent effect on Vδ1 T cell proliferation [32].

Somewhat surprisingly, very few studies have addressed the role of CD2 in murine γδ T cell activation. Nonetheless, it seems clear that for both lymph node [33] and IEL [34] γδ T cells, CD2 expression correlates with increased proliferative capacity. Additional functional studies are required to understand the importance of CD2 signals in murine γδ T cell responses, particularly in models of infection or autoimmunity.

Other Ig superfamily coreceptors

ICOS

After being induced by T cell activation, inducible costimulator (ICOS) binds to ICOS-L (B7 h) to provide positive signals that control αβ T cell proliferation and differentiation. Recently it was shown that ICOS signals can differentially impact on Th1/Th17/Tfh (follicular helper T) $CD4^+$ cell responses, depending on the inflammatory milieu [35]. Moreover, ICOS deficiency results in impaired immune responses [36–38], while mice doubly deficient for ICOS and CD28 are even more immune-compromised [39].

Human peripheral blood Vγ9Vδ2 T cells have been shown to induce ICOS expression upon activation, with >50% of cells becoming $ICOS^+$ after a few days in culture in the presence of phosphoantigens and IL-2 [40]. On the other hand, while freshly isolated blood Vγ9Vδ2 T cells lacked ICOS expression, a subpopulation of Vγ9Vδ2 T cells present in the tonsils readily exhibited high levels of ICOS [41]. This subset, originally defined as $CXCR5^+$, also expressed various other activations (like CD25 and HLA-DR) and memory markers, and promoted IgG, IgA and IgM secretion in co-cultures with B cells. In fact, tonsil $CXCR5^+$ $ICOS^+$ Vγ9Vδ2 T cells displayed overt Tfh properties [41], unlike blood Vγ9Vδ2T cells that are strongly Th1-biased [42]. Despite these interesting findings associated with ICOS expression on activated human γδ T cells, it remains to be elucidated whether ICOS signals contribute to proliferation or cytokine secretion following TCRγδ stimulation.

PD-1

Unlike ICOS, programmed death-1 (PD-1 or CD279) provides negative signals that limit the activation and expansion of TCR-triggered T cells, thus contributing to the maintenance of tolerance. PD-1-deficient mice develop autoimmune syndromes that include cardiomyopathy and a lupus-like disease [43]. The expression of PD-1 is upregulated after αβ T cell activation to deliver, upon ligation by PD-L1 (B7-H1) or PD-L2 (B7-DC), inhibitory signals (often along the TCR signaling cascade) that suppress proliferation and cytokine secretion. More recently, PD-1 was shown to play a crucial role in tumour evasion of $CD8^+$ T cell surveillance [44, 45].

Two sets of unpublished findings suggest that PD-1 may also control γδ T cell activation and anti-tumour responses. Resting human Vγ9Vδ2 T cells express PD-1 and further upregulate it upon phosphoantigen treatment. Moreover, PD-1 is recruited to the immunological synapse during Vγ9Vδ2 T cell activation, and PD-1 blockade enhanced proliferation and the secretion of Th1-type cytokines (Daniel Olive, personal communication).

Furthermore, data obtained in the mouse suggest that inhibitory PD-1 signals may play a key role in the exhaustion of tumour-reactive γδ-T cells in vivo. PD-1 is expressed to a greater extent on γδ-T cells isolated from tumour-bearing mice as compared to γδ-T cells from healthy controls (Richard Lopez, personal communication). In addition, a far greater proportion of the PD-1-positive γδ T cells failed to proliferate and underwent apoptosis, when compared to the PD-1-negative population. Most importantly, PD-L1 blockade substantially restored γδ-T cell proliferation in these assays (Richard Lopez, personal communication). This suggests that suppressing the PD-1 pathway may possibly "rescue" or "revive" γδ T-cell expansion in tumour-bearing hosts. Thus, the manipulation of PD-1 signals may prove to be of significant value for (γδ-)T cell-based immunotherapy.

JAML

Junctional adhesion molecule-like (JAML) was initially identified as a player in leukocyte transmigration, as its ectopic expression in myeloid leukemia cells resulted in enhanced cell adhesion to endothelial cells [46]. Recent data from the Havran group have demonstrated a key costimulatory function of JAML in murine dendritic epidermal T cells (DETC), a murine IEL subset that expresses an oligoclonal Vγ5 Vδ1 TCR [6]. JAML binds to Coxsackie and adenovirus receptor (CAR) expressed on keratinocytes [17]. This induces JAML clusters on the DETC surface and recruits PI3 K through a mechanism analogous to that employed by CD28 [47].

While JAML is present at low levels in resting DETC, as well as in intestinal (but not in thymic or splenic) γδ T cells, its expression is significantly upregulated after stimulation with a mitogen [17]. JAML costimulation was shown to induce DETC proliferation and the secretion of IFN-γ, TNF-α and IL-2. Furthermore, JAML blockade after wounding decreased local DETC activation and impaired

the healing process [17]. These data established JAML as a key coreceptor in mouse DETC activation. It is still unknown whether JAML plays any role in the costimulation of other (including human) γδ T cell subsets.

TNFR superfamily coreceptors in γδ T cell activation

CD27 costimulation of IFN-γ-producing γδ T cells

Extensive work by the Borst and van Lier groups, among others, have clearly shown that CD27 (TNFRSF7) plays critical roles in αβ T cell activation [48, 49]. For example, mice lacking CD27 showed reduced numbers of virus-specific $CD8^+$ T cells in the lung and spleen following primary and secondary infection with Influenza [48, 50]. Conversely, CD70 (CD27-ligand) transgenic mice displayed increased αβ T-cell responses to viral or to tumour challenge [51, 52], and chronic immune activation resulted in immunodeficiency [51, 53]. Recent work has demonstrated that CD70–CD27 interactions promote the survival of virus-specific $CD8^+$ T cells (particularly in the infected tissue) and has identified IL-2 as a key positive target of CD27 signalling [54].

We have shown that CD27 levels define two distinct and stable subsets of γδ T cells in naïve C57Bl/6 mice [55]. In the spleen, lymph nodes and various other tissues (such as lung or gut) of adult mice, approximately 75–90% of γδ T cells are $CD27^+$. Upon activation, these cells make IFN-γ, whereas IL-17 is only produced by their $CD27^-$ counterparts. Interestingly, these distinct phenotypes are established in the thymus, where $CD27^+$ γδ cells express genes associated with a Th1 differentiation programme, while $CD27^-$ thymocytes constitutively express IL-17 [55, 56]. In fact, the "developmental pre-programming" of γδ T cells [57] could be observed already in the foetal thymus [55, 58]. In brief, the current model of γδ T cell development proposes that ligation of both TCRγδ [59] and CD27 [55] are required for the differentiation of IFN-γ-producing γδ T cells, whereas the Th17-like pathway is preferentially driven by cytokines such as TGF-β [56].

Having identified CD27 as a thymic determinant of γδ T cell differentiation, we went on to explore its role in the peripheral activation of "pre-programmed" IFN-γ-producing γδ T cells. We observed that CD27 signals are absolutely required for the expansion of these cells upon infection with herpes viruses or malaria parasites in mice [60]. We further showed that CD70-CD27 interactions provide survival and proliferative signals that control TCRγδ-driven activation. Thus, CD27 signalling activated the non-canonical NF-kB pathway and enhanced the expression of anti-apoptotic and cell cycle-related genes [60].

While the data above were obtained with murine γδ T cells, we have more recently addressed the role of CD27 costimulation in human γδ T cell activation. An average of 80% of Vγ9Vδ2 T cells express CD27 [61] and are considered to include both naive and central memory cells [62]. Upon activation with PMA and ionomycin, the vast majority of $CD27^+$ Vγ9Vδ2 T cells produced IFN-γ, whereas less than 1% made IL-17 [61]. Importantly, the proliferation of these cells was sensitive to CD70-CD27 modulation: administration of soluble recombinant CD70 enhanced, whereas anti-CD27 (or anti-CD70) mAbs reduced, Vγ9Vδ2 T cell expansion in vitro. Moreover, CD27 signals induced calcium flux and upregulated the expression of *cyclin D2* and the anti-apoptotic gene *Bcl2a1*. In fact, a major role of CD27 costimulation appeared to be the protection from AICD following phosphoantigen stimulation [61]. These data suggest that the modulation of CD70-CD27 signals may be of great value for T cell-based immunotherapy. Consistent with this, work on $CD8^+$ T cells has demonstrated that the expansion of tumour-specific cytotoxic T lymphocytes (CTLs) is critically dependent on CD27 costimulation [63, 64].

Regulation of cytokine production by CD30 costimulation

CD30 is a lymphoid-restricted receptor, widely used as a Hodgkin's lymphoma marker, which is physiologically involved in T cell differentiation. Although initially associated with Th2 cells and clones [65], recent work has proposed a role for CD30 in both Th1 [66] and Th17 [67] differentiation of $CD4^+$ T cells.

In human γδ T cells, CD30 is expressed upon activation, usually declining in long-term (>2 weeks) cultures [68]. Moreover, CD30 can be shed (as soluble CD30, sCD30) from the surface of γδ T cells stimulated with anti-CD3 and anti-CD30 mAbs [69]. Of note, sCD30 is regarded as a marker of chronic B or T cell activation, and it has significant predictive value for transplant rejection [70].

Consistent with earlier studies on αβ T cells [65], high levels of CD30 expression in human γδ T cells were linked to a Th2-like cytokine profile [71–73]. However, CD30 costimulation has been shown to enhance transcription and cytokine production of both IL-4 and IFN-γ in TCR-activated γδ T cells [72]. Moreover, CD30 signalling, which prolonged the calcium flux induced by TCR activation, appeared to have a selective effect on cytokine production, but not on γδ T cell survival or proliferation [72].

Although CD30 function is clearly important in various infection and inflammation models, such as mycobacterial infection [66] and inflammatory colitis [74], its specific relevance to the γδ T cell response in vivo remains to be elucidated.

Other TNFR superfamily coreceptors

Based on their important roles in the activation of other lymphocyte lineages, the TNFR superfamily members OX40 (CD134) [75] and 4-1BB (CD137) [76] would be strong candidates to impact on $\gamma\delta$ T cell costimulation. 4-1BB is expressed on a variety of murine and human $\gamma\delta$ T cell subsets [72] (our unpublished data), whereas OX40 is upregulated following phosphoantigen activation of Vγ9Vδ2 T cells, with up to 90% of cells expressing OX40 after a few days in culture [40]. However, the functional implication of these coreceptors in $\gamma\delta$ T cell activation is still unknown.

Interestingly, activated Vγ9Vδ2 T cells also express high levels of 4-1BBL (CD137L) [77], which besides acting as a ligand for 4-1BB on T and NK cells, may also participate in $\gamma\delta$ T cell activation because of its known reverse signalling ability [78]. This in fact also applies to CD70 (CD27-ligand), which is highly induced upon phosphoantigen-mediated stimulation of Vγ9Vδ2 T cells [40, 61]. These issues deserve further investigation.

NKG2D as "signal 2" in $\gamma\delta$ T cell activation

While costimulation is traditionally attributed to members of the Ig or TNFR superfamilies, unrelated proteins can also make important contributions to T cell activation. For $\gamma\delta$ T cells, the most notable case is the C-type lectin-like NKG2D (natural killer group 2, member D) receptor (Fig. 1). This protein is highly expressed on NK, $\gamma\delta$ and CD8$^+$ T cells, and provides activating signals upon ligation to one of its multiple ligands. In humans, these belong to the MIC (A-B) and ULBP (1-6) families, whereas in the mouse there are H60, MULT1 and various RAE1 molecules [79]. Interestingly, and although NKG2D is 70% identical between the two species, its ligands are not necessarily orthologous. NKG2D ligands are induced in response to cellular stress, for example, downstream of the DNA-damage response pathway in tumour cells [80, 81]. Consequently, NKG2D-expressing lymphocytes are activated to kill tumour [82] or pathogen-infected cells [83]. The biological significance of this recognition system is testified to by the increased susceptibility of NKG2D-deficient mice to tumour development [84].

NKG2D can either directly activate lymphocytes, as happens for NK cells, or "play second fiddle" (coreceptor) to the TCR, as described for CD8$^+$ T cells [85]. A costimulatory function of NKG2D in $\gamma\delta$ T cells was first reported by Das et al. [86]. They showed that MICA-NKG2D interactions enhanced the response (i.e., cytokine production) of Vγ9Vδ2 T cells upon TCR activation, and proposed that NKG2D could be a functional equivalent of CD28, which was absent in many of these cells [86]. More recently, Scotet and coworkers have performed a detailed study on intracellular calcium mobilisation following Vγ9Vδ2 T cell activation [87]. The authors observed that while NKG2D per se could not induce calcium flux, its co-engagement significantly augmented the intensity of TCR/CD3-mediated responses, which also translated into enhanced cytotoxic activity. By contrast, the production of IFN-γ was unaffected by NKG2D costimulation [87].

Other researchers have defended that NKG2D signals can activate $\gamma\delta$ T cells in the absence of TCR engagement. Thus, NKG2D ligation in Vγ9Vδ2 T cells upregulated the activation marker CD69 independently of (and to similar extent as) TCR stimulation [88]. Moreover, mouse DETCs, which are very sensitive to NKG2D ligand expression [89, 90], were suggested to kill some tumours solely based on NKG2D engagement [91].

To complicate matters further, some studies have suggested that NKG2D ligands can also bind to TCR$\gamma\delta$. Thus, MIC proteins were shown to bind to Vδ1$^+$ TCR [92], whereas ULBP4 was reported to bind to the Vγ9Vδ2 TCR [93]. In this scenario, NKG2D ligands could provide both "signal 1" and "signal 2" in $\gamma\delta$ T cell activation.

Our own data have dissociated the roles of NKG2D and TCR$\gamma\delta$ in the context of anti-tumour Vγ9Vδ2 T cell responses. We thus favour a two-step model where the two receptors act independently (unlike costimulation): TCR$\gamma\delta$ mediates bona fide activation, i.e., proliferation and cytokine production, for which the contribution of NKG2D is negligible; however, the recognition of "stressed self" is critically mediated by NKG2D rather than TCR$\gamma\delta$ [94]. This view is supported by our findings that although TCR-mediated activation is a prerequisite for effector Vγ9Vδ2 T cell function [42], the definition of killing targets segregates with ULBP1 expression in haematopoietic tumours [95] and is selectively abrogated upon NKG2D, but not TCR$\gamma\delta$, antibody-mediated blockade [96].

While future research will undoubtedly clarify how much and what type of "signal 2" is provided by NKG2D engagement on $\gamma\delta$ T cells, its positive impact on functional properties should be promptly applied in the clinic [94].

Other receptors (potentially) involved in $\gamma\delta$ T cell costimulation

CD5

CD5 is a type-I transmembrane glycoprotein found on essentially all T cells; it was actually used as a T cell marker before the discovery of CD3. The extracellular region of CD5 consists of three domains belonging to the scavenger receptor cysteine-rich (SRCR) superfamily. The

SRCR domain is an ancient and highly conserved protein module of approximately 100-110 amino acids, present in a series of soluble or membrane-bound receptors found on hematopoietic and non-hematopoietic cells [97].

CD5 is known to inhibit $\alpha\beta$ T cell activation, presumably by interfering with the signalling machinery used by the positive regulators TCR$\alpha\beta$, CD28 and CD2. Thus, CD5-deficient T cells are hyper-responsive to TCR/CD3 stimulation, which has inspired the concept of CD5 signalling being protective against autoimmunity [98].

Although almost all human $\gamma\delta$ T cells express CD5, specific antibody blockade had no effect on their proliferation [21]. In mice, whereas most splenic $\gamma\delta$ T cells express CD5, only small subsets do so in the gut intraepithelial and lamina propria compartments. Intestinal CD5$^+$ $\gamma\delta$ T cells were proposed to exert a regulatory role in colitis [99], but this, and more generally CD5 function on $\gamma\delta$ T cells, requires further investigation. A novel piece of data that should be taken into account is that CD5 is involved in species-specific homophilic interactions, i.e., CD5 binds to CD5 [100] (Fig. 2).

CD46

CD46 is an ubiquitously expressed type I glycoprotein that regulates complement activity by binding to C3b and C4b, and acting as a cofactor for their proteolytic degradation by serine protease factor I [101]. On the other hand, CD46 is known to serve as a receptor for seven pathogens (viral and bacterial) that infect humans. More recently, CD46 was shown to act as a costimulatory receptor for $\alpha\beta$ T cells. Co-engagement of TCR and CD46 on human CD4$^+$ T cells induces substantial IL-10 production, at the expense of IFN-γ secretion [102, 103]. In fact, CD46 signalling attenuates IL-2 production and upregulates IL-10 expression in polarised Th1 cultures [103]. However, unlike CD4$^+$ T cells, Vγ9Vδ2 T cells do not express the BC1 isoform of CD46 (linked to Th1 switch to IL-10 producers), but an alternative BC2 version of the protein, which contains a distinct cytoplasmic domain. Signalling through this isoform fails to induce IL-10 expression, but reduces IFN-γ and TNF-α secretion in HMB-PP-stimulated Vγ9Vδ2 T cell cultures [103]. These data suggest a novel and unique role for CD46 in regulating the production of (Th1-like) pro-inflammatory cytokines in human $\gamma\delta$ T cells.

LFA-1

Lymphocyte function-associated antigen (LFA-1) is an integrin expressed on T and B cells, and granulocytes/macrophages. LFA-1 is critical for cell-cell adhesion and thus affects many aspects of T cell physiology. One study has suggested that LFA-1 blockade partially inhibits Vδ1 T cell proliferation in co-cultures with DCs [32]. By contrast, work on Vγ9Vδ2 T cells showed that blocking LFA-1 had no effect on proliferation or cytokine secretion, but inhibited cytolytic activity against lymphoma cells [9]. By analogy to $\alpha\beta$ CD8$^+$ T cells, it is very plausible that LFA-1 is important to stabilise lytic synapses between $\gamma\delta$ T cells and their targets, without directly affecting $\gamma\delta$ T cell activation.

Concluding remarks and future directions

We have made significant progress over the past 2 decades towards understanding the roles of $\gamma\delta$ T cells in immunity to pathogens and tumours. However, some key aspects of the basic biology of the cells still elude us. Thus, most TCR$\gamma\delta$ ligands are yet to be identified, and the functional relevance of many costimulatory molecules expressed on the surface of $\gamma\delta$ T cells remains unclear (or even controversial).

We believe it will be critical to clarify the individual roles of costimulatory receptors during distinct phases of $\gamma\delta$ T cell responses, both in murine models and in human diseases. Some of these studies are underway. However, while some receptors, such as NKG2D, CD27 and the newly implicated JAML and CD46, are under the spotlight, many others have been forgotten over the last 10 years. These include proteins such as ICOS, OX40 or 4-1BB, known to play crucial roles in $\alpha\beta$ T cell activation. Therefore, it would be important to (re)visit the function of these molecules using experimental tools such as antagonist/agonist monoclonal antibodies or fusion proteins, and genetically modified mice. These could provide novel functional insight based on infection [60] or autoimmune models [104, 105] that illicit $\gamma\delta$ T cell responses.

From a fundamental point of view, we still need to understand exactly which costimulatory molecules impact on survival, proliferation or cytokine production (considering that different rules may apply to IFN-γ-producing versus IL-17-producing $\gamma\delta$ T cells); and to what extent coreceptors that provide negative signals (like PD-1) contribute to the regulation of $\gamma\delta$ T cell function. On the other hand, aiming at translational benefit, it will be key to manipulate the activity of costimulatory receptors in pre-clinical models and in clinical trials. We think the success of $\gamma\delta$ T cell-based immunotherapy could be currently limited by the lack of a costimulation component and hope this caveat can be overcome by future research.

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