Review Article

Repertoire Development and the Control of Cytotoxic/Effector Function in Human $\gamma\delta$ T Cells

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T cells develop into two major populations distinguished by their T cell receptor (TCR) chains. Cells with the $\alpha\beta$ TCR generally express CD4 or CD8 lineage markers and mostly fall into helper or cytotoxic/effector subsets. Cells expressing the alternate $\gamma\delta$ TCR in humans generally do not express lineage markers, do not require MHC for antigen presentation, and recognize nonpeptidic antigens. We are interested in the dominant Vγ2Vδ2+ T cell subset in human peripheral blood and the control of effector function in this population. We review the literature on $\gamma\delta$ T cell generation and repertoire selection, along with recent work on CD56 expression and defining a cytotoxic/effector lineage within the phosphoantigen-reactive Vγ2Vδ2 cells. A unique mechanism for MHC-independent repertoire selection is linked to the control of effector function that is vital to the role for $\gamma\delta$ T cells in tumor surveillance. Better understanding of these mechanisms will improve our ability to exploit this population for tumor immunotherapy.

1. Introduction

The idea of using the immune system to combat cancer dates back to 1890, when Paul Ehrlich proposed vaccines against cancer in the wake of various successful immunizations to protect against microbial diseases. The importance of immune surveillance against tumor emergence and progression was reinforced with the observation that immune deficiency states, including iatrogenic immune suppression, severe combined immunodeficiency (SCID), common variable immunodeficiency (CVID), and acquired immunodeficiency syndrome (AIDS), greatly increased patient susceptibility to many types of malignancies [1–4]. Targeting the immune system to combat tumors is in principle, a promising therapeutic strategy [5] although progress has been slow and success is limited. Malignant cells are often difficult to distinguish from normal cells making it difficult to achieve acceptable therapies and there are few schemes for generating immune treatments with sufficient potency to overcome the tumor burden.

The recent discovery of tumor associated antigens, expressed at higher levels or uniquely expressed by tumors cells, provided a means for targeting immune responses to specific malignancies [5, 6]. Efforts have focused on generating major histocompatibility (MHC)-restricted, tumor specific $\alpha\beta$ T cells, through vaccination, ex vivo activation or expansion of cytotoxic lymphocytes, or various methods of redirected cytolysis. The efficacy of T cell immunotherapies continues to be limited because tumor neoantigens are usually weak immunogens except in some cases of viral transformation where virus antigens are expressed on malignant cells. Further, MHC tumor-associated antigens or costimulatory molecules can be downregulated to evade detection and tumors can kill or inactivate responding T cells [7–9].

Alternatives to conventional T cell responses might include the use of innate-like lymphocytes, such as $\gamma\delta$ T cells, which have non-MHC restricted recognition of tumor cells. The $\gamma\delta$ T cell population was first described in 1986 with reports of a new heterodimeric T cell receptor [10] that was
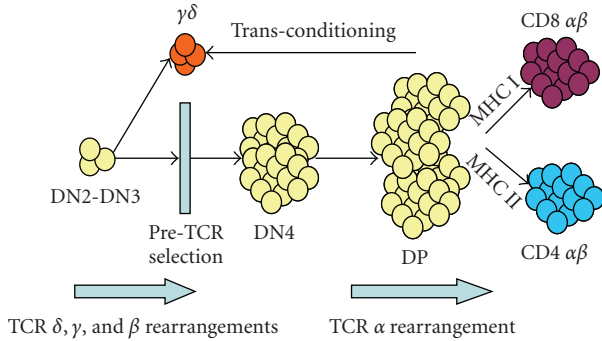
Figure 1: T Cell Development. $\gamma\delta$ T cells most often arise from the CD4−CD8− (double negative, DN) stages of thymocyte development as a result of successful rearrangements of both the γ and δ TCR chains. Little, if any, proliferation occurs between these steps, thus limiting diversity. Signals delivered in *trans* from the CD4+CD8+ (double positive, DP) population is crucial for the development of effector functions in $\gamma\delta$ cells. Figure adapted from Hayday and Pennington [18].

associated with CD3 [11]. Rapid growth and evolution of this field lead to the identification of important roles for $\gamma\delta$ T cells in immune regulation, response to infectious disease, and participation in tumor surveillance. A large and growing interest centers on the possibility of exploiting $\gamma\delta$ T cells for cancer immunotherapy [12–17]. However, key questions about the activation and cytotoxic activities of these cells remain unanswered, especially regarding the mechanisms controlling cytotoxic effector activities that are limited to a subset or lineage of cells found in adult human beings.

2. $\gamma\delta$ T Cell Development

Most of our knowledge about T cell development and especially about the evolution of cytotoxic $\gamma\delta$ T cells comes from murine studies. T cells develop normally from pluripotent precursor cells in the thymus. A complex series of signaling events direct developing thymocytes to become either $\alpha\beta$ or $\gamma\delta$ T cells (Figure 1). Most progenitors entering the thymus, first from fetal liver and later from bone marrow, are "double negative" (DN) expressing neither CD4 nor CD8, the lineage markers of $\alpha\beta$ T cells [19, 20]. Thymocytes progress through at least 4 DN stages [21, 22]. Murine $\gamma\delta$ T cells emerge mostly from the DN2 and DN3 stages, consistent with their predominantly CD4−CD8− phenotype in the periphery. This is also true for humans, though some plasticity remains even late into $\alpha\beta$ differentiation [23].

Somatic rearrangement of genes encoding the TCR chains δ, γ, and β begins in DN2 [24]. Several lines of evidence suggest the Tcrd locus (encoding the δ TCR chain) rearranges before other TCR genes. In SCID mice, T cell development is arrested just after recombination at Tcrd [25]. In humans, the earliest thymic progenitors (CD34+CD1a−) have a rearranged Tcrd locus, while the Tcrb locus remains in germline configuration [26]. Additionally, partial allelic exclusion is evident at the Tcrg locus

[27], indicating that γ chains are pairing with preexisting δ chains.

Successful recombination of δ and γ chains leads to expression of $\gamma\delta$ TCR on the cell surface. Here, signaling events through the $\gamma\delta$ TCR are thought to drive the developing thymocytes away from the CD4+CD8+ "double positive" (DP) stage of $\alpha\beta$ maturation and onto the $\gamma\delta$ track. Successful rearrangement of the TCR β chain allows pairing of the β chain with a surrogate pre-TCRα chain, forming the pre-TCR. Signaling through this pre-TCR allows survival, extensive proliferation, and differentiation towards the DP stage of $\alpha\beta$ development, followed by rearrangement of the Tcra locus [18]. Great diversity is generated in the $\alpha\beta$ population by expansion of pre-TCR expressing cells since multiple daughter cells, bearing identical β chains, will then rearrange unique α chains and expand repertoire.

There is no evidence for a pre-TCR in $\gamma\delta$ T cell development [28], indicating that commitment to the $\gamma\delta$ fate requires a complete TCR with γ and δ chains successfully rearranged and paired. Little proliferation occurs during γ and δ chain rearrangement [28]. The requirement for two successful recombination events before $\gamma\delta$ TCR expression biases thymocytes towards a $\alpha\beta$ T cell fate since only the β chain needs to be rearranged before expansion of the population, and limits diversity of the $\gamma\delta$ population [29].

In $\alpha\beta$ T cells, negative selection prevents autoimmunity by deleting or inactivating cells that express a self-reactive TCR. Selection is accomplished when self ligands are expressed by medullary thymic epithelial cells [30]. For $\gamma\delta$ T cells, the criteria for negative selection are poorly defined. Many of the known $\gamma\delta$ T cell antigens in both humans and mice are self or ubiquitous molecules, making negative selection for these cells more complex perhaps using subtle differences in the strength of signal delivered through the TCR/CD3 complex.

Positive selection in $\alpha\beta$ T cells restricts the TCR repertoire by selecting for cells that recognize MHC class I or II molecules. Selection also defines lineage, marked by expression of CD4 or CD8, depending on whether MHC Class I or II molecules are recognized by the $\alpha\beta$ TCR [31]. Cells recognizing MHC class I downregulate expression of CD4, become single positive for CD8, and evolve as cytolytic effectors. Those cells recognizing MHC class II will downregulate expression of CD8, become single positive for CD4, and adopt cytokine-secreting helper functions [32]. Additional signals, received either during development or upon activation, push $\alpha\beta$ T cells down other paths including Th1, Th2, Th17, and regulatory lineages, each with distinct functions.

For $\gamma\delta$ T cells, evidence suggests that signaling through the expressed TCR is important for commitment to the $\gamma\delta$ lineage, but whether this is accomplished through ligand-dependent or independent signaling is unknown [18]. Thymic signals may also affect function. It was discovered recently that a nonclassical MHC molecule T10/T22 binds directly to mouse $\gamma\delta$ T cell heterodimers mainly in the CDR3 region [33–35]. Mice lacking expression of T10 or T22 develop normal numbers of $\gamma\delta$ T cells able to recognize these ligands, suggesting that ligand-dependent selection may not

be required for differentiation [36]. While the number of $\gamma\delta$ T cells remained unchanged in T10/T22 knock-outs, the cells are altered functionally. Those that developed in the presence of T10/T22 secreted IFN-γ upon activation, while those that developed without antigen secreted IL-17 [36]. Another example of the importance for thymic signaling is seen in the FvB·Tac strain of mice, where the epidermal Vγ5Vδ1 TCR repertoire is depleted [37]. The FvB strain defect has been mapped as a mutation in the SkinT gene. SkinT is an Ig superfamily member that encodes a protein in the butyrophilin family. This protein positively selects epidermal $\gamma\delta$ T cells; a selection does not occur in the mutant FvB strain and the repertoire has a lower frequency of Vγ5Vδ1+ cells [38].

In transconditioning [39–41], the functions of $\gamma\delta$ T cells are partly dependent on the presence of $\alpha\beta$ double positive (DP, CD4+CD8+) T cells in the thymus. Mice either lacking the Tcrb gene locus or unable to make the pre-TCRα have few double positive $\alpha\beta$ cells present in the thymus. $\gamma\delta$ T cells arising in these mice show altered gene expression profiles [41]. Activated splenic $\gamma\delta$ T cells in these mice adopt a regulatory phenotype, resembling skin-resident $\gamma\delta$ cells rather than the usual cytotoxic, IFN-γ producing effector phenotype [40]. Since neither $\gamma\delta$ T cells nor DN2 cells have a cell-autonomous requirement for TCR β, these effects are thought to be *in trans* [41].

In 2009, Ribot et al. reported that developing $\gamma\delta$ T cells in mice could be separated into populations based upon CD27 expression [42]; differences were apparent even at early stages of embryonic development (days 14-15). CD27+ $\gamma\delta$ T cells that engaged the ligand CD70 became effector cells able to secrete IFN-γ. Cells that did not express CD27 developed a regulatory phenotype and secreted IL-17. These data suggested that distinct lineages exist among $\gamma\delta$ T cells, not unlike CD8 and CD4 $\alpha\beta$ T cells, and lineage differences impact effector functions.

Overall, evidence suggests that signals received in the thymus can alter the function of developing $\gamma\delta$ T cells in mice, either through the expressed TCR or through transconditioning events. Much less is known about the distinct developmental stages and unique signaling requirements for human $\gamma\delta$ T cells and CD27 expression was noted [43] in cytotoxic and noncytotoxic subpopulations of Vγ2Vδ2+ cells, indicating it may not be a strict marker for cytotoxic subsets in man.

3. Vγ2Vδ2 T Cells

Human $\gamma\delta$ T cells can be subdivided into two main populations based upon δ chain expression. $\gamma\delta$ T cells expressing the Vδ1 chain are most often found in mucosal tissues, where they are thought to be involved in maintaining epithelial tissue integrity in the face of damage, infection, or transformation [44, 45]. A second population of $\gamma\delta$ T cells expresses the Vδ2 chain and makes up about 1%–10% of circulating lymphocytes in healthy human adults [46]. The Vδ2 chain pairs almost exclusively with Vγ2 (called Vγ9 in an alternative nomenclature). The Vγ2Vδ2 pairing is only present in humans and nonhuman primates [44, 47].

$\gamma\delta$ T cells are often termed innate-like lymphocytes, due to their rapid, antigen-triggered responses, and lack of classical MHC restriction. However, they possess a TCR composed of rearranging germline elements [46], require antigen presentation [48], and undergo peripheral selection [49], arguing they should be classified as components of adaptive immunity. Functionally, they demonstrate cytotoxic responses against cells infected with a variety of viruses, bacteria, or protozoa and they also recognize and kill many human tumors [44, 50]. Cytotoxicity is mediated in much the same manner as for $\alpha\beta$ T cells, namely, through perforin/granzyme and Fas/FasL pathways or the production of TNF-α [51, 52].

$\gamma\delta$ T cells are preserved evolutionarily in all jawed vertebrates [53] indicating that their role in immune defense is not redundant [44]. Indeed, the $\alpha\beta$ and $\gamma\delta$ T cell populations recognize vastly different types of antigens. $\alpha\beta$ T cells recognize nonself peptide fragments presented by MHC molecules. $\gamma\delta$ T cells, on the other hand, recognize a wide variety of self-antigens including stress molecules like MICA and MICB, heat shock proteins, and intriguingly, nonpeptidic metabolites of isoprenoid biosynthesis [54–58]. They do not require conventional antigen presentation in the context of MHC [59].

The differences in antigen recognition and specificities between the two T cell types define their unique roles in immunity. Studies using both $\alpha\beta$ and $\gamma\delta$ T cell-depleted mice showed qualitative and quantitative differences in clearance of infections [60, 61]. Human patients with microbial diseases, such as tuberculosis or malaria, often have large expansions of the peripheral $\gamma\delta$ T cell subset, sometimes comprising up to 80% of all T cells [62–64]. In the case of malaria, these expanded cells are believed to mediate pathogen elimination. Some viral infections, such as Hepatitis C Virus and Coxsackie B, induce high numbers of $\gamma\delta$ T cells that home to the site of infection and contribute to pathology [50, 65]. Human Immunodeficiency Virus type 1 (HIV-1) infection results in rapid and specific depletion of the peripheral Vδ2 T cell population with consequences for the host's ability to resist intercurrent infections [66, 67]. Interestingly, $\gamma\delta$ T cell levels and activity are high among patients who have natural control of HIV without the use of antiretroviral therapy [68]. These unique protective or pathogenic responses of $\gamma\delta$ T cells in diverse infectious diseases highlight their unique roles in immunity.

The γ and δ TCR chains share an Ig-like structure similar to the $\alpha\beta$ T cell receptor; chain expression is determined by randomly rearranging V, (D) and J segments. In the case of γ and δ chains, the germline repertoire is restricted severely, due to a limited number of rearranging elements [69]. For instance, the Tcrb locus has 48 functional V and 13 functional J elements, compared to the 8 V and 5 J elements of the Tcrg locus [70]. Much of the CDR3 region diversity is due to N nucleotide addition at the V-J and V-D-J junctions [46]. Due to multiple D segment rearrangements in the δ chain, actual diversity may be even greater than that seen in $\alpha\beta$ T cells [71, 72]. Despite the potential for diversity, greater than 60% of circulating $\gamma\delta$ T cells bear the Vγ2Vδ2 TCR [73, 74], suggesting either restricted
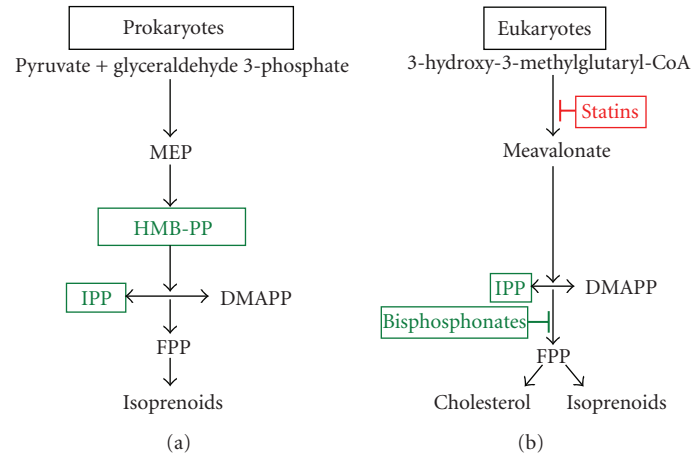
FIGURE 2: Human Vγ2Vδ2 T cells respond to stimulatory phosphoantigens produced during bacterial or mammalian isoprenoid synthesis. Isoprenoid synthesis in many prokaryotes and protists produces the intermediate (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) from pyruvate and glyceraldehyde 3-phosphate via the erythritol 4 phosphate pathway. Eukaryotes use the mevalonate pathway that produces isoprenoids and colesterol. HMBPP and IPP are phosphoantigens; HMBPP is unique to the erythritol 4 phosphate pathway while IPP is produced in both pathways. Phosphoantigens stimulate cytokine secretion and cytotoxicity in human Vγ2Vδ2 T cells. Statins block the eukaryotic pathway prior to mevalonate synthesis, and decrease the production of IPP. Bisphosphonates are a class of drugs that inhibit farnesyl pyrophosphate synthase and cause the accumulation of IPP in mammalian cells. When mammalian cells of myeloid origin and some tumor cells are treated with bisphosphonate, they are more stimulatory for Vγ2Vδ2 T cells due to increased IPP production.

chain pairing or a selective chronic expansion of this cell population. Studies comparing the genotypes of γδ clones derived from the thymus with those derived from peripheral blood found that thymic clones possessed nearly all possible Vγ-Vδ combinations, with the Vγ2Vδ2 pairing making up only 5%, indicating that physical restriction on chain pairing was not a critical factor [46]. Nearly all peripheral γδ T cells acquire memory markers by the time an individual reaches 2 years of age [75, 76]. These data suggest that selective activation and expansion in response to ubiquitous or self-antigens is the mechanism responsible for overrepresentation of the Vγ2Vδ2 T cell subset among adults [71, 77].

Vγ2Vδ2 T cells respond to low-molecular weight, non-peptidic, phosphate-containing molecules termed phosphoantigens [78, 79]. The best characterized of these antigens is isopentenyl pyrophosphate (IPP) [54], a substrate in the mevalonate pathway for cholesterol synthesis (Figure 2) in eukaryotes and some bacteria [80]. Physiologic levels of IPP, however, are not stimulatory [81]. A more potent antigen is (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate, or HMBPP [82], a substrate in the nonmevalonate pathway found in plants and prokaryotic organisms [83, 84]. The difference in potency is thought to provide a mechanism for self-recognition, wherein γδ T cells respond to local bacterial infections, but not to normal tissues [85].

Phosphoantigen stimulation causes a polyclonal expansion of Vγ2Vδ2 T cells and selects for the Vγ2-Jγ1.2 rearrangement but does not select specific complementary determining region 3 sequences (CDR3). Spectratyping analysis of the Vγ2 chain demonstrated a strong bias for Vγ2-Jγ1.2 rearrangements, reflecting chronic expansion of the population in response to phosphoantigens. Spectratyping of the Vδ2 chain, on the other hand, showed a normal distribution of chain lengths [86], suggesting less bias in the Vδ2 chain repertoire and arguing that phosphoantigen recognition is influenced mainly by Vγ chain sequences.

X-ray crystal structures of a human Vγ2Jγ1.2 Vδ2 T cell receptor revealed a potential positively charged binding pocket made of arginine residues from the Vγ2 segment and lysine residues from the Jγ1.2 segment, of which Lys109 appeared to be most important [70]. Site-directed mutagenesis confirmed the importance of these lysine residues, as mutations in the KKIK amino acid stretch encoded in Jγ1.2 either partially or completely abolished responsiveness to phosphoantigens [87]. No particular rearrangements were selected in the δ chain [86], though a conserved hydrophobic residue at position 97 was correlated with antigen recognition [88]. Perhaps the most compelling argument for TCR interactions with phosphoantigen is that transfection of the Vγ2-Jγ1.2Vδ2 TCR into a TCR negative JRT3 T cell line conferred responsiveness to phosphoantigen [89].

Despite evidence implicating the Vγ2Jγ1.2 chain in phosphoantigen reactivity, all attempts to show physical interactions between receptor and antigen have been unsuccessful [59]. While neither phosphoantigen-driven activation nor the cytotoxic activity of γδ T cells requires MHC molecules, activation does depend upon cell-cell contact with an antigen presenting cell (APC) [48, 90]. Stimulated γδ T cells themselves seem to act as APC, but self-activation is not optimal for proliferation responses [91]. Antigen processing by APC is not required; paraformaldehyde-fixed cells are still able to stimulate γδ T cell responses [90]. This suggests that an antigen presentation molecule is involved in TCR recognition of phosphoantigen. In fact, tetramers of murine γδ TCR bind to cells in an antigen-dependent manner [34] and similar data are emerging for human Vγ2Vδ2 TCR [92, 93].

4. $\gamma\delta$ Recognition of Tumor Cells

In addition to their defensive role in microbial infections, $\gamma\delta$ T cells are cytotoxic against a variety of tumor cell lines including B cell lymphomas, multiple myeloma, and solid tumors of the kidneys, colon, prostate, breast and head and neck [94–97]. Attempts to define a specific antigen among this diverse group of malignancies have pointed to overproduction of metabolic intermediates like IPP, by transformed cells [98]. Repertoire analysis of $\gamma\delta$ T cells expanded by exposure to IPP or Daudi cells demonstrated very similar Vγ2 chain repertoire, indicating a substantial overlap of phosphoantigen and tumor cell recognition [99]. Furthermore, bisphosphonates, a class of drugs used to treat certain bone diseases, inhibit farnesyl synthase in the mevalonate pathway, lead to overproduction of IPP [100], and these drugs also stimulate $\gamma\delta$ T cell proliferation in PBMC cultures. Patients taking these drugs often show significant expansions of the Vγ2Vδ2 peripheral T cell subset [14, 101].

Not all evidence supports the idea of IPP as a tumor antigen. While all tumors have increased metabolic activity and would therefore produce increased metabolic intermediates such as IPP, not all tumors stimulate Vγ2Vδ2 T cells [99]. $\gamma\delta$ tumor responses are also species specific, despite the fact that tumors from mice or other species will have increased metabolic activity and also produce excess IPP [102]. Moreover, it is not certain that finely tuned tumor surveillance could be accomplished by recognizing an ubiquitous, metabolic intermediate. Freshly isolated $\gamma\delta$ T cells demonstrate little to no basal cytolytic activity against most tumor cell lines; ex vivo expansion in response to phosphoantigen stimulation is needed to generate broad lytic activity [103, 104]. All of these data imply that the $\gamma\delta$ TCR is important for recognizing phosphoantigen and proliferating in response to antigen, but its mechanism for discriminating transformed from normal tissues remains unclear.

Given the conflicting evidence for IPP as tumor antigen, an extensive search for conclusive Vγ2Vδ2 antigens has been underway. Comparing cell surface markers expressed on tumor cell lines susceptible to $\gamma\delta$ T cell lysis with those expressed by resistant tumor cell lines suggested that a structure related to the mitochondrial ATP-synthase molecule may be an antigen [105] The F1-ATPase bound directly to the Vγ2Vδ2 TCR, as shown by surface plasmon resonance, and binding induced IFN-γ and TNF-α release from clones. Apolipoprotein A that binds both the TCR and the ATPase [105], enhanced cell activation. While this study provided the first physical evidence for TCR binding to another molecule, the significance of these interactions is unknown. Additionally, blocking of the ATPase by a monoclonal antibody reduced specific tumor cell lysis by $\gamma\delta$ T cells but did not abolish it, suggesting a role for other cell:cell interactions.

Gene transfer studies demonstrated the dependence of phosphoantigen recognition on the Vγ2Vδ2 TCR, as mentioned previously. However, when this system was used to analyze activation by Daudi lymphoma cells, a much weaker response was generated [89]. Studies using Daudi cells fused with melanoma or lymphoma cell lines, resulted in a significant depression of Vγ2Vδ2 T cell expansion and a resistance to cytotoxicity, presumably due to expression of MHC class I on the surface of hybrid cells [106]. This suggests a system of self recognition similar to NK cells. There is conflicting evidence regarding antibody blockade of the Vγ2Vδ2 TCR. In some reports, TCR blocking resulted in a significant decrease in the ability of cells to lyse tumor targets [107, 108]. Others showed that blocking of the TCR resulted in little or no decrease in cytolysis against a variety of tumor cell types [103, 109], again calling into the question the role for the Vγ2Vδ2 TCR and hinting at the presence of other cytotoxicity-mediating molecules on the surface of activated $\gamma\delta$ T cells.

In addition to the characteristic TCR, $\gamma\delta$ T cells also possess a variety of NK cell receptors including the activating receptor NKG2D [110], the inhibitory NKG2A, and killer immunoglobulin-like receptors (KIR) [111, 112]; the KIR family of receptors can be activating or inhibitory [113]. NKG2D recognizes the MHC-class I-related molecules MICA and MICB, as well as the UL-16 binding proteins (ULBP), all of which are expressed frequently on transformed cells and deliver activating signals [55, 114]. NKG2D is highly expressed on $\gamma\delta$ T cells before and after phosphoantigen stimulation. The inhibitory receptors recognize MHC class I molecules on the surface of normal cells and act to inhibit cytotoxic responses, making sure they are directed only against infected or transformed cells. Although $\gamma\delta$ T cells possess many of the same receptors, NK and $\gamma\delta$ T cells do not lyse all of the same tumor targets [115] implying unique recognition systems in these two cell types.

It is likely that NK receptors play a role in $\gamma\delta$ T cell tumor killing [116], but their exact contribution is uncertain and likely influenced by the type of tumor target. As mentioned previously, Daudi cells lack MHC Class I expression on their surface and are highly susceptible to $\gamma\delta$ T cell lysis [117, 118]. Cytotoxicity is significantly reduced though not abolished, when Daudi cells are engineered to express MHC [106], indicating that inhibitory NK or KIR receptors negatively regulate $\gamma\delta$ T cell function. In fact, expression of inhibitory KIR on $\gamma\delta$ T cells correlates with the level of cytotoxicity, a pattern already known for NK and $\alpha\beta$ T cells [112, 119].

Antibody blocking studies directed against the $\gamma\delta$ TCR indicate that some of the tumoricidal activity seen in expanded cells is TCR-independent [103, 109, 120]. This finding, combined with high levels of NKG2D expressed on expanded $\gamma\delta$ cells, suggested that activating NKR has a major role in tumor cytolysis [120]. Ligation of NKG2D leads to cytolytic responses, Th1 cytokine secretion, and release of cytotoxic granules [55, 121]. Lytic activity of Vγ2Vδ2 cell lines against MICA-expressing targets can be inhibited by up to 50% when blocked by anti-MICA or anti-NKG2D monoclonal antibodies [121]. Unstimulated peripheral blood Vγ2+ cells also express NKR [112] but still require phosphoantigen stimulation before demonstrating potent lytic activity [121].

While NKR and other costimulatory molecules are likely involved in $\gamma\delta$ T cell tumor recognition and lysis, cells possessing the specific Vγ2Vδ2 TCR are selectively expanded and maintained in peripheral blood and show the highest

cytotoxicity toward particular tumor targets. This implies a definitive role for the TCR, but the control over $\gamma\delta$ T cell-mediated killing remains unclear. The TCR could be the sole signal required for lytic function or might contribute to various activating signals received through other receptors much like NK cells. Alternatively, the TCR could act as a growth factor receptor, much like the TCR in invariant NKT cells [122], recognizing a metabolite and mediating activation of the population without a direct role in tumor cell recognition.

5. CD56 as a Marker of Cytotoxicity

As discussed previously, developing thymocytes in the murine system segregated into effector and regulatory $\gamma\delta$ T cell lineages at early embryonic stages with a prominent role for IL-17 in $\gamma\delta$ T cells that are naïve to antigen [36, 42]. These distinct subsets were preserved even during antigen stimulation and expansion [42]. In humans, no distinct $\gamma\delta$ T cell thymocyte subsets are known. When mature cells were expanded with phosphoantigen, potent cytotoxic Vγ2Vδ2 effectors could be distinguished from weakly lytic cells by the level of CD56 expression [43]. A low proportion (1%–2%) of adult Vγ 2Vδ2 T cells express IL-17 upon phosphoantigen stimulation [123] and might have a regulatory role during responses to infections or tumors. However, little is known about the requirement for $\gamma\delta$ T cell-produced IL-17 in tumor cytotoxicity.

CD56, also known as (NCAM), is a calcium-independent adhesion molecule, discovered originally in the nervous system [124]. CD56 has been detected on a number of cell types, most notably NK cells and certain cytotoxic T cells (including some $\gamma\delta$ T cells). CD56 undergoes alternative splicing to generate multiple isoforms depending on cell type and stage of development. Both NK and T cells express exclusively the transmembrane-anchored, 140-kD form [125]. CD56 expression on cytotoxic lymphocytes correlates with lack of MHC restriction, reduced TCR dependence [126], and senescence [127]. The restricted oligoclonal Vβ repertoire present in the CD56+ $\alpha\beta$ lymphocytes and their memory cell phenotype is consistent with antigen selection and expansion [128]. CD56 does not appear to play a role in the killing activity of CTL, although crosslinking of the molecule does induce cell signaling as evidenced by increased total phosphorylation [129]. In the nervous system, CD56 is involved in cell adhesion through homotypic interactions between CD56-expressing neurons [130]. Studies examining CD56 as an adhesion molecule in NK lysis of tumor cells were inconclusive [129, 131] and the exact function of CD56 in the immune system remains unknown.

Recently, CD56 expression was used as a marker to divide NK cells into highly cytotoxic CD56$^{\text{dim}}$CD16$^+$KIR$^{\text{hi}}$ and weakly cytotoxic, cytokine-producing CD56$^{\text{bright}}$ CD16$^-$KIR$^{\text{low}}$ populations. Once believed to be a uniform effector population, it is now thought that NK cells can be activated and progress along a differentiation path similar to T cells, maturing from CD56$^{\text{bright}}$ to CD56$^{\text{dim}}$ upon activation [132].

We and others found that CD56 expression marks a subset of peripheral Vγ2Vδ2 T cells that are potently cytotoxic for tumor cells [43, 133]. Following phosphoantigen expansion, about 50% of peripheral $\gamma\delta$ T cells express CD56 [43]. These cells display potent cytotoxicity against squamous cell carcinoma of the head and neck (SCCHN) cell lines among others, and are resistant to FasL-mediated apoptosis. Expression of CD56 can be induced on CD56 negative fresh $\gamma\delta$ T cells after treating with IL-2 or IL-15 alone but interestingly, these cells do not gain lytic potential [43] suggesting that stimulation through the TCR and proliferation are together necessary for gain of cytotoxic function.

In Vγ2Vδ2 T cells, CD56 is an unusual activation marker. Its expression is not sufficient for cytotoxic activity, since freshly isolated CD56+ $\gamma\delta$ T cells and cells treated with IL-2 alone are not lytic for tumor cells [43, 104]. After expansion, CD56 expression is a marker for cytotoxicity. When Vγ2Vδ2 T cells were expanded by phosphoantigen and separated into CD56+ and CD56− fractions, we found sharp differences in the Vγ2 chain repertoire among these subsets [134]. Cells in both fractions proliferated rapidly after phosphoantigen/IL-2 stimulation, but the subset expressing CD56 uniquely possessed cytolytic activity against tumor cell targets. Public Vγ2 chain sequences common to most healthy adults were present in CD56+ or CD56− fractions and their distribution bias was different for each donor, arguing that Vγ2 chains were not selected to be in the CD56+, cytotoxic subset based on antigen-recognition properties. Further, the reproducible, biased distribution of clones into CD56+ or CD56− fractions showed that control of CD56 expression was not random and the marker did not behave as an activation antigen. The pattern of CD56 expression seems to define a lineage within circulating Vγ2Vδ2 T cells. Phosphoantigen triggers proliferation among all cells expressing the Vγ2Jγ1.2Vδ2 TCR, but only the precursor CTL, defined by their capacity to express CD56 after activation, develop cytotoxic effector activities. The mechanisms controlling lineage, whether this occurs before or after TCR expression, and whether mature clones can acquire or lose the capacity to express CD56, all remain as open questions.

The analysis of CD56 expression and TCR sequences on Vγ2Vδ2 T cells shows that T cell populations harbor precursors to cytotoxic and noncytotoxic subsets, and the CD56+ subset tends to oligoclonality. The picture for $\gamma\delta$ T cells is similar to what was observed previously for CD8+ $\alpha\beta$ T cells [128, 135] where the CD56+ subset was oligoclonal and required for cytotoxicity. A similar finding was reported previously for CD8+ $\alpha\beta$ T cells. In that study [128], human CD8+ $\alpha\beta$ T cells were sorted into CD56+ and CD56− fractions before assaying redirected cytotoxicity against murine P815 cells (FcR+) using anti-CD3 monoclonal antibody. The CD56+ fractions had higher value for specific lysis and tended to oligoclonality be compared with CD56− cells from the same donors. Thus, CD56 is a marker for both $\alpha\beta$ and $\gamma\delta$ CTL, and the trend to oligoclonality suggests these cells have been selected and maintained. For Vγ2Vδ2 cells, we know that the Vγ2 chain repertoire is stable for years and possibly

decades in healthy donors [99], with a tendency to become more oligoclonal over time. Clearly, selection mechanisms exist to expand and maintain CD56+ subsets and preserve the capacity for CTL function.

The finding that CD56 expression is clonally-restricted but not linked to specific Vγ2 chains [134] clarifies the mechanism controlling Vγ2Vδ2 T cell tumor cytotoxicity but does not solve fundamental problems of tumor cell recognition. Indeed, it is likely that the capacity for expressing CD56 was adopted at an early stage of γδ T cell differentiation. Such a mechanism would be consistent with our finding [134] that multiple cells expressing the same Vγ2 chain nucleotype (indicating that they all derived from the same original clone) segregate together into CD56+ or CD56− subsets. Thus, CD56+ clones within the mature γδ T cell repertoire but are not distinguished by TCR specificity. We also know from cell cloning studies [134] that CD56+ cells are heterogeneous with respect to KIR expression. These insights are leading to a view that early lineage marking and heterogeneous expression of KIR limit the proportion of phosphoantigen-responsive Vγ2Vδ2+ cells that are capable of tumor cell lysis. In a sense, we are describing NK tumor cell cytolysis except that initial cell activation depends on a rearranged TCR and phosphoantigen recognition. Mature Vγ2Vδ2 T cells represent between 1/40 and 1/400 of circulating; CD3+ cells in healthy adults and limitations on the capacity for CTL function may be necessary to prevent lethal autoimmunity during γδ responses to infection or malignant transformation. Virtually all Vγ2Vδ2 cells produce proinflammatory cytokines IFN-γ and TNF-α after stimulation, and this function also figures prominently in their immune response activities.

6. Summary

Human γδ T cells present fascinating challenges to our understanding of T cell selection, repertoire maintenance, and the control of effector functions. Because they have a limited γ chain repertoire in adults and they include a high proportion of clines responding to a single antigen, this system permits unique experimental studies not always possible with αβ T cells. An important example is the recent discovery of a cytotoxic effector lineage within the adult population that expresses CD56 and kills tumor cells upon activation. This γδ T cell lineage has similarities to CD8+ αβ CTL, but is selected in the absence of lineage marker (CD8) expression and without conventional MHC restriction. In ways that are not yet clear, γδ T cells (especially the human Vγ2Vδ2+ subset) are selected for response to self-antigens including isopentenylpyrophosphate made by all mammalian cells, without triggering lethal autoimmunity. This produces a T cell subset poised for rapid responses to infected or malignant cells. Much remains to be learned about this interesting subset of T cells, though it is increasingly clear that γδ T cells are important for many immune responses and are novel targets for new for new immunotherapies in cancer and infectious disease.

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