# Heterogeneous yet stable Vδ2$^{(+)}$ T cell profiles define distinct cytotoxic effector potentials in healthy human individuals

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## Abstract

Human γδ T cells display potent responses to pathogens and malignancies. Of particular interest are those expressing a γδ T cell receptor (TCR) incorporating TCRδ-chain variable-region-2 (Vδ2$^{(+)}$ T cells), that are activated by pathogen-derived phosphoantigens (pAgs), or host-derived pAgs that accumulate in transformed cells or in cells exposed to aminobisphosphonates. Once activated, Vδ2$^{(+)}$ T cells exhibit multiple effector functions that have made them attractive candidates for immunotherapy. Despite this, clinical trials have reported mixed patient responses, highlighting a need for better understanding of Vδ2$^{(+)}$ T cell biology. Here, we reveal previously unappreciated functional heterogeneity between the Vδ2$^{(+)}$ T cell compartments of 63 healthy individuals. In this cohort, we identify distinct "Vδ2-profiles" that are stable over time, that do not correlate with age, gender, or history of phosphoantigen activation, and that develop after leaving the thymus. Multiple analyses suggest these Vδ2-profiles consist of variable proportions of two dominant but contrasting Vδ2$^{(+)}$ T cell subsets that have divergent transcriptional programs and that display mechanistically distinct cytotoxic potentials. Importantly, an individual's Vδ2-profile predicts defined effector capacities, demonstrated by contrasting mechanisms and efficiencies of killing of a range of tumour cell lines. In short, these data support patient stratification to identify individuals with Vδ2-profiles that have effector mechanisms compatible with tumour killing, and suggest that tailored Vδ2-profile-specific activation protocols may maximise the chances of future treatment success.

**Keywords:** Human γδ T cells; Vδ2$^{(+)}$ T cells; anti-tumour cytotoxicity; functional heterogeneity

## Significance Statement

A type of human white blood cell, known as the Vδ2$^{(+)}$ T cell, has shown promise in immunotherapies against a range of tumours. However, in recent clinical trials patient responses and clinical outcomes have been variable and unpredictable. To address this, we here reveal a significant variability in Vδ2$^{(+)}$ T cell functional potential between individuals in the general population, which develops shortly after birth, is stable over time, and is manifest by differential mechanistic capacities to kill tumour targets. These results support personalised clinical approaches to identify patients with "Vδ2-profiles" that are compatible with killing of their particular tumour, and suggest that tailored Vδ2-profile-specific activation protocols may maximise the chances of future treatment success.

## Introduction

Human γδ T cells display potent responses to pathogens and malignancies (1-3). Of particular interest are those that express TCRδ-chain variable-region-2 ($V\delta2^{(+)}$ T cells) (4). These cells are uniquely activated by low-molecular-weight non-peptide phosphoantigens (pAgs), such as microbial-derived (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) (5, 6). $V\delta2^{(+)}$ T cells are also activated by isopentyl pyrophosphate (IPP), a pAg that accumulates in transformed eukaryotic cells, or cells exposed to aminobisphosphonates such as zoledronate (7). The mechanism of pAg-mediated activation of $V\delta2^{(+)}$ T cells is still unclear, but likely involves pAg association with butyrophilin 3A1 (BTN3A1) (8, 9).

Consequent to pAg stimulation, $V\delta2^{(+)}$ T cells can display significant cytokine-dependent functional plasticity. Indeed, Th1-like, Th2-like, Th17-like, and Treg-like effector characteristics have all been reported (10-14). Moreover, $V\delta2^{(+)}$ T cells demonstrate efficient cytolytic capacity (15-17), and function as antigen presenting cells if activated under appropriate conditions (18). These functional potentials underpin the role of $V\delta2^{(+)}$ T cells in diverse immune scenarios. $V\delta2^{(+)}$ T cells respond vigorously to *Mycobacterium tuberculosis* (19) and *Plasmodium falciparum* (20), often expanding to >50% of total blood T cells (21), and show responses to both HIV (22) and influenza (23). $V\delta2^{(+)}$ T cells also kill a spectrum of malignant cells that includes leukaemias and lymphomas, and solid tumours such as renal cell, breast, prostate and colorectal carcinomas (24). Indeed, meta-analysis of gene expression signatures from ~18,000 human tumours across 39 malignancies indicated a tumour-associated γδ T cell profile as the best predicator of patient survival (25). Thus, there appears enormous potential to harness these anti-pathogen and anti-tumour effector functions for clinical applications.

Despite this therapeutic promise, results from phase I/II clinical trials that have activated $V\delta2^{(+)}$ T cells with aminobisphosphonates are mixed. While objective clinical outcomes were observed in some patients with relapsed/refractory low-grade non-Hodgkin's lymphoma, multiple myeloma, metastatic hormone-refractory prostate cancer or advanced metastatic breast cancer (26-28), numerous patients failed to demonstrate effective $V\delta2^{(+)}$ T cell responses. Clearly, understanding this person-to-person heterogeneity in $V\delta2^{(+)}$ T cell responsiveness, correlated with subsequent clinical outcome, is critical, not only for optimisation of $V\delta2^{(+)}$ T cell-related therapies, but also for predicting disease progression where $V\delta2^{(+)}$ T cell responses are involved.

In this study, we reveal functional $V\delta2^{(+)}$ T cell heterogeneity, between individuals, in a large cohort of healthy volunteers. The effector potentials of these "Vδ2-profiles" are characterised by two dominant but qualitatively contrasting phenotypes. At one extreme, $V\delta2^{(+)}$ T cells display high proliferative capacity, express several cytokine and chemokine receptors, and demonstrate unusual granzyme K-mediated target cell killing. At the other extreme, $V\delta2^{(+)}$ T cells have lower expansion potential, but possess a dominantly cytotoxic nature characterized by granzyme B-mediated cytotoxicity. This inter-individual $V\delta2^{(+)}$ T cell

heterogeneity develops after birth, although acquisition of a particular Vδ2-profile does not correlate with gender, age, country of birth, or chronic $V\delta2^{(+)}$ T cell stimulation *in vivo*. Moreover, these Vδ2-profiles were stable in individuals over the three-year study period, suggesting an active homeostatic maintenance. Importantly, an individual's Vδ2-profile predicts functional potential that we demonstrate by differential killing of various tumour cell lines. Thus, these data highlight a phenotypic and functional heterogeneity in the human $V\delta2^{(+)}$ T cell pool that has profound clinical implications, such that individuals with different Vδ2-profiles would be predicted to respond differently to $V\delta2^{(+)}$ T cell-targeted immunotherapies, or in response to infections.

## Results

### Significant functional heterogeneity in Vδ2$^{(+)}$ T cell subsets between healthy individuals

We had regularly observed phenotypic heterogeneity when using the commonly utilised markers CD27 and CD45RA to assess human Vδ2$^{(+)}$ T cells from small numbers of healthy volunteers (Fig. 1*A*). As this compromised our interpretation of Vδ2$^{(+)}$ T cell involvement in disease, the nature and extent of this heterogeneity was investigated in a much larger cohort of healthy individuals (n=63). In our hands, CD45RA staining of Vδ2$^{(+)}$ T cells (but not other T cell subsets) does not give distinct demarcation of positive and negative subsets (Fig. 1*A*). Thus, we instead assessed Vδ2$^{(+)}$ T cells using CD27, CD28 and CD16 that consistently identified four distinct Vδ2$^{(+)}$ populations; γδ$^{(28+)}$ cells [CD28$^{(+)}$CD27$^{(+)}$CD16$^{(-)}$]; γδ$^{(28-)}$ cells [CD28$^{(-)}$CD27$^{(+)}$CD16$^{(-)}$]; γδ$^{(16-)}$ cells [CD28$^{(-)}$CD27$^{(-)}$CD16$^{(-)}$], and; γδ$^{(16+)}$ cells [CD28$^{(-)}$CD27$^{(-)}$CD16$^{(+)}$] (Fig. 1*B*). The γδ$^{(28+)}$ population was most prevalent across the cohort (Fig. 1*C*) comprising on average ~54% of total Vδ2$^{(+)}$ T cells, followed by the γδ$^{(28-)}$ subset (~22%), γδ$^{(16+)}$ subset (~11%) and γδ$^{(16-)}$ subset (~8%). However, dominance of a particular subset did not correlate with age, gender, or developmental index of country of birth (Fig. S1). The four Vδ2$^{(+)}$ T cell subsets were functionally assessed after stimulation with bisphosphonate (zoledronate) and IL-2. Proliferative potential segregated with CD27 expression, as the γδ$^{(28+)}$ and γδ$^{(28-)}$ subsets, but less so the γδ$^{(16-)}$ and γδ$^{(16+)}$ subsets, divided multiply after activation (Fig. 1*D*). Consistent with this, γδ$^{(16+)}$ cells expressed the highest level of CD57 that has been reported to correlate with replicative senescence (29), although other markers of exhaustion, such as PD-1, were similar across all populations (Fig. S2). All subsets had potential to secrete both IFNγ and TNFα (Fig. S3). However, cytolytic capacity, assessed by intracellular staining for perforin and granzyme B (Fig. 1*E*), and surface staining of CD56 (Fig. S4), was greater in the γδ$^{(16-)}$ and γδ$^{(16+)}$ subsets. Finally, the γδ$^{(28+)}$ subset demonstrated a reduced capacity to degranulate, judged by surface CD107a expression after 8hr of activation (Fig. 1*F*). Thus, CD27, CD28, and CD16 unambiguously identify four subsets of Vδ2$^{(+)}$ T cells in normal healthy individuals. Importantly, as these subsets have distinct functional capacities, the overall effector potential of the Vδ2$^{(+)}$ T cell compartment will reflect the relative contributions of these subsets in any given individual.

### Individuals possess stable Vδ2-profiles

The 63 healthy individuals could be stratified into six "Vδ2-profiles" defined by relative distribution of the γδ$^{(28+)}$, γδ$^{(28-)}$, γδ$^{(16-)}$, and γδ$^{(16+)}$ subsets (Fig. 2*A*), that also did not correlate with gender, with developmental index of country of birth, or as yet, with a limited assessment of ethnicity (Fig. S5). Profile #2 (50-70% γδ$^{(28+)}$ cells with 10-35% γδ$^{(28-)}$ cells) was observed most frequently (*n*=28), and only two profiles featured a single dominant subset; γδ$^{(28+)}$ cells were dominant in profile #1 whereas γδ$^{(16+)}$ cells dominated profile #6 (Fig. 2*A*). This is consistent with γδ$^{(28+)}$ and γδ$^{(16+)}$ cells being at opposite ends of a

differentiation pathway, with $\gamma\delta^{(28-)}$ and $\gamma\delta^{(16-)}$ cells in-between. However, the prevalence of any particular profile (e.g. profile #6) did not increase with age (Fig. 2*B*), suggesting that regular and cumulative exposure to phosphoantigen-producing pathogens is not sufficient to drive differentiation of $V\delta2^{(+)}$ T cells from one subset phenotype to another. This stable nature of an individual's Vδ2-profile over time was supported by a longitudinal analysis of $V\delta2^{(+)}$ T cells from three individuals over 36-months, in which each person's Vδ2-profile remained largely constant (Fig. 2*C*). Moreover, analysis of blood from osteoporotic patients on long-term oral or intravenous bisphosphonates (essentially the chronic activation of $V\delta2^{(+)}$ T cells, *in vivo*), did not reveal a selective accumulation of any $V\delta2^{(+)}$ T cell subset or profile (Fig. 2*D*). *In vitro*, monitoring over 12-days of $V\delta2^{(+)}$ T cells after phosphoantigen/IL-2 activation of PBMCs from healthy individuals demonstrated significant recovery of the initial Vδ2-profiles (Fig. S6). And even short-term stimulation of sorted $V\delta2^{(+)}$ T cell subsets by phosphoantigen/IL-2 for 3 days, in the presence of sorted $CD14^{(+)}$ autologous accessory cells, demonstrated little change to the initial $V\delta2^{(+)}$ T cell phenotypes (Fig. S7). Thus, the $V\delta2^{(+)}$ T cell compartments of normal healthy adult individuals can be stratified into distinct Vδ2-profiles, that are stable over time, and that do not essentially change in response to acute or chronic $V\delta2^{(+)}$ T cell activation. To investigate whether these Vδ2-profiles are "set" during early $V\delta2^{(+)}$ T cell development, Vδ2-profiles were assessed from ten neonatal (4-22 months) thymuses obtained from cardiac surgeries. Only the $CD28^{(+)}CD27^{(+)}CD16^{(-)}$ phenotype of profile #1 was evident in all samples (Fig. 2E). Such initial uniformity of $V\delta2^{(+)}$ T cell phenotype suggests that the range of distinct Vδ2-profiles is established post-thymically, presumably in response to early interactions with the neonatal and/or infant environment.

**Vδ2-profiles are polarized towards two dominant phenotypes**

Each Vδ2-profile consists of different proportions of $\gamma\delta^{(28+)}$, $\gamma\delta^{(28-)}$, $\gamma\delta^{(16-)}$, and $\gamma\delta^{(16+)}$ cells, the combination of which will differentially dictate overall $V\delta2^{(+)}$ T cell responses. To explore these functional potentials further, we sorted $\gamma\delta^{(28+)}$, $\gamma\delta^{(28-)}$, $\gamma\delta^{(16-)}$, and $\gamma\delta^{(16+)}$ cells to high purity from three different individuals to perform microarray expression analysis (using Illumina's HumanHT-12 v4 beadchip array). After multiple comparisons for genes expressed at least 2-fold higher or lower between any two subsets the $\gamma\delta^{(28+)}$ and $\gamma\delta^{(16+)}$ populations appeared most different (469 genes), consistent with these subsets having distinct functional potentials (Fig. 3A). By contrast, only 78 genes differed between $\gamma\delta^{(28-)}$ and $\gamma\delta^{(16-)}$ cells.

We also re-analysed our flow cytometry data using Gemstone software that assesses multiple flow parameters from multiple flow cytometry data files simultaneously (http://www.vsh.com/products/gemstone/). This is used to order or group cells by phenotypic similarity. Using assumptions that CD27, CD28 and CD16 expression can be high or low on any cell, the software analysed all collected events from all 63 individuals to generate a summary representation of common cell phenotypes from all samples (Fig. 3B). This analysis supported the idea of dominant $\gamma\delta^{(28+)}$ and $\gamma\delta^{(16+)}$ subsets, and again suggested less well-defined distinctions between $\gamma\delta^{(28-)}$ and $\gamma\delta^{(16-)}$ cells with several additional intermediate

phenotypes. Taken together with our data indicating that Vδ2-profiles are stable over time, it suggests that the Vδ2$^{(+)}$ T cell compartments of healthy adult individuals are polarized, to different extents, toward one of two major effector potentials; dominated respectively by either the γδ$^{(28+)}$ or γδ$^{(16+)}$ subset.

**Demarcation of dominant Vδ2$^{(+)}$ T cell phenotypes by CCR6 and CX3CR1**

To explore Vδ2-profile effector potentials further, a table of immune-related genes that differed in expression by ≥2-fold between γδ$^{(28+)}$ and γδ$^{(16+)}$ cells was compiled (Fig. 3*C*). This highlighted differential expression in γδ$^{(16+)}$ cells of genes related to cytotoxic function, including perforin, granzyme B, granzyme H, granulysin, and eight members of the killer cell Ig-like receptor (KIR) family. By contrast, γδ$^{(28+)}$ cells expressed higher levels of granzyme K (30), and cytokine and chemokine receptors such as IL-7Rα, IL-18Rα, IL-23R, CCR2, CCR7, and CCR6 (see below).

Of particular interest was differential expression of CCR6 in γδ$^{(28+)}$ cells (~4.4-fold higher), and CX3CR1 (fractalkine receptor) in γδ$^{(16+)}$ cells (~10-fold higher), which was validated in 12 individuals (Fig. 4*A*). CX3CR1 was expressed at high levels by all γδ$^{(16+)}$ cells, but only by a fraction of the γδ$^{(28+)}$ subset, and at a lower per-cell level. Conversely, CCR6 was not detected on γδ$^{(16+)}$ cells, but identified a distinct population of CCR6$^{(+)}$CX3CR1$^{(-)}$ γδ$^{(28+)}$ cells. An identifiable subset of CCR6$^{(+)}$CX3CR1$^{(+)}$ double positive cells was never truly evident, suggesting a mutually exclusive relationship that may have functional implications. Consistent with this, the intensity of CX3CR1 expression correlated with cytolytic potential as judged by co-expression of granzyme B and perforin (Fig. 4*B*). By contrast, CCR6$^{(+)}$ γδ$^{(28+)}$ cells were GzmB$^{(-)}$Pfn$^{(-)}$ but expressed granzyme K (Fig. 4*C*). CCR6$^{(+)}$ γδ$^{(28+)}$ cells also expressed higher surface levels of both CCR2 and CCR5 (Fig. 4*D*), and the IL-18Rα chain (Fig. 4*E*), compared with CX3CR1$^{(+)}$ cells. CCR6 and granzyme K were also expressed (in the absence of CX3CR1 and granzyme B), in a sizable proportion of Vδ2$^{(+)}$ T cells from all our neonatal thymus samples (Fig. 4F), supporting the idea that freshly generated Vδ2$^{(+)}$ T cells initially adopt a profile #1 phenotype.

CCR6, along with RORγt and IL-23R that are also differentially up-regulated in γδ$^{(28+)}$ cells (Fig. 3*C*), are associated with an IL-17A-secreting phenotype (31, 32). Unlike in mice, human γδ cells with IL-17A-secreting potential are not abundant in adult peripheral blood (12). However, they are present in cord blood (33), and in inflammatory lesions of immunopathologies such as meningitis and psoriasis (12, 34); thus, their origin are of particular importance. As expected, very few IL-17A-secreting Vδ2$^{(+)}$ T cells were observed in peripheral blood of any individual tested. Nonetheless, when these cells were observed they expressed CCR6 (Fig. 4*G*) and resided in the γδ$^{(28+)}$ subset (Fig. S8). Moreover, CCR6$^{(+)}$ γδ$^{(28+)}$ cells expressed higher levels of CD161, the upregulation of which was recently demonstrated on IL-17A-secreting Vδ2$^{(+)}$ T cells *in vitro* (12) (Fig. 4*H*).

**Distinct Vδ2-profiles display distinct cytotoxic effector potentials**

The data above predict that healthy adult individuals with distinct Vδ2-profiles will display qualitatively different effector responses to phosphoantigen challenge. To explore this, PBMC were isolated from individuals with either Vδ2-profile #1 or #2, in which $\gamma\delta^{(28+)}$ cells dominate, or Vδ2-profile #5 or #6, in which $\gamma\delta^{(16+)}$ cells dominate. As expected, total $V\delta2^{(+)}$ T cells from profiles #1/2 expanded on average >10-fold more than those from profiles #5/6 in response to HMB-PP/IL-2 (Fig. 5*A*). Next, we assessed cytotoxic potential by first confirming, in 5 further subjects, that $V\delta2^{(+)}$ T cells from profile #6 individuals express high levels of granzyme B and granulysin, whereas $V\delta2^{(+)}$ T cells from profile #1 individuals instead express granzyme K (Fig. 5*B*). Consistent with this contrast in cytotoxic potential, HMB-PP/IL-2-expanded $V\delta2^{(+)}$ T cell lines from profile #6 subjects efficiently lysed MOLT-4 cells (acute lymphoblastic leukaemia), DOHH-2 cells (B cell lymphoma), and HL-60 cells (acute promyelocytic leukaemia), to a much greater extent than expanded $V\delta2^{(+)}$ T cell lines from profile #1 individuals (Fig. 5*C*). Nonetheless, $V\delta2^{(+)}$ T cell lines from both profiles efficiently killed Jurkat cells (acute T cell leukaemia), while $V\delta2^{(+)}$ T cell lines from profile #1 individuals killed HCT-116 cells (colon carcinoma) more effectively than those from profile #6 (Fig. 5*C*). HCT-116 cells are reported to express proteinase inhibitor 9 (PI-9), a potent inhibitor of granzyme B (but not granzyme K) (35), possibly explaining the reduced cell killing in profile #6 cultures. This differential killing was confirmed using sorted $V\delta2^{(+)}$ T cells from profile #1 and #6 individuals activated with HMB-PP/IL-2 for 4 hours and then co-cultured with HCT-116 cells (Fig. 5*D*). Importantly, the greater killing of HCT-116 cells in profile #1 cultures could be inhibited by pre-incubation of the $V\delta2^{(+)}$ T cells with nafomostat mesylate (NM) that inhibits granzyme K (36) (Fig. 5*D*). NM also reduced killing of MOLT-4 cells by freshly isolated HMB-PP/IL-2-activated $V\delta2^{(+)}$ T cells from profile #1 individuals, whereas granzyme B inhibitor Z-Ala-Ala-Asp(OMe)-chloromethyl ketone (Z-AAD) (37) had minimal effect (Fig. 5*E*). Conversely however, Z-AAD, but not NM, was able to inhibit MOLT-4 cell killing in similar cultures of activated $V\delta2^{(+)}$ T cells from profile #6 individuals, confirming a granzyme B-mediated mode of cytotoxicity (Fig. 5*E*). $V\delta2^{(+)}$ T cells from profile #6 subjects also express abundant CD16 that can mediate antibody-dependent cellular cytotoxicity. Consistent with this, DAUDI cells (Burkitt's Lymphoma) that are killed to some degree by the anti-CD20 antibody rituximab, show almost complete cell lysis in the presence of activated $V\delta2^{(+)}$ T cells from profile #6 individuals (Fig. 5*F*). By contrast, $V\delta2^{(+)}$ T cells from profile #1 subjects in the presence of rituximab, and $V\delta2^{(+)}$ T cells from either profile in the absence of rituximab, displayed no significant DAUDI cell killing above control (Fig. 5*F*). In sum, our data demonstrate that individuals with distinct Vδ2-profiles display qualitatively distinct $V\delta2^{(+)}$ T cell-mediated cytotoxic effector function, that is mediated in large part by differential expression of cytotoxic mediators such as granzyme B and K. Importantly, this suggests that only individuals possessing Vδ2-profiles that are compatible with particular features of their tumour targets are likely to show promising clinical outcomes.

## Discussion

Current understanding of Vδ2$^{(+)}$ T cell biology does not adequately explain the variability in objective clinical responses in Vδ2$^{(+)}$ T cell-based immunotherapies against cancer (26-28). In addressing this, we demonstrate significant inter-individual heterogeneity in Vδ2$^{(+)}$ T cell phenotype that translates to distinct Vδ2$^{(+)}$ T cell effector potentials. Our analysis of 63 healthy individuals revealed several "Vδ2-profiles"; each defined by differing proportions of Vδ2$^{(+)}$ T cell subsets with distinct functional characteristics. Using multiple analyses, our data suggest that these Vδ2-profiles consist of varying contributions from two dominant but contrasting Vδ2$^{(+)}$ T cell populations; identified as γδ$^{(28+)}$ and γδ$^{(16+)}$ cells. Moreover, these opposing phenotypes segregate by mutually exclusive expression of chemokine receptors CCR6 and CX3CR1. CX3CR1 correlates with increased granzyme B/perforin-associated cytotoxic capacity but decreased proliferative potential, whereas CCR6 characterises γδ$^{(28+)}$ cells with increased expression of cytokine receptors (e.g. IL-18Rα) and chemokine receptors (e.g. CCR2 and CCR5), and expression of granzyme K. CCR6$^{(+)}$ γδ$^{(28+)}$ cells also include the few IL-17A-secreting Vδ2$^{(+)}$ T cells found in peripheral blood.

A key finding is that distinct Vδ2-profiles appear stable over time, as highlighted in three individuals over 36-months. It had been suggested from studies *in vitro* that phosphoantigen stimulation of Vδ2$^{(+)}$ T cells resulted in differentiation from a CD27$^{(+)}$CD45RA$^{(+)}$ phenotype to either a CD27$^{(-)}$CD45RA$^{(-)}$ or CD27$^{(-)}$CD45RA$^{(+)}$ phenotype, via a CD27$^{(+)}$CD45RA$^{(-)}$ intermediate (15). However, in our cohort, older individuals, who inevitably will have been exposed to more phosphoantigen-producing pathogens than younger individuals, did not favour any particular Vδ2-profile. Moreover, patients on long-term (>36 months) bisphosphonate therapies, either orally or intravenously, did not present with any specific Vδ2-profile, as would be expected if repeated stimulation drove Vδ2$^{(+)}$ T cell differentiation. Together, these findings suggest Vδ2-profiles change little over time despite repeated stimulation. This evokes a model in which Vδ2$^{(+)}$ T cell activation expands short-lived effector cells, but preserves proportions of longer-lived "memory" cells to maintain the Vδ2-profile. The underlying mechanism for this requires further investigation but may involve "memory stem cell" activity, akin to that described for memory CD8$^{(+)}$ αβ T cells (38), although whether Vδ2$^{(+)}$ T cells demonstrate true memory is still uncertain (39).

The stable yet heterogeneous nature of Vδ2-profiles between healthy individuals raises the question of how they develop. The neonatal (4-22 months) thymus data demonstrate a uniformity of phenotype that resembles profile #1, with a predominance of γδ$^{(28+)}$ cells, and expression of CCR6 and granzyme K. This suggests that, presumably early in life (40), an individual's Vδ2$^{(+)}$ T cell compartment transitions from a uniform thymic profile to a particular peripheral profile that constitutes a stable set-point. The drivers of this transition may be genetic. Alternatively, it may result from childhood exposure to specific pathogens, such as *Mycobacterium tuberculosis* (19), *Plasmodium falciparum* (20), or CMV (41). Such infections may cause aggressive, or qualitatively different, Vδ2$^{(+)}$ T cell responses that force a change

in Vδ2-profile. Such profile-changing responses could also depend on certain Vγ9Vδ2TCR specificities that are restricted to certain individuals. These ideas are currently under investigation.

A corollary to identification of distinct stable Vδ2-profiles across healthy subjects is that these individuals should demonstrate distinct $V\delta2^{(+)}$ T cell-mediated effector responses. Indeed, we demonstrate differences in both efficiency and mechanism by which $V\delta2^{(+)}$ T cells, from profile #1 versus profile #6 individuals, kill a range of tumour lines. Profile #6 $V\delta2^{(+)}$ T cells show Granzyme B-mediated cytotoxicity, but are not efficient at killing tumour lines that express the Granzyme B inhibitor PI-9. However, these cells can additionally deploy ADCC to kill tumour targets in the presence of tumour-binding antibodies. By contrast, $V\delta2^{(+)}$ T cells from profile #1 individuals favour Granzyme K-mediated cytotoxicity, and appear more effective at killing PI-$9^{(+)}$ tumour cells than profile #6 $V\delta2^{(+)}$ T cells. These data thus have important clinical implications for patient stratification in $V\delta2^{(+)}$ T cell-based therapies, as there is likely an optimal $V\delta2^{(+)}$ T cell response for any given malignancy. Although the $V\delta2^{(+)}$ T cell compartment is in theory capable of making a range of responses, a particular patient may be restricted in the responses they can actually make. If this effector potential is mismatched to that required to target a particular tumour, treatment is likely to fail, which may have contributed to inconsistent patient responses in recent clinical trials (26-28, 42). By contrast, selecting patients with defined $V\delta2^{(+)}$ T cell effector potentials that complement features of a tumour, and tailoring activation protocols to optimise specific responses, may maximise the chances of future treatment success.

## Materials and Methods

**Study population.** Informed consent and ethical approval was obtained for blood (London - City and East Research Ethics Committee (REC) 13/LO/0548) and thymus samples (London - Queen Square REC 14/LO/2132).

The isolation of peripheral blood mononuclear cells (PBMC), flow cytometry, proliferation assays, cytotoxicity assays, degranulation assay, Gemstone™ probability state modelling (PSM), microarray analysis, and statistical analyses are described in the *SI Methods*.

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## Authorship

Contribution: P.L.R., N.S., C.J.H., C.B., N.I., and C.L.A.G. performed experiments; P.L.R., N.S and C.J.H analyzed results and made the figures; L.A.B., A.S.J. and D.J.P. supervised the research; D.J.P. designed the study and P.L.R and D.J.P wrote the paper.
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## Figure legends

**Fig. 1.** Inter-individual Vδ2$^{(+)}$ T cell phenotypic variation. (A) CD27/CD45RA plots of blood Vδ2$^{(+)}$ cells from 5 healthy individuals. Quadrant % are indicated. (B) Non-naïve CCR7$^{(-)}$ Vδ2$^{(+)}$ cells comprise 4 subsets; **γδ$^{(28+)}$** [CD28$^{(+)}$CD27$^{(+)}$CD16$^{(-)}$]; γδ$^{(28-)}$ [CD28$^{(-)}$CD27$^{(+)}$CD16$^{(-)}$]; γδ$^{(16-)}$ [CD28$^{(-)}$CD27$^{(-)}$CD16$^{(-)}$], and; γδ$^{(16+)}$ [CD28$^{(-)}$CD27$^{(-)}$CD16$^{(+)}$]. (C) Vδ2$^{(+)}$ subset distribution for 63 healthy individuals (mean +/- s.d.). (D) Representative plots of eFluor®670 dilution in sorted Vδ2$^{(+)}$ subsets after culture with Vδ2-depleted mononuclear accessory cells (1:1) 7-days after stimulation with zoledronate (1μM) + IL-2 (100U/mL); proliferating cells (P), undivided cells (UD). (E) Intracellular Perforin and Granzyme B in Vδ2$^{(+)}$ subsets after 4h stimulation of PBMC *ex-vivo* with PMA/ionomycin. Summary chart for % of Vδ2$^{(+)}$ subset producing; Perforin (Pfn$^{(+)}$), Granzyme B (GzmB$^{(+)}$) or both (Pfn$^{(+)}$GzmB$^{(+)}$) (n=3). (F) MFI of CD107a on Vδ2$^{(+)}$ subsets 8h after activation of PBMCs with HMB-PP (20nM) + IL-2 (100U/mL). Multiple comparison testing using one-way ANOVA with Tukey's post-test used in E and F, **P* < .05, ***P* < .01 and ****P* < .001.

**Fig. 2.** Healthy individuals have stable Vδ2-profiles. (A) Top rows: Representative individuals possess distinct Vδ2-profiles. Bottom charts: Individuals (n=63) were assigned to a Vδ2-profile. (B) Chart of mean age (years) for Vδ2-profiles (#1 to #6). (C) Longitudinal analysis of Vδ2-profiles #3, #4 and #6 at t=0 and 36-mths. (D) Plots of Vδ2-profiles from osteoporotic patients on weekly oral (oral BPs; n=3), or annual (>3yr) intravenous (iv BPs; n=3), bisphosphonates. (E) Plots of Vδ2$^{(+)}$ T cells from four infant thymuses (4-22-mths old). % of gated cells is indicated.

**Fig. 3.** Vδ2-profiles polarize towards two dominant phenotypes. (A) Number of differentially expressed genes (≥2 fold difference and *P* < 0.05) between Vδ2$^{(+)}$ subsets. Multiple comparison tests were with False Discovery Rate (FDR) Benjamini-Hochberg *P*-value correction. (B) Average probability state model (Gemstone™ software) for CD28, CD27, CD16, CD45RA and CD11a on Vδ2$^{(+)}$CD3$^{(+)}$ cells (n=63). Lines show average relative intensity of marker along a cumulative progression axis (x-axis). (C) Differentially expressed immune-related genes with ≥ 2-fold expression difference (*P* < 0.05) between γδ$^{(28+)}$ and γδ$^{(16+)}$ subsets. Dark shaded bars indicate in which subset gene expression is highest.

**Fig. 4.** Demarcation of Vδ2$^{(+)}$ T cell phenotypes by CCR6 and CX3CR1. (A) Representative plots (left) and summary charts (right) showing (A) CCR6 and CX3CR1 on γδ$^{(28+)}$ and γδ$^{(16+)}$ T cells in healthy individuals (n=12), gating CCR6$^{(+)}$ γδ$^{(28+)}$ cells, CX3CR1$^{(+)}$ γδ$^{(28+)}$ cells, and CX3CR1$^{(+)}$ γδ$^{(16+)}$ cells. Subsets from (A), further stained for intracellular Perforin and Granzyme B (B), and Granzyme K and Granzyme B (C), or surface CCR2 and CCR5, following 4h PMA/Ionomycin stimulation (n=6-9). (E) MFI for IL-18Rα for subsets described in (A) (n=5). (F) Plots from four infant thymuses (4-22-mths old) for CCR6, CX3CR1, and intracellular Granzyme K and Granzyme B. (G) CCR6 and intracellular IL-17A expression in

Vδ2$^{(+)}$ and Vδ2$^{(-)}$ (predominantly αβ T cell) subsets from blood (n=2). (H) MFI for CD161 for Vδ2$^{(+)}$ subsets described in (A) (n=6). Mean values with error bars (s.d.). Multiple comparisons using one-way ANOVA with Tukey's post-test *$P$ < .05, **$P$ < .01, ***$P$ < .001, ****$P$ < .0001.

**Fig. 5.** Distinct Vδ2-profiles display distinct cytotoxic effector potentials. (A) Fold-change in Vδ2$^{(+)}$ cells as % of CD3$^{(+)}$ cells 7-days after stimulation of PBMC with zoledronate (1μM) & IL-2 (100U/mL), comparing Vδ2-profiles #1 and #2 (n=10) with #5 and #6 (n=4). (B) Representative plots and summary MFIs (n=6) for intracellular Granzyme B, Granzyme K, and Granulysin from sorted Vδ2$^{(+)}$ cells from Vδ2-profiles #1 or #6, after 4h stimulation with PMA/ionomycin. (C) Chart showing % apoptosis of tumour lines after 4h co-culture with sorted Vδ2$^{(+)}$ T cells (Vδ2-profiles #1 or #6) from PBMC cultures stimulated for 12-days with 1nM HMB-PP & 100U/mL IL-2. Effector:target ratios were 5:1. Error bars are s.d. (D-F) Charts showing % apoptosis of PI-9$^{(+)}$ HCT-116 (D), MOLT-4 (E), or DAUDI (F) cells after 4h co-culture with sorted and activated (1nM HMB-PP and 100U/mL IL-2) Vδ2$^{(+)}$ T cells from Vδ2-profiles #1 or #6 (n=3) *ex vivo*. Pre-treatment (16h) of Vδ2$^{(+)}$ cells with 100μM nafamostat mesylate (NM) was used to inhibit Granzyme K (D and E). Granzyme B was inhibited by 100μM Z-AAD (E). Rituximab (anti-CD20 mAb) was included to induce antibody-dependent cellular cytotoxicity (F). Effector:target ratios were 5:1. Error bars are s.d. Differences compared with two-tailed, unpaired student's t-tests; *$P$ < .05, **$P$ < .01 and ***$P$ < .001.