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The capacity of CD4⁺ Vγ9Vδ2 T cells to kill cancer cells correlates with co-expression of CD56

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ABSTRACT

Human Vγ9Vδ2 T cells are a unique T-cell type, and data from recent studies of Vγ9Vδ2 T cells emphasize their potential relevance to cancer immunotherapy. Vγ9Vδ2 T cells exhibit dual properties since they are both antigen-presenting cells and cytotoxic toward cancer cells. The majority of Vγ9Vδ2 T cells are double-negative for the co-receptors CD4 and CD8, and only 20–30% express CD8. Even though they are mostly neglected, a small fraction of Vγ9Vδ2 T cells also express the co-receptor CD4. Here the authors show that CD4⁺ Vγ9Vδ2 T cells comprise 0.1–7% of peripheral blood Vγ9Vδ2 T cells. These cells can be expanded *in vitro* using zoledronic acid, pamidronic acid or CD3 antibodies combined with IL-2 and feeder cells. Unlike most conventional CD4⁺ αβ T cells, CD4⁺ Vγ9Vδ2 T cells are potentially cytotoxic and can kill cancer cells, which is here shown by the killing of cancer cell lines of different histological origins, including breast cancer, prostate cancer and melanoma cell lines, upon treatment with zoledronic acid. Notably, the killing capacity of CD4⁺ Vγ9Vδ2 T cells correlates with co-expression of CD56.

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Introduction

The γδ T cells represent a subset of T cells that express a T-cell receptor (TCR) composed of a γ chain and δ chain rather than α and β TCR chains [1–3]. The γδ T cells are much less abundant than αβ T cells, and in human peripheral blood the major γδ T-cell subset is the Vγ9Vδ2 T cell. These cells are only found in humans and higher primates (and alpaca) and constitute 0.5–5% of the lymphocytes in peripheral blood [4,5].

In contrast to αβ T cells, which require HLA class I and II molecules for antigen recognition, Vγ9Vδ2 T cells respond in an HLA non-restricted manner to a group of small, non-peptide molecules called phosphoantigens (pAgs). The exact mechanism by which Vγ9Vδ2 T cells recognize pAgs is not completely understood. Yet the interaction between pAgs and butyrophilin molecules, especially butyrophilin 3A1 and 2A1, is necessary and sufficient for proper recognition of pAg by the Vγ9Vδ2 TCR [6–9]. Isopentenyl pyrophosphate (IPP) is a pAg

and the end product of both the mevalonate and non-mevalonate pathway utilized by all living cells [10]. To this end, stress-induced, elevated IPP levels can activate Vγ9Vδ2 T cells, whereas naturally occurring IPP levels are insufficient. Cancer cells produce higher levels of IPP than normal cells and can consequently be sensed by Vγ9Vδ2 T cells [11]. This suggests that Vγ9Vδ2 T cells may have a role as stress-sensing T cells and play a potentially crucial role in recognizing cancer cells [12].

A group of aminobisphosphonates (nBPs) that includes zoledronic acid (ZOL) has also been shown to activate Vγ9Vδ2 T cells in a more indirect fashion by inhibiting the enzyme farnesyl pyrophosphate synthase in isoprenoid biosynthesis, leading to accumulation of IPP, and stimulating cytokine production in other cells, such as monocytes and B cells [13,14]. Importantly, treatment of human peripheral blood mononuclear cells (PBMCs) with pAgs or nBPs leads to the selective expansion of Vγ9Vδ2 T cells. From a clinical perspective, the nBP ZOL is especially interesting since it can activate Vγ9Vδ2 T cells and is already approved by the Food and Drug Administration for the treatment of osteoporosis patients and cancer patients with bone metastases [15]. The approval of ZOL for clinical use renders it an attractive drug within the field of γδ T-cell research given a relative absence of barriers to its employment in clinical studies.

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V γ 9V δ 2 T cells, selectively expanded by ZOL, possess cytotoxic capacity across a broad spectrum of cancer cell lines [11]. The cytotoxic capacity of $\alpha\beta$ T cells is primarily coupled to the CD8⁺ phenotype; however, this does not seem to be the case for V γ 9V δ 2 T cells. The majority of V γ 9V δ 2 T cells are double-negative (DN) for CD4 and CD8, and only 20–30% of V γ 9V δ 2 T cells express CD8 [16]. Consequently, V γ 9V δ 2 T-cell cultures that are capable of killing cancer cell lines are mainly comprised of DN V γ 9V δ 2 T cells [17].

In the current study, the authors examined CD4⁺ V γ 9V δ 2 T cells and found that these cells constitute a minor fraction of PBMCs in healthy donors (HDs) as well as cancer patients. Moreover, using purified cultures, the authors demonstrated that these CD4⁺ V γ 9V δ 2 T cells can kill cancer cells, and that this capacity correlates with expression of CD56.

Methods

Samples from patients and HDs

Collection of patient samples was approved by the local ethics committee, and informed consent was obtained from melanoma, breast cancer and renal cell carcinoma patients before participation. PBMCs from HDs were obtained from the blood bank at Rigshospitalet, Copenhagen, Denmark. Processing was completed within 6 h for all sample specimens. PBMCs were isolated by centrifugation with Lymphoprep (Axis-Shield PoC, Scotland, UK) for 30 min at 1200 RPM without breaks and cryopreserved at -150°C in fetal bovine serum (FBS) (Gibco-BRL) plus 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA) using a CoolCell (Biocision, USA) gradual freezing device. Cells were thawed in pre-warmed 37°C RPMI (Gibco-BRL) and counted after thawing using Trypan Blue staining and a microscope.

Cancer cell lines

The cancer cell lines PC3 (prostate cancer) and MDA-MB-231 (breast cancer) were purchased from American Type Culture Collection. The FM82 (melanoma, ESTDAB-027) cancer cell line was generated from patients by the Danish Cancer Society. All cancer cells were grown in RPMI 1640 medium with GlutaMAX-I (Gibco) supplemented with 10% FBS (R10) at 37°C without antibiotics. With regard to the passages, the authors grew cells from fresh ampules only until the required number of cells had been reached. To ensure a mycoplasma-free laboratory, tests for mycoplasma are performed as a standard operational procedure at the authors' facility when a new cancer cell line is obtained. An additional mycoplasma test was performed using the PanReac PCR mycoplasma test kit (A3744; AppliChem) for the cancer cell lines used in this study (PC3, MDA-MB-231 and FM82). All cancer cell lines tested negative, and results are shown in supplementary Figure 2.

Expansion of V γ 9V δ 2 T cells

V γ 9V δ 2 T cells were expanded *in vitro* from PBMCs. On day 0, PBMCs were thawed, washed twice and counted. The cells were set up using 24-well plates at 1×10^6 PBMCs/well in 2 mL X-VIVO 15 medium (Lonza, Basel, Switzerland) plus 5% human serum (HS) (Sigma-Aldrich). At the same time, PBMCs were stimulated with 10 μM ZOL (Zometa 4 mg/5 mL; Novartis, Copenhagen, Denmark) or 20 μM pamidronic acid (PAM) (Hospira, Lake Forest, IL, USA). On day 2, the cells were given IL-2 at 100 U/mL (in PAM-expanded cultures) or 1000 U/mL (in ZOL-expanded cultures) (PeproTech, Rocky Hill, NJ, USA). Every second to third day from this point on, 900 μL of medium was replaced with 1 mL fresh medium and fresh IL-2 according to the noted concentration. Purity of the cultures was analyzed by flow cytometry after 2 weeks of culture.

Table 1

List of antibodies used in this study.

Antibody	Fluorochrome	Clone	Company
$\alpha\beta$ TCR	PE-Cy7	IP26	BioLegend, San Diego, CA, USA
CD3	BV421	UCHT1	BioLegend, San Diego, CA, USA
CD4	BV510	OKT4	BioLegend, San Diego, CA, USA
CD8	FITC	L307.4	BioLegend, San Diego, CA, USA
CD56	PE	NCAM16.1	BD Biosciences, Franklin Lakes, NJ, USA
CD80	FITC	L307.4	BioLegend, San Diego, CA, USA
CD86	APC	IT2.2	BioLegend, San Diego, CA, USA
CD107a	BV421	H4A3	BD Biosciences, Franklin Lakes, NJ, USA
DNAM-1	FITC	11A8	BioLegend, San Diego, CA, USA
HLA-DR	HV500	G46-6	BD Biosciences, Franklin Lakes, NJ, USA
IFN γ	BV510	4S.B3	BioLegend, San Diego, CA, USA
NKG2D	PE	1B11	BioLegend, San Diego, CA, USA
NKp30 (NCR ^a)	APC	AF29-4D12	Miltenyi Biotec, Bergisch Gladbach, Germany
NKp44 (NCR ^a)	APC	Clone 2.29	Miltenyi Biotec, Bergisch Gladbach, Germany
NKp46 (NCR ^a)	APC	REA808	Miltenyi Biotec, Bergisch Gladbach, Germany
TNF α	PE-CF594	Mab11	BD Biosciences, Franklin Lakes, NJ, USA
V δ 2 TCR	PE	RUO	BD Biosciences, Franklin Lakes, NJ, USA
V γ 9	PC5	IMMU360	Beckman Coulter, Brea, CA, USA

APC, allophycocyanin; BV, brilliant violet; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

^a Natural cytotoxicity receptor.

Flow cytometry

Flow cytometry was used to analyze the expression of surface and intracellular markers on cells. For extracellular staining, PBMCs or $\gamma\delta$ T cells were either fresh or thawed and rested overnight for analysis. Cells were washed twice in phosphate-buffered saline (PBS) plus 2% FBS before and after staining. Antibodies (Table 1) were mixed to a total volume of 50 μL diluted in PBS or Brilliant stain buffer (BD Biosciences, Franklin Lakes, NJ, USA), and cells were stained on ice for 30 min. Flow analysis was performed on the BD FACSCanto II (BD Biosciences). Near-infrared fixable dead cell stain was obtained from Invitrogen (Carlsbad, CA, USA).

Intracellular staining

V γ 9V δ 2 cells were co-cultured with PC3 cells (cancer cells), either non-stimulated or stimulated with 10 μM ZOL, at a ratio of 2:3. The cells were incubated for 5 h in the presence of brefeldin A (BioLegend) and anti-CD107a antibody. Addition of cell culture medium served as a negative control, whereas 5 ng/mL phosphomolybdic acid (Sigma-Aldrich) plus 75 nM ionomycin (Sigma-Aldrich) was used as a positive control. After incubation, cells were centrifuged and washed twice with PBS plus 2% FBS. Staining with surface antibodies was performed as described earlier. Then cells were fixed and permeabilized as described in detail in the manual of the intracellular fixation and permeabilization buffer set (eBioscience, San Diego, CA, USA). In short, cells were fixed overnight at 4°C in 200 μL fixation buffer per well. After centrifugation, cells were washed twice with 150 μL permeabilization buffer per well. Staining with intracellular antibodies was then performed in the same manner as surface antibody staining. Subsequently, cells were washed twice with permeabilization buffer, resuspended in 150 μL PBS plus 2% FBS and acquired on the NovoCyte Quanteon.

Cell sorting

Sorting of V γ 9V δ 2 T cells was performed on the BD FACSAria (BD Biosciences). The procedure for surface staining of V γ 9V δ 2 T cells

was performed as described earlier with the following antibodies: CD3, V γ 9, $\alpha\beta$ TCR, CD4, CD8 and CD56 (Table 1). Cells were sorted according to the expression of the marker of interest. All cells were initially sorted from V γ 9V δ 2 T-cell cultures that had been in culture for 2–3 weeks. Cells were sorted directly into 15-mL tubes containing 5 mL preheated 37°C X-VIVO plus 5% HS medium and afterward transferred to the rapid expansion protocol (REP) mix for expansion. For most cultures, sorting and REP were repeated one to three times to obtain adequate purity. This means that cells were sorted from an initial REP culture and expanded in a new REP culture with the purpose of increasing purity.

Rapid expansion protocol (REP)

Sorted $\gamma\delta$ T cells were expanded *in vitro* using REP. The REP mix included feeder cells, 6000 U/mL IL-2 (Proleukin; PeproTech) and 0.6 μ g/20 mL anti-CD3 (clone OKT3; eBioscience). The detailed protocol is described elsewhere [18,19]. In short, feeder cells were irradiated at 30 Gy and counted to a concentration of 20×10^6 PBMCs/20 mL medium (X-VIVO plus 5% HS), to which IL-2 and anti-CD3 were added. The REP mix was then ready for the sorted $\gamma\delta$ T cells to be added. At this point, the cells were given fresh IL-2 (3000 U/mL) and medium (X-VIVO plus 5% HS) three times a week until the $\gamma\delta$ T cells started growing, allowing for further analysis.

Cytotoxicity assay

Conventional 4-h chromium-51 release assays for cell-mediated cytotoxicity were carried out as described elsewhere [20]. Target cells were the cancer cell lines PC3 (prostate cancer), MDA-MB-231 (breast cancer) and FM82 (melanoma). Cancer cell lines were pre-treated with 10 μ M ZOL overnight for the cytotoxicity assay.

Results

CD4⁺ V γ 9V δ 2 T cells among PBMCs

Flow cytometry was used to study the presence and frequency of CD4⁺ V γ 9V δ 2 T cells among PBMCs of HDs ($n = 15$) and leukapheresis samples from cancer patients (renal cell carcinoma, $n = 5$, melanoma, $n = 10$). Representative results are shown in Figure 1A, demonstrating a small but distinct population of CD4⁺ V γ 9V δ 2 T cells. Compiled data of all flow cytometry analyses from Figure 1A are depicted in Figure 1B and show that 0.1% to approximately 7% of the V γ 9V δ 2 T-cell population expressed CD4. Since V γ 9 may be paired with variable δ chains beyond the V δ 2 chain, the authors verified the V δ 2 expression of these cells using a V δ 2-specific antibody, demonstrating that the clear majority of the V γ 9 cells were indeed V γ 9V δ 2 T cells (see supplementary Figure 1).

V γ 9V δ 2 T-cell cultures expanded by ZOL or PAM comprise CD4⁺ as well as CD8⁺ and DN V γ 9V δ 2 T cells

For selective expansion of V γ 9V δ 2 T cells, PBMCs from HDs were stimulated with ZOL or PAM, in combination with IL-2. In both cases, a small but distinct population of CD4⁺ V γ 9V δ 2 T cells was preserved (Figure 1C). The authors conducted several experiments comparing ZOL and PAM and saw no significant differences ($P = 0.87$) in the capacity to expand either bulk V γ 9V δ 2 T cells or CD4⁺ V γ 9V δ 2 T cells (Figure 1C). Similarly, fractions of CD8⁺ and DN V γ 9V δ 2 T cells were expanded equally well by ZOL and PAM (data not shown).

Pure CD4⁺ V γ 9V δ 2 T-cell cultures were established using REP

To scrutinize the functional capacity of CD4⁺ V γ 9V δ 2 T cells, these cells were sorted and expanded *in vitro*. Specifically, V γ 9V δ 2 T-cell

cultures from five HDs were sorted using BD FACSAria into three populations: (i) CD4⁺ V γ 9V δ 2 T cells, (ii) CD8⁺ V γ 9V δ 2 T cells and (iii) DN V γ 9V δ 2 T cells. Sorted cells were then expanded using REP [18,19]. Representative dot plots of sorted CD4⁺, CD8⁺ and DN V γ 9V δ 2 T-cell cultures are shown in Figure 2A. The purity of five sorted cultures is shown in Figure 2B for each of the three populations: CD4, CD8 and DN. In general, the authors observed that pure CD4⁺ V γ 9V δ 2 T-cell cultures kept a stable expression of CD4 (as well as CD3 and V γ 9), whereas CD8 expression seemed to vary in both the sorted CD8⁺ and DN V γ 9V δ 2 T-cell cultures (Figure 2A,B). Finally, the authors showed that all three populations could be expanded using REP, with a median fold expansion of 132.6-fold for all cultures.

CD56 expression is associated with cytotoxic capacity of CD4⁺ V γ 9V δ 2 T cells

It has previously been shown that V γ 9V δ 2 T cells efficiently kill cancer cells upon sensitization of cancer cells with, for example, ZOL [11]. Having sorted CD4⁺ V γ 9V δ 2 T cells and expanded these to high cell numbers while maintaining high purity ($>95\%$ CD4⁺), the authors tested the capacity of these cells to kill cancer cell targets. However, during the expansion of these cultures, flow cytometry analysis revealed that the cultures consisted of distinct CD56[−] and CD56⁺ populations. In comparison, all CD8⁺ and DN V γ 9V δ 2 T cells were CD56⁺ (Figure 3A). Since CD56 is associated with cytotoxic capacity [21], the next step was to test CD56[−] and CD56⁺ CD4⁺ V γ 9V δ 2 T-cell cultures sorted by fluorescence-activated cell sorting (FACS) for their ability to kill cancer cells. When tested in a 4-h chromium-51 release assay, only CD56⁺ cultures were cytotoxic against prostate (PC3), melanoma (FM82) and breast (MDA-MB-231) cancer cell lines (Figure 3D). To further verify these data, a total of five CD4⁺ V γ 9V δ 2 T-cell cultures were sorted according to their CD56 expression. The expression of CD56 ranged from 10% to 75% in the CD56⁺ cultures and 1% to 3% in the CD56[−] cultures (Figure 3B). In all five cases, the CD56⁺ cells killed the prostate cancer cell line (PC3) sensitized with ZOL (ratio 1:10) more efficiently than the CD56[−] CD4⁺ V γ 9V δ 2 T cells (Figure 3C).

To further verify these data, CD107a, interferon gamma (IFN γ) and tumor necrosis factor alpha (TNF α) were measured using intracellular flow cytometry in a co-culture between CD4⁺ V γ 9V δ 2 T cells, either CD56⁺ or CD56[−], and PC3 cells. The authors found a significantly higher expression of CD107a when the CD56⁺ CD4⁺ V γ 9V δ 2 T cells were co-cultured with PC3 cells sensitized with ZOL compared with CD56[−] CD4⁺ V γ 9V δ 2 T cells. The same was true for cytokine production of both IFN γ and TNF α , where the CD56⁺ cells had a much higher production upon meeting their target compared with CD56[−] cells. Furthermore, expression of DNAX accessory molecule-1 (DNAM-1), natural killer group 2D (NKG2D) and natural cytotoxicity receptors (NCRs), representing a pool of NCRs, including Nkp30, Nkp44 and Nkp46, was also measured and compared between CD56⁺ and CD56[−] CD4⁺ V γ 9V δ 2 T cells ($n = 4$). All cells expressed DNAM-1 at a high level (range, 84–92%), whereas low levels (0.5–5%) of NCR expression were found, but no significant difference in expression could be found between CD56⁺ and CD56[−] CD4⁺ V γ 9V δ 2 T cells. Expression of NKG2D varied a lot between donors (28–99%), but again, no significant difference in expression could be found according to expression of CD56. Overall, these data suggest that the ability of CD4⁺ V γ 9V δ 2 T cells to kill cancer cells correlates with the expression of CD56.

Discussion

V γ 9V δ 2 T cells are the major and best characterized $\gamma\delta$ T-cell subset among human PBMCs. These cells are easily expanded to cultures of high purity and cell number. We strive to develop cellular therapies that can complement and potentially replace our current clinical application of *in vitro*-expanded tumor-infiltrating lymphocytes in

Figure 1. Vγ9Vδ2 T cells express CD4. (A) Analysis of PBMC samples from one HD and one melanoma and one RCC patient using flow cytometry. Top row shows the frequency of Vγ9Vδ2 T cells among CD3⁺ T cells. Bottom row shows the frequency of CD4⁺, CD8⁺ and DN within the Vγ9Vδ2 T-cell population (compiled data from three experiments). (B) Compiled data of the screening for CD4⁺ Vγ9Vδ2 T cells in PBMCs from HDs (n = 15) and melanoma (n = 10) and RCC (n = 5) patients. Blue dots represent the plots in (A) (compiled data from three experiments). (C) Vγ9Vδ2 T-cell cultures were expanded *in vitro* using initial stimuli with either ZOL or PAM. Presented here are the frequencies of CD4⁺ Vγ9Vδ2 T cells in Vγ9Vδ2 T-cell cultures after 2 weeks of culture (n = 6 HD setup for expansion with both ZOL and PAM, compiled data of three experiments). Wilcoxon signed-rank test $P = 0.87$. RCC, renal cell carcinoma.

cancer therapy [18]. Therefore, the authors studied Vγ9Vδ2 T cells given their ease of expansion directly from PBMCs and their capacity to specifically kill cancer cells.

In the authors' studies of expanded cultures, a small subpopulation of CD4⁺ γδ T cells was repeatedly observed. Since Vγ9Vδ2 T cells can be expanded with different nBPs, the authors investigated whether ZOL and PAM could differentially expand CD4⁺ Vγ9Vδ2 T cells *in vitro* (Figure 1C). Distinct populations of CD4⁺ cells were found in both PAM- and ZOL-expanded cultures. Nevertheless, no significant differences could be observed in the frequencies of CD4⁺ Vγ9Vδ2 T cells, although ZOL appeared to favor CD4⁺ Vγ9Vδ2 T cells in some cultures (Figure 1C).

To evaluate whether the presence of CD4⁺ Vγ9Vδ2 T cells was indicative of a true subset of Vγ9Vδ2 T cells in human peripheral

blood or merely a property of *in vitro*-expanded cultures, the authors performed flow cytometry on PBMCs from HDs. The authors found that a small but distinct population of CD4⁺ Vγ9Vδ2 T cells (0.1–7% of Vγ9Vδ2 T cells) was indeed present across all tested HDs (Figure 1A,B). Since Vγ9Vδ2 T cells, as also shown in the current study, are capable of recognizing cancer cells, the authors looked for CD4⁺ Vγ9Vδ2 T cells among leukapheresis samples from cancer patients. This revealed an equally distinct population with frequencies similar to those found in HDs (Figure 1B). Thus, it seems clear that CD4⁺ Vγ9Vδ2 T cells are directly detectable *ex vivo*, and that these cells are also present in cancer patients.

For αβ T cells, the CD8 molecule plays a co-stimulatory role by interacting with the HLA class I molecule. Likewise, for CD4⁺ αβ T cells, the CD4 molecule interacts with the HLA class II molecule and

Figure 2. Sorting and rapid expansion of CD4⁺ V γ 9V δ 2 T-cell cultures *in vitro*. To test the functional abilities, V γ 9V δ 2 T cells were sorted by BD FACSARIA according to CD4 or CD8 or cells not expressing CD4 or CD8 (DN). Afterward, the sorted V γ 9V δ 2 T cells were expanded using REP. (A) Representative examples of one such CD4⁺, CD8⁺ or DN V γ 9V δ 2 T-cell culture (compiled data from two experiments). (B) Flow analysis showing purity of REP V γ 9V δ 2 T-cell cultures from five different HDs (n = 5 CD4, 5 CD8 and 5 DN, compiled data of five different experiments). Mean is indicated with SD. (C) Fold expansion of sorted and REP V γ 9V δ 2 T-cell cultures after 3 weeks of culture (n = 5 CD4, 5 CD8 and 5 DN, compiled data of five different experiments). Mean is represented by a line. SD, standard deviation. (Color version of figure is available online.)

provides a co-stimulatory signal to the T cell [21]. Little is known about the role of CD8 in V γ 9V δ 2 T cells, and no differences have been described between DN and CD8⁺ V γ 9V δ 2 T cells. As a consequence, the potential role of both CD8 and CD4 in V γ 9V δ 2 T cells should be studied in more detail. Given the low frequencies of CD4⁺ V γ 9V δ 2 T cells *in vivo*, the authors aimed to study these cells in expanded cultures to permit a more extensive study of phenotype and functionality. The authors took advantage of FACS and REP [19] to establish pure cultures of CD4⁺ V γ 9V δ 2 T cells (Figure 2A). These cultures remained pure with a stable expression of CD4 during *in vitro* culturing. Throughout the course of the current study, the authors made several attempts to purify CD8⁺ V γ 9V δ 2 T cells. However, in contrast to CD4, this was not as successful (Figure 2A). These results were unexpected since the number of cells available in a culture is much higher for CD8⁺ (20–30%) compared with CD4⁺ (0.1–7%) T cells. In short, the authors made several pure FACS-sorted CD8⁺ V γ 9V δ 2 T-cell cultures, but post-expansion the purity settled at 45–85%, and the remaining cells were DN (Figure 2A,B). Similarly, the authors also observed that sorted DN V γ 9V δ 2 T cells shifted toward 1–20% CD8⁺ cells post-expansion. CD4⁺ V γ 9V δ 2 T cells remained undetectable in the sorted CD8⁺ and DN V γ 9V δ 2 T-

cell cultures. Despite the use of a conservative gating strategy while sorting by BD FACSARIA flow cytometer (which has always proven sufficient when sorting CD4⁺ $\gamma\delta$ T cells) and the sorting of five different cultures, contamination during sorting cannot be formally excluded. Although the authors have no formal proof of this notion, the data could suggest a continuous conversion between CD8⁺ and DN V γ 9V δ 2 T cells and vice versa.

As mentioned, several studies have shown that V γ 9V δ 2 T cells can kill cancer cells upon sensitization with nBPs. Careful scrutiny of the literature revealed a single study that described the existence of CD4⁺ V γ 9V δ 2 T cells among PBMCs and expanded bulk cultures of V γ 9V δ 2 T cells from HDs, and that demonstrated that these cultures displayed a Th1 cytokine profile [22]. However, functional assays beyond cytokine release were not conducted. Using pure and stable CD4⁺ V γ 9V δ 2 T-cell cultures, the authors demonstrated that the prostate cancer, melanoma and breast cancer cell lines were killed upon nBP sensitization (Figure 3B). Although conventional CD4⁺ T cells are generally not cytotoxic, CD4⁺ $\alpha\beta$ T cells with cytotoxic properties have been described, either as direct killers by recognizing tumor antigen in the context of HLA class II molecules [23,24] or indirectly by activating other immune cells by cytokine release [24]. Direct killing requires

Figure 3. Expression of CD56 correlates with the ability of CD4⁺ V γ 9V δ 2 T cells to kill cancer cells. During sorting and REP of V γ 9V δ 2 T-cell cultures, a difference in expression of CD56 was observed in flow analysis. (A) Example of one sorted but not yet pure CD4⁺ V γ 9V δ 2 T-cell culture, comparing expression of CD56 in the CD4⁺, CD8⁺ and DN compartments (n = 1). (B) Five pure CD4⁺ V γ 9V δ 2 T-cell cultures that were sorted, and processed using REP, according to CD56 expression (CD4⁺ expression >95%). Statistical significance was tested using the paired *t*-test. Error bars represent mean $\pm$ SD (n = 5, combined data from four experiments). (C) The cytotoxic ability of CD4⁺ V γ 9V δ 2 T-cell cultures was tested against melanoma (FM-82), breast cancer (MDA-MB-231) and prostate cancer (PC3) cell lines in a 4-h chromium-51 release assay. The top and bottom rows compare CD4⁺ V γ 9V δ 2 T-cell cultures expressing or negative for CD56, first shown as expression of CD56 in flow cytometry diagrams and then comparing corresponding cytotoxic abilities in chromium-51 release assays. Error bars represent mean $\pm$ SD of technical replicates (n = 3, combined data from two experiments). (D) The cytotoxic ability of the five cultures in (C) was also tested in a 4-h chromium-51 release assay against PC3 plus ZOL at an effector:target ratio of 1:10. Significance of differences was calculated with the paired *t*-test (n = 5, combined data from three experiments). (E) Intracellular flow analysis was used to measure CD107a, IFN γ and TNF α in a co-culture between CD4⁺ V γ 9V δ 2 T cells negative or positive for CD56 and PC3 cells pre-treated with or without ZOL. Significance of differences was calculated with the paired *t*-test (n = 4 CD56⁻ cultures and CD56⁺ cultures from one experiment). (F) Flow analysis comparing expression of NKG2D, DNAM-1 and NCRs, including NKp30, NKp44 and NKp46, in CD4⁺ V γ 9V δ 2 T-cell cultures negative or positive for CD56 (n = 4 CD56⁻ cultures and CD56⁺ cultures from one experiment). **P* < 0.05, ***P* $\leq$ 0.01. SD, standard deviation. (Color version of figure is available online.)

expression of HLA class II molecules, which is a frequent phenomenon in melanoma; however, most other solid cancers are negative for HLA class II. In the current study, the authors could not reveal any information regarding the recognition elements for recognition of cancer cells by the CD4⁺ V γ 9V δ 2 T-cell cultures, which may in fact rely on more than a single molecule [20].

CD56 is expressed on a number of cell types but most notably on natural killer cells and cytotoxic T cells [25]. In the process of elucidating the phenotype and function of CD4⁺ V γ 9V δ 2 T cells, the authors also looked at the CD56 marker. A bimodal expression of CD56 was displayed for CD4⁺ V γ 9V δ 2 T cells (Figure 3A), whereas in REP cultures, all CD8⁺ and DN V γ 9V δ 2 T cells were CD56⁺. The establishment of CD56⁺ and CD56[−] CD4⁺ V γ 9V δ 2 T-cell cultures (Figure 3C) allowed the authors to investigate whether the cytolytic capacity of these cells was associated with expression of CD56. The authors' data demonstrated that expression of CD56 correlated with the cytolytic activity of CD4⁺ V γ 9V δ 2 T-cell cultures (Figure 3D), as shown with cultures established from five HDs targeting the prostate cancer cell line (Figure 3C). This supports data shown by others, including Urban *et al.* [21], demonstrating that V γ 9V δ 2 T-cell populations expressing CD56 show increased cytotoxic effector function compared with the CD56[−] compartment, and that this cytotoxic function is acquired when the population is stimulated to proliferate [25]. Alexander *et al.* [26] showed that CD56⁺ V γ 9V δ 2 T cells killed human squamous cell carcinoma cells more efficiently. Also, $\alpha\beta$ T cells expressing CD56 have a greater cytotoxic effector function and pro-inflammatory cytokine production, such as IFN γ and TNF α [25,27,28]. The authors also observed an increase in the cytokines IFN γ and TNF α , together with CD107a, for V γ 9V δ 2 T cells expressing CD56 (Figure 3E). However, no difference was found in the expression of DNAM-1, NKG2D and NCRs. Taken together, this supports the finding that the ability of CD4⁺ V γ 9V δ 2 T cells to kill correlates with expression of CD56, as shown in Figure 3.

Gentles *et al.* [29] studied tumor biopsy samples (approximately 18 000 samples from 39 cancer types) and characterized $\gamma\delta$ T cells as the number one immune cell population out of 22 associated with favorable prognosis. Despite this, clinical trials with V γ 9V δ 2 T cells have shown limited clinical success. There are potentially many reasons for this, and deeper insight into this biological enigma is hampered by our limited understanding of V γ 9V δ 2 TCR diversity, restricted knowledge of receptor–ligand interactions and underestimation of the impact of functional diversity on the V γ 9V δ 2 T-cell population [30]. A study by Gründer *et al.* [31] found an unexpectedly high variability in tumor recognition between clones in the V γ 9V δ 2 T-cell repertoire, suggesting that diversity of V γ 9V δ 2 T cells can be functionally important. V γ 9V δ 2 T cells are among the first T cells to develop in the human fetus, and the repertoire of V γ 9V δ 2 T cells narrows during adolescence through individual encounters with micro-organisms (i.e., microbial education) [32]. As a consequence, each adult has a unique $\gamma\delta$ T-cell repertoire in terms of $\gamma\delta$ TCR diversity and potentially also functional diversity. Therefore, a general focus is being put on understanding diversity within the V γ 9V δ 2 T-cell repertoire. The authors' data provide knowledge about a minor population of CD4⁺ V γ 9V δ 2 T cells existing within the V γ 9V δ 2 T-cell population in both HD and cancer patients, thus contributing to a better understanding of the diversity of V γ 9V δ 2 T cells. To this end, it should be noted that the V γ 9V δ 2 T-cell cultures in this study were treated with high amounts of IL-2. Therefore, the weakness of this study is that the authors cannot rule out whether the reactivity correlated with CD56 for CD4⁺ V γ 9V δ 2 T cells is potentially an artifact due to the high concentration of IL-2 used *in vitro*. Additional studies are needed to characterize whether these cells are engaged with unique functions in the immune system *in vivo*, including whether these cells play a role in spontaneous or treatment-induced immune responses against cancer.

Conclusions

A small fraction of V γ 9V δ 2 T cells express the co-receptor CD4, and their existence is supported by data from Dunne *et al.* [22]. Based on the data from the current study, the authors conclude that CD4⁺ V γ 9V δ 2 T cells can be found in the PBMCs of both HDs and cancer patients, comprising between 0.1% and 7% of peripheral blood V γ 9V δ 2 T cells. To the authors' knowledge, we are the first to describe that CD4⁺ V γ 9V δ 2 T cells can kill cancer cells, and that killing *in vitro* correlates with expression of CD56. The authors hereby suggest CD56 expression as a marker of cytotoxicity for CD4⁺ V γ 9V δ 2 T cells, which supports data suggesting CD56 as a cytotoxic marker of V γ 9V δ 2 T cells [25].

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: GHO, IMS, MI, PtS. Acquisition of data: GHO, SRP, PA. Analysis and interpretation of data: GHO, MI, PtS. Drafting or revising the manuscript: GHO, PtS. All authors have approved the final article.

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Supplementary materials

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