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Phenotypic and Functional Alterations of V γ 2V δ 2 T Cell Subsets in Patients with Active Nasopharyngeal Carcinoma

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Abstract

Introduction—Human V γ 2V δ 2 T cells play important role in immunity to infection and cancer by monitoring self and foreign isoprenoid metabolites with their $\gamma\delta$ T cell antigen receptors. Like CD4 and CD8 $\alpha\beta$ T cells, adult peripheral V γ 2V δ 2 T cells represent a pool of heterogeneous cells with distinct functional capabilities.

Purpose—The aim of this study was to characterize the phenotypes and functions of various V γ 2V δ 2 T cell subsets in patients with nasopharyngeal carcinoma (NPC). We sought to develop a better understanding of the role of these cells during the course of disease and to facilitate the development of immunotherapeutic strategies against NPC.

Results—Although similar total percentages of peripheral blood V γ 2V δ 2 T cells were found in both NPC patients and normal donors, V γ 2V δ 2 T cells from NPC patients showed decreased cytotoxicity against tumor cells whereas V γ 2V δ 2 T cells from normal donors showed potent cytotoxicity. To investigate further, we compared the phenotypic characteristics of V γ 2V δ 2 T cells from 96 patients with NPC and 54 healthy controls. The fraction of late effector memory V γ 2V δ 2 T cells (T_{EMRA}) was significantly increased in NPC patients with corresponding decreases in the fraction of early memory V γ 2V δ 2 T cells (T_{CM}) compared with those in healthy controls. Moreover, T_{EMRA} and T_{CM} V γ 2V δ 2 cells from NPC patients produced significantly less IFN- γ and TNF- α , potentially contributing to their impaired cytotoxicity. Radiotherapy or concurrent chemo-radiotherapy further increased the T_{EMRA} V γ 2V δ 2 T cell population but did not correct the impaired production of IFN- γ and TNF- α observed for T_{EMRA} V γ 2V δ 2 T cells.

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Conclusion—We have identified distinct alterations in the V γ 2V δ 2 T cell subsets of patients with NPC. Moreover, the overall cellular effector function of $\gamma\delta$ T cells is compromised in these patients. Our data suggest that the contribution of V γ 2V δ 2 T cells to control NPC may depend on the activation state and differentiation of these cells.

Keywords

CD27; CD28; Nasopharyngeal carcinoma; Memory V γ 2V δ 2 T cell subsets; Peripheral V γ 2V δ 2 T cells; IFN- γ ; TNF- α

Introduction

Human $\gamma\delta$ T cells are a unique subset of T cells that constitute 1–5% of peripheral blood T cells. The V δ 2 chain of the T cell receptor (TCR) usually pairs with the V γ 2 chain to form the V γ 2V δ 2 TCR (also termed V γ 9V δ 2 TCR), which is expressed by the majority of circulating $\gamma\delta$ T cells [31,32]. V γ 2V δ 2 T cells are present in low numbers at birth but expand during infancy due to environmental stimuli [36]. Preferential expansion of V γ 2V δ 2 T cells has been observed during a number of microbial infections [31]. The stimulating microbial antigens have been shown to be small nonpeptide metabolites (commonly termed phosphoantigens) essential for isoprenoid biosynthesis. We and others have demonstrated that the most potent natural microbial antigen for V γ 2V δ 2 T cells is (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) [21,37,39]. HMBPP is a microbial metabolite in the methyl erythritol phosphate pathway responsible for isoprenoid biosynthesis that is used by most Eubacteria and Apicomplexan parasites [31]. The major human antigen reported for V γ 2V δ 2 T cells is isopentenyl pyrophosphate (IPP) [51], which is increased in some malignant cells and in almost all cells upon pharmacological treatment with bisphosphonates [16,52] or alkylamines[31].

Unlike CD4 and CD8 $\alpha\beta$ T cells, the majority of adult peripheral blood V γ 2V δ 2 T cells express various phenotypic markers generally found on memory cells [8]. Similar to CD4 and CD8 $\alpha\beta$ T cells, these memory V γ 2V δ 2 T cells are heterogeneous and multiple cell subsets can be distinguished based on their maturation surface markers and functional activities [5]. Various cell surface receptors have been used to divide memory cells into subsets. One criterion primarily used for human CD4 $\alpha\beta$ T cells has been the expression of the CCR7 chemokine receptor, which is required for lymph node homing [41,42]. CD4 central memory T cells (T_{CM}) express CCR7, whereas effector memory cells (T_{EM}) do not. In addition to CCR7 [14], we and others have found that the expression of CD27, CD28, CD45RA, and CD45RO molecules also serves to distinguish memory $\gamma\delta$ T cell and CD8 $\alpha\beta$ T cells subsets [1,2,19, 50]. Central memory CD8 $\alpha\beta$ T cells (T_{CM}, also termed early memory cells by Appay[1]) were identified as CD28⁺ CD27⁺ CD45RA[−] cells and also included early effector cells [50] that secrete IFN- γ and TNF- α but have little or no cytolytic activity due to low perforin expression [19,50]. On rare occasions, CD8 $\alpha\beta$ T cells in the central memory pool lose CD27, but retain CD28 expression as well as other central memory cell properties (T_{CM27−}) [2]. In contrast, effector memory T cells (T_{EM}, also termed intermediate memory cells) lack CCR7 and CD28, but continue to express CD27. Additionally, T_{EM} also produce low levels of cytokines, but intermediate levels of perforin [50]. Late effector memory T cells (T_{EMRA}, also termed CD45RA⁺ late memory cells) lack both CD28 and CD27 expression and represent terminally differentiated effector T cells that express a high level of perforin, but demonstrate limited proliferative capacity.

Upon activation, V γ 2V δ 2 T cells kill a broad range of tumor cells, including lymphoma cells, leukemia cells, and other malignant hematological cells. V γ 2V δ 2 T cells can also kill a variety of solid tumors, including nasopharyngeal carcinoma [57], breast carcinoma [17], hepatocellular carcinoma [23], lung carcinoma [13], renal cell carcinoma [6,24,53], pancreatic

adenocarcinoma [22], prostate carcinoma [28], and neuroblastoma [44]. Moreover, adoptively transferred V γ 2V δ 2 T cells exhibit anti-tumor activity in various xenograft mouse models [22,30,57]. There is increasing evidence for the use of V γ 2V δ 2 T cells in cancer immunotherapy. In one study, stimulation of V γ 2V δ 2 T cells with the bisphosphonate drug pamidronate in combination with IL-2 led to partial remission and stable disease in some patients with refractory or relapsing B cell malignancies [55]. In a second study, the bisphosphonate, zoledronate, together with IL-2 was used to treat patients with metastatic prostate carcinoma [12]. A higher proportion of the treated patients demonstrated partial remission or stable disease and those patients with the highest numbers of V γ 2V δ 2 T cells exhibited the lowest levels of prostate-specific antigen, a cancer cell marker [12]. These findings suggest that V γ 2V δ 2 T cells may play a role in tumor immunosurveillance, as well as the possibility of employing these $\gamma\delta$ T cells in cancer immunotherapy.

Materials and Methods

Patients and healthy controls

Venous blood samples were obtained from patients with nasopharyngeal carcinoma diagnosed at the National Cancer Center of Singapore. Healthy controls (NOR) were recruited from the Center for Transfusion Medicine, Health Sciences Authority of Singapore. Approval from the Institutional Review Board was obtained for the present protocol prior to the collection of blood samples from healthy individuals and NPC patients. A total of 96 NPC patients and 54 healthy donors were included in this study. The diagnosis of NPC was determined based on histopathological analysis. Clinical characteristics including gender, age, and tumor size and staging of cancer patients at the time of diagnosis were obtained from medical records (Table 1). Of the 96 NPC patients, 69 were male and 27 were female with a median age of 50 (range: 29–77); whereas the healthy donors comprised 45 males and 9 females with a median age of 37.5 (range: 22–59).

Isolation of PBMC and FACS staining

PBMC were isolated by Ficoll-Paque density centrifugation. The following monoclonal antibodies were used: FITC- or PE-labeled V δ 2 (B6.1), FITC-labeled CD8 (RPA-T8), PE-labeled CD27 (M-T271), APC-labeled CD27 (0323), PE-Cy5-labeled CD28 (28.2), PE-Cy7-labeled CD28 (28.2), R-PE-labeled CD3 (HIT3a), and PE-Cy7-labeled CD3 (SK7). All Abs used in the present study were purchased from BD Biosciences (San Jose, CA, USA). Three- and four-color flow cytometry were performed and analyzed using a FACSCalibur or a FACSCantoII flow cytometer (BD Biosciences).

MACS isolation of V γ 2V δ 2 T cells

PBMC were incubated with FITC anti- V δ 2 TCR mAb on ice for 30 min. Cells were washed once with chilled 2% FCS to remove excess unbound mAb. Anti-FITC MicroBeads were added and the cells were incubated on ice for another 30 min. The magnetic isolation of V δ 2 T cells was performed as described in the manufacturer's instructions (Miltenyi Biotech). These cells were subsequently passed through magnetic columns that separate non- V δ 2 T cells from V δ 2 T cells. The purity of the V δ 2 T cells was determined by flow cytometry.

Cytotoxicity assay

CTL assays were performed using DELFIA EuTDA Cytotoxicity Reagents (Perkin-Elmer) according to the manufacturer's instructions. Briefly, 5×10^3 K562 cells were labeled with a fluorescence enhancing ligand, (bis(acetoxymethyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate (BATDA), in 1 ml of culture medium at 37°C for 30 min. The cells were washed 4 times with PBS and adjusted to 5×10^4 cells/ml. One hundred microliter aliquots containing 5×10^3 cells

each were then plated in 96-well round bottom plates. Purified V γ 2V δ 2 T cells were added at the indicated E:T ratio and centrifuged at 100 $\times$ g for 2 min. The plates were incubated for 2 h at 37°C. After incubation, the plates were centrifuged at 100 $\times$ g for 2 min. Twenty microliters of supernatant was transferred to 96-well flat bottom plates, followed by the addition of 180 μ l of the provided europium solution. The plates were then shaken for 15 min at RT. The fluorescence intensity was measured using a time-resolved fluorometer (VICTOR³). The percentage of specific lysis was calculated as: % specific lysis = 100 $\times$ (experimental release - spontaneous release)/(maximum release - spontaneous release). The background signal was subtracted from the maximum and spontaneous release values before the values were used to calculate the percentage of specific lysis.

Flow cytometric detection of intracellular cytokines

PBMC were plated at 1×10^5 cells/well in 96-well round-bottom tissue culture plates in complete RPMI 1640 medium with or without HMBPP (0.316 μ M) for 4 hr in the presence of Brefeldin A (GolgiPlug, BD Biosciences). To determine the full cytokine capability of CD8 T cells, PBMC were stimulated with PMA (10 ng/ml) and ionomycin (1 μ M) under the same conditions. After stimulation, the cells were harvested and stained with FITC-labeled anti-V δ 2 or FITC-labeled CD8, APC-labeled anti-CD27, and PE-Cy7-labeled anti-CD28. Cells were washed twice in staining buffer and subsequently fixed in 100 μ l of 1X BD Cytofix/Cytoperm for 20 min on ice. After fixation, the cells were washed twice with 1X permeabilization buffer, followed by cytoplasmic staining with PE-conjugated anti-TNF- α (MAB11) or PE-conjugated anti-IFN- γ (4S.B3), both from BD Biosciences. The percentage of cytokine-producing V δ 2 or CD8 T cells among the four V γ 2V δ 2 T cell subsets was determined by four-color cytometry. A total of 100,000 to 150,000 events were acquired for each sample and analyzed using CellQuest Pro (BD Biosciences) and FlowJo 8.5.2 (TreeStar).

Statistical analyses

Data obtained for different V γ 2V δ 2 T cell subsets were analyzed by one-way ANOVA using GraphPad Prism 4.0 software. Results are presented as scatter plots in which each dot represents either a healthy donor or a NPC patient. Comparisons between groups were performed using a non-parametric Mann-Whitney U test. A p value < 0.05 was considered significant.

Results

V γ 2V δ 2 T cells from NPC patients exhibit a significant reduction in tumor-specific cytotoxicity

To compare the cytolytic activity of V γ 2V δ 2 T cells from NPC patients and healthy controls (NOR), highly purified $\gamma\delta$ T cells were incubated with K562 (a $\gamma\delta$ T cell-sensitive cell line) or HK-1 (a human NPC cell line). Due to their low frequencies in peripheral blood, V γ 2V δ 2 T cells were isolated from 6 NPC patients and 5 NOR and pooled into their respective groups. The $\gamma\delta$ T cells from NPC patients demonstrated lower cytolytic activity against both K562 and HK-1 compared with those from NOR (Figure 1a and 1b). The cytotoxicity assay represents a single experiment that was performed using pooled V γ 2V δ 2 T cells from NPC patients and NOR.

A comparison of the frequency of V γ 2V δ 2 T cells from 96 patients with active NPC disease and 54 NOR revealed no significant difference in the total percentage of V γ 2V δ 2 T cells between NPC patients and NOR (Figure 1c). The median frequency of V γ 2V δ 2 T cells detected in NPC patients and NOR was 1.8% (range: 0.03%–16.2%) and 2.0% (range: 0.3%–10.7%) respectively. Although the incidence of NPC is 2–3 times more common in males than in females, we could not detect any sex-related difference in the total number of V γ 2V δ 2 T cells between the two genders (Figure 1d).

Differential regulation of the various T cell subsets of V γ 2V δ 2 T cells in NPC patients

Using CD27 and CD28, V γ 2V δ 2 T cells can be delineated into four distinct subsets: T_{CM} (CD27⁺ CD28⁺), T_{CM 27-} (CD27⁻ CD28⁺), T_{EM} (CD27⁺ CD28⁻), and T_{EM RA} (CD27⁻ CD28⁻) (Figure 2). Similar to CD8⁺ $\alpha\beta$ T memory cells, we and others have previously suggested that T_{CM} cells predominantly represent early memory V γ 2V δ 2 T cells, whereas T_{EM RA} cells are predominantly late memory V γ 2V δ 2 T cells [1]. In support of this, T_{EM RA} cells have been shown to possess the highest level of perforin expression among the four $\gamma\delta$ T cell subsets, suggesting that T_{EM RA} cells have higher cytolytic activity towards tumor cells than the other T cell subsets. Circulating V γ 2V δ 2 T cells are very heterogeneous, and the ability to identify $\gamma\delta$ cellular subsets based on CD27 and CD28 expression (Figure 2a) may offer an opportunity to “fine-tune” the effector immune functions of $\gamma\delta$ T cells during immunotherapy for NPC.

We examined the proportions of the various V γ 2V δ 2 T cell subsets in NPC patients. The major V γ 2V δ 2 cell subset present in the peripheral blood of both NPC patients and NOR was T_{CM} cells. The median frequency of T_{CM} V γ 2V δ 2 cells in NOR was 61.8% (range: 12.1%–91.7%). The median frequency of T_{CM} V γ 2V δ 2 cells in NPC patients was 42.7% (range: 0.7%–86.8%), a significantly lower frequency compared to NOR (Figure 2b). In contrast, the median frequency of late effector memory cells (T_{EM RA}) was 25.2% (range: 0%–98.4%), which was significantly higher in comparison to NOR, because the median frequency of T_{EM RA} in NOR was 10.7% (range: 0.3%–69.4%). The increased T_{EM RA} cells and decreased T_{CM} cells in NPC patients were not related to age (Figure 2c) because linear regression analyses showed no correlation between the frequencies of these two subsets and age in both NPC patients and NOR. Interestingly, the median frequency of T_{CM} cells in NOR was marginally lower ($p=0.0447$) in males than in females (Figure S1). However, this was not observed in NPC patients. Overall, there was no observable difference in the total circulating V γ 2V δ 2 T cells between NPC patients and NOR; however, a specific and significant reduction in the T_{CM} cells and an increase in the T_{EM RA} cells could be detected in NPC patients.

Differential expression of the various T cell subsets of V γ 2V δ 2 T cells is independent of the histological subtype and tumor stage of NPC

Undifferentiated carcinoma (UNC, WHO type III) and non-keratinizing squamous cell carcinoma (SCC, WHO type II) are the two most common types of NPC in southeast Asia, including Singapore. Among the NPC specimens studied, 73 were UNC type and 23 were SCC type. When compared individually, there was no significant difference in the total percentage of V γ 2V δ 2 T cells among patients with UNC or SCC compared with normal controls (Figure S2a). However, when the various V γ 2V δ 2 T cell subsets were studied, T_{CM} cells were significantly reduced in patients with UNC or SCC compared with normal controls (Figure S2b). The median frequency of T_{CM} cells in patients with UNC was 43.5% (range: 0.7%–86.8%), whereas patients with SCC exhibited a median frequency of 41.3% (range: 10.5%–85.1%). The median frequency of T_{EM RA} cells in UNC patients was 23.1% (range: 0%–98.4%), a significant increase compared to NOR (Figure 4b). A similar increase was noted in T_{EM RA} cells in SCC patients (median frequency of 29.5% (range: 1.2%–71%)). This difference was slightly below statistical significance ($p=0.0759$) (Figure S2b).

Similar comparisons were performed after segregating the NPC specimens according to their clinical, nodal, and overall disease staging. Although a similar trend of decreased T_{CM} cells and increased T_{EM RA} cells was observed (Figures S3), these differences were not statistically significant due to the limited number of specimens available for each of the clinical sub-groups.

Perforin is highly expressed in the T_{EM} and T_{EM RA} V γ 2V δ 2 T cell subsets of NOR and NPC patients

V γ 2V δ 2 T cell-mediated killing of tumor targets is mediated by the release of stored perforin upon contact with tumor cells. To determine if there were differences in perforin expression in V γ 2V δ 2 T cells between patients with NPC and normal controls, we performed intracellular perforin staining on blood V γ 2V δ 2 T cells. In V γ 2V δ 2 T cells from normals, perforin expression was higher in T_{EM RA} cells than T_{CM} cells (Figure 3a, left panel). The median proportions of T_{EM} and T_{EM RA} V γ 2V δ 2 T cells that expressed perforin were 77.2% (range: 31.1%–94.0%) and 89.7% (range: 40.0%–96.7%), respectively. These proportions were significantly higher than those of early memory T_{CM} cells (median: 31.3%, range: 5.1%–83.4%) and T_{CM 27}⁺ cells (median: 37.5%, range: 11.9%–89.4%) (Figure 3a, right panel). Similar to healthy controls (NOR), a greater proportion of T_{EM} and T_{EM RA} V γ 2V δ 2 T cells expressed perforin compared with T_{CM} and T_{CM 27}⁺ cells from NPC patients (n=10) (Figure 3b). However, a significantly greater proportion of T_{CM} cells from NPC patients expressed perforin compared with T_{CM} cells from healthy controls ($p=0.029$). No statistically significant differences were detected in the other $\gamma\delta$ T cell subsets (Figure 3b).

Production of IFN- γ and TNF- α by T_{CM} and T_{EM RA} V γ 2V δ 2 T cells is significantly impaired in patients with active NPC disease

To determine the functional capabilities of $\gamma\delta$ T cells from NPC patients compared with healthy controls, V γ 2V δ 2 T cells were stimulated with HMBPP and IFN- γ and TNF- α production measured by intracellular staining (Figure 4a, left panel). The proportion of V γ 2V δ 2 T cells producing IFN- γ and TNF- α in NPC patients was significantly lower than in healthy controls (Figure 4a, right panel). For NPC patients, the median percentage of IFN- γ - and TNF- α -positive V γ 2V δ 2 T cells was 17.4% (range: 3.4%–46.4%) and 17.0% (range: 3.4%–45.2%), respectively (Figure 4a, right panel). In contrast, the median percentage of IFN- γ - and TNF- α -positive V γ 2V δ 2 T cells in NOR was 26.9% (range: 10.5%–46.4%) and 23.4% (range: 11.7%–56.2%), respectively (Figure 4a, right panel).

To further delineate whether a specific T cell subset within the V γ 2V δ 2 T cells contributed to the overall impairment of cytokine production, IFN- γ and TNF- α production by the various $\gamma\delta$ T cell subsets was evaluated in NPC patients and NOR. In NPC patients, the proportion of IFN- γ -positive V γ 2V δ 2 T cells was significantly reduced in all four subsets, with the T_{CM} and T_{EM RA} subsets demonstrating the most severe reductions (Figure 4b). Whereas the median proportion of IFN- γ -positive T_{CM} cells in NPC patients was 18.6% (range: 5.4%–57.1%), it was 32.7% (range: 17.3%–58.4%) in NOR. The median proportion of IFN- γ -positive T_{EM RA} cells in NPC patients was 9.6% (range: 5.4%–22.5%) compared with 19.8% (range: 4.8%–47.4%) in NOR (Figure 4b). When compared to NOR, the proportion of TNF- α -positive T_{CM} and T_{EM RA} cells in NPC patients was also significantly reduced (Figure 4b). The median percentage of TNF- α -positive T_{CM} cells detected in NPC patients was 19.2% (range: 3.5%–49.4%) compared with 28.7% (range: 13.2%–66.6%) in NOR. Similarly, the median percentage of TNF- α -positive T_{EM RA} cells detected in NPC patients was 7.18% (range: 0%–67.1%), in contrast to 18.8% (range: 0%–40.6%) in NOR (Figure 4b).

Linear regression analyses showed no correlation between the number of cytokine-producing $\gamma\delta$ T cells and the age of the NPC patients or healthy controls (Figure 4c). This shows that the reduction in cytokine-producing $\gamma\delta$ T cells in NPC patients was not due to differences in the ages of the NPC patients and the healthy controls.

To determine the maximum cytokine-producing capability of $\gamma\delta$ T cells and CD8 T cells in response to mitogenic stimuli, PBMC from NPC patients and NOR were stimulated with PMA and ionomycin. Similar proportions of IFN- γ and TNF- α -positive CD8 T cells were detected

in NPC patients and NOR (Figure 4d). There was also no significant difference in the proportion of cytokine-positive V γ 2V δ 2 T cells between NPC patients and NOR. These results suggest that the reduced cytokine production of $\gamma\delta$ T cells from NPC patients in response to the HMBPP antigen is caused by a defect in TCR signaling.

Radiotherapy or concurrent chemo-radiotherapy increases the proportion of T_{EM RA} cells in NPC patients but fails to correct cytokine production

NPC patients with low-grade tumors respond well to radiotherapy. However, the response rate for NPC patients with tumor grades of 3–4 is poor and concurrent chemo-radiotherapy is usually introduced to improve the clinical outcome for these patients. To determine whether these treatments could normalize the $\gamma\delta$ T cell subset distributions and $\gamma\delta$ T cell function in NPC patients to those observed in healthy controls, PBMC were obtained from NPC patients 3–6 months after receiving their last treatment and the various V γ 2V δ 2 T cell subsets assessed. Instead of normalizing, the percentage of V γ 2V δ 2 T_{CM} cells dropped even further to very low levels with a corresponding rise in the percentage of T_{EM RA} cells (Figure 5a).

When the total proportion of IFN- γ - and TNF- α -positive V γ 2V δ 2 T cells was analyzed in healthy controls, NPC patients prior to therapy, and NPC patients post-therapy, the proportion of IFN- γ - and TNF- α -positive V γ 2V δ 2 T cells continued lower post-therapy than healthy controls with no evidence of improvement when compared with patients prior to therapy (Figure 5b). Further analysis of the various $\gamma\delta$ T cell subsets showed slight restoration of IFN- γ -positive T_{CM} cells in NPC patients following treatment ($p=0.0478$). However, the impaired IFN- γ production observed for T_{EM RA} cells and the production of TNF- α by both T_{CM} and T_{EM RA} cells were not corrected following treatment (Figure 5c).

Discussion

V γ 2V δ 2 T cells have recently been exploited to provide protective immunity against intracellular pathogens and cancer [4]. In the present study, we show that V γ 2V δ 2 T cells from NPC patients are functionally impaired with respect to both their ability to kill tumor cells and their capacity to produce IFN- γ and TNF- α . These functional defects are associated with alterations in the distribution of the various V γ 2V δ 2 T cell subsets. Immunophenotypic analyses of PBMC from NPC patients and healthy controls demonstrated that, although there was no difference in the total percentage of V γ 2V δ 2 T cells, the distribution of V γ 2V δ 2 T cell subsets was altered in NPC patients compared with healthy controls.

Differences in the expression of the costimulatory receptors CD28 and CD27, define 4 subsets of V γ 2V δ 2 T cells in NPC patients and healthy controls. Both naïve and central memory CD8 $\alpha\beta$ T cells express CD28 and CD27 [40]. Naïve T cells can be further discriminated from central memory cells because naïve T cells express both the CD45RA isoform and the lymph node homing receptor, CCR7, whereas central memory cells retain CCR7 but lack CD45RA [40] [42]. In most healthy controls and some NPC patients, CD28⁺ CD27⁺ V γ 2V δ 2 T cells represent the majority of $\gamma\delta$ T cells. However, unlike CD8 $\alpha\beta$ T cells, very few naïve V γ 2V δ 2 T cells are present in the peripheral blood of adult individuals. De Rosa *et al.* showed that naïve V γ 2V δ 2 T cells were CD45RO⁺, CD11a^{low}, and CD27^{high} and the percentage of CD27^{high} cells falls below 10% after 1 year of age [8]. Because it is technically difficult to consistently discriminate CD27^{high} from CD27^{low} based on CD28 and CD27 staining, we are unable to determine the exact proportion of naïve V γ 2V δ 2 T cells. We determined CD28⁺ CD27⁺ V γ 2V δ 2 T cells to represent predominantly memory cells. This is in agreement with an earlier study showing that the predominant population of V γ 2V δ 2 T cells in the peripheral blood was CD45RO⁺ CD45RA⁺ CD27⁺ [11]. Similar to CD8 $\alpha\beta$ central memory T cells, CD45RA⁺ CD27⁺ V γ 2V δ 2 T cells also express CD62L and CCR7 [42]. Our results showed that in NPC

patients the CD28⁺ CD27⁺ (T_{CM}) V γ 2V δ 2 T cell subset was markedly reduced whereas the CD28⁻ CD27⁻ (T_{EM RA}) V γ 2V δ 2 T cell subset was significantly increased.

Similar CD8 $\alpha\beta$ T cells differentiation has been shown to correlate with disease progression in patients with viral infections [1]. For instance, HIV-infected long-term non-progressors are characterized by an increase of highly proliferative CD27⁺ CD28⁺ HIV-specific memory CD8 $\alpha\beta$ T_{CM} cells [35]. In contrast, other persistent viral infections deplete CD8 $\alpha\beta$ T_{CM} cells by driving their differentiation to the CD27⁻ CD28⁻ terminally differentiated phenotype [35]. The observed reductions in T_{CM} cells and increases in T_{EM RA} V γ 2V δ 2 T cells in NPC patients is likely due to a similar differentiation of T_{CM} cells. However, it is unclear whether this differentiation is due to a concurrent viral infection or is driven by the presence of the NPC only.

EBV infection is one possible viral cause of terminal differentiation of V γ 2V δ 2 T cells in NPC patients. The development of NPC in EBV-endemic regions is highly associated with latent EBV infections. Primary EBV infection in childhood usually manifests as an asymptomatic infection. In developed countries, however, not all individuals are infected in childhood. Primary EBV infection in adolescents and adults can then result in acute infectious mononucleosis. Acute infectious mononucleosis is associated with $\gamma\delta$ T cell expansions [7, 20]. Moreover, V γ 1 T cells proliferate in response to stimulation by EBV-transformed B lymphocytes [18,34]. However, when we compared the percentage of V γ 1⁺ cells in NPC patients and healthy controls, we did not observe a significant expansion in the V γ 1⁺ population in NPC patients ($p=0.2291$). It is therefore unlikely that the decline in T_{CM} V γ 2V δ 2 T cells in NPC patients is caused by changes in V γ 1 T cells.

High frequencies of CD8⁺ $\alpha\beta$ T_{EM RA} cells (CD28⁻ [29] or CD45RA⁺ CD27⁻ [25]) are found in individuals who have been infected by CMV [1], likely due to continued stimulation of CD8 $\alpha\beta$ T cells by chronic CMV infection. Increasing evidence suggests that this type of chronic immune activation can alter the overall composition of T cells, culminating in dysfunctional T cell responses. Our data demonstrate an increase in the T_{EM RA} subset of V γ 2V δ 2 T cells in NPC patients that parallels the increase in late-differentiated CMV-specific CD8 $\alpha\beta$ T cells observed in patients with CMV infection. Thus, CMV infection is another possible cause for the terminal differentiation of V γ 2V δ 2 T cells in NPC patients. Consistent with this hypothesis, CMV infection is endemic in Singapore. For example, when 120 antenatal Singaporean women were screened for CMV infection, 87% tested seropositive [56]. Moreover, $\gamma\delta$ T cells expand in kidney transplant recipients developing CMV infection. However, against this hypothesis, these expansions are restricted to V γ 1 and V γ 3 T cells, not V γ 2V δ 2 T cells [9]. In our study, NPC patients were not tested for CMV seropositivity. Therefore, we were unable to determine if CMV seropositivity correlated with the observed changes in the V δ 2 T cell subset distribution.

The increased levels of T_{EM RA} cells in NPC patients could be due to increased apoptosis of T_{CM} V δ 2 T cells. However, this explanation seems less likely because T_{CM} cells express higher levels of the anti-apoptotic protein bcl-2 compared with T_{EM} and T_{EM RA} cells [5]. This argues against the suggestion that the increased sensitivity of T_{CM} cells to apoptosis could account for the loss of T_{CM} cells in NPC patients. Furthermore, gag-specific CD27⁺, but not CD27⁻, CD8 T cells persist in HIV-infected patients following adoptive transfer suggesting that the expression of CD27 on memory T cells may confer a survival advantage *in vivo* [33]. Therefore, the increased number of T_{EM RA} cells in NPC patients may result from the differentiation of T_{CM} to T_{EM RA} cells with the loss of CD27 and CD28 expression due to viral or tumor effects. Alternatively, the increased levels of T_{EM RA} cells could simply be a consequence of the preferential expansion of T_{EM RA} cells in NPC patients although this has never been described.

Previously, an age-related decrease in $\gamma\delta$ T cells [15] and an increase in the CD27⁺ CCR7⁺ effector/memory V γ 2V δ 2 cells was reported for healthy donors [38]. Furthermore, the increased number of CD27⁺ V δ 2 T cells negatively correlated with the ability to expand in response to IPP and IL-2 [38]. In the present study, there was no correlation between the levels of T_{CM} or T_{EM RA} V δ 2 T cells and the age of the NPC patients or healthy controls. Similarly, there was no correlation between the proportion of cytokine-positive V γ 2V δ 2 T cells and the age of NPC patients or healthy controls. Thus, the reduced number of cytokine-producing T_{CM} and T_{EM RA} in NPC patients compared with healthy controls is not due to differences in their ages.

V γ 2V δ 2 T cells can be activated to produce large amounts of TNF- α and IFN- γ after stimulation by phosphoantigens [26,27,45]. Here we demonstrated that fewer V γ 2V δ 2 T cells from NPC patients produced IFN- γ and TNF- α upon stimulation with the HMBPP antigen compared with V γ 2V δ 2 T cells obtained from healthy controls. However, mitogenic stimulation using PMA and ionomycin did not show the same decline in the numbers of IFN- γ producing cells suggesting that the decrease in cytokine production by V γ 2V δ 2 T cells is due to alterations in T cell receptor signaling following antigen presentation. The impaired production of cytokines by T_{CM} and T_{EM RA} $\gamma\delta$ T cells from NPC patients may reflect general V γ 2V δ 2 T cell dysfunction. There also may be an increased susceptibility to tumorigenesis since IFN- γ deficiency in mice makes them more susceptible to the development of lymphomas [48] and epithelial malignancies [49].

IFN- γ secretion does not always correlate with cytotoxicity [47]. Using a single-cell assay that simultaneously measures cytotoxicity and IFN- γ secretion, Snyder *et al.* found that these two effector functions could be regulated independently [46]. In the present study, however, the reduced frequency of IFN- γ - and TNF- α -producing V γ 2V δ 2 T cells in NPC patients was also associated with lower cytotoxicity against tumor cells. Thus, the impairments that lead to the decreased production of IFN- γ and TNF- α by V γ 2V δ 2 T cells from NPC patients may also affect V γ 2V δ 2 T cell cytotoxicity. These functional defects could potentially limit the ability of V δ 2 T cells to mount effective responses against tumor cells. However, it remains unclear whether the loss of function and altered subset distribution of V γ 2V δ 2 T cells in NPC patients reflect a general alteration in all circulating T cells including $\alpha\beta$ T cells. Further studies are needed to address this possibility.

NPC are relatively radiosensitive and, therefore, radiation is a standard modality of treatment for NPC. For NPC patients with advanced disease, combined radiotherapy and chemotherapy is often administered to improve clinical outcome and survival rates. Although these are useful modalities for NPC treatment, neither treatment restored V γ 2V δ 2 T cell function or returned V γ 2V δ 2 T cell subset distributions to normal.

Recent advances in immunotherapy specifically targeting $\gamma\delta$ T cells have shown promise for the treatment of multiple myeloma [54], renal cell carcinoma [3], and prostate cell carcinoma [10]. Large-scale *ex vivo* expansion of V γ 2V δ 2 T cells to provide an adequate source of cells for adoptive immunotherapy is now feasible [43]. The present study shows that alterations in the proportions of the V γ 2V δ 2 T cell subsets and their functions could have profound effects on overall V γ 2V δ 2 T cell responses. Thus, monitoring V γ 2V δ 2 T cell subset distributions and functions may allow predictions about the effectiveness of V γ 2V δ 2 T cell immunotherapy. Such monitoring offers a rational basis for assessing the potential effectiveness of immunotherapy strategies using V γ 2V δ 2 T cells to optimize for the best clinical outcome.

Supplementary Material

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Abbreviations

HMBPP	(<i>E</i>)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
IPP	isopentenyl pyrophosphate
NOR	healthy controls
NPC	nasopharyngeal carcinoma
SCC	non-keratinizing squamous cell carcinoma
TCR	T cell antigen receptor
T_{CM}	CD28 ⁺ CD27 ⁺ central memory T
T_{CM 27-}	CD28 ⁺ CD27 ⁻ central memory T
T_{EM}	CD28 ⁻ CD27 ⁺ effector memory T
T_{EM RA}	CD28 ⁻ CD27 ⁻ CD45RA ⁺ effector memory T
UNC	undifferentiated carcinoma

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Figure 1. V γ 2V δ 2 T cells from NPC patients demonstrate decreased tumor cytolytic activity but are present in normal numbers

(a) Purity of MACS-purified V γ 2V δ 2 T cells. V γ 2V δ 2 T cells were isolated from 6 NPC patients and 5 NOR controls as described in Materials and Methods and analyzed by flow cytometry. (b) Effector V δ 2 T cells from NPC patients exhibit reduced tumor cytolytic activity. Purified V γ 2V δ 2 T cells were incubated with K562 or HK-1 tumor cells for 4 hr at an E:T of 10:1. Note that V δ 2 T cells from healthy controls (NOR) showed higher cytolytic activity against K562 and HK-1 compared with V δ 2 T cells from NPC patients. (c) No significant difference in the frequency of V γ 2V δ 2 T cells in NPC patients and healthy controls (NOR). The percentage of peripheral blood V γ 2V δ 2 T cells among CD3 positive T cells was measured

by flow cytometry as described in the Materials and Methods. (d) No significant differences observed in the percentage of V δ 2 T cells in males and females in the NPC and NOR cohorts. V γ 2V δ 2 T cells were assessed as in (c).

Figure 2. The proportion of T_{EMRA} $V\gamma 2V\delta 2$ T cells is increased in patients with active NPC compared with healthy controls

(a) $V\gamma 2V\delta 2$ T cells can be divided into 4 distinct populations based on CD27 and CD28 surface expression. Representative staining is shown. (b) $V\gamma 2V\delta 2$ T cell subset distributions differ between NPC patients and healthy controls. An example of the $\gamma\delta$ T cell subset distribution in an NPC patient and a NOR is shown (left panel). T_{CM} ($CD27^+CD28^+$) $V\gamma 2V\delta 2$ T cells were significantly reduced and T_{EMRA} ($CD27^-CD28^-$) cells significantly increased in NPC patients compared with NOR (right panel). (c) The increase in T_{EMRA} cells, and decrease in T_{CM} cells in NPC patients was not related to the age of the patients compared with NOR. No relationship

exists between age and T_{CM} and T_{EMRA} cells among NPC patients (closed symbols) and healthy controls (open symbols).

Figure 3. Perforin is highly expressed in T_{EM} and $T_{EM RA}$ $V\gamma 2V\delta 2$ T cells

(a) Perforin expression in $V\gamma 2V\delta 2$ T cell subsets. PBMC from NOR were surface stained with anti- $V\delta 2$ TCR, CD27, and CD28 prior to fixation and permeabilization with BD Cytofix/CytoPerm. Intracellular perforin was detected using anti-perforin mAb. A mouse isotype control was included to determine background staining. (Left panel) Representative perforin staining. Perforin expression was higher in $T_{EM RA}$ cells (orange shaded) compared with T_{CM} cells (violet shaded). Isotype controls for $T_{EM RA}$ (orange unshaded) and T_{CM} (violet unshaded) are shown. (Right panel) Increased frequency of perforin expressing cells in the T_{EM} and $T_{EM RA}$ $V\gamma 2V\delta 2$ T cell subsets in NOR. (b) Comparison of perforin expression among $V\gamma 2V\delta 2$ T cell subsets in NPC patients and healthy controls (NOR). Note that with the exception of T_{CM} , there were no statistically significant differences in perforin expression between NOR and NPC patients.

a

b

Figure 4. IFN- γ and TNF- α production is impaired in V γ 2V δ 2 T cells from patients with active NPC

(a) IFN- γ and TNF- α production in response to HMBPP stimulation is reduced in total V γ 2V δ 2 T cells from NPC patients. PBMC from NPC patients (n=29) and NOR (n=28) were stimulated with or without HMBPP (0.316 μ M) for 4 hr followed by intracellular cytokine staining for IFN- γ and TNF- α as described in the Materials and Methods. Representative staining is shown in the left panel. Note that the frequency of IFN- γ or TNF- α -producing V γ 2V δ 2 T cells in NPC was decreased compared with V γ 2V δ 2 T cells from NOR. (b) IFN- γ and TNF- α production in response to HMBPP stimulation by T_{CM} and T_{EMRA} cells is reduced in NPC patients compared with NOR. Stimulation was as described in (a). (c) No correlation between cytokine-producing $\gamma\delta$ T cells and age in NPC patients or NOR. Linear regression analyses was done to assess the frequencies of cytokine-producing $\gamma\delta$ T cells in NPC patients (filled circles) and NOR (empty circles) with their ages. (d) IFN- γ and TNF- α production in response to mitogen stimulation is similar between NPC patients and healthy controls (NOR). PBMC from NPC patients and NOR were stimulated with PMA and ionomycin followed by intracellular cytokine staining for IFN- γ and TNF- α for V δ 2 and CD8 T cells to evaluate antigen-independent IFN- γ and TNF- α production. The frequencies of IFN- γ -(left panel) and

TNF- α -(right panel) positive cells are shown. Note that there were no significant differences in cytokine production by V δ 2 and CD8 T cells obtained from NPC patients or NOR.

Figure 5. Radiotherapy or concurrent chemo-radiotherapy further increases TEM RA and decreases TCM V γ 2V δ 2 T cells but fails to correct cytokine production

(a) The frequency of TCM and TEM RA V γ 2V δ 2 cells was compared between NOR, NPC patients prior to receiving treatment (PRE), and post-therapy patients (PT). For PT patients, the percentages of TEM RA V γ 2V δ 2 T cells were significantly higher than for NOR and PRE subjects. Increased TEM RA in PT was accompanied by a decline in early memory TCM V γ 2V δ 2 T cells. (b) Treatment failed to correct the defective IFN- γ and TNF- α production detected for total V δ 2 T cells. (c) IFN- γ and TNF- α production by V γ 2V δ 2 T cell subsets remained defective, although IFN- γ production by TCM cells from PT patients showed a marginal increase compared with NPC patients before therapy.

Table 1
Characteristics of NPC patients included in this study.

NPC Patients (n=96)	
Age (yr)	
Median (range)	50 (29–77)
Sex	
Male	69
Female	27
Tumor stage *	
I	21
II/IIa/IIb	2/5/32
III/IIIb	20/1
IV	15
Nodal status	
N0	31
N1	24
N2/N2c	31/1
N2c	1
N3/N3a/3b	5/1/3
Histological type	
Non-keratinizing squamous carcinoma	23
Undifferentiated carcinoma	73

* The staging is based on the 6th edition American Joint Committee on Cancer (AJCC) classification for NPC.