The Primary Response of Human γ/δ^+ T Cells to *Mycobacterium tuberculosis* Is Restricted to Vγ9-bearing Cells

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Summary

We have previously reported that peripheral blood γ/δ^+ T cells proliferate in high frequency (1 in 2–20) in response to heat-killed *Mycobacterium tuberculosis* (M.tb.). In the present study, the T cell receptor phenotype of mycobacteria-responsive human γ/δ^+ T cells was analyzed in primary cultures with a set of monoclonal antibodies (mAbs) directed against Vγ9, Vδ1, and Vδ2. When unseparated T cells were stimulated with M.tb., all proliferating γ/δ^+ T cells expressed Vγ9 (and Vδ2) after culture. Selective depletion of Vγ9-bearing cells before culture completely abolished the proliferative response of all γ/δ^+ T cells (but did not inhibit reactivity of α/β^+ T cells). In addition, when $CD4^-CD8^-$ thymocytes were stimulated with M.tb., there was again selective outgrowth of $V\gamma9^+$ cells. In this case, the starting responder population contained few (0.5–1.8%) $V\gamma9^+$ and many (11.5–31.5%) $V\delta1^+$ cells that did not coexpress Vγ9. These $V\delta1^+$ cells were not activated by M.tb. but could be readily stimulated by anti-Vδ1 mAb A13. Finally, a Vγ9-specific mAb selectively suppressed the proliferative response of γ/δ^+ T cells to M.tb. Taken together, our results demonstrate that, within γ/δ^+ T cells, reactivity towards M.tb. is an exclusive property of $V\gamma9^+/V\delta2^+$-bearing cells.

A minor subset of human and murine T cells expresses a TCR composed of γ and δ chains (1–7). γ/δ^+ T cells account for 1–10% of $CD3^+$ peripheral blood T cells, and appear to be evenly distributed throughout the lymphoid tissue in humans (6–9). In striking contrast to the TCR-α/β, there is only a small number of functional Vγ and Vδ gene segments, which results in limited germline diversity of the TCR-γ/δ when compared with the TCR-α/β (6, 7, 10). In addition, it appears that only a few of the available Vγ and Vδ segments are actually used to construct the expressed TCR-γ/δ. Thus, >60% of human peripheral blood γ/δ^+ T cells express a Vγ9 (nomenclature of Vγ genes according to reference 10*a*) chain paired with a δ chain using the Vδ2 element (11–15). The second most frequent (10–20%) subset of peripheral blood γ/δ^+ T cells utilizes a Vδ1 gene segment that is usually paired with a Vγ element other than Vγ9 (14–17). The extensive occurrence of Vγ9 paired with Vδ2 among peripheral blood γ/δ^+ T cells is not genetically determined, because $V\gamma9^+/V\delta2^+$ cells constitute only a minor fraction of γ/δ^+ T cells in postnatal human thymus (9, 18). In addition, the molecular analysis of a large number of human thymic γ/δ^+ clones did not reveal any evidence for the selective Vγ9/Vδ2 pairing as being due to constraints at the level of DNA rearrangements or protein pairing (19). It is thus possible that the extrathymic expansion of $V\gamma9^+/V\delta2^+$ T cells in human peripheral blood is driven by specific antigenic pressure (18–20).

The nature of the ligands recognized by γ/δ^+ T cells remains elusive (7, 21, 22). A striking feature of both murine and human γ/δ^+ T cells, however, is their frequent reactivity towards mycobacterial antigens, including in some cases the 65-kD mycobacterial heat shock protein (hsp)[1] (23–29). We and others have shown that human peripheral blood γ/δ^+ T cells proliferate in high frequency in response to killed mycobacteria (30, 31). The surprisingly high frequencies of mycobacteria-reactive γ/δ^+ cells (1 in 2–20) measured in our previous study (30) suggested that a major fraction of all peripheral blood γ/δ^+ T cells was activated by killed *Mycobacterium tuberculosis* (M.tb.) bacteria. The present experiments were designed to identify the mycobacteria-reactive subset of human γ/δ^+ T cells.

[1] *Abbreviations used in this paper:* DN, double negative; hsp, heat shock protein; MACS, magnet-activated cell sorter; MNC, mononuclear cell; M.tb., *Mycobacterium tuberculosis*; NA, nonadherent; SEA, staphylococcal enterotoxin A.

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Materials and Methods

Isolation of Lymphocyte Subpopulations. PBMC from normal donors were isolated by Ficoll-Hypaque density gradient centrifugation. Monocytes/macrophages were removed from PBMC by plastic adherence for 60 min at 37°C. T cells were purified from plastic-nonadherent (NA) cells by the E rosette method as described (30). Vγ9-expressing γ/δ$^+$ T cells were depleted from either NA or separated T cells by FACS® (Becton Dickinson & Co., Mountain View, CA) using mAb 7A5 developed in our laboratory. mAb 7A5 precipitates the TCR solubilized from the surface membrane of cloned γ/δ$^+$ T cells (32). As tested on a large panel of γ/δ$^+$ clones, its specificity is identical to mAb TiγA, which has been shown to recognize a Vγ9-encoded epitope (11). The Vγ9 specificity of mAb 7A5 has been confirmed by molecular analysis of 7A5$^+$ clones (M.B. Brenner and H. Band, personal communication). NA or T cells were incubated for 20 min on ice with biotinylated mAb 7A5. After being washed, cells were incubated with PE-labeled streptavidin (Becton Dickinson & Co.) as a second-step reagent. 7A5$^-$ and 7A5$^+$ subsets were sorted on a FACStar Plus® cell sorter (Becton Dickinson & Co.) based on forward light scatter and fluorescence intensity. Dead cells were excluded by propidium iodide staining. In some experiments, 7A5$^+$ cells were depleted by using a magnet-activated cell sorting device (see below).

Samples of postnatal thymus tissue were obtained from children undergoing corrective heart surgery, and were kindly made available through Prof. S. Hagl (University of Heidelberg). Thymus tissue was minced, and the single cell suspension was subsequently centrifuged on Ficoll-Hypaque gradients. CD4$^-$CD8$^-$ double-negative (DN) cells were enriched from thymus mononuclear cells (MNC) by depleting most CD4$^+$ and CD8$^+$ cells by means of a magnet-activated cell sorter (MACS; Miltenyi Biotechnik, Bergisch-Gladbach, Germany). To this end, thymus MNC were incubated with saturating amounts of biotinylated anti-CD4 (OKT4; American Type Culture Collection [ATCC], Bethesda, MD) plus biotinylated anti-CD8 (OKT8; ATCC) mAbs. After being washed, the cells were incubated for additional 20 min with biotinylated goat anti-mouse IgG (Tago, Inc., Burlingame, CA). After two washing steps, FITC-labeled avidin (Dianova GmbH, Hamburg, Germany) was added as a second-step reagent, and incubation was continued for 20 min on ice. The cells were washed, resuspended on ice in biotinylated magnetic beads, and passed through a magnetic column.

Cell Cultures. Responder cells were cultured in round-bottomed microtiter plates (Nunc, Roskilde, Denmark) at 5–10 × 10^4 cells per well. The culture medium was RPMI 1640 (Biochrom KG, Berlin, Germany) supplemented with 2 mM L-glutamine, antibiotics, 10 mM Hepes, and 10% heat-inactivated male human serum. In most experiments, 10^5 irradiated (2,000 rad from a cesium source) autologous PBMC were added as APC. In the case of thymocyte responder cells, allogeneic irradiated PBMC were used as APC. The following stimuli were included to activate resting T cell. PHA-P (Wellcome, Burgwedel, Germany) at 0.5 μg/ml, anti-CD3 mAb OKT3 (Cilag AG, Sulzbach, Germany) at 1 μg/ml, allogeneic irradiated PBMC stimulator cells at 10^5 cells per well, or 0.01% (vol/vol) of a suspension of killed M.tb. strain H37RV. Mycobacteria were autoclaved for 20 min at 120°C, sonicated for 15 min, and stored at −70°C. Immediately before use, bacteria were again sonicated for 5 min. In some experiments, DN thymocytes were stimulated with soluble anti-Vδ1 mAb A13 (ascites diluted at 1:2,000 final; see below). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Where indicated, 1 ng/ml rIL-2 (EuroCetus, Amsterdam, The Netherlands) was added after 3 d (see Results). After 3–8 d of culture, 1 μCi [^{3}H]TdR (sp act, 6.7 Ci/mmol) was added per microculture, and incubation was continued for another 6 h. Cell cultures were harvested and prepared for counting in a liquid scintillation counter as described (30). Results are presented as mean cpm of triplicate cultures. SD were always <15%.

Phenotypic Analysis of Fresh and Cultured T Cells. The following mAbs were used to characterize the expressed TCR on T cells before and after culture. BMA031 (TCR-α/β; reference 33), TCR-δ1 (C_δ; references 18 and 34), δTCS1 (Vδ1-Jδ1, Vδ1-Jδ2; reference 35), A13 (Vδ1; reference 36), BB3 (Vδ2; reference 15), 7A5 (Vγ9; reference 32). In indirect staining procedures, FITC-conjugated F(ab)$_2$ goat anti-mouse IgG (Tago, Inc.) was used as a second-step reagent. Commercial mAbs (TCR-δ1, δTCS1; both from T Cell Sciences, Cambridge, MA) were used as FITC conjugates. In two-color analyses, biotinylated 7A5 mAb was visualized with PE-streptavidin. After being stained, all samples were resuspended in 1% paraformaldehyde. All analyses were measured on a FACScan® cytofluorometer (Becton Dickinson & Co.).

Results

Stimulation of Unseparated T Cells with M.tb. Induces Selective Expansion of Vγ9$^+$/Vδ2$^+$ Cells. We and others (30, 32) have recently reported that stimulation of unfractionated peripheral blood T cells (containing from 0.5 to 8% γ/δ$^+$ T cells) with killed M.tb. preferentially triggers the activation of γ/δ$^+$ T cells, thus giving rise to a population of 40–90% γ/δ$^+$ T cells after 6–9 d of culture. We have now explored the TCR phenotype of M.tb.-stimulated γ/δ$^+$ T cells with mAbs directed against variable regions of γ (Vγ9:

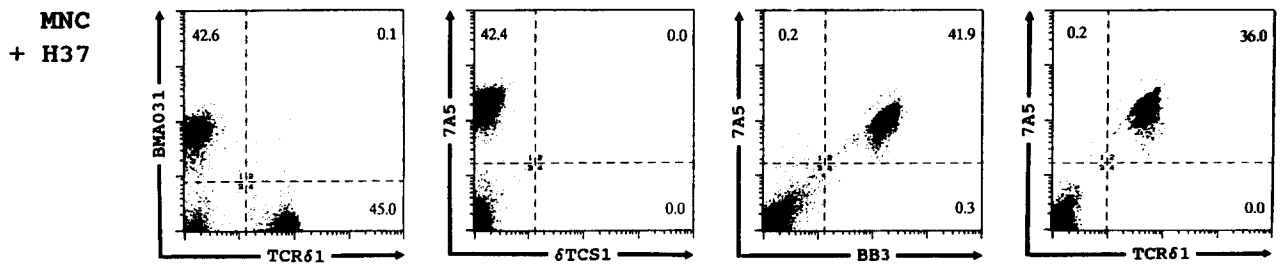

Figure 1. TCR phenotype of PBMC after stimulation with M.tb. PBMC containing 0.3% γ/δ$^+$ (TCR-δ1$^+$) T cells were cultured with M.tb. (H37). After 9 d, proliferating cells were stained with the indicated mAbs and analyzed on a FACScan®. *x*-axis represents log green fluorescence (FITC); *y*-axis indicates log red fluorescence (PE).

mAb 7A5) and δ chains (Vδ1: mAb δTCS1; Vδ2: mAb BB3). Results of a representative two-color cytofluorimetric analysis are shown in Fig. 1. Before culture, PBMC responder cells consisted of 90.0% α/β^+ and 0.3% γ/δ^+ T cells. After culture for 9 d with M.tb., 42.6% of proliferating cells were α/β^+, whereas 45.0% were γ/δ^+. Importantly, all γ/δ^+ T cells after culture expressed the Vγ9 epitope as shown by two-color staining with mAbs TCR-δ1 and 7A5 (Fig. 1). Further two-color analyses revealed that all $V\gamma9^+$ ($7A5^+$) cells coexpressed the BB3 (Vδ2) epitope (Fig. 1) but failed to stain with mAb δTCS1 (Vδ1) (Fig. 1). Qualitatively identical results were obtained in six additional experiments.

Depletion of $V\gamma^+$ Cells Abrogates the γ/δ^+ T Cell Response to M.tb. It is well established that $V\gamma9^+/V\delta2^+$ cells account for a major fraction (~60–70%) of adult peripheral blood γ/δ^+ T cells, while the mutually exclusive subset of $V\delta1^+$ cells represents ~10–20% of peripheral γ/δ^+ T cells (17). To address the issue of whether the selective growth of $V\gamma9^+$ cells after M.tb. stimulation was simply due to $V\gamma9^+$ cells outnumbering $V\delta1^+$ cells severalfold in the starting population, we performed depletion experiments in which $V\gamma9^+$ cells were selectively removed from E-rosetted T cells by MACS or FACS® cell sorting. As detailed in Table 1, the starting population of NA or E-rosetted T cells consisted of 61.4–89.3% α/β^+ T cells and 3.5–8.1% γ/δ^+ T cells, of which a major fraction was $7A5^+$. Depletion of $7A5^+$ cells by MACS or FACS® resulted in cell populations with greatly diminished, yet detectable numbers (0.2–4.0%) of γ/δ^+ cells as revealed by staining with mAb TCR-δ1. Reanalysis of sorted cells ensured that $V\gamma9^+$ ($7A5^+$) cells were efficiently depleted. As expected, the majority of remaining γ/δ^+ cells was $\delta TCS1^+$. When unfractionated T cells (E^+ or NA) and 7A5-depleted cells ($E^+/7A5^-$ or NA/$7A5^-$) were stimulated with M.tb., dramatic differences were observed with respect to the TCR phenotype of proliferating cells. As noted in previous experiments, a large fraction (42.8–88.1%) of unseparated responder cells displayed the $7A5^+/\delta TCS1^-$ phenotype after culture with M.tb., while the percentage of α/β^+ cells ranged from only 9.5–54.0%. As reported in Table 1, depletion of $7A5^+$ cells before culture with M.tb. completely abolished the proliferative response of all γ/δ^+ T cells, thus giving rise to cell populations that were predominantly (63.5–97.7%) α/β^+, with only minor contaminations (0.1–2.3%) of γ/δ^+ T cells. Note that this was also true when the unfractionated T cell population contained unusually high numbers of $V\gamma9^-/V\delta1^+$ ($7A5^-/\delta TCS1^+$) γ/δ^+ T cells, such as in exp. 3 of Table 1. Here, the 7A5-depleted T cells still contained 4.0% γ/δ^+ cells, of which 3.7% were $\delta TCS1^+$. These cells were unable, however, to proliferate in response to M.tb. Thus, the phenotypic analysis of cultured cells revealed the proliferation of exclusively α/β^+ T cells with negligible contamination (0.3%) by $\delta TCS1^+$ γ/δ^+ T cells.

Anti-Vγ9 mAb 7A5 Inhibits the Proliferative Response to M.tb. The next experiments were aimed at analyzing whether the selective activation of $V\gamma9^+$ T cells was initiated through TCR-dependent recognition of M.tb.. To this end, the effect of anti-Vγ9 mAb 7A5 on the activation of PBMC responder cells by M.tb., PHA, or allogeneic stimulator cells (MLR) was investigated. As shown in Fig. 2, mAb 7A5 inhibited in a dose-dependent manner specifically the response of PBMC (containing 2.3% $7A5^+$ cells) to M.tb., while it

Table 1. *Depletion of $7A5^+$ ($V\gamma9^+$) T Cells Abrogates the Proliferative Response of all γ/δ^+ T cells to M.tb.*

		Phenotype (day 0)				Phenotype after culture (days 6–9)			
Exp.	Responder	BMA031$^+$	TCR-δ1$^+$	7A5$^+$	δTCS1$^+$	BMA031$^+$	TCR-δ1$^+$	7A5$^+$	δTCS1$^+$
			%				%		
1	E^+	78.3	7.4	7.2	1.2	26.5	69.9	69.6	0.1
	$E^+/7A5^-$	83.6	1.2	0.1	1.2	84.1	2.3	1.4	0.8
2	E^+	85.5	5.4	5.0	0	15.8	79.4	79.2	0.1
	$E^+/7A5^-$	90.2	0.3	0.1	0.1	74.8	0.1	0	0.1
3	E^+	83.5	8.1	4.8	3.0	54.0	44.7	42.8	0.3
	$E^+/7A5^-$	86.2	4.0	0	3.7	97.7	0.5	0.9	0.3
4	E^+	89.3	3.5	2.9	0.2	11.7	87.8	88.1	0
	$E^+/7A5^-$	80.7	0.5	0	0.3	83.5	0.2	0	0.2
5	NA	61.4	4.8	4.8	0	9.5	83.0	81.1	0
	NA/$7A5^-$	70.2	0.2	0	0.1	63.5	0.1	0	0

E-rosetted T cells (E^+; exp. 1–4) or plastic-nonadherent cells (NA^+; exp. 5) were depleted of $7A5^+$ γ/δ^+ T cells by MACS (exp. 1) or FACS® (exp. 2–5), and cultured with autologous PBMC feeder cells and M.tb. [^{3}H]-TdR uptake was determined after 4–6 d. The TCR phenotype was analyzed before (day 0) and after culture (days 6–9) with the indicated mAbs.

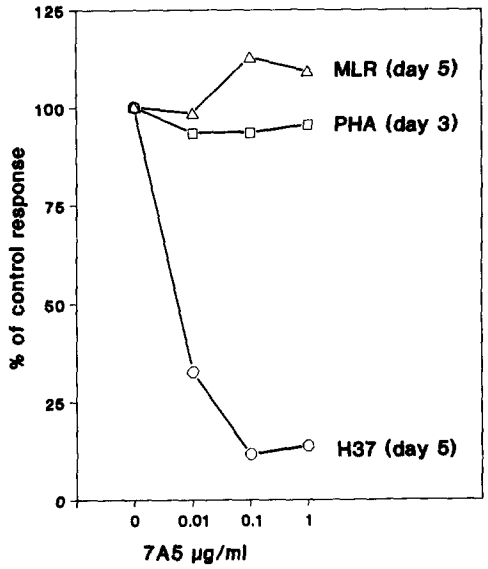

Figure 2. Inhibition of M.tb. reactivity by anti-Vγ9 mAB 7A5. 10^5 PBMC (containing 3.2% TCR-δ1$^+$ and 2.3% 7A5$^+$ cells) were cultured per microwell with 10^5 irradiated allogeneic PBMC stimulator cells (MLR), PHA (0.5 µg/ml), or M.tb. (H37). mAb 7A5 was added as indicated. [^{3}H]TdR uptake was determined after 3 d (PHA) or 5 d (MLR; H37). Mean control cpm of triplicate cultures in the absence of mAb were as follows: PHA, 77,100 cpm; MLR, 22,000 cpm; H37, 51,500 cpm.

did not have any inhibitory effect on PHA or MLR responses. In additional experiments (data not shown), time course kinetics revealed significant [^{3}H]TdR incorporation also in the presence of 7A5 (1 µg/ml) in M.tb.-stimulated cultures at later time points (day 7). As shown by phenotypic analysis, however, the vast majority of M.tb.-stimulated T cells cultured in the presence of mAb 7A5 were α/β$^+$.

M.tb. Selectively Triggers Vγ9$^+$/Vδ2$^+$ Cells among CD4$^-$ CD8$^-$ Thymocytes. The above experiments with unseparated and Vγ9-depleted peripheral blood T cells showed that Vγ9$^+$ but not Vδ1$^+$ γ/δ$^+$ T cells proliferate in response to M.tb. stimulation. The strength of this argument is weakened, however, by the fact that very few (0.1–3.7%; Table 1) Vδ1$^+$ cells remain among peripheral blood T cells after removal of Vγ9$^+$ cells; therefore, it may be argued that, for technical reasons, these few Vδ1$^+$ γ/δ$^+$ T cells could not be activated under our culture conditions. To definitively prove that responsiveness to M.tb. is an exclusive property of Vγ9$^+$ and not of Vδ1$^+$ cells among γ/δ$^+$ T cells, we resorted to responder cell populations where the Vγ9/Vδ1 ratio usually found in adult peripheral blood (4–6:1) is inversed. To this end, CD4$^-$CD8$^-$ double-negative cells were enriched from thymus MNC obtained from three children undergoing corrective heart surgery. A comparative phenotypic analysis of MNC and DN cells from a representative thymus is shown in Fig. 3. Unfractionated thymus MNC contained 68.1% α/β$^+$ and 0.5% γ/δ$^+$ T cells. After MACS depletion of most CD4$^+$ and CD8$^+$ cells, the remaining DN cells contained 5.1% α/β$^+$ and 21.8% γ/δ$^+$. In line with published reports (18), the majority (13.5%) of DN γ/δ$^+$ cells were δTCS1$^+$, while only a minor fraction (1.7%) expressed the Vγ9-encoded 7A5 epitope. In contrast to adult peripheral blood, 7A5$^+$/δTCS1$^+$ double-positive cells were readily detectable (3.1%; Fig. 3). When DN thymocytes were cultured

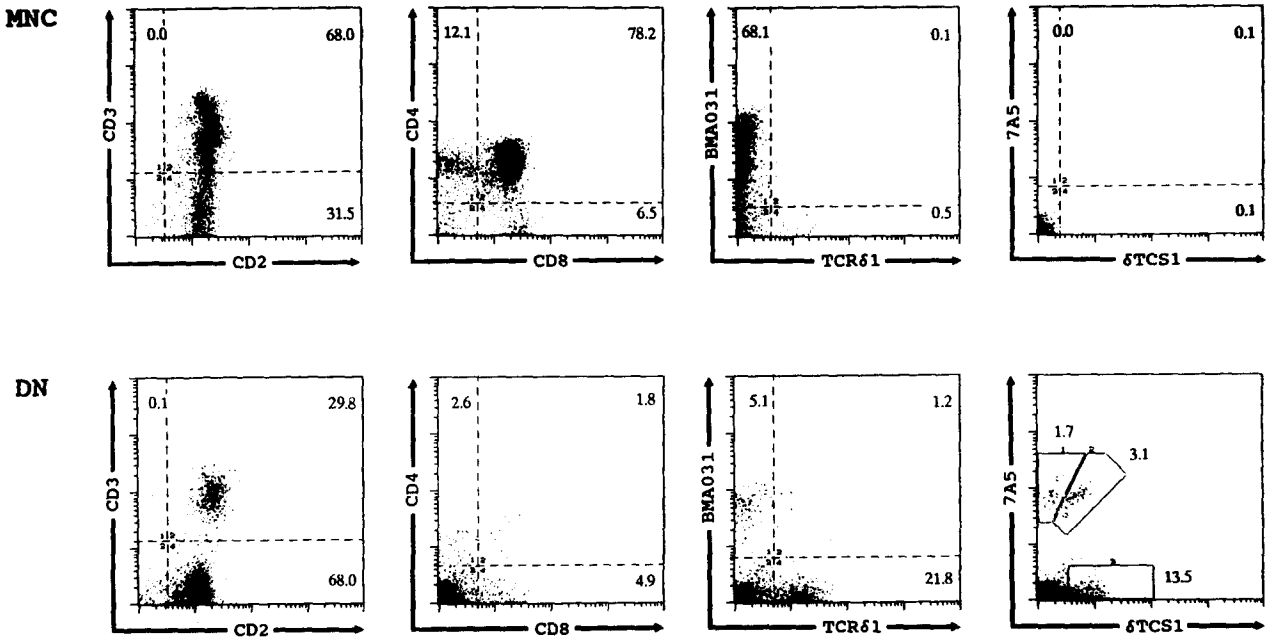

Figure 3. Phenotypic characterization of freshly isolated unseparated and CD4$^-$CD8$^-$ double-negative thymocytes. Thymus (sample A140) MNC and DN cells were stained with the indicated mAbs and analyzed on a FACScan®. *x*-axis indicates log green fluorescence (FITC); *y*-axis indicates log red fluorescence (PE).

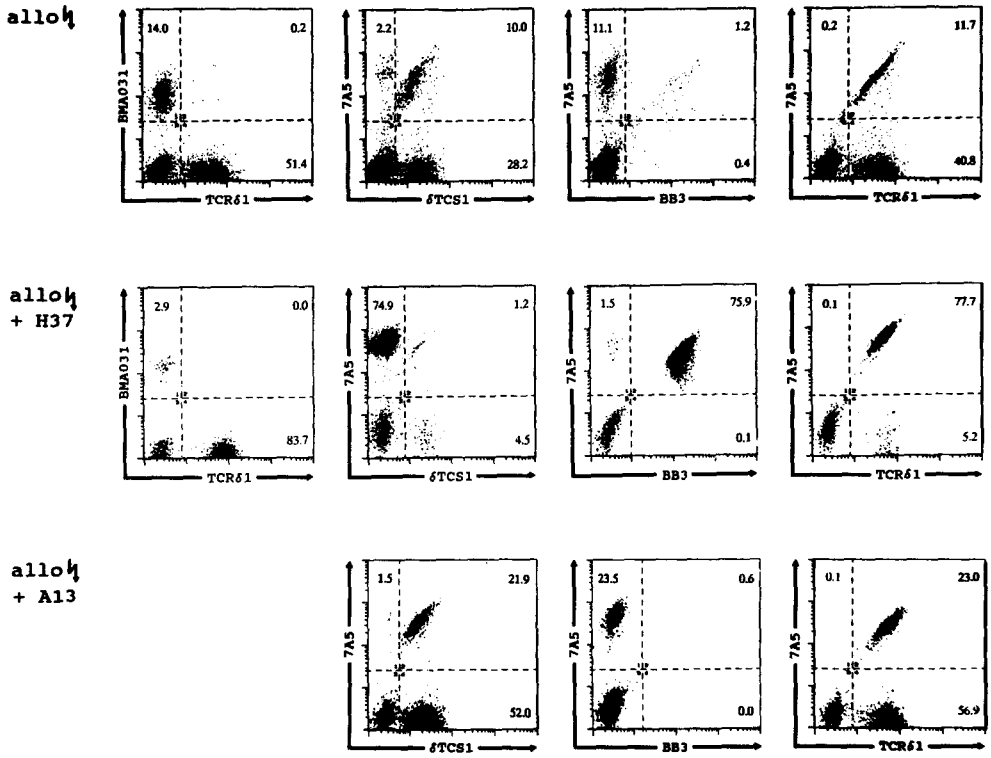

Figure 4. Phenotypic characterization of CD4$^-$CD8$^-$ thymocytes after culture. 5 × 10^4 thymus DN cells (sample A140) were cultured per microwell with 10^5 irradiated allogeneic PBMC feeder cells in the absence (*top row*) or presence of M.tb. (H37; *middle row*) or anti-Vδ1 mAb A13 (*bottom row*). After 2 d, rIL-2 (1 ng/ml) was added to all cultures. On day 12, the proliferating cells were analyzed by two-color analysis using the indicated combinations of mAbs directed against TCR-α/β (BMA031), TCR-γ/δ (TCRδ1), Vγ9 (7A5), Vδ1 (δTCS1), and Vδ2 (BB3). *x*-axis indicates log green fluorescence (FITC); *y*-axis indicates log red fluorescence (PE).

in the presence of allogeneic feeder cells and exogenous IL-2, significant proliferation was observed (data not shown). Under these conditions, the relative proportion of 7A5$^+$ and δTCS1$^+$ after 8 d of culture did not change; i.e., δTCS1$^+$ were far in excess (28.2%) of 7A5$^+$ cells (2.2%; Fig. 4, *top row*). Quite different results were obtained when M.tb. was added at the initiation of culture. In this case, the phenotypic analysis of cultured cells revealed the presence of large numbers of 7A5$^+$ cells (74.9%), while the percentage of Vδ1$^+$ cells was as low as 4.5% (Fig. 4, *middle row*). In accordance with results obtained with peripheral blood cells, the vast majority of 7A5$^+$ DN thymocytes stimulated by M.tb. coexpressed the BB3 (Vδ2) epitope (see Fig. 4, *middle row*); the very few 7A5$^+$/δTCS1$^+$ cells detected after M.tb. stimulation were most likely derived from the few 7A5$^+$/δTCS1$^+$ cells present in the starting population (compare Figs. 3 and 4). In the experiment shown in Fig. 4, an aliquot of DN thymocytes was also cultured with allogeneic feeder cells and anti-Vδ1 mAb A13 (36) to selectively trigger the activation of Vδ1$^+$ T cells. As can be seen in Fig. 4 (*bottom row*), this protocol resulted in the preferential expansion of two subsets of Vδ1$^+$ subsets that did (21.9%) or did not (52.0%) express the Vγ9 epitope. In striking contrast to M.tb.-stimulated cultures (Fig. 4, *middle row*), Vδ2$^+$ (BB3$^+$) γ/δ$^+$ T cells were at almost undetectable levels (<1%) in A13-stimulated cultures. Comparable results were obtained with the other two thymus samples. It is thus clear that the failure of Vδ1$^+$ cells to proliferate in response to M.tb. stimulation is not due to a general lack of responsiveness to activation signals of Vδ1$^+$ cells.

Discussion

A striking feature γ/δ$^+$ T cells is their frequent reactivity towards mycobacterial antigens (23–31). The high frequencies of mycobacteria-reactive γ/δ$^+$ T cells determined in our previous study (30) suggested that this reactivity might be a property of a major proportion of peripheral blood γ/δ$^+$ T cells. Therefore, we attempted to characterize the responsive subset with the aid of Vγ9-, Vδ1-, and Vδ2-specific mAbs. To ensure that the results would be representative for the whole population of γ/δ$^+$ T cells, we investigated the reactivity of unmanipulated primary (i.e., polyclonal) cells rather than that of individual established clones. We found that all γ/δ$^+$ T cells proliferating in response to killed M.tb. coexpressed Vγ9$^+$ and Vδ2$^+$. More importantly, T cells selectively depleted of Vγ9-bearing cells before culture (yet still containing other subsets of γ/δ$^+$ cells such as Vδ1$^+$) proliferated vigorously after stimulation with M.tb. In this case, however, the response was mediated exclusively by α/β$^+$ T cells (with some contribution of CD2$^+$CD3$^-$ cells), indicating that Vδ1$^+$ cells were nonresponsive to M.tb. The failure of Vδ1$^+$ cells to respond to M.tb. stimulation was even more obvious when CD4$^-$CD8$^-$ thymocytes were analyzed. Coculture of DN thymocytes with M.tb. again resulted in the preferential outgrowth of Vγ9$^+$/Vδ2$^+$ cells, although these cells were present in only small numbers in the starting responder population. In contrast, Vδ1$^+$ cells accounting for a major proportion of γ/δ$^+$ T cells among DN thymocytes did not expand in response to M.tb. stimulation. These cells could be activated, however, by soluble anti-Vδ1 mAb A13 (Fig. 4), indicating that Vδ1$^+$ thymocytes are not generally

refractory to activation stimuli. Therefore, we conclude that the proliferative response of primary human γ/δ^+ T cells to killed M.tb. is an exclusive property of Vγ9-bearing cells. A similarly limited receptor repertoire of purified protein derivative–reactive γ/δ^+ T cells has been previously observed with hybridomas derived from newborn murine thymus (37). However, it cannot be inferred from our data that the antimycobacteria reactivity of human V$\gamma9^+$ cells is an (oligo) clonal response. Despite the limited germline diversity of mycobacteria-responsive Vγ and Vδ TCR elements, it is possible that the proliferating V$\gamma9^+$ cells display significant diversity in the junctional regions between V and J (for the γ chain), and V and D, D and D, and D and J (for the δ chain). To address this issue, junctional regions of a large number of M.tb.-reactive V$\gamma9^+$/Vδ^+ clones will have to be sequenced (20).

Our study has not addressed the nature of the mycobacterial antigen(s) or ligand(s) that stimulate V$\gamma9^+$ human T cells. In this context, it is of interest that others have described protease-resistant low molecular weight fractions of mycobacterial cell lysates that exclusively stimulate γ/δ^+ but not α/β^+ T cells (31). Apart from mycobacterial ligands, two other antigens that selectively activate Vγ9-bearing T cell clones have recently been described. Fisch et al. (38) reported that all of their tested V$\gamma9^+$ clones specifically lysed Daudi but not Raji target cells, whereas simultaneously derived CD3/TCR$^-$ NK clones lysed both targets. Furthermore, Rust et al. (39) demonstrated that cytotoxic effector activity of all analyzed Vγ9-bearing clones could be triggered by staphylococcal enterotoxin A (SEA), whereas V$\gamma9^-$ γ/δ^+ clones were resistant to SEA stimulation. Taken together with our present results, it appears that at least three seemingly different ligands (killed mycobacteria, Daudi tumor cells, and SEA) are recognized by most if not all V$\gamma9^+$ cells. Stimulation of the whole γ/δ^+ T cell subset expressing a V$\gamma9^+$/V$\delta2^+$ receptor by a given ligand is reminiscent of the recently described "superantigen" reactivity of α/β^+ T cells bearing a particular Vβ (40). Further studies are required to investigate the nature of the mycobacterial ligand that activates Vγ9-bearing cells. Based on our previous results (30) and those obtained by others (31), it is unlikely that the 65-kD hsp is the major Vγ9-stimulating antigen. As shown in Fig. 2, anti-Vγ9 mAb 7A5 efficiently blocked the primary response of γ/δ^+ T cells to M.tb. Although no formal proof, this is strong evidence that activation was mediated through TCR-dependent ligand recognition, and not simply through TCR-independent mitogenic effects. However, in view of our recents results that mAb 7A5 can induce programmed cell death (apoptosis) in IL-2-dependent 7A5$^+$ clones (32), we cannot exclude the possibility that 7A5 killed M.tb.-reactive V$\gamma9^+$ cells shortly after activation.

In conclusion, we have shown that the primary response of peripheral blood and thymus γ/δ^+ T cells to killed mycobacteria is an exclusive property of Vγ9-bearing cells that coexpress Vδ2. In view of the fact that V$\gamma9^+$/V$\delta2^+$ cells dominate in adult peripheral blood but neither in cord blood nor in postnatal thymus (18), our results support the hypothesis that the extrathymic expansion of this particular γ/δ^+ subset might be due to postnatal exposure to certain bacterial antigens such as M.tb. or SEA (39).

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