

γ/δ T Lymphocytes Express CD40 Ligand and Induce Isotype Switching in B Lymphocytes

By Anthony A. Horner, Haifa Jabara, Narayanaswamy Ramesh, and Raif S. Geha

From the Division of Immunology, Children's Hospital, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Summary

T cells expressing γ/δ T cell receptors home to epithelial tissue and may play a role in immunity to infectious agents and foreign antigens. In an effort to understand the role of γ/δ T cells in directing B cell responses, we investigated the capacity of human γ/δ T cells to express CD40 ligand (CD40L) and to drive immunoglobulin (Ig) isotype switching in B cells. A multiple step purification procedure resulted in the recovery of highly pure populations of peripheral blood CD4⁻CD8⁻ γ/δ T cells. Neither CD40L surface expression nor CD40L mRNA were detected in unstimulated γ/δ T cells. Stimulation with phorbol ester and ionomycin induced CD40L mRNA and surface CD40L expression by γ/δ T cells. Both the percentage of CD40L⁺ cells and the cell surface density of CD40L were significantly lower in γ/δ T cells compared to unselected T cells. We further demonstrated that in the presence of neutralizing monoclonal antibody to interferon γ (IFN- γ), γ/δ T cells could induce IgE synthesis in B cells, albeit to a lesser extent than unselected T cells. Furthermore, IgE synthesis driven by γ/δ T cells was inhibited by monoclonal antibody to CD40L. These observations demonstrate that activated γ/δ T cells express CD40L and can induce isotype switching in B cells.

γ/δ T cells represent a substantial percentage of the lymphocyte population in areas of the body exposed to large amounts of foreign antigens, such as the large intestine in humans, and the lung, skin, and intestines in mice. This localization makes them well suited for the orchestration of an early immune response to a variety of pathogens and foreign antigens (1).

γ/δ T cells proliferate to Con A, anti-CD3 mAb, heat shock proteins and other bacterial mycobacterial antigen, superantigen, tetanus toxin, and herpes simplex virus glycoprotein I and can recognize both classical and nonclassical MHC molecules (1). Little is known about the capacity of γ/δ T cells in directing B cell function. CD4⁺ γ/δ T cell clones have been shown to induce IgM, IgG, and IgA synthesis by B cells in the presence of IL-2, and IgE synthesis in the presence of IL-4, whereas CD4⁻CD8⁻ and CD8⁺ clones were ineffective (2, 3). Membrane preparations derived from activated CD4⁺ γ/δ T cells could replace whole cells in the induction of Ig synthesis (2, 3). Recently, mice deficient in α/β ⁺ T cells were found to make Ig of all isotypes with high levels of IgE and IgG1, suggesting a role for γ/δ T cells in directing isotype switching (4).

Interaction between the B cell surface antigen CD40 and its ligand (CD40L) plays a critical role in T cell-dependent isotype switching in vitro (5). CD40L is expressed on activated CD4⁺ T cells with only low level expression on acti-

vated CD8⁺ T cells (6), and is also expressed on mast cells and basophils. These cells have been shown to support isotype switching in B cells (7). In this paper, we demonstrate that highly purified CD4⁻CD8⁻ γ/δ T cells isolated from normal peripheral blood express CD40L after stimulation with PMA and ionomycin and are able to induce isotype switching in B cells.

Materials and Methods

mAbs. Anti-CD3, anti-CD4, and anti-CD8 mAbs were purified from ascites raised by injecting mice with OKT3, OKT4, and OKT8 hybridomas obtained from the American Type Culture Collection (Rockville, MD). FITC- and PE-conjugated mAbs to TCR- α/β (WT-31), TCR- γ/δ , CD3, CD4, CD8, CD14, CD19, and CD56 were purchased from Becton Dickinson & Co. (Mountain View, CA). The anti-CD40 mAb 626.1 was a gift of Dr. S. M. Fu (University of Virginia, Charlottesville, VA). The anti-CD40L mAb 5c8 was a generous gift of Drs. S. Lederman and L. Chess (Columbia University, New York).

Cell Populations. Peripheral blood γ/δ T and B cells were purified from healthy adult blood donors. Mononuclear cells were separated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation and rosetted with sheep red blood cells (E) for 2 h. The E⁺ cells were suspended at 10⁷ cells/ml in RPMI 1640 medium supplemented with 10% AB⁺ serum (Sigma Chemical Co., St. Louis, MO), and adhered overnight on plastic plates at 37°C and 5% CO₂. Monocyte-depleted E⁺ cells were incubated with anti-CD4 and anti-CD8 mAbs (1:100 dilution of ascites), washed, rein-

cubated with goat anti-mouse Ig linked magnetic beads (Advanced Magnetics, Cambridge, MA), and subjected to magnetic separation as per the manufacturer's instructions. The negatively sorted cells were stained with FITC-conjugated anti-TCR- α/β mAb and with PE-conjugated anti-TCR- γ/δ mAb and subjected to FACS[®] (Becton Dickinson & Co.) sorting. The γ/δ T cells obtained were uniformly >97% pure and contained <0.5% α/β T cells. E⁺ cells isolated after adherence were used as control unselected T cells.

B cell-enriched populations were prepared as described (8). The cell preparation obtained contained <0.5% α/β T cells as assessed by flow cytometry.

Cell Surface Expression of CD40. T cells were stimulated with 20 ng/ml PMA and 0.5 μ M ionomycin (Calbiochem-Novabiochem Corp., San Diego, CA). The cells were then stained with a fusion protein constructed from the extracellular domain of CD40 and the Fc portion of human IgG1 (sCD40) or with a control CD44:IgG1 fusion protein, both constructs a kind gift of Dr. Ivan Stamenkovic (Massachusetts General Hospital, Boston, MA) followed by FITC-conjugated goat anti-human Ig (Becton Dickinson & Co.), and analyzed by FACSscan[®] (Becton Dickinson & Co.) as previously described (9).

Northern Blot Analysis. T cells were incubated with PMA and ionomycin for 3 h or left unstimulated. Total RNA was extracted by the method of Chomczynski and Sacchi (10). 3 μ g of RNA was fractionated on a 1.5% formaldehyde/agarose gel, blotted on nitrocellulose, and hybridized with random primer ³²P-labeled (Pharmacia) full-length human CD40L cDNA probe (gift of Dr. Alessandro Aruffo, Bristol-Myers Squibb, Seattle, WA).

Cell Culture and IgE Assay. B cells and autologous irradiated (2,500 rad) T cells were incubated in 12 \times 75-mm tubes (1:2 ratio, final concentration 1.5 \times 10⁶ cells/ml) in RPMI 1640 with 10% heat-inactivated FCS (Hyclone Sterile Systems Inc., Logan, UT). Cells were cultured at 37°C and 5% CO₂. IL-4 and IL-6 (R & D Systems, Minneapolis, MN) were added to all cultures at 5 ng/ml each. Neutralizing anti-IFN- γ (Genzyme, Cambridge, MA) was added at 20 U/ml. Supernatants from cultured cells were collected on day 12 of culture, and IgE was measured by a RIA, with a limit of sensitivity of 150 pg/ml.

Results

Cell Purity. Using the protocol outlined in Materials and Methods, we routinely obtained γ/δ T cells with >97% purity and <0.5% α/β T cell contamination as assessed by two-color flow cytometry. A typical FACS[®] profile of purified γ/δ T cells is presented in Fig. 1. In 10 experiments, the

mean purity of γ/δ T cells was 98.3 \pm 0.55% with 0.19 \pm 0.09% contamination by α/β T cells. Fig. 1 also shows that <0.5% of the purified γ/δ T cells expressed CD4 and <1.5% expressed CD8. This is not unexpected given that the purification process included negative selection against CD4 and CD8 expression.

B cell preparations contained >70% CD19⁺ B cells and <0.5% CD3⁺ cells. The majority of contaminating cells were CD14⁺ monocytes (~5%) and CD56⁺ NK (~20–25%) cells. More importantly, the B cell preparations failed to proliferate in response to PHA (data not shown).

CD40L Expression on γ/δ T Cells. It has been shown previously that CD40L is expressed on T cells only after activation, with peak surface expression 6 h after stimulation with PMA + ionomycin (9). Fig. 2 compares CD40L expression on γ/δ T cells and unselected T cells isolated from the same individual. Freshly isolated unstimulated γ/δ T cells expressed no detectable CD40L as assessed by their capacity to bind sCD40 and did not bind the control fusion protein CD44:IgG1. Fig. 2 shows that stimulation with PMA + ionomycin induced CD40L expression on γ/δ T cells. This expression was vigorous at 6 h and waned by 24 h after stimulation. The percentage of CD40L⁺ cells and the surface density of CD40L protein, as measured by mean fluorescence intensity (MFI) were consistently lower on γ/δ T cells compared to unselected T cells derived from the same donor and stimulated in parallel. In three experiments, the mean percentage of CD40L⁺ cells after 6 h of stimulation with PMA + ionomycin was 43% for γ/δ T cells and 81% for unselected T cells ($p = 0.012$), with average mean fluorescence intensities of 81 and 108, respectively ($p < 0.01$). The lower capacity of γ/δ T cells to express CD40L was unlikely to be due to prior engagement of the TCR during purification because incubation of unselected T cells with anti-TCR- α/β mAb did not affect their capacity to express CD40L after stimulation with PMA + ionomycin (data not shown).

We next compared CD40L mRNA expression in γ/δ T cells and unselected T cells. We have previously shown that stimulation of unselected peripheral blood T cells with PMA + ionomycin induces CD40L mRNA expression that peaks at 3 h (9). Because of the limited number of highly purified γ/δ T cells available, we examined CD40L mRNA expres-

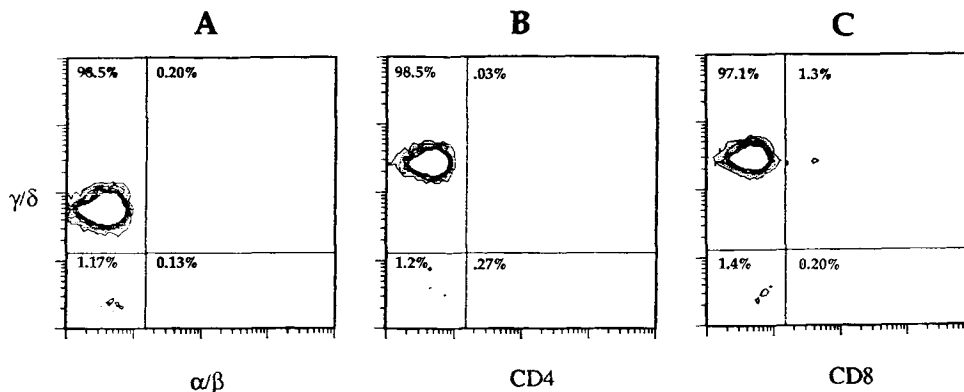


Figure 1. FACS[®] analysis of purified γ/δ T cells. Purified peripheral blood γ/δ T cells (see Materials and Methods) were stained with PE-conjugated anti-TCR- γ/δ and with FITC-conjugated anti-TCR- α/β mAb (A), FITC-conjugated anti-CD4 (B), or FITC-conjugated anti-CD8 (C). The data were analyzed using Consort 30 software (Becton Dickinson & Co.). Numerical values describe the percentage of cells contained in each quadrant. The data shown are representative of multiple experiments.

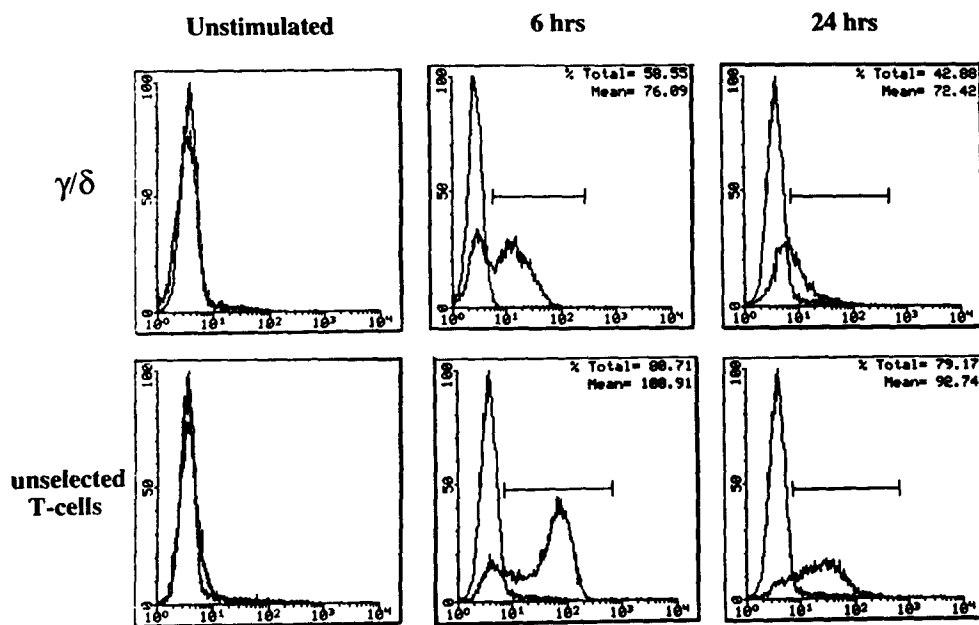


Figure 2. CD40L expression on γ/δ T cells. γ/δ T cells and unselected T cells from the same donor were either left unstimulated or were stimulated in parallel with PMA (20 ng/ml) and ionomycin (0.5 μ M). At the indicated times, cells were harvested and stained with sCD40 (see Materials and Methods). sCD44 was used as a control for nonspecific staining. The data were analyzed using LYSYS software (Becton Dickinson & Co.), and regions were set to exclude 95% of the cells staining with sCD44. The percent CD40L⁺ cells, and the mean fluorescence intensity for the region are given in the upper right hand corner of each histogram. The data shown are representative of three experiments.

sion at this time point. Fig. 3 shows a Northern blot analysis for CD40L mRNA in γ/δ T cells and unselected T cells from the same donor stimulated in parallel. No CD40L mRNA was detected in either cell population at rest. 3 h after stimulation with PMA + ionomycin, CD40L mRNA was expressed by both cell populations, however the level of CD40L mRNA was much lower in γ/δ T cells compared to unselected T cells. Taken together, these results indicate that γ/δ T cells are able to express CD40L, but to a lesser degree than unselected T cells.

IgE Synthesis in Cultures of B Cells and γ/δ T Cells. Given the capacity of γ/δ T cells to express CD40L, we next examined their ability to help autologous B cells undergo IgE isotype switching in vitro. Irradiated T cells were mixed with B cells at a ratio of 2:1 and the cells were cultured for 12 d in the presence of IL-4 (5 ng/ml) and IL-6 (5 ng/ml) to optimize IgE production. Under these conditions, unselected T cells consistently induced IgE synthesis, whereas γ/δ T cells failed to do so (data not shown, $n = 3$ experiments). Moreover, no IgE synthesis could be detected at ratios of γ/δ T cells to B cells ranging from 0.5:1 to 4:1 (data not shown). The failure of γ/δ T cells to induce isotype switching under

these culture conditions was unlikely due to prior engagement of their antigen receptor by mAb during purification because incubation of unselected T cells with anti-TCR- α/β mAb did not diminish their capacity to induce isotype switching. More importantly, γ/δ T cells purified by negative selection from CD4⁻CD8⁻ T cell populations without γ/δ TCR engagement were unable to drive isotype switching (data not shown).

T cell dependent IgE synthesis is sensitive to inhibition by IFN- γ (11). It has been previously reported that human γ/δ T cell clones secrete higher (four- to fivefold more) amounts of IFN- γ than α/β T cell clones (12). We therefore examined the capacity of γ/δ T cells to induce IgE synthesis in B cells cultured with IL-4 and IL-6, in the presence of neutralizing mAb to IFN- γ . Table 1 shows that in the presence of neutralizing IFN- γ mAb, both γ/δ T cells and unselected T cells induced substantial IgE synthesis in B cells, but that B cells by themselves produced little or no IgE. This was unlikely to be due to α/β T cell contamination because our preparations of γ/δ T and B cells uniformly contained <0.5% α/β T cells. To further prove that IgE synthesis in cultures of γ/δ T and B cells was not due to contaminating α/β T cells, we cultured B cells with increasing numbers of unselected T cells. No IgE synthesis could be detected in the presence of up to 5% unselected T cells (data not shown). In another approach, we examined cultures containing γ/δ T and B cells for their content of α/β T cells at the beginning and at the end of the 12-d culture period, and detected no increase in the percentage of contaminating α/β T cells (data not shown).

Induction of IgE synthesis by γ/δ T cells was consistently and significantly less than for unselected cells. In three experiments, the mean IgE synthesis induced by γ/δ T cells was $2,994 \pm 1,476$ pg/ml versus $17,560 \pm 7,363$ pg/ml induced by unselected T cells ($p < 0.03$). We considered the

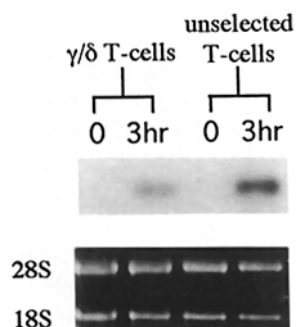


Figure 3. CD40L mRNA expression by γ/δ T cells. T cells were incubated with PMA (20 ng/ml) and ionomycin (0.5 μ M) for 3 h or left unstimulated. Northern blot analysis was performed using a human CD40L cDNA probe (top). Ethidium bromide staining of 28S and 18S RNA illustrates the relative amounts of RNA loaded in each lane (bottom).

Table 1. Induction of IgE Synthesis in B Cells by γ/δ T Cells

T cells	IL-4	IgE		
		Exp 1	Exp 2	Exp 3
-	-	<150	<150	<150
-	+	349 ± 24	<150	259 ± 6
γ/δ	-	<150	<150	<150
γ/δ	+	3,149 ± 510	1,446 ± 44	4,386 ± 310
Unselected	-	<150	<150	<150
Unselected	+	23,972 ± 1,256	9,519 ± 543	19,189 ± 440

T cells were irradiated with 2,500 rad and added to autologous B cells at a ratio of 2:1. IL-4 was added at 5 ng/ml. All cultures received IL-6 (5 ng/ml) and neutralizing anti-IFN- γ mAb (40 U/ml). Supernatants were harvested on day 12, and IgE content was measured by RIA. Samples were run in duplicate and the results are expressed as mean ± SD.

possibility that the lower capacity of γ/δ T cells to induce IgE synthesis was due to incomplete neutralization of IFN- γ , however, increasing the concentration of anti-IFN- γ mAb up to 1,000 U/ml did not result in increased in IgE synthesis (data not shown). Furthermore, we compared IFN- γ production by γ/δ T cells and unselected T cells after stimulation with PMA + ionomycin or with insolubilized anti-CD3 and after a 12-d incubation with B cells and IL-4 + IL-6 to mimic the conditions used in the IgE assay. Under all these conditions, γ/δ T cells and unselected T cells produced equivalent amounts of IFN- γ (data not shown). We also considered the possibility that γ/δ T cells may express cell-associated molecules or cytokines other than IFN- γ which exert an inhibitory effect on IgE production. Addition of γ/δ T cells at a 2:1 ratio to B cells cultured with anti-CD40 mAb (5 μ g/ml) and IL-4 (5 ng/ml) did not inhibit IgE synthesis. These observations suggest that the lower capacity of γ/δ T cells in inducing IgE synthesis is unlikely to be due to excessive produc-

tion of IFN- γ or to an inhibitory effect of these cells on IgE production.

To ascertain the role of CD40L-CD40 interactions in the induction of IgE synthesis by γ/δ T cells, we examined the effect of the anti-CD40L mAb 5c8 in this system. Table 2 shows that addition of 5c8 mAb (10 μ g/ml) completely abrogated γ/δ T cells driven IgE synthesis in IL-4 treated B cells. These results indicate a functional role for CD40L expressed on γ/δ T cells.

Discussion

In this paper we demonstrate that γ/δ T cells express CD40L after activation and are able to induce isotype switching in B cells.

We have used a multiple step purification procedure that yielded highly pure populations of γ/δ T cells (>97% pure) with <0.5% contamination by α/β T cells (Fig. 1). We used double negative γ/δ T cells because positive selection of γ/δ T cells is extremely difficult given their low percentage. We had first to enrich E⁺ cells for γ/δ T cells by depleting α/β T cells. We did this by negative selection of CD4⁺ and CD8⁺ cells rather than by negative selection of α/β T cells because the hybridoma needed to generate sufficient anti-TCR- α/β mAb was not available. Freshly isolated γ/δ T cells expressed neither CD40L on their surface nor CD40L mRNA. Stimulation with PMA + ionomycin for 6 h resulted in CD40L surface expression. Both the percentage of CD40L⁺ cells and intensity of CD40L surface expression were lower for γ/δ T cells than for unselected T cells (Fig. 2). This difference is not likely to be due to different kinetics of CD40L expression in γ/δ T cells because it was seen both at 6 and 24 h after stimulation with PMA + ionomycin, nor was it likely to be due to engagement of the γ/δ T cell antigen receptor by mAb during purification, as such engagement did not effect CD40L expression on unselected T cells. IFN- γ has been reported to inhibit CD40L expression on murine

Table 2. Antibody to CD40L Inhibits Induction of IgE Synthesis by γ/δ T cells

T cells	IL-4	mAb	IgE	
			Exp 1	Exp 2
-	-	-	<150	578
-	+	-	<150	575
+	+	-	21,250	5,653
+	+	5C8	191	<150
+	+	IgG2a	22,328	9,126

Culture conditions were as described in Table 1. mAbs were used at 10 μ g/ml.

T cells (13), but addition of neutralizing mAb to IFN- γ did not enhance CD40L expression on γ/δ T cells (data not shown). However we cannot rule out the possibility that cytokines other than IFN- γ may have played a role in down-regulating CD40L expression by γ/δ T cells.

The lower surface expression of CD40L on γ/δ T cells was accompanied by lower level CD40L mRNA expression compared to unselected T cells. This lower expression may be due to a lower rate of transcription or to a higher rate of mRNA degradation in γ/δ T cells. The 3' untranslated CD40L mRNA contains AUUUA sequences (14) which are known to confer instability to cytokine mRNAs (15). Run on assays and measurements of mRNA half life will help clarify the mechanisms responsible for the decreased expression of CD40L by γ/δ T cells.

CD40L expressed on γ/δ T cells was biologically active because γ/δ T cells were able to induce isotype switching to IgE in B cells (Table 1) and this was inhibited by anti-

CD40L mAb (Table 2). Even with the addition of neutralizing mAb to IFN- γ , γ/δ T cells induced significantly lower IgE production than unselected T cells. Previous reports have shown that CD4⁻CD8⁻ γ/δ T cell clones secrete higher levels of IFN- γ than α/β T cell clones (12). In our studies, however, IFN- γ production was equivalent for freshly isolated γ/δ and unselected T cells, suggesting that the lower capacity of γ/δ T cells to drive IgE synthesis was not due to their overproduction of IFN- γ . We have previously reported that interaction between CD4 and MHC class II molecules is important for T cell-dependent isotype switching (16). Absence of CD4 expression in our γ/δ T cell preparations also may have contributed to their lower capacity to induce isotype switching. It has been previously suggested that the density of CD40L expression on the surface of T cells determines their capacity to activate B cells (6). The lower capacity of γ/δ T cells to express CD40L and to induce isotype switching is consistent with this notion.

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Address correspondence to Dr. R. S. Geha, Division of Immunology, Children's Hospital, Department of Pediatrics, Harvard Medical School, 300 Longwood Avenue, Boston, MA 02115

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