*Journal of Clinical Immunology, Vol. 14, No. 4, 1994*

# Elevated Numbers of Gamma–Delta (γδ+) T Lymphocytes in Children with Immune Thrombocytopenic Purpura

RUSSELL E. WARE[1,2] and THAD A. HOWARD[1]

*Accepted: March 14, 1994*

Immune thrombocytopenic purpura (ITP) in childhood is a heterogeneous clinical disorder characterized by immune-mediated platelet destruction. Although generally considered to involve autoreactive B lymphocytes which produce antiplatelet antibodies, there is increasing evidence that T lymphocytes also play an important role in this autoimmune process. We studied 11 children with acute ITP and 19 children with chronic ITP and observed elevated numbers of TCRγδ+ T lymphocytes in several patients. In the three children with the highest elevations (TCRγδ+/CD3+ percentage ranging from 37.8 to 48.1% at initial evaluation), the expanded cell population exclusively expressed the surface Vδ2/Vγ9 heterodimer and had enhanced *in vitro* proliferation to mycobacterial extracts and IL-2. Analysis of the nucleotide sequences used by these TCRγδ+ cells demonstrated a diverse set of VDDJC gene rearrangements, indicating polyclonal expansion of cells reminiscent of a superantigen response. There was a close correlation between the number of TCRγδ+ T lymphocytes and the degree of thrombocytopenia in each patient. TCRγδ+ T lymphocytes may be important in the pathogenesis of immune-mediated platelet destruction in some children with ITP.

**KEY WORDS:** Immune thrombocytopenic purpura; T lymphocytes; autoimmunity; gamma–delta lymphocytes; heat shock protein.

## INTRODUCTION

Immune thrombocytopenic purpura (ITP) is a hematologic disorder characterized by immune-mediated destruction of platelets (1). In over 90% of cases, there is measurable autoantibody on the surface of circulating platelets (2, 3), which leads to clearance by the reticuloendothelial system and consequent thrombocytopenia (1, 4). In adults, ITP is typically characterized by persistent thrombocytopenia. ITP in childhood is a more heterogeneous disorder; most children have a self-limited form known as acute ITP, in which the thrombocytopenia resolves within 6 to 12 months. In contrast, 10–20% of children will have a prolonged clinical course termed chronic ITP, in which the thrombocytopenia persists for over 12 months (1, 5).

The pathogenesis of immune dysregulation which leads to antiplatelet autoantibody production in ITP is not well understood. Platelet autoantibodies typically are IgG (2, 6), although IgM and IgA antibodies can be detected on the platelets of some patients. The antibodies are usually polyclonal but may be oligoclonal (7) and have reactivity against a variety of platelet surface glycoproteins (8, 9). We and others have demonstrated that peripheral T lymphocytes from some patients with ITP have *in vitro* reactivity against platelets, which is measurable at both the polyclonal (10, 11) and the clonal level (11). It is therefore likely, at least in a subset of patients with ITP, that peripheral blood T lymphocytes play a direct role in the B lymphocyte-mediated autoimmune response (5, 12). However, the T lymphocyte subsets which are important in the pathophysiology of ITP and the mechanisms by which they contribute to immune platelet destruction have not been elucidated.

Recently, T lymphocytes which express the gamma/delta (γδ) T cell receptor (TCRγδ+ lymphocytes) have been implicated in a variety of autoimmune human diseases. Increased numbers of TCRγδ+ T lymphocytes have been reported in rheumatoid arthritis (13, 14), polymyositis (15), Sjogren's syndrome, and systemic lupus erythema-

[1]Department of Pediatrics, Division of Hematology/Oncology, Duke University Medical Center, Durham, North Carolina 27710.
[2]To whom correspondence should be addressed at P.O. Box 2916, Duke University Medical Center, Durham, North Carolina 27710.



0271-9142/94/0700-0237$07.00/0 

tosus (16). An adult patient with chronic neutropenia and an elevated number of T lymphocytes with a hybrid CαVδ1 surface receptor has been reported (17). To date, however, there have been no descriptions of TCRγδ+ T lymphocytes in children or adults with ITP.

In this study, we report that increased numbers of peripheral blood TCRγδ+ T lymphocytes are present in some children with ITP. Three children with the highest elevations (37.8, 40.2, and 48.1% of CD3+ T lymphocytes, respectively) had additional investigation with the following observations: (i) predominant expression of the Vδ2/Vγ9 heterodimer, indicating a restricted TCR variable gene expression in the expanded cell population; (ii) enhanced *in vitro* proliferative responses to mycobacterial extracts and IL-2, but not against human heat shock protein or allogeneic platelets; (iii) polyclonal expansion of TCRγδ+ cells, with polymerase chain reaction (PCR) analysis demonstrating both Vδ2–Jδ1 and Vδ2–Jδ3 gene rearrangements and nucleotide analysis demonstrating unique V–D–D–J sequences; and (iv) a close correlation between the number of circulating TCRγδ+ T lymphocytes and the degree of thrombocytopenia. We hypothesize that TCRγδ+ T lymphocytes may be important in the pathogenesis of immune-mediated platelet destruction in some children with ITP.

## METHODS

*Patients.* Patients were grouped into three categories: children with acute ITP, children with chronic ITP, and young adult normal volunteers. A total of 11 children with acute ITP (4 males and 7 females) were studied, with a mean age of 5.5 years, mean platelet count of $19 \times 10^9$/L, and median platelet count of $8 \times 10^9$/L. A total of 19 children with chronic ITP (11 males and 8 females) was studied, with a mean age of 10.7 years, mean platelet count of $49 \times 10^9$/L, and median platelet count of $40 \times 10^9$/L excluding 2 patients with postsplenectomy normalization. Ten young adult volunteers (six males and four females) were also studied. Hematologic characteristics of some of these children have been previously described (11). All blood was obtained in accordance with a protocol approved by the Duke University Institutional Review Board.

*Antibodies.* Directly labeled monoclonal antibodies (mAbs) conjugated to either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used for single- and double-color analysis of lymphocyte subpopulations. The mAbs included CD2-PE, CD3-FITC, CD3-PE, CD4-FITC, CD4-PE, CD8-FITC, CD8-PE, CD20-FITC, and CD45RO-PE (Dako Corporation, Carpinteria, CA). Additional mAbs CD7, CD45RA (2H4), CD29 (4B4), and CD56 (NKH1), all conjugated to phycoerythrin, were kind gifts from Meryl Forman (Coulter Corporation, Hialeah, FL). Analysis of T cell receptor (TCR) antigens included Pan-TCRαβ, TCRδ1, δV1, δV2, and γV2 (T cell Diagnostics, Cambridge, MA). Control antibodies included IgG-FITC and IgG-PE from Dako. All antibodies were used according to the manufacturers' recommendations.

*Immunophenotyping.* Peripheral blood mononuclear cells (PBMC) were obtained from heparinized venous blood using Ficoll–Hypaque density centrifugation (18). Approximately $0.5 \times 10^6$ PBMC were incubated with saturating amounts of one or two mAbs for 30 min at 4°C as described previously (19). After washing with phosphate-buffered saline (PBS) with 2% bovine serum albumin (Sigma, St. Louis, MO), cells were fixed in paraformaldehyde and stored at 4°C in the dark until analysis. Samples were analyzed on a Profile II flow cytometer (Coulter) using forward- and side-scatter lymphocyte gating and color compensation.

In Vitro *Proliferation Assays.* PBMC from patients and controls were resuspended in RPMI with 15% human A serum (HAS; Worldwide Biologicals, Cincinnati, OH) at $1.0 \times 10^6$/ml. Final concentrations of stimuli included 0.5–2.0 μg/ml phytohemagglutinin (PHA; Burroughs Wellcome, Research Triangle Park, NC), 0.5–2.0 μg/ml concanavalin A (Con A; Sigma), 0.5–2.0 μl/ml pokeweed mitogen (PWM; GIBCO, Grand Island, NY), tetanus toxoid (Connaught Laboratory, Toronto Canada), 0.125–1.0 mg/ml *Candida albicans* (Greer Lab, Boone, NC), 100 U/ml recombinant interleukin-2 (rIL-2; gift from Chiron Corporation, Emeryville, CA), 10 μg/ml mycobacterial extract (mTB; gift from Dr. Drew Pardoll), 10 μg/ml recombinant *Mycobacterium bovis* BCG 65-kD protein (gift from the World Health Organization), 10 μg/ml 65-kD recombinant human heat shock protein (hsp65; gift from Satish Jindal of Procept, Cambridge, MA), and $100 \times 10^6$/ml allogeneic platelets which were purified as described (20).

For blastogenesis assays, 100 μl ($10^5$) cells were placed in triplicate into 96-well U-bottom plates (Costar, Cambridge, MA) along with 100 μl stimuli and incubated at 37°C (3–4 days for PHA, 4–5 days

for Con A, 5–6 days for PWM, 6 days for *Candida* and tetanus, and 7 days for all other stimuli). Cells were pulsed with 0.4 μCi $^3$H-thymidine (New England Nuclear, Boston, MA), harvested on a cell harvester (Cambridge Technologies, Watertown, MA), and counted on a beta scintillation counter. For bulk culture assays, 750 μl of cells was placed into 24-well plates (Costar) along with 750 μl of stimuli. After 7–9 days, 100-μl aliquots of cells were removed for immunophenotype analysis.

*Reverse Transcriptase Reaction.* Total RNA was harvested from PBMC of patients and controls using a cesium centrifugation method as described previously (19), then used in a reverse transcriptase (RT) reaction to generate cDNA. Total RNA (2.0 μg) was added to a 20-μl mixture containing 30 U of AMV RT (Stratagene, La Jolla, CA), 20 U of RNase inhibitor (Promega, Madison, WI), 1 μg of oligo(dT) (Pharmacia, Piscataway, NJ), 20 nmol (1 m*M* final concentration) of dNTPs (Pharmacia), 2 μl of 10× buffer (500 m*M* Tris pH 8.3, 200 m*M* KCl, 100 m*M* $MgCl_2$, and 50 m*M* dithiothreitol), and $ddH_2O$. The RT reaction was incubated at room temperature for 10 min, then 42°C for 60 min, followed by 70°C for 10 min and quenching on ice.

*Polymerase Chain Reaction.* The Vδ2 primer from 5′ to 3′ was GGGGTCGACCCTCAGGTGCTCCATGAA, which contains a 5′ *Sal*I restriction endonuclease site to allow subcloning of the PCR fragment. The Jδ1 primer, GGGGAATTCCACAGTCACACGGGTTC, and the Jδ3 primer, GGGGAATTCCACGAAGAGTTTGATGCC, each contain a 5′ *Eco*RI cutting site. A 50-μl PCR reaction contained 1.0 μl of the RT reaction as a source of template cDNA, 1 μg of each primer, 2.5 units of *Taq* polymerase (Stratagene), 10 nmol of dNTPs, 5 μl of 10× reaction buffer (Stratagene), and $ddH_2O$. The reaction mixture was heated to 94°C for 3 min for initial denaturation, followed by 35 cycles at 94°C for 1 min, 51°C for 2 min, and 72°C for 2 min. Negative controls included the following reactions: (i) RT, no primers added; (ii) no RT, primers added; and (iii) no RT, no primers added.

*Analysis of Vδ2 Gene Rearrangements.* The PCR product was purified using Prep-a-gene (Bio-Rad) prior to restriction endonuclease digestion with *Eco*RI and *Sal*I, then purified again prior to ligation. Bluescript vector (Stratagene) was also cut with both restriction endonucleases, and ligation was performed in a 10-μl reaction containing T4 ligase (Pharmacia), dATP (Pharmacia), and 10× ligase buffer. The ligated product was transformed into TG-1 bacteria, and positive colonies were first identified using blue–white β-galactosidase selection and then confirmed by PCR using flanking T3 and T7 primers. Individual clones which contained inserts of the correct size were then grown overnight in a 10-ml culture, and the amplified plasmids were purified using Magicprep (Promega) and chemically denatured. Sequencing reactions were performed in both directions using the T3 and T7 primers and Sequenase (United States Biochemical, Cleveland, OH); sequencing gels were 6% acrylamide and run at 50°C.

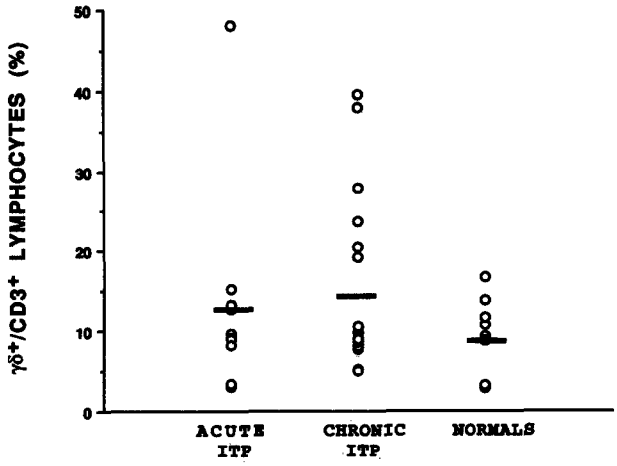


**Fig. 1.** Gamma–delta (TCRγδ+) T lymphocytes in childhood ITP. Two-color analysis of PBMC from 11 children with acute ITP, 19 with chronic ITP, and 10 normal volunteers demonstrated elevated numbers of TCRγδ+ T lymphocytes in several affected children. The horizontal bars represent the mean values, which were not significantly different among the three groups. The three children with the highest values (37.8, 40.2, and 48.1% of CD3+ lymphocytes, respectively) underwent additional investigation.

*Statistical Analysis.* Statistical tests were performed using the Primer of Biostatistics (McGraw-Hill, New York) software package. The *t* test was used to compare values among the three groups.

## RESULTS

### *Enumeration of TCR γδ+ T Lymphocytes*

The number of TCRγδ+ lymphocytes as a percentage of peripheral blood CD3+ T lymphocytes for each patient group is shown in Fig. 1. The 11 children with acute ITP had an average (mean ± SE) of 13.1 ± 3.7% TCRγδ+ lymphocytes, the 19 children with chronic ITP had 14.7 ± 2.4%, and the normal volunteers had 8.3 ± 1.6% TCRγδ+ lym-

**Table I.** Phenotypic and Functional Analysis of Peripheral Blood Lymphocytes at Initial Patient Evaluation[a]

| | Patient 1 | Patient 2 | Patient 3 | Control |
|---|---|---|---|---|
| T lymphocyte enumeration (%) | | | | |
| TCRαβ/CD3 | 58.4 | 69.7 | 38.9 | 95.4 ± 7.5 |
| TCRγδ/CD3 | 40.2 | 37.8 | 48.1 | 8.5 ± 2.0 |
| Vδ1/TCRγδ | 7.7 | 4.7 | 9.8 | 36.7 ± 10.0 |
| Vδ2/TCRγδ | 84.2 | 80.3 | 91.1 | 60.0 ± 10.0 |
| Vγ9/TCRγδ | 94.7 | 92.0 | 93.3 | 66.7 ± 10.0 |
| Lymphocyte proliferation (cpm) | | | | |
| Medium | 2,292 | 1,713 | 2,445 | 3,116 |
| PHA | 214,368 | 169,828 | 225,100 | 207,383 |
| Con A | 261,533 | 211,120 | 218,812 | 218,275 |
| PWM | 183,273 | 168,933 | 115,315 | 125,294 |
| Tetanus | 7,351 | 50,126 | 157,785 | 120,067 |
| Candida | 2,471 | 89,275 | 81,737 | 41,774 |
| mTB | 139,244 | 10,618 | 51,547 | 56,252 |
| BCG | 12,769 | 4,038 | 151,941 | 9,748 |
| rIL-2 | 286,744 | 151,941 | 364,519 | 216,753 |
| rIL-2 + cyclosporin A | 143,760 | 87,188 | 160,358 | 66,036 |
| hsp65 | 2,494 | 2,640 | 1,887 | 3,360 |
| Platelets | 1,187 | 2,695 | 2,016 | 1,943 |

[a]Control values (mean ± SE) for lymphocyte enumeration are from nine normal volunteers, while control values for lymphocyte proliferation are the mean of three to five normal volunteers for each point.

phocytes. There were no statistically significant differences in the number of TCRγδ+ lymphocytes among the three groups, but several children with increased numbers of TCRγδ+ lymphocytes were identified (Fig. 1). Two children with chronic ITP and one with acute ITP had highly elevated numbers of TCRγδ+ lymphocytes on initial evaluation and were evaluated further.

### *Case Reports*

*Patient 1.* A 23-month-old white male in good health developed a minor upper respiratory infection and, 2 weeks later, had bruising and petechiae; his peripheral blood counts were normal except for a platelet count of $3 \times 10^9$/L. He was diagnosed with ITP and treated with intravenous immunoglobulin (IVIG) with a good response, but required monthly treatments to maintain a platelet count above $30 \times 10^9$/L. Seven months later, the thrombocytopenia worsened and he became less responsive to IVIG therapy. Bone marrow aspirate showed slightly increased numbers of megakaryocytes and normal myeloerythroid maturation. Therapy with oral corticosteroids was effective, but thrombocytopenia recurred after steroid taper.

The patient was referred to Duke University Medical Center (DUMC) 12 months after diagnosis. Peripheral blood counts were normal except for a platelet count of $8 \times 10^9$/L. HIV ELISA was negative, and bone marrow aspirate revealed a normal 46XY karyotype. Table I lists his initial PBMC immunophenotype, which included an abnormally high number of TCRγδ+ T lymphocytes (40.2% of CD3+ T lymphocytes; absolute count, 1266 cells/μl). Figure 2 depicts his platelet counts and TCRγδ+ lymphocyte percentages over time and demonstrates that the elevation was documented on seven occasions over the next 5 months. A trial of cyclosporin A (4–11 mg/kg/day orally divided b.i.d. to achieve a serum level of 200 μg/ml) led to a slightly increased percentage of peripheral blood TCRγδ+ T lymphocytes. Following elective splenectomy, the platelet count and the number of TCRγδ+ T lymphocytes normalized.

*Patient 2.* A 10-year-old white male in good health presented with a 1- to 2-week history of epistaxis, bruising, petechiae, and oral mucosal bleeding. Peripheral blood count included a platelet count of $5 \times 10^9$/L, and he was referred to DUMC for further evaluation. Medical history and family history were negative for hematologic or autoimmune diseases, but the child had received a booster immunization of diphtheria/tetanus 12 days prior to presentation. Physical examination was normal except for bruising and petechiae, and peripheral blood counts were normal except for thrombocytopenia. Bone marrow aspirate was normal including 46XY karyotype, and HIV ELISA was negative. Table I shows the patient's initial PBMC

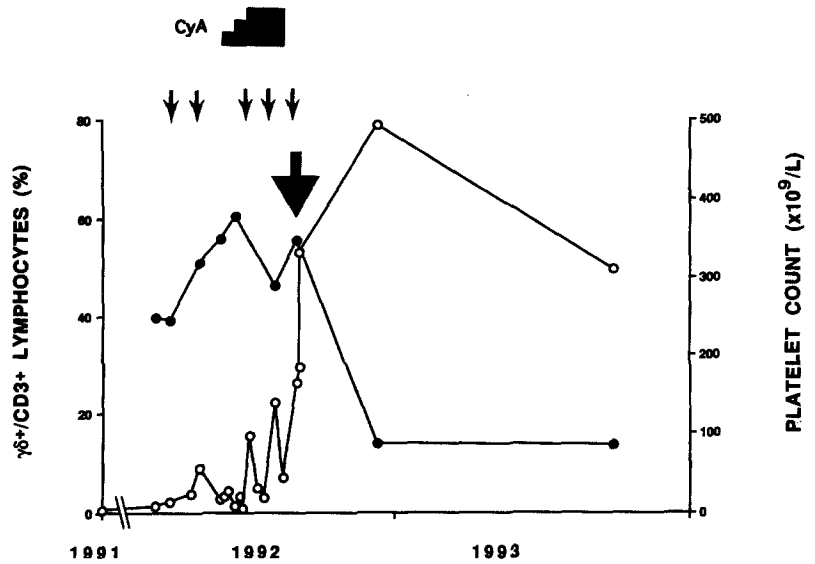


**Fig. 2.** Serial measurements of TCRγδ+ T lymphocytes and platelet counts in patient 1 with chronic ITP. The platelet counts (open circles) were below 100 × $10^9$/L from the time of diagnosis, despite therapy with IVIG (small vertical arrows) and cyclosporin A (CyA). Following elective splenectomy in late 1992 (large vertical arrow), the platelet count normalized. Measurement of TCRγδ+ T lymphocytes (filled circles) demonstrated 40–60% TCRγδ+ cells on multiple occasions despite therapy but normal values (<15%) following splenectomy.

immunophenotype, with an elevated number of TCRγδ+ T lymphocytes (37.8% of CD3+ T lymphocytes; absolute count, 539 cells/μl). He was initially treated with IVIG and has received periodic additional treatments for recurrent thrombocytopenia. He is currently 11 months from diagnosis (6 months since last IVIG), and his most recent platelet count was 97 × $10^9$/L. Coincident with the amelioration of his thrombocytopenia, the number of circulating TCRγδ+ T lymphocytes diminished and, at last measurement, was 21.2% of CD3+ lymphocytes.

*Patient 3.* A healthy 13-year-old white male presented with a 10-day history of spontaneous bruises on his trunk and extremities. Peripheral blood counts included a platelet count of 45 × $10^9$/L but were otherwise normal. Medical history and family history were negative. At DUMC 3 days later, his platelet count had risen to 110 × $10^9$/L and his erythrocyte sedimentation rate was 10 mm/hr; fluorescent antinuclear antibody and HIV ELISA were negative. Bone marrow aspirate revealed increased numbers of megakaryocytes, normal myeloerythroid maturation, and 46XY karyotype. Table I shows the PBMC immunophenotype at diagnosis and demonstrates an elevated number of TCRγδ+ T lymphocytes (48.1% of CD3+ T lymphocytes; absolute count, 643 cells/μl). His platelet count rose spontaneously over the next month to 241 × $10^9$/L and, most recently (13 months from diagnosis), was 299 × $10^9$/L. Figure 3 depicts the patient's platelet counts and percentage of TCRγδ+ T lymphocytes over time and shows that the number of TCRγδ+ T lymphocytes decreased toward the normal range (13–20%) coincident with resolution of his thrombocytopenia.

### *Phenotypic Subset Analysis of TCRγδ+ T Lymphocytes*

Because TCRγδ+ T lymphocytes are heterogeneous and can be categorized on the basis of their variable gene usage, we performed additional immunophenotypic analysis. In each case, the TCRγδ+ T lymphocytes were almost exclusively of the Vδ2 subset, with few cells reacting with the Vδ1 mAb. In addition, the cells also were strongly positive with the Vγ9 mAb, demonstrating that the cells expressed the Vδ2/Vγ9 heterodimer (Table II).

Two-color analysis further demonstrated that the TCRγδ+ T lymphocytes of each patient were phenotypically similar to those of controls (Table II). Patient TCRγδ+ T lymphocytes expressed CD2, CD3, CD7, and CD29 antigens but were

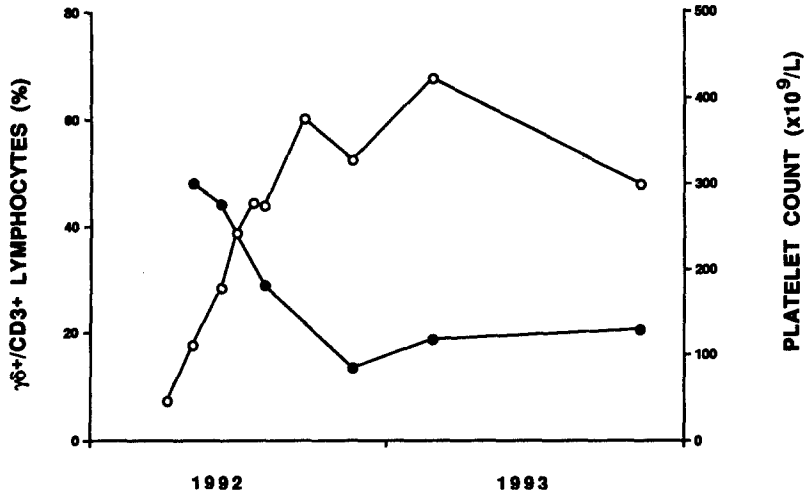


**Fig. 3.** Serial measurements of TCRγδ+ T lymphocytes and platelet counts in patient 3 with acute ITP. The platelet counts (open circles) were initially low at the time of diagnosis, quickly exceeded 100 × $10^9$/L, and have remained normal. Measurement of TCRγδ+ T lymphocytes (filled circles) demonstrated >40% TCRγδ+ cells on two occasions early in the disease but much lower values following the spontaneous resolution of the thrombocytopenia.

negative for CD4 and CD56 expression. CD8 expression was negative for patient 1 and low for patients 2 and 3, similar to CD8 expression in controls. Analysis of CD45 isoforms demonstrated that the majority of TCRγδ+ cells was CD45RO+ in patients 1 and 2, while patient 3 (the child with acute ITP) had lower numbers of CD45RO+ TCRγδ+ T lymphocytes at diagnosis, but predominantly CD45RO+ cells (82.6%), after resolution of thrombocytopenia.

**Table II.** Phenotypic Analysis of TCRγδ+ T lymphocytes[a]

| mAb | Patient 1 | Patient 2 | Patient 3 | Controls (mean ± SE) |
|---|---|---|---|---|
| Vδ1 | 5.8 | 4.7 | 10.0 | 31.6 ± 4.4 |
| Vδ2 | 86.3 | 80.3 | 91.1 | 67.7 ± 4.9 |
| Vγ9 | 95.2 | 92.0 | 93.1 | 74.2 ± 4.8 |
| CD2 | 87.4 | 94.7 | 93.9 | 95.2 ± 1.0 |
| CD3 | 99.6 | 93.8 | 99.6 | 99.0 ± 0.5 |
| CD4 | 2.3 | 0.0 | 0.8 | 1.5 ± 0.3 |
| CD7 | 99.0 | 98.6 | 97.4 | 93.0 ± 3.0 |
| CD8 | 3.7 | 10.2 | 30.1 | 19.6 ± 1.7 |
| CD29 | 89.9 | 84.9 | 80.7 | 79.5 ± 2.4 |
| CD45RA | 13.2 | 11.9 | 19.7 | 34.5 ± 6.6 |
| CD45RO | 61.9 | 60.9 | 22.5 | 45.0 ± 5.0 |
| CD56 | 4.5 | 15.6 | 27.0 | 14.6 ± 2.7 |

[a]PBMC from each patient at initial examination were stained with TCRδ1-FITC to identify all TCRγδ+ T lymphocytes and with additional mAbs to analyze and calculate TCRγδ subsets. Results are the percentage of TCRγδ+ T lymphocytes which were positive for each additional mAb. Control values derive from other children with ITP ($n$ = 16) and normal volunteers ($n$ = 6).

*Lymphocyte Proliferative Responses*

To determine the functional characteristics of the TCRγδ+ T lymphocytes, PBMC from each patient were tested in blastogenesis assays for proliferation to various stimuli. Table I shows that at initial evaluation, each child demonstrated normal [$^3$H]thymidine incorporation against mitogens including PHA, Con A, and pokeweed mitogen. Responses to recall antigens tetanus and candida were normal in patients 2 and 3 but low in patient 1, who was very young.

*In vitro* proliferative responses of patient PBMC to mycobacterial extract were positive as measured by [$^3$H]thymidine incorporation, similar to values obtained with controls (Table I). However, the mTB stimulus induced an enhanced proliferation of patient TCRγδ+ cells in bulk culture (Fig. 4); these results were much greater than those obtained from controls. Similarly, recombinant IL-2 led to strong [$^3$H]thymidine incorporation in both patient and control PBMC but induced enhanced growth of patient TCRγδ+ T lymphocytes compared with controls (Fig. 4).

*In vitro* blastogenesis using recombinant bovine BCG and human heat shock protein (hsp65) showed no proliferative responses (Table I) and no selective proliferation of TCRγδ+ cells in bulk culture (data not shown). Stimulation with allogeneic platelets also induced no proliferation.

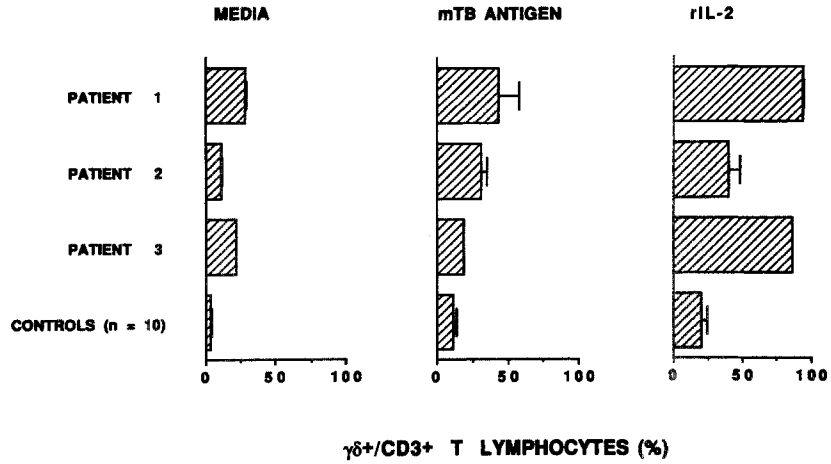


**Fig. 4.** Enhanced *in vitro* proliferation of TCRγδ+ T lymphocytes in childhood ITP. PBMC from patients and controls were incubated in bulk culture with stimuli for 7–9 days, then immunophenotyped for the number of TCRγδ+ cells as a percentage of CD3+ T lymphocytes. The three children with high values of TCRγδ+ T lymphocytes demonstrated an enhanced TCRγδ+ cell proliferation when stimulated with mTB antigen or rIL-2. The control values included both normal volunteers and other children with ITP. The data for patients 1 and 2 (with chronic ITP) were obtained from experiments on two different occasions, while data for patient 3 (with acute ITP) were obtained at the time of initial evaluation.

### *Analysis of Vδ2 Gene Rearrangements*

To determine whether the elevated numbers of Vδ2+/Vγ9+ TCRγδ+ T lymphocytes in our three patients represented a monoclonal, oligoclonal, or polyclonal expansion, Vδ2–Jδ gene rearrangements were directly analyzed. RT-PCR using the Vδ2 primer with either the Jδ1 or the Jδ3 primer demonstrated that the patients utilized both Jδ1 and Jδ3 gene segments in combination with the Vδ2 gene (Fig. 5). These results showed that the TCRγδ+ T lymphocytes did not represent a monoclonal population of cells. Next RT-PCR was performed using Vδ2 and Cδ primers, followed by cloning of individual PCR products and sequencing of the VDDJ gene rearrangements. Figure 6 shows the nucleotide sequence of multiple clones from each patient and demonstrates that each Vδ2–D–D–Jδ1 nucleotide sequence was unique. Similar results were obtained for the Vδ2–D–D–Jδ3 nucleotide sequences (data not shown). These results demonstrate unequivocally that the elevated numbers of TCRγδ+ T lymphocytes in the three children represented, in each case, a polyclonal expansion of cells.

## DISCUSSION

Cells which express the gamma–delta T cell receptor (TCRγδ+ lymphocytes) typically constitute 1–15% of the peripheral blood lymphocytes and are evenly distributed throughout the lymphoid system (21). TCRγδ+ T lymphocytes accumulate in the granulomatous lesions of leprosy (22), proliferate in response to *Mycobacterium tuberculosis* (23) and *Plasmodium falciparum* (24), and are increased in HIV (25) and EBV (26) infection. Some TCRγδ+ T lymphocytes recognize hsp (27, 28), while TCRγδ+ clones may display cytolytic activity (29–31). It has been postulated, therefore, that TCRγδ+ T lymphocytes have a distinct role in the primary immune response to infectious organisms (reviewed in Ref. 32). However, TCRγδ+ T lymphocytes may proliferate *in vivo* against endogenous hsp and lead to autoimmune processes (33).

TCRγδ+ T lymphocytes normally express CD2, CD3, and CD7, but surface CD8 is variable and CD4 is usually absent (21). Despite the presence of multiple human Vδ genes, the Vδ1 and Vδ2 gene products form the two major subsets of TCRγδ+ cells (34, 35), and the Vδ2 protein almost always forms a surface heterodimer with the Vγ9 gene product (36). Vδ2+/Vγ9+ T lymphocytes specifically proliferate against purified protein derivative (PPD) of *Mycobacterium tuberculosis* and, to a lesser extent, to recombinant purified hsp (37). It is likely that this limited peripheral blood repertoire is

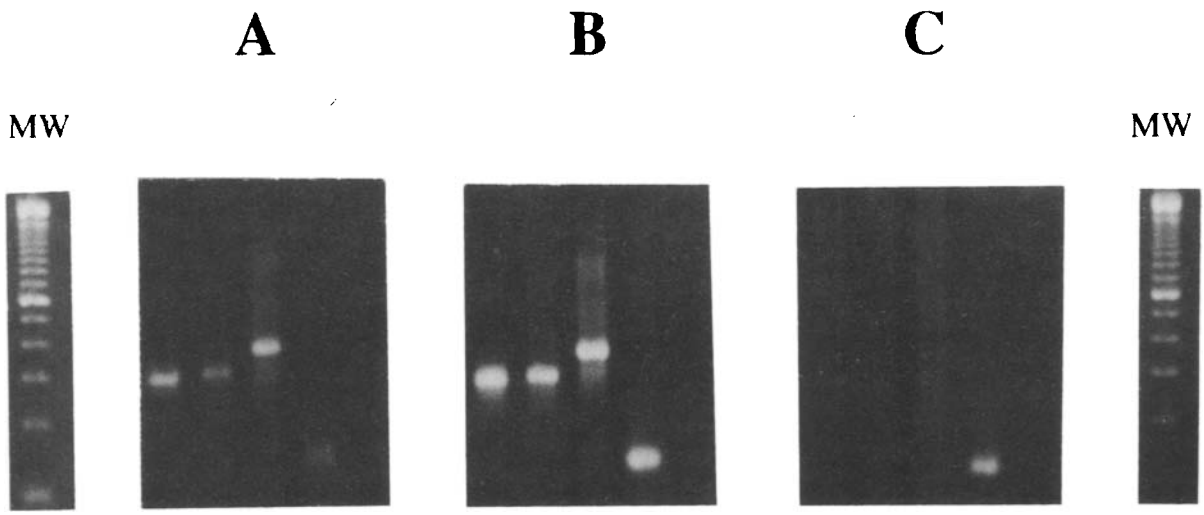


**Fig. 5.** Analysis of V–J gene rearrangements in the TCRγδ+ T lymphocytes of children with ITP. As the immunophenotype had demonstrated exclusive surface expression of the Vδ2 antigen, we performed RT-PCR using a Vδ2 primer with either a Jδ1 (lane 1), Jδ3 (lane 2), or Cδ primer (lane 3). The positive control (lane 4) used primers for the CD7 gene, while a negative control is shown in lane 5. Molecular weight (MW) markers on each side have a bright band at 600 bp for size reference. (A) Patient 1, (B) patient 2, and (C) another child with chronic ITP who had normal numbers of circulating TCRγδ+ T lymphocytes. Both Vδ2–Jδ1 and Vδ2–Jδ3 gene rearrangements were present in each child, indicating that the expanded TCRγδ+ T lymphocyte population was not monoclonal.

due to postthymic selection and stimulation of this specific subset.

To understand T lymphocyte abnormalities in children with immune-mediated platelet destruction, we analyzed the peripheral blood of children with acute or chronic ITP. Three children had highly elevated numbers of TCRγδ+ T lymphocytes at initial evaluation, ranging from 37.8 to 48.1% of the total CD3+ T lymphocytes. These expanded cell populations had a common surface immunophenotype which was CD2+, CD3+, CD7+, CD29+, CD4−, and CD56− (Table II, Fig. 2). These TCRγδ+ cells also expressed the Vδ2+/Vγ9+ phenotype almost exclusively, consistent with an enhanced expansion of this subset. Analysis of CD45 isoforms in patients 1 and 2 (with chronic ITP) had predominantly CD45RO+ (memory) TCRγδ+ T lymphocytes. The TCRγδ+ cells at initial evaluation in patient 3 (with acute ITP) were primarily CD45RA+ (naive) cells but converted to the CD45RO+ phenotype. We hypothesize that the TCRγδ+ lymphocytes of patients 1 and 2 had previously encountered antigen and undergone *in vivo* proliferation and expansion. The TCRγδ+ T lymphocytes of patient 3 had recently encountered antigen at the time of diagnosis and initial evaluation and later acquired the memory phenotype.

There was no evidence of generalized immune deficiency which might account for the elevation of TCRγδ+ T lymphocytes (38). The enhanced proliferative responses of the TCRγδ+ T lymphocytes to mycobacterial antigen and IL-2 are similar to those previously reported (39) and suggest that these cells were activated *in vivo*. As there was no *in vitro* proliferation to recombinant hsp, however, we were unable to identify potential antigens which might have induced *in vivo* proliferation of TCRγδ+ T lymphocytes.

These expanded cell populations do not represent a monoclonal malignant proliferation of TCRγδ+ T lymphocytes. PCR performed on TCRγδ+ T lymphocyte mRNA identified both Vδ2–Jδ1 and Vδ2–Jδ3 gene rearrangements (Fig. 5). Nucleotide sequencing of VDDJ sequences further identified diverse gene rearrangements (Fig. 6), confirming a polyclonal expansion of TCRγδ+ T lymphocytes. Selection and proliferation of lymphocytes which express a single variable gene region, but have extensive junctional diversity, are reminiscent of a superantigen response (40, 41). We speculate that a superantigen (possibly from an infectious organism) may have been the initiating immune stimulus and led to the tremendous polyclonal expansion of Vδ2+/Vγ9+ TCRγδ+ T lymphocytes.

There was a close correlation between the number of TCRγδ+ T lymphocytes and the degree of thrombocytopenia in each of the three children with ITP. Patient 1 had elevated TCRγδ+ T lympho-

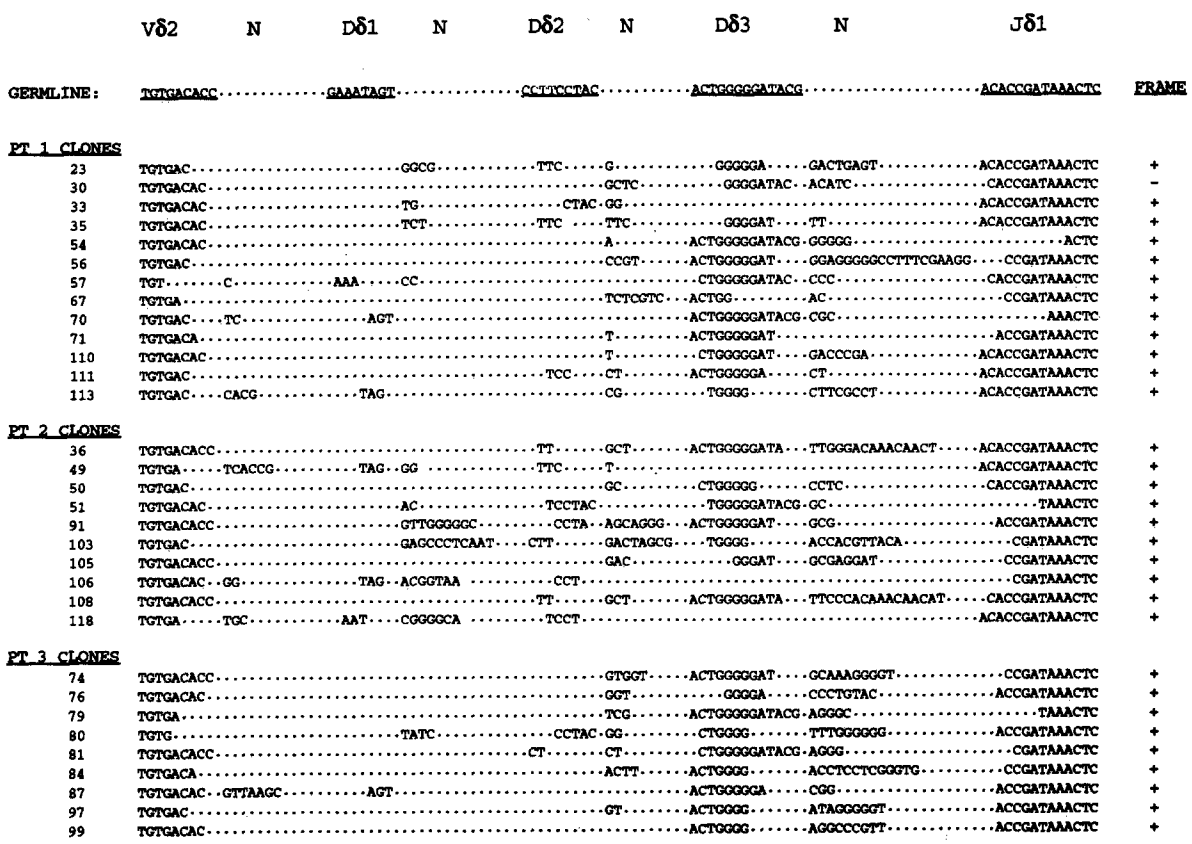


**Fig. 6.** Analysis of VDDJC nucleotide sequences in the TCRγδ+ T lymphocytes of children with ITP. To determine if the elevated number of TCRγδ+ T lymphocytes represented an oligoclonal expansion of cells, RT-PCR followed by DNA sequencing was performed for each of the three children. Each sequence used in Vδ2–D–D–Jδ1 rearrangements was unique, indicating a polyclonal expansion of TCRγδ+ T lymphocytes. Similar results were obtained from sequence analysis of Vδ2–D–D–Jδ3 rearrangements.

cytes for several months, along with severe thrombocytopenia. However, following splenectomy, both his platelet count and the number of TCRγδ+ T lymphocytes normalized (Fig. 2). Patient 2, also with chronic ITP, had a definite diminution in the number of TCRγδ+ T lymphocytes as his disease process has ameliorated. Finally, patient 3, with acute ITP, had 48.1% TCRγδ+ T lymphocytes at initial evaluation, but the percentage of TCRγδ+ T cells has nearly normalized coincident with resolution of his thrombocytopenia (Fig. 3). These clinical observations support the hypothesis that the expanded polyclonal population of TCRγδ+ T lymphocytes played a direct role in the pathogenesis of ITP.

The therapeutic use of cyclosporin A (CyA) in Patient 1 was based in part on the *in vitro* observation that CyA inhibited his PBMC proliferation to IL-2 (Table I). Many immunosuppressive effects of CyA on T lymphocytes relate to inhibition of TCR-mediated activation events (42). However, CyA has been shown not to affect the intrathymic development of TCRγδ+ cells (42, 43). Therapeutic CyA levels actually increased the number of TCRγδ+ T lymphocytes (perhaps by preferentially inhibiting TCRαβ+ lymphocytes) and had no effect on his thrombocytopenia.

The mechanisms by which TCRγδ+ T lymphocytes could mediate or participate in immune-mediated platelet destruction are only speculative. First, TCRγδ+ T lymphocytes may produce cytokines which are important in the pathogenesis of ITP (5). TCRγδ+ cells secrete a variety of cytokines following activation, including IL-2, GM-CSF, and IFN-γ (44–46). Alternatively, TCRγδ+ T lymphocytes could enhance antibody production by autoreactive B lymphocytes. TCRγδ+ cells can help B cells produce specific antibodies (47), even in the absence of nominal antigen (48). Finally, it is possible that TCRγδ+ T lymphocytes produce a

direct cytolytic effect on platelets. TCRγδ+ cells express killer activity against microbial organisms (45, 49), although the ligands which direct this effect are not known. In our study, no PBMC *in vitro* proliferative responses were observed against platelets, although the availability of autologous platelets might have provided a different result. The dramatic normalization of TCRγδ+ cells in patient 1 following splenectomy suggests that these cells were proliferating within the spleen, perhaps in response to platelet antigenic stimulation.

## ACKNOWLEDGMENTS

R.E.W. is the recipient of a James S. McDonnell Scholarship in Molecular Medicine. The authors are grateful to Dr. Barton Haynes and Dr. Michael Krangel for helpful discussions and to Kathy Greenwell for secretarial assistance.

## REFERENCES

1. Ware R, Kinney TR: Immunopathology of childhood idiopathic thrombocytopenic purpura. CRC Rev Oncol/Hematol 7:169–181, 1987
2. Dixon R, Rosse W, Ebbert L: Quantitative determination of antibody in idiopathic thrombocytopenic purpura. N Engl J Med 292:230–236, 1975
3. Luiken G, McMillan R, Lightsey A, Gordon P, Zevely S, Schulman I, Gribble T, Longmire R: Platelet-associated IgG in immune thrombocytopenic purpura. Blood 50:317–325, 1977
4. Lightsey A, McMillan R: The role of the spleen in "autoimmune" blood disorders. Am J Pediat Hematol/Oncol 1:331–341, 1979
5. Ware RE: Platelets and thrombocytopenia. *In* Samter's Immunological Diseases, 5th ed, MM Frank, KF Austen, HN Claman, ER Unanue (eds). Boston, Massachusetts, Little, Brown, pp. 919–932, in press
6. LoBuglio A, Court W, Vinocur L: Immune thrombocytopenic purpura: Use of a $^{125}$I labeled antihuman IgG monoclonal antibody to quantify platelet-bound IgG. N Engl J Med 309:459–463, 1983
7. Leber B, Brown C, Kelton JG: Oligoclonal B cell populations in severe, chronic immune thrombocytopenic purpura: Use of fetally related V-H gene segments with somatic hypermutation. Blood 80:48a, 1992
8. Van Leeuwen EF, Van der Ven J, Engelfriet CP, von dem Borne AK: Specificity of autoantibodies in autoimmune thrombocytopenia. Blood 59:23–26, 1982
9. Kunicki TJ, Newman PJ: The molecular immunology of human platelet proteins. Blood 80:1386–1404, 1992
10. Semple JW, Freedman J: Increased antiplatelet T helper lymphocyte reactivity in patients with autoimmune thrombocytopenia. Blood 78:2619–2625, 1991
11. Ware RE, Howard TA: Phenotypic and clonal analysis of T lymphocytes in childhood immune thrombocytopenic purpura. Blood 82:2137–2142, 1993
12. Semple JW, Freedman J: Cellular immune mechanisms in chronic autoimmune thrombocytopenic purpura (ATP). Autoimmunity 13:311–319, 1992
13. Olive C, Gatenby PA, Serjeantson SW: Variable gene usage of T cell receptor γ- and δ-chain transcripts expressed in synovia and peripheral blood of patients with rheumatoid arthritis. Clin Exp Immunol 87:172–177, 1992
14. Jacobs MR, Haynes BF: Increase in TCRγδ T lymphocytes in synovia from rheumatoid arthritis patients with active synovitis. J Clin Immunol 12:130–138, 1992
15. Hohlfeld R, Engel AG, Ii K, Harper MC: Polymyositis mediated by T lymphocytes that express the γ/δ receptor. N Engl J Med 324:877–881, 1991
16. Gerli R, Agea E, Bertotto A, Tognellini R, Flenghi L, Spinozzi F, Velardi A, Grignani F: Analysis of T cells bearing different isotypic forms of the γ/δ T cell receptor in patients with systemic autoimmune diseases. J Rheumatology 18:1504–1510, 1991
17. Bank I, Book M, Cohen L, Kneller A, Rosental E, Pras M, Bassat IB, Ben-Nun A: Expansion of a unique subpopulation of cytotoxic T cells that express a CαVδ1 T-cell receptor gene in a patient with severe persistent neutropenia. Blood 80:3157–3163, 1992
18. Denning SM, Tuck DT, Singer KH, Haynes BF: Human thymic epithelial cells function as accessory cells for autologous mature thymocyte activation. J Immunol 138:680–686, 1987
19. Ware RE, Hart MK, Haynes BF: Induction of T cell CD7 gene transcription by nonmitogenic ionomycin-induced transmembrane calcium flux. J Immunol 147:2787–2794, 1991
20. Rosse WF, Devine D, Ware RE: Reactions of immunoglobulin G-binding ligands with platelets and platelet-associated immunoglobulin G. J Clin Invest 73:489–496, 1984
21. Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, Warnke RA, Bhan AK, Strominger JL, Brenner MB: Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. J Exp Med 169:1277–1294, 1989
22. Modlin RL, Pirmez C, Hofman FM, Torigian V, Uyemura K, Rea TH, Bloom BR, Brenner MB: Lymphocytes bearing antigen-specific γ/δ T cell receptors accumulate in human infectious disease lesions. Nature 339:544–548, 1989
23. Janis EM, Kaufmann SHE, Schwartz RH, Pardoll DM: Activation of γδ T cells in the primary immune response to *Mycobacterium tuberculosis*. Science 244:713–716, 1989
24. Goodier M, Fey P, Eichmann K, Langhorne J: Human peripheral blood γδ T cells respond to antigens of *Plasmodium falciparum*. Int Immunol 4:33–41, 1991
25. De Maria A, Ferrazin A, Ferrini S, Ciccone E, Terragna A, Moretta L: Selective increase of a subset of T cell receptor γδ T lymphocytes in the peripheral blood of patients with human immunodeficiency virus Type 1 infection. J Infect Dis 165:917–919, 1992
26. DePaoli P, Gennari D, Martelli P, Cavarzerani V, Comoretto R, Santini G: γδ T cell receptor-bearing lymphocytes during Epstein-Barr virus infection. J Infect Dis 161:1013–1016, 1990
27. Haregewoin A, Soman G, Hom RC, Finberg RW: Human γδ+ T cells respond to mycobacterial heat-shock protein. Nature 340:309–312, 1989

28. Born W, Hall L, Dallas A, Bormel J, Shinnick T, Young D, Brennan P, O'Brien R: Recognition of a peptide antigen by heat shock-reactive γδ T lymphocytes. Science 249:67–69, 1990
29. Brenner MB, McLean J, Scheft H, Riberdy J, Ang S-L, Seidman JG, Devlin P, Krangel MS: Two forms of the T cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. Nature 325:689–694, 1987
30. Fisch P, Malkovsky M, Braakman E, Sturm E, Bolhuis RLH, Prieve A, Sosman JA, Lam VA, Sondel PM: γ/δ T cell clones and natural killer cell clones mediate distinct patterns of non-major histocompatibility complex-restricted cytolysis. J Exp Med 171:1567–1579, 1990
31. Bluestone JA, Cron RQ, Cotterman M, Houlden BA, Matis LA: Structure and specificity of T cell receptor γ/δ on major histocompatibility complex antigen-specific CD3+, CD4−, CD8− T lymphocytes. J Exp Med 168:1899–1916, 1988
32. Haas W, Pereira P, Tonegawa S: Gamma/delta cells. Annu Rev Immunol 11:637–685, 1993
33. Winfield JB: Stress proteins, arthritis, and autoimmunity. Arth Rheum 32:1497–1504, 1989
34. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, Moretta L, Moretta A: Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. J Exp Med 168:491–505, 1988
35. Triebel F, Hercend T: Subpopulations of human gamma delta lymphocytes. Immunol Today 10:186–188, 1989
36. Casorati G, De Libero G, Lanzavecchia A, Migone N: Molecular analysis of human γ/δ+ clones from thymus and peripheral blood. J Exp Med 170:1521–1535, 1989
37. O'Brien RL, Happ MP, Dallas A, Palmer E, Kubo R, Born WK: Stimulation of a major subset of lymphocytes expressing T cell receptor γδ by an antigen derived from Mycobacterium tuberculosis. Cell 57:667–674, 1989
38. Carbonari M, Cherchi M, Paganelli R, Giannini G, Galli E, Gaetano C, Papetti C, Fiorilli M: Relative increase of T cells expressing the gamma/delta rather than the alpha/beta receptor in ataxia-telangiectasia. N Engl J Med 322:73–76, 1990
39. Fisch P, Malkovsky M, Kovats S, Sturm E, Braakman E, Klein BS, Voss SD, Morrissey LW, DeMars R, Welch WJ, Bolhuis RLH, Sondel PM: Recognition by human Vγ9/Vδ2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. Science 250:1269–1273, 1990
40. Choi Y, Kotzin B, Herron L, Callahan J, Marrack P, Kappler J: Interaction of *Staphylococcus aureus* toxin "superantigens" with human T cells. Proc Natl Acad Sci USA 86:8941–8945, 1989
41. Marrack P, Kappler J: The Staphylococcal enterotoxins and their relatives. Science 248:705–711, 1990
42. Jenkins MK, Schwartz RH, Pardoll DM: Effects of cyclosporine A on T cell development and clonal deletion. Science 241:1655–1658, 1988
43. Bader P, Bendigs S, Wagner H, Heeg K: Cyclosporin A (CsA) prevents the generation of mature thymic α/β T cells but spares γ/δ T lymphocytes. Current Topics Microbiol Immunol 173:81–89, 1991
44. Munk ME, Gatrill AJ, Kaufman SHE: Target cell lysis and interleukin-2 secretion by g/d T lymphocytes after activation with bacteria. J Immunol 145:2434–2439, 1990
45. Follows GA, Munk ME, Gatrill AJ, Conradt P, Kaufmann SHE: Gamma interferon and interleukin 2, but not interleukin 4, are detectable in γ/δ T cell cultures after activation with bacteria. Infect Immun 60:1229–1231, 1992
46. Krangel MS, Yssel H, Brockelhurst C, Spits H: A distinct wave of human T cell receptor γ/δ lymphocytes in the early fetal thymus: Evidence for controlled gene arrangement and cytokine production. J Exp Med 172:847–859, 1990
47. Vidovic O, Dembic Z: Qa-1 restricted γδ T cells can help B cells. Current Topics Microbiol Immunol 173:239–244, 1991
48. Sperling AI, Wortis HH: CD4−, CD8− γ/δ cells from normal mice respond to a syngeneic B cell lymphoma and can induce its differentiation. Int Immunol 1:434–442, 1989
49. Raulet DH: The structure, function, and molecular genetics of the γ/δ T cell receptor. Annu Rev Immunol 7:175–207, 1989