

Human $\gamma\delta$ T Cells Recognize Alkylamines Derived from Microbes, Edible Plants, and Tea: Implications for Innate Immunity

Jack F. Bukowski,*[†] Craig T. Morita,[†]
and Michael B. Brenner*

*Lymphocyte Biology Section
Division of Rheumatology, Immunology, and Allergy
Department of Medicine
Brigham and Women's Hospital
and Harvard Medical School
Boston, Massachusetts 02115

[†]Division of Rheumatology
Department of Internal Medicine
University of Iowa
Iowa City, Iowa 52242

Summary

Approximately 4% of peripheral blood T cells in humans express a T cell receptor with markedly restricted germline gene segment usage ($V\gamma 2V\delta 2$). Remarkably, these T cells expand 2- to 10-fold (8%–60% of all circulating T cells) during many microbial infections. We show here that these T cells recognize a family of naturally occurring primary alkylamines in a TCR-dependent manner. These antigenic alkylamines are secreted to millimolar concentrations in bacterial supernatants and are found in certain edible plants. Given the large numbers of memory $V\gamma 2V\delta 2$ T cells in adult humans, recognition of alkylamine antigens offers the immune system a response of the magnitude of major superantigens for $\alpha\beta$ T cells and may bridge the gap between innate and adaptive immunity.

Introduction

Recent studies in mice suggest that $\gamma\delta$ T cells have diverse functions, not only mediating host defense in preventing lethal HSV-1 encephalitis (Sciannas et al., 1997), protective immunity against malaria (Tsuji et al., 1994), and antibacterial effects in tuberculosis (Hiro-matsu et al., 1992) and listeriosis (Ladel et al., 1995), but also in immunoregulation by ameliorating the severity of adjuvant arthritis (Peterman et al., 1993) and enhancing immune tolerance in several model systems (McMenamin et al., 1994; Mengel et al., 1995; Harrison et al., 1996; Ke et al., 1997).

Several lines of evidence also implicate $\gamma\delta$ T cells as important in human immunity. Polyclonal $V\gamma 2V\delta 2$ T cells expand *in vivo*, typically becoming 8%–60% of all peripheral blood T cells during a variety of infectious diseases such as tuberculosis (mean = 14%) (Balbi et al., 1993), salmonellosis (mean = 18%) (Hara et al., 1992), tularemia (mean = 31%) (Sumida et al., 1992), brucellosis (mean = 29%) (Bertotto et al., 1993), listeriosis (mean = 12%) (Munk et al., 1990), erlichiosis (mean = 57%) (Caldwell et al., 1996), leishmaniasis (mean = 13%)

(Raziuddin et al., 1992), malaria (mean = 11%–16%) (Perera et al., 1994), toxoplasmosis (mean = 9%) (Sca-lise et al., 1992), and HIV (early stages; mean = 8%) (De Maria et al., 1992).

Given the emerging examples of how $\gamma\delta$ T cells mediate host defense and immunoregulation, it is surprising that few natural antigens that $\gamma\delta$ T cells recognize have been identified. Recent insights into antigen recognition by $\gamma\delta$ T cells have focused on the immunoglobulin-like structure of the $\gamma\delta$ TCR (Li et al., 1998) and on the recognition of small organic phosphate molecules (Tanaka et al., 1994, 1995) or unprocessed protein antigens (Schild et al., 1994). Such antigen specificity is remarkably different from MHC-restricted recognition of bound peptides by $\alpha\beta$ TCRs. Here we show that a previously unrecognized class of naturally occurring antigens, the alkylamines, stimulate the $V\gamma 2V\delta 2$ bearing $\gamma\delta$ T cells and are secreted by bacteria, found in certain foods, and present in human body fluids. Thus, a large pool of memory T cells reactive to alkylamine antigens exists in humans. Here we show their specificity for a range of ubiquitous alkylamine antigens encountered in nature.

Results and Discussion

$\gamma\delta$ T Cells Respond to Secreted Bacterial Alkylamine Antigens

Despite the large number of organisms known to stimulate $\gamma\delta$ T cells, only a few $\gamma\delta$ T cell antigens have been identified, and these are small anionic molecules that invariably contain a phosphate moiety. We reasoned that other major bacterial products might also contain antigens recognized by $\gamma\delta$ T cells. One antigen preparation tested was a crude supernatant from *Proteus morganii*, an important cause of urinary tract infections and urosepsis. This bacterium secretes large amounts of the aliphatic alkylamines *iso*-butylamine and *iso*-amylamine (Ghenghesh and Drucker, 1989). To determine if these abundant secreted products of bacterial metabolism could stimulate $V\gamma 2V\delta 2$ T cells, fresh supernatants from bacterial broth cultures and supernatants that were extracted to enrich for alkylamines were cultured with peripheral blood mononuclear cells (PBMC) for 12 days, and the cells were analyzed by flow cytometry to enumerate $\gamma\delta$ T cells. The crude bacterial supernatant and its alkylamine-enriched extract induced in a dose-dependent manner a 3-fold and a 7-fold increase, respectively, in $V\gamma 2V\delta 2$ T cell numbers, whereas the bacteriological media alone had no effect (Figure 1A). Quantitative headspace GC-mass spectrometry analysis of the crude bacterial supernatant revealed the presence of 3.4 mM *iso*-butylamine and 3.9 mM *iso*-amylamine, whereas the uninoculated culture media had no detectable alkylamines. The alkylamine-enriched extract of the bacterial supernatant contained 4.0 mM *iso*-butylamine and 8.4 mM *iso*-amylamine. The results suggested that these alkylamines might be antigens for $V\gamma 2V\delta 2$ T cells.

To determine directly whether *iso*-butylamine found in bacterial supernatants could stimulate the $V\gamma 2V\delta 2$

[†]To whom correspondence should be addressed (e-mail: jbukowski@rics.bwh.harvard.edu).

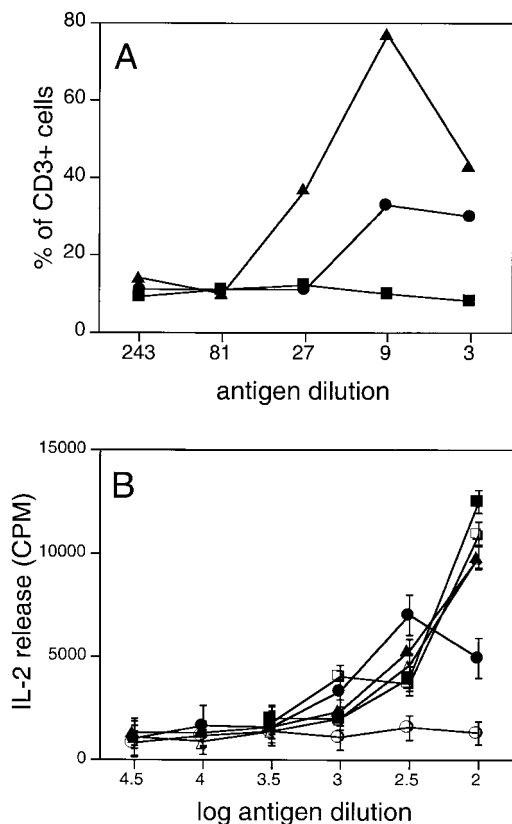


Figure 1. *Proteus morganii* Supernatants and Their Extracts Caused Expansion of V γ 2V δ 2 T Cells from PBMC

(A) Supernatants from a broth culture of *Proteus morganii* (circles), an extract from this supernatant enriched for amines (triangles), and uninoculated bacterial culture media (squares) were mixed with PBMC in 0.5 ml cell culture medium. On day 3, 0.5 ml cell culture medium containing 0.3 nM IL-2 was added to all cultures. On day 12, cells were analyzed by flow cytometry with anti- $\gamma\delta$ TCR mAbs to enumerate $\gamma\delta$ T cell numbers. The total number of cells in the cultures remained constant over the 12 day period. Data are expressed as the percentage of CD3⁺ cells with $\gamma\delta$ TCRs. Greater than 99% of $\gamma\delta$ T cells were V γ 2V δ 2⁺ T cells as assessed using V chain specific antibodies.

(B) Alkaline phosphatase treatment of extracts of *Proteus morganii* broth culture supernatants had no effect on their antigenic activity. Monoethylphosphate (circles), isobutylamine (squares), or extracts of *Proteus* supernatants (triangles) were treated with alkaline phosphatase (open symbols), or mock-treated (closed symbols) and used as antigens to stimulate IL-2 release from the V γ 2V δ 2 TCR transfectant DBS43. Half-log dilutions of alkylamine antigen stock solutions were added to 10⁵ responder TCR transfectants in the presence of 10 nM phorbol myristate acetate as a costimulator. After 24 hr, supernatants were harvested and tested at a final dilution of 1:8 for their ability to stimulate the growth of the IL-2-dependent HT-2 cell line.

TCR, we tested $\gamma\delta$ TCR transfectants for their ability to release IL-2 in response to bacterial supernatants and to pure *iso*-butylamine. TCR transfectant DBS43 was made by cotransfecting cDNA constructs encoding the V γ 2 and V δ 2 TCR chains from the T cell clone DG.SF13 into a TCR-deficient mutant of Jurkat T cells. For comparison, another TCR transfectant, 27/3.62, is specified by cDNA derived from the T cell clone F7 encoding a V γ 1V δ 1 TCR chain pair (Bukowski et al., 1995). An extract of *Proteus* supernatant enriched for alkylamines, pure

iso-butylamine, and monoethylphosphate (an example of a phosphate antigen) were either mock treated or treated with alkaline phosphatase. Alkaline phosphatase treatment of monoethylphosphate reduced by 7-fold its ability to induce IL-2 release from the V γ 2V δ 2 TCR transfectant (Figure 1B) but not the V γ 1V δ 1 TCR transfectant (data not shown), thus totally abrogating antigenic activity. In contrast, this treatment failed to significantly reduce the antigenic activity of the *Proteus* extract or pure *iso*-butylamine, emphasizing that alkylamines and not phosphate antigens were responsible for this activity (Figure 1B).

Specificity of $\gamma\delta$ T Cells for Alkylamine Antigens

We then assessed directly the ability of a series of alkylamines to induce the expansion of $\gamma\delta$ T cells from human peripheral blood (Figure 2). The alkylamines ethyl- (5- to 6-fold), *n*-propyl- (2- to 4-fold), *n*-butyl- (3-fold), *iso*-propyl- (4- to 5-fold), *iso*-butyl- (4- to 5-fold), *sec*-butyl- (4- to 8-fold), and *iso*-amyl- (5- to 6-fold) expanded $\gamma\delta$ T cells compared to either media alone or to tetanus toxoid, which did not expand $\gamma\delta$ T cells. The magnitude of the expansion induced by *sec*-butylamine was comparable to that induced by isopentenyl pyrophosphate (IPP) (Figure 2). Methyl- and *n*-amylamines, putrescine, di- and trimethylamines, ethanolamine, and phosphoethanolamine, histamine, and tyramine failed to expand $\gamma\delta$ T cells from PBMC of several donors (data not shown). $\alpha\beta$ T cells were not expanded by alkylamines, and flow cytometry using V γ - and V δ -specific mAbs revealed that all the expanded $\gamma\delta$ T cells expressed V γ 2 and V δ 2 gene segments. This response to alkylamines was unexpected, since they represent a class of naturally occurring compounds that are chemically distinct from previously known $\gamma\delta$ T cell antigens (Davodeau et al., 1993; Schoel et al., 1994; Tanaka et al., 1994, 1995; Bürk et al., 1995).

These results show that alkylamines recognized by $\gamma\delta$ T cells are characterized by a straight or branched alkyl chain of two to five carbons with a single primary amine group as the only substituent (Figure 3). Conversely, alkylamines with one carbon (methylamine) or more than five carbons, or any substituent in addition to the primary amino group were not antigenic (Figure 3). These structural constraints on alkylamine antigens are reminiscent of those of alkyl phosphates and prenyl pyrophosphates (Tanaka et al., 1994, 1995). However, there are exceptions to these general observations. For instance, the five carbon *iso*-amyl phosphate was not recognized, while *iso*-amylamine was recognized. Whereas methylphosphate was antigenic, methylamine was not. In summary, straight or branched chain alkyl phosphates of one to four or five carbons and primary alkylamines of two to five carbons were antigenic for V γ 2V δ 2 T cells (Figure 3) (Tanaka et al., 1994, 1995).

Representatives of a number of the biologically active alkylamine antigens are produced by human pathogens. For example, *Salmonella typhimurium* (Ghenghesh and Drucker, 1989; Hara et al., 1992) and *Listeria monocytogenes* (Daneshvar et al., 1989; Jouen-Beades et al., 1997) produce the antigenic alkylamines *iso*-butylamine and *iso*-amylamine, and *n*-butylamine, respectively, and these bacterial infections cause the in vivo $\gamma\delta$ T cell expansions in humans. Further examples such as *Yersinia enterocolitica* and *Escherichia coli* that produce

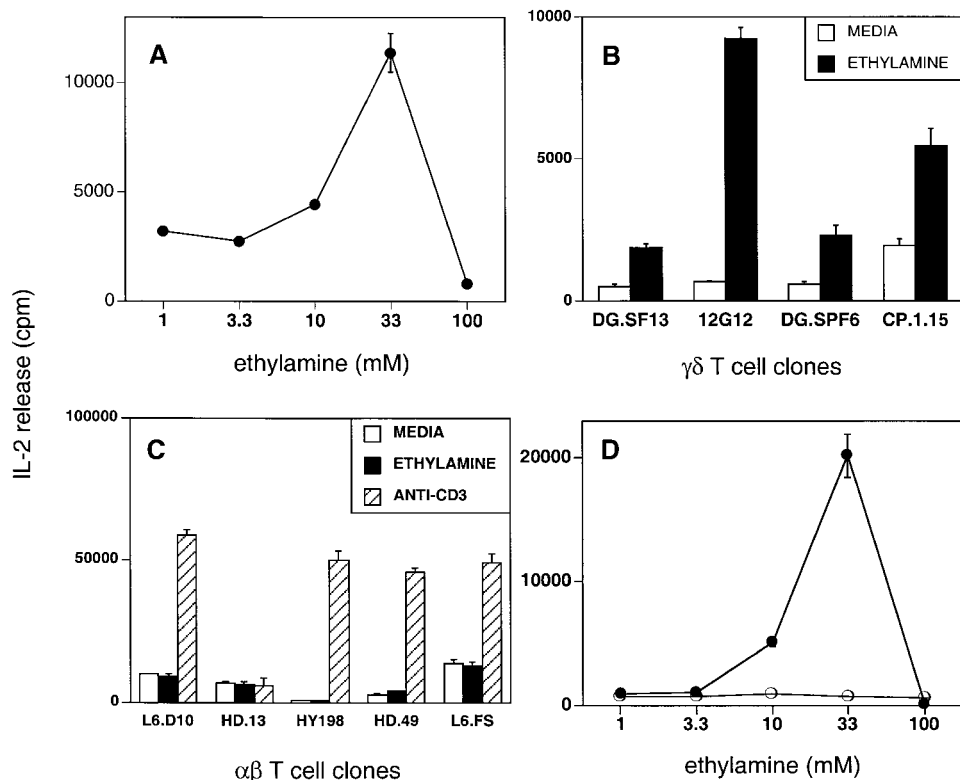


Figure 4. Specificity of Ethylamine for $V\gamma 2V\delta 2$ T Cells

Ethylamine caused IL-2 release in a polyclonal $V\gamma 2V\delta 2$ T cell line G2D2a (A) and $V\gamma 2V\delta 2$ T cell clones (B) but not in $\alpha\beta$ T cell clones (C). Ethylamine treatment resulted in IL-2 release from a $V\gamma 2V\delta 2$ (closed circles) but not a $V\gamma 1V\delta 1$ (open circles) TCR transfectant (D). Both transfectants released similar levels of IL-2 in response to anti-CD3 mAb in a dose-dependent manner (data not shown). Half-log dilutions of ethylamine starting at 100 mM (A and D) or a fixed dose of 30 mM (B and C) or, as a positive control, anti-CD3 mAb (striped bars) were added to responder cells, and IL-2 release was measured (see legend to Figure 1B). The decreased IL-2 responses at high concentrations of ethylamine were due to toxicity.

recognition, we measured the IL-2 release in response to ethylamine of a panel of $\gamma\delta$ and $\alpha\beta$ T cells including a polyclonal $\gamma\delta$ T cell line (G2D2a) coexpressing $V\gamma 2V\delta 2$ variable region T cell receptor (TCR) chains, four $\gamma\delta$ T cell clones coexpressing $V\gamma 2V\delta 2$ (DG.SF13, 12G12, DG.SPF6, and CP.1.15), five $\alpha\beta$ T cell clones (L6. D10, HD.13, HY198, HD.49, and L6. FS), and the $V\gamma 2V\delta 2$ and $V\gamma 1V\delta 1$ TCR transfectants (DBS43 and 27/3.62, respectively). The $V\gamma 2V\delta 2$ T cell line showed a 4-fold greater response to ethylamine as compared to media (Figure 4A), and the $V\gamma 2V\delta 2$ T cell clones had up to a 5-fold greater response to ethylamine as compared to media (Figure 4B). The $V\gamma 2V\delta 2$ TCR transfectant (Figure 4D) had a response to ethylamine comparable to that of the $V\gamma 2V\delta 2$ T cell line (Figure 4A). In contrast, there was no significant difference in the amount of IL-2 released in response to ethylamine by a series of $\alpha\beta$ T cell clones when compared to media (Figure 4C) or by the $V\gamma 1V\delta 1$ TCR transfectant (Figure 4D). The alkylamines that expanded $V\gamma 2V\delta 2$ T cells from peripheral blood also caused IL-2 release from the $V\gamma 2V\delta 2$ TCR transfectant (Figure 5), emphasizing that recognition of alkylamines is TCR dependent. *iso*-butyl- and *sec*-butylamines were the most potent antigens on a molar basis for this particular transfectant TCR. *n*-propyl-, *iso*-propyl-, *n*-butyl-, and *iso*-amylamines had intermediate potency, and

ethylamine was the least potent antigen. These findings provide strong evidence for the role of the $V\gamma 2V\delta 2$ TCR in alkylamine recognition.

These results indicate that the same $V\gamma 2V\delta 2$ TCR can recognize positively charged molecules (alkylamines, Figure 3) as well as the previously characterized negatively charged molecules such as ethyl phosphate and isopentenyl pyrophosphate (Tanaka et al., 1994, 1995). This suggests that the TCR might bind these two different molecules at different locations on the TCR. This is easily possible, given the small sizes of these antigens. Alternatively, the TCR may recognize the alkyl chain of either alkyl phosphates or alkylamines at one TCR site, while distinct TCR residues at adjacent sites may accommodate either the positive or the negative charges and result in TCR dependent signaling.

Several lines of evidence suggest that $\gamma\delta$ T cells recognize antigens in a way that is more like immunoglobulins than like $\alpha\beta$ TCRs. In the mouse, $\gamma\delta$ T cells directly recognize the MHC class II molecule I-E^s and the non-classical MHC molecules T10 and T22 without peptide dependence (Schild et al., 1994). Moreover, $\gamma\delta$ T cells recognize native HSV glycoprotein I even when bound to plastic without antigen-presenting molecules (Sciama et al., 1994). Analysis of CDR3 length distributions of TCR δ chains indicates that they are more similar to

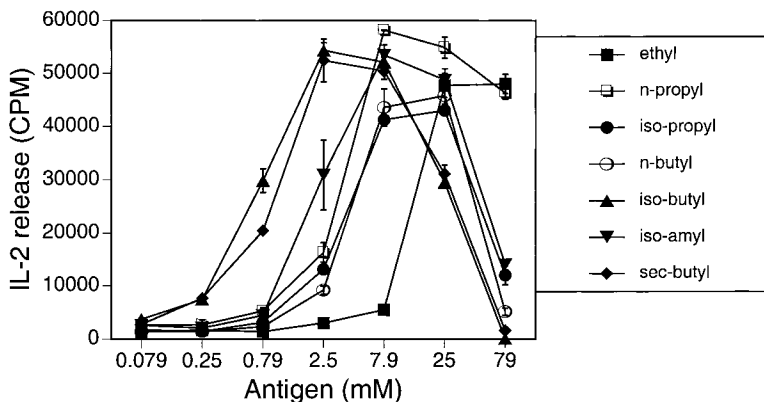


Figure 5. Alkylamines Cause IL-2 Release from a $V\gamma 2V\delta 2$ TCR Transfectant in a Dose-Dependent Manner

The decreased IL-2 responses at high antigen doses were due to toxicity (see legend to Figure 1B).

those of IgH than to TCR α and TCR β chains (Rock et al., 1994), and, finally, atomic structure analysis of human $V\delta 3$ indicates that this TCR models more closely on Ig V domains than on TCR α or $-\beta$ V domains (Li et al., 1998). Crystallographic studies have shown that immunoglobulins recognize small phosphate-containing molecules such as phosphorylcholine in a way that is highly dependent on both the heavy and light chain CDR3 regions (Padlan et al., 1976). Thus, the $V\gamma 2V\delta 2$ TCR-alkylamine antigen complex may be very similar to an Ig-hapten complex. However, preliminary evidence in our laboratory shows that cell-to-cell contact is necessary for IL-2 release by $\gamma\delta$ T cells in response to alkylamines. It is not known whether this requirement for cell-to-cell contact indicates a role for an unidentified alkylamine antigen presenting molecule or merely a requirement for costimulation.

L-Theanine, Precursor of Ethylamine, a $\gamma\delta$ T Cell Antigen Found in Tea

Alkylamines are found also in plants. Ethylamine is a breakdown product of tea digestion in humans that is found in the urine of tea drinkers but not in non-tea drinkers. Ethylamine is produced by acid hydrolysis of L-theanine in the gut and by enzymatic hydrolysis mediated by amidases in the liver (Figure 6) (Asatoor, 1966). Tea beverage typically contains a 2–10 mM concentration of L-theanine, an amino acid that has been found only in tea (*Camellia sinensis*). Black, green, and oolong teas are different preparations of *C. sinensis* but each contains L-theanine (Sakato, 1949; Cartwright et al., 1954; Graham, 1992). To determine if components of tea beverage could cause $\gamma\delta$ T cell expansion in vitro, we mixed unfractionated PBMC isolated from healthy donors with green or black tea leaf aqueous extracts. Neither green nor black tea extracts directly added to cultures expanded the number of $\gamma\delta$ T cells (data not shown). To mimic the in vivo breakdown of L-theanine in vitro, we acid hydrolyzed green and black tea extracts by reflux in 5 N HCl to liberate ethylamine from L-theanine and brought these extracts to pH 7.4 (Asatoor, 1966). These hydrolyzed tea extracts induced a 3- to 5-fold expansion of $\gamma\delta$ T cells (Figure 7A). Similarly, purified intact L-theanine failed to cause $\gamma\delta$ T cell expansion, but L-theanine that was acid hydrolyzed, brought to pH 7.4, and diluted in media caused a 15-fold expansion of $\gamma\delta$ T cells (5%–75%) from PBMC (Figure 7B). To verify

that ethylamine was released by acid hydrolysis from the L-theanine in tea or from purified L-theanine, quantitative headspace GC-mass spectrometry analysis was performed on acid hydrolyzed or unhydrolyzed tea extract or L-theanine. In unhydrolyzed samples, ethylamine was not detected. In contrast, 7.2 mM ethylamine was detected in the biologically active acid hydrolyzed tea sample that was used to stimulate $\gamma\delta$ T cells. GC-mass spectrometry analysis of a purified acid hydrolyzed 1 mM L-theanine sample revealed the presence of a roughly equimolar amount (1.1 mM) of ethylamine, confirming the observation of Sakato (1949). Taken together, these results show that ethylamine such as that produced in vivo by L-theanine hydrolysis in tea drinkers (Asatoor, 1966) is capable of expanding $\gamma\delta$ T cells.

The alkylamines here shown to be capable of expanding $\gamma\delta$ T cells are found in apples (*n*-butylamine) (Hartmann, 1967) and in red and white wine (*iso*-amylamine) (Ibe et al., 1991). Various human secretions and fluids also contain antigenic alkylamines, including urine of tea drinkers (ethylamine) (Perry et al., 1962), breast milk and amniotic fluid (ethylamine, *n*-propylamine, *n*-butylamine, *iso*-butylamine), (Lichtenberger et al., 1991),

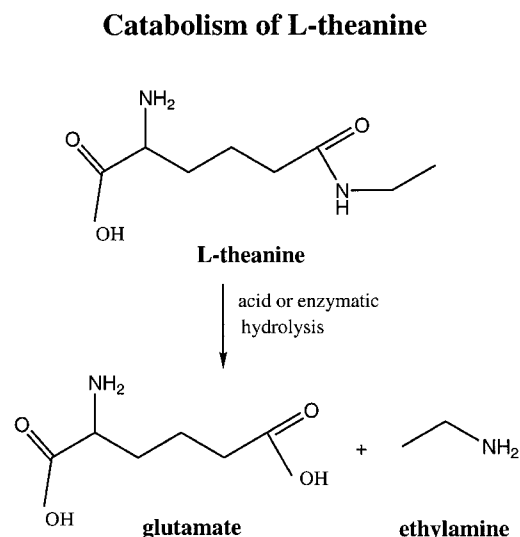


Figure 6. Catabolism of L-Theanine, an Amino Acid Found Only in Tea

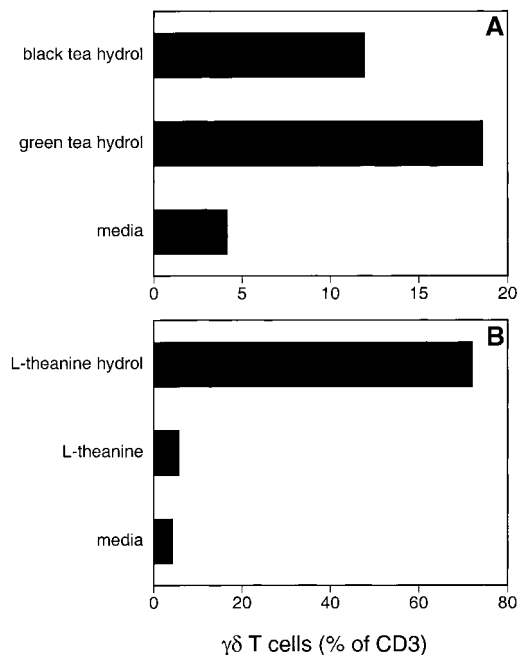


Figure 7. Catabolic Products of Green and Black Tea Cause Expansion of V γ 2V δ 2 T Cells

Acid hydrolyzed green and black tea extracts (A) and hydrolyzed L-theanine (B) caused expansion of V γ 2V δ 2 T cells from PBMC (see legend to Figure 1A).

and vaginal secretions (*iso*-butylamine) from healthy women (Jones et al., 1994). Thus, alkylamine antigens may be derived from either plant foodstuffs or from bacteria and are ubiquitously found in human body fluids. It is important to emphasize that antigenic alkylamines are found *in vivo* in humans in concentrations of up to 10 mM (Perry et al., 1962; Lichtenberger et al., 1991; Jones et al., 1994), well above the 400 μ M concentrations that elicited the $\gamma\delta$ T cell expansions from PBMC *in vitro* (Figure 2).

Proposed Role of V γ 2V δ 2 T Cells in the Immune Response

Detailed knowledge of the antigenic structures recognized by $\alpha\beta$ T cells has deepened our understanding of their role in immunity. An essential key to understanding the role of $\gamma\delta$ T cells in immunity is to identify the nature of the antigens they recognize. The alkylamines in plant foods such as tea, apples, and wine may enhance immunity or provide for immunomodulation. A potential role for $\gamma\delta$ T cells in autoimmunity is underscored by the production of these alkylamine antigens by bowel flora and their presence in various body fluids. Alkylamine antigens secreted by pathogenic bacteria point to a role in host defense. Thus, the alkylamines are a newly identified class of V γ 2V δ 2 T cell antigens with chemical and biological properties distinct from protein, lipid, or phosphate antigens. The widespread occurrence of alkylamine antigens in nature offers ample opportunities for them to be encountered by the immune system under normal conditions such as in the gut in pathological states during microbial infection. As shown by the *in vitro* experiments here, such encounters would likely result in the activation of this major subset of $\gamma\delta$ T cells.

in vitro experiments here, such encounters would likely result in the activation of this major subset of $\gamma\delta$ T cells.

V γ 2V δ 2 T cells may be considered part of the adaptive immune system in that they have a memory phenotype (Parker et al., 1990), junctionally diverse TCRs (Panchamoorthy et al., 1991) that require gene rearrangement for their cell surface expression (Band et al., 1987), and the ability to undergo either anergy or expansion depending on the availability of costimulation (Pawelec et al., 1995; Hara et al., 1996). On the other hand, V γ 2V δ 2 T cells also may be considered part of the innate immune response, since their frequently paired TCR variable region genes V γ 2 and V δ 2 reflect limited germ-line diversity. This V gene pairing enables each V γ 2V δ 2 TCR to immediately recognize families of unprocessed antigens with conserved molecular patterns such as the alkylamines and alkyl phosphates. This TCR-dependent recognition of the conserved one- to five-carbon straight and branched-chain alkyl groups found in antigenic alkylamines and alkyl phosphates that are products of multiple pathogens is reminiscent of the CD14-mediated pattern recognition of pathogen-associated molecular patterns, such as the repeating sugar residues found in various bacterial lipopolysaccharides (Medzhitov and Janeway, 1997). This pattern recognition by the V γ 2V δ 2 TCR allows the expansion of memory $\gamma\delta$ T cells to large numbers in normal adults (mean 4.5% of CD3⁺ T cells) and to 2- to 10-fold higher levels (8%–60% of CD3⁺ T cells) during a host of microbial infections. These large numbers of memory T cells capable of responding to alkylamine antigens produced by microbes thus may bridge the gap between innate and adaptive immune responses.

Experimental Procedures

Transformed Cell Lines

Transformed cell lines were maintained on RPMI-1640 supplemented with 10% bovine calf serum, 10 mM HEPES, penicillin-streptomycin, 5×10^{-5} M β -mercaptoethanol, and L-glutamine (cell culture medium). EBV-transformed lymphoblastoid cell lines (LCL) were derived as previously described and maintained on RPMI. SH-5YSY neuroblastoma cells were obtained from Dr. Gloria Lee, Harvard Medical School, and maintained on RPMI.

Antibody and Antigen Reagents

mAb ascites against T cell antigens used were as follows: control mAb (P3), pan $\gamma\delta$ TCR (anti-TCR δ 1), V δ 1/V δ 1 (dTCS1), V δ 2 (BB3), V γ 2 (7A5), and CD3 (OKT3), CD4 (OKT4), and TCR β (BMA-031). The specificity of these mAbs is reviewed in Porcelli et al. (1991). FITC-conjugated (Fab'); goat anti-mouse IgG was purchased from Tago. The mycobacterial supernatant was obtained by culturing *M. fortuitum* in Middlebrook 7H9 broth (Difco) with 0.5 g/L Tween 80 and 2 ml/L glycerol under agitation at 37°C until stationary phase growth was observed. Two weeks later, mycobacteria were removed by centrifugation followed by 0.2 μ m filtration. This mycobacterial supernatant was then purified as previously described (Tanaka et al., 1995). Ethyl pyrophosphate (EPP) was synthesized as described. L-theanine, methyl-, ethyl-, *n*-propyl-, *iso*-propyl-, *n*-butyl-, *iso*-butyl-, *sec*-butyl-, *iso*-amyl-, and *n*-amylamines were purchased from Sigma. One molar stock solutions were brought to pH 7.4 with HCl and used in half-log dilutions as antigens. Tetanus toxoid (TT) was obtained from the Massachusetts Department of Public Health.

Acid Hydrolysis of Tea Extracts

A concentrated green tea or black tea extract was made by mixing 15 g green or black tea leaves (Gloria Jean's Gourmet Coffees and Teas, Boca Raton, FL) with 500 ml boiling water and steeping for

10 min. The mixture was centrifuged at $200 \times g$ for 20 min and filtered to remove large particles, yielding 400 ml of concentrated tea extract. Five milliliters of these extracts or 500 mg of L-theanine (Sigma) were refluxed in 5N HCl for 2 hr and brought to pH 7.4 with NaOH. These substances were then tested for their ability to expand $\gamma\delta$ T cells from PBMC. Hydrolyzed tea extracts were used at a 1:30 dilution, and hydrolyzed L-theanine was used at 10 mM.

Bacterial Supernatants

Proteus morganii, strain 235, (National Collection of Type Cultures, London, UK) was grown in LB broth at 37°C overnight. The culture supernatant was obtained by centrifugation at $2000 \times g$ for 10 min. In some experiments, this supernatant was added directly to PBMC or to $\gamma\delta$ TCR transfectants to assess its antigenic activity. To enrich for alkylamines, 10 ml of supernatant was saturated with NaCl and brought to pH 1.5 with concentrated sulfuric acid. After centrifugation at $2000 \times g$ for 10 min, the supernatant was extracted thrice with 7 ml diethyl ether, and the aqueous extract was brought to pH 13 with 10 N NaOH. This fraction was then extracted thrice with 7 ml chloroform, and the organic phase was extracted with 3 ml 5 N HCl. The aqueous phase was then dried in an oven at 95°C, reconstituted with 1 ml H₂O, brought to pH 7.4, and passed through a .45 μ m filter (Ghenghesh and Drucker, 1989).

Alkaline Phosphatase Treatment of Antigens

Five-hundred microliters of either undiluted bacterial supernatant, 10 mM isobutylamine, 10 mM monoethylphosphate, or a bacterial supernatant extracted to enrich for amines (see above) were mock treated or treated with 5 U shrimp alkaline phosphatase (Sigma) for 2 hr at 37°C and used as antigens (Tanaka et al., 1994).

GC-Mass Spectrometry

Samples were analyzed for alkylamine content by quantitative headspace gas chromatography and mass spectrometry. To each sample we added 0.13 mM *tert*-butylamine, an alkylamine not known to occur in nature, as an internal standard. One milliliter samples of alkylamine standards, bacterial supernatants, or extracts of bacterial supernatants in headspace vials were treated with 100 μ l 10 N NaOH to volatilize alkylamines and were brought to 80°C for 10 min. One milliliter of headspace was injected at 250°C into a PTA-5 column. Analysis was carried out with a 3 min hold at -20°C, followed by a temperature increase at 10°C per minute up to 280°C, using 1 ml/min (10 psi) flow rate of helium carrier gas. Mass spectrometry analysis was performed with a Finnigan MAT 8230 mass spectrometer. Peak areas were integrated and results were reported as parts per million (ppm) per 1 ml headspace and converted to molarity based on the original 1 ml liquid sample volume.

Antigen Stimulation of PBMC

PBMC obtained from random healthy donors were cultured at one million cells per well in 24-well flat-bottom plates with or without antigen in RPMI media with the additives described above at 37°C. On day 3 IL-2 was added to 0.5 nM, and on day 12-14 the cells were counted and analyzed by flow cytometry using TCR V gene specific mAbs.

V γ 2V δ 2 T Cell Line

PBMC from a normal healthy donor were treated with the pan $\gamma\delta$ mAb anti-TCR δ 1, and positive selection was carried out using magnetic beads charged with goat anti-mouse Ig (Dyna). Positively selected cells (100,000) were placed in 24-well plates with an equal number of γ -irradiated PBMC and 500,000 γ -irradiated LCL with PHA-P (Difco), and 1 nM IL-2. These cells were subjected to a negative selection procedure using mAbs OKT4 and BMA-031 treatment followed by magnetic beads charged with goat anti-mouse Ig. The resulting population of cells was >99% V γ 2V δ 2 TCR⁺ and is named G2D2a.

V γ 2V δ 2 T Cell Clones

The V γ 2V δ 2 T cell clones were DGSF.13, which was derived from the synovial fluid of a patient with rheumatoid arthritis, and 12G12, DG.SPF6, and CP.1.15, which were derived from the PBMC of healthy donors by stimulation with a mycobacterial extract (Morita

et al., 1991). The $\alpha\beta$ T cell clones L6.D10, HD.13, HY198, HD.49, and L6.FS were isolated from the PBMC of healthy donors by limiting dilution and expanded with PHA (Tanaka et al., 1995).

Transfectants

The DBS43 and 27/3.62 transfectants were made as previously described (Bukowski et al., 1995). DBS43 was made by transfecting TCR⁻ J.RT3-T3.5 cells with cDNA made from DG.SF13, a V γ 2V δ 2 TCR⁺ phosphate antigen-reactive $\gamma\delta$ T cell clone obtained by stimulation of synovial fluid mononuclear cells with a mycobacterial extract. 27/3.62 was obtained by transfecting J.RT3-T3.5 cells with cDNA made from F7, a V γ 1V δ 1 TCR⁺ $\gamma\delta$ T cell clone.

Stimulation of T Cell Clones and Transfectants

Stimulation of T cell clones and transfectants was performed in 96-well flat-bottom plates with 1×10^5 responder cells per well in 0.2 ml of cell culture medium (Bukowski et al., 1995). In some experiments, 5×10^4 mitomycin-treated or glutaraldehyde-fixed B LCL or SH-5YSY neuroblastoma cells per well were used as feeders or antigen-presenting cells, but these antigen-presenting cells were not necessary to obtain IL-2 release from the transfectant Jurkat cells. Half-log dilutions of antigen or, as a positive control, the calcium ionophore ionomycin (at 1 μ g/ml) were added in the presence of 10 ng/ml PMA as a costimulator (Saito et al., 1987). After 24 hr, supernatants were harvested and tested at a final dilution of 1:8 for their ability to stimulate the growth of the IL-2-dependent HT-2 cell line. Proliferation assays were performed in triplicate using 5×10^3 HT-2 cells per flat-bottom well of a 96-well plate. After 18 hr, the cells were pulsed with [³H]thymidine (1 μ Ci/well), harvested at 24 hr, and counted by liquid scintillation on a Betaplate system. The standard deviation of the triplicate determination was less than 10% of the mean.

Acknowledgments

The authors thank Dr. Thomas Hartman, Center for Advanced Food Technology, Rutgers University, New Brunswick, NJ, USA for performing GC-mass spectrometry analysis. This research was supported by grants from the National Institute of Allergy and Infectious Disease, the National Institute of Arthritis, Musculoskeletal, and Skin Diseases, the Office of Alternative Medicine, and the Arthritis Foundation.

Received April 28, 1999.

References

- Allison, C., and Macfarlane, G.T. (1989). Influence of pH, nutrient availability, and growth rate on amine production by *Bacteroides fragilis* and *Clostridium perfringens*. Appl. Environ. Microbiol. 55, 2894-2898.
- Asatoor, A.M. (1966). Tea as a source of urinary ethylamine. Nature 210, 1358-1360.
- Balbi, B., Valle, M.T., Oddera, S., Giunti, D., Manca, F., Rossi, G.A., and Allegra, L. (1993). T-lymphocytes with $\gamma\delta^+$ V δ 2⁺ antigen receptors are present in increased proportions in a fraction of patients with tuberculosis or with sarcoidosis. Am. Rev. Respir. Dis. 148, 1685-1690.
- Band, H., Hochstenbach, F., McLean, J., Hata, S., Krangel, M.S., and Brenner, M.B. (1987). Immunogenetic proof that a novel rearranging gene encodes the T cell receptor δ subunit. Science 238, 682-684.
- Bertotto, A., Gerli, R., Spinuzzi, F., Muscat, C., Scalise, F., Castellucci, G., Sposito, M., Candio, F., and Vaccaro, R. (1993). Lymphocytes bearing the $\gamma\delta$ T cell receptor in acute *Brucella melitensis* infection. Eur. J. Immunol. 23, 1177-1180.
- Bukowski, J.F., Morita, C.T., Tanaka, Y., Bloom, B.R., Brenner, M.B., and Band, H. (1995). V γ 2V δ 2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. J. Immunol. 154, 998-1006.
- Burk, M.R., Mori, L., and De Libero, G. (1995). Human V γ 9-V δ 2 cells

- are stimulated in a cross-reactive fashion by a variety of phosphorylated metabolites. *Eur. J. Immunol.* **25**, 2052–2058.
- Caldwell, C.W., Everett, E.D., McDonald, G., Yesus, Y.W., Roland, W.E., and Huang, H.M. (1996). Apoptosis of γ/δ T cells in human ehrlichiosis. *Am. J. Clin. Pathol.* **105**, 640–646.
- Cartwright, R., Roberts, E., and Wood, D. (1954). Theanine, an amino acid *N*-ethylamide, present in tea. *J. Sci. Food Agric.* **5**, 597–599.
- Daneshvar, M.I., Brooks, J.B., Malcolm, G.B., and Pine, L. (1989). Analyses of fermentation products of *Listeria* species by frequency-pulsed electron-capture gas-liquid chromatography. *Can. J. Microbiol.* **35**, 786–793.
- Davodeau, F., Peyrat, M.A., Hallet, M.M., Houde, I., Vie, H., and Bonneville, M. (1993). Peripheral selection of antigen receptor junctional features in a major human γ/δ subset. *Eur. J. Immunol.* **23**, 804–808.
- De Libero, G., Casorati, G., Giachino, C., Carbonara, C., Migone, N., Matzinger, P., and Lanzavecchia, A. (1991). Selection by two powerful antigens may account for the presence of the major population of human peripheral γ/δ T cells. *J. Exp. Med.* **173**, 1311–1322.
- De Maria, A., Ferrazin, A., Ferrini, S., Ciccone, E., Terragna, A., and Moretta, L. (1992). Selective increase of a subset of T cell receptor γ/δ T lymphocytes in the peripheral blood of patients with human immunodeficiency virus type 1 infection. *J. Infect. Dis.* **165**, 917–919.
- Eschenbach, D.A. (1993). History and review of bacterial vaginosis. *Am. J. Obstet. Gynecol.* **169**, 441–445.
- Ghenghesh, K.S., and Drucker, D.B. (1989). Gas liquid chromatography of amines produced by the *Enterobacteriaceae*. *Brazilian J. Med. Biol. Res.* **22**, 653–665.
- Graham, H.N. (1992). Green tea composition, consumption, and polyphenol chemistry. *Prev. Med.* **21**, 334–350.
- Hara, T., Mizuno, Y., Takaki, K., Takada, H., Akeda, H., Aoki, T., Nagata, M., Ueda, K., Matsuzaki, G., Yoshikai, Y., and Nomoto, K. (1992). Predominant activation and expansion of V γ 9-bearing γ/δ T cells in vivo as well as in vitro in Salmonella infection. *J. Clin. Invest.* **90**, 204–210.
- Hara, T., Ohashi, S., Yamashita, Y., Abe, T., Hisaeda, H., Himeno, K., Good, R.A., and Takeshita, K. (1996). Human V δ 2⁺ γ/δ T-cell tolerance to foreign antigens of *Toxoplasma gondii*. *Proc. Natl. Acad. Sci. USA* **93**, 5136–5140.
- Harrison, L.C., Dempsey-Collier, M., Kramer, D.R., and Takahashi, K. (1996). Aerosol insulin induces regulatory CD8 γ/δ T cells that prevent murine insulin-dependent diabetes. *J. Exp. Med.* **184**, 2167–2174.
- Hartmann, T. (1967). [Detection of n-butylamine in apples]. *Experientia* **23**, 680–681.
- Hermann, E., Lohse, A.W., Mayet, W.J., van der Zee, R., Van Eden, W., Probst, P., Poralla, T., Meyer zum Buschenfelde, K.H., and Fleischer, B. (1992). Stimulation of synovial fluid mononuclear cells with the human 65-kD heat shock protein or with live enterobacteria leads to preferential expansion of TCR- γ/δ ⁺ lymphocytes. *Clin. Exp. Immunol.* **89**, 427–433.
- Hiromatsu, K., Yoshikai, Y., Matsuzaki, G., Ohga, S., Muramori, K., Matsumoto, K., Bluestone, J.A., and Nomoto, K. (1992). A protective role of γ/δ T cells in primary infection with *Listeria monocytogenes* in mice. *J. Exp. Med.* **175**, 49–56.
- Ibe, A., Saito, K., Nakazato, M., Kikuchi, Y., Fujinuma, K., and Nishima, T. (1991). Quantitative determination of amines in wine by liquid chromatography. *J. AOAC Int.* **74**, 695–698.
- Jones, B.M., Al-Fattani, M., and Gooch, H. (1994). The determination of amines in the vaginal secretions of women in health and disease. *Int. J. STD AIDS* **5**, 52–55.
- Jouen-Beades, F., Paris, E., Dieulois, C., Lemeland, J.F., Barre, D.V., Marret, S., Humbert, G., Leroy, J., and Tron, F. (1997). In vivo and in vitro activation and expansion of γ/δ T cells during *Listeria monocytogenes* infection in humans. *Infect. Immun.* **65**, 4267–4272.
- Ke, Y., Pearce, K., Lake, J.P., Ziegler, H.K., and Kapp, J.A. (1997). Gamma delta T lymphocytes regulate the induction and maintenance of oral tolerance. *J. Immunol.* **158**, 3610–3618.
- Ladel, C.H., Blum, C., Dreher, A., Reifenberg, K., and Kaufmann, S.H. (1995). Protective role of γ/δ T cells and $\alpha\beta$ T cells in tuberculosis. *Eur. J. Immunol.* **25**, 2877–2881.
- Li, H., Lebedeva, M.I., Liera, A.S., Fields, B.A., Brenner, M.B., and Mariuzza, R.A. (1998). Crystal structure of the V δ domain of a human γ/δ T cell antigen receptor. *Nature* **391**, 502–506.
- Lichtenberger, L.M., Gardner, J.W., Barreto, J.C., and Morriss, F.H., Jr. (1991). Evidence for a role of volatile amines in the development of neonatal hypergastrinemia. *J. Pediatr. Gastroenterol. Nutr.* **13**, 342–346.
- Lundqvist, C., and Hammarstrom, M.L. (1993). T-cell receptor gamma delta-expressing intraepithelial lymphocytes are present in normal and chronically inflamed human gingiva. *Immunology* **79**, 38–45.
- McMenamin, C., Pimm, C., McKersey, M., and Holt, P.G. (1994). Regulation of IgE responses to inhaled antigen in mice by antigen-specific gamma delta T cells. *Science* **265**, 1869–1871.
- Medzhitov, R., and Janeway, C.A. (1997). Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295–298.
- Mengel, J., Cardillo, F., Aroeira, L.S., Williams, O., Russo, M., and Vaz, N.M. (1995). Anti-gamma delta T cell antibody blocks the induction and maintenance of oral tolerance to ovalbumin in mice. *Immunol. Lett.* **48**, 97–102.
- Mincheva, N.L., Hammarstrom, S., and Hammarstrom, M.L. (1992). Human decidual leukocytes from early pregnancy contain high numbers of gamma delta+ cells and show selective down-regulation of alloreactivity. *J. Immunol.* **149**, 2203–2211.
- Morita, C.T., Verma, S., Aparicio, P., Martinez, C., Spits, H., and Brenner, M.B. (1991). Functionally distinct subsets of human γ/δ T cells. *Eur. J. Immunol.* **21**, 2999–3007.
- Munk, M.E., Gatrill, A.J., and Kaufmann, S.H.E. (1990). Target cell lysis and IL-2 secretion by γ/δ T lymphocytes after activation with bacteria. *J. Immunol.* **145**, 2434–2439.
- Padlan, E.A., Davies, D.R., Rudikoff, S., and Potter, M. (1976). Structural basis for the specificity of phosphorylcholine-binding immunoglobulins. *Immunochemistry* **13**, 945–949.
- Panchamoorthy, G., McLean, J., Modlin, R.L., Morita, C.T., Ishikawa, S., Brenner, M.B., and Band, H. (1991). A predominance of the T cell receptor V γ 2/V δ 2 subset in human mycobacteria-responsive T cells suggests germline gene encoded recognition. *J. Immunol.* **147**, 3360–3369.
- Parker, C.M., Groh, V., Band, H., Porcelli, S.A., Morita, C., Fabbi, M., Glass, D., Strominger, J.L., and Brenner, M.B. (1990). Evidence for extrathymic changes in the T cell receptor γ/δ repertoire. *J. Exp. Med.* **171**, 1597–1612.
- Pawelec, G., Friccius, H., Boshell, M., Siegels-Hubenthal, P., Rehbein, A., Schlotz, E., Pohla, H., Schaudt, K., and Sansom, D. (1995). Human γ/δ T cells are resistant to induction of anergy but not to induction of cell death in vitro. *Cell. Immunol.* **162**, 8–15.
- Perera, M.K., Carter, R., Goonewardene, R., and Mendis, K.N. (1994). Transient increase in circulating γ/δ T cells during *Plasmodium vivax* malarial paroxysms. *J. Exp. Med.* **179**, 311–315.
- Perry, T.L., Shaw, N.F., Walker, D., and Redlich, D. (1962). Urinary excretion of amines in normal children. *Pediatrics* **30**, 576–584.
- Peterman, G.M., Spencer, C., Sperling, A.I., and Bluestone, J.A. (1993). Role of γ/δ T cells in murine collagen-induced arthritis. *J. Immunol.* **151**, 6546–6558.
- Porcelli, S., Brenner, M.B., and Band, H. (1991). Biology of the human γ/δ T-cell receptor. *Immunol. Rev.* **120**, 137–183.
- Raziuddin, S., Telmasani, A.W., El-Awad, M.E., Al-Amari, O., and Al-Janadi, M. (1992). γ/δ T cells and the immune response in visceral leishmaniasis. *Eur. J. Immunol.* **22**, 1143–1148.
- Rock, E.P., Sibbald, P.R., Davis, M.M., and Chien, Y.-H. (1994). CDR3 length in antigen-specific immune receptors. *J. Exp. Med.* **179**, 323–328.
- Saito, T., Weiss, A., Miller, J., Norcross, M.A., and Germain, R.N. (1987). Specific antigen-Ia activation of transfected human T cells expressing murine T $\alpha\beta$ -human T3 receptor complexes. *Nature* **325**, 125–130.
- Sakato, Y. (1949). Studies on the chemical constituents of tea. III. On a new amide theanine. *Nippon Goei Kagaku Kaishi* **23**, 262–271.
- Scalise, F., Gerli, R., Castellucci, G., Spinozzi, F., Fabietti, G.M.,

- Crupi, S., Sensi, L., Britta, R., Vaccaro, R., and Bertotto, A. (1992). Lymphocytes bearing the $\gamma\delta$ T-cell receptor in acute toxoplasmosis. *Immunology* 76, 668–670.
- Schild, H., Mavaddat, N., Litzberger, C., Ehrlich, E.W., Davis, M.M., Bluestone, J.A., Matis, L., Draper, R.K., and Chien, Y.-H. (1994). The nature of major histocompatibility complex recognition by $\gamma\delta$ T cells. *Cell* 76, 29–37.
- Schoel, B., Sprenger, S., and Kaufmann, S.H.E. (1994). Phosphate is essential for stimulation of V γ 9V δ 2 T lymphocytes by mycobacterial low molecular weight ligand. *Eur. J. Immunol.* 24, 1886–1892.
- Sciammas, R., Johnson, R.M., Sperling, A.I., Brady, W., Linsley, P.S., Spear, P.G., Fitch, F.W., and Bluestone, J.A. (1994). Unique antigen recognition by a herpesvirus-specific TCR- $\gamma\delta$ cell. *J. Immunol.* 152, 5392–5397.
- Sciammas, R., Kokukula, Q., Tang, R.L., Hendricks, R.L., and Bluestone, J.A. (1997). T cell receptor $\gamma\delta$ cells protect mice from herpes simplex type 1-induced lethal encephalitis. *J. Exp. Med.* 185, 1969–1975.
- Sumida, T., Maeda, T., Takahashi, H., Yoshida, S., Yonaha, F., Sakamoto, A., Tomioka, H., Koike, T., and Yoshida, S. (1992). Predominant expansion of V γ 9/V δ 2 T cells in a tularemia patient. *Infect. Immun.* 60, 2554–2558.
- Tanaka, Y., Sano, S., Nieves, E., De Libero, G., Roca, D., Modlin, R.L., Brenner, M.B., Bloom, B.R., and Morita, C.T. (1994). Nonpeptide ligands for human $\gamma\delta$ T cells. *Proc. Natl. Acad. Sci. USA* 91, 8175–8179.
- Tanaka, Y., Morita, C.T., Tanaka, Y., Nieves, E., Brenner, M.B., and Bloom, B.R. (1995). Natural and synthetic non-peptide antigens recognized by human $\gamma\delta$ T cells. *Nature* 375, 155–158.
- Tanner, A., and Stillman, N. (1993). Oral and dental infections with anaerobic bacteria: clinical features, predominant pathogens, and treatment. *Clin. Infect. Dis.* 4, S304–S309.
- Tsuji, M., Mombaerts, P., Lefrancois, L., Nussenzweig, R.S., Zavala, F., and Tonegawa, S. (1994). $\gamma\delta$ T cells contribute to immunity against the liver stages of malaria in $\alpha\beta$ T-cell-deficient mice. *Proc. Natl. Acad. Sci. USA* 91, 345–349.
- Zenka, J., Hulinska, D., and Jegorov, A. (1989). An analysis of *Trichinella pseudospiralis* excretions and study of their effect on striated muscles of mouse. *Folia Parasitol.* 36, 177–183.