

# Activation-induced Modification in the CD3 Complex of the $\gamma\delta$ T Cell Receptor

Sandra M. Hayes,<sup>1</sup> Karen Laky,<sup>2</sup> Dalal El-Khoury,<sup>1</sup> Dietmar J. Kappes,<sup>3</sup>  
B.J. Fowlkes,<sup>2</sup> and Paul E. Love<sup>1</sup>

<sup>1</sup>Laboratory of Mammalian Genes and Development, National Institute of Child Health and Human Development and <sup>2</sup>Laboratory of Molecular and Cellular Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

<sup>3</sup>Division of Basic Science, Fox Chase Cancer Center, Philadelphia, PA 19111

## Abstract

The T cell antigen receptor complexes expressed on  $\alpha\beta$  and  $\gamma\delta$  T cells differ not only in their respective clonotypic heterodimers but also in the subunit composition of their CD3 complexes. The  $\gamma\delta$  T cell receptors (TCRs) expressed on ex vivo  $\gamma\delta$  T cells lack CD3 $\delta$ , whereas  $\alpha\beta$  TCRs contain CD3 $\delta$ . While this result correlates with the phenotype of CD3 $\delta^{-/-}$  mice, in which  $\gamma\delta$  T cell development is unaffected, it is inconsistent with the results of previous studies reporting that CD3 $\delta$  is a component of the  $\gamma\delta$  TCR. Since earlier studies examined the subunit composition of  $\gamma\delta$  TCRs expressed on activated and expanded peripheral  $\gamma\delta$  T cells or  $\gamma\delta$  TCR<sup>+</sup> intestinal intraepithelial lymphocytes, we hypothesized that activation and expansion may lead to changes in the CD3 subunit composition of the  $\gamma\delta$  TCR. Here, we report that activation and expansion do in fact result in the inclusion of a protein, comparable in mass and mobility to CD3 $\delta$ , in the  $\gamma\delta$  TCR. Further analyses revealed that this protein is not CD3 $\delta$ , but instead is a differentially glycosylated form of CD3 $\gamma$ . These results provide further evidence for a major difference in the subunit composition of  $\alpha\beta$ - and  $\gamma\delta$  TCR complexes and raise the possibility that modification of CD3 $\gamma$  may have important functional consequences in activated  $\gamma\delta$  T cells.

Key words: T cell receptor • structure • glycosylation • CD3 • activation

## Introduction

The antigen receptor on T cells is a multisubunit complex composed of a clonotypic heterodimer and four invariant signaling subunits, CD3 $\gamma$ , CD3 $\delta$ , CD3 $\epsilon$ , and TCR- $\zeta$ . The generally accepted stoichiometry of the  $\alpha\beta$  TCR is non- $\gamma\delta$  TCR- $\alpha\beta$ , CD3 $\gamma\epsilon$ , CD3 $\delta\epsilon$ , and TCR- $\zeta\zeta$  (1). Previous data obtained from expanded populations of peripheral  $\gamma\delta$  T cells suggested a similar structure for the  $\gamma\delta$  TCR, except that the  $\gamma\delta$  TCR could contain the TCR- $\zeta$  family member, Fc $\epsilon$ R1  $\gamma$  (FcR $\gamma$ ), as a homodimer or as a heterodimer with TCR- $\zeta$  in lieu of the TCR- $\zeta$  homodimer (2). However, recent data have called for a revision in this model of  $\gamma\delta$  TCR structure. For example, the observation that FcR $\gamma$  is a component of the  $\gamma\delta$  TCR conflicts with the findings that FcR $\gamma$  transcripts are not detected in purified thymic and peripheral  $\gamma\delta$  T cells (3) and that  $\gamma\delta$  T cell de-

velopment is unaffected in FcR $\gamma^{-/-}$  mice (4, 5). We extended these studies by analyzing the subunit composition of  $\gamma\delta$  TCR complexes on ex vivo  $\gamma\delta$  T cells from  $\gamma\delta$  TCR Tg and non $\gamma\delta$ -TCR Tg mice (6). Biochemical analyses showed that TCR- $\zeta$  is a component of the  $\gamma\delta$  TCR on ex vivo  $\gamma\delta$  T cells, while FcR $\gamma$  is not. However, after in vitro activation and expansion, FcR $\gamma$  is expressed and incorporated into the  $\gamma\delta$  TCR complex (6). These data show that activation and expansion induce changes in the  $\gamma\delta$  TCR complex and demonstrate that the subunit composition of  $\gamma\delta$  TCRs expressed by activated and expanded populations of  $\gamma\delta$  T cells does not accurately represent the subunit composition of TCR complexes expressed by primary  $\gamma\delta$  T cells.

A more striking inconsistency with the previous model of  $\gamma\delta$  TCR structure was the finding that the  $\gamma\delta$  TCRs expressed on ex vivo thymic and lymph node  $\gamma\delta$  T cells do not contain CD3 $\delta$  (6). This result appears to contradict previous studies, in which the CD3 subunit composition of the  $\alpha\beta$  TCR and  $\gamma\delta$  TCR was found to be identical (2, 7,

Address correspondence to Paul E. Love, Laboratory of Mammalian Genes and Development, NICHD/NIH Bldg. 6B, Rm. 2B-210, 9000 Rockville Pike, Bethesda, MD 20892-2780. Phone: 301-402-4946; Fax: 301-480-6302; E-mail: pel@helix.nih.gov

8). As these previous studies used expanded populations of peripheral  $\gamma\delta$  T cells,  $\gamma\delta$  TCR<sup>+</sup> intestinal intraepithelial lymphocytes (iIELs)\* and  $\gamma\delta$  T cell hybridomas as sources of  $\gamma\delta$  T cells, it is possible that activation and expansion induced a change in the subunit composition of the CD3 complex. To test this, we compared the subunit composition of TCR complexes expressed by  $\gamma\delta$  T cells before and after in vitro activation and expansion. Here, we report the detection of a protein, equivalent in mass to CD3 $\delta$ , in the  $\gamma\delta$  TCR after activation and expansion. Biochemical analyses revealed that this protein was not CD3 $\delta$  but a differentially glycosylated form of CD3 $\gamma$ . In addition, modification of the CD3 $\gamma$  subunit was observed in in vitro-activated  $\alpha\beta$  T cells and in iIELs.

Together, these results demonstrate that the protein initially thought to be CD3 $\delta$  in the  $\gamma\delta$  TCR complex is, instead, a differentially glycosylated form of CD3 $\gamma$  and provide further substantiation for the conclusion that the  $\alpha\beta$  TCR and  $\gamma\delta$  TCR complexes differ in their subunit composition.

## Materials and Methods

**Mice.** B6.129-TCR- $\beta^{-/-}$  (TCR- $\beta^{-/-}$ ) mice (9) were purchased from The Jackson Laboratory. C57BL/6 (B6), C57BL/6-V $\gamma$ 6-J $\gamma$ 1-C $\gamma$ 1/V $\delta$ 1-D $\delta$ 2-J $\delta$ 2-C $\delta$   $\gamma\delta$  TCR Tg ( $\gamma\delta$  TCR Tg, line 134) (10), and C57BL/6-CD3 $\delta^{-/-}$  (CD3 $\delta^{-/-}$ ) (11) mice were maintained in our animal facility. All experimental mice were killed at 5–8 wk of age.

**Antibodies.** Antibodies used for cell separation were FITC-labeled anti-CD19 (1D3), FITC-labeled anti-I-A<sup>b</sup> (AF6–120.1), FITC-labeled anti-CD4 (RM4–5), FITC-labeled anti-CD8 $\alpha$  (53–6.7), and FITC-labeled anti-TCR- $\beta$  (H57–597), all of which were purchased from BD PharMingen. Antibodies used for stimulation and biochemical analysis were anti- $\gamma\delta$ TCR (UC7–13D5 and GL4), anti-TCR- $\beta$  (H57–597), and CD3 $\epsilon$  (145–2C11) (BD PharMingen), hamster anti-TCR- $\zeta$  (H146) and hamster anti-CD3 $\gamma$  (H25) (provided by D. Wiest, Fox Chase Cancer Center, Philadelphia, PA), rabbit anti-CD3 $\delta$  (R9) (provided by L. Samelson, National Institutes of Health, Bethesda, MD), goat anti-CD3 $\gamma$  and goat anti-CD3 $\epsilon$  (Santa Cruz Biotechnology, Inc.), HRP-conjugated Protein A (Transduction Laboratories), and donkey anti-goat IgG (H + L)-HRP (Jackson ImmunoResearch Laboratories).

**Isolation of iIELs.** iIELs were isolated as described previously (12).

**Cell Separation.** Peripheral  $\alpha\beta$  and  $\gamma\delta$  T cells were purified from lymph node cells from B6 and  $\gamma\delta$  TCR Tg mice, respectively, using the MACS<sup>®</sup> magnetic bead separation system (Miltenyi Biotec) as described previously (6). The purity of the resulting cell populations was typically 98% for  $\alpha\beta$  T cells and 99% for  $\gamma\delta$  T cells.

**Stimulation and Expansion of T Cells In Vitro.**  $3 \times 10^6$  unfractionated lymph node cells were stimulated on 6-well plates coated with anti- $\gamma\delta$ TCR mAb (10  $\mu$ g/ml GL4), anti-TCR- $\beta$  mAb (10  $\mu$ g/ml H57–597), or anti-V $\gamma$ 1 mAb (10  $\mu$ g/ml 2.11; reference 13) for 2 d and then expanded in the presence of 40

U/ml of murine rIL-2 (Peprotech, Inc.) for 2–12 more days to replicate conditions used in previous studies (2, 6). Unless otherwise noted, stimulated  $\alpha\beta$  and  $\gamma\delta$  T cells were harvested and analyzed 6 d after activation. Phenotypic analysis on day 6 revealed that cultures stimulated with anti- $\gamma\delta$ TCR mAb or with anti-V $\gamma$ 1 mAb were 100%  $\gamma\delta$  TCR<sup>+</sup> or V $\gamma$ 1<sup>+</sup>, respectively. Similarly, cultures of B6 and CD3 $\delta^{-/-}$  lymph node cells stimulated with anti- $\alpha\beta$ TCR mAb were 100%  $\alpha\beta$  TCR<sup>+</sup> and were predominantly CD4<sup>+</sup> CD8<sup>+</sup>. The only major difference detected between the B6 and CD3 $\delta^{-/-}$ -stimulated  $\alpha\beta$  T cells was in the level of  $\alpha\beta$  TCR expression, with the level of surface TCR on CD3 $\delta^{-/-}$   $\alpha\beta$  T cells being seven to eightfold less than that observed on B6  $\alpha\beta$  T cells.

**Surface Biotinylation.** Biotinylation of surface proteins on purified  $\alpha\beta$  and  $\gamma\delta$  T cells was performed as described previously (6).

**Deglycosylation of TCR Subunits.** Removal of N-linked glycans from glycoproteins in the TCR complex has been described previously (14). In brief, immunoprecipitated TCR proteins were denatured by boiling in 1% SDS/1% 2-mercaptoethanol for 5 min. The eluted proteins were neutralized by the addition of 10% NP-40 and then resuspended in the reaction buffer provided by the manufacturer (New England Biolabs). Each sample was treated with 2,500 U peptide-N-glycosidase F (PNGase F; New England Biolabs) and then allowed to incubate overnight at room temperature. The digestion reaction was quenched with 2 $\times$  SDS sample buffer containing 2-mercaptoethanol.

**Protein Analysis.** Immunoprecipitations and immunoblotting were performed as described previously (15), except that cells were lysed in a buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, protease inhibitors (Roche Laboratories), and 1% Triton X-100 (Calbiochem). Two-dimensional (2-D) SDS-PAGE analysis of TCR subunits has been described previously (6). Biotinylated surface proteins were detected using ABC-HRP (Vector Laboratories) and the ECL Western blot detection system (Amersham Pharmacia Biotech).

## Results and Discussion

**Alteration in the CD3 Subunit Composition of the  $\gamma\delta$  TCR After TCR Stimulation.** To determine whether in vitro activation and expansion lead to changes in the CD3 subunit composition of the  $\gamma\delta$  TCR, we compared the subunit composition of surface CD3 complexes expressed by primary and in vitro activated and expanded  $\gamma\delta$  T cells from V $\gamma$ 6-J $\gamma$ 1-C $\gamma$ 1/V $\delta$ 1-D $\delta$ 2-J $\delta$ 2-C $\delta$   $\gamma\delta$  TCR Tg ( $\gamma\delta$  TCR Tg) mice (10). As shown in Fig. 1, 2-D SDS-PAGE analysis of anti- $\gamma\delta$ TCR immunoprecipitates revealed the presence of a 26-kD protein in CD3 complexes expressed on stimulated but not on unstimulated ex vivo  $\gamma\delta$  T cells. This protein was also observed in surface CD3 complexes when anti-TCR- $\zeta$  and anti-CD3 $\epsilon$  mAbs (Fig. 1 and unpublished data) were used to immunoprecipitate TCR complexes from stimulated  $\gamma\delta$  T cells. Examination of the kinetics of appearance of the 26-kD protein in the  $\gamma\delta$  TCR after TCR activation and expansion showed that it first appeared in surface  $\gamma\delta$  TCRs at 5 d after activation and was still observed at the latest time point of the experiment (12 d after activation; unpublished data). The 26-kD protein was also detected in the CD3 complexes expressed by polyclonal  $\gamma\delta$  T cell populations from TCR- $\beta^{-/-}$  mice after TCR activation and expansion (unpublished data). Comparison of

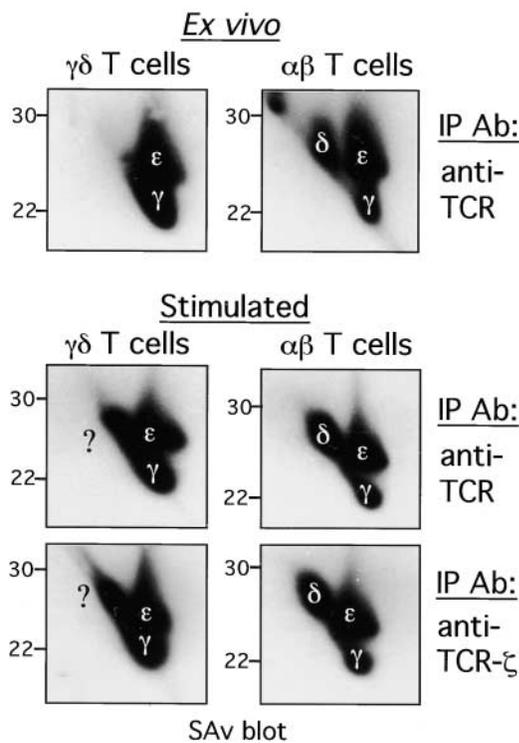
\*Abbreviations used in this paper: 2-D, two-dimensional; Fc $\gamma$ R, Fc $\epsilon$ R1  $\gamma$  chain; iIEL, intestinal intraepithelial lymphocyte; PNGase, peptide-N-glycosidase.

the CD3 complexes on stimulated  $\alpha\beta$  and  $\gamma\delta$  T cells by 2-D SDS-PAGE analysis demonstrated that the 26-kD protein, detected in TCR complexes on activated  $\gamma\delta$  T cells, migrated similarly to CD3 $\delta$  (Fig. 1). Therefore, these results demonstrated that activation and expansion induce the inclusion of a protein, comparable in mass and mobility to CD3 $\delta$ , in the  $\gamma\delta$  TCR.

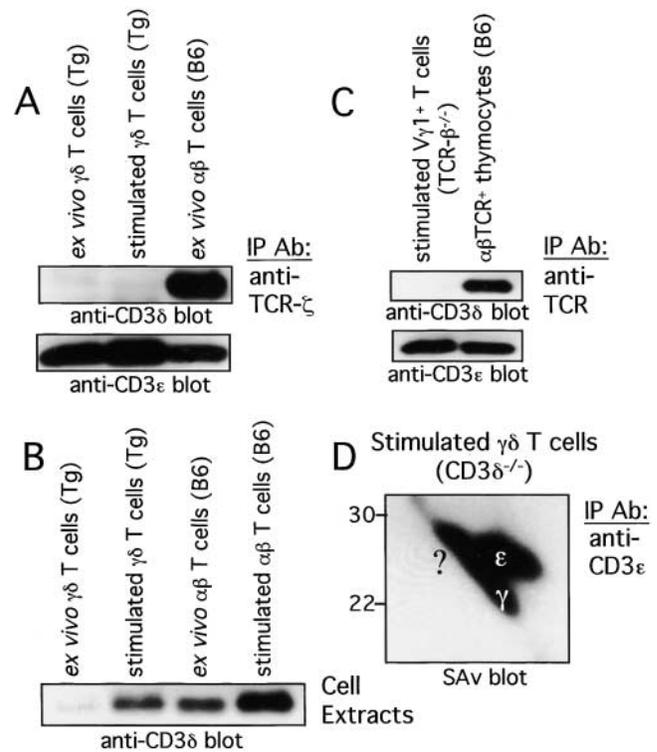
*CD3 $\delta$  Is Not a Component of the TCR Expressed by In Vitro Activated and Expanded  $\gamma\delta$  T Cells.* To determine whether the 26-kD protein in the  $\gamma\delta$  TCR was in fact CD3 $\delta$ , fully assembled TCR complexes from stimulated  $\gamma\delta$  T cells were recovered by immunoprecipitation with anti-TCR- $\zeta$  mAb and then immunoblotted with anti-CD3 $\delta$  serum. As shown previously (6) and in Fig. 2 A, little or no CD3 $\delta$  was detected in anti-TCR- $\zeta$  immunoprecipitates from ex vivo  $\gamma\delta$  T cells. In contrast, CD3 $\delta$  was detected in anti-TCR- $\zeta$  immunoprecipitates from ex vivo  $\alpha\beta$  T cells (Fig. 2 A). Interestingly, the anti-CD3 $\delta$  serum did not react with any protein in the  $\gamma\delta$  TCRs recovered from stimulated  $\gamma\delta$  T cells (Fig. 2 A). This finding is even more striking given the fact that total cellular CD3 $\delta$  pro-

tein was increased in stimulated  $\gamma\delta$  T cell extracts relative to ex vivo  $\gamma\delta$  T cell extracts (Fig. 2 B). These data agree with those of our previous study, in which we reported that the TCR- $\gamma$  and TCR- $\delta$  chains are unable to pair efficiently with CD3 $\delta\epsilon$  dimers (6).

As the TCR- $\gamma$  chain in the transgenic  $\gamma\delta$  TCR utilizes the C $\gamma$ 1 gene segment, we next determined whether TCR- $\gamma$  chains using the C $\gamma$ 4 gene segment (the other major C $\gamma$  gene segment expressed in peripheral  $\gamma\delta$  T cells; reference 13) could pair with CD3 $\delta\epsilon$  dimers. Using the anti-V $\gamma$ 1 mAb (13), we selectively activated V $\gamma$ 1-J $\gamma$ 4-C $\gamma$ 4-bearing  $\gamma\delta$  T cells from TCR- $\beta^{-/-}$  mice. 2-D SDS-PAGE analysis of surface biotinylated TCR subunits from stimu-



**Figure 1.** Effect of in vitro activation and expansion on the subunit composition of  $\alpha\beta$ - and  $\gamma\delta$  TCR complexes. Ex vivo  $\alpha\beta$  and  $\gamma\delta$  T cells were purified from the lymph nodes of B6 and  $\gamma\delta$  TCR Tg mice, respectively. Stimulated  $\alpha\beta$  and  $\gamma\delta$  T cells, from B6 and  $\gamma\delta$  TCR Tg mice, respectively, were generated as described in Materials and Methods. Surface proteins were labeled with biotin and  $\alpha\beta$  and  $\gamma\delta$  TCR complexes were immunoprecipitated using anti-TCR mAbs (H57-597 and UC7-13D5, respectively) or anti-TCR- $\zeta$  mAb (H146). Immunoprecipitated proteins were resolved by nonreducing/reducing 2-D SDS-PAGE. ABC-HRP and chemiluminescence were used to visualize surface biotinylated proteins. The positions of the CD3 subunits and the unknown 26-kD subunit (?) are marked.



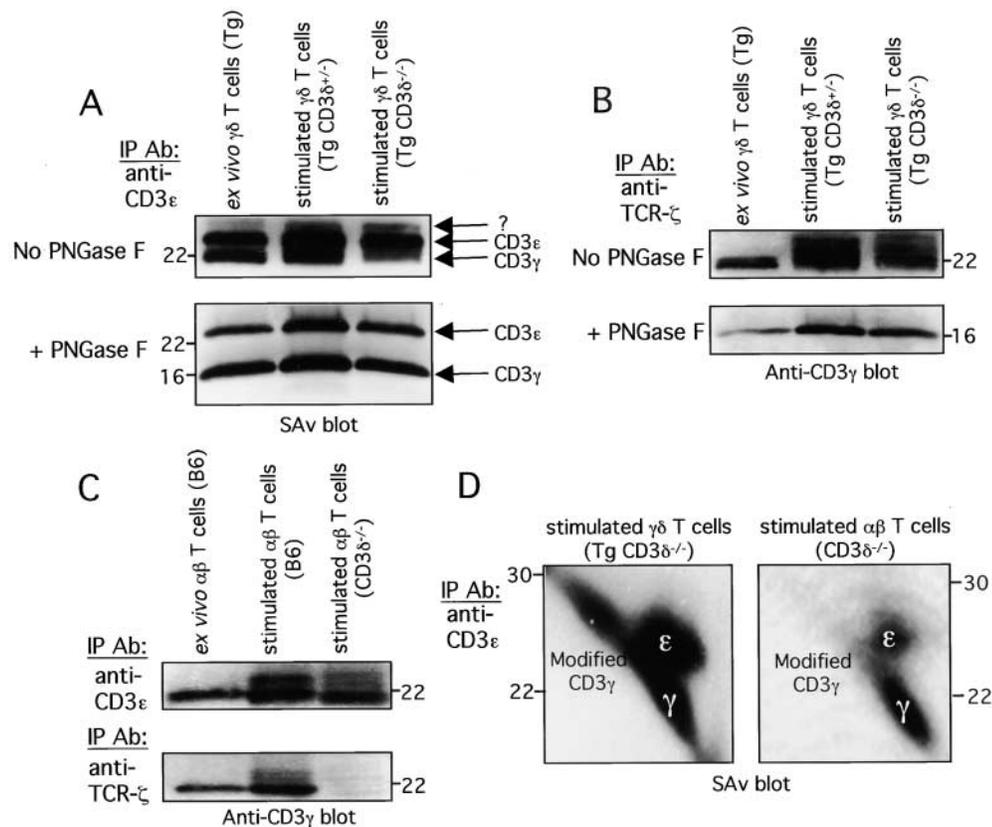
**Figure 2.** Biochemical analysis of CD3 complexes expressed by stimulated  $\gamma\delta$  T cells. (A) Lysates from  $20 \times 10^6$  ex vivo  $\alpha\beta$  and  $\gamma\delta$  T cells and stimulated  $\gamma\delta$  T cells were immunoprecipitated with anti-TCR- $\zeta$  mAb (H146). Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-CD3 $\delta$  serum. The blot was subsequently stripped and probed with anti-CD3 $\epsilon$  to assess the efficiency of immunoprecipitation. (B) Total cellular CD3 $\delta$  protein levels in ex vivo and in stimulated  $\alpha\beta$  and  $\gamma\delta$  T cells. Extracts were made from  $10 \times 10^6$  cells and  $0.3 \times 10^6$  cell equivalents were then analyzed by immunoblotting with anti-CD3 $\delta$  serum. (C) Lysates from  $25 \times 10^6$  stimulated V $\gamma$ 1-J $\gamma$ 4-C $\gamma$ 4 $^+$   $\gamma\delta$  T cells and B6 thymocytes were immunoprecipitated with anti-TCR mAbs (UC7-13D5 and H57-597, respectively). Immunoprecipitated proteins were resolved by SDS-PAGE and immunoblotted with anti-CD3 $\delta$  serum. The blot was subsequently stripped and probed with anti-CD3 $\epsilon$  to assess the efficiency of immunoprecipitation. (D)  $20 \times 10^6$  stimulated  $\gamma\delta$  T cells from nontransgenic CD3 $\delta^{-/-}$  mice were surface biotinylated, lysed, and incubated with anti-CD3 $\epsilon$  mAb (145-2C11). Immunoprecipitated proteins were resolved by nonreducing/reducing 2-D SDS-PAGE. Biotinylated TCR subunits were detected with ABC-HRP and chemiluminescence. The positions of the CD3 subunits and the unknown 26-kD subunit (?) are marked.

lated  $C_{\gamma}4$ -bearing  $\gamma\delta$  T cells also demonstrated the inclusion of a 26-kD protein in the  $\gamma\delta$  TCR after activation and expansion (unpublished data). However, this protein was not CD3 $\delta$  as assessed by Western blot analysis of anti- $\gamma\delta$  TCR immunoprecipitates (Fig. 2 C). Taken together, these results suggested that most  $\gamma\delta$  T cells, whether stimulated or unstimulated, do not contain CD3 $\delta$  as a component of their TCRs.

To verify that the 26-kD protein detected in the  $\gamma\delta$  TCR after activation and expansion was not CD3 $\delta$ , we analyzed the subunit composition of TCRs expressed by stimulated  $\gamma\delta$  T cells from CD3 $\delta^{-/-}$  mice (11). Significantly, the surface CD3 complexes on stimulated CD3 $\delta^{-/-}$   $\gamma\delta$  T cells were similar in composition to those on stimulated CD3 $\delta^{+/+}$   $\gamma\delta$  T cells (Figs. 1 and 2 D, and unpublished data), thereby confirming that the 26-kD protein detected in the  $\gamma\delta$  TCR after TCR activation and expansion was not CD3 $\delta$ . Based on these results, we conclude that activation and expansion induce changes in the CD3 complex of the  $\gamma\delta$  TCR but do not induce the inclusion of CD3 $\delta$ .

*CD3 $\gamma$  Is Modified After TCR Stimulation in Both  $\alpha\beta$  and  $\gamma\delta$  T Cells.* The 26-kD protein identified in the  $\gamma\delta$  TCR after activation and expansion could potentially be a

modified form of one of the other invariant TCR subunits, with the modification being differential *N*-glycosylation, ubiquitination, and/or phosphorylation. To determine whether this protein is a differentially glycosylated form of an invariant TCR subunit, we treated immunoprecipitated TCR proteins with PNGase F, a glycosidase that removes *N*-linked glycans at the asparagine residue. As shown in Fig. 3 A, three surface biotinylated proteins were detected in the CD3 complex in untreated anti-CD3 $\epsilon$  immunoprecipitates from stimulated  $\gamma\delta$  T cells, as predicted by the 2-D SDS-PAGE analysis (Fig. 1). However, only two proteins, identical in mass to CD3 $\epsilon$  and CD3 $\gamma$ , were detected in the CD3 complex after PNGase F treatment. This finding suggested that the 26-kD protein may be a differentially glycosylated form of CD3 $\gamma$ , because CD3 $\gamma$  is the only invariant subunit other than CD3 $\delta$  to have an *N*-linked glycosylation site (16). To test this, we repeated the experiment using anti-TCR- $\zeta$  mAb to immunoprecipitate mature  $\gamma\delta$  TCR complexes and anti-CD3 $\gamma$  serum to immunoblot for CD3 $\gamma$  in the  $\gamma\delta$  TCR complex. In anti-TCR- $\zeta$  immunoprecipitates that were not treated with the glycosidase, we detected at least four forms of CD3 $\gamma$ , ranging in mass from 21–26-kD, in stimulated  $\gamma\delta$  T cells (Fig. 3 B).



**Figure 3.** Modification of the CD3 $\gamma$  subunit after in vitro activation and expansion. (A)  $30 \times 10^6$  ex vivo  $\gamma\delta$  ( $\gamma\delta$  TCR Tg [Tg] CD3 $\delta^{+/+}$  mice) and stimulated  $\gamma\delta$  T cells (Tg CD3 $\delta^{+/+}$  and Tg CD3 $\delta^{-/-}$  mice) were surface biotinylated, lysed, and incubated with anti-CD3 $\epsilon$  mAb (145–2C11). Immunoprecipitated proteins were either treated with PNGase F or left untreated and resolved by reducing SDS-PAGE. ABC-HRP and chemiluminescence were used to visualize surface biotinylated proteins. The positions of the CD3 subunits and the unknown 26-kD subunit (?) are marked. (B) Lysates from  $30 \times 10^6$  ex vivo  $\gamma\delta$  (Tg CD3 $\delta^{+/+}$  mice) and stimulated  $\gamma\delta$  T cells (Tg CD3 $\delta^{+/+}$  and Tg CD3 $\delta^{-/-}$  mice) were immunoprecipitated with anti-TCR- $\zeta$  mAb (H146) and then treated with PNGase F or left untreated. Digested and undigested TCR proteins were resolved by reducing SDS-PAGE and immunoblotted with anti-CD3 $\gamma$  serum. (C) Lysates from  $30 \times 10^6$  ex vivo  $\alpha\beta$  (B6 mice) and stimulated  $\alpha\beta$  T cells (B6 and CD3 $\delta^{-/-}$  mice) were immunoprecipitated with anti-

CD3 $\epsilon$  (145–2C11) or anti-TCR- $\zeta$  mAb (H146). Immunoprecipitated proteins were resolved by reducing SDS-PAGE and immunoblotted with anti-CD3 $\gamma$  serum. (D) Stimulated  $\alpha\beta$  and  $\gamma\delta$  T cells from CD3 $\delta^{-/-}$  mice were generated as described in Materials and Methods. Surface proteins on equivalent numbers of cells were labeled with biotin and TCR complexes were immunoprecipitated using the anti-CD3 $\epsilon$  mAb (145–2C11). Immunoprecipitated proteins were resolved by nonreducing/reducing 2-D SDS-PAGE. ABC-HRP and chemiluminescence were used to visualize surface biotinylated proteins. The exposure time for each blot is identical. The positions of the CD3 $\epsilon$  and the modified forms of CD3 $\gamma$  are marked.

After PNGase F treatment, all forms of CD3 $\gamma$  were reduced to 16 kD, the core protein size of CD3 $\gamma$ . Interestingly, when we immunoprecipitated biotinylated TCR proteins with the anti-CD3 $\gamma$  mAb (H25), we did not detect the modified forms of CD3 $\gamma$  while we did detect the 21-kD form of CD3 $\gamma$  (unpublished data), suggesting that the differential glycosylation of CD3 $\gamma$  affects the binding of the H25 mAb. Therefore, these results suggested that the 26-kD protein in the CD3 complex of the  $\gamma\delta$  TCR is a differentially glycosylated form of CD3 $\gamma$ . As PNGase F treatment of this 26-kD protein removed only N-linked sugars and reduced its mass to 16 kD, these results indicate that CD3 $\gamma$  is modified only by differential glycosylation and not additionally by ubiquitination or phosphorylation, which are covalent modifications that are not affected by glycosidase treatment.

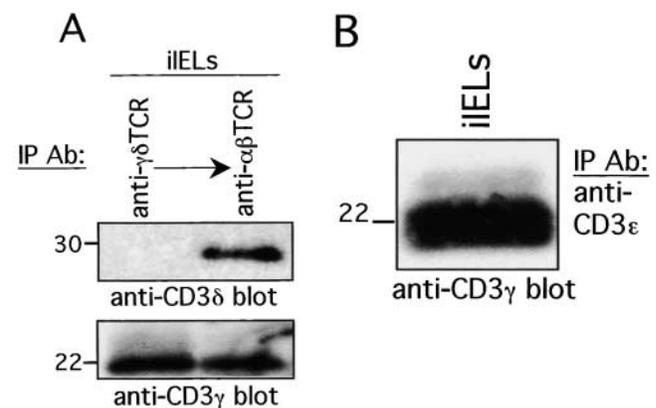
Because activation and expansion induced changes in the glycosylation pattern of the CD3 $\gamma$  subunit in  $\gamma\delta$  T cells, it was of particular interest to determine whether this modification also occurred in stimulated  $\alpha\beta$  T cells. To test this, we repeated the preceding experiment using  $\alpha\beta$  T cells that were activated and expanded in conditions similar to those used for  $\gamma\delta$  T cells. Western blot analysis was performed on anti-CD3 $\epsilon$  immunoprecipitates from stimulated  $\alpha\beta$  T cells from CD3 $\delta^{+/+}$  mice. Interestingly, while differentially glycosylated forms of CD3 $\gamma$  were detected in stimulated  $\alpha\beta$  T cells, we did not observe the 26-kD form (Fig. 3 C). Next, we determined whether any of the modified forms of CD3 $\gamma$  were incorporated into fully assembled  $\alpha\beta$  TCRs by immunoblotting anti-TCR- $\zeta$  immunoprecipitates with anti-CD3 $\gamma$  serum. As shown in Fig. 3 C, only very low amounts of the modified forms of CD3 $\gamma$  were detected in fully assembled TCRs expressed by  $\alpha\beta$  T cells.

Because the TCRs on stimulated  $\gamma\delta$  T cells lack CD3 $\delta$  and contain the differentially glycosylated forms of CD3 $\gamma$ , it is conceivable that the presence of CD3 $\delta$  hinders the incorporation of modified CD3 $\gamma$  into the  $\alpha\beta$  TCR complex. To test this, we examined  $\alpha\beta$  TCRs from stimulated  $\alpha\beta$  T cells from CD3 $\delta^{-/-}$  mice for the presence of CD3 $\gamma$  modification. While we detected modified forms of CD3 $\gamma$  in CD3 $\delta^{-/-}$   $\alpha\beta$  T cells, we did not detect any CD3 $\gamma$  (including the 21-kD form) associated with TCR- $\zeta$  (Fig. 3 C). The inability to detect CD3 $\gamma$  in the anti-TCR- $\zeta$  immunoprecipitate may be due to the instability and/or extremely low surface expression of CD3 $\delta$ -deficient  $\alpha\beta$  TCR complexes (reference 11 and unpublished data). Therefore, we analyzed the subunit composition of  $\alpha\beta$  TCRs expressed on stimulated CD3 $\delta^{-/-}$   $\alpha\beta$  T cells by surface biotinylation and 2-D SDS-PAGE. The amount of modified CD3 $\gamma$  detected in surface CD3 $\delta$ -deficient  $\alpha\beta$  TCRs was negligible, similar to the amount detected in intact  $\alpha\beta$  TCRs (Figs. 1 and 3, C and D). These findings suggest that modified forms of CD3 $\gamma$  are major components of TCRs on stimulated  $\gamma\delta$  T cells but not on stimulated  $\alpha\beta$  T cells. The reason why these modified forms of CD3 $\gamma$  are incorporated at a high frequency into the fully assembled  $\gamma\delta$  TCR but not into the fully assembled  $\alpha\beta$  TCR is unknown, but it may be due to the ability of the TCR- $\gamma$ /TCR- $\delta$  chains to bind

more efficiently than the TCR- $\alpha$ /TCR- $\beta$  chains to differentially glycosylated CD3 $\gamma\epsilon$  dimers. This ability of the  $\gamma\delta$  TCR to accommodate CD3 $\gamma\epsilon$  dimers with bulkier sugar groups demonstrates yet another fundamental difference in  $\alpha\beta$  and  $\gamma\delta$  TCR structure.

**Modified CD3 $\gamma$  Is Detected in iIELs.** While our results established that in vitro activation and expansion are able to induce modification of the CD3 $\gamma$  chain in both  $\alpha\beta$  and  $\gamma\delta$  T cells, it remained unclear whether this modification occurs in vivo. To address this, we analyzed the subunit composition of the TCRs expressed by iIELs, which are 50%  $\gamma\delta$  TCR $^{+}$  and display an activated phenotype (unpublished data and reference 17). As predicted from our analysis of TCR complexes on ex vivo and activated  $\gamma\delta$  T cells, little or no CD3 $\delta$  was detected in TCR complexes on  $\gamma\delta$  TCR $^{+}$  iIELs (Fig. 4 A). When we assayed for modified forms of CD3 $\gamma$ , higher molecular weight forms of CD3 $\gamma$  were detected in anti- $\gamma\delta$  TCR immunoprecipitates of iIEL lysates, albeit at a much lower level than that observed for in vitro activated and expanded  $\gamma\delta$  T cells (Figs. 4 A and 3 B).

The lower level of CD3 $\gamma$  modification may be due to the fact that  $\gamma\delta$  TCR $^{+}$  iIELs are not homogeneous in their state of activation and, consequently, there may be fewer cells that express the differentially glycosylated forms of CD3 $\gamma$ . To examine the extent of CD3 $\gamma$  modification in iIELs, we recovered CD3 dimers by immunoprecipitating unfractionated iIEL lysates with anti-CD3 $\epsilon$  mAb and assayed for the presence of modified CD3 $\gamma$  to determine whether a change in the glycosylation pattern of CD3 $\gamma$  occurred in iIELs. As shown in Fig. 4 B, differentially glycosylated forms of CD3 $\gamma$ , but not the 26-kD form, were detected in iIELs (Fig. 4 B). These results demonstrated that



**Figure 4.** Detection of differentially glycosylated forms of CD3 $\gamma$  in iIELs. (A) Lysate from  $30 \times 10^6$  iIELs from B6 mice was subjected to serial immunoprecipitations with anti- $\gamma\delta$ TCR and then anti- $\alpha\beta$ TCR mAbs (UC7-13D5 and H57-597, respectively). Immunoprecipitated proteins were resolved by reducing SDS-PAGE and immunoblotted with anti-CD3 $\delta$  serum. The blot was subsequently stripped and immunoblotted with anti-CD3 $\gamma$  serum. (B) Lysates from  $20 \times 10^6$  ex vivo iIELs from B6 mice were immunoprecipitated with anti-CD3 $\epsilon$  (145-2C11). Immunoprecipitated proteins were resolved by reducing SDS-PAGE and immunoblotted with anti-CD3 $\gamma$  serum.

CD3 $\gamma$  modification is detected and therefore, can occur in activated mature T cells in vivo.

Many surface glycoproteins have been reported to undergo changes in glycosylation patterns during development and after activation (for reviews, see references 18 and 19). For example, two recent studies have shown that the O-linked sugars on CD8 play a role in cognate/non-cognate binding of MHC class I tetramers to thymocytes (20, 21). The CD8 expressed on immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes is capable of binding MHC class I tetramers from various MHC alleles and haplotypes. This is not the case for mature CD8<sup>+</sup> thymocytes and peripheral CD8<sup>+</sup> T cells, as their CD8 is sialylated and can only bind to the appropriate peptide–MHC class I complex (20, 21). Although the importance of this change in CD8 glycosylation is unknown, both groups speculated that the differential binding of CD8 molecules to MHC class I molecules during development may play a role in selection by affecting the span of time a developing thymocyte spends interacting with class I-bearing thymic stroma (20, 21).

Similarly, it is not known how the glycosylation status of CD3 $\gamma$  affects  $\gamma\delta$  TCR signaling and  $\gamma\delta$  T cell function. However, using mice with a targeted mutation in a carbohydrate-modifying enzyme as a guide (22), we can speculate as to the role of this modification in  $\gamma\delta$  T cell biology. It is known that a typical N-glycan is 30 Å in length, comparable to an Ig domain (23). Because in vitro activation and expansion increase the mass of the carbohydrate group on CD3 $\gamma$ , it is conceivable that the carbohydrate chain on CD3 $\gamma$  is elongated through addition of branched sugars (18). An increase in carbohydrate chain length on CD3 $\gamma\epsilon$  dimers could cause the  $\gamma\delta$  TCR complexes in the cell membrane of stimulated  $\gamma\delta$  T cells to be spaced further apart, making it more difficult to cross-link surface  $\gamma\delta$  TCR complexes and thereby, increasing the threshold of signaling for the  $\gamma\delta$  TCR (22, 23). In practice, this may be a mechanism by which  $\gamma\delta$  T cells that recognize self-heat shock proteins or stress antigens establish a baseline threshold for signaling after their first encounter with self-antigens and then are able to distinguish between normal and stressed cells in subsequent encounters.

Modification in CD3 $\gamma$  glycosylation may not result in carbohydrate chain elongation but may result in the linkage of different sugar residues to the CD3 $\gamma$  chain. Such changes in carbohydrate moieties may affect the binding of endogenous lectins, such as the galectin family of carbohydrate-binding proteins, to the TCR complex (22). Because galectins have been shown to regulate T cell signaling and apoptosis (for a review, see reference 24), it is conceivable that the inclusion of modified CD3 $\gamma$  in the  $\gamma\delta$  TCR may facilitate the binding of galectins and increase the susceptibility of stimulated  $\gamma\delta$  T cells to apoptosis. If this is true, this may explain why, unlike  $\alpha\beta$  T cells, no memory response has been described for murine  $\gamma\delta$  T cells (for a review, see reference 25). While future studies are required to determine the importance of this modification, the fact that another alteration in  $\gamma\delta$  TCR subunit composition, inclusion of FcR $\gamma$ , occurs after activation and expansion

(6) suggests that the signaling potential of the  $\gamma\delta$  TCR may be profoundly affected.

In summary, we have described an activation and expansion induced modification in the CD3 $\gamma$  subunit of the TCR that consists of differential glycosylation at its single site of N-linked glycosylation. While this modification could occur in all T cells, the modified forms of CD3 $\gamma$  were only incorporated at a high frequency into the  $\gamma\delta$  TCR. One of the differentially glycosylated forms of CD3 $\gamma$  that was included in the  $\gamma\delta$  TCR had a mass and mobility in 2-D SDS-PAGE similar to CD3 $\delta$ . We believe that it is this modified form of CD3 $\gamma$  that was initially identified as CD3 $\delta$  in early studies of  $\gamma\delta$  TCR structure, because CD3 $\delta$  is not detected in  $\gamma\delta$  TCR complexes on primary and in vitro activated and expanded  $\gamma\delta$  T cells.

We thank Drs. Karl Pfeifer, Elizabeth Shores, and Connie Sommers for helpful advice and critical review of the manuscript.

Submitted: 12 July 2002

Revised: 9 September 2002

Accepted: 10 October 2002

## References

1. Punt, J.A., J.L. Roberts, K.P. Kearse, and A. Singer. 1994. Stoichiometry of the T cell antigen receptor (TCR) complex: each TCR/CD3 complex contains one TCR $\alpha$ , one TCR $\beta$ , and two CD3 $\epsilon$  chains. *J. Exp. Med.* 180:587–593.
2. Qian, D., A.I. Sperling, D.W. Lanki, Y. Tatsumi, T.A. Barrett, J.A. Bluestone, and F.W. Fitch. 1993. The  $\gamma$  chain of the high-affinity receptor for IgE is a major functional subunit of the T-cell antigen receptor complex in  $\gamma\delta$  T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 90:11875–11879.
3. Guy-Grand, D., B. Rocha, P. Mintz, M. Malassis-Seris, F. Selz, B. Malissen, and P. Vassalli. 1994. Different use of T cell receptor transducing modules in two populations of gut intraepithelial lymphocytes are related to distinct pathways of T cell differentiation. *J. Exp. Med.* 180:673–679.
4. Heiken, H., R.-J. Schulz, J.V. Ravetch, E.L. Reinherz, and S. Koyasu. 1996. T lymphocyte development in the absence of Fc $\epsilon$  receptor I $\gamma$  subunit: analysis of thymic-dependent and independent  $\alpha\beta$  and  $\gamma\delta$  pathways. *Eur. J. Immunol.* 26:1935–1943.
5. Shores, E.W., M. Ono, T. Kawabe, C.L. Sommers, T. Tran, L. Lui, M.C. Udey, J. Ravetch, and P.E. Love. 1998. T cell development in mice lacking all T cell receptor family members ( $\zeta$ ,  $\eta$ , and Fc $\epsilon$ R1 $\gamma$ ). *J. Exp. Med.* 187:1093–1101.
6. Hayes, S.M., and P.E. Love. 2002. Distinct structure and signaling potential of the  $\gamma\delta$ TCR complex. *Immunity.* 16:827–838.
7. Park, S.W., H. Arase, K. Wakizaka, N. Hirayama, S. Masaki, S. Sato, J.V. Ravetch, and T. Saito. 1995. Differential contribution of the FcR $\gamma$  chain to the surface expression of the T cell receptor among T cells localized in epithelia: analysis of FcR $\gamma$ -deficient mice. *Eur. J. Immunol.* 25:2107–2110.
8. Cron, R.Q., F. Koning, W.L. Maloy, D. Pardoll, J.E. Coligan, and J.A. Bluestone. 1988. Peripheral murine CD3<sup>+</sup>, CD4<sup>-</sup>, CD8<sup>-</sup> T lymphocytes express novel T cell receptor  $\gamma\delta$  structures. *J. Immunol.* 141:1074–1082.
9. Mombaerts, P., E. Mizoguchi, M.J. Grusby, L.H. Glimcher, A.K. Bhan, and S. Tonegawa. 1993. Spontaneous develop-

- ment of inflammatory bowel disease in T cell receptor mutant mice. *Cell*. 75:274–282.
10. Sim, G.-K., C. Olsson, and A. Augustin. 1995. Commitment and maintenance of the  $\alpha\beta$  and  $\gamma\delta$  T cell lineages. *J. Immunol.* 154:5821–5831.
  11. Dave, V.P., Z. Cao, C. Browne, B. Alarcon, G. Fernandez-Miguel, J. Lafaille, A. de la Hera, S. Tonegawa, and D.J. Kappes. 1997. CD3 $\delta$  deficiency arrests development of the  $\alpha\beta$  but not  $\gamma\delta$  T cell lineage. *EMBO J.* 16:1360–1370.
  12. Laky, K., L. Lefrançois, E.G. Lingenheld, H. Ishikawa, J.M. Lewis, S. Olson, K. Suzuki, R.E. Tigelaar, and L. Puddington. 2000. Enterocyte expression of interleukin 7 induces development of  $\gamma\delta$  T cells and Peyer's Patches. *J. Exp. Med.* 191:1569–1580.
  13. Pereira, P., D. Gerber, S.Y. Huang, and S. Tonegawa. 1995. Ontogenic development and tissue distribution of  $V_{\gamma}1$ -expressing  $\gamma/\delta$ -T lymphocytes in normal mice. *J. Exp. Med.* 182:1921–1930.
  14. Berger, M.A., M. Carleton, M. Rhodes, J.M. Sauder, S. Trop, R.L. Dunbrack, P. Hugo, and D.L. Wiest. 2000. Identification of a novel preTCR isoform in which the accessibility of the TCR- $\beta$  subunit is determined by occupancy of the missing V domain of pre-T $\alpha$ . *Int. Immunol.* 12:1579–1591.
  15. Sommers, C.L., R.L. Rabin, A. Grinberg, H.C. Tsay, J. Farber, and P.E. Love. 1999. A role for the Tec family tyrosine kinase Txk in T cell activation and thymocyte selection. *J. Exp. Med.* 190:1427–1438.
  16. Klausner, R.D., J. Lippincott-Schwartz, and J.S. Bonifacino. 1990. The T cell antigen receptor: insights into organelle biology. *Annu. Rev. Immunol.* 6:403–431.
  17. Sydora, B.C., P.F. Mixer, H.R. Holcombe, P. Eghtesady, K. Williams, M.C. Amaral, A. Nel, and M. Kronenberg. 1993. Intestinal intraepithelial lymphocytes are activated and cytolytic but do not proliferate as well as other T cells in response to mitogenic signals. *J. Immunol.* 150:2179–2191.
  18. Lowe, J.B. 2001. Glycosylation, immunity, and autoimmunity. *Cell*. 104:809–812.
  19. Baum, L.G. 2002. Developing a taste for sweets. *Immunity*. 16:5–8.
  20. Moody, A.M., D. Chui, P.A. Reche, J.J. Priatel, J.D. Math, and E.L. Reinherz. 2001. Developmentally regulated glycosylation of the CD8 $\alpha\beta$  coreceptor stalk modulates ligand binding. *Cell*. 107:501–512.
  21. Daniels, M.A., L. Devine, J.D. Miler, J.M. Moser, A.E. Lukacher, J.D. Altman, P. Kavathas, K.A. Hogquist, and S.C. Jameson. 2001. CD8 binding to MHC class I molecules is influenced by T cell maturation and glycosylation. *Immunity*. 15:1051–1061.
  22. Demetriou, M., M. Granovsky, S. Quaggin, and J.W. Dennis. 2001. Negative regulation of T cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature*. 409:733–739.
  23. Rudd, P.M., M.R. Wormald, R.L. Stanfield, M. Huang, N. Mattsson, J.A. Speir, J.A. DiGennaro, J.S. Fetrow, R.A. Dwek, and I.A. Wilson. 1999. Roles for glycosylation of cell surface receptors involved in cellular immune recognition. *J. Mol. Biol.* 293:351–366.
  24. Rabinovich, G.A., L.G. Baum, N. Tinari, R. Paganelli, C. Natoli, F.-T. Liu, and S. Iacobelli. 2002. Galectins and their ligands: amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol.* 23:313–320.
  25. Hayday, A.C. 2000.  $\gamma\delta$  cells: a right time and a right place for a conserved third way of protection. *Annu. Rev. Immunol.* 18: 975–1026.