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33. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
34. The soluble mouse T22^b heavy chain and human β_2M heterodimer complex was produced in *E. coli* and purified as described (*6*). The T22^b preparation was then concentrated to 13 mg/ml and extensively dialyzed against 20 mM Hepes (pH 7.2) and 25 mM sodium chloride before crystallization.
35. M. P. Crowley and Y. Chien, data not shown.
36. We thank J. Speir for critical reading of the manuscript and helpful discussions; R. Stanfield, S. Greasley, and A. Heine for help with data collection and data processing; and the staff of SSRL beamline 9-1. This study was supported by NIH grants CA58896 (I.A.W.) and AI33431 (Y.C.), postdoctoral fellowships from the Swedish Foundation for International Cooperation in Research and Higher Education (C.W.), the Foundation BLANCEFLOR Boncompagni-Ludovisi, nee Bildt (C.W.), and an NSF predoctoral fellowship (M.C.). Coordinates have been deposited in the PDB with accession code 1c16.

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A Population of Murine γδ T Cells That Recognize an Inducible MHC Class Ib Molecule

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Although γδ T cells are implicated in regulating immune responses, γδ T cell–ligand pairs that could mediate such regulatory functions have not been identified. Here, the expression of the major histocompatibility complex (MHC) class Ib T22 and the closely related T10 molecules is shown to be activation-induced, and they confer specificity to about 0.4% of the γδ T cells in normal mice. Thus, the increased expression of T22 and/or T10 might trigger immunoregulatory γδ T cells during immune responses. Furthermore, the fast on-rates and slow off-rates that characterize this receptor/ligand interaction would compensate for the low ligand stability and suggest a high threshold for γδ T cell activation.

αβ and γδ T cells contribute differently to host immune defense. Mice deficient in γδ T cells generally exhibit more profound defects in the regulation of immune function than in the clearance of intracellular pathogens (*1*). However, neither the ligands nor a normal population of γδ T cells responsible for mediating such immunoregulatory functions have been identified in any of the systems studied.

The closely related nonclassical MHC class I molecules T10 and T22 (94% amino acid identity) have been identified as ligands for two independently isolated γδ T cell clones KN6 and G8 (*2, 3*). T10-β_2-microglobulin (β_2M) and T22-β_2M heterodimers do not bind peptide and adopt a structure distinct from that of classical MHC molecules (*4, 5*). Both heterodimers are recognized directly by G8, without a requirement for other component (*3, 4*). Lipopolysaccharide (LPS)– or concanavalinA (conA)–activated splenocytes stimulate G8 and KN6 better than resting cells (*6*), implying that activation of lymphocytes results in increased expression of T10 and/or T22 on the cell surface. To monitor T10 and T22 expression and to understand its relationship to γδ T cells, we generated a monoclonal antibody (mAb), 7H9, specific for T10 and T22 (*7*). The 7H9 mAb recognizes in vitro folded T10-β_2M, T22-β_2M proteins and stains T10 and T22 transfected cell lines [CHO-T10 and T2-T22 (*8*)], but not cells transfected with the MHC class I molecule L^d or the MHC class II molecule I-E^k (Fig. 1A) (*9*). Both T10 and T22 are recognized equally by 7H9 with a high affinity around 0.1 nM determined by surface plasmon resonance with immobilized 7H9 (*9*).

In B10.BR (H-2^k) mice in which the T22 gene is nonfunctional (*10*), cell surface expression of T10 was only observed if splenic cells were activated by LPS or conA. An increase in T10 expression was also observed, after antigenic stimulation, on αβ T cells from B10.BR mice transgenic for the 5CC7 αβ T cell receptor (TCR), which is specific for the cytochrome c–I-E^k (Fig. 1B). The induction of T10 or T22 on splenocytes, including B cells and cells other than B and T cells, has also been observed in a peripheral tolerance induction model (*11, 12*). Because γδ T cells can recognize their ligands directly, without a requirement for antigen processing and presentation, the induction of T10 and T22 on the cell surface of lymphocytes may provide a mechanism by which γδ T cells specific for T10 and/or T22 to regulate immune cells.

If T10 and T22 are biologically important γδ T cell ligands, then the γδ T cells specific for these molecules should be detectable in unimmunized mice. Tetrameric peptide-MHC reagents have been used to track both MHC class I and class II restricted αβ T cell populations (*11, 13*). We therefore used the T22 protein to produce a tetrameric flow cytometry staining reagent (*14*). This reagent stains the G8 hybridoma but not αβ T cell hybridomas or the γδ T cell LBK5 (specific for I-E^k) (*9*). Nearly all splenic γδ T cells in G8 γδ TCR transgenic mice were stained by the tetramer (Fig. 2A). Staining of αβ T cells was not observed in either spleen or intestine (*9, 15*).

In normal animals, approximately 0.3 to 0.6% of splenic γδ T cells stained with the tetramer (Fig. 2C) (*16*). Greater than 90% of these cells are CD4$^-$8$^-$, while the rest are either CD4 or CD8 single positive (about 3 to 4% each) (*15*). A similar frequency of tetramer-positive γδ T cells was also found in the intestinal intraepithelial lymphocyte (iIEL) population (*9*). Tetramer binding was abolished when cells were first incubated with monomeric T22-β_2M (Fig. 2D), further demonstrating the specificity of the tetramer-positive cells for T22. Further, splenic γδ T cells from normal mice activated by plate-bound T22-β_2M complex, or by Chinese hamster ovary (CHO) cells expressing T10, showed an induced activated phenotype on tetramer-positive, but not on the tetramer-negative cell populations (Fig. 3) (*15*). Taken together, these results demonstrate that a population of γδ T cells in the spleen respond to T22 and/or T10 and that they can be identified by the T22 tetramer staining reagent. It should be noted that MICA/B, human inducible nonclassical MHC class I molecules, have been shown to trigger human γδ T cell lines. Subsequent experiments demonstrated that MICA/B are ligands for the activating natural killer cell receptor NKG2D and that the reactivity of γδ T cell lines to MICA/B-expressing cells is inhibited by antibodies to NKG2D. It was proposed that MICA/B might also act as ligands for the γδ TCR, because antibodies to the receptor also inhibited the reactivity (*17, 18*). A direct interaction between MICA/B

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and the γδ TCR, however, remains to be demonstrated.

Kinetic measurements of αβ TCR binding to peptide-MHC complexes have provided insight into the mechanisms of αβ T cell activation (*19*). To perform similar direct binding experiments, we produced a soluble G8 γδ TCR (*20*). Using surface plasmon resonance, binding curves were generated for surfaces possessing different densities of G8 TCR (500-2500RU) by titrating each with T10-β_2M or T22-β_2M (Fig. 4) (*21*). Similar results were obtained for both T10 and T22 heterodimer binding to G8. The dissociation rate for the interaction (k_d = 8.1 ± 2.3 × 10^{-3} s^{-1}) is

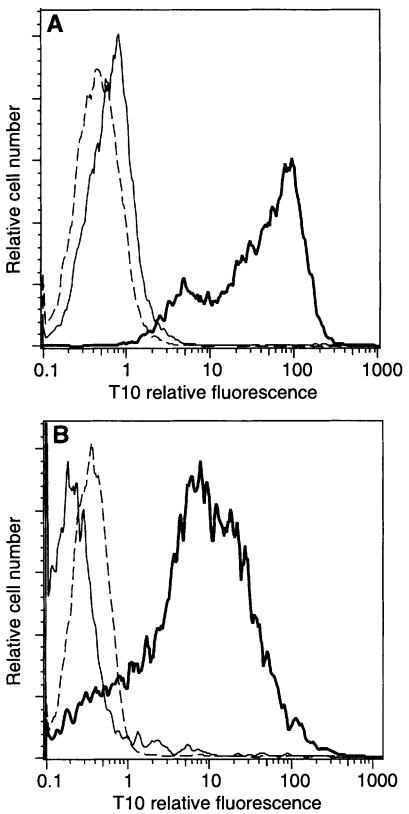

Fig. 1. (**A**) Monoclonal antibody 7H9 is specific for T10. CHO cells expressing T10 (bold line) or the MHC class I molecule L^d (solid line) were stained with biotinylated 7H9 and avidin-PE, cychrome anti TNP antibody (A19-3, Pharmingen) and propidium iodide (PI, 1 μg/ml in the final wash). Cells positive for A19-3 and PI were excluded from the analyses. The dotted line represents background staining of both CHO cell transfectants with the avidin-PE secondary reagent only. Data shown are representative of at least three independent experiments. (**B**) Expression of T10 is increased on αβ T cells following antigenic activation. The 5CC7 αβ TCR transgenic splenocytes were cultured at 2 × 10^6 cells/ml for 68 hours with (bold line) or without (solid line) 1 μM pigeon cytochrome c then assayed for T10 expression by FACS. Cells were stained with antibodies against T10/T22 (biotinylated 7H9, with avidin-PE secondary), TCR β chain (H57, fluorescein coupled, Pharmingen), and TNP (A19-3, cychrome coupled) as well as PI. Cells positive for A19-3 and PI are excluded from analyses. The dotted line represents background staining of PCC-activated spleen cells with avidin-PE secondary alone. Bold lines are pregated for βTCR^+ cells. T10 up-regulation was demonstrated in at least three different experiments covering a range of time points.

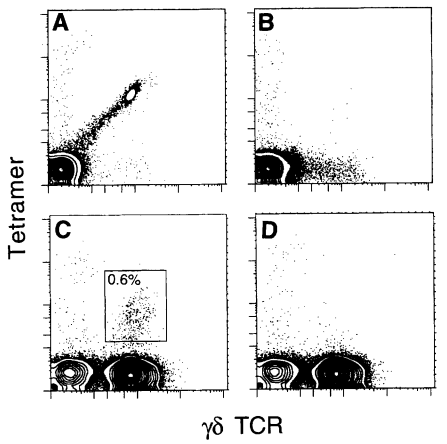

Fig. 2. The T22 tetramer stains nearly all γδ T cells in G8 γδ TCR transgenic mice and identifies a population of γδ T cells in normal mice. Splenocytes from (**A**) G8 transgenic (H-2^d) and (**B**) BALB/c (H-2^d) mice were stained with a T22 avidin-PE conjugated tetramer, and antibodies against TCR δ chain (GL3, fluorescein coupled) and TNP (A19-3, cy-chrome coupled) as well as PI (*16*). Cells positive for A19-3 and PI were excluded from analyses. Splenic γδ T cells were enriched by staining with a GL3–fluorescein isothiocyanate (FITC) conjugate and positively selecting with anti-FITC MACS beads (Miltenyi Biotec). Typical enrichments are by 20- to 50-fold. The γδ T cell–enriched splenocytes from B10.BR were incubated for 45 min with (**C**) phosphate-buffered saline or (**D**) unlabeled T22-β_2M monomer (*16*) prior to tetramer and antibody staining. The magnetic bead selection step does not alter the staining results. B10.BR, BALB/c, C3H, and C57BL6 mice were tested and gave similar results (*9*). Data shown is representative of three independent experiments.

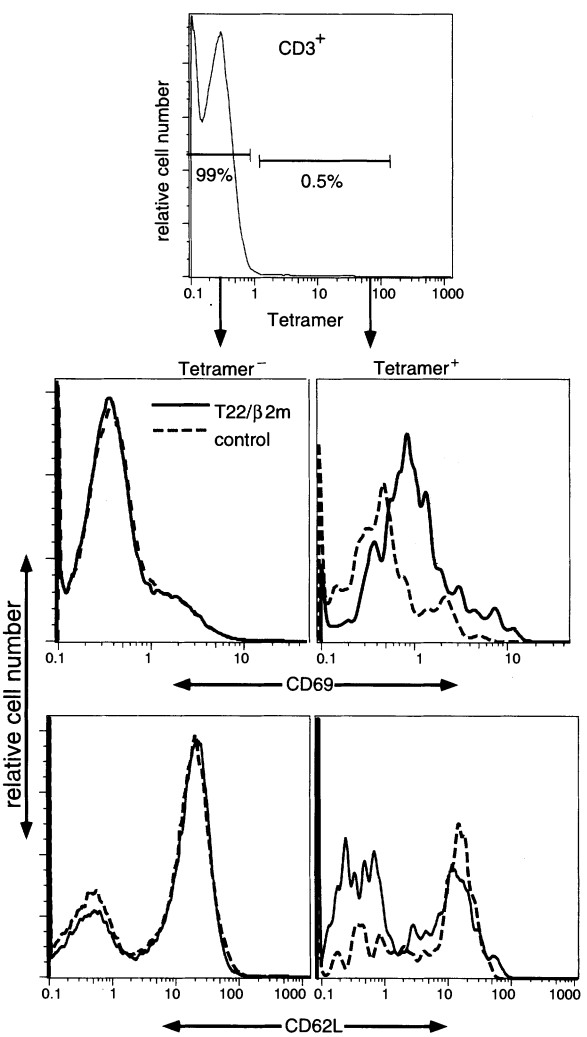

Fig. 3. γδ T cells responding to T22-β_2M can be identified by the tetramer. Enriched populations of splenic γδ T cells from BALB/c mice were obtained by depleting other major cell populations by magnetic cell separation with antibodies to CD19 (clone 1D3); αβ TCR (H57-597); GR-1 (RB6-8C5); CD11b (M1/70), and F4/80-antigen. The remaining cells were cultured for 6 hours in Dulbecco's modified Eagle's medium with 10% fetal bovine serum in the presence of a plate-bound control antibody (anti-human CD20, 5 μg/ml) or plate-bound T22-β_2M (5 μg/ml). Cells were stained for nine-color FACS analysis (*26*) with the following conjugates: CD69-FITC; T10-Tetramer-phycoerythrin (PE); CD4-Cy7PE; γδTCR-allophycocyanin (APC), CD8-Cy7APC; CD62L-TexasRed; CD19-Cy5.5APC; and CD3 Cascade Blue. Top graph shows histogram plots of live $CD3^+$ cells after gating for lymphocyte forward and site scatter and exclusion of propidium iodide. Frequencies of tetramer-positive cells were determined as indicated on the plots. Comparable frequencies of tetramer-positive γδT cells were found in cultures containing control antibody and T22-β_2M (0.5% and 0.6%). Tetramer positive and negative $CD3^+$ cells from the two cultures were analyzed for expression of the activation markers CD69. Histogram overlays show a two-fold increase in the expression of the very early activation marker CD69 (MFI 0.5 and 1.2) of tetramer-expressing cells, but not of tetramer nonexpressing cells (MFI 0.4 and 0.4) after stimulation. At this time point, about 50% of the tetramer-positive cells showed decreased CD62L expression. At least 5 × 10^5 events were collected and analyzed using FlowJo software (Treestar, San Carlos, CA).

slower than those observed for most αβ TCR and MHC-peptide complex interactions. The association rate ($k_a = 6.53 \pm 1.73 \times 10^4$ 1/ms) is among the fastest reported for αβ T cell receptor and ligand pairs (*19*). Consequently, compared to αβ TCRs, the affinity of the G8 interaction with T10 and T22 is rather high ($K_D = 0.13 \pm 0.05$ μM). Equilibrium binding studies produced results similar to the kinetic analysis ($K_D = 0.11 \pm 0.07$ μM) (Web figure 1) (*22*).

A high-affinity receptor is likely a characteristic of the T22 responding γδ T cell population in general, not a feature restricted to this specific receptor and ligand pair. Despite the heterogeneity in ligand affinities among normal γδ T cells specific for T10/T22, as inferred from the tetramer staining, the T22 tetramer stain can be effectively competed off with monomeric T22, thus suggesting that this receptor-ligand interaction is of higher affinity than most αβ TCR–ligand interactions, which have K_D values in the 10^{-4} to 10^{-6} M range. Heterodimers of T10 or T22 with β_2M are much less stable than classical MHC class I molecules complexed with an appropriate peptide (*4*). T10- and T22-reactive γδ T cells may therefore require TCRs with unusually high on-rates in order to "catch" these ligands, which are only transiently expressed at the cell surface.

The dissociation rate of the G8 TCR-T10 or TCR-T22 complex (0.008 s^{-1}), is orders of magnitude smaller than the dissociation rates reported for most αβ TCR-peptide-MHC interactions (*19*). The only comparable value is that of the αβ TCR 2C-QL9/L^d complex (0.003 s^{-1}). However, to reach the half-maximal value for cytolysis by 2C T cells requires fewer than five MHC-peptide complexes per target cell (*23*). In comparison, more than a hundred T10-T22 complexes are required to stimulate G8 to a half-maximal value (*3, 4*). These observations suggest that the avidity of the TCR and ligand interaction required to trigger a γδ T cell response to T10 or T22 molecules may be higher than that required to trigger αβ T cells. A high threshold for activation may in part reflect a general mechanism by which γδ T cells avoid activation by self antigens under inappropriate circumstances.

One of the perplexities of the proposed "cross talk" between αβ and γδ T cells has been the apparent scarcity of γδ T cells relative to the much larger population of αβ T cells. However, the frequency of T10- and T22-reactive γδ T cells (approximately 0.4%) is considerably higher than the one in 10^5 to 10^6 αβ T cells recognizing any given peptide-MHC complex prior to immunization. Thus, the frequency of T10- and T22-specific γδ T cells in normal mice is consistent with a regulatory function for these cells. Although the existence of specific regulatory T cells has been postulated for many years, their precise nature and particularly the identity of the molecules that guide their activities have proven elusive. The system we describe here has many of the salient features expected for such regulatory cells.

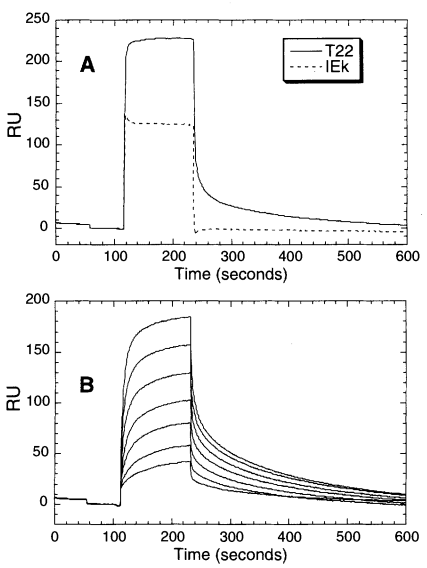

Fig. 4. Affinity measurements of T22-β_2M heterodimer and G8 γδ T cell receptor. (**A**) The T22 heterodimer, but not a soluble MCC–I-E^k molecule, binds specifically to immobilized G8 TCR. (**B**) Binding curve profiles for a kinetic analysis of T22 association with and dissociation from immobilized G8 TCR. The flow rate was 25 μl/min. The T22 analyte concentration ranges from 0.04 to 2.0 μM.

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15. N. Baumgarth and Y. Chien, unpublished data.
16. γδ T cells from G8 transgenic mice (BALB/c background) stain along a diagonal with the pan-γδ antibody GL3, indicating that cells with higher T cell receptor levels are also brighter for tetramer staining. We incubated 4 to 6 μg/ml of T22 tetramer with cells for 45 min on ice. Blocking of T22 tetramer staining with T22 monomer was performed at 100 to 200 μg/ml of unlabeled monomer protein, preincubated with cells for 45 min and present during tetramer incubation.
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20. Soluble G8 γδ TCR was produced in a *Drosophila melanogaster* cell expression system, using γ and δ TCR chain constructs to which the leucine zipper acid and base peptide segments were added following the COOH-terminal interchain disulfide bond (*25*). Purification was carried out by a succession of nickel-agarose, anion-exchange (MonoQ, Pharmacia), and gel filtration chromatography (Superdex 200, Pharmacia). Each step was monitored by SDS-PAGE.
21. The G8 TCR protein was immobilized by amine coupling, and size-purified T10-β_2M and T22-β_2M heterodimers were used as analytes in surface plasmon resonance analysis. The G8 TCR protein binds T22-β_2M protein but not soluble MCC88-103–I-E^k (Fig. 4A). The T10 and T22 proteins also do not interact with a null surface or with soluble 2B4 αβ TCR (specific for MCC88-103–I-E^k), immobilized identically on a sensorchip (*9*). Association and dissociation constants for T10 and T22 binding to immobilized G8 TCR were calculated simultaneously using BIAevaluation 3.0 software, fitting to a simple 1:1 Langmuir model.
22. Scatchard analysis of specific binding of T22-β_2M to immobilized G8 TCR (after subtracting nonspecific association of T22 to an immobilized 2B4 αβ TCR surface). The flow rate was 5 μl/min. All data shown are representative of at least three independent experiments. Web figure 1 can be found at *Science* Online at www.sciencemag.org/feature/data/1043813.shl
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