of mass in such a way as to conserve its position inertially. The present separation of Earth and moon is $3.564 \times 10^{10} \leq 3.84 \times 10^{10} \leq 4.007 \times 10^{10}$ cm [see (*18*)]. The BCC angular momentum is partitioned between the moon (h_m) and Earth (h_e); Earth has 1.23% of the total system angular momentum, and the moon has the remaining 98.8%. Thus, the moon's present-epoch share of orbital angular momentum must be reduced by 1.23%, the fraction belonging to Earth. For the earlier time periods, the barycenter shifts closer to Earth's center. For the Earth-sun barycenter, the sun is taken as infinitely massive and the moon's mass is ignored. The obliquity of the lunar orbit to Earth's equator is necessarily ignored here.

14. D. S. Broomhead and G. P. King, *Physica D* **20**, 217 (1986); R. P. Vautard, P. Yiou, M. Ghil, *Physica D* **58**, 95 (1992).
15. G. L. Bretthorst, *Bayesian Spectrum Analysis and Parameter Estimation*, Vol. 48 of *Lecture Notes in Statistics*, J. Berger, S. Fienberg, J. Gani, K. Krickeberg, B. Singer, Eds. (Springer-Verlag, Berlin, 1988).
16. In MLE computations, we assume noise is Gaussian (normally distributed). Additionally, as the frequency scale is based on neap-spring laminae count (index) rather than on true frequency, errors attributable to lost laminae can contract the time scale.
17. J. O. Dickey *et al.*, Science **265**, 482 (1994).
18. C. W. Allen, *Astrophysical Quantities* (Althone/Univ. of London Press, London, 1973).
19. G. W. Platzman, *J. Phys. Oceanogr.* **2**, 117 (1972); K. S. Hansen, *Rev. Geophys. Space Phys.* **20**, 457 (1982).
20. W. H. Munk and G. J. F. MacDonald, *The Rotation of the Earth* (Cambridge Univ. Press, Cambridge, 1960).
21. Of the conservation laws for momentum and energy, the former is easiest to deal with because momentum is partitioned primarily between terrestrial rotation and the lunar orbit, whereas energy is partitioned three ways, between these two parameters and frictional loss $f(k_2,Q)$. For constant f, $d\xi/dt$ varies as $\xi^{11/2}$. Conversely, for constant or approximately constant $d\xi/dt$ (Fig. 3), $f \sim \xi^{-11/2}$. Moreover, for constant f, the ratio of k_2 to Q (*20*) must be constant. It is difficult to assess the invariance of k_2 over the past eon, and perhaps it has changed little, but the tidal lead angle has almost certainly not been fixed—and indeed has probably changed by a large factor—because the terrestrial gravity disturbance field caused by the presence of the moon varies as ξ^{-3}.
22. G. Faure, *Principles of Isotope Geology* (Wiley, New York, 1977); J. S. Kargel and J. S. Lewis, *Icarus* **105**, 1 (1993).
23. H. Takeuchi, *J. Fac. Sci. Univ. Tokyo Sect. 2*, 7 (1951) [quoted in (*20*)]; *Trans. Am. Geophys. Union* **31**, 651 (1950) [quoted in (*10*)].

24. In memoriam S. K. Runcorn. Supported in part by the NASA Innovative Research Program (to C.P.S.), the NSF Atmospheres program (to G. E. Williams and C.P.S.) for the Elatina data reduction, and NSF grant EAR-9315937 (to M.A.C. and E.P.K.) for the BCC data. We thank H. R. Johnson and A. Zawostoski for technical support.

19 January 1996; accepted 17 April 1996

T Cell Activation Determined by T Cell Receptor Number and Tunable Thresholds

Antonella Viola and Antonio Lanzavecchia*

The requirements for T cell activation have been reported to vary widely depending on the state of the T cell, the type of antigen-presenting cell, and the nature of the T cell receptor (TCR) ligand. A unitary requirement for T cell responses was revealed by measurement of the number of triggered TCRs. Irrespective of the nature of the triggering ligand, T cells "counted" the number of triggered TCRs and responded when a threshold of ~8000 TCRs was reached. The capacity to reach the activation threshold was severely compromised by a reduction in the number of TCRs. Costimulatory signals lowered the activation threshold to ~1500 TCRs, thus making T cells more sensitive to antigenic stimulation.

T cells are activated when their TCRs are engaged and triggered by ligands on the surface of antigen-presenting cells (APCs). The natural ligand is a complex of a peptide bound to a major histocompatibility complex (MHC) molecule (*1*), but T cells can also be activated by bacterial superantigens (*2*) and by antibodies to the TCR-CD3 complex (*3*). In different experimental systems, the amount of ligand required to activate T cells varies according to the state of the T cells (naïve or memory) and the number of adhesion and costimulatory molecules on the APC (*4*). A limitation of this type of analysis is that the T cell response can be correlated only to the relative amount of ligand offered, whereas the most relevant parameter—the number of triggered TCRs—remains unknown. This number can now be measured by TCR down-regulation (*5*). We have used this method to determine the number of triggered TCRs required for the induction of T cell activation and to explain how T cells can respond to very few ligands.

We compared three ligands—complexes of specific peptide and MHC molecules, complexes of the bacterial superantigen TSST (toxic shock syndrome toxin) and MHC molecules, and monovalent antibodies to CD3 (anti-CD3)—with respect to their ability to trigger TCRs and activate T cells (*6*). All three ligands induced a dose-dependent TCR down-regulation, but the efficiencies and kinetics differed (Fig. 1A).

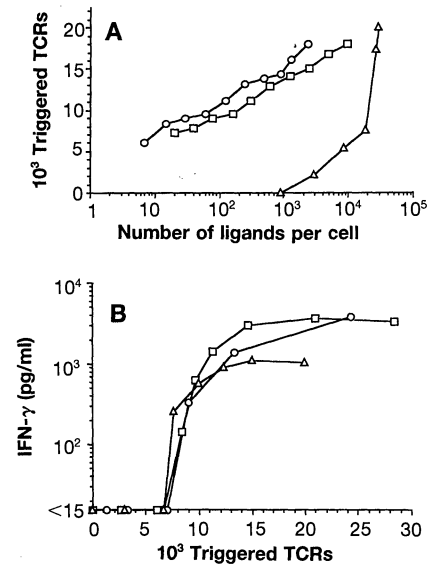

Fig. 1. T cells produce IFN-γ above a threshold of triggered TCRs, regardless of the nature of the stimulus. A $V_\beta 2^+$ T cell clone was stimulated with specific peptide pulsed on autologous EBV-B cells (○), with TSST pulsed on autologous EBV-B cells (□), or with monovalent anti-CD3 in the presence of FcR^+ THP1 cells (△). (**A**) Number of triggered TCRs, measured by TCR down-regulation, as a function of the number of ligands per cell. (**B**) IFN-γ production as a function of the number of triggered TCRs.

As previously shown (*5*), a small number of peptide-MHC complexes were able to trigger serially a large number of TCRs. Complexes of TSST bound to MHC class II molecules were similarly effective, possibly because TSST-MHC complexes (*2*), like the peptide-MHC complex (*7*), bind the TCR with low affinity, thus allowing both triggering and dissociation. Anti-CD3 was much less efficient; the number of TCRs down-regulated was always lower than the number of antibodies offered. The high affinity of binding prevents antibodies from serially triggering TCRs (*8*).

Although the mode of interaction with TCR-CD3, the kinetics of triggering, and the stoichiometries were different for the three ligands, the responses of the T cells were strikingly similar relative to the number of triggered TCRs. Regardless of the stimulus, T cells produced interferon-γ (IFN-γ) only when ≥ ~8000 TCRs were triggered, and this production rapidly reached a plateau at higher numbers of triggered TCRs (Fig. 1B). The plateau was lower in cells triggered by anti-CD3 (*9*). The same threshold of activation, both for IFN-γ production and for cell proliferation, was observed with four different $CD4^+$ antigen-specific T cell clones that normally produce IFN-γ in the range of 400 to 20,000 pg/ml. We conclude that T cells "count" the number of triggered TCRs and

Basel Institute for Immunology, Grenzacherstrasse 487, CH 4005 Basel, Switzerland.

*To whom correspondence should be addressed. E-mail: Lanzavecchia@bii.ch

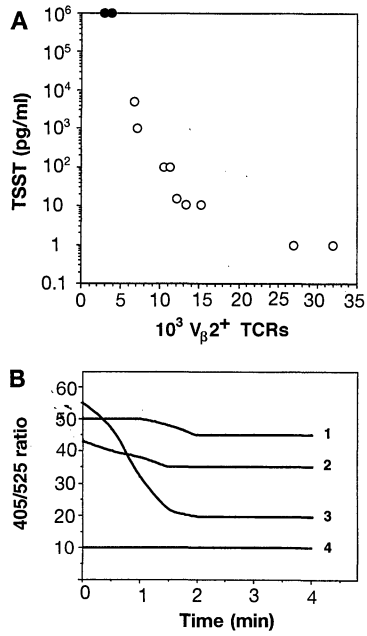

Fig. 2. The absolute number of TCRs determines the capacity to respond to different concentrations of superantigen. (**A**) Relation between the number of $V_\beta2^+$ TCRs and the concentration of TSST required to induce 30% of the maximum IFN-γ production. ○, individual responsive clones; ●, clones that express very small amounts of $V_\beta2$ and fail to produce IFN-γ even at the highest TSST concentrations. The TSST concentrations used for stimulation are below the 50% saturation (*17*). The thresholds of triggered TCRs required for T cell activation were comparable in responding clones with large or small amounts of $V_\beta2$. All clones produced IFN-γ when triggered by anti-CD3. Similar results were obtained when the proliferative response was measured. (**B**) Kinetics of $[Ca^{2+}]_i$ increase (mean values) in T cell clones expressing small or large numbers of $V_\beta2^+$ TCRs. **1**, clone 2.3.3 (expressing 32,000 $V_\beta2^+$ TCRs) conjugated with APCs pulsed with TSST (100 ng/ml); **2**, clone 2.3.3 conjugated with APCs pulsed with TSST (0.1 ng/ml); **3**, clone 2.3.33 (expressing 4000 $V_\beta2^+$ TCRs) conjugated with APCs pulsed with TSST (100 ng/ml); **4**, baseline of T cells conjugated with unpulsed APCs. In T cell clones with normal numbers of TCRs, the signal is sustained for ≥30 min (*11*). The 405/525 ratio is the ratio of emission at 405 nm (Ca^{2+}-bound Indo 1) to that at 525 nm (free Indo 1) and measures $[Ca^{2+}]_i$ independent of dye loading.

respond above an activation threshold, and that this response is independent of the nature of the triggering ligand.

Because many TCRs must be triggered, T cells that express fewer TCRs might be less responsive. To analyze the influence of the extent of TCR expression on T cell responsiveness, we used a panel of dual-receptor T cell clones that express various amounts of $V_\beta2$ in a stable fashion together with a second V_β (*10*). This experimental system enabled us to use normal T cells, all in the same state and with similar activation requirements, to examine the effect of small numbers of TCRs. The capacity of T cells to respond to the $V_\beta2$-specific superantigen TSST correlated with the amount of $V_\beta2$ expressed (Fig. 2A). T cell clones with ~30,000 $V_\beta2^+$ TCRs responded to very low doses of TSST, whereas clones expressing ~7000 $V_\beta2^+$ TCRs required up to 1000 times as much TSST. Despite the different requirements for ligand concentration, all responding clones had comparable activation thresholds of ~6000 to 8000 TCRs. Two clones that express very small numbers of $V_\beta2^+$ TCRs (<5000) neither proliferated nor produced IFN-γ, even when exposed to an extremely high dose of TSST. These clones underwent a transient increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) after exposure to TSST-pulsed APCs. On the contrary, at all TSST doses, the Ca^{2+} response was sustained in clones expressing large numbers of TCRs (Fig. 2B) (*11*). Thus, T cells with very few TCRs are triggered transiently but fail to be activated, because the number of TCRs is insufficient to allow these cells to sustain the signal and reach the activation threshold.

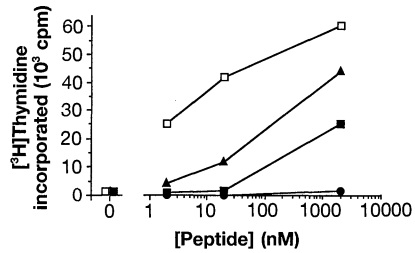

Fig. 3. TCR down-regulation results in a reduced response to antigen. Clone KS140 was cultured for 3 days with autologous EBV-B cells that were either unpulsed (□) or pulsed with 2 nM (▲), 20 nM (■), or 2 μM (●) tetanus toxin 830–843 peptide. At the time of the assay, the T cells expressed 100%, 65%, 50%, and 10% of CD3, respectively. The data show the proliferative response of T cells (as measured by [^{3}H]thymidine incorporation) in the presence of fresh EBV-B cells pulsed with different peptide concentrations. The possibility that unresponsive T cells might undergo activation-induced cell death was ruled out in parallel experiments by propidium iodide staining.

TCR down-regulation is a physiological consequence of antigenic stimulation and is long-lasting, because the original numbers of TCRs are recovered only after a few days (*12*). To investigate whether TCR down-regulation would result in a decreased responsiveness, we induced different amounts of TCR down-regulation by stimulating a T cell clone with APCs pulsed with various peptide concentrations. After 3 days, T cells with down-regulated TCRs were tested for their capacity to proliferate in response to antigenic stimulation (*13*). Down-regulation of as few as 35% of the TCRs resulted in a marked shift in the dose response curve (Fig. 3), which indicated that the T cells were still responsive but required a much higher antigen concentration. T cells expressing only 10% of the original number of TCRs were completely unresponsive. The results obtained with dual-receptor T cell clones and clones with down-regulated TCRs are comparable, which indicates that the absolute number of TCRs is a key factor in determining T cell sensitivity to antigen. Normal numbers of TCRs ensure maximum sensitivity, whereas reduced numbers lead to a disproportionate increase in the amount of antigen required to reach the activation threshold.

Naïve T cells require higher concentrations of antigen than do activated T cells and are more dependent on costimulation (*4*). We sought to determine whether this difference was attributable to a higher activation threshold of naive cells and whether costimulation could modify this threshold. Peripheral blood T cells were stimulated with TSST or monovalent anti-CD3 in the presence or absence of costimulation provided by B7, the CD28 ligand, expressed on APCs (*14*). In the absence of costimulation, resting T cells responded to the same concentration of anti-CD3 as did T cell clones, but they required more than 100 times as much TSST to be activated (*15*). The threshold for activation of resting T cells was similar to that of T cell clones (~8000 TCRs) (Fig. 4). However, in the presence of costimulation, there was a marked shift of the response curve toward a threshold one-fifth as great (Fig. 4). Similar results were obtained with short-term $CD28^+$ T cell clones (Fig. 4C) (*14*). Taken together, these results indicate that the activation threshold is comparable in T cell clones and resting T cells and can be decreased by costimulation through CD28-B7 interaction.

Our results suggest that T cells "count" the number of triggered TCRs and respond when this number reaches an appropriate threshold. This threshold is independent of the nature of the triggering ligand and can be decreased by costimulation. The capacity to reach the activation threshold is severely compromised in cells that have few TCRs and cannot sustain the triggering process. We suggest that after conjugate formation, the triggered TCRs are rapidly consumed at the contact site between T cells and APCs, so that a sustained signal depends on the continuous recruitment of new TCRs. This hypothesis may explain why, in T cells with fewer TCRs, an increase in ligand concentration can compensate for the initial rate of triggering but cannot sustain the signal, which depends on the recruitment of new TCRs into the contact site. The relation

between T cell responsiveness and the number of TCRs has two implications: (i) TCRs expressed in small numbers in dual-receptor T cells may be functionally excluded, because they will never find enough ligand. (ii) TCR down-regulation after antigenic stimulation results in T cell unresponsiveness.

The mechanism by which costimulation lowers the activation threshold requires further investigation; it is possible that the TCR and CD28 pathways synergize at some point along the signal transduction cascade. The tuning of the activation threshold by costimulation has important implications for the control of the immune response. Professional APCs provide the most efficient conditions for T cell activation by small numbers of peptide-MHC complexes, because they express large numbers of adhesion and costimulatory molecules that facilitate TCR triggering and lower the activation threshold. Conversely, nonprofessional APCs, lacking adhesion and costimulatory molecules, may induce TCR down-regulation below the triggering threshold, leading to T cell unresponsiveness (*16*).

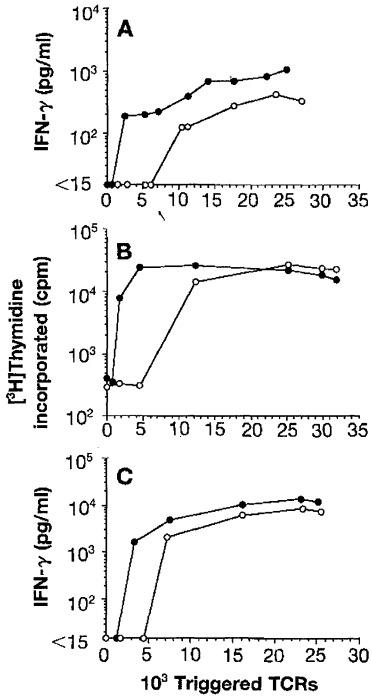

Fig. 4. Costimulation lowers the activation threshold in resting T cells and short-term T cell clones. (**A**) Peripheral blood T cells were conjugated with B7$^-$ (○) or B7$^+$ (●) THP1 cells pulsed with various concentrations of TSST. The minimum stimulatory concentration of TSST was 7 ng/ml in the presence of costimulation and 70 ng/ml in the absence of costimulation. (**B**) Peripheral blood T cells were pulsed with various concentrations of monovalent anti-CD3 and cultured with B7$^-$ (○) or B7$^+$ (●) THP1 cells. (**C**) A CD28$^+$ $V_\beta2^+$ short-term T cell clone was conjugated with EBV-B cells pulsed with various concentrations of TSST in the presence (○) or absence (●) of CTLA4-Ig (5 μg/ml). (A) and (C) show the number of triggered TCRs (as measured by down-regulation of $V_\beta2$) versus the amount of IFN-γ produced; (B) shows the number of triggered TCRs (as measured by down-regulation of CD3) versus T cell proliferation (as measured by [^{3}H]thymidine incorporation). Comparable results were obtained with five additional short-term clones.

REFERENCES AND NOTES

1. A. Townsend *et al.*, *Cell* **62**, 285 (1990); B. P. Babbitt, P. M. Allen, G. Matsueda, E. Haber, E. R. Unanue, *Nature* **317**, 359 (1985); M. M. Davis and P. J. Bjorkman, *ibid.* **334**, 395 (1988).
2. J. Kappler *et al.*, *Science* **244**, 811 (1989); J. Thibodeau *et al.*, *ibid.* **266**, 1874 (1994).
3. W. J. M. Tax, H. W. Willems, P. P. M. Reekers, P. J. A. Capel, R. A. P. Koene, *Nature* **304**, 445 (1983); J. Kaye, S. Porcelli, J. Tite, B. Jones, C. A. Janeway, *J. Exp. Med.* **158**, 836 (1983); S. C. Meuer *et al.*, *ibid.*, p. 988.
4. K. Inaba and R. M. Steinman, *J. Exp. Med.* **160**, 1717 (1984); M. Luqman and K. Bottomly, *J. Immunol.* **149**, 2300 (1992); M. Croft, D. D. Duncan, S. L. Swain, *J. Exp. Med.* **176**, 1431 (1992).
5. S. Valitutti, S. Müller, M. Cella, E. Padovan, A. Lanzavecchia, *Nature* **375**, 148 (1995).
6. T cells from a $V_\beta2^+$ DR13-restricted T cell clone (AL4.3) specific for the tetanus toxin 830–843 peptide were stimulated with autologous Epstein-Barr virus transformed B (EBV-B) cells pulsed for 2 hours with different concentrations of peptide or TSST. The number of peptide-MHC and TSST-MHC complexes per APC was estimated as described (*5*, *17*). For stimulation with anti-CD3, the T cells were pulsed for 30 min on ice with different concentrations of the purified bispecific anti-CD3–anti-ovarian carcinoma antibody OCTR1 (*18*), and the amount of antibody bound to T cells was estimated from the saturation curve measured by indirect immunofluorescence for a known total number of TCRs (*5*). The bound antibody did not dissociate during the assay. The pulsed T cells were cultured with Fc receptor–expressing (FcR$^+$) cells (THP1). Cultures containing 5×10^4 T cells and 10^5 APCs were placed in 200 μl of RPMI 1640 and 5% fetal bovine serum in 96 U-bottom microplates, centrifuged to allow conjugate formation, and incubated at 37°C for 5 hours. TCR down-regulation was measured by indirect immunofluorescence (*5*) with an excess of OCTR1 followed by a second antibody. The data were calculated using the mean fluorescence value of the cell population (SE, <0.5). IFN-γ production was measured as described (*5*). The data represent the mean of triplicate wells (SD, <10%).
7. K. Matsui *et al.*, *Science* **254**, 1788 (1991); S. Weber, A. Traunecker, F. Oliveri, W. Gerhard, K. Karjalainen, *Nature* **356**, 793 (1992); K. Matsui, J. J. Boniface, P. Steffner, P. A. Reay, M. M. Davis, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 12862 (1994); M. Corr *et al.*, *Science* **265**, 946 (1994).
8. For stimulation by peptide-MHC or TSST-MHC, the data fit a logarithmic curve, $y = a + b(\log x)$, which indicates that serial triggering is optimal at low ligand density and becomes less effective as the number of complexes increases. For stimulation by anti-CD3, the data fit a linear curve, $y = ax$ (where $a < 1$), which indicates that the process has the same efficiency over the range of antibody concentrations tested. The latter conclusion was confirmed in experiments in which the same EBV-B cells used for peptide or TSST presentation were pulsed with known amounts of a bispecific anti-CD3–anti-MHC class II antibody (A. Viola, unpublished data).
9. The smaller amount of IFN-γ produced after stimulation with anti-CD3 is characteristic of CD4$^+$ but not CD8$^+$ T cell clones and correlates with a lower capacity of antibody to recruit CD4 in the triggered TCR complex, as shown by decreased CD4 down-regulation (A. Viola, in preparation). At subthreshold numbers of TCRs, no IFN-γ production was detected by a sensitive enzyme-linked immunosorbent assay (ELISA), whereas above the threshold the IFN-γ production rapidly reached a plateau. Because the clones used were CD28$^-$, the threshold was not affected by the type of APC or by the presence or absence of costimulation. Nor was the threshold affected by addition of interleukin-12, which doubled the amount of IFN-γ produced.
10. Dual β T cell clones were isolated as described [E. Padovan *et al.*, *J. Exp. Med.* **181**, 1587 (1995)]. The clones expressed different numbers of $V_\beta2^+$ TCRs, as follows:

Clone	TCRs
2.3.3	32,000
2.3.19	27,000
2.22.25	15,000
2.22.30	13,000
2.22.39	12,000
2.52.40	11,000
2.52.39	10,000
2.3.37	7,000
2.3.29	7,000
2.3.15	3,000
2.3.33	4,000

$V_\beta2$ was detected with monoclonal antibody MPB2/D5 (Immunotech, Marseille, France) and the number of TCRs was calculated as described (*5*). The second V_β ($V_\beta3$, $V_\beta22$, or $V_\beta5.2$) did not react with TSST.
11. $[Ca^{2+}]_i$ was measured as described [S. Valitutti, M. Dessing, K. Aktories, H. Gallati, A. Lanzavecchia, *J. Exp. Med.* **181**, 577 (1995)]. For T cells expressing normal amounts of $V_\beta2$, the $[Ca^{2+}]_i$ increase is sustained for ≥30 min.
12. A. Lanzavecchia, in *The Tolerance Workshop 1986*, P. Matzinger *et al.*, Eds. (Roche, Basel, 1986), pp. 293–298. T cell clones retained small numbers of TCRs for as long as 5 to 8 days after stimulation.
13. T cells from a DR11-restricted T cell clone (KS140) specific for the tetanus toxin 830–843 peptide were stimulated at 1:1 ratio with mitomycin C–treated autologous EBV-B cells pulsed with different concentrations of peptide. After 3 days, CD3 concentrations were measured and the cells were tested for their capacity to proliferate in response to fresh EBV-B cells pulsed with different concentrations of peptide.
14. THP1 cells transfected with B7-1 and mock-transfected cells were produced as described (*19*). Short-term T cell clones were isolated by sorting $V_\beta2^+$ CD28$^+$ peripheral blood T cells and cloning, as described (*20*). In some experiments, peripheral blood mononuclear cells (PBMCs) were conjugated with THP1 pulsed with various concentrations of TSST. In other experiments, the PBMCs were first pulsed for 30 min on ice with various concentrations of OCTR1 and cultured with FcR$^+$ THP1. Separately, T cell clones were conjugated with EBV-B cells pulsed with various concentrations of TSST in the presence or absence of CTLA4-immunoglobulin (Ig) fusion protein (*19*). All long-term T cell clones expressed very little or no CD28. Comparable results were obtained when THP1 was used for presentation of both TSST and antibody.
15. H. Fischer *et al.*, *J. Immunol.* **148**, 1993 (1992); N. Bhardwaj, J. W. Young, A. J. Nisanian, J. Baggers, R. M. Steinman, *J. Exp. Med.* **178**, 633 (1993).
16. G. Schönrich *et al.*, *Cell* **65**, 293 (1991); I. Ferber *et al.*, *Science* **263**, 674 (1994).
17. P. Panina-Bordignon, X. T. Fu, A. Lanzavecchia, R. W. Karr, *J. Exp. Med.* **176**, 1779 (1992).
18. E. Roosnek and A. Lanzavecchia, *ibid.* **170**, 297 (1989).
19. C. Döhring, L. Angman, G. Spagnoli, A. Lanzavecchia, *Int. J. Cancer* **57**, 754 (1994).
20. A. Lanzavecchia, *Nature* **314**, 537 (1985).

21. We thank K. Karjalainen, G. De Libero, and S. Valitutti for critical reading and comments, and M. Dessing for help in flow cytometry. A.L. is associate professor of immunology at the University of Genova, Genova, Italy. The Basel Institute for Immunology was founded and is supported by F. Hoffmann–La Roche, Basel, Switzerland.

8 March 1996; accepted 13 May 1996