Brief Definitive Report

Costimulation by B7 Modulates Specificity of Cytotoxic T Lymphocytes: A Missing Link That Explains Some Bystander T Cell Activation

By Pan Zheng and Yang Liu

From the Michael Heidelberger Division of Immunology, Department of Pathology and Kaplan Cancer Center, New York University Medical Center, New York 10016

Summary

It has been proposed that some bystander T cell activation may in fact be due to T cell antigen receptor (TCR) cross-reactivity that is too low to be detected by the effector cytotoxic T lymphocyte (CTL). However, this hypothesis is not supported by direct evidence since no TCR ligand is known to induce T cell proliferation and differentiation without being recognized by the effector CTL. Here we report that transgenic T cells expressing a T cell receptor to influenza virus A/NT/68 nucleoprotein (NP) 366-374:D^b complexes clonally expand and become effector CTLs in response to homologous peptides from either A/PR8/34 (H1N1), A/AA/60 (H2N2), or A/NT/68 (H3N2). However, the effector T cells induced by each of the three peptides kill target cells pulsed with NP peptides from the H3N2 and H2N2 viruses, but not from the H1N1 virus. Thus, NP366–374 from influenza virus H1N1 is the first TCR ligand that can induce T cell proliferation and differentiation without being recognized by CTLs. Since induction of T cell proliferation was mediated by antigen-presenting cells that express costimulatory molecules such as B7, we investigated if cytolysis of H1N1 NP peptide–pulsed targets can be restored by expressing B7-1 on the target cells. Our results revealed that this is the case. These data demonstrated that costimulatory molecule B7 modulates antigen specificity of CTLs, and provides a missing link that explains some of the bystander T cell activation.

Bystander activation of T cells, or the expansion and functional differentiation of T cells with no apparent reactivity to the immunizing antigen, has been described in several models (1–5). Several groups have recently revisited the mechanisms of bystander T cell activation, and at least two hypotheses have been proposed (6–8). First, bystander activation may be due to nonspecific effects of cytokines with no involvement of TCR recognition. This hypothesis is supported by a recent report that type 1 interferon induced by virus can induce T cell proliferation and/or acquisition of memory cell markers (6). Second, bystander T cell activation may be due to antigen-driven proliferation of lymphocytes, but such cross-reactivity is too low to be detected by conventional cytotoxic T cell assay. Recent studies using transgenic T cells demonstrated that if the TCR is definitely unrelated to the challenging antigen, then bystander T cell activation is insignificant during CTL immune responses (7, 8). Although the findings in this study favor the second hypothesis, it remains to be demonstrated if there are low-affinity TCR ligands that induce T cell proliferation and maturation but not cytolysis.

TCR ligand density required for inducing T cell proliferation and IL-2 production appears to be significantly higher than that required for cytolysis (9, 10). These findings (9, 10) demonstrated that the total number of signals required for the induction is higher than that required to trigger the effector function. However, to compare the requirement for TCR ligand density it was necessary to use the same cells both as targets for cytolysis and as stimulators for T cell activation (9, 10). The professional APCs that induce the immune response are distinct from the cells on which the effector function of the CTL is executed. One of the distinct features is that APCs express multiple costimulatory molecules, the most potent of which are B7 family members B7-1 and B7-2 (for review see reference 11), whereas most target cells used for CTL assay do not express B7 molecules at significant levels. Since B7-CD28/CTLA4 interaction has been demonstrated to reduce the threshold for T cell activation (12), it is possible that some TCR ligands that cannot trigger cytolysis in the absence of B7 may do so in its presence. In this study, we compared the specificity of a TCR in the induction of T cell proliferation, differentiation into effector CTL, and cytolysis of target cells. We report here a viral peptide that induces clonal expansion and functional maturation of CD8 T cells without triggering cytolysis in CTL assay. These data provide the first direct evidence that clonal expansion and functional maturation of T cells can be induced by a ligand whose cross-reactivity

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/11/1787/05 $2.00
Volume 186, Number 10, November 17, 1997 1787–1791
http://www.jem.org

Table 1. *Sequence of Peptides Used in this Study*

Origin	Viral subtype	Peptide sequence (366–374)
A/PR8/34	H1N1	ASNENMETM
A/Ann Arbor/60	H2N2	ASNENMDTM
A/NT/68	H3N2	ASNENMDAM
P1A (AA35–43)	control	LPYLGWLVF

cannot be detected by a conventional CTL assay. More importantly, expression of B7-1 on the target cells restores CTL recognition of the viral peptide. These data demonstrate that costimulation by B7 modulates CTL specificity, and provide a missing link that explains some bystander T cell activation.

Materials and Methods

Experimental Animals. F5 transgenic mice (13) expressing T cell receptor for influenza nucleoprotein (NP) peptide 366–374 and F5 mice with a targeted mutation of the RAG-1 gene (14) were provided by Dr. Eugenia Spanopoulou (Mt. Sinai Medical Center, New York). C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Generally, 6–12-wk-old mice were used for the study, except for the Rag-1–deficient mice, which were used at the age of 4 wk old.

Viral Peptides. Three influenza viral peptides were used for this study. An L^d-binding tumor antigen P1A peptide was used as the control in most experiments. The sequences, viral origin, and viral subtypes of the peptides are listed in Table 1. All peptides were synthesized by Research Genetics (Huntsville, AL). Although influenza viruses of the same subtype can have different NP sequences, for simplicity we have used the subtypes to indicate the peptide used for the study.

Transfection of EL4 Cells. EL4 cells were transfected with either pSV vector or the pSV vector containing murine B7-1 cDNA as has been previously described (15). After selecting with G418 (0.6 μg/ml), the viable cells were screened for B7-1 expression using anti–B7-1 mAb 3A12 (16). A single clone expressing a high level of B7-1 and a control drug-resistant clone, EL4-Neo, were used for the study.

Proliferation of T Cells to Antigenic Peptides. Total spleen cells (2×10^5/well) from F5 transgenic mice were cultured with the given concentrations of peptides in Click's EHAA medium for 48 h. In some experiments, 10^4/well of RAG-1–deficient F5 transgenic spleen cells and 2×10^5 mitomycin C–treated C57BL6/j spleen cells were stimulated by given concentration of the viral peptides for 72 h. The proliferation of T cells was determined by incorporation of [^{3}H]TdR pulsed (1.25 μCi/well) during the last 6 h of culture. The data presented are means of duplicates with variation from the means <15%.

CTL Assay. For target cells, we used EL-4 ($H\text{-}2^b$) target cells, or EL-4- cells transfected with either vector alone (EL4-Neo) or with B7-1 (EL4-B7). These targets were labeled with ^{51}Cr for 1 h at 37°C. After three washes, the labeled target cells were added to a 96-well plate containing varying concentrations of synthetic peptides corresponding to amino acid (AA) 366–374 of influenza virus nucleoprotein or a control peptide. The effector cells were added and incubated for 6 h, and the released ^{51}Cr in the supernatants was determined. The specific percentage of lysis was calculated by the following formula: Specific % lysis = $[(cpm_{samples} - cpm_{medium})/(cpm_{maxim} - cpm_{medium})] \times 100$. As effector cells, we used spleen cells (0.5×10^6/ml) from F5 transgenic mice after they were stimulated with 0.1 μg/ml of viral peptides for 4 d in vitro.

Results and Discussion

Split Responses of F5 Transgenic T Cells to NP Peptides from H1N1 Virus. The α and β chains for TCR in the F5 transgenic mice were cloned from a CTL clone, F5, which kills target cells pulsed with peptide from a H3N2 virus (NP366–374) but not those pulsed with the homologous peptide from a H1N1 virus (17). To test whether naive transgenic T cells have the same specificity, we isolated spleen cells from the F5 mice and tested their proliferative responses to the NP366–374 peptides from H1N1, H2N2, and H3N2 virus. The NP peptides from H3N2 and H1N1 viruses each differ from H2N2 NP peptide in one amino acid, at either position 7 or 8. As shown in Fig. 1 *a*, the H1N1 NP peptide induces significant proliferative responses of the transgenic T cells. However, 100-fold more H1N1 peptide than H3N2 peptide is needed to achieve similar levels of proliferation. Interestingly, H2N2 NP peptides are ~100-fold more efficient than the peptide from H3N2 virus that induced the F5 T cells in the first place.

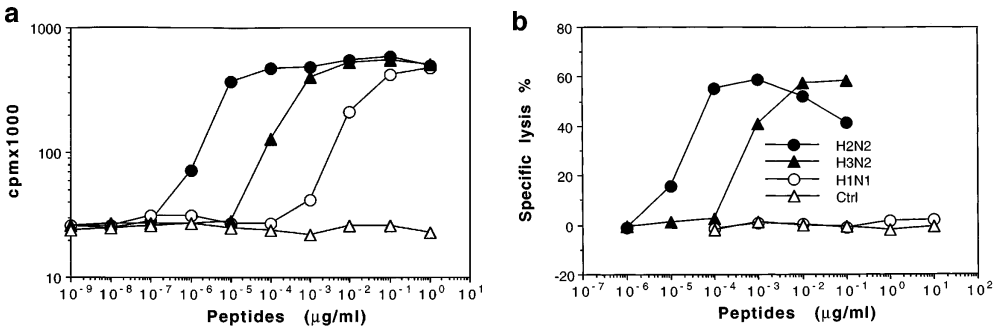

Figure 1. Split responses of the F5 transgenic T cells to peptides corresponding to AA366–374 of nucleoprotein from influenza virus A/PR8/34 (H1N1), A/Ann Arbor/60 (H2N2), and A/NT/68 (H3N2). (*a*) Proliferative T cell response. F5 spleen cells (2×10^5/well) were stimulated with given concentration of the viral peptides or control P1A peptides for 48 h. Proliferation of T cells was determined by incorporation of [^{3}H]TdR pulsed during the last 6 h of culture. (*b*) Specificity of activated F5 CTLs. F5 spleen cells (5×10^5/ml) were stimulated with 0.1 μg/ml of the H3N2 NP peptide for 4 d. Viable cells were isolated and used as effector cells. EL4 cells were labeled using ^{51}Cr and added to 96-well plates containing varying concentrations of peptides. Effector/target ratio is 60:1 for all groups.

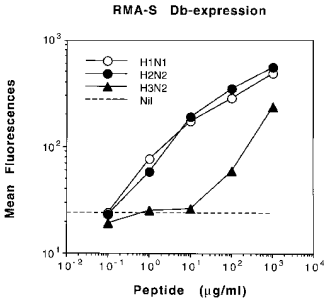

Figure 2. Relative D^b-binding activity of the viral peptides used for the study as determined by MHC stabilization assay using RMA-S cells. RMA-S cells (10^5/well) were incubated overnight with varying concentrations of viral peptides in RPMI medium containing 20% FCS. The cell surfaces of H-2D^b were determined by flow cytometry using biotinylated anti-D^b mAb (KH95) and PE-labeled streptavidin. Data presented are mean fluorescences, as measured by flow cytometry. The dotted line indicates expression of D^b in the absence of exogenous peptide.

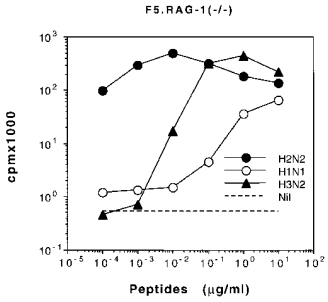

Figure 3. Proliferation of RAG-$1^{-/-}$ F5 T cells to NP peptides from all three strains of influenza viruses. RAG-$1^{-/-}$ spleen cells (10^4/well) were stimulated by varying concentrations of the viral peptides and 2×10^5 mitomycin C–treated syngeneic spleen cells per well as accessory cells. The dotted line depicts T cell proliferation when no peptide is added.

The proliferation is specific, as control peptide from a tumor antigen P1A, which binds H-2L^d but not D^b, induces no proliferative responses at all doses tested.

Next we activated the spleen cells with the H3N2 NP peptide and tested the specificity of the effector T cells. As shown in Fig. 1 *b*, the F5 effector T cells efficiently lysed the target cells pulsed with either H3N2 or H2N2 NP peptides, but at all doses tested no lysis of the H1N1 NP peptide–pulsed target cells was detected. A comparison of the dose responses in Fig. 1, *a* and *b*, reveals that differential specificity of the F5 T cells detected by proliferation and CTL assay was not due to the differential sensitivity of the assays. First, with regards to the doses of peptides, the sensitivities of both assays were very similar; 50% maximal response was achieved at 10^{-3} μg/ml of H3N2 peptide and 10^{-5} μg/ml of H2N2 peptide. Second, in proliferation assay, it took only 100-fold more H1N1 than H3N2 peptide to induce a comparable proliferation as the H3N2 peptides, whereas 10,000-fold more H1N1 peptide than is required for maximal lysis of H3N2 peptide–pulsed targets still does not trigger cytolysis.

The difference in the ability of the three peptides to induce F5 T cell proliferation and sensitize target cell lysis by CTL may be due either to the differential ability of these peptides to bind H-2D^b, or to differential recognition of these peptides by the F5 TCR. We performed RMA-S D^b-stabilization experiments to differentiate these possibilities. RMA-S cells lack functional TAP(transporter associated with antigen processing)-2 gene, and express empty MHC class I that can be stabilized by adding exogenous peptides (18). MHC stabilization assay has been widely used to measure MHC–peptide interaction on live cells. As shown in Fig. 2, NP peptides from H1N1 and H2N2 viruses are comparable in stabilizing H-2D^b, whereas the peptide from H3N2 virus is ~100-fold less efficient. Thus, the difference between the H3N2 and H2N2 NP peptides at position 8 affects their ability to bind D^b, whereas the difference between the H1N1 and two other NP peptides at position 7 affects F5 TCR recognition. This is consistent with the three-dimensional structure of D^b:NP peptide complex (19) in which the main chain and part of the side chain of the H1N1 peptide at position 8 are buried in the MHC, and residue E at position 7 is fully accessible to solvent. It is therefore likely that the unique split T cell response to the H1N1 NP peptide is caused by TCR ligand structure rather than ligand density.

Since most of the experiments were carried out in transgenic mice which may undergo endogenous V-D-J/V-J rearrangement, it is possible to explain the distinct specificity at the inductive and the effector phase by postulating the existence of two populations of T cells: one expresses the transgenic receptors that have the specificity of the original F5 clones, whereas the other gains the specificity for the H1N1 peptide as a result of pairing a transgenic TCR chain with an endogenous chain. We used spleen cells from the Rag-$1^{-/-}$ F5 mice as the responder T cells to rule out this possibility. Again, the H1N1 NP peptide induces significant proliferation of the Rag-1–deficient F5 T cells (Fig. 3). This result confirmed that the F5 TCR has reactivity to the H1N1 NP peptide.

To test if all three related peptides are capable of inducing cytotoxicity from the transgenic T cells, we stimulated F5 spleen cells with NP peptides from all three strains of influenza viruses and tested the influenza-specific CTLs generated. In addition, we also compared the fine specificity of the CTL generated in each culture by using a target cell pulsed with three NP peptides and an unrelated control peptide. As shown in Fig. 4, *a–c*, all three influenza peptides induce mature CTLs capable of lysis H3N2 and H2N2 NP peptide–pulsed targets. These results demonstrated that the H1N1 NP peptide is capable of inducing the maturation of CTLs. Furthermore, although the H1N1 NP peptide-induced CTL is less potent than those induced by the H2N2 and H3N2 NP peptides, the relative lysis of the CTL towards three different peptides remains the same. These results substantiate the notion that T cells with the same fine specificity are stimulated by three different NP peptides.

Taken together, the results presented in this section indicate that NP 366–374 from the H1N1 virus can induce F5 T cell proliferation and maturation into CTLs. However, the F5 CTL cannot recognize target cell pulsed with the H1N1 NP peptide. To our knowledge, this is the first TCR ligand known to have such properties. This type of TCR ligand can be the underlying cause of some of the so-called bystander T cell activation in vivo.

Modulation of CTL Specificity by Costimulatory Molecule B7-1. Proliferation assays used spleen accessory cells expressing multiple costimulatory molecules. In contrast, the

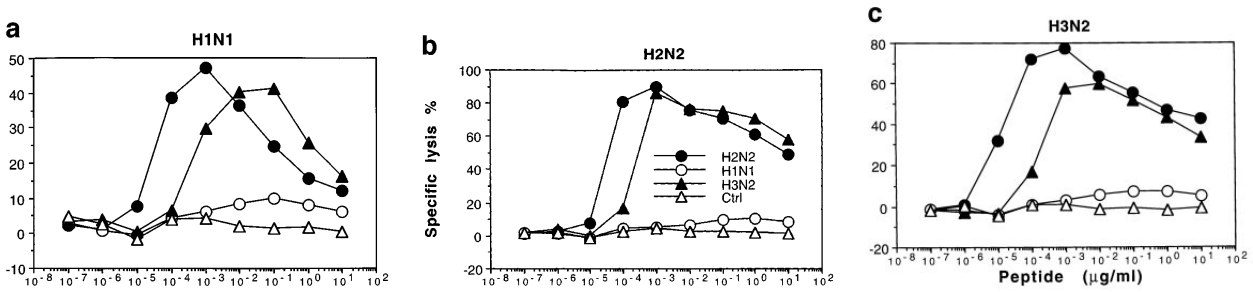

Figure 4. Induction of cytotoxicity of F5 T cells by viral peptides. Fine specificity of T cells induced by three different peptides. RAG-$1^{+/+}$ F5 spleen cells (5×10^5/ml) were stimulated with 0.1 μg/ml of H1N1 (*a*), H2N2 (*b*) or H3N2 (*c*) viral peptides for 4 d, and the viable cells were isolated and used as effectors. Effector/target ratio is 60:1 for all groups. See Fig. 1 legend for details.

CTL assay used EL4 thymoma devoid of costimulatory activity as targets (20). Therefore, it is possible that the split T cell response was due to the presence or absence of costimulatory activity in the cells used to measure cross-reactivity. To test this possibility, we transfected B7-1 into the EL4 cells. As shown in Fig. 5 *a*, EL4 cells transfected with vector alone have no detectable B7-1, while B7-1–transfected EL4 cells express high level of B7-1. Therefore, we compared cytolysis of EL4-Neo and EL4-B7 in the presence of various viral peptides. As shown in Fig. 5 *b*, EL4-Neo cells are lysed in the presence of H2N2 and H3N2 NP peptides, but not in the presence of H1N1 or control P1A peptides. In contrast, EL4-B7 targets are lysed in the presence of H1N1 NP peptide. Thus, expression of B7-1 on target cells restores F5 CTL recognition of H1N1 NP peptide–pulsed target cells. To our knowledge, this is the first evidence that costimulation by B7-1 modulates CTL specificity.

It has been demonstrated that costimulatory molecule B7 reduces the threshold of signals required for T cell activation, as evidenced by the reduction of density (12) and duration (21) of the ligand required to trigger a TCR in the presence of T cell costimulation. Moreover, we (15) and others (22, 23) have shown that costimulation enhances CTL effector function in vitro and in vivo in antitumor immunity and autoimmunity. The results presented here extend this notion by showing that one outcome of lowering the threshold of T cell activation is to increase cross-reactivity of TCRs.

Despite the recent interest in the subject (6–8), the molecular basis for bystander T cell activation remains unclear. Tough et al. (6) reported massive proliferation of memory CD8 T cells mediated by type 1 interferon, which is produced in response to viral infection. Ehl et al. (7) showed that when the overwhelming number of naive T cells are specific for an unrelated antigen, a low level of bystander CTL activation, mediated by IL-2, can be detected. However, Ehl et al. concluded that this level of bystander activation is unlikely to be biologically significant in nontransgenic systems. Zarozinski and Welsh (8) demonstrated that minimal proliferation of T cells that are not cross-reactive to the challenging antigen during a strong immune response, and, by inference, suggested that the majority of T cell expansion during viral infection must be due to some degree of TCR engagement. Our study demonstrates that presence of costimulatory molecules, such as B7-1, on the target cells, can increase the sensitivity of TCR for low-affinity ligands. Given the existence of multiple costimulatory molecules on the professional APCs (for review see

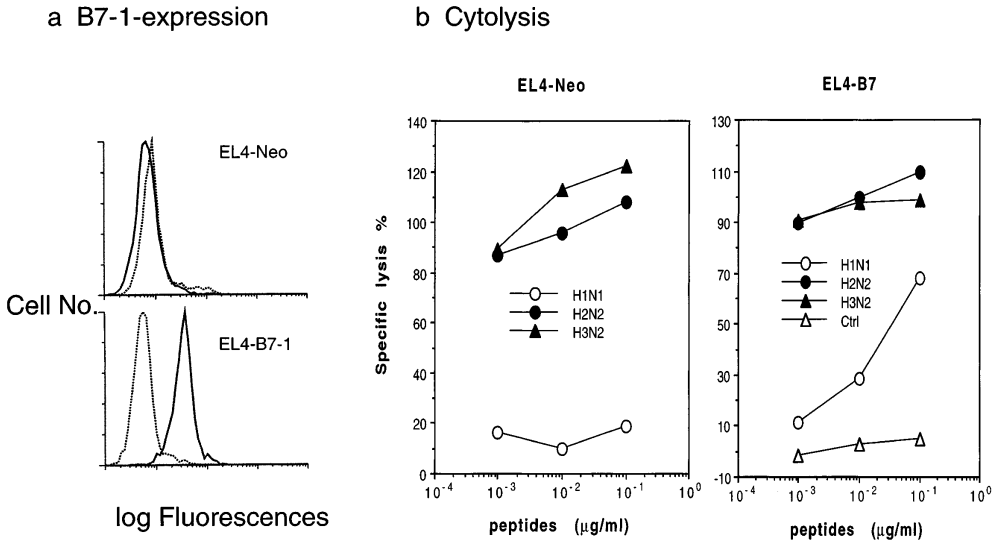

Figure 5. Modulation of CTL specificity by costimulatory molecule B7-1. (*a*) Expression of B7-1 in EL4 cells transfected with vector alone (*top*) or B7-1 cDNA (*bottom*). Data presented are FACS‟ histograms of fluorescences in the presence (*solid lines*) and absence of anti–B7-1 mAb 3A12. (*b*) Fine specificity of F5 CTL using EL4-Neo or EL4-B7 targets. See Fig. 1 legends for details of the CTL assay.

reference 11), the conclusion from this study provides a mechanism by which viral antigens induce TCR-mediated expansion of multiple T cell clones, even if their TCR–cross-reactivity is too low to be detected by conventional CTL assays.

We thank Dr. Tom Moran for helpful discussion, Dr. Eugenia Spanopoulou for providing Rag-1–deficient F5 transgenic mice, and Dr. John Hirst for assistance in flow cytometry.

This study was supported by a grant from the National Institutes of Health (AI-32981) and a grant from the Council for Tobacco Research, U.S.A., Inc.

Address correspondence to Yang Liu, Michael Heidelberger Division of Immunology, Department of Pathology and Kaplan Cancer Center, New York University Medical Center, 550 First Ave, New York, NY 10016. Phone: 212-263-7838; FAX: 212-263-8179.

Received for publication 15 August 1997 and in revised form 16 September 1997.

References

1. Nahill, S.R., and R.M. Welsh. 1993. High frequency of cross-reactive cytotoxic T lymphocytes elicited during virus-induced polyclonal cytotoxic T lymphocyte response. *J. Exp. Med.* 177:317–327.
2. Yang, H., and R. Welsh. 1986. Induction of alloreactive cytotoxic T cells by acute virus infection of mice. *J. Immunol.* 136:1186–1193.
3. Yang, H., P. Dunson, S. Nahill, and R. Welsh. 1989. Virus-induced polyclonal cytotoxic T lymphocyte stimulation. *J. Immunol.* 142:1710–1718.
4. Schendel, D., C. Reinhardt, P. Nelson, B. Maget, L. Pullen, G. Bornkamm, and A. Steinle. 1992. Cytotoxic T lymphocytes show HLA-C–restricted recognition of EBV-bearing cells and allorecognition of HLA class I molecules presenting self-peptides. *J. Immunol.* 149:2406–2414.
5. Stang, G., and A. Rickinson. 1987. Multiple HLA class I–dependent cytotoxicities constitute the "non-HLA-restricted" response to infectious mononucleosis. *Eur. J. Immunol.* 17:1007–1014.
6. Tough, D.L., P. Borrow, and J. Sprent. 1996. Induction of bystander T cell proliferation by virus and type I interferon in vivo. *Science (Wash. DC).* 251:1225–1228.
7. Ehl, S., J. Hombach, P. Aichele, H. Hengartner, and R. Zinkernagel. 1997. Bystander activation of cytotoxic T cells: studies on the mechanism and evaluation of in vivo significance in a transgenic mouse model. *J. Exp. Med.* 185:1241–1251.
8. Zarozinski, C.C., and R. Welsh. 1997. Minimal bystander activation of CD8 T cells during the virus-induced polyclonal T cell response. *J. Exp. Med.* 185:1629–1639.
9. Valitutti, S., S. Muller, M. Dessing, and A. Lanzavecchia. 1996. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J. Exp. Med.* 183:1917–1921.
10. De Bruijn, M.L., T.N. Schumacher, J.D. Nieland, H.L. Ploegh, W.M. Kast, and C.J. Melief. 1991. Peptide loading of empty major histocompatibility complex molecules on RMA-S cells allows the induction of primary cytotoxic T lymphocyte responses. *Eur. J. Immunol.* 21:2963–2970.
11. Liu, Y., and P.S. Linsley. 1992. Costimulation of T-cell growth. *Curr. Opin. Immunol.* 4:265–270.
12. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science (Wash. DC).* 273:104–106.
13. Mamalaki, C., J. Elliott, T. Norton, N. Yanoutsos, A.R. Townsend, P. Chandler, E. Simpson, and D. Kioussis. 1993. Positive and negative selection in transgenic mice expressing a T cell receptor specific for influenza nucleoprotein and endogenous superantigen. *Dev. Immunol.* 3:159–174.
14. Corbella, P., D. Moskophidis, E. Spanopoulou, C. Mamalaki, M. Tolaini, A. Itano, D. Lans, D. Baltimore, E. Robey, and D. Kioussis. 1994. Functional commitment to helper T cell lineage precedes positive selection and is independent of T cell MHC receptor specificity. *Immunity.* 1:269–276.
15. Ramarathinam, L., M. Castle, Y. Wu, and Y. Liu. 1994. T cell costimulation by B7/BB1 induces CD8 T cell–dependent tumor rejection: an important role of B7/BB1 in the induction, recruitment, and effector function of antitumor T cells. *J. Exp. Med.* 179:1205–1214.
16. Wu, Y., Y. Guo, and Y. Liu. 1993. A major costimulatory molecule on antigen-presenting cells, CTLA4 ligand A, is distinct from B7. *J. Exp. Med.* 178:1789–1793.
17. Townsend, A.R., J. Rothbard, F.M. Gotch, G. Bahadur, D. Wraith, and A.J. McMichael. 1986. The epitope of influenza nucleoprotein recognized by cytotoxic lymphocytes can be defined with short synthetic peptides. *Cell.* 44:959–968.
18. Powis, S.J., A.R. Townsend, E.V. Deverson, J. Bastin, G.W. Butcher, and J.C. Howard. 1991. Restoration of antigen presentation to the mutant cell line RMA-S by an MHC-linked transporter. *Nature (Lond.).* 354:528–531.
19. Young, A.C.M., W. Zhang, J.C. Sacchettini, and S.G. Nathenson. 1994. The three-dimensional structure of H-2Db at 2.4 A resolution: implication for antigen-determinant selection. *Cell.* 76:39–50.
20. Zuckerman, L.A., A.J. Sant, and J. Miller 1995. Identification of a unique costimulatory activity to murine T helper 1 T cell clones. *J. Immunol.* 154:4503–4512.
21. Kündig, M., A. Shahinian, K. Kawai, H.-W. Mittrücker, E. Sebzda, M.F. Bachmann, T.W. Mak, and P.S. Ohashi. 1996. Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity.* 5:41–52.
22. Soldevila, G., T. Geiger, and R.A. Flavell. 1995. Breaking immunologic ignorance to an antigenic peptide of simian virus 40 large T antigen. *J. Immunol.* 155:5590–5600.
23. Harlan, D. M., H. Hengartner, M.L. Huang, Y.H. Kang, R. Abe, R.W. Moreadith, H. Pircher, G.S. Gray, P.S. Ohashi, G.J. Freeman et al. 1994. Mice expressing both B7-1 and viral glycoprotein on pancreatic beta cells along with glycoprotein-specific transgenic T cells develop diabetes due to a breakdown of T-lymphocyte unresponsiveness. *Proc. Natl. Acad. Sci. USA.* 91:3137–3141.