Partial Signaling by CD8$^+$ T Cells in Response to Antagonist Ligands

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Summary

Structural variants of an agonist peptide–major histocompatibility complex (MHC) molecule ligand can show partial agonist and/or antagonist properties. A number of such altered ligands appear to act as pure antagonists. They lack any detectable ability to induce T cell effector function and have been described as unable to induce calcium transients and turnover of inositol phosphates. This has been interpreted as an inability of these ligands to initiate any T cell receptor (TCR)–dependent signal transduction, with their antagonist properties ascribed to competition with offered agonist for TCR occupancy. Yet antagonists for mature CD8$^+$ T cells can induce positive selection of thymocytes, implying active induction of T cell differentiation events, and partial agonists or agonist/antagonist combinations elicit a distinctive pattern of early TCR-associated tyrosine phosphorylation events in CD4$^+$ T cells. We have therefore directly examined proximal TCR signaling in a CD8$^+$ T cell line in response to various related ligands. TCR engagement with natural peptide–MHC class I agonist resulted in the same pattern of early TCR-associated tyrosine phosphorylation events as seen with CD4$^+$ cells, including accumulation of both the p21 and p23 forms of phosphorylated ζ, phosphorylation of CD3ϵ, and association of phosphorylated ZAP-70 with the TCR. Two antagonists that lacked the ability to induce any detectable CTL effector response (cytolysis, esterase release, γ interferon secretion, interleukin-2 receptor α upregulation) were nevertheless found to also induce TCR-dependent phosphorylation events. In these cases, there was preferential accumulation of the p21 form of phospho-ζ without net phosphorylation of CD3ϵ, as well as the association of nonphosphorylated ZAP-70 kinase with the receptor. These data show that variant ligands induce similar TCR-dependent phosphorylation events in CD8$^+$ T cells as first observed in CD4$^+$ cells. More importantly, they demonstrate that some putatively pure antagonists are actually a subset of partial agonists able to induce intracellular biochemical changes through the TCR. This delivery of a partial signal by antagonists raises the possibility that antagonism in some cases may result from active interference with stimulation of effector activity by agonist in mature T cells, while the same variant signal could selectively trigger intracellular events that allow positive without negative selection in thymocytes.

Over the last few years the traditional TCR occupancy model for T cell activation has been challenged by the observation that subtle changes in the MHC or peptide component of a TCR ligand can give rise to antagonist ligands that no longer elicit any measurable T cell effector function but can diminish the response to the original ligand (1–5). Consistent with their inability to elicit T cell effector functions, several peptide antagonists fail to induce TCR-dependent signaling events such as the generation of Ca^{2+} fluxes and turnover of inositol phosphates (1, 3). These observations have led some investigators to conclude that antagonism is due to nonproductive engagement of the TCR by antagonist ligands, which competes with productive engagement by real agonists and spoils the formation of signaling oligomers (1, 6, 7). However, peptides that act as pure antagonists for mature T cells can positively select immature thymocytes bearing the same TCR, whereas agonist ligands act as negatively selecting elements in the same system (8–10). These results are difficult to reconcile with the presumed failure of antagonists to deliver signals through the TCR, unless it is assumed that these ligands positively select thymocytes by antagonizing a deletional signal induced by other thymic ligand(s) (11).

Partial agonists, which selectively induce certain effector functions or anergy in T cells (2, 12–15), have recently been shown to deliver signals through the TCR that give rise to a distinct pattern of phosphorylation of TCR-associated and recruited proteins (16, 17). These observations

were made using $CD4^+$ T cell clones and have not been extended to either $CD8^+$ cells, for which almost no data on peptide–MHC ligand–induced tyrosine phosphorylation is available, or to antagonist ligands that induce no measurable mature T cell effector function. In the present study, we have examined whether TCR signaling can be detected in $CD8^+$ T cells in response to antagonists and report that ligands able to inhibit but not induce effector responses of a $CD8^+$ T cell line elicit a distinctive subset of biochemical signals through the TCR. These signals are characterized by a selective increase in the p21 form of phosphorylated ζ, little or no steady-state phosphorylation of CD3ϵ chains, and recruitment of ZAP-70 to the TCR complex without subsequent stable phosphorylation, results strikingly similar to those obtained with $CD4^+$ T cell clones in response to partial agonists or mixtures of agonist and antagonist (16, 17). These findings demonstrate that the failure to observe effector functions or downstream events such as Ca^{2+} transients or inositol triphosphate hydrolysis in response to peptide antagonists does not necessarily indicate an absolute lack of TCR signaling induced by these ligands. Our observations raise the possibilities that first, partial signals transduced in response to antagonists may in some cases actively interfere with signaling by agonist ligands, and second, the advantage of antagonists over agonists in inducing net positive selection may be due to the delivery of qualitatively different signals to developing thymocytes that promote differentiation without death.

Materials and Methods

Medium. RPMI 1640 medium (Biofluids, Rockville, MD) was supplemented with 2 mM glutamine, 10 mM nonessential amino acids, 10 mM HEPES, 10 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS (all from Biofluids), and 10 μM 2-ME.

Peptides. The OVA peptide 257–264 (SIINFEKL) (18) was synthesized by Dr. J. Coligan, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD. Variant peptides P6 (SIINFPKL), D7 (SIINFEDL), and V-OVA (RGYNYEKL, containing the three dominant TCR contact residues from SIINFEKL in the context of an unrelated, K^b-binding peptide from vesicular stomatitis virus [3]) were obtained from Chiron Mimotopes Peptide Systems (San Diego, CA). These peptides have previously been used to study antagonism of CTL clones specific for SIINFEKL plus K^b (3). The control K^b-binding peptide SENDAI (FAPGNYPAL; Sendai virus nucleoprotein 324–332 [19]) was a gift from Dr. David H. Margulies, NIAID, NIH, and has an affinity for K^b similar to that of SIINFEKL (20).

Cell Lines. MCW, a CTL line specific for SIINFEKL plus K^b, was generated from B6 mice immunized with vaccinia expressing the SIINFEKL epitope and has been in culture for over a year (21). It expresses high levels of CD3ϵ, TCR, CD8α, and CD8β and is uniformly $V\beta6^+$ despite the fact that it has not been cloned by limiting dilution (data not shown). The line was maintained in 24-well plates (Costar Corp., Cambridge, MA) in medium containing 7.5% T-Stim™ (Collaborative Biomedical Products–Becton Dickinson Labware, Bedford, MA) and was stimulated biweekly with irradiated (2,000 rad) C57BL/6 spleen cells pulsed for 30–60 min with 10 μM SIINFEKL. The MCW line was expanded for experiments by transferring cells from each well to 25-cm^2 flasks (Corning Inc., Corning, NY) with peptide-pulsed irradiated stimulators and culturing upright for 3 d as for stimulation on plates. On day 3, fresh medium with T-Stim™ to a final concentration of 15% was added, the cells were resuspended, and the cultures were incubated lying down for a further 4 d.

RMA cells ($H\text{-}2^b$) (22) or 1-3, an L cell line transfected with K^b (23), were used as APCs for MCW in functional and biochemical tests.

Cytolysis Assays. Targets were labeled for 1–2 h at 37°C with ~100 μCi of [^{51}Cr]sodium chromate (Amersham, Arlington Heights, IL) in the presence (antagonism assay) or absence (agonism assay) of the concentrations of SIINFEKL peptide indicated in the figure legends. Following washing in PBS, $0.8\text{–}2.0 \times 10^4$ targets were added to a titration of test peptides in 96-well round-bottom plates in a final volume of 100 μl of complete medium and incubated for 30–60 min at 37°C (3). MCW CTLs were subsequently added in 100 μl of complete medium to the final indicated E/T ratio, the plates were centrifuged to promote cell contact, and the pelleted cells were left at 37°C for 4–6 h. Supernatants were harvested, and radioactivity was counted and converted to percent specific release as described previously (21). In some variations of the experiment, labeled targets were pulsed with or without SIINFEKL followed by the test peptides and washed before incubating with the CTLs.

For prolonged assays in which multiple parameters of MCW activation were measured, cytolysis of APCs was assessed by the release of lactate dehydrogenase (LDH)[1] into the supernatant by use of an enzymatic assay (CytoTox 96 kit; Promega, Madison, WI) per the manufacturer's instructions. Data are expressed as percentage of the maximal release observed following subtraction of the release induced by targets pulsed with no peptide.

IFN-γ Production. MCW cells were incubated in 24-well plates overnight with peptide-pulsed APCs in 1 ml of complete medium. Supernatants were assayed for the presence of IFN-γ by ELISA in triplicate, using the XMG1.2 mAb (PharMingen, San Diego, CA) as a capture reagent. Bound cytokine was detected with a rabbit polyclonal serum against mouse IFN-γ (kind gift from Dr. W.E. Paul, NIAID, NIH), followed by goat anti–rabbit conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The plates were developed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO), and the absorbance at 405 nm was recorded after 0.5–2.0 h. Data are expressed as percentage of the maximal secretion observed after subtraction of the secretion induced by cells pulsed with no peptide.

Serine Esterase Release. MCW cells were either cultured in 24-well plates overnight with APCs as for the IFN-γ assay or were cultured in 96-well plates for 6 h with (unlabeled) targets as for the chromium release assays. Serine esterase release by MCW was measured in triplicate as previously described (3). The mean absorbance at 405 nm is expressed as a percentage of the maximal release observed in response to APCs pulsed with SIINFEKL peptide after subtraction of the release induced by targets pulsed with no peptide.

Flow Cytometry. Propidium iodide–gated cells were analyzed with a FACScan® cytometer and Lysys™ software (Becton Dickinson & Co., Mountainview, CA). To determine IL-2 receptor α (IL-2Rα; CD25) upregulation following MCW activation by

[1] *Abbreviation used in this paper:* LDH, lactate dehydrogenase.

peptide-pulsed 1-3 cells, CTL–APC conjugates were dissociated by washing in PBS containing 1 mM EDTA, and cells were double-stained with mAbs (from PharMingen), 3C7 (αCD25; rat IgG2b), followed by PE-conjugated goat anti–rat (Caltag, San Francisco, CA) and FITC-AF3-12.1 (αH-2K^k; mouse IgG1). Because 1-3 but not MCW cells express H-2^k antigens, APCs were excluded from the analysis by live gating on FITC-negative cells.

Tyrosine Phosphorylation Assays. A modification of a previously described assay was employed to detect tyrosine-phosphorylated proteins involved in TCR signaling (17). In brief, 5–10 × 10^6 MCW cells were added to 1–3 × 10^6 peptide-pulsed APCs in Eppendorf tubes, cells were pelleted by centrifugation, and the tubes were placed in a water bath at 37°C for 5 or 10 min. At the end of the incubation period, the tubes were transferred to ice, cells were resuspended in ice-cold PBS containing 0.4 mM EDTA and 0.4 mM sodium orthovanadate, and they were then re-pelleted and lysed in lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris–HCl, pH 7.6, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM orthovanadate). ZAP-70 was immunoprecipitated from lysates with a rabbit polyclonal antiserum (generous gift of Dr. L. Samelson, National Institute of Child Health and Human Development, NIH, or purchased from Upstate Biotechnology, Lake Placid, NY). Immunoprecipitates were separated by SDS-PAGE on precast, 12 or 15% Mini-PROTEAN® II gels (Bio-Rad, Richmond, CA) under reducing conditions and Western blotted onto nitrocellulose. The blots were probed with a mouse mAb against phosphotyrosine (4G10; Upstate Biotechnology), followed by peroxidase–sheep anti–mouse (Amersham International, Buckinghamshire, England), and developed by chemiluminescence (Renaissance kit; DuPont–New England Nuclear, Boston, MA). Quantification of bands on film exposures was performed with a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Results

SIINFEKL Variant Peptides V-OVA, P6, and D7 Have No Detectable Agonist Function for MCW CD8$^+$ CTLs. We made use of a CD8$^+$, Vβ6$^+$ CTL line generated in our laboratory, specific for SIINFEKL plus K^b (21), to investigate the response of CD8$^+$ T cells to altered ligands. Jameson and Bevan have mapped the primary TCR contact sites of the SIINFEKL epitope to peptide residues 4, 6, and 7 and reported that a large proportion of variants at these positions can act as antagonist ligands for one or more CTL clones (3, 24). We therefore tested three of these variants we had available, V-OVA, P6, and D7, for their ability to sensitize target cells for lysis by MCW in a standard cytotoxicity assay. In various assays, using ^{51}Cr or LDH release from 1-3 or RMA targets as a measure of cytolysis and testing peptide concentrations up to 50 μM and assay lengths up to 6 h, we were unable to detect any killing of targets pulsed with the variant peptides, despite the fact that SIINFEKL was able to sensitize targets for killing at concentrations down to the picomolar range (data not shown). However, because variant peptide ligands can selectively induce some T cell effector functions and not others (2, 12, 13, 15, 25), we examined other parameters of MCW activation for evidence of partial agonist function by the SIINFEKL analogues. MCW cells were incubated overnight with APCs prepulsed with a titration of SIINFEKL or the test peptides. The variant peptides failed to induce MCW cells to release serine esterase, secrete IFN-γ, or upregulate the α chain of the receptor for IL-2, despite the fact that 10^7 times less SIINFEKL peptide (5 pM) than the maximal tested concentration of variant peptide (50 μM) was capable of inducing a measurable response for each of these parameters (Table 1). As before, killing of the variant peptide–pulsed target cells was also not induced in this long assay, despite the fact that it was the most sensitive effector function measurable (Table 1). D7 was also completely unable to sensitize 1-3 or RMA targets to form conjugates with MCW, even though conjugates with SIINFEKL-pulsed targets were easily detectable by flow cytometry (data not shown). Taken together, these data demonstrate that V-OVA, P6, and D7 are unable to elicit nuclear transcription–independent (cytolysis, serine esterase release, adhesion) or transcription-dependent (IFN-γ production, CD25 upregulation)

Table 1. *SIINFEKL Variant Peptides Fail to Act as Agonists for MCW CTL*

	Killing		Serine esterase		IFN-γ		IL-2Rα
[SIINFEKL]	Mean*	SD*	Mean*	SD*	Mean*	SD*	Mean‡
0	0	0	0	0	0	0	0
5 pM	98	1	30	0	13	1	6
50 pM	96	2	33	0	27	3	9
500 pM	89	3	47	0	49	2	18
5 nM	95	2	89	1	58	3	61
50 nM	97	1	97	1	58	2	77
500 nM	99	2	98	0	57	3	91
5 μM	96	1	100	1	60	1	100
50 μM	100	2	100	1	100	12	80
50 μM SENDAI	1	0	0	0	0	0	0
50 μM V-OVA	0	0	0	0	0	0	1
50 μM P6	0	0	0	0	0	0	0
50 μM D7	8	0	0	0	0	0	0

10^6 1-3 APC were pulsed for 2 h at 37°C with the indicated peptide concentrations, washed in PBS, and plated with 10^6 MCW CTL in 1 ml complete medium in 24-well plates. After overnight incubation (17 h), supernatants were recovered and assayed for the presence of LDH (released by the target cells) and serine esterase and IFN-γ (released by the CTL), as detailed in Materials and Methods. The cells were harvested, dissociated into a single-cell suspension in the presence of EDTA, and double-stained for IL-2Rα (CD25) and H-2K^k. IL-2Rα expression by MCW was assessed by flow cytometry after propidium iodide gating to exclude dead cells and live gating to exclude H-2K^{k+} 1-3 targets. Data for all parameters are expressed as a percentage of the maximum response after subtraction of the response to unpulsed 1-3. Negative values are expressed as zero.

*Mean and standard deviation of triplicate determinations.

‡Mean fluorescence of 10,000 collected events.

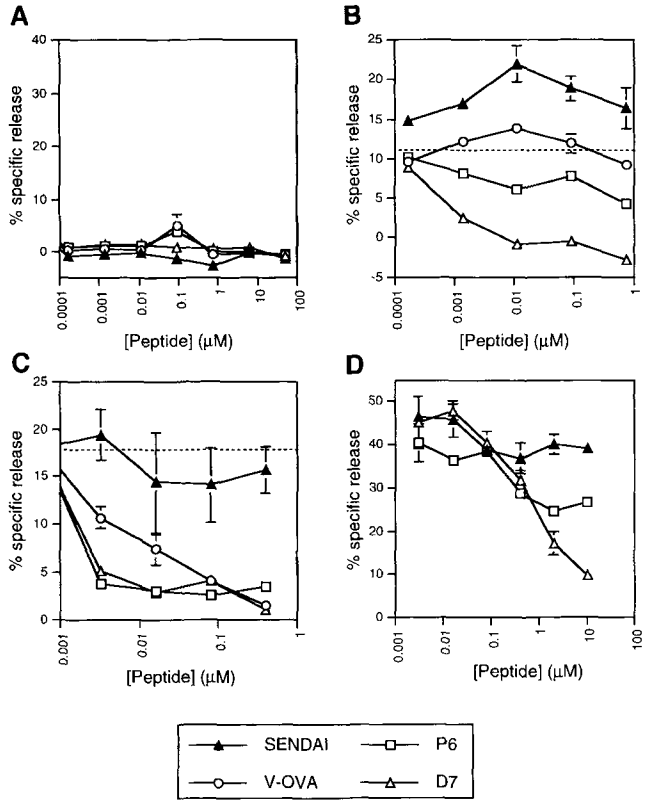

Figure 1. Peptide analogues modulate cytolysis of SIINFEKL-pulsed target cells. (*A*) agonism assay (no SIINFEKL pre-pulse); and (*B–D*) antagonism assays (SIINFEKL pre-pulse). *A* and *B* are from the same experiment; *C* and *D* are from separate experiments. RMA (*A*, *B*, and *D*) or 1-3 (*C*) targets were labeled with ^{51}Cr for 1–1.5 h in the absence (*A*) or presence (*B–D*) of SIINFEKL (*B* and *C*, 1 nM; *D*, 100 nM), washed twice in PBS, and added in 50 μl of complete medium to wells of a microtiter plate containing 50 μl of a serial dilution of the indicated peptides, to give the indicated final peptide concentration. After preincubation for 30–45 min at 37°C, MCW cells were added in 100 μl of medium, halving the concentration of peptide. The plates were centrifuged and incubated at 37°C for 4–6 h. Data are expressed as the mean ^{51}Cr release of triplicate wells converted to percent specific release ±SD. Dotted lines in *B* and *C* indicate the level of killing in the absence of test peptide during the assay. Number of targets used and E/T ratios were as follows: *A* and *B*, 8 × 10^3 targets, E/T 5:1; *C*, 15 × 10^3 targets, E/T 5:1; *D*, 15 × 10^3 targets, E/T 17:1. Spontaneous and maximum release values ±SD (in cpm): *A*, 2517 ± 387 and 18394 ± 1355; *B*, 2345 ± 229 and 15530 ± 963; *C*, 3275 ± 363 and 29239 ± 1526; *D*, 726 ± 112 and 11755 ± 1153.

effector functions and by these criteria would typically be classified as non-agonists for MCW cells.

V-OVA, P6, and D7 Can Act as Antagonists for MCW Cells. Jameson and Bevan have demonstrated that some OVA variant peptides that do not act as agonists in functional assays can nonetheless act as specific TCR antagonists (3). To test for antagonist activity, ^{51}Cr-labeled targets were either pulsed or not with a limiting concentration of SIINFEKL and were plated with different concentrations of the variant peptides. CTLs were added, and cytolysis was measured 4–6 h later. As before, unpulsed targets could not be sensitized for lysis by the variant peptides present in the assay (Fig. 1 *A*), although, in control experiments, the inclusion of SIINFEKL resulted in target cell lysis (data not shown). Three antagonism experiments using targets pulsed with a limiting concentration of SIINFEKL peptide are shown in Fig. 1, *B–D*. D7 and P6 consistently inhibited lysis in a dose-dependent manner (Fig. 1, *B–D*). No such effect was seen with the control K^b-binding peptide, SENDAI, demonstrating that this effect was due to TCR antagonism rather than MHC competition (Fig. 1, *B–D*). V-OVA was not an antagonist in most experiments (e.g., Fig. 1 *B*), although it occasionally inhibited lysis (e.g., Fig. 1 *C*). Antagonism by D7 was seen in four out of four experiments and in three out of three experiments for P6. D7 was always the most and V-OVA the least potent antagonist of the three (Fig. 1, *B–C*). As previously reported (3), antagonism was not due to a direct effect of the peptide on the CTL, because it could also be seen in experiments in which targets were pulsed sequentially with SIINFEKL and D7 and washed before addition of MCW killers (data not shown). The fact that V-OVA, P6, and D7 are able to modulate the response to the agonist peptide demonstrates that these analogues are capable of interacting specifically with MCW TCRs, despite being unable to act as effector agonists per se.

P6 and D7 Signaling Results in Accumulation of the p21 Form of Phospho-ζ without Detectable Amounts of Tyrosine-Phosphorylated CD3ε, and in ZAP-70 Recruitment without Its Net Phosphorylation. The earliest events after TCR engagement, before the hydrolysis of phosphatidylinositol 4,5-bisphosphate and generation of Ca^{2+} fluxes, are the rapid tyrosine phosphorylation of TCR subunit chains and recruitment of the tyrosine kinase ZAP-70, followed by its phosphorylation and concomitant activation (26, 27). Some partial agonist ligands for $CD4^+$ T cells have been shown to induce an altered pattern of these early phosphorylation events as detected by anti-phosphotyrosine immunoblotting that may result in the selective induction of downstream events (16, 17). Little information is available on the early tyrosine phosphorylation events that follow receptor engagement by agonists or variant ligands in $CD8^+$ T cells. Höllsberg et al. have examined stimulation of a CTL clone with an altered peptide ligand and concluded that, as in $CD4^+$ T cells (15), a partial agonist was capable of activating coreceptor-associated $p56^{lck}$ in a manner similar to that of the wild-type agonist peptide (25). However, those authors did not report studies with antagonist ligands or, for the partial agonist examined, the phosphorylation status of ζ or ZAP-70. Therefore, to evaluate whether the antagonist peptides were capable of delivering a biochemical signal through the TCR, MCW cells were exposed to peptide-pulsed 1-3 cells for 10 min at 37°C. ZAP-70 and associated molecules were immunoprecipitated from lysates of these cells; then the proteins in the precipitates were resolved by SDS-PAGE and blotted for the presence of phosphorylated tyrosine residues. MCW cells exposed to unpulsed APCs showed few phosphorylated protein species coprecipitating with ZAP-70: only the p21 form of phospho-ζ was constitutively present (Fig. 2, lane *2*). In contrast, incubation of MCW with APCs pulsed with 10 μM SIINFEKL resulted

in the appearance of prominent tyrosine-phosphorylated species that coprecipitated with ZAP-70: two isoforms of phospho-ζ, p23 and p21, appeared as bands of roughly equal intensity, and a third isoform, p18, was also present as a fainter band; CD3ε was also phosphorylated, and a distinct band of phosphorylated ZAP-70 was evident (Fig. 2, lane *1*). Thus, these $CD8^+$ CTLs show a pattern of early TCR-associated phosphorylation events in response to agonist ligand similar to that observed with $CD4^+$ T cells (16, 17, 28). V-OVA did not induce phosphorylation of any ZAP-70 coprecipitable proteins above background (Fig. 2, lane *5*). However, the two more potent antagonist peptides, P6 and D7, clearly induced an increase in the level of ζ phosphorylation (Fig. 2, lanes *3* and *4*). The signal induced by P6 and D7 was distinct from that seen upon exposure to full agonist in several ways: the two variant peptides induced a disproportionate accumulation of the p21ζ form over the p18ζ and p23ζ forms, even after correcting for background p21ζ levels, and clearly allowed the association of ZAP-70 with the receptor complex while failing to induce its subsequent stable phosphorylation, because anti–ZAP-70 coprecipitated the ζ chains even though no phosphorylated ZAP-70 was detectable. ζ phosphorylation was also not accompanied by visible phosphorylation of the CD3ε chains. Identical patterns of phosphorylation were seen in samples immunoprecipitated with anti-CD3ε instead of anti–ZAP-70 (data not shown). Thus, these $CD8^+$ CTLs show a pattern of signaling in response to variant ligands indistinguishable from that previously reported for $CD4^+$ T cell clones in response to partial agonists or certain agonist/antagonist combinations (16, 17).

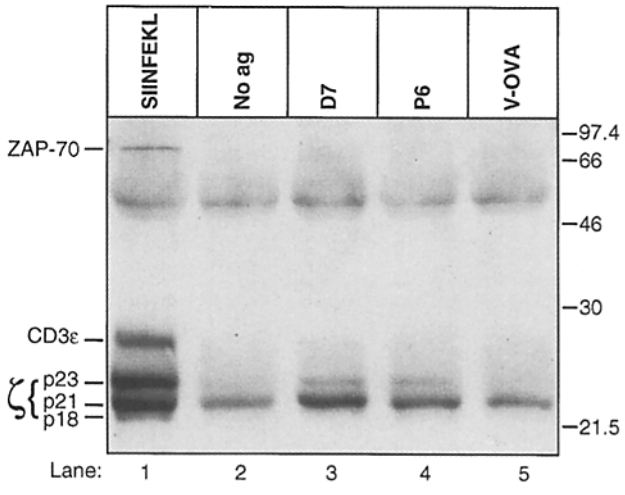

Figure 2. Variant signaling by MCW CTL in response to stimulation by K^b + variant peptides, P6 and D7. 1-3 APC (1.5×10^6/lane) were pulsed in complete medium for 1.5 h with medium alone (No ag) or the indicated peptides at 100 μM (V-OVA, P6, D7) or 10 μM (SIINFEKL). 10^7 MCW cells were added to each tube and stimulated for 5 min at 37°C, as detailed in Materials and Methods. Proteins in lysates were immunoprecipitated with an antiserum against ZAP-70, separated on a 15% polyacrylamide gel, and Western blotted onto nitrocellulose. The blot was probed with a mAb against phosphotyrosine and developed using chemiluminescence. The migration of prestained molecular weight markers is indicated on the right. The marks on the left identify the major tyrosine-phosphorylated species.

Phospho-ζ induction above background in response to D7 and P6 was observed in six out of seven experiments with D7 and two of three experiments with P6, using a variety of APCs and stimulation conditions. In contrast, phospho-ζ accumulation was not detectable in any of three experiments in response to V-OVA stimulation. Interestingly, the degree of ζ phosphorylation obtained with the variant peptides correlated with their potency in the antagonism assay (see above). Thus, D7 consistently gave the highest level of ζ phosphorylation and was also the most powerful antagonist, whereas the P6 peptide was of intermediate antagonist potency and elicited a weaker phosphorylation response (Figs. 1, *B–D*, and 2). Antagonism by V-OVA was inconsistent, and this peptide also did not elicit a biochemical signal from MCW cells. Thus, P6 and D7 act as partial agonists at the level of signaling through the TCR despite being nonagonists at the level of multiple effector functions.

The Pattern of Partial Signaling Induced by Antagonists Appears to Be Distinct from Low Level Signaling Induced by Agonist. Although the pattern of early phosphorylation of TCR complex chains induced by P6 and D7 appeared distinctly different from that in response to the SIINFEKL agonist peptide, it was possible that it simply represented weak signaling events insufficient to induce a functional response. If so, titrating the SIINFEKL peptide down to a dose where it no longer elicited a measurable response should result in a pattern of phosphorylation identical to that seen in response to P6 or D7. To address this issue, a phosphorylation assay was run in parallel with the effector assays shown in Table 1. MCW cells were incubated for 10 min with the same peptide-pulsed 1-3 cells used as APCs in the functional assay, and ZAP-70 and associated proteins were immunoprecipitated from lysates and blotted for phosphotyrosine residues as before. Fig. 3 shows that the set of phosphorylated species associated with ZAP-70 decreased in parallel with decreasing doses of offered SIINFEKL (lower gel, lanes *1–8*). Once again, D7 but not V-OVA or the control SENDAI peptide induced ζ chain phosphorylation above background (Fig. 3 *top*). Importantly, whereas 5 pM of SIINFEKL gave a barely detectable biochemical signal (Fig. 3, lower gel, lane *2*), it still induced maximal killing in the cytolysis assay that was run in parallel (Table 1). Thus, when using conventional agonist ligand, the cytolysis assay is more sensitive that the phosphorylation assay as a measure of TCR signaling. Yet at the same time, we observe definite TCR-dependent phosphorylation of the ζ chain when using D7, in the absence of any measurable cell killing. Because this latter pattern of detectable ζ phosphorylation without cytolysis is not observed across a full dose–response range for SIINFEKL, these data clearly imply a qualitative change in the relationship between TCR-induced biochemical changes and biological responses in the case of D7 presentation.

Further support for qualitative differences in signaling between SIINFEKL and P6 and D7 came from visual and

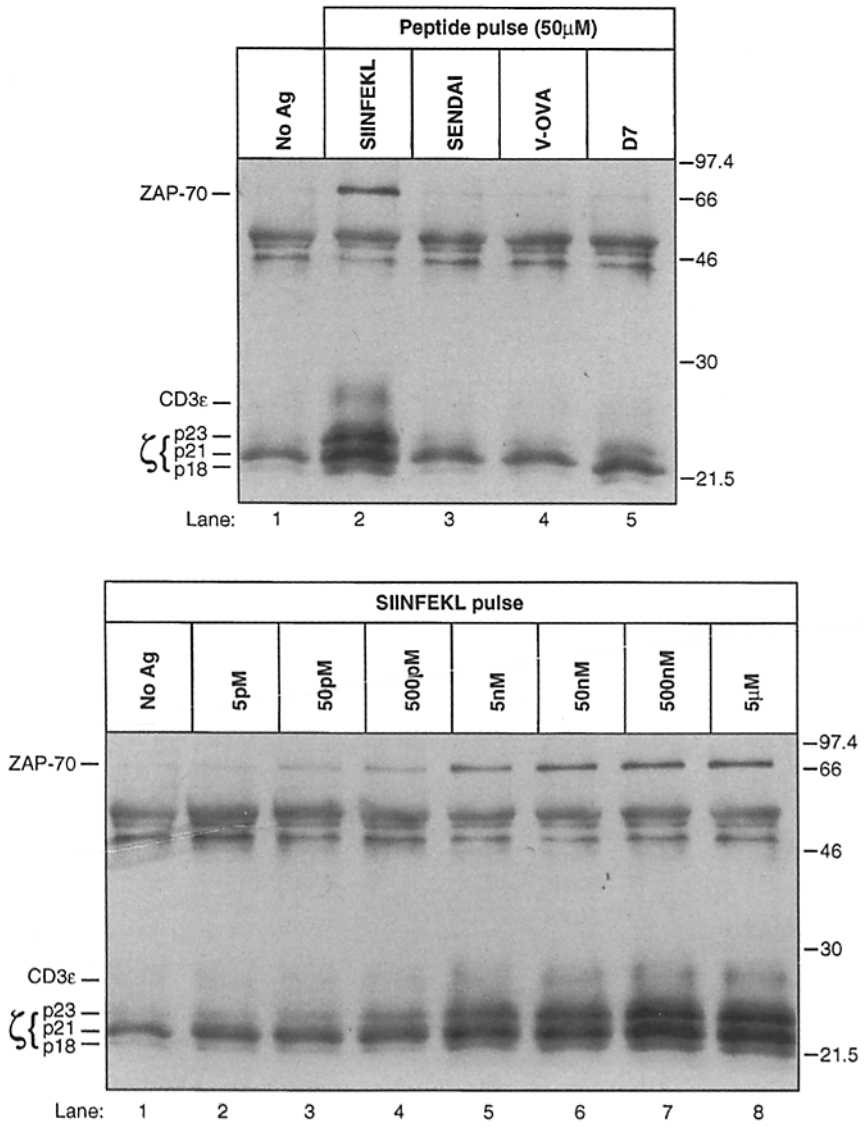

Figure 3. The signaling pattern induced by variant peptides is not seen with low amounts of full agonist. 12×10^6 MCW cells were stimulated for 10 min with 3×10^6 1-3 APC pulsed for 2 h with medium alone (No ag), the test peptides at 50 μM (SENDAI, V-OVA, D7), or varying concentrations of SIINFEKL (5 pM–50 μM). Proteins in lysates were immunoprecipitated with anti–ZAP-70, separated on 12% polyacrylamide gels, and analyzed as in Fig. 2. In producing the figure, the "No ag" lane in the top gel was duplicated and added to the lower gel to facilitate comparison. Note that the same peptide-pulsed 1-3 APC were used to stimulate MCW for the functional part of the experiment, which is detailed in Table 1. The sample corresponding to P6 stimulation was lost in this experiment.

densitometric analysis of the patterns of phosphorylation induced by the two categories of ligands (Fig. 3 and Table 2). As before, D7 signaling led to a preferential accumulation of the p21 form of phospho-ζ over the p18 and p23 forms without phosphorylation of CD3ϵ and with association of ZAP-70 with the receptor without its net phosphorylation (Fig. 3, upper gel, lane 5). Although decreasing concentrations of offered SIINFEKL also resulted in some skewing toward the p21 form of phospho-ζ, in part because of its background constitutive phosphorylation in MCW cells, the accumulation of p21 phospho-ζ in D7-stimulated samples was still accompanied by proportionately less p23ζ than in samples stimulated with 0.5–5.0 nM SIINFEKL that have a similar level of p21 accumulation (Fig. 3 and Table 2). Such skewing toward p21 phospho-ζ in response to partial agonist stimulation has been observed previously in $CD4^+$ T cell clones (16, 17). In addition, clear differences in phosphorylation of CD3ϵ chains and ZAP-70 could be seen between the D7 and the SIINFEKL-stimulated samples (Fig. 3). Although p21 phospho-ζ induction by 50 μM D7 matched that by 500 pM SIINFEKL, the variant peptide only induced 6 densitometric U of phosphorylated ZAP-70 and 0 U of phosphorylated CD3ϵ, whereas the agonist induced 33 and 36 U, respectively (Table 2). These results clearly indicate that the interactions of D7 and P6 with the T cell receptors of MCW cells result in an overall pattern of signaling events that differs significantly from that induced by low levels of agonist ligand.

Discussion

In contrast to partial agonist ligands that induce a subset of T cell functions and can, under certain conditions, also act as TCR antagonists, pure antagonists have been claimed

Table 2. *Densitometric Quantitation of Signaling Induced by SIINFEKL and Variant Peptides*

[SIINFEKL]	ZAP-70	CD3ϵ	p23ζ	p21ζ	p18ζ
0	0	0	0	0	0
5 pM	5	0	103	211	0
50 pM	16	26	88	272	18
500 pM	33	36	208	438	60
5 nM	126	115	773	1113	192
50 nM	210	138	1190	1264	297
500 nM	279	256	1413	1898	611
5 μM	351	234	2025	1881	678
50 μM	385	216	1972	1747	375
50 μM SENDAI	2	0	79	55	0
50 μM V-OVA	2	0	80	100	0
50 μM D7	6	0	120	426	92

Densitometric quantitation of the bands in Fig. 2 was performed using a grid to select bands across several lanes and volume integration using Molecular Dynamics IQ software. Data are expressed as arbitrary densitometric units after subtraction of the response to unpulsed APC. Negative values are expressed as zero.

to deliver no signals through the TCR (1, 6, 7, 29). Here we have demonstrated that several such inhibitory ligands, which by all tested measures lack any ability to induce mature CTL effector function, nonetheless deliver a biochemical signal upon TCR engagement. These findings support the hypothesis that many antagonists are a subset of partial agonist peptides that elicit TCR-dependent signals that fail to reach a quantitative or qualitative threshold necessary for stimulation of the effector functions of a mature T cell, but whose interaction with the TCR may in some cases actively interfere with the response to full agonists or induce differentiation events in immature lymphocytes (8–10, 30–32). They also emphasize the similarity in early TCR signaling events between mature $CD4^+$ and $CD8^+$ T cells, despite earlier results suggesting that tyrosine phosphorylation events in response to TCR cross-linking differed between $CD4^+$ and $CD8^+$ T cells (33), presumably due to the reportedly stronger association of $p56^{lck}$ with CD4 than CD8 (34).

Although biochemical evidence of signaling was not seen in response to V-OVA, which also had weak antagonist activity, this may reflect the limitations of the technique used to detect signaling rather than the identification of an antagonist lacking all ability to initiate intracellular biochemical changes through the TCR. We raise this possibility because variant ligands that lacked measurable effector-inducing activity on their own were found to be able to contribute to effector function in a positive way. At very high concentrations of the antagonist peptides studied here (in particular V-OVA), enhanced lysis of targets prepulsed with a limiting concentration of SIINFEKL was seen, in contrast with the antagonism normally found at lower concentrations of the variant peptides (Reis e Sousa, C., unpublished observations). These results suggest that although modified signaling by an antagonist alone does not normally reach the threshold needed for induction of T cell effector function, it can augment stimulation by an agonist, that is, it can act as a "cryptic" agonist. Competition between the antagonistic and cryptic agonistic functions of a variant peptide may also explain why, in some assays, a plateau of inhibition is seemingly reached with P6, where increasing the concentration of analogue beyond a certain threshold does not result in further inhibition (Fig. 1, *B* and *D*). Cryptic agonist function is in some ways similar to the mixed antagonists/partial agonists described by Jameson and Bevan, which inhibit cell lysis in response to agonist at low concentrations, but lead to effector activity at high concentrations (3). Cryptic agonism by V-OVA implies some ability to contribute to TCR-dependent signal generation, even if this cannot be measured biochemically under our current conditions.

Related technical limitations of the methods available to evaluate protein phosphorylation in living cells are also relevant to interpretation of the specific phosphorylation patterns seen with variant and wild-type ligands. Immunoblotting detects the state of TCR-associated phosphorylation events resulting from the simultaneous activities of tyrosine kinases and phosphatases over a time scale (minutes) that is long compared with that for the rate of enzymatic function of these proteins. It is thus possible that certain phosphorylation events occurring following TCR engagement by antagonist ligands are both too transient and too weak to be detected by this method. A number of models have been proposed to explain how altered signaling in response to variant ligands can result from a low-affinity interaction with the TCR (7, 30–32, 35). We have recently emphasized the notion that a lack of a stable state of phosphorylation of some substrates such as ZAP-70, rather than a failure to phosphorylate these substrates at all, may explain the altered pattern of phosphorylation seen using such variant ligands (32). Transient signaling may be at the heart of anergy induction by partial agonist ligands (35a), and may also lead to changes in kinase function or substrate availability that actively inhibit signaling by agonist-engaged TCR. Together with our findings here that the degree of ζ phosphorylation elicited by the SIINFEKL analogues correlates with their potency as antagonists, these findings make it tempting to speculate that TCR-associated biochemical changes, acting in a dominant negative fashion (36), could be at the root of the ability of certain variant ligands to antagonize the response to agonist.

Finally, the demonstration here that so-called pure antagonists for the TCR of $CD8^+$ T cells, like partial agonists studied previously using $CD4^+$ T cells (16, 17), do induce TCR-dependent biochemical changes that differ from those seen with low levels of typical agonist, may help explain the potency of such altered ligands in inducing net positive se-

lection of transgenic thymocytes (8–10). The advantage of antagonists or partial agonists defined using mature T cell activation in inducing positive selection of immature cells with the same receptor may depend on their ability to deliver qualitatively different signals to developing thymocytes that allow maturation with a lower likelihood of signaling for apoptosis. This proposal is in accord with suggestions made recently based on studies of transgenic mice in which positive selection was disrupted using dominant negative $p21^{ras}$ and mitogen-activated protein kinase mutations (37, 38). Thus, both activation of mature T cells and development of immature T cells may be affected by altered signaling that accompanies variant ligand engagement of the TCR. A more complete description of such altered signaling and its intracellular effects will clearly help in understanding the molecular events underlying normal agonist signaling in T cells and in determining how T cell behavior is modified by variants of these more typical ligands.

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References

1. De Magistris, M.T., J. Alexander, M. Coggeshall, A. Altman, F.C. Gaeta, H.M. Grey, and A. Sette. 1992. Antigen analog-major histocompatibility complexes act as antagonists of the T cell receptor. *Cell.* 68:625–634.
2. Racioppi, L., F. Ronchese, L.A. Matis, and R.N. Germain. 1993. Peptide-major histocompatibility complex class II complexes with mixed agonist/antagonist properties provide evidence for ligand-related differences in T cell receptor–dependent intracellular signaling. *J. Exp. Med.* 177:1047–1060.
3. Jameson, S.C., F.R. Carbone, and M.J. Bevan. 1993. Clone-specific T cell receptor antagonists of major histocompatibility complex class I–restricted cytotoxic T cells. *J. Exp. Med.* 177:1541–1550.
4. Klenerman, P., S. Rowland-Jones, S. McAdam, J. Edwards, S. Daenke, D. Lalloo, B. Koppe, W. Rosenberg, D. Boyd, A. Edwards et al. 1994. Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature (Lond.).* 369:403–407.
5. Bertoletti, A., A. Sette, F.V. Chisari, A. Penna, M. Levrero, M. De Carli, F. Fiaccadori, and C. Ferrari. 1994. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature (Lond.).* 369:407–410.
6. Ruppert, J., J. Alexander, K. Snoke, M. Coggeshall, E. Herbert, D. McKenzie, H.M. Grey, and A. Sette. 1993. Effect of T-cell receptor antagonism on interaction between T cells and antigen-presenting cells and on T-cell signaling events. *Proc. Natl. Acad. Sci. USA.* 90:2671–2675.
7. Sette, A., J. Alexander, J. Ruppert, K. Snoke, A. Franco, G. Ishioka, and H.M. Grey. 1994. Antigen analogs/MHC complexes as specific T cell receptor antagonists. *Annu. Rev. Immunol.* 12:413–431.
8. Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell.* 76:17–27.
9. Jameson, S.C., K.A. Hogquist, and M.J. Bevan. 1994. Specificity and flexibility in thymic selection. *Nature (Lond.).* 369: 750–752.
10. Hogquist, K.A., S.C. Jameson, and M.J. Bevan. 1995. Strong agonist ligands for the T cell receptor do not mediate positive selection of functional $CD8^+$ T cells. *Immunity.* 3:79–86.
11. Williams, O., Y. Tanaka, M. Bix, M. Murdjeva, D.R. Littman, and D. Kioussis. 1996. Inhibition of thymocyte negative selection by T cell receptor antagonist peptides. *Eur. J. Immunol.* 26:532–538.
12. Evavold, B.D., and P.M. Allen. 1991. Separation of IL-4 production from Th cell proliferation by an altered T cell receptor ligand. *Science (Wash. DC).* 252:1308–1310.
13. Evavold, B.D., J. Sloan-Lancaster, B.L. Hsu, and P.M. Allen. 1993. Separation of T helper 1 clone cytolysis from proliferation and lymphokine production using analog peptides. *J. Immunol.* 150:3131–3140.
14. Sloan-Lancaster, J., B.D. Evavold, and P.M. Allen. 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature (Lond.).* 363:156–159.
15. Windhagen, A., C. Scholz, P. Höllsberg, H. Fukaura, A. Sette, and D.A. Hafler. 1995. Modulation of cytokine patterns of human autoreactive T cell clones by a single amino acid substitution of their peptide ligand. *Immunity.* 2:373–380.
16. Sloan-Lancaster, J., A.S. Shaw, J.B. Rothbard, and P.M. Allen. 1994. Partial T cell signaling: altered phospho-zeta and lack of zap70 recruitment in APL-induced T cell anergy. *Cell.* 79:913–922.
17. Madrenas, J., R.L. Wange, J.L. Wang, N. Isakov, L.E. Samelson, and R.N. Germain. 1995. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science (Wash. DC).* 267:515–518.
18. Falk, K., O. Rötzschke, S. Stevanovic, G. Jung, and H.G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature (Lond.).* 351:290–296.

19. Schumacher, T.N., M.L. De Bruijn, L.N. Vernie, W.M. Kast, C.J. Melief, J.J. Neefjes, and H.L. Ploegh. 1991. Peptide selection by MHC class I molecules. *Nature (Lond.).* 350: 703–706.
20. Matsumura, M., Y. Saito, M.R. Jackson, E.S. Song, and P.A. Peterson. 1992. In vitro peptide binding to soluble empty class I major histocompatibility complex molecules isolated from transfected Drosophila melanogaster cells. *J. Biol. Chem.* 267:23589–23595.
21. Reis e Sousa, C., and R.N. Germain. 1995. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J. Exp. Med.* 182:841–851.
22. Ljunggren, H.G., and K. Kärre. 1985. Host resistance directed against H-2–deficient lymphoma variants. *J. Exp. Med.* 162:1745–1759.
23. Allen, H., D. Wraith, P. Pala, B. Askonas, and R.A. Flavell. 1984. Domain interactions of H-2 class I antigens alter cytotoxic T-cell recognition sites. *Nature (Lond.).* 309:279–281.
24. Jameson, S.C., and M.J. Bevan. 1992. Dissection of major histocompatibility complex (MHC) and T cell receptor contact residues in a K^b-restricted ovalbumin peptide and an assessment of the predictive power of MHC-binding motifs. *Eur. J. Immunol.* 22:2663–2667.
25. Höllsberg, P., W.E. Weber, F. Dangond, V. Batra, A. Sette, and D.A. Hafler. 1995. Differential activation of proliferation and cytotoxicity in human T-cell lymphotropic virus type I tax-specific CD8 T cells by an altered peptide ligand. *Proc. Natl. Acad. Sci. USA.* 92:4036–4040.
26. Weiss, A., and D.R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell.* 76:263–274.
27. Chan, A.C., D.M. Desai, and A. Weiss. 1994. The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction. *Annu. Rev. Immunol.* 12:555–592.
28. Qian, D., I. Griswold-Prenner, M.R. Rosner, and F.W. Fitch. 1993. Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation. *J. Biol. Chem.* 268:4488–4493.
29. Snoke, K., J. Alexander, A. Franco, L. Smith, J.V. Brawley, P. Concannon, H.M. Grey, A. Sette, and P. Wentworth. 1993. The inhibition of different T cell lines specific for the same antigen with TCR antagonist peptides. *J. Immunol.* 151: 6815–6821.
30. Evavold, B.D., L.J. Sloan, and P.M. Allen. 1993. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol. Today.* 14:602–609.
31. Jameson, S.C., and M.J. Bevan. 1995. T cell receptor antagonists and partial agonists. *Immunity.* 2:1–11.
32. Madrenas, J., and R.N. Germain. 1996. Variant TCR ligands: new insights into the molecular basis of antigen-dependent signal transduction and T cell activation. *Semin. Immunol.* 8: 83–101.
33. Shi, J., and R.A. Miller. 1993. Differential tyrosine-specific protein phosphorylation in mouse T lymphocyte subsets. Effect of age. *J. Immunol.* 151:730–739.
34. Wiest, D.L., L. Yuan, J. Jefferson, P. Benveniste, M. Tsokos, R.D. Klausner, L.H. Glimcher, L.E. Samelson, and A. Singer. 1993. Regulation of T cell receptor expression in immature $CD4^+CD8^+$ thymocytes by p56lck tyrosine kinase: basis for differential signaling by CD4 and CD8 in immature thymocytes expressing both coreceptor molecules. *J. Exp. Med.* 178:1701–1712.
35. Germain, R.N., E.H. Levine, and J. Madrenas. 1995. The T-cell receptor as a diverse signal transduction machine. *Immunologist.* 3/4:113–121.
35a. Madrenas, J., R.H. Schwartz, and R.N. Germain. 1996. IL-2 production, not the pattern of early TCR-dependent tyrosine phosphorylation, controls anergy induction by both agonists and partial agonists. *Proc. Natl. Acad. Sci. USA.* In press.
36. Tite, J.P., A. Sloan, and C.A. Janeway, Jr. 1986. The role of L3T4 in T cell activation: L3T4 may be both an Ia-binding protein and a receptor that transduces a negative signal. *J. Mol. Cell Immunol.* 2:179–190.
37. Swan, K.A., J. Alberola-Ila, J.A. Gross, M.W. Appleby, K.A. Forbush, J.F. Thomas, and R.M. Perlmutter. 1995. Involvement of $p21^{ras}$ distinguishes positive and negative selection in thymocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:276–285.
38. Alberola-Ila, J., K.A. Forbush, R. Seger, E.G. Krebs, and R.M. Perlmutter. 1995. Selective requirement for MAP kinase activation in thymocyte differentiation. *Nature (Lond.).* 373:620–623.