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

By Luigi Racioppi,* Franca Ronchese,* Louis A. Matis,‡ and Ronald N. Germain*

*From the *Lymphocyte Biology Section, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health; and ‡Biological Response Modifier Branch, National Cancer Institute, Bethesda, Maryland 20892*

Summary

Clonal activation of $CD4^+$ and $CD8^+$ T lymphocytes depends on binding of peptide–major histocompatibility complex (MHC) molecule complexes by their α/β receptors, eventually resulting in sufficient aggregation to initiate second messenger generation. The nature of intracellular signals resulting from such T cell receptor (TCR) occupancy is believed to be independent of the specific structure of the ligand being bound, and to vary quantitatively, not qualitatively, with the concentration of ligand offered and the affinity of the receptor for the peptide–MHC molecule complex. In contrast to the expectations of this model, the analysis of the response of a T helper type 1 clone to mutant $E\alpha E\beta^k$ molecules in the absence or presence of a peptide antigen revealed that peptide inhibited the interleukin 2 (IL-2) response to an otherwise allostimulatory mutant form of this MHC class II molecule. The inhibition was not due to competition for formation of alloantigen, it required TCR recognition of peptide–mutant MHC molecule complexes, and it decreased IL-2 production without affecting receptor-dependent IL-3, IL-2 receptor α, or size enlargement responses. This preferential reduction in IL-2 secretion could be correlated with the costimulatory signal dependence of this cytokine response, but could not be overcome by crosslinking the CD28 molecule on the T cell. These results define a new class of TCR ligands with mixed agonist/antagonist properties, and point to a ligand-related variation in the quality of clonotypic receptor signaling events or their integration with other signaling processes. It was also found that a single TCR ligand showed greatly different dose thresholds for the elicitation of distinct effector responses from a cloned T cell population. The observations that changes in ligand structure can result in qualitative alterations in the effects of receptor occupancy and that quantitative variations in ligand density can be translated into qualitative differences in T cell responses have important implications for models of intrathymic selection and control of the results of active immunization.

The antigen specificity of T lymphocyte responses resides in the recognition properties of clonally distributed, Ig-like TCRs. For $CD8^+$ and $CD4^+$ T cells, the physiological ligand for the clonotypic α/β receptors (TCR) on these cells consists of a peptide bound to the polymorphic region of plasma membrane–associated MHC class I or II molecules, respectively (1, 2). Signals essential to T cell triggering are elicited through the α/β receptor as a consequence of its interactions with other proteins having the capacity to generate intracellular second messengers (3). Recent data indicate that the CD3 γ, δ, ϵ:ζ/η complex stably associated with the TCR α/β consists of a minimum of two signal transduction modules with distinct and independent capacities to initiate second messenger cascades (4, 5) that include primary events such as tyrosine phosphorylation (6) and secondary events such as phosphoinositol bis-phosphate (PIP_2) hydrolysis and elevation of $[Ca^{2+}]_i$ (7, 8).

The effect of these biochemical events on T cell differentiation is heavily influenced by other receptor–ligand interactions generating signals differing qualitatively from those evoked by TCR–ligand interaction (9–13). The participation of such costimulatory signals is most evident in control of production of IL-2 (14), the cytokine principally responsible for the clonal expansion that follows T cell activation. Metabolically inactivated cells bearing recognizable ligands for TCRs on $CD4^+$ cells are poor or ineffective stimulators of IL-2-dependent T cell proliferation (15–17). The component missing in such circumstances contributes to T cell IL-2 produc-

tion by a mechanism independent of augmenting TCR occupancy and TCR-regulated second messenger generation (18). Several candidate receptor–ligand pairs have been proposed as the source of this critical "second signal," but many studies have failed to distinguish molecular interactions contributing to receptor occupancy by augmenting cell–cell adhesion from those providing a unique second signal in the face of adequate receptor occupancy, without which IL-2 production and clonal expansion does not occur (19). When analyzed in this manner, the binding of B7 on the APC by CD28 on the responding T cell (11–13, 20–22) and the recognition of the heat-stable antigen (19, 23) on APC by an uncharacterized T cell counter-receptor appear to be the best candidates for providing the costimulatory signal(s) needed for IL-2 production.

Despite the complex biochemical events associated with TCR-dependent intracellular signaling, a relatively simple model for the relationship between peptide–MHC ligand and TCR in the initiation of T cell triggering remains the primary paradigm in the field (24, 25). Activation is generally believed to occur when an adequate number of TCRs are aggregated at the adhesion receptor–generated membrane interface between the T cell and the presenting cell (26). The extent of this receptor–ligand aggregation depends on the number of available receptors on the T cell, the number of available peptide–MHC complexes on the APC, and the affinity of the TCR for the ligand. It is commonly assumed that when a high level of MHC–peptide complexes on the APC fails to induce measurable T cell activation, this is due to a low affinity of the TCR for the ligand that prevents receptor occupancy from exceeding the threshold needed for second messenger generation within the T cell itself (27).

This affinity-based occupancy model predicts that in the presence of intact, metabolically active APC known to be capable of delivering costimulatory signals, peptide–MHC complexes should fall into two categories: agonists that can induce full T cell activation and nonagonists that are ignored by the T cell because even at saturation of the surface MHC molecules with peptide antigen, the number of occupied TCR is below the triggering threshold, due to a low TCR affinity for the peptide–MHC complex (24). However, the recent work of Evavold and Allen (28) has cast into doubt the validity of this simple occupancy model for T cell activation. They observed that a single residue substitution in the peptide ligand for the TCR on a mouse Th2 clone prevented stimulation of proliferative responses, while permitting IL-4 cytokine production. This indicates that a ligand can stimulate T cell second messenger generation without evoking the full repertoire of effector responses. We report here the ability of certain peptide–MHC complexes interacting with the TCR to actively and selectively block IL-2 production by a mouse Th1 clone, without preventing IL-3 secretion, IL-2Rα upregulation, or cell size enlargement induced by an available TCR agonist. Our studies demonstrate that this effect does not result from competition for receptor occupancy, but rather is related to alteration in the quality of T cell signaling that may interfere with the development of, or response to, costimulation. These results define a new class of receptor ligands with the properties of mixed agonists/antagonists able to selectively modulate certain T cell effector activities in a TCR-specific manner. These same studies also showed that the occupancy thresholds for secretion of distinct cytokines can vary markedly using a single ligand–TCR combination, and that the difference appears to reflect an unequal contribution of costimulatory signals to the development of specific effector responses. These results have important implications for models of thymic selection and peripheral T cell activation, and they may provide new pharmacological approaches to the treatment of autoimmune disease and in vaccine design.

Materials and Methods

cDNA Constructs and L Cell Transfectants. cDNA expression constructs encoding wild-type and mutant Eβ^k chains were created as previously described (29). These plasmids were cotransfected into the DAP.3 subline of mouse L cells (30) together with a construct encoding wild-type Eα and a plasmid containing a marker gene for drug selection. Drug-resistant clones expressing suitable surface levels of EαEβ dimers were isolated as described (29).

Peptides. The CNBr fragment 81–104 from pigeon cytochrome *c* (PCC 81–104)[1] was prepared as previously described (31). DASP, an analogue of moth cytochrome *c* (residues 86–90 and 94–103; KKANELIAYLKQATK), as well as PCC 88–104, hen egg lysozyme (HEL) 81–96, and HEL 46–61 with or without an NH_2-terminal long-chain biotin (32) were synthesized and purified by Dr. John Coligan (Biological Resources Branch, NIAID, NIH, Bethesda, MD). DASP conjugated to an NH_2-terminal long-chain biotin was prepared and kindly donated by Dr. Jonathan Rothbard (ImmuLogic Corp., Palo Alto, CA). PCC 81–104 [99Q] was the kind gift of Dr. Ronald Schwartz, (LCMI, NIAID, NIH).

T Cell Clones and Hybridomas. The 3C6 CD4$^+$ T cell clone was produced as previously described (33) from pigeon cytochrome *c*–immune spleen cells of a B10.A mouse. The C6E1 CD4$^+$ T cell hybridoma was produced from the 3C6 clone by fusion to BW1100 (34), which lacks its own functionally rearranged TCR α and β gene loci. The hybridoma and the T cell clone were grown in RPMI 1640 with 10% FCS, 2 mM glutamine, and nonessential amino acids. The 3C6 cytochrome *c*–specific T cell clone was maintained in vitro by stimulation with antigen and irradiated spleen cells, followed by a period of rest in the absence of antigen. Cells collected at the end of the resting phase were centrifuged over a Ficoll gradient and treated with a cocktail of I-A- and I-E-specific mAbs. Antibody-treated T cells were negatively selected by sorting with magnetic beads (Dynal, Inc., Great Neck, NY) and an MPC-1 magnetic concentrator as recommended by the supplier.

T Cell Functional Assays. Production of IL-2 and IL-3 in response to L cell transfectants in the presence or absence of added peptide was measured as previously described (35). In brief, 2–5 $\times$ 10^4 T cells were incubated with 2–5 $\times$ 10^4 transfected L cells in the wells of 96-well flat-bottomed culture plates in 200 μl of complete medium with or without various concentrations of peptide antigen. Supernatants were collected after 24 h and assayed for IL-2 content using CTLL indicator cells or for IL-3 content using FDC.1 cells. IL-2 and IL-3 units were calculated as the inverse of the dilution giving 50% of the maximum [^{3}H]thymidine incorporation by CTLL (IL-2) or FDC.1 (IL-3) cells observed with reference IL-2

[1] *Abbreviations used in this paper:* HEL, hen egg lysozyme; PCC, pigeon cytochrome *c*; SSC, side scatter.

and IL-3 preparations. Proliferative responses were measured by assessing [^{3}H]thymidine incorporation between 48 and 66 h of culture. For experiments in which the data are expressed as "percent max" or "percent alloresponse," the actual absolute 100% responses were: IL-2 alloresponse, 10–40 U/ml; IL-3 alloresponse, 100–500 U/ml; IL-2 response to EαEβ^k plus PCC peptide, 150–300 U/ml; IL-3 response to EαEβ^k plus PCC peptide, 1,000–10,000 U/ml; IL-2 response to fixed cells bearing EαEβ^k plus PCC peptide, 30–50 U/ml; IL-3 response to fixed cells bearing EαEβ^k plus PCC peptide, 50–200 U/ml.

For analysis of the effects on anti-CD28 antibody on the 3C6 response, 5×10^4 3C6 T cells were cocultured with 5×10^4 transfected L cells expressing the allostimulatory mutant EαEβ molecule in the presence or absence of varying dilutions (1:250–16,000) of ascites containing hamster anti-mouse CD28 mAb (36). IL-2 and IL-3 accumulation in these cultures was measured as described above. Where indicated, the anti-CD28 was crosslinked by first preincubating the 3C6 cells with the anti-CD28 for 1 h at 4°C, washing and then adding polyclonal anti-hamster IgG antisera (Caltag Labs, San Francisco, CA) to a final dilution of 1:40, incubating for 1 h at 4°C, then washing the cells before use in a standard stimulation culture.

Flow Cytometric Analysis. Analysis of cell surface class II MHC molecule expression by transfected cells was carried out using the anti-Eα mAb 14.4.4S (37) and FITC goat anti-mouse F(ab')$_2$ as the detection reagent (38). Cells were analyzed using either an EPICS V (Coulter Electronics, Hialeah, FL), FACSAnalyzer®, or FACScan® (Becton Dickinson & Co., Mountain View, CA). For multiparameter analysis of 3C6 T cells incubated with APC with or without PCC peptide, 3C6 cells (0.5×10^5) were cocultured with FT 27.2.A2 I-E-expressing L cells (0.5×10^5) that had been preincubated for 1 h at 37°C with PCC 81–104 peptide (20 μM). After 24 h of incubation, cells recovered from the 48-well plate were stained using an anti-Thy-1.2 mAb labeled with FITC (Becton Dickinson & Co.) and mAb 7D4, an anti-IL2-Rα-specific reagent (39). Detection of bound 7D4 was with R-PE-labeled goat anti-rat antibody (Caltag Labs). Cells were analyzed on a FACScan® flow cytometer. Cells were gated on Thy-1.2 staining, and positive cells (3C6 T cells) were analyzed for 7D4 expression and cell size (side-scatter [SSC] parameter). Supernatants of these cultures were assayed for IL-2 and IL-3 content. Parallel cultures were analyzed for [^{3}H]thymidine incorporation.

Measurement of Peptide Binding Using Biotinylated Peptides. A modified version of the assay of Busch et al. (32) was used to measure peptide binding to cell-associated class II MHC molecules. After extensive washing in PBS/1% BSA, 2×10^5 DAP.3 cells or DAP.3 transfectants expressing various EαEβ molecules were incubated at 37°C in 200 μl of PBS/2% FCS containing the indicated concentration of biotinylated peptide. In the competition experiments competitors were added at the same time as labeled peptide and at the indicated concentrations. After 4 h, the cells were washed and stained with a sandwich of FITC-avidin (Vector Laboratories, Inc., Burlingame, CA), biotinylated antiavidin (Vector Laboratories, Inc.), and FITC-avidin (Vector Laboratories, Inc.). After washing, the cells were analyzed on a FACScan® flow cytometer for fluorescence. Only viable cells were considered in the analysis, as determined by propidium iodide (PI) staining. The data are expressed as net mean fluorescence intensity (MFI), calculated as: actual MFI minus MFI obtained by staining in the absence of biotinylated peptide. Percent inhibition in the competition experiments was calculated as: 100× (net MFI without competitor − net MFI with competitor/net MFI without competitor).

Measurement of Cytokine mRNA Levels. 3C6 T cells were cocultured with FT 27.2.A2 cells expressing the allostimulatory mutant EαEβ class II molecule that had previously been incubated with PCC 81–104 peptide for 1 h at 37°C. After 4 h of coculture, total RNA was extracted by the guanidinium thiocyanate-phenol-chloroform method (40), analyzed on a denaturating agarose gel, and quantified spectrophotometrically. Reverse transcription of 1 μg of total RNA with 37.5 μg/ml of oligo(dT)$_{12-18}$ (Collaborative Research, Lexington, MA) was performed for 1 h at 42°C using 600 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO BRL, Gaithersburg, MD) in 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 60 mM KCl, 10 mM DDT, 75 μg/ml of acetylated BSA, 1 U/ml of RNasin (Promega Biotec, Madison, WI), and 1 mM of dATP, dGTP, dCTP, and dTTP. 1 μCi of [^{32}P]dCTP (3,000 μCi/mM; Amersham Corp., Arlington Heights, IL) was added in the reaction mixture. The efficiency of the reverse transcription reaction was assayed by comparing the TCA-precipitable radiolabeled cDNA present in each sample. PCR analysis was performed on cDNA samples adjusted to contain the same amounts of TCA-precipitable labeled material. 10% of the product cDNA was combined with 1 μM of each of the specific IL-2 and IL-3 primers (Cytokine Mapping Amplimers™; Clontech Laboratories, Inc., Palo Alto, CA), 200 μM of each dNTP, and 1.25 U of Taq DNA polymerase (5 U/ml; Perkin Cetus) in 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA). Total volume was 50 μl. PCRs were performed as described in the Cytokine Mapping Amplimers™ manual (Clontech). The number of cycles was adjusted to fall in the log linear range of signal for these amplimers and cDNA source. PCR products were analyzed on 5% polyacrylamide precast Tris-Borate-EDTA gels (Bio-Rad Laboratories, Richmond, CA). Gels were dried and radiolabeled bands were quantified using a Phosphor-Imager (Molecular Dynamics, Inc., Sunnyvale, CA). Images were analyzed by Image Quant™ version 3.15 software (Molecular Dynamics, Inc.).

Results

Peptide Inhibition of the IL-2 Response of a T Cell Clone to a Mutant MHC Class II Molecule. 3C6 is a Th1-type (41) cloned cell line derived from a PCC-immunized B10.A mouse that produces IL-2 when stimulated by antigen and splenic APC. In addition to the expected specific activation of 3C6 by COOH-terminal cytochrome peptides presented by cells expressing the wild-type, self EαEβ^k MHC class II molecule, this clone also responds in the absence of added antigen to APC expressing the closely related EαEβ^s molecule. This constitutes an alloantigenic crossreaction that has been observed among such cytochrome *c*–reactive T cells (33). Transfected L cells expressing MHC class II molecules with mutant Eβ^k proteins containing various Eβ^s allelic substitutions were produced to examine the relationships among MHC molecule structure, peptide antigen presentation, and allorecognition (29). Transfectants expressing molecules with Eβ chains containing substitutions of E^s allelic residues at positions 75 and 79 in the putative helix of the peptide-binding regions (referred to as β75^s, 79^s) or at positions 72, 75, and 79 evoked a substantial peptide-independent (alloantigenic) response (Fig. 1 *A*).

3C6 was then tested for responses to PCC peptide presented by the mutant molecules capable of stimulating an alloantigenic response. In addition to inducing a peptide-independent alloresponse, a clear dose-dependent stimulation of the clone

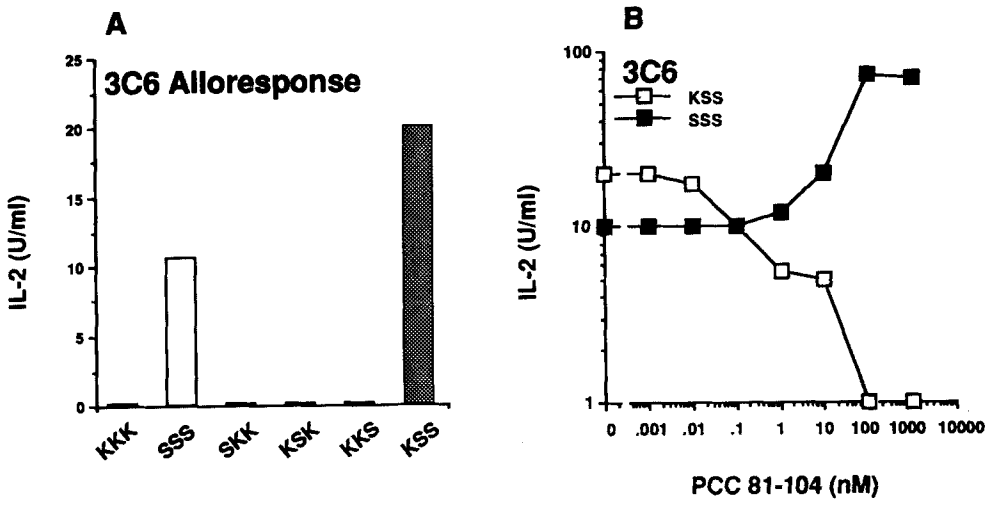

Figure 1. Response of the 3C6 Th1 clone to PCC 81–104 peptide presented by EαEβ^k molecules mutated in the βHV3 region. (*A*) IL-2 responses (alloreactivity) of 3C6 to EαEβ^k molecules with HV3 mutations in the absence of PCC 81–104 antigen. (*B*) Effect of addition of PCC 81–104 peptide on the 3C6 IL-2 response to EαEβ molecules with β72^s,75^s,79^s (*SSS*) or β75^s,79^s (*KSS*) β chains.

by PCC peptide was observed using APC expressing an Eβ^k chain with s residues at positions 72, 75, and 79 (Fig. 1 *B*). Unexpectedly, addition of the same peptide to cells expressing a mutant EαEβ^k molecule with β75^s, 79^s not only failed to stimulate a response above that seen in the absence of peptide, but such peptide addition inhibited the IL-2 response in a dose-dependent fashion (Fig. 1 *B*).

Competition for MHC Peptide-Binding Does Not Account for the PCC Peptide Inhibition of Alloreactivity. There is now substantial evidence that many T cell responses to allogeneic MHC molecules involve recognition of the peptide(s) bound to the non-self MHC molecules (42–45). Thus, a simple explanation for the ability of the PCC peptide to inhibit the 3C6 alloresponse to the β75^s, 79^s mutant molecule would be competition for MHC class II molecule binding of a peptide necessary for the formation of the alloantigenic ligand recognized by the 3C6 receptor. Such competition at the level of the MHC molecule has been suggested as an explanation for previous observations of exogenous peptide inhibition of allogeneic stimulation (46–48). This possibility seemed unlikely to us, however, as it is generally difficult to block MHC-dependent responses by addition of competing peptide after the stimulatory peptide has had an opportunity to bind (49, 50). If the culture medium or the transfectant itself were the source of any putative peptide needed for the allostimulation, the continuously cultured cells would have had ample time to form the stimulatory peptide–MHC complexes before the introduction of the potentially competing PCC peptide. Nevertheless, this possibility was directly assessed by examining the effects on 3C6 alloresponses of other peptides able to bind to the mutant EαEβ molecule to a similar extent as the inhibitory PCC 81–104 peptide (Fig. 2 *A*). Fig. 2 *B* shows that despite only minor differences in direct MHC molecule binding by three cytochrome-related peptides, they varied over two to three orders of magnitude in their ability to stimulate the 3C6 clone when presented by the wild-type EαEβ^k molecule. These data confirm the assignment of position 99 as a key epitopic residue in the PCC determinant (51–53), and show that the change at this position from lysine to glutamine affects TCR-dependent recognition, not MHC molecule binding. Fig. 2 *C* demonstrates that the ability of these same peptides to mediate inhibition of the 3C6 alloresponse to the β75^s, 79^s mutant was directly related to their capacity to stimulate the clone in the context of the wild-type EαEβ^k molecule. Thus, the fine specificity of the 3C6 TCR for the peptide, and not the ability of the peptide to bind to the mutant MHC molecule, clearly dictated the capacity to mediate inhibition of alloantigen-stimulated IL-2 responses. This argues against direct peptide competition at the MHC molecule level as the cause of IL-2 response inhibition by PCC 81–104. Further evidence for this conclusion comes from similar experiments using an unrelated HEL peptide. Although it bound well to the mutant EαEβ molecule (Fig. 3 *A*), this peptide also lacked the ability to inhibit alloantigen-driven IL-2 production by 3C6 (Fig. 3 *B*).

Peptide Inhibition Is Not Due to High-Dose Suppression. Mouse Th1 clones like 3C6 exhibit a characteristic decline in antigen-stimulated proliferation as the concentration of offered antigen is increased to high levels (24, 54). This phenomenon is termed "high-dose suppression" and appears to be related to prolonged or repetitive TCR engagement. It was thus formally possible that the low apparent response of 3C6 to the mutant EαEβ molecule was in fact due to a very high level of allostimulation, and that additional TCR ligand in the form of PCC peptide–MHC complexes drove the response even lower. 3C6 did exhibit high-dose suppression when stimulated with wild-type EαEβ^k plus PCC peptide (Fig. 4 *A*). However, the pattern observed was a reduced proliferative response in the face of increasing IL-2 cytokine accumulation in the culture. The inhibitory effect of PCC peptide on 3C6 in the presence of alloantigen showed a different phenotype (Fig. 4 *B*). Added PCC peptide decreased IL-2 accumulation with only marginal effects on proliferation. Consistent with these data, addition of small amounts of anti-EαEβ mAb to cultures of 3C6 stimulated in the absence of PCC peptide did not result in augmentation of IL-2 production (not shown). Such an increase would have been expected if the alloresponse were in the high-dose suppres-

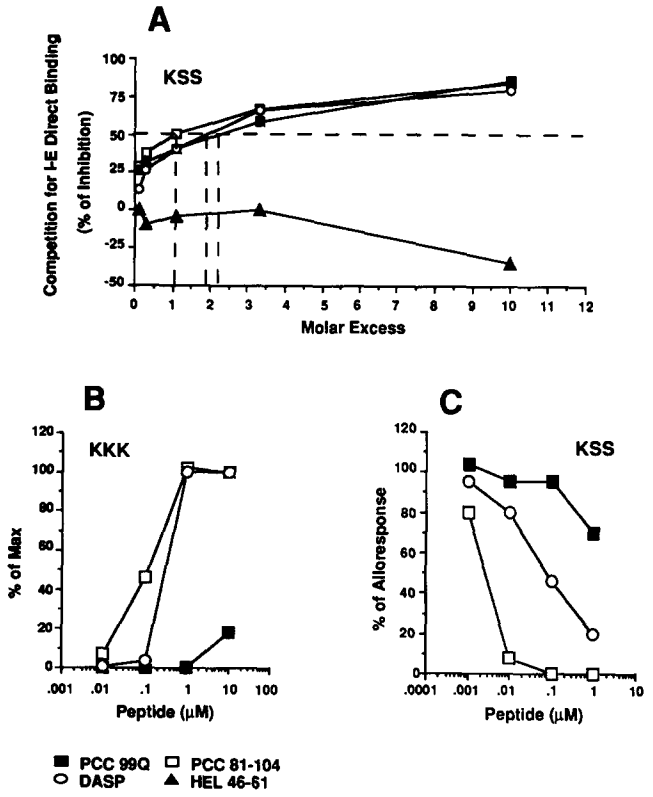

Figure 2. The inhibitory effect of EαEβ-binding peptides on 3C6 alloresponses to the β75s,79s Eβ–expressing transfectant is related to their ability to be recognized by the 3C6 receptor. (*A*) Binding of PCC 81–104, PCC 81–104 (99Q), DASP, and HEL 46–61 peptides to transfected L cells expressing EαEβ molecules containing the β75s,79s Eβ chain as assayed by competition for the binding of biotinylated-DASP (25 μM). Data are expressed as percent inhibition at each competitor concentration, calculated as described in Materials and Methods. (*B*) IL-2 response of 3C6 to PCC 81–104, PCC 81–104 (99Q), and DASP peptides presented by an L cell transfectant expressing wild-type EαEβk molecules. (*C*) Effect of PCC 81–104, PCC 81–104 (99Q), and DASP peptides on IL-2 production induced by an L cell transfectant expressing the β75s,79s Eβ chain. Data are expressed as percent of IL-2 production obtained in the absence of any cytochrome *c* peptide in the culture.

sion range, because of the reduction in receptor engagement caused by antibody addition. Thus, we conclude that the inhibitory effect of peptide is not due to excessive TCR-dependent stimulation of 3C6.

Inhibition Is Selective for IL-2 Production and Does Not Involve Abrogation of All TCR-dependent Signaling. Additional experiments were performed to determine if any effective TCR-dependent signal transduction occurred when 3C6 was exposed to alloantigen plus PCC peptide. As shown in the flow cytometry profiles of Fig. 5 *A* and summarized quantitatively in Fig. 5 *B*, under conditions of PCC peptide addition that blocked virtually all IL-2 accumulation in the culture, alloantigen-stimulated, TCR-dependent IL-3 production, IL-2Rα chain upregulation, and cell size enlargement proceeded as they did in the absence of added PCC peptide. Thus, 3C6 continued to receive biologically active signals through the TCR under the same conditions that abrogated the IL-2 response. This indicates that the activity of PCC–mutant EαEβ complexes in downregulating the IL-2 response did not result from the elimination of effective TCR occupancy by the agonist alloantigen. The inability of a cytochrome-unrelated I-E binding peptide like HEL to affect either IL-2 (Fig. 3) or IL-3 (not shown) also indicates that the divergent effect of PCC on IL-2 vs. IL-3 is not the result of replacing the alloantigen agonist with a new ligand capable of only IL-3 stimulation. If this were the case, addition of the HEL peptide should have resulted in loss of both responses, rather than the observed lack of any effect on cytokine production. Fig. 5, *C* and *D*, demonstrates that the decrease in IL-2 measured in the inhibited cultures was not the result of increased IL-2 consumption by proliferating cells, but reflected a decrease in steady-state IL-2 mRNA levels. IL-3 mRNA levels were similar in the presence or absence of added PCC peptide, as expected from the bioassays of IL-3 activity.

Different Antigen Dose Thresholds for IL-2 vs. IL-3 Responses Based on Varying Synergy between Costimulatory and TCR Signals. Neither inhibition of alloantigen formation, replace-

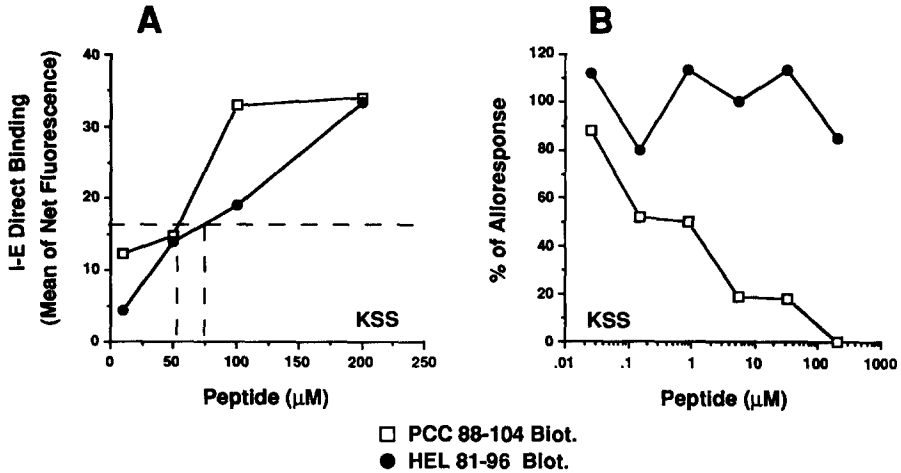

Figure 3. The capacity of a peptide to bind EαEβ molecules containing the β75s,79s Eβ chain does not correlate with its capacity to inhibit the 3C6 IL-2 response. (*A*) Direct binding of biotinylated PCC 88–104 and biotinylated HEL 81–96 to an L cell transfectant expressing EαEβ molecules with the β75s,79s Eβ chain. Data are expressed as mean net fluorescence calculated as described in Materials and Methods. (*B*) Effect of biotinylated PCC 88–104 and biotinylated HEL 81–96 peptides on 3C6 IL-2 production induced by an L cell transfectant expressing EαEβ molecules with the β75s,79s Eβ chain. Data are expressed as percent of the IL-2 production obtained in the absence of added peptide.

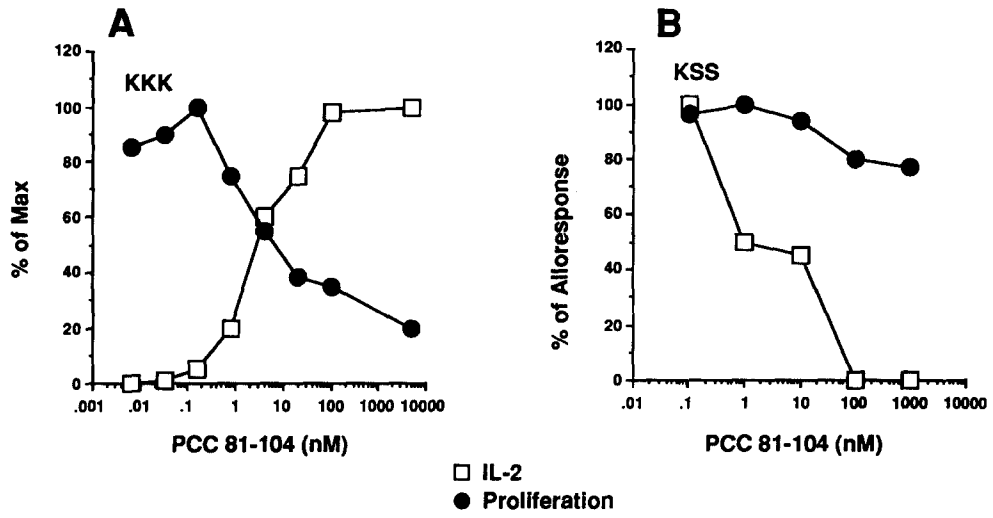

Figure 4. High-dose inhibition does not account for the inhibitory effect of PCC 81–104 on the IL-2 alloresponse of 3C6 to L cells expressing EαEβ molecules with the β75s,79s Eβ chain. (*A*) IL-2 and proliferative responses of the 3C6 clone to PCC 81–104 presented by an L cell transfectant expressing wild-type EαEβk molecules. (*B*) IL-2 and proliferative responses of the 3C6 clone to L cells expressing EαEβ molecules containing the β75s,79s Eβ chain in the presence of PCC 81–104.

ment of agonist alloantigen with partial agonist complexes, nor high-dose suppression explained the inhibitory effect of PCC peptide on the 3C6 IL-2 alloresponse. The effect was clearly related to receptor engagement by the PCC peptide–mutant EαEβ complexes, but did not result from a blockade of all TCR-dependent signal transduction. Although it would violate the standard occupancy model of TCR stimulation, one could imagine the PCC peptide–mutant EαEβ

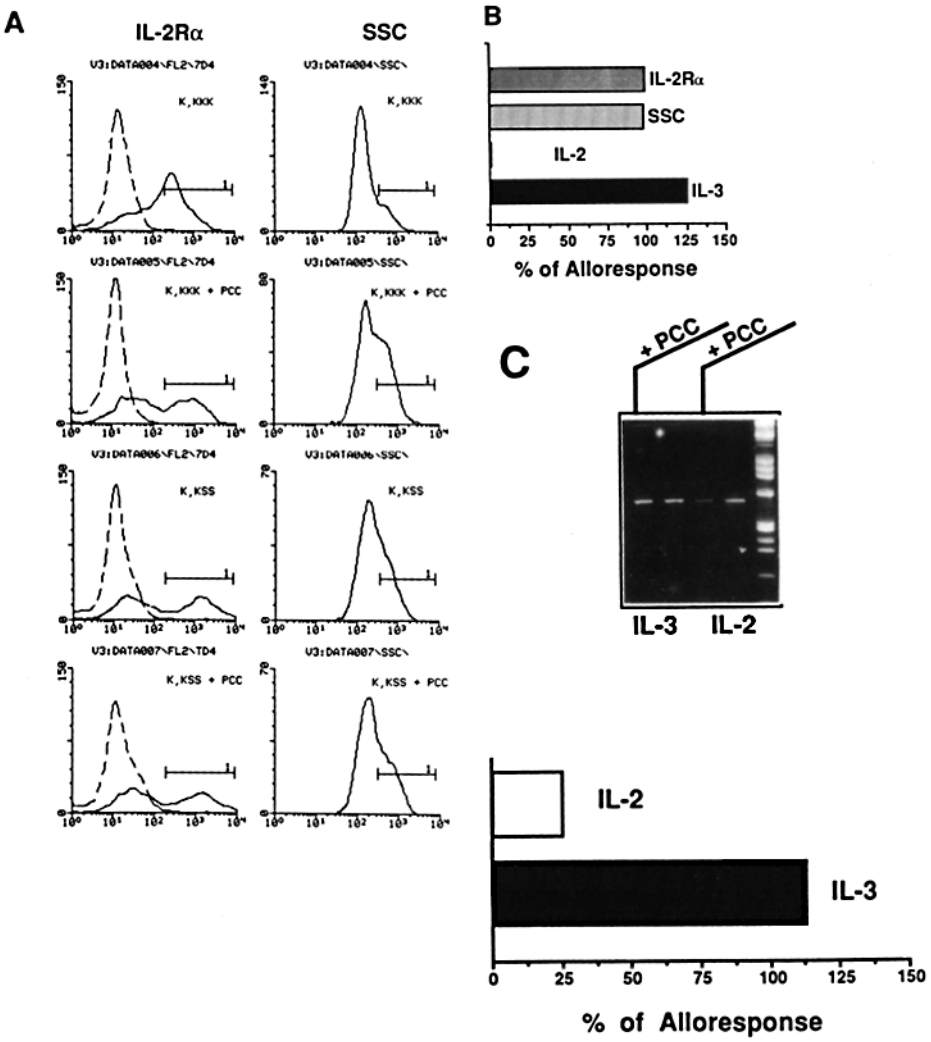

Figure 5. The inhibitory effect of PCC 81–104 on IL-2 alloresponses of 3C6 is selective and does not prevent several other TCR-dependent activation events. (*A*) Two-color flow cytometry profiles of 3C6 cells cocultured for 24 h with transfected L cells expressing either wild-type EαEβk or EαEβ with the β75s,79s Eβ chain. (*Left*) 7D4 (anti-IL-2R α chain) staining of G7 (Thy-1)-positive (*solid lines*) and negative (*dotted lines*) cells. (*Right*) SSC (size) of G7-positive cells. The Eβ chain of the EαEβ molecule expressed by the L cell used in culture and the presence or absence of PCC 81–104 are indicated in each panel. (*B*) Relative change in IL-2Rα expression for cells included in the gate 1 subset of Thy-1+ cells in *A*, *left*, comparing cultures with transfectants expressing the β75s,79s Eβ chain in the absence and presence of PCC 81–104; change in SSC (size) was calculated as change in percent of cells in gate 1 of *A*, *right*, comparing cultures with transfectants expressing the β75s,79s Eβ chain in the absence and presence of PCC 81–104; IL-2 and IL-3 production was expressed as relative production comparing cultures with transfectants expressing the β75s,79s Eβ chain in the absence and presence of PCC 81–104. (*C*) Reverse transcription PCR analysis of RNA extracted from cocultures of 3C6 and transfected L cells expressing EαEβ molecules with the β75s,79s Eβ chain in the absence or presence of PCC 81–104 (1 μM). (*Top*) Ethidium stain of PCR products; (*bottom*) calculated relative amounts of IL-2 and IL-3 mRNA determined as described in Materials and Methods.

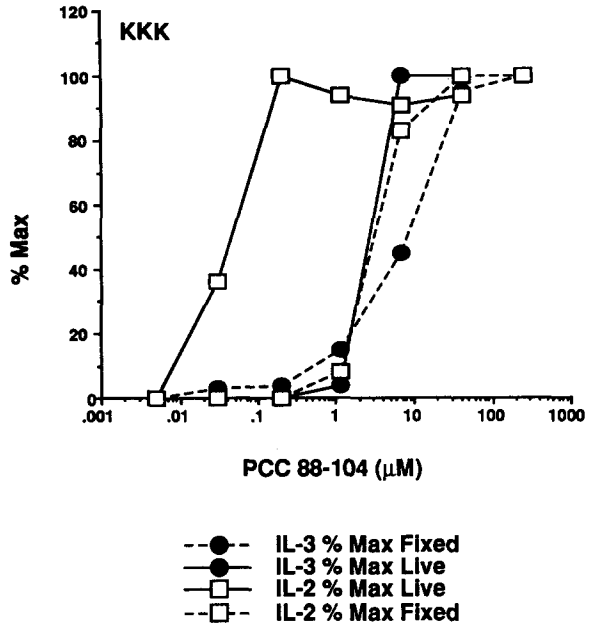

Figure 6. Antigen dose-dependent patterns of IL-2 and IL-3 production by 3C6 to peptide presented on live or fixed L cell transfectants expressing wild-type EαEβ^k. Results are expressed as percent of the maximum IL-2 or IL-3 response, respectively, obtained with live or fixed presenting cells.

complexes acting to reduce the number of available TCR for alloantigen recognition by engaging them without contributing to signal generation. One could then explain the selective inhibition by PCC peptide of the IL-2 response by postulating that a lower level of residual second messenger generation in the presence of competing PCC-MHC complexes sufficed for IL-3 secretion, in comparison with that needed for IL-2 production. We therefore examined the relative sensitivity of 3C6 to peptide-MHC molecule stimulation of IL-2 and IL-3 responses. Fig. 6 shows the IL-2 and IL-3 responses of 3C6 to peptide presented by wild-type EαEβ^k-expressing APC. Attaining the same fraction of maximal response for IL-2 required 100-fold less peptide then needed for IL-3, implying that IL-3 production under these conditions required higher, not lower, TCR occupancy than did IL-2 production. This is the opposite of what would be required to explain selective peptide-MHC complex inhibition of alloantigen-driven IL-2 but not IL-3 production on the basis of differential sensitivity to residual TCR signaling, because at any level of reduced occupancy based on TCR blockade, a greater reduction in IL-3 than IL-2 production should have been seen.

The phenotype of TCR-dependent IL-3 production in the absence of IL-2 production by Th1 clones has been previously reported. Th1 clones stimulated by peptide-MHC complexes in planar membranes gave significant though subnormal IL-3 production, whereas IL-2 production was not observed under these conditions (55). The defect in IL-2 production has been attributed to an absence of (an) essential costimulatory signal(s) provided by viable APC that is (are) separate and distinguishable from ligand-receptor interactions that affect the occupancy of the TCR or its production of second messengers (56). If the ability of 3C6 to produce IL-2 at low ligand densities with viable APC resulted from a potent synergy between TCR signaling and costimulatory signaling that did not apply equally to IL-3 gene activation, the differences in relative dose-responses for the two cytokines should be reduced or eliminated using aldehyde-fixed APC with greatly reduced levels of costimulatory signals. Fig. 6 shows that this was the case; although as previously reported (17), the absolute cytokine responses elicited by fixed APC were reduced >90%, the antigen concentration needed for half-maximal IL-3 responses was only slightly increased (~3×) compared with that needed with viable APC, whereas the dose needed for half-maximal IL-2 production increased close to 100-fold to approach that required for IL-3.

Evidence for a Relationship between Costimulatory Signaling and Selective Inhibition of IL-2 Responses by Cytochrome Peptide. These data on cytokine responses to viable and fixed cells suggest that the selective inhibition by PCC-mutant EαEβ complexes of alloantigen-stimulated IL-2 but not IL-3

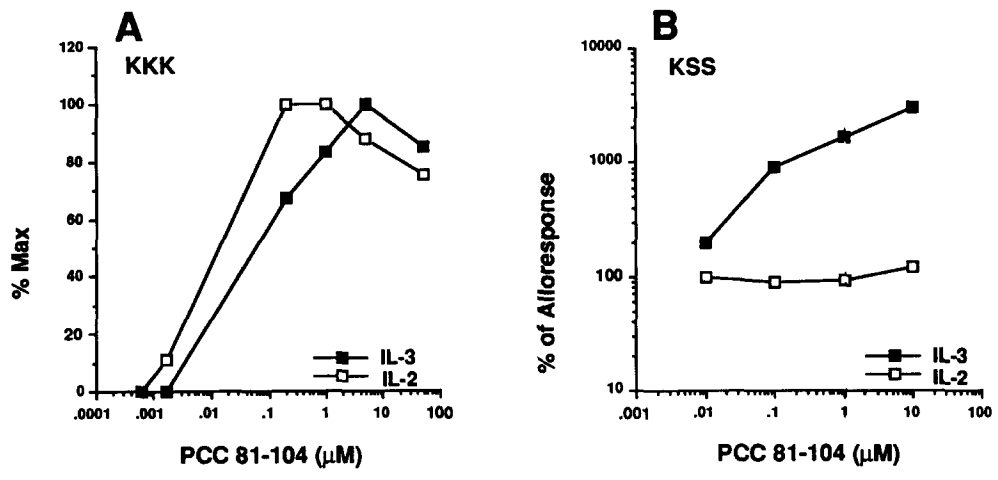

Figure 7. PCC 81-104 does not inhibit the IL-2 response and can increase the IL-3 response of the C6E1 hybridoma to L cell transfectants expressing EαEβ molecules with the β75^s,79^s Eβ chain. (*A*) IL-2 and IL-3 responses of the C6E1 hybridoma to PCC 81-104 peptide presented by L cells expressing wild-type EαEβ^k molecules. Data are expressed as percent of maximal stimulation of IL-2 and IL-3, respectively. (*B*) IL-2 and IL-3 responses of the C6E1 hybridoma to PCC 81-104 peptide presented by L cells expressing EαEβ molecules with the β75^s,79^s Eβ chain. Data are expressed as percent of the response obtained in the absence of PCC 81-104 peptide.

responses could reflect an interference with the generation of, or response to, costimulation in the cultures. The L cell transfectant used in the experiment shown in Fig. 6 constitutively expresses B7 (57, and our unpublished observations), a ligand for the CD28 costimulatory pathway, and the capacity to observe low but detectable IL-2 production using these APC after fixation appears to relate to a low level of residual costimulation mediated by preexisting B7 present on these fixed cells. However, experiments with purified peptide–MHC complexes in planar membranes have shown that Th1 clones do not produce IL-2 in response to peptide–MHC molecule ligands in the total absence of costimulatory signals (55). Thus, we could not test this hypothesis concerning the locus of the peptide inhibitory effect by examining 3C6 IL-2 production in response to alloantigen in the complete absence of costimulation. However, T cell hybridomas do produce IL-2 in the absence of costimulatory signals, such as in response to peptide–MHC complexes in planar membranes (58). We therefore examined the effect of added PCC peptide on the mutant EαEβ alloresponse of a T cell hybridoma bearing the same TCR as 3C6. This hybrid was derived by fusion of a TCR chain–negative T lymphoma cell with the 3C6 clone and it has a peptide and alloantigen response profile indistinguishable from the 3C6 clone (not shown).

Evidence that costimulation-related signals do not play a major role in IL-2 production by the hybridoma comes from a dose-response analysis comparing IL-2 and IL-3 generation (Fig. 7 *A*). In the presence of viable APC, the hybridoma did not exhibit the difference in IL-2 and IL-3 dose-responses characteristic of the 3C6 Th1 clone, but instead responded almost precisely as the clone did to fixed APC lacking almost all costimulatory activity. This is consistent with the hypothesis that the difference in the cytokine dose-responses seen using the clone reflected the asymmetric contribution of costimulation to the IL-2 response of normal T cells, and suggested that if the inhibitory effect of PCC peptide on the alloresponse of the clone were due to interference with exogenously provided costimulation, such inhibition should not be seen with the hybridoma. In agreement with this prediction, Fig. 7 *B* shows that the hybridoma's alloantigen-stimulated IL-2 response was not decreased by addition of PCC peptide.

These data also indicate that the PCC peptide–mutant MHC class II complexes are not pure antagonists of, but actually weak agonists for, TCR signal generation, because at high antigen concentrations they could clearly elicit an effector response from the hybridoma (increased IL-3 production). Given the slight dose-response advantage seen for IL-2 vs. IL-3 production in Fig. 7 *A*, it was somewhat surprising that we did not observe a similar increase in IL-2 secretion at these antigen concentrations. This result may be explained by generation of qualitatively different signals upon TCR engagement of wild-type MHC–PCC peptide vs. mutant MHC–PCC peptide ligand, which in the latter case is inadequate to evoke IL-2 responses. Alternatively, although costimulation is not required for IL-2 production by T hybridomas, it can modestly increase such responses (our unpublished observations), a phenomenon consistent with the slight dose-response advantage seen for IL-2 compared with IL-3. In the presence of peptide–mutant MHC molecule complexes, a balance between weak receptor signaling and simultaneous inhibition of the small costimulation effect would then explain the failure to see an increase in IL-2 production in a fashion fully concordant with the results obtained using the 3C6 clone.

Effects of Anti-CD28 Antibody. Interaction of the CD28 molecule on T cells with the B7 membrane protein on APC appears to activate a major costimulatory pathway involved in regulating IL-2 production (11–13, 20–22). Antibody to the CD28 molecule on mouse or human T cells can modulate the cytokine response of such cells to TCR stimulation (36, 59, 60), presumably by altering delivery of a critical costimulatory signal. Because of the above observations suggesting that the PCC-induced downregulation of IL-2 responses was related to the special contribution of costimulation to IL-2 production, we examined the role of CD28 in IL-2 responses of the 3C6 clone and the possibility that antibody-mediated activation of the CD28 signaling pathway might counteract the inhibitory effect of the peptide–mutant MHC complexes.

Fig. 8 *A* shows that inclusion of soluble anti-CD28 in a coculture of 3C6 and the allostimulatory L cell transfectant inhibited IL-2 production almost completely, without de-

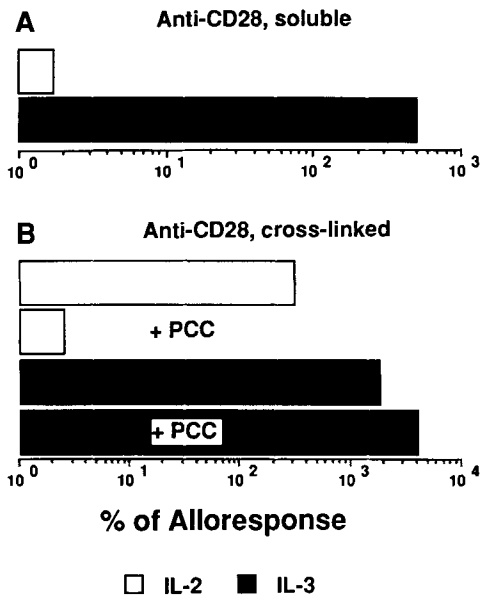

Figure 8. Antibody crosslinking of CD28 on the 3C6 clone does not prevent selective PCC 81–104–mediated inhibition of IL-2 alloresponses. (*A*) IL-2 and IL-3 responses of the 3C6 clone in the absence or presence of anti-CD28 mAb (1:250 dilution of ascites) to an L cell transfectant expressing EαEβ molecules containing the β75^s,79^s Eβ chain. Data are expressed as percent of the response obtained in the absence of the anti-CD28 antibody. (*B*) Effect of anti-CD28 antibody–mediated crosslinking on the IL-2 and IL-3 responses of 3C6 to an L cell expressing EαEβ molecules with the β75^s,79^s Eβ chain in the presence of PCC 81–104 peptide (1 μM). Data are expressed as percent of the response obtained in the absence of both the anti-CD28 antibody and the PCC peptide.

creasing IL-3 production. This confirmed prior data on the effect of soluble anti-CD28 on IL-2 secretion in response to alloantigen (59) and replicated the phenotype seen when PCC peptide was added to similar cultures. The ability of soluble anti-CD28 to inhibit IL-2 responses by the 3C6 clone suggests a critical contribution of this molecule to costimulation of IL-2 production, presumably via interaction with the B7 surface protein present on the transfected L cells. Anti-Ig-mediated crosslinking could overcome the inhibitory effect of soluble anti-CD28 on IL-2 responses of 3C6 in the absence of PCC peptide. The increase in IL-3 production seen using crosslinked anti-CD28 antibody is consistent with the known ability of costimulation to augment this response without being required for it, and provides additional evidence for the efficacy of the crosslinking procedure in activating the CD28 pathway. Nevertheless, even though CD28-dependent signaling appeared necessary for IL-2 production by 3C6 cells, antibody-dependent stimulation of this pathway did not reverse the inhibition of IL-2 secretion mediated by PCC peptide addition (Fig. 8 *B*). These results suggest that peptide-mediated inhibition of IL-2 secretion either involves interference with effective CD28 costimulatory signal transduction at a site within the T cell or the disruption of a crucial costimulatory pathway distinct from that evoked by CD28 aggregation (see Discussion).

Discussion

The results reported here describe a new class of TCR α/β ligands that might best be called mixed agonists/antagonists, whose interaction with TCRs results in differential stimulation or suppression of various effector functions. Previous studies (55, 61) have shown that it is possible to separate effector activities of both $CD4^+$ and $CD8^+$ T cells into those that do (IL-2 secretion) and do not (IL-3 secretion, cell killing) show a nearly absolute requirement for costimulatory signals. In these prior reports, the dichotomy was achieved by manipulation of the potential of the APC to provide costimulation, and did not vary according to the ligand used for TCR engagement. The present study shows that changing the structure of the TCR ligand by even a single amino acid can interfere with the elicitation of costimulation-dependent effector responses to ligand presented by a physiologically intact APC. Although we have mutated the MHC class II molecule in the present case, current understanding of T cell recognition implies that a similar effect could be mediated by appropriate changes in the peptide. This would be in accord with the results of Evavold and Allen (28), who found that a peptide–MHC complex differing from the wild type in a single residue of the peptide lost the capacity to induce proliferative responses by a Th2 clone, while remaining able to evoke IL-4 production.

However, in marked contrast to the results of these investigators, the mutant EαEβ–peptide ligand studied here did not simply fail to elicit certain T cell responses, but actively interfered with some but not other differentiation events mediated by TCR occupancy with typical agonist ligand. IL-2 secretion was decreased without substantial interference with the development of signals in the T cell that suffice for induction of IL-3 secretion, IL-2Rα upregulation, and size enlargement responses. A peptide able to bind to the mutant EαEβ molecule but unrelated to the 3C6 receptor specificity did not have any significant inhibitory function. If the loss of IL-2 response were due simply to loading alloantigenic mutant EαEβ molecules (either empty or containing some naturally processed antigen) with the cytochrome peptide, eliminating these fully stimulatory ligands, and producing partial agonist complexes able to trigger IL-3 but not IL-2 responses, the unrelated HEL peptide should have inhibited both responses as a result of such alloantigen replacement. Because this was not observed, the mechanism of IL-2 inhibition cannot be simple removal of the stimulatory ligand from the culture.

The selective blockade of IL-2 production seen here in the face of persistent TCR signaling differs markedly from the results reported by De Magistris et al. (62). These authors observed a similar dominant interference by variant peptide–MHC complexes with agonist ligand-induced IL-2 responses. However, they concluded that these inhibitory complexes were pure TCR antagonists, lacking the capacity to generate any intracellular signals themselves and acting by simple receptor competition to completely block all signal generation by available agonist complexes. There may well be two distinct classes of inhibitory peptide–MHC complexes, one with pure antagonist activity and the other with mixed agonist/antagonist potential, that interfere with T cell responses by distinct means. The former would prevent intracellular messenger generation in the T cell by removing TCRs from the functional pool, thus allowing no effector responses. The latter would interfere with certain effector activities based on qualitative differences in requirements for intracellular signaling, possibly related to the costimulation dependence of the functions analyzed. However, only IL-2 responses were measured in the simultaneous presence of agonist and inhibitory peptides by De Magistris et al. (62). It is therefore possible that the conclusion we reach here that the defective response does not result from simple TCR blockade, but rather from a qualitative difference in signaling, may also apply to the peptide–MHC combinations studied by these other investigators.

Several observations, including the selective inhibitory effect of peptide on IL-2 production, the dramatic effect of fixation on IL-2 but not IL-3 dose-response relationships, and the failure of peptide to inhibit IL-2 production by a T cell hybridoma, all suggested that the peptide–mutant MHC complexes might be acting to interfere with the production of, or response to, costimulatory signals. Numerous reports indicate that the interaction of CD28 on responding T cells with B7 on APC provides important signals for the generation of high-level IL-2 production (11–13, 20–22). Soluble anti-CD28 antibody affected the 3C6 response to alloantigen in a manner similar to inclusion of PCC peptide, in that IL-3 responses were maintained in the face of inhibition of IL-2 production. This result is also consistent with a model in which the inhibitory effect of PCC peptide on alloresponses is related to alteration

in costimulatory signalling. Crosslinking the anti-CD28 antibody reversed the inhibition of alloresponses by the soluble CD28, in agreement with the previous report of Damle et al. (59), and this maneuver increased IL-3 responses, indicating effective stimulation of the CD28 pathway. However, such crosslinking of the CD28 molecules on 3C6 cells did not prevent the inhibition of IL-2 production mediated by exposure to PCC–mutant MHC class II complexes. These observations imply that CD28–B7 interaction may be necessary but not sufficient for IL-2 responses under the present conditions. It is possible that the intracellular signals delivered through CD28 are not effective when the 3C6 TCR is engaged with the PCC peptide–mutant EαEβ complexes, implying the generation of dominant-negative intracellular messengers by the mixed agonist/antagonist complexes. This hypothesis is consistent with preliminary data on the ability of peptide–MHC complexes to inhibit IL-2 production by 3C6 in the presence of PMA and ionophore (L. Racioppi and R. N. Germain, unpublished observations). The effects of such interfering signals would have to be gene or transcript specific, as IL-3 production was actually increased under the same conditions. Alternatively, costimulatory receptor–ligand interactions distinct from CD28–B7 may be important. The heat-stable antigen (23) has recently been suggested as another APC-expressed molecule regulating IL-2 production (19), and experiments are in progress to evaluate the relationship of this costimulatory pathway to selective peptide–MHC molecule–mediated inhibition of IL-2 responses.

Exploration of the basis for the ability of PCC peptide to inhibit alloresponses by the 3C6 clone led to additional insights concerning T cell activation and signaling. Careful dose-response titrations showed that distinct effector activities had grossly different quantitative thresholds for elicitation. In particular, 100-fold less peptide–MHC ligand was needed to evoke IL-2 than IL-3 responses in the presence of wild-type EαEβ^k. This difference appeared to be due to the more potent synergy of costimulation with TCR-dependent induction of IL-2 secretion. An appealing explanation of this effect comes from considering downstream signaling events. Ultimately, the readout of surface receptor engagement is gene activation mediated by various transcription factor complexes (63). An emergent theme of promoter function is the role of a complex array of short motifs acted upon by a number of distinct constitutive and regulated factors, both positive and negative, in determining gene activity (64). Evidence exists that costimulation results in the enhanced function of transcription factors distinct from those invoked by TCR-dependent signals alone (65). If the gene of interest can have its transcriptional activity markedly increased via a costimulation-dependent factor, the consequent effector activity will be very costimulation sensitive, and may show a decreased requirement for TCR signals due to amplification of a small amount of these latter signals. Alternatively, the apparent gene specificity might be mediated by selective costimulator-dependent changes in mRNA lifetime. Either model is fully consistent with the relationship between IL-2 and IL-3 responses observed here using viable vs. fixed APC. This argues that qualitative differences in effector function that depend on the mix and proportion of cytokines produced by a T cell may be influenced in a nonlinear way by the balance between TCR and costimulation signaling. This will not only vary according to the APC and T cells involved, but also in accord with the properties of the ligand, as shown here. This model can explain the data reported by Soloway et al. (66) in which alterations in peptide ligand or dose affected the quality of the immune response, and also the results of Murray et al. (67) in which a change in qualitative effector activity accompanied alteration in ligand concentration.

The molecular mechanism by which a modified receptor ligand can change integration of TCR and costimulatory signaling is unclear. A simple view of the occupancy model of T cell activation would argue that independent of affinity, any ligand able to engage the receptor would contribute in a positive sense to development of intracellular signals. This is because the presence of ligand of any quality would always serve to increase the steady-state number of occupied receptors and thus the number of aggregated (signaling) TCR complexes. Furthermore, no matter what the affinity of the interaction with the TCR, ligands that do not reduce the available number of agonist complexes on the APC itself should not interfere with T cell activation by the good ligand. This is because the poor ligand must at equilibrium itself occupy each TCR that is prevented from interacting with the good (agonist) ligand (25). Therefore, although the nature of the ligand occupying the TCR will change as more and more poor ligand is added, the total number of occupied receptors will not be altered and the same aggregation-dependent signal generation should occur. As this does not fit the present data, a more complex model must be considered.

TCR signal transduction requires participation of a number of membrane components that are not in tight or permanent association. Interaction among these components results from ligand binding by the TCR and other molecules, such as the CD4 or CD8 coreceptors (26, 68). In this regard, formation of an active signaling complex by the T cell is like association of univalent growth factor receptors that need to dimerize to signal (69). Conformational changes induced by ligand binding have been suggested to play a role of growth factor association and subsequent signaling, and the pattern of second messengers generated by TCR occupancy could vary with the quality rather than binding affinity of the ligand on such a conformation-dependent basis. In the T cell case, however, the TCR seems to have a substantially lower affinity for its ligand than a typical growth factor receptor for its (70, 71). Ligand-engaged TCRs therefore exist as a pool of loosely associated complexes in the same membrane region, with rapid dissociation and reassociation among components. Reduction in effective binding affinity for a specific ligand could thus result in an inability to maintain an adequate local pool of engaged complexes, and TCR/TCR or TCR/coreceptor (CD4, CD8) association might not persist for the time needed for full development of cytosolic multiprotein assemblies involved in signal propagation (26, 72). This kinetic model of TCR signaling is very similar in principle to the explana-

tion put forth by Metcalf and Klinman (73) to explain why modest differences in B cell Ig receptor affinity for structurally related haptens resulted in an all-or-nothing difference in signaling function that could not be compensated for by appropriate increases in ligand concentration.

Generation of only a subset of TCR-dependent intracellular signals due to either conformation- or affinity-related effects of altered ligand structure could thus result in selective gene activation. The production of excess negative mediators by the PCC peptide–mutant EαEβ molecules that could inhibit CD28-dependent intracellular signaling might explain why CD28 crosslinking could not overcome the peptide-induced inhibition of IL-2 production. Alternatively, even with decreased TCR–ligand affinity, the incomplete agonist we have studied might generate a normal pattern of intracellular messengers in the T cell itself. There is accumulating evidence that the costimulatory function of many APC is initially low and needs to be upregulated during T cell–APC interaction (12, 57). If so, then a reduced TCR–ligand affinity might interfere with aggregation-based signaling (perhaps via the MHC class II molecules of the APC), preventing needed costimulator upregulation. This would lead to intracellular T cell signaling under conditions in which APC second messenger generation, and hence, APC costimulatory activity, is deficient. Irrespective of whether the site of defective signaling is the T cell or the APC, the ability of relatively small numbers of incomplete agonist complexes to interfere with responses in the presence of complete agonist ligand implies that introduction of ineffective TCR–ligand complexes into the forming signaling assemblies at the T cell–APC interface interferes in a nonlinear way with signal generation.

Our data on different thresholds for distinct T cell differentiation events and on the ability of incomplete agonists to dominantly interfere with T cell effector function have implications for understanding intrathymic T cell development and for the creation of new approaches to autoimmune disease treatment and vaccine design. Exposure of immature thymocytes to both TCR and costimulatory signals may be responsible for intrathymic deletion (74). If some ligands can generate intracellular signals in T cells adequate for certain gene activation events without permitting effective costimulation, these would constitute ligands with the proper characteristics for positive selection of thymocytes without subsequent negative selection. In addition, the marked difference in threshold stimulation of different effector activities seen in comparing IL-2 vs. IL-3 secretion would suggest that in and of itself low level receptor occupancy could evoke differentiation without deletion in developing thymocytes, just as it would stimulate IL-2 without IL-3 production in mature cells. This suggestion is in full accord with the recent report of Robey et al. (75) on the effect of changing CD8 levels on thymic selection.

The present results also suggest an alternative explanation for some instances of immune downregulation reported upon coimmunization with related peptides that have been previously ascribed to competition for peptide binding to MHC molecules (76, 77). Because T cell signaling in the absence of costimulation frequently leads to a state of unresponsiveness termed anergy (78), not only would a properly altered self-peptide with incomplete agonist properties potentially be able to block ongoing autoimmune T effector activity, as might a true MHC blocking peptide, but administration of antigens generating mixed agonist/antagonist complexes might lead to a lasting decrease in autoimmune disease due to anergy induction among the self-reactive T cells. Finally, the split in cytokine production seen with such mixed agonist/antagonist complexes might also be used to deviate immune responses after vaccination away from those that sometime potentiate disease upon subsequent infection and towards those that are highly protective. Additional study of this novel class of TCR ligand is thus likely to produce new insights in both basic T cell biology and applied immunology.

We thank R. Schwartz, J. Ashwell, R. Koenig, J. van Meerwijk, and M. Lenardo for their careful reading of this manuscript and helpful suggestions for its improvement. We also thank L. Samelson for discussions concerning T cell signal transduction, members of the Lymphocyte Biology Section for help with various molecular techniques, P. Linsley for providing CTLA4-Ig for identifying B7 on the L cell transfectants, J. Allison for providing the hamster anti–mouse CD28 antibody, R. Schwartz for the PCC 99Q peptide, and J. Rothbard for the biotinylated DASP peptide.

Address correspondence to Ronald N. Germain, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11N311, 9000 Rockville Pike, Bethesda, MD 20892. Franca Ronchese's present address is the Basel Institute for Immunology, Basel, Switzerland. Louis A. Matis's current address is Alexion Pharmaceuticals, New Haven, CT. Luigi Racioppi's current address is Universita degli Studi di Napoli Federico II, Naples, Italy.

Received for publication 18 September 1992 and in revised form 14 January 1993.

References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I–restricted T lymphocytes. *Annu. Rev. Immunol.* 7:601.

2. Rothbard, J.B., and M.L. Gefter. 1991. Interactions between immunogenic peptides and MHC proteins. *Annu. Rev. Immunol.*

9:527.
3. Ashwell, J.D., and R.D. Klausner. 1990. Genetic and mutational analysis of the T-cell antigen receptor. *Annu. Rev. Immunol.* 8:139.
4. Letourneur, F., and R.D. Klausner. 1991. T-cell and basophil activation through the cytoplasmic tail of T-cell-receptor zeta family proteins. *Proc. Natl. Acad. Sci. USA.* 88:8905.
5. Wegener, A.M., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton, and B. Malissen. 1992. The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules. *Cell.* 68:83.
6. Samelson, L.E., M.D. Patel, A.M. Weissman, J.B. Harford, and R.D. Klausner. 1986. Antigen activation of murine T cells induces tyrosine phosphorylation of a polypeptide associated with the T cell antigen receptor. *Cell.* 46:1083.
7. Weiss, A., J. Imboden, D. Shoback, and J. Stobo. 1984. Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. *Proc. Natl. Acad. Sci. USA.* 81:4169.
8. June, C.H., M.C. Fletcher, J.A. Ledbetter, and L.E. Samelson. 1990. Increases in tyrosine phosphorylation are detectable before phospholipase C activation after T cell receptor stimulation. *J. Immunol.* 144:1591.
9. Weaver, C.T., C.M. Hawrylowicz, and E.R. Unanue. 1988. T helper cell subsets require the expression of distinct costimulatory signals by antigen-presenting cells. *Proc. Natl. Acad. Sci. USA.* 85:8181.
10. Mueller, D.L., M.K. Jenkins, L. Chiodetti, and R.H. Schwartz. 1990. An intracellular calcium increase and protein kinase C activation fail to initiate T cell proliferation in the absence of a costimulatory signal. *J. Immunol.* 144:3701.
11. Linsley, P.S., W. Brady, L. Grosmaire, A. Aruffo, N.K. Damle, and J.A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J. Exp. Med.* 173:721.
12. Koulova, L., E.A. Clark, G. Shu, and B. Dupont. 1991. The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of $CD4^+$ T cells. *J. Exp. Med.* 173:759.
13. Vandenberghe, P., G.J. Freeman, L.M. Nadler, M.C. Fletcher, M. Kamoun, L.A. Turka, J.A. Ledbetter, C.B. Thompson, and C.H. June. 1992. Antibody and B7/BB1-mediated ligation of the CD28 receptor induces tyrosine phosphorylation in human T cells. *J. Exp. Med.* 175:951.
14. Jenkins, M.K., D.M. Pardoll, J. Mizuguchi, H. Quill, and R.H. Schwartz. 1987. T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol. Rev.* 95:113.
15. Bach, F.H., C.C. Grillot, O.J. Kuperman, H.W. Sollinger, C. Hayes, P.M. Sondel, B.J. Alter, and M.L. Bach. 1977. Antigenic requirements for triggering of cytotoxic T lymphocytes. *Immunol. Rev.* 35:76.
16. Germain, R.N. 1981. Accessory cell stimulation of T cell proliferation requires active antigen processing, Ia-restricted antigen presentation, and a separate nonspecific second signal. *J. Immunol.* 127:1964.
17. Jenkins, M.K., and R.H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J. Exp. Med.* 165:302.
18. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J. Immunol.* 142:2617.
19. Liu, Y., B. Jones, A. Aruffo, K.M. Sullivan, P.S. Linsley, and C.A. Janeway, Jr. 1992. Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. *J. Exp. Med.* 175:437.
20. Freeman, G.J., G.S. Gray, C.D. Gimmi, D.B. Lombard, L.J. Zhou, M. White, J.D. Fingeroth, J.G. Gribben, and L.M. Nadler. 1991. Structure, expression, and T cell costimulatory activity of the murine homologue of the human B lymphocyte activation antigen B7. *J. Exp. Med.* 174:625.
21. Gimmi, C.D., G.J. Freeman, J.G. Gribben, K. Sugita, A.S. Freedman, C. Morimoto, and L.M. Nadler. 1991. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc. Natl. Acad. Sci. USA.* 88:6575.
22. Reiser, H., G.J. Freeman, Z. Razi-Wolf, C.D. Gimmi, B. Benacerraf, and L.M. Nadler. 1992. Murine B7 antigen provides an efficient costimulatory signal for activation of murine T lymphocytes via the T-cell receptor/CD3 complex. *Proc. Natl. Acad. Sci. USA.* 89:271.
23. Kay, R., F. Takei, and R.K. Humphries. 1990. Expression cloning of a cDNA encoding M1/69-J11d heat-stable antigens. *J. Immunol.* 145:1952.
24. Matis, L.A., L.H. Glimcher, W.E. Paul, and R.H. Schwartz. 1983. Magnitude of response of histocompatibility-restricted T-cell clones is a function of the product of the concentrations of antigen and Ia molecules. *Proc. Natl. Acad. Sci. USA.* 80:6019.
25. Ashwell, J.D., B.S. Fox, and R.H. Schwartz. 1986. Functional analysis of the interaction of the antigen-specific T cell receptor with its ligands. *J. Immunol.* 136:757.
26. Singer, S.J. 1992. Intercellular communication and cell-cell adhesion. *Science (Wash. DC).* 255:1671.
27. Fiering, S., J.P. Northrop, G.P. Nolan, P.S. Mattila, G.R. Crabtree, and L.A. Herzenberg. 1990. Single cell assay of a transcription factor reveals a threshold in transcription activated by signals emanating from the T-cell antigen receptor. *Genes & Dev.* 4:1823.
28. 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.
29. Racioppi, L., F. Ronchese, R.H. Schwartz, and R.N. Germain. 1991. The molecular basis of class II MHC allelic control of T cell responses. *J. Immunol.* 147:3718.
30. Margulies, D.H., G.A. Evans, K. Ozato, R. Camerini-Otero, K. Tanaka, E. Appella, and J.G. Seidman. 1983. Expression of $H\text{-}2D^d$ and $H\text{-}2L^d$ mouse major histocompatibility antigen genes in L cells after DNA-mediated gene transfer. *J. Immunol.* 130:463.
31. Corradin, G., and H.A. Harbury. 1970. Cleavage of cytochrome *c* with cyanogen bromide. *Biochim. Biophys. Acta.* 221:489.
32. Busch, R., G. Strang, K. Howland, and J.B. Rothbard. 1990. Degenerate binding of immunogenic peptides to HLA-DR proteins on B cell surfaces. *Int. Immunol.* 2:443.
33. Matis, L.A., D.L. Longo, S.M. Hedrick, C. Hannum, E. Margoliash, and R.H. Schwartz. 1983. Clonal analysis of the major histocompatibility complex restriction and the fine specificity of antigen recognition in the T cell proliferative response to cytochrome *c*. *J. Immunol.* 130:1527.
34. White, J., M. Blackman, J. Bill, J. Kappler, P. Marrack, D.P. Gold, and W. Born. 1989. Two better cell lines for making hybridomas expressing specific T cell receptors. *J. Immunol.* 143:1822.
35. Ronchese, F., R.H. Schwartz, and R.N. Germain. 1987. Functionally distinct subsites on a class II major histocompatibility

complex molecule. *Nature (Lond.).* 329:254.
36. Harding, F.A., J.G. McArthur, J.A. Gross, D.H. Raulet, and J.P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature (Lond.).* 356:607.
37. Ozato, K., N.M. Mayer, and D.H. Sachs. 1980. Hybridoma cell lines secreting monoclonal antibodies to mouse H-2 and Ia antigens. *J. Immunol.* 124:533.
38. Ronchese, F., M.A. Brown, and R.N. Germain. 1987. Structure-function analysis of the $A\beta^{bm12}$ mutation using site-directed mutagenesis and DNA-mediated gene transfer. *J. Immunol.* 139:629.
39. Malek, T.R., R.J. Robb, and E.M. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA.* 80:5694.
40. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
41. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
42. Heath, W.R., M.E. Hurd, F.R. Carbone, and L.A. Sherman. 1989. Peptide-dependent recognition of H-$2K^b$ by alloreactive cytotoxic T lymphocytes. *Nature (Lond.).* 341:749.
43. Lombardi, G., S. Sidhu, J.R. Lamb, J.R. Batchelor, and R.I. Lechler. 1989. Co-recognition of endogenous antigens with HLA-DR1 by alloreactive human T cell clones. *J. Immunol.* 142:753.
44. Cotner, T., E. Mellins, A.H. Johnson, and D. Pious. 1991. Mutations affecting antigen processing impair class II-restricted allorecognition. *J. Immunol.* 146:414.
45. Rötzschke, O., K. Falk,, S. Faath, and H.G. Rammensee. 1991. On the nature of peptides involved in T cell alloreactivity. *J. Exp. Med.* 174:1059.
46. Rock, K.L., and B. Benacerraf. 1984. Selective modification of a private I-A allo-stimulating determinant(s) upon association of antigen with an antigen-presenting cell. *J. Exp. Med.* 159:1238.
47. Eckels, D.D., J. Gorski, J. Rothbard, and J.R. Lamb. 1988. Peptide-mediated modulation of T-cell allorecognition. *Proc. Natl. Acad. Sci. USA.* 85:8191.
48. Adorini, L., J. Moreno, F. Momburg, G.J. Hämmerling, J.C. Guery, A. Valli, and S. Fuchs. 1991. Exogenous peptides compete for the presentation of endogenous antigens to major histocompatibility complex class II–restricted T cells. *J. Exp. Med.* 174:945.
49. Maryanski, J.L., P. Pala, J.C. Cerottini, and G. Corradin. 1988. Synthetic peptides as antigens and competitors in recognition by H-2-restricted cytolytic T cells specific for HLA. *J. Exp. Med.* 167:1391.
50. Adorini, L., and Z. Nagy. 1989 Competition for antigen presentation in living cells involves exchange of peptides bound by class II MHC molecules. *Nature (Lond.).* 342:800.
51. Hansburg, D., T. Fairwell, R.H. Schwartz, and E. Appella. 1983. The T lymphocyte response to cytochrome *c*. IV. Distinguishable sites on a peptide antigen which affect antigenic strength and memory. *J. Immunol.* 131:319.
52. Fox, B.S., C. Chen, E. Fraga, C.A. French, B. Singh, and R.H. Schwartz. 1987. Functionally distinct agretopic and epitopic sites. Analysis of the dominant T cell determinant of moth and pigeon cytochromes *c* with the use of synthetic peptide antigens. *J. Immunol.* 139:1578.
53. Jorgensen, J.L., U. Esser, B. Fazekas de St. Groth, P.A. Reay, and M.M. Davis. 1992. Mapping T-cell receptor-peptide contacts by variant peptide immunization of single-chain transgenics. *Nature (Lond.).* 335:224.
54. Suzuki, G., Y. Kawase, S. Koyasu, I. Yahara, Y. Kobayashi, and R.H. Schwartz. 1988. Antigen-induced suppression of the proliferative response of T cell clones. *J. Immunol.* 140:1359.
55. Quill, H., and R.H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J. Immunol.* 138:3704.
56. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7:445.
57. Razi-Wolf, Z., G.J. Freeman, F. Galvin, B. Benacerraf, L. Nadler, and H. Reiser. 1992. Expressing and function of the murine B7 antigen, the major costimulatory molecule expressed by peritoneal exudate cells. *Proc. Natl. Acad. Sci. USA.* 89:4210.
58. Watts, T.H., A.A. Brian, J.W. Kappler, P. Marrack, and H.M. McConnell. 1984. Antigen presentation by supported planar membranes containing affinity-purified I-A^d. *Proc. Natl. Acad. Sci. USA.* 81:7564.
59. Damle, N.K., L.V. Doyle, L.S. Grosmaire, and J.A. Ledbetter. 1988. Differential regulatory signals delivered by antibody binding to the CD28 (Tp44) molecule suring activation of human T lymphocytes. *J. Immunol.* 140:1753.
60. Ledbetter, J.A., J.B. Imboden, G.L. Schieven, L.S. Grosmaire, P.S. Rabinovitch, T. Lindsten, C.B. Thompson, and C.H. June. 1990. CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood.* 75:1531.
61. Otten, G.R., and R.N. Germain. 1991. Split anergy in a $CD8^+$ T cell: receptor-dependent cytolysis in the absence of interleukin-2 production. *Science (Wash. DC).* 251:1228.
62. De Magistris, M., 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.
63. Crabtree, G.R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science (Wash. DC).* 243:355.
64. Johnson, P.F., and S.L. McKnight. 1989. Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* 58:799.
65. Fraser, J.D., B.A. Irving, G.R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science (Wash. DC).* 251:313.
66. Soloway, P., S. Fish, H. Passmore, M. Gefter, R. Coffee, and T. Manser. 1991. Regulation of the immune response to peptide antigens: differential induction of immediate-type hypersensitivity and T cell proliferation due to changes in either peptide structure or major histocompatibility complex haplotype. *J. Exp. Med.* 174:847.
67. Murray, J.S., C. Pfeiffer, J. Madri, and K. Bottomly. 1992. Major histocompatibility complex (MHC) control of CD4 T cell subset activation. II. A single peptide induces either humoral or cell-mediated responses in mice of distinct MHC genotype. *Eur. J. Immunol.* 22:559.
68. Dianzani, U., A. Shaw, B. al-Ramadi, R.T. Kubo, and C.A. Janeway, Jr. 1992. Physical association of CD4 with the T cell receptor. *J. Immunol.* 148:678.
69. Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell.* 61:203.

70. Matsui, K., J.J. Boniface, P.A. Reay, H. Schild, B. Fazekas de St. Groth, and M.M. Davis. 1991. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science (Wash. DC).* 254:1788.
71. Weber, S., A. Traunecker, F. Oliveri, W. Gerhard, and K. Karjalainen. 1992. Specific low-affinity recognition of major histocompatibility complex plus peptide by soluble T-cell receptor. *Nature (Lond.).* 356:793.
72. Koch, C.A., D. Anderson, M.F. Moran, C. Ellis, and T. Pawson. 1991. SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. *Science (Wash. DC).* 252:668.
73. Klinman, N.R., 1972. The mechanism of antigenic stimulation of primary and secondary clonal precursor cells. *J. Exp. Med.* 136:241.
74. Vasquez, N.J., J. Kaye, and S.M. Hedrick. 1992. In vivo and in vitro clonal deletion of double-positive thymocytes. *J. Exp. Med.* 175:1307.
75. Robey, E.A., F. Ramsdell, D. Kioussis, W. Sha, D. Loh, R. Axel, and B.J. Fowlkes. 1992. The level of CD8 expression can determine the outcome of thymic selection. *Cell.* 69:1089.
76. Adorini, L., S. Muller, F. Cardinaux, P.V. Lehmann, F. Falcioni, and Z.A. Nagy. 1988. In vivo competition between self peptides and foreign antigens in T-cell activation. *Nature (Lond.).* 334:623.
77. Lock, C., D. Smilek, A. Gautam, M. Vaysburd, S. Dwivedy, and H. McDevitt. 1991. Competitive inhibition of antigen presentation in animal models of autoimmune disease. *Semin. Immunol.* 3:247.
78. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science (Wash. DC).* 248:1349.