Modulation of Naive CD4 T Cell Activation with Altered Peptide Ligands: The Nature of the Peptide and Presentation in the Context of Costimulation Are Critical for a Sustained Response

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Modulation of Naive CD4 T Cell Activation with Altered Peptide Ligands: The Nature of the Peptide and Presentation in the Context of Costimulation Are Critical for a Sustained Response

Paul R. Rogers, Howard M. Grey, and Michael Croft

Altered peptide ligands containing single amino acid substitutions have the potential to be used for modulating immune function. Using a panel of moth cytochrome c peptides, we demonstrate that different phases of naive CD4 T cell response are alternately modulated depending on altered peptide ligand dose and accessory molecule expression by APC. Weak agonists presented at high concentration, and with costimulation, efficiently induced early phase naive T cell activation as assessed by IL-2R/CD69 expression, but could only promote sufficient IL-2 for a short-lived proliferative response. In contrast, strong agonists and heteroclitic peptides induced early phase T cell activation even at low concentrations with costimulation, and allowed sustained IL-2 secretion and proliferation. In the absence of accessory molecule help, early and late phase activation was impaired with weak agonists, whereas strong agonists partially compensated for a lack of costimulation for early phase activation, and also promoted enhanced IL-2 with sustained proliferation. These studies support the hypothesis that the naive T cell response will be determined by the balance between provision of accessory molecule help and the affinity of peptide/MHC complexes for individual TCRs, and suggest that extended IL-2 production is the main facet of naive CD4 activation that is affected by altering the nature of the peptide. The Journal of Immunology, 1998, 160: 3698–3704.

Effective stimulation of naive CD4 T cells is critical for a productive and long-lasting primary immune response. Studies to date have largely suggested that the nature of the APC may play an important role in promoting efficient activation of naive cells (1, 2). Thus, low level cytokine secretion and little proliferation result in vitro, and tolerance results in vivo, unless Ag is presented on mature or highly activated APC such as dendritic cells (3–8). The stringent requirements for response largely appear to relate to provision of help from accessory molecules on the APC that interact with T cell coreceptors. These interactions may function in both an adhesive capacity to augment cell-cell interaction and also provide costimulatory signals that appear to be required for promoting IL-2 secretion (9, 10). This requirement for accessory molecule help has also been observed in vivo, in that efficient priming and expansion only occur if an inflammatory situation is generated, such as with adjuvant, which appears to at least in part reflect the need to up-regulate accessory molecules (11, 12). Similarly, knockout mice, deficient in either B7-CD28 or ICAM-LFA interactions, cannot be primed effectively for a T cell response (13–15).

In contrast to APCs, modulation of naive CD4 response by altering the nature of the peptide Ag has received little attention. Many studies have established that single amino acid substitutions, largely at TCR contact residues, can alter responses of long-term T cell clones (16–18). A range of activities has been described from studies of clones, such as antagonism and anergy induction due to differential signal transduction, and cytokine production in the absence of proliferation (19–26). It is not clear, however, whether the parameters of activation and responses observed in these studies equally apply to the naive cell. In addition, the majority of studies on peptide ligands to date have not taken into account the characteristics of the APC and the contribution of costimulatory accessory molecules to the overall response.

In the present study, we have used naive CD4 T cells specific for pigeon cytochrome c (PCC) and moth cytochrome c (MCC), obtained from Vβ3/Vα11 TCR transgenic mice, to analyze aspects of the naive CD4 response by altering the nature of the peptide Ag. Using a panel of altered peptide ligands containing single amino acid substitutions, we demonstrate that different phases of naive CD4 T cell response are alternately modulated depending on altered peptide ligand dose and accessory molecule expression by APC. Weak agonists presented at high concentration, and with costimulation, efficiently induced early phase naive T cell activation as assessed by IL-2R/CD69 expression, but could only promote sufficient IL-2 for a short-lived proliferative response. In contrast, strong agonists and heteroclitic peptides induced early phase T cell activation even at low concentrations with costimulation, and allowed sustained IL-2 secretion and proliferation. In the absence of accessory molecule help, early and late phase activation was impaired with weak agonists, whereas strong agonists partially compensated for a lack of costimulation for early phase activation, and also promoted enhanced IL-2 with sustained proliferation. These studies support the hypothesis that the naive T cell response will be determined by the balance between provision of accessory molecule help and the affinity of peptide/MHC complexes for individual TCRs, and suggest that extended IL-2 production is the main facet of naive CD4 activation that is affected by altering the nature of the peptide.

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3. Abbreviations used in this paper: PCC, pigeon cytochrome c; MCC, moth cytochrome c; APL, altered peptide ligand.
Table I. Altered peptide ligands

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>I-E(^b) Binding (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC</td>
<td>ANERADLIAYLKQATK</td>
<td>48</td>
</tr>
<tr>
<td>L98A</td>
<td>ANERADLIAYLKQATK</td>
<td>79</td>
</tr>
<tr>
<td>T102S</td>
<td>ANERADLIAYLKQATK</td>
<td>91</td>
</tr>
<tr>
<td>K99E</td>
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<tr>
<td>Y97K</td>
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<td>28</td>
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<tr>
<td>K99A</td>
<td>KAERADLIAYLKQATK</td>
<td>19</td>
</tr>
<tr>
<td>T102L</td>
<td>ANERADLIAYLKQATK</td>
<td>25</td>
</tr>
<tr>
<td>PCC</td>
<td>KAERADLIAYLKQATK</td>
<td>63</td>
</tr>
</tbody>
</table>

*Peptides of moth (MCC) and pigeon cytochrome c (PCC) were selected for similar binding to I-E\(^b\) as assessed by dose needed for 50% inhibition of binding of iodinated PCC. Data shown is the average from two to eight experiments. All APLs are variants of MCC, except K99A. All APLs possessed single amino acid substitutions at TCR contact residues (positions 97, 99, or 102), except L98A.

demonstrate that the nature of the APC and the nature of the peptide ligand are both important mediators of naive T cell activation, and optimization of both is critical for determining the extent of the primary T cell response through support of long-lasting IL-2 production.

Materials and Methods

Mice

AND TCR transgenic mice expressing the V\(\beta3/V\alpha11\) TCR were bred on a B10.BR background (H2\(^d\)) as previously described (5, 10).

Altered peptide ligands (APLs)

Peptides of MCC or PCC were synthesized in the peptide facility at La Jolla Institute for Allergy and Immunology. Each peptide was selected based on similar binding to purified I-E\(^b\) as shown in Table I, with single amino acid substitutions as indicated. Binding assays were as previously described (27) using a competition assay with radiiodinated PCC. Values represent concentrations of unlabeled peptides required to inhibit 50% of the binding of the iodinated peptide.

T cells

CD4\(^+\) T cells were purified from spleen and lymph nodes of TCR transgenic mice as before (5, 10) by nylon wool depletion, followed by complement treatment with Abs to CD8 (3.155), heat-stable Ag (J11D), class II MHC (M5/114 and CA-4.A12), macrophages (M1/70), and dendritic cells (33D1), cross-linked with mouse anti-rat \(\kappa\) (MAR 18.5). Any residual APC and any in vivo-activated T cells were removed by incubation with high density cells spun through a Percoll gradient (45, 53, and 80%). The resultant cells were resting (low forward scatter, CD69\(^-\), CD95\(^-\), CD71\(^-\), CD40L\(^-\), CD25\(^-\)) and >95% CD4\(^+\). More than 95% of these cells possessed a phenotype associated with naive CD4 cells (CD45RB\(^+\)/CD44low) along with expression of the V\(\beta3/V\alpha11\) TCR (5, 10). In most experiments, T cells were further purified by positive selection with anti-CD26L (Mel 14) using magnetic beads (Miltenyi Biotech, Sunnyvale, CA) as in previous studies (5).

Ag-presenting cells

Fibroblast cells transfected with I-E\(^b\) (originally generated by Dr. R. Germain, National Institutes of Health, Bethesda, MD) were used as APC. Two fibroblast lines were used as before (9, 10) that expressed, or lacked, B7-1 and ICAM-1 (DCEK.ICAM, referred to as ICAM\(^+\)B7\(^−\); DCEK\(^−\), referred to as ICAM\(^−\)B7\(^+\)). These cells do not express vascular cell adhesion molecule-1, very late Ag-4, 0-2X, 0-40L, 4-1BBL, LFA-1, heat-stable Ag, or CD48 by FACS analysis. APC populations were treated with mitomycin \(c\) (75–100 \(\mu\)g/ml; Sigma, St. Louis, MO) for 30 min at 37°C before use.

Cell cultures

Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) with penicillin, streptomycin, glutamine, 2-ME, sodium pyruvate, and 7% FCS (HyClone, Logan, UT, and Irvine Scientific). Cultures were generally set up in 0.2-ml vol in 96-well plates (Costar, Cambridge, MA) in triplicate. Naive CD4 cells were plated at a concentration of 2.5 \(\times\) 10^5/ml with half as many APC. APLs of MCC or PCC were added into culture at varying concentrations, or APC were pulsed with the indicated concentrations at 2 \(\times\) 10^5/ml for 2 h at 37°C.

Fluorescence analysis

CD25 expression was assessed using FITC-labeled anti-CD25 (rat IgM; Pharmingen, San Diego, CA) and CD69 with FITC-labeled anti-CD69 (hamster IgG; Pharmingen). Controls were FITC-labeled purified rat IgM or hamster IgG (Caltag, Burlingame, CA). CD4\(^+\) T cells were identified using phycocerythrin-conjugated anti-CD4 (PharMingen). FACS analyses were performed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) with Cellquest software. Data were gated for viable CD4\(^+\) T cells and are plotted as the number of cells positive for each marker at 22 h.

Proliferation

Cell division was assessed by addition of 1 \(\mu\)Ci of tritiated thymidine (ICN Pharmaceuticals, Costa Mesa, CA) to 0.2-ml cultures for approximately 12 h at the end of culture. Response was assessed at different times depending on the experiment, either between 36 to 48 h, 60 to 72 h, or 84 to 96 h.

Cytokine secretion

Triplicate supernatants were recovered 20 to 24 h and 35 to 40 h after T cell stimulation and pooled to assess cytokine content. IL-2 production was determined as before (5, 9) by titrating pooled replicate supernatants onto NK.3 cells, in duplicate, in the presence of anti-IL-4 (purified from the 11B11 cell line; American Type Culture Collection, Rockville, MD). Standard curves were constructed with purified IL-2 (supernatant from the X63.Ag.IL-2 cell line).

Results

APLs modulate the ability of highly costimulatory APC to promote naive T cell proliferation and IL-2 secretion

Peptides of MCC and PCC were screened for reactivity with naive CD4 cells expressing the V\(\beta3/V\alpha11\) TCR and isolated from AND transgenic mice. Initial screening was conducted with presentation on a highly stimulatory fibroblast APC expressing I-E\(^b\) along with B7-1 and ICAM-1, as used in previous studies (9, 10). Several peptides were selected to give a range of activities compared with the native peptides from nonstimulatory to heteroclitic agonists (agonists that are more stimulatory than the native peptide). Because each peptide displayed similar binding to I-E\(^b\) (Table I), reactivities are most likely attributable to varying affinities of interaction between the TCR and the peptide/MHC complex.

Figure 1 shows proliferation and IL-2 secretion of naive CD4 cells assessed at times previously found to give maximal responses to the native peptides. Generally, substitutions at the dominant (positions 99 and 102) and subdominant (position 97) TCR contact residues result in peptides that produce altered T cell responses (28), and largely these have been utilized in the studies here. However, substitutions at adjacent residues can also produce APLs of differential activity, e.g., an L to A substitution at position 98 resulted in a heteroclitic peptide that induces proliferation and IL-2 secretion from naive T cells at lower doses than the native peptide (Fig. 1, left hand graphs). In addition, different amino acid substitutions at the same position can result in tremendous variations in activity. Thus, T to S at position 102 results in a better agonist peptide than MCC, whereas T to L produces a peptide that can antagonize responses induced by the native peptide (P. R. Rogers, unpublished observations using B7\(^+\)ICAM\(^+\) fibroblasts as APC) and does not induce proliferation or IL-2 secretion from naive cells even with optimal costimulation (Fig. 1).

Peptides of different reactivities were therefore utilized to dissect parameters of naive T cell responsiveness to define more closely the relative contributions of overall TCR-MHC affinity and...
accessory molecule help in eliciting downstream events of activation. Initial experiments in Figure 1 were conducted with B7+1ICAM+ APCs (1.25 × 10^6/ml) and varying doses of soluble peptides in triplicate. Supernatants were collected at 24 h, pooled together, and assayed for IL-2. Proliferation was measured between 60 to 72 h with data representing the mean cpm from triplicates. Errors for individual points were less than 15% of the means. Proliferation and IL-2 in the absence of Ag is shown by the isolated points on the γ-axes. Similar results were seen in four repeated experiments.

**FIGURE 1.** Identification of APLs with increased or decreased ability to activate transgenic CD4 T cells. Naive T cells (2.5 × 10^6/ml) were cultured with B7+1ICAM+ fibroblast APCs that we have previously shown are equivalent in stimulatory capacity to dendritic cells (10). These APCs therefore potentially provide optimal accessory molecule help as relates to provision of both adhesion to maximize the ability to form conjugates and costimulation to promote late events of T cell response. Even with such APC, naive T cell responses were dramatically different depending on the peptide. Heteroclitic peptides (L98A and T102S) induced similar maximum levels of proliferation to the native peptide, but responses were induced with approximately 10- to 50-fold less Ag. Similar results were apparent assessing IL-2 production, which is largely responsible for the extent of naive T cell proliferation (our unpublished observations), although these peptides induced higher total levels of IL-2 in most experiments as well as shifting the dose-response curves. In contrast, weak agonist peptides displayed two activities with the highly costimulatory APC. Some peptides such as K99E were also capable of promoting responses of similar magnitude to the native and heteroclitic peptides but only when present at doses 10- to 100-fold above those with the native peptide. In contrast, other peptides such as Y97K and K99A only initiated naive CD4 responses at concentrations in excess of 1000-fold more than those required with the native peptide, and the responses obtained were far lower in magnitude than that with the MCC regardless of the concentration. This was evident for both IL-2 secretion taken at 24 h and proliferation from 60 to 72 h, suggesting that the extent of IL-2 produced was critical for the overall response and could be governed by the nature of the peptide.

**APLs modulate the ability of highly costimulatory APC to promote naive T cell activation**

The data presented in Figure 1 suggested that APLs affected the extent of IL-2 production from naive CD4 cells and that this dictated the later proliferative response, but did not address whether the peptides varied in capacity to promote early phases of naive T cell activation. We therefore assessed expression of the activation Ags CD69 and IL-2Rα (CD25), which during the initial stages of naive T cell response are not dependent on costimulatory signaling or the extent of IL-2 produced (our unpublished observations). This analysis represents a reliable method for determining the number of T cells that are activated in culture. Several different phenomena were observed depending on the peptide analyzed (Fig. 2). The antagonist peptide, T102L, was virtually incapable of promoting T cell activation at any dose tested correlating with its inability to promote IL-2 and proliferation. In contrast, the very weak agonist, K99A, which only induced IL-2 and proliferation at minimal levels, was capable of activating virtually all of the T cells present in culture at doses of 10 to 100 μM, which was 10,000-fold more than that required for a similar response with the native Ag (0.001 μM). The other weak agonists, Y97K and K99E, which induced greater proliferative and IL-2 responses than K99A but also varied in stimulatory capacity with respect to each other, were identical in their ability to promote naive activation as assessed by CD69 and IL-2R expression. The threshold of response occurred between 0.01 and 0.1 μM. Heteroclitic peptides (T102S, Fig. 2, and L98A not shown) were equivalent to the native peptide in their capacity to promote early activation, the majority of cells expressing CD69 and IL-2R at doses of 0.0001 to 0.001 μM. Interestingly, significant IL-2 production and proliferation were only observed at Ag doses that resulted in the majority of T cells in culture being activated (compare dose responses of Figs. 1 and 2), but early activation of naive T cells was not strictly correlated with the later phases such as the extent of IL-2 produced. Thus, weak agonist peptides varied from the native peptide in the ability to promote all aspects of naive CD4 activation at low Ag doses, but at higher doses were largely defective in the ability to induce IL-2 production and promote later responses such as T cell proliferation. In contrast, heteroclitic peptides functioned primarily by promoting enhanced IL-2 when present at low doses.

**Weak agonist peptides can promote efficient naive T cell proliferation at early times but cannot sustain the response**

Because the majority of T cells were activated with weak agonist peptides at higher Ag concentrations and some IL-2 was produced depending on the nature of the peptides, we investigated whether...
this would affect the kinetics of proliferation compared with the native peptide. Figure 3 shows proliferation over time, induced by APLs presented on highly costimulatory APC (B7 ICAM fibroblasts). As seen in previous studies (5, 9) and shown in Figure 1, optimal proliferation of naive T cells in response to native MCC was between 3 and 4 days after activation. Differing kinetics were seen with weak agonists. K99E, which was the most stimulatory of the weak agonists, maintained proliferation at levels similar to MCC over the 3 days studied, with the dose required for similar response being relatively constant at approximately 10- to 50-fold more. Y97K, which could not induce equivalent IL-2 secretion regardless of concentration, also induced equivalent proliferative responses at days 2 and 3, but could not maintain a strong response past this time. High doses of K99A, able to promote only minimal IL-2 secretion, induced similar levels of proliferation to the native peptide at day 2 (36–48 h) but were incapable of sustaining the response beyond this time point. Thus, peptides of presumed low affinity, when expressed on highly stimulatory APC, were capable of promoting naive CD4 activation as assessed by early induction of CD69/IL-2R (Fig. 2) and early proliferation (Fig. 3) but were lacking in ability to induce the high levels of IL-2 required for sustaining T cell expansion.

The ability of APLs to activate naive T cells is determined by accessory molecule expression on the APC

We have previously shown that the extent of proliferation and IL-2 secretion from naive CD4 cells in response to native PCC is determined by the expression of accessory molecules on the APC, and that an APC lacking one or more of these molecules induces minimal proliferation and little IL-2 (9). To investigate the role of accessory molecule help in response to APLs, we examined naive CD4 activation (IL-2R expression) by APCs that lack B7 and ICAM but are still able to present Ag (Fig. 4). Expression of IL-2Rα was assessed at 21 h, with similar results obtained at 15- and 42-h time points. Identical results were obtained when analyzing CD69 expression (data not shown). Similar to ICAM B7 cells, the APC lacking those accessory molecules were capable of activating the majority of T cells in culture with native MCC; however, about 100-fold more peptide was required. The heteroclitic peptide, T102S, when compared with native Ag, did not further enhance the ability of ICAM B7 APC to promote early T cell activation as assessed by IL-2R expression. However, weak agonist peptides presented by these APC became much less effective than when presented by APC with accessory molecules. Thus, K99A that induced all T cells to become activated when present at high concentrations with B7 and ICAM help was at least 100-fold less efficient in the absence of B7 and ICAM and capable of promoting activation of only a small fraction of T cells at the maximum dose tested (50 μM). A similar lower effectiveness was seen with other weak agonist peptides (K99E, Y97K) when presented in the absence of B7 and ICAM. Significantly, a peptide such as K99A...
Heteroclitic peptides can partially compensate for a lack of accessory molecule help and promote enhanced naive response at late times

The heteroclitic peptide, T102S, was next assessed for its ability to promote later phases of naive activation with and without help from B7 and ICAM. Naive CD4 cells were stimulated with peptide-pulsed ICAM$^+$B7$^-$ APC, and proliferation and IL-2 secretion were assessed over time compared with that elicited with ICAM$^+$B7$^+$ APC (Fig. 5). In the absence of B7 and ICAM, IL-2 production at 20 h was similar regardless of whether T102S or the native peptide was used, with naive cells producing only minimal quantities even at a high Ag dose. This low level of IL-2 induced only minimal proliferation at day 2, which again was similar regardless of peptide. With accessory molecule help at this early time, T102S did not enhance proliferation compared with MCC even though T102S promoted higher levels of IL-2 at lower doses. In contrast, late phase proliferation was enhanced with the heteroclitic peptide, regardless of the APC, and this correlated with significant differences in IL-2 production at 37 h. Although T102S presented on the accessory molecule-deficient APC enhanced IL-2 secretion somewhat, and proliferation to a large extent, responses were still less than those of the native peptide presented in the presence of B7 and ICAM. Therefore, heteroclitic peptides can partially overcome the need for multiple accessory molecules on APC and can result in enhanced naive responses at late times, but cannot totally compensate for the costimulatory signals and adhesion provided by these molecules.

**FIGURE 4.** Effect of APC accessory molecules on naive T cell CD25 expression. Naive CD4 T cells were cultured as in previous figures with B7$^-$/ICAM$^+$ (filled symbols) or B7$^+$ICAM$^-$ (open symbols) APC and varying doses of soluble peptide. Cells were harvested at 22 h and stained for CD25 expression. Gated CD4 cells were analyzed and the percentage of CD25-positive cells was determined by flow cytometry. Similar results were seen when analyzing CD69 induction. Data are representative of three experiments.

**FIGURE 5.** Heteroclitic peptides induce sustained proliferation and IL-2 secretion from naive T cells. Naive cells were cultured with B7$^+$ICAM$^+$ (filled symbols) or B7$^-$ICAM$^-$ (open symbols) APCs that had been pre pulsed for 2 h with the indicated concentrations of peptides MCC and T102S. Supernatants were collected from triplicate cultures at 20 and 37 h and assayed for IL-2. Proliferation was measured between 36 to 48 h and 60 to 72 h and mean values calculated. Similar results were seen in two repeated experiments.

**Discussion**

In this study, we have investigated the interrelationship between the nature of the peptide ligand and the nature of the APC in determining the extent of response of naive CD4 T cells. Single amino acid substitutions at major TCR contact residues and at secondary residues were able to either enhance or diminish the ability of APC to promote early naive cell activation as assessed by CD69 and IL-2R induction, and modulate later phases of response including IL-2 secretion and proliferation. In particular, weak agonist peptides were shown to reduce the activating capacity of a normally highly stimulatory APC expressing B7 and ICAM to that of a normally weakly stimulatory APC lacking B7 and ICAM. Conversely, heteroclitic peptides enhanced the capacity of weakly stimulatory APC to activate naive T cells to an extent, but could only partially compensate for the lack of accessory molecule help for IL-2 production and T cell expansion. Thus, the ultimate outcome of naive T cell interaction with an APC is equally governed by both the affinity of peptide/MHC complexes for TCRs and whether T cell coreceptors engage APC accessory molecules. The results show conclusively that for a maximal naive T cell response, both parameters must be optimized, and that these are largely required to promote IL-2 secretion that is sustained over time and allow T cell expansion that does not terminate prematurely.

Our previous studies on Ag presentation to naive CD4 cells using native PCC demonstrated that the later phase response was highly dependent on expression of accessory molecules by the APC. Much lower levels of both proliferation and IL-2 secretion were produced if only one accessory molecule was expressed (e.g., B7 or ICAM alone) and very little response was seen in the absence of B7 or ICAM (9). Data in Figure 4 assessing CD69/IL-2R expression extend these observations and show, in striking contrast...
to IL-2 and proliferation, that equivalent early phenotypic changes of activation are induced regardless of whether the APCs possess several of the major accessory molecules (e.g., B7, ICAM, 4-1BBL, Ox-40L, or heat-stable Ag). However, this only applies to situations in which sufficient quantities of native peptide are presented. At low peptide concentrations that are otherwise insufficient for induction of a discernible response, the presence of accessory molecules is required for an APC to activate T cells. How these molecules function in this regard is not clear. Both adhesion and costimulatory functions have been described for many such molecules, including B7 and ICAM. Therefore, it is feasible that when peptide/MHC complexes are rare, an increased stability of the T cell-APC interaction may result in the presence of accessory molecules that is not achieved in their absence. In this case, the overall effect may be translated into a higher level of TCR signaling over time. Alternatively, costimulating may purely compensate for the lack of TCR signaling. Recent observations from Valitutti et al. (29) and Viola and Lanzavecchia (30) suggested that a threshold number of TCRs needed to be engaged to produce a response in a T cell, and that this number was diminished approximately fivefold (from 8000 to 1500) if the interacting APC expressed B7. Thus, in our studies, it is possible that additional signals generated through both CD28 and LFA-1 may compensate for low level TCR signaling with limiting Ag, and produce a level of total signals that is equivalent to the level induced with higher doses of Ag presented without CD28 and LFA-1 engagement. The enhanced responses at low dose Ag were apparent regardless of the peptide ligand, with APC expressing multiple accessory molecules inducing CD25 and CD69 expression at 10- to 100-fold lower concentrations (Fig. 4).

Accessory molecules had a profound bearing on T cell activation by weak agonist peptides. APC with B7 and ICAM were able to efficiently induce early events of naive activation with all peptides tested as long as sufficient concentrations could be achieved (Fig. 2). The only exceptions were peptides that could actively antagonize responses of the naive peptides (e.g., T102L). In contrast, peptides that were very weak agonists on B7+ICAM+ APC were either unable to activate naive cells on B7+ICAM+ APC (as assessed by CD69/IL-2R expression), at least with the maximal amounts used here (e.g., K99A at 50 μM; Fig. 4), or only resulted in activation if at least 100-fold more peptide was available (e.g., K99E, Y97K; Fig. 4). The stimulatory capacity of the peptides is likely to be a function of the affinity of interaction between the peptide/MHC complex and the TCR, as each peptide was chosen for similar binding to MHC. Based on studies with soluble agonist peptide/MHC complexes binding to immobilized TCR, it has recently been proposed that the affinity of each complex is largely due to the rate of dissociation from the individual TCRs (31, 32). Therefore, it could be argued that accessory molecule-coreceptor engagement may compensate for the intrinsic fast dissociation rate of the weak agonist ligands and allow T cell activation. Thus, peptides such as K99A may have an off-rate that is too high, if presented in the context of minimal accessory help, to induce sufficient signals for the T cell to express surface activation markers such as IL-2R. The off-rate may not be altered unless co-signaling induces conformational changes in the TCR; however, the ability to associate may be increased due to enhanced T cell-APC avidity, which ultimately could result in greater densities of TCR/MHC complexes and overall increased TCR signaling. Another question is additionally raised, and that is whether expression of costimulatory accessory molecules has a bearing on the ability of a peptide to antagonize T cell activation. Antagonist peptides appear to function by competing with agonist/MHC complexes for binding to TCRs (20) and inducing incomplete intracellular signaling (22, 23, 26), the latter potentially being related to the high dissociation rates discussed above. In line with this concept, two recent studies showed that weak agonist peptides can become antagonists if CD4 is unable to bind MHC (33, 34), again suggesting that the overall avidity of TCR-MHC interaction and length of engagement is crucial for determining the outcome. Although CD4 is known as an accessory molecule, because of binding to MHC, it is not classically viewed as costimulatory although signaling through the molecule can obviously occur. Therefore, whether lack of engagement of conventional costimulatory accessory molecules such as B7 and ICAM can turn a weak agonist peptide such as K99A into an antagonist peptide is unclear. If B7 and ICAM are primarily important for delivering co-signals, this phenomenon may not be seen, whereas if they are equally important for stabilizing MHC/TCR engagement, antagonism may occur. These and other questions are currently being pursued.

As well as focussing on early phases of naive T cell activation, the studies presented here also highlight the stringent requirements of these cells for making large quantities of IL-2 and proliferating, and show that T cell activation per se can be largely dissociated from the production of IL-2. Thus, weak agonist peptides such as K99A and Y97K promoted efficient T cell activation at a high dose when presented on B7+ICAM+ APC (as assessed by CD69/IL-2R expression; Fig. 2), but were unable to induce IL-2 at the same levels as those seen with the native MCC peptide (Fig. 1). Using intracellular staining to detect IL-2-secreting cells, we also found a correlation between peptide dose and the number of IL-2-secreting cells (data not shown). A total of 100 (K99E) to 1000 (K99A) times more peptide was required to obtain an equivalent number of IL-2-secreting cells compared with agonist (MCC) peptide. Therefore, even in the presence of multiple accessory molecules and potentially high levels of costimulation, the ultimate outcome of Ag presentation was still dependent on the extent of TCR ligation. Similar conclusions were reached in our earlier studies of naive T cell activation using anti-CD3 to vary the magnitude of TCR signals (9), showing that IL-2 secretion is critically controlled by the balance between TCR signaling and co-signaling. This was also exemplified in the studies with the heteroclitic peptide, T102S. This peptide could partially compensate for the lack of B7 and ICAM on the APCs and produced an enhanced response compared with the weak proliferation and IL-2 secretion observed with native MCC, but could not promote responses of similar magnitude to those induced in the presence of B7 and ICAM (Fig. 5).

The final evidence that demonstrated that IL-2 production was the main facet of naive T cell activation that distinguished strong from weak agonists were the kinetic studies of proliferation (Figs. 3 and 5). High doses of weak agonists (K99A, Y97K) promoted similar levels of proliferation to native MCC at earlier times (day 2, and days 2 and 3, respectively), but were unable to sustain these responses, a phenomenon that directly correlated with the differences in the extent of IL-2 production observed. Similarly, the heteroclitic peptide (T102S) did not possess a greater ability to initiate early activation of the T cells compared with the native peptide (Figs. 2 and 4), regardless of APC, but did promote higher levels of IL-2 that resulted in greater proliferation at late times. We also obtained a similar pattern of responses using activated B cells rather than the fibroblast transfectants. Weak agonists such as K99A induced higher proliferative responses on day 2 vs day 3, whereas agonist or heteroclitic peptides induced higher proliferation on day 3. These studies therefore suggest that the magnitude of IL-2 induction is critical for determining the overall extent of naive responses. Such a conclusion is supported by a recent study of APLs and cloned T cells that suggested that the major difference between peptides that vary in their capacity to induce anergy is the
ability to promote IL-2 secretion rather than early intracellular signaling events (35).

In summary, the data in this report demonstrate that the ultimate outcome of naive T cell interaction with an APC is a function of both the affinity of interaction between peptide/MHC and the TCR, and the engagement of accessory molecules by T cell coreceptors. The data also indirectly support the contention that there is a hierarchy of steps during T cell activation that is governed by the overall level of signaling achieved, with early phases of naive response having less stringent requirements than later phases. Such a phenomenon was recently observed with a T cell clone using various altered peptides of MCC, with the conclusion that events such as \( \zeta \)-chain phosphorylation required a shorter duration of TCR ligation than Ca flux, which in turn was more easily triggered than proliferation (36). Although our studies cannot discriminate between a requirement for enhanced TCR ligation over time and the provision of costimulatory signals, they do highlight the fact that accessory molecule-coreceptor engagement plays a critical role in determining the stimulatory capacity of a peptide.

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References


