Potent T Cell Activation with Dimeric Peptide-Major Histocompatibility Complex Class II Ligand: The Role of CD4 Coreceptor

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Summary

The interaction of the T cell receptor (TCR) with its cognate peptide-major histocompatibility complex (MHC) on the surface of antigen presenting cells (APCs) is a primary event during T cell activation. Here we used a dimeric IEK-MCC molecule to study its capacity to activate antigen-specific T cells and to directly analyze the role of CD4 in physically stabilizing the TCR-MHC interaction. Dimeric IEK-MCC stably binds to specific T cells. In addition, immobilized dimeric IEK-MCC can induce TCR downregulation and activate antigen-specific T cells more efficiently than anti-CD3. The potency of the dimeric IEK-MCC is significantly enhanced in the presence of CD4. However, CD4 does not play any significant role in stabilizing peptide-MHC-TCR interactions as it fails to enhance binding of IEK-MCC to specific T cells or influence peptide-MHC-TCR dissociation rate or TCR downregulation. Moreover, these results indicate that dimerization of peptide-MHC class II using an IgG molecular scaffold significantly increases its binding avidity leading to an enhancement of its stimulatory capacity while maintaining the physiological properties of cognate peptide-MHC complex. These peptide-MHC-IgG chimeras may, therefore, provide a novel approach to modulate antigen-specific T cell responses both in vitro and in vivo.

Key words: T cell stimulation • CD4 coreceptor • major histocompatibility complex multimerization • T cell receptor downregulation
Association of the CD4 and CD8 coreceptors with engaged TCR represents an additional process that dramatically affects TCR signaling and T cell activation (2). Recruitment of the coreceptor to the occupied TCR complex allows cytoplasmically associated Lck to tyrosine phosphorylate CD3ε and/or ζ-associated proteins, thereby helping to initiate a kinase signaling cascade (11). Indirect evidence has implicated the coreceptors in modulating additional components of signal 1 generation including stabilization and/or multimerization of the peptide-MHC-TCR complex as well as TCR downregulation associated with serial TCR engagement (11–14). Defining the interaction between the coreceptors and these different parameters of TCR signaling will provide an enhanced ability to efficiently manipulate antigen-specific T cell responses.

Production of soluble MHC and TCR molecules has markedly increased our understanding of the basic mechanism of T cell recognition through crystallographic and biophysical studies (1). However, the low affinity of these molecules for cognate ligands has limited their usefulness in studying the interactions of the TCR with other surface molecules that act in concert to produce immune responses or in tracking antigen-specific T cells in vivo. To overcome the low-affinity problem, multivalent peptide-MHC class I ligands have been generated recently and have been used successfully for visualization of antigen-specific T cells in several different systems (15–17).

In this study we examined the capacity of a dimeric peptide-MHC class II-IgG chimera to bind to and activate cognate T cell lines. We found that, in contrast to monomeric peptide-MHC class II, the dimeric peptide-MHC class II-IgG complexes bound stably and specifically to cognate T cells. Further analyses showed that peptide-MHC class II-IgG was significantly more potent than anti-CD3 mAb in stimulating T cell activation (2). Recruitment of the coreceptor to the occupied TCR complex directly affects TCR signaling and T cell activation (2). Recruitment of the coreceptor to the occupied TCR complex directly affects TCR signaling and T cell activation (2).

**Materials and Methods**

**Mice.** C57BL/6 × SJL founder AND transgenic (Tg)2 mice, specific for moth cytochrome C peptide (MCC 91–103) in the context of IEα, backcrossed to B10.BR (H-2k) mice for six to eight generations, were bred and maintained in the animal care facility at the Cancer Center of the Johns Hopkins School of Medicine (18, 19).

**T Cell Hybridomas and IL-2 Production.** The two hybridomas used in this study were a generous gift of Drs. K. Appler and M. Rack (National Jewish Medical and Research Center, Denver, CO). 5KC73.8 is specific for MCC 91–103 in the context of IEα (20). DO 11.10 is specific for chicken ovalbumin peptide (cOVA 327–339) in the context of IAβ (21). Since SK C73.8 cells have a tendency to lose expression of the CD4 molecule, limiting dilutions were used to obtain a CD4-positive (No. 9) and a CD4-negative (No. 10) clone of the hybridoma. These two clones expressed similar levels of TCR.

For stimulation assays, wells of Immulon3 microtiter plates (Dynatech Laboratories Inc., Chantilly, VA) were coated with different proteins in PBS overnight at 4°C. Plates were then washed and coated with 10% FCS for 1 h. Wells coated with monomeric IEα-MCC, dimeric IEα-MCC (described below), or anti-CD3 mAb were then used to activate T cell hybridomas at a density of 5 × 104 cells per well. After overnight incubation at 37°C, IL-2 was measured using an ELISA minikit (Endogen, Cambridge, MA).

**Production of Soluble Dimeric MHC Class II IEα-MCC Protein.** Soluble monomeric IEα-covalently linked to the MCC 91–103 peptide was produced and purified as described before (22). Dimeric IEα-MCC was produced by inserting the cDNA encoding the extracellular domains of the IEα and β chain upstream of the IgG light and heavy chains, respectively. A Kpn1 restriction site and a linker were inserted immediately 3' to the interface between the extracellular and transmembrane domains of IEβ polypeptide. The 5' regions of the gene had been modified already to encode the MCC peptide and an EcoR1 restriction site. The IEα chain was modified by introduction of a HindIII restriction site immediately 3' to the codon at the interface between the extracellular and transmembrane domains. Soluble proteins were produced in a baculovirus expression system and purified on 14.4.4.5 (anti-IEα) affinity column as described previously (22).

**Antibodies and Flow Cytometric Analysis.** The following antibodies were used in this study: H597 and 2C11 mAbs specific for murine TCR Cβ chain and CD3ε, respectively, were produced following standard techniques. Fluoresceinated RM4-5 specific for mouse CD4 and biotin-A85-1 specific for murine IgG1 were purchased from Pharmingen (San Diego, CA) whereas PE-labeled goat anti–mouse IgG1 was purchased from Southern Biotech, Association, Inc. (Birmingham, AL). The IgG light and heavy chains, respectively, were produced by recombinant techniques. Fluoresceinated RM4-5 specific for mouse CD4 and biotin-A85-1 specific for murine IgG1 were purchased from Pharmingen (San Diego, CA) whereas PE-labeled goat anti–mouse IgG1 was purchased from Southern Biotech, Association, Inc. (Birmingham, AL). The IgG light and heavy chains, respectively, were produced by recombinant techniques. Fluoresceinated RM4-5 specific for mouse CD4 and biotin-A85-1 specific for murine IgG1 were purchased from Pharmingen (San Diego, CA) whereas PE-labeled goat anti–mouse IgG1 was purchased from Southern Biotech, Association, Inc. (Birmingham, AL). The IgG light and heavy chains, respectively, were produced by recombinant techniques. Fluoresceinated RM4-5 specific for mouse CD4 and biotin-A85-1 specific for murine IgG1 were purchased from Pharmingen (San Diego, CA) whereas PE-labeled goat anti–mouse IgG1 was purchased from Southern Biotech, Association, Inc. (Birmingham, AL). The IgG light and heavy chains, respectively, were produced by recombinant techniques.

**In Vitro Proliferation Assays.** Single cell suspensions from spleens of AND × B10. BR Tg mice were harvested and cultured at 2.5 × 105 in 96-well Immulon4 microtiter plates that contained various concentrations of immobilized monomeric IEα-MCC, dimeric IEα-MCC, or anti-CD3. Cultures were incubated in 200 μl of Cymte (complete tissue culture medium) at 37°C for 72 h and

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1Abbreviation used in this paper: Tg, transgenic.
then pulsed with 1 μCi of ³H-TdR for 6-12 h. Cultures were then harvested and ³H-TdR uptake was determined by scintillation counting. Results are expressed as the mean of triplicate cultures (background subtracted).

Measurement of TCR downregulation. The CD4-positive and CD4-negative clones of the 5KC73.8 T cell hybridoma were incubated with varying concentrations of immobilized monomeric IE<sup>k</sup>-MCC, dimeric IE<sup>k</sup>-MCC, or anti-CD3 in CTM for 5 h at 37°C. Control cells were incubated in parallel cultures in PBS. All cultures were then recovered and stained, as described above, with anti-β-mAb H597 to determine the level of TCRs that remained on the cell surface after stimulation. Results were expressed as percentages of TCRs on surfaces of the PBS-stimulated control clones.

Effect of CD4 on Binding of IE<sup>k</sup>-MCC Dimer to 5KC73.8 Clones. Various concentrations of IE<sup>k</sup>-MCC dimer were incubated with 2 x 10<sup>5</sup> cells on ice for 2 h. Cells were washed and bound dimer was detected by PE-labeled goat anti-mouse IgG1 antibodies. As a control, tested clones were stained with anti-TCR (H597) and anti-CD4 (RM4-5) to confirm the level of TCR and CD4 at the time of each experiment.

To study the effect of CD4 on dissociation of the dimeric IE<sup>k</sup>-MCC from 5KC73.8 cells, various concentrations of soluble dimer were incubated with the CD4-positive (No. 9) and the CD4-negative (No. 10) clones for 2 h on ice. All cultures were then placed at 4°C, washed once, and resuspended in 180 μl of staining buffer for the indicated period of time at 4°C. Cells were immediately washed at 4°C and the bound dimer was detected using PE-conjugated goat anti-murine IgG1, as described above.

Results

Stable and Specific Binding of Dimeric IE<sup>k</sup>-MCC to Specific T Cells. We used the well characterized I-E<sup>k</sup>-restricted MHC system to study T cell activation by dimeric peptide-MHC complexes. Using IgG as a molecular scaffold we expressed a soluble IE<sup>k</sup>-MCC dimer. For the production of this chimeric molecule, two chimeric genes were constructed and introduced into a baculovirus transfer vector. The first chimeric gene linked DNA encoding the extracellular domains of IE<sup>k</sup> α gene to the 5′ end of a gene encoding the mature Ig light chain. The second chimeric gene linked DNA encoding the extracellular domains of IE<sup>k</sup> β gene to the 5′ end of a gene encoding the mature Ig heavy chain. The 5′ sequences of the β chain had been altered already by the addition of DNA sequences encoding the antigenic peptide MCC 91–103 via an alanine/glycine encoded linker (22). The constructs were cloned into the dual promoter baculovirus expression vector and used to infect Sf9 cells. Infected cells produced ~0.5 mg of chimeric protein per liter of culture. A monomeric IE<sup>k</sup>-MCC, which utilized the same MHC 91–103 peptide and a linker sequence as the dimeric IE<sup>k</sup>-MCC, was used for comparative purposes (22).

We first sought to determine whether dimeric IE<sup>k</sup>-MCC would bind stably and specifically to MCC-specific T cells. For these purposes, we used the IE<sup>k</sup> + MCC-specific T cell hybridoma, 5KC73.8, and, as a specificity control, the IA<sup>α</sup> + OVA-specific T cell hybridoma, DO 11.10. Analysis by flow cytometry showed significant binding of dimeric IE<sup>k</sup>-MCC to 5KC73.8 T cell hybridoma. No binding of the dimeric IE<sup>k</sup>-MCC to control DO 11.10 T cell hybridoma was detected although it expressed a high level of TCR, comparable to those expressed by the 5KC73.8 T cell hybridoma (Fig. 1, a and b). Not surprisingly, there was no stable binding of monomeric IE<sup>k</sup>-MCC to either 5KC73.8 or to DO 11.10 T cell hybridomas at all doses tested (data not shown). Therefore, dimerization of IE<sup>k</sup>-MCC on the IgG scaffold augmented affinity significantly, allowing for binding to cognate T cells that was detectable by flow cytometry.

Dimeric IE<sup>k</sup>-MCC Is a Potent T Cell Stimulatory Molecule. We next compared the ability of monomeric IE<sup>k</sup>-MCC, dimeric IE<sup>k</sup>-MCC, and anti-CD3 to activate MCC-specific T cells. To assay T cell stimulation, the different molecules were coated at varying concentrations onto wells of Immulon<sup>4</sup> plates and used to stimulate IL-2 release by the MCC-specific T cell hybridoma. Both monomeric IE<sup>k</sup>-MCC and dimeric IE<sup>k</sup>-MCC stimulated specific IL-2 release by 5KC73.8 T cells, but with considerably different potency (Fig. 2). Dimeric IE<sup>k</sup>-MCC was significantly better at stimulating the 5KC73.8 T cell hybridoma than monomeric IE<sup>k</sup>-MCC. At least 40-fold, or more, immobilized monomeric IE<sup>k</sup>-MCC was required to achieve a similar response as that elicited by the dimer. Interestingly, dimeric IE<sup>k</sup>-MCC was even more potent than divalent anti-CD3 mAb, by ~5–10-fold. As a negative control, we showed that DO 11.10 T cell hybridoma responded to anti-CD3 mAb but not to either monomeric IE<sup>k</sup>-MCC or dimeric IE<sup>k</sup>-MCC to 5KC73.8 T cell hybridoma. No binding of the dimeric IE<sup>k</sup>-MCC to control DO 11.10 T cell hybridoma was detected although it expressed a high level of TCR, comparable to those expressed by the 5KC73.8 T cell hybridoma (Fig. 1, a and b). Not surprisingly, there was no stable binding of monomeric IE<sup>k</sup>-MCC to either 5KC73.8 or to DO 11.10 T cell hybridomas at all doses tested (data not shown). Therefore, dimerization of IE<sup>k</sup>-MCC on the IgG scaffold augmented affinity significantly, allowing for binding to cognate T cells that was detectable by flow cytometry.

![Figure 1. Specific binding of the dimeric IE<sup>k</sup>-MCC to 5KC73.8 T cell hybridoma. (a) Binding of the IE<sup>k</sup>-MCC dimer to the 5KC73.8 T cell hybridoma but not to the negative control, DO 11.10 T cell hybridoma. Cells were incubated with 100 μl of 100 μg/ml of the dimer for 2 h on ice. Cells were then washed, and bound dimer was detected using biotin-H597 followed by PE-conjugated streptavidin. (b) Both 5KC37.8 and DO 11.10 T cell hybridomas express high levels of TCR. Cells were stained with a saturating amount of biotin-H597 for 2 h on ice and bound antibody was detected with PE-streptavidin conjugate.](image-url)
**IEk-MCC**, confirming the specificity of these molecules for their cognate TCR.

The stimulatory capacity of the dimeric IEk-MCC was also evident using naive untransformed T cells from MCC-specific AND transgenic mice (Fig. 3a). Under these conditions, immobilized monomeric IEk-MCC was the least stimulatory for T cells, while dimeric IEk-MCC was roughly 10-fold more stimulatory than anti-CD3. No significant activation of splenocytes from naive B10.BR mice was observed using monomeric or dimeric IEk-MCC, indicating the response was antigen specific (data not shown). An additional advantage of the dimeric IEk-MCC molecules is their ability to bind through the Fc portion of the IgG to FcR+ splenocytes and activate specific T cells. As expected, the soluble dimer added to splenocytes from AND × B10.BR Tg mice elicited strong IL-2 response, comparable to that induced by anti-CD3, whereas a small amount of IL-2 was produced in response to the soluble monomer (Fig. 3b).

Recent experiments indicate that T cell response correlates in magnitude and kinetics with the downregulation of the TCR (10, 23). To further compare the physiological properties of monomeric IEk-MCC, dimeric IEk-MCC, and anti-CD3, the capacity of immobilized forms of these complexes to induce TCR downregulation of the CD4-positive clone of 5KC73.8 T cell hybridoma was tested. We found that dimeric IEk-MCC was more efficient than anti-CD3 in triggering TCR downregulation (Fig. 4a). However, we did not observe any significant downregulation of TCRs in response to the immobilized monomer at concentrations sufficient to induce IL-2 production by 5KC73.8 T cell hybridoma. Whether failure of monomeric IEk-MCC to induce TCR downregulation is due to reduced sensitivity of the assay or multimerization is needed for this process requires further investigation. On the other hand, only anti-CD3, but not monomeric IEk-MCC or dimeric IEk-MCC, was able to effectively induce TCR downregulation of the specificity control DO11.10 T cell hybridoma (Fig. 4b). Thus, the enhanced stimulatory capacity of dimeric IEk-MCC correlates very well with its ability to downregulate TCR associated with serial triggering.

**The Enhanced T Cell Stimulation by Dimeric IEk-MCC Is Partly Mediated by the CD4 Molecule.** Engagement of CD4 by the MHC class II molecules is necessary for optimum T cell response to specific antigens. If this is the reason behind the enhanced potency of IEk-MCC relative to anti-CD3, then responses of the 5KC73.8 T cell hybridoma to dimeric IEk-MCC, but not to anti-CD3, should significantly diminish when the CD4 effect is removed. To test
this hypothesis, CD4-positive (No. 9) and CD4-negative (No. 10) clones of the 5KC73.8 T cell hybridoma that expressed identical levels of TCRs were isolated (Fig. 5a). Dose-response curves of these clones to all immobilized antigens (monomeric IE\(^{k}\)-MCC, dimeric IE\(^{k}\)-MCC, and anti-CD3) were significantly shifted to the right in the absence of CD4, indicating that the 5KC73.8 T cell hybridoma response to MHC antigen is CD4 dependent (Fig. 5b). Interestingly, dimeric IE\(^{k}\)-MCC was more potent than anti-CD3 only when used to stimulate the CD4-positive clone. However, when the CD4-negative clone was stimulated, the dimeric IE\(^{k}\)-MCC and anti-CD3 elicited equivalent responses. This result also indicates that dimerization of MHC class II by IgG scaffold does not interfere with CD4/MHC binding as manifested by the major impact of CD4 on the ability of dimeric IE\(^{k}\)-MCC, but not anti-CD3, to activate specific T cells. Thus, it appears that the enhanced stimulatory capacity of dimeric IE\(^{k}\)-MCC, relative to anti-CD3, depends on its ability to recruit the CD4 coreceptor to engaged TCR complexes.

### CD4 Does Not Stabilize TCR–peptide–MHC Complex Interactions or Alter TCR Downregulation.

The ability to directly measure binding of dimeric IE\(^{k}\)-MCC to the T cell surface, as well as the finding that 5KC73.8 T cell hybridoma activation is CD4 dependent, allowed us to examine the role of CD4 in stabilizing TCR–MHC class II–peptide binding. A potential adhesion role for CD4 has been derived from indirect functional data and from cell–cell adhesion assays using B cells expressing high levels of MHC class II and fibroblasts transfected with supraphysiological levels of CD4 (12, 13, 24). In addition, some structural models have implicated the CD4 molecule in binding and multimerizing TCR–MHC–peptide complexes (7).

To determine if CD4 can enhance binding of soluble dimeric IE\(^{k}\)-MCC to 5KC73.8 cells, we compared binding of various concentrations of dimeric IE\(^{k}\)-MCC to the CD4-positive and CD4-negative 5KC73.8 clones. The binding of IE\(^{k}\)-MCC to both the CD4-positive and CD4-negative 5KC73.8 clones was virtually identical at all concentrations tested (Fig. 6a). The binding specificity was confirmed by the failure of the dimer to bind to the negative control, DO 11.10 T cell hybridoma. Similar results were obtained with cells stained with dimeric IE\(^{k}\)-MCC at 37°C (data not shown). Consistent with this binding data, we did not see any significant difference in the rate of dissociation of the dimer from the CD4-positive and CD4-negative clones of 5KC73.8 (Fig. 6b). These results demonstrated that CD4 does not play any significant role in physically stabilizing TCR–MHC–peptide complexes even though it is required for optimum T cell activation.

We next examined the influence of the CD4 molecule on TCR downregulation, a phenomenon that has been used recently to measure T cell activation (9). We reasoned that if CD4 binding to TCR–peptide–MHC complex is required, then removal of CD4 should delay or inhibit antigen-induced TCR downregulation. Therefore, the ability of dimeric IE\(^{k}\)-MCC to induce TCR downregulation in the presence or absence of the CD4 molecule was determined. The results in Fig. 7 showed that both the CD4-positive and CD4-negative 5KC73.8 clones downregulated their TCR to the same extent, regardless of the level of CD4 expression. We conclude that antigen-induced TCR downregulation, at least in this system, can occur in a CD4-independent manner.

### Discussion

In this study, a dimeric peptide–MHC class II molecule was made, its capacity to bind and activate antigen-specific T cells was analyzed, and then it was used to directly examine the adhesion role of the CD4 coreceptor. We demonstrated that dimeric IE\(^{k}\)-MCC could stably bind to cognate T cells and efficiently stimulate them due to its ability to cross-link TCR, engage CD4 molecule, and induce serial reaction.

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**Figure 5.** (a) Isolation of CD4-positive and CD4-negative clones of 5KC73.8 T cell hybridoma. 5KC73.8 T cell hybridoma was cloned by limiting dilution. A CD4-positive (No. 9) and a CD4-negative (No. 10) clone of 5KC73.8 T cell hybridoma that expressed almost identical levels of TCRs were selected. (b) CD4 expression significantly enhances the response of 5KC73.8 T cell hybridoma to dimeric IE\(^{k}\)-MCC but not to anti-CD3. IL-2 produced by the CD4-positive clone No. 9 (top) and the CD4-negative clone No. 10 (bottom) incubated with various concentrations of immobilized monomeric IE\(^{k}\)-MCC, dimeric IE\(^{k}\)-MCC, or anti-CD3 was measured as described in the legend for Fig. 2. Monomeric IE\(^{k}\)-MCC elicited an IL-2 response from only the CD4-positive but not the CD4-negative clone, whereas dimeric IE\(^{k}\)-MCC induced IL-2 responses by both the CD4-positive and CD4-negative clones. However, dimeric IE\(^{k}\)-MCC was ∼10-fold better than anti-CD3 at stimulating the CD4-positive clone. Results are expressed as the mean of duplicate cultures. A representative of three experiments with similar results is shown.
TCR triggering. In addition, we found that CD4 did not play a significant adhesion function as it failed to enhance binding of IEk-MCC to specific T cell hybridoma, influence MHC-TCR dissociation rate, or influence TCR downregulation.

The MCC system has been used widely to study peptide-MHC class II interactions with specific T cells. Numerous T cell hybridomas that recognize MCC in the context of IEk have been made and affinity of their soluble TCRs for IEk-MCC has been measured. Generally, the affinity of specific TCRs for IEk-MCC complex is weak, with $K_d$ in between 30 and 90 μM (1, 25). This low affinity makes it difficult to study and visualize binding of soluble peptide-MHC class II complexes to specific T cells. Dimerization of IEk-MCC by the immunoglobulin scaffold significantly increased the avidity of the complex resulting in stable binding detectable by flow cytometry. Affinity measurements of dimeric MHC class II complexes with the TCRs on the T cell surface has been derived from indirect functional data and cell-cell adhesion assays (12, 13, 24). In this report we showed that stable binding of the dimer to specific T cells was independent of CD4 expression. Furthermore, the dissociation of peptide-MHC class II from T cell is CD4 independent. The fact that the response of 5KC73.8 to IEk-MCC is CD4 dependent, especially at low antigen dose, and the generally low affinity of T cells for MCC antigen provide stringent conditions that should have allowed us to unmask any significant adhesion property of the CD4 molecule.

Our observation that TCR downregulation induced by dimeric IEk-MCC is CD4 independent contrasts with the result of Viola et al., who found that peptide-MHC-mediated TCR downregulation was inhibited by the addition of antibodies against CD4 (14). However, interpretation of the anticoreceptor antibody blocking studies is probably complicated by the potential direct effect of the antibody-mediated coreceptor cross-linking. In addition, in the same study the authors found that inhibitors of p56ilk kinase blocked T cell activation but not TCR downregulation, indicating that TCR downregulation can be dissociated from T cell activation. This latter result is consistent with our observation that lack of CD4 expression significantly impaired the ability of 5KC73.8 T cell hybridoma to produce IL-2 (Fig. 5 b), but not to downregulate its TCR (Fig. 7).

Several previous reports have concluded indirectly that CD4 has a minimal adhesion effect. For example, Weber...
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