THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 30, Issue of July 27, pp. 28068–28074, 2001

Printed in U.S.A.

Receptor Proximity, Not Intermolecular Orientation, Is Critical for Triggering T-cell Activation*

Received for publication, April 12, 2001, and in revised form, May 18, 2001
Published, JBC Papers in Press, May 30, 2001, DOI 10.1074/jbc.M103280200

Jennifer R. Cochran, Thomas O. Cameron, Jennifer D. Stone, Jodi B. Lubetsky‡, and Lawrence J. Stern§

From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Engagement of antigen receptors on the surface of T-cells with peptides bound to major histocompatibility complex (MHC) proteins triggers T-cell activation in a mechanism involving receptor oligomerization. Receptor dimerization by soluble MHC oligomers is sufficient to induce several characteristic activation processes in T-cells including internalization of engaged receptors and up-regulation of cell surface proteins. In this work, the influence of intermolecular orientation within the activating receptor dimer was studied. Dimers of class II MHC proteins coupled in a variety of orientations and topologies each were able to activate $CD4^+$ T-cells, indicating that triggering was not dependent on a particular receptor orientation. In contrast to the minimal influence of receptor orientation, T-cell triggering was affected by the inter-molecular distance between MHC molecules, and MHC dimers coupled through shorter cross-linkers were consistently more potent than those coupled through longer cross-linkers. These results are consistent with a mechanism in which intermolecular receptor proximity, but not intermolecular orientation, is the key determinant for antigen-induced $CD4^+$ T-cell activation.

Helper ($CD4^+$) T-cells play a key role in the adaptive immune response, by detecting and responding to foreign antigens bound to class II major histocompatibility complex (MHC)[1] proteins found on the surface of B cells, macrophages, and other specialized antigen presenting cells of the immune system (1). T-cells express on their surface clonotypic antigen receptors (TCR), which bind complexes of MHC protein and specific antigenic peptides (2). TCR engagement by MHC-peptide complexes induces T-cell signaling cascades. Once triggered, helper T-cells elicit a variety of characteristic processes, including cytokine secretion, up-regulation of adhesion and costimulatory molecules, and T-cell proliferation, which leads to activation of effector functions in antigen presenting cells, recruitment of other immune cells, and eventually to clearance of the foreign material (3–5). Some characteristic T-cell activation responses can be triggered by soluble agents that multivalently engage TCR, including anti-TCR antibodies (6, 7) and oligomeric MHC-peptide complexes (reviewed in Ref. 8). Monomeric reagents generally are not able to induce a response in T-cells, although some exceptions have been reported, particularly for $CD8^+$ T-cells (7, 9). These considerations have led to the understanding that T-cells can be triggered by oligomerization of their surface TCR, although the full cellular activation response requires additional (co-stimulatory) signals from the antigen-presenting cell in addition to the antigenic signal transduced by the TCR (10).

Several studies have pointed to formation of a TCR dimer as the key event for triggering T-cell activation. Quantitative analyses of the dependence of T-cell activation on the surface density of MHC-peptide complexes presented on the surface antigen presenting cells (11, 12) or incorporated into planar lipid bilayers (13), have suggested a crucial role of dimer formation in triggering a T-cell response. For soluble class II MHC oligomers, the minimal MHC valency required to initiate signaling in $CD4^+$ T-cells appears to be a dimer (14, 15). Higher valency MHC oligomers can activate more potently than dimers, but this is due only to their increased binding avidity (15).

The mechanism by which dimerization of TCR triggers cytoplasmic signaling cascades is unknown. Receptor dimerization as the proximal activating stimulus is consistent with several potential molecular mechanisms, including formation of a specific TCR dimer in an activating conformation (as observed for receptor tyrosine kinases (16)), molecular rearrangement of a pre-existing receptor dimer (as observed for the bacterial aspartate receptor (17)), or nonspecific co-localization of receptor cytoplasmic domains (18). Experimental discrimination between these potential mechanisms can be difficult, and recent evidence has suggested that some systems originally thought to occur through a mechanism of receptor oligomerization may in fact involve allosteric rearrangements in a pre-existing receptor oligomer (reviewed in Ref. 19). In support of a nonspecific dimerization model, T-cell activation has been induced by many different anti-receptor antibodies (6, 7), and by oligomerization of a variety of chimeric TCR cytoplasmic domains (20–22). The exact stoichiometry of the unliganded form of the TCR complex is unknown, but has been proposed to contain two $\alpha\beta$ TCRs (23–25), raising the possibility that molecular rearrangement of a pre-existing receptor oligomer may be a potential activation mechanism. However, it is possible that the presence of two antigen-binding domains could serve only to facilitate the large-order clustering and oligomerization of receptors on the cell-surface that has been observed physiologically (26).

To investigate these possible mechanisms, dimeric MHC-peptide complexes were prepared in a variety of intermolecular

* This work was supported by National Institutes of Health Grants N01AI95361 (to L. J. S.) and T32 GM08334 (to J. R. C., T. O. C., and J. D. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Current address: Dept. of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06527.

§ To whom correspondence should be addressed: Dept. of Chemistry, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-253-2849; Fax: 617-258-7847; E-mail: stern@mit.edu.

[1] The abbreviations used are: MHC, major histocompatibility complex; TCR, T-cell receptor; R_S, Stokes radius; PAGE, polyacrylamide gel electrophoresis; IL, interleukin; PE, phycoerythrin; SA, streptavidin.

orientations and topologies, and used to probe the effects of receptor orientation and topology on T-cell triggering. Receptor orientation was not critical for T-cell signaling, as a variety of conformationally constrained MHC dimers coupled through either the α- or β-subunit each were able to induce T-cell activation processes. However, efficient T-cell triggering was dependent on receptor proximity, as activation was diminished when MHC dimers were coupled through longer cross-links. Collectively, these results suggest that T-cells are triggered by a mechanism of generalized intermolecular receptor proximity that does not depend on the intermolecular receptor orientation.

EXPERIMENTAL PROCEDURES

HLA-DR1 Expression and Folding—HLA-DR1 (A*0101, B1*0101) α- and β-subunits, or modified versions carrying a C-terminal cysteine residue ($_{Cys}\alpha$,β_{Cys}) (27), were expressed as insoluble inclusion bodies in *E. coli* BL21(DE3) cells as described (28). Subunits included the peptide binding and membrane-proximal immunoglobulin domains (α1–182 and β1–190). In some experiments a longer version of the β-subunit ($\beta_{L\text{-}Cys}$) was used, which included also the "connecting peptide" β191–198 (RSESAQSK). Recombinant MHC-peptide complexes were folded by dilution of urea-solubilized subunits in the presence of peptide and redox buffers, and isolated by ion-exchange chromatography, as previously described (28). HLA-DR1 complexes carried antigenic peptide Ha (resiudes 306–318) (PKYVKQNTLKLAT), derived from influenza virus hemagglutinin (29), or control endogenous peptide A2 (residues 103–117) (VGSDWRFLRGYHQYA), derived from class I MHC HLA-A2 (30). HLA-DR1 used in preparation of MHC dimers carried a cysteine residue at either the α or β C terminus. To prevent oxidation of the introduced cysteine residues, MHC-peptide complexes containing introduced cysteines were purified in 5 mM dithiothreitol, which was removed immediately prior to cross-linking. The introduced cysteines undergo facile reaction with thiol-specific reagents, allowing specific cross-linking at the α- or β-subunit termini.

Synthesis of Cross-linking Reagents—Polypeptide-based cross-linkers were synthesized by Fmoc chemistry on a solid-phase peptide synthesizer as previously described (27) and verified by matrix-assisted laser desorption ionization-time of flight mass spectrometry. All cross-linker peptides were capped at their N termini by reaction with fluorescein isothiocyanate. To introduce thiol-reactive maleimide groups, purified peptides (2–5 mg) were reacted through their lysine ϵ-amino groups with *N*-(ϵ-maleimidocaproyloxy)succinimide ester (Pierce), by dissolving 5-fold molar excess in *N*,*N*-dimethylformamide and adding it to peptide in 10 mM Na-phosphate buffer (pH 7), 150 mM NaCl. After 1.5 h at room temperature, the modified peptides were purified by reverse phase high performance liquid chromatography using a C18 column (Vydac), and the presence of both maleimide functional groups was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

Dimerization of MHC-Peptide Complexes—For direct disulfide bond formation between the introduced thiols, 0.25 mM $CuSO_4$ and 1.3 mM 1,10-phenanthroline (Sigma-Aldrich) were added to MHC-peptide complexes in 50 mM HEPES, (pH 8) (31), for at least 1 h at room temperature. For dimerization through the thiol-reactive maleimide groups on the synthetic peptide-based cross-linkers X3X, X9X, and X14X (Fig. 1*A*), the cross-linker was added in small batches to MHC-peptide complexes in 10 mM Na-phosphate (pH 7), 150 mM NaCl, 5 mM EDTA over ~5 h at room temperature, to a final cross-linker:MHC ratio of 1:2. Cross-linked MHC-peptide complexes were isolated by gel filtration chromatography on Superdex-200, using two HR 10/30 FPLC columns (Amersham Pharmacia Biotech) linked in series. The integrity of covalent thiol linkages and the presence of bound antigenic peptide were confirmed by SDS-PAGE (12.5%). Maleimide-to-maleimide distances for cross-linkers in extended conformations were estimated using molecular models.

Hydrodynamic studies of MHC-Peptide Dimers—Apparent M_r ($M_{r,app}$) values for MHC-peptide dimers were determined from elution volumes obtained by gel filtration chromatography, by reference to a calibration curve obtained from the elution volume of known M_r standards (Bio-Rad). The Stokes radii (R_S) of the MHC dimers were derived from the $M_{r,app}$ as previously described (32), except the following equation was used to calculate the hydrated volume (V_H),

$$V_H = \frac{M_{r,\,app}(\mathrm{psv} + (\mathrm{hyd}_{protein})(\rho_{water}))}{N} \qquad \text{(Eq. 1)}$$

where $hyd_{protein}$ is the estimated extent of hydration in the protein (0.35 g of water/g of protein), ρ_{water} is the density of water at 20 °C (0.998 g/cm^3), N is Avogadro's number, and psv is the partial specific volume of HLA-DR1-Ha (0.738 cm^3/g) calculated from the amino acid composition. Confidence intervals ($\pm\sigma$) reported in Table I reflect the standard deviation from the mean of replicate samples in separate experiments.

T-cell Lines—HA1.7 (33) is a well studied human CD4$^+$ T-cell clone that responds to the Ha peptide bound to HLA-DR1. The HLA-DR1-restricted, Ha peptide-specific polyclonal T-cell line (designated 1H) was raised from the mononuclear cell fraction of peripheral blood from an HLA-DR1 homozygous individual, by repeated *in vitro* stimulation with Ha peptide. HA1.7 and 1H were maintained in RPMI containing 5% human AB serum (Sigma-Aldrich) and 5% fetal bovine serum (Sigma-Aldrich), with antigenic stimulation every 2 weeks using 120 IU/ml IL-2 (BIOSOURCE) and an irradiated mixture of nonspecific peripheral blood lymphocytes and EBV1.24, a DR1$^+$ B-cell line, pulsed with 1 μM Ha peptide. T-cells were rested for a minimum of 7 days after stimulation before use in activation and binding assays.

T-cell Activation Assays—Soluble MHC-peptide complexes were added to 5×10^4 T-cells in complete medium in round-bottom polypropylene 96-well plates. After the desired incubation time at 37 °C and 7% CO_2, cells were placed on ice and levels of cell surface markers were determined using the following fluorescent monoclonal antibodies: phycoerythrin (PE)-labeled anti-CD3 (clone UCHT-1, Pharmingen), or allophycocyanin (APC)-labeled anti-CD25 (M-A251, Pharmingen), APC-anti-CD69 (FN50, Pharmingen), and APC-anti-CD71 (T56/14, Leinco Technologies). After 1 h at 4 °C, cells were washed with phosphate-buffered saline containing 1% fetal bovine serum and 0.1% sodium azide, and analyzed by flow cytometry. Fluorescence data were obtained with a Becton-Dickinson FACS Calibur flow cytometer and analyzed using Cell Quest software. The number of CD3 molecules per cell was determined from the mean PE fluorescence using SPHERO rainbow calibration particles (Spherotech) containing known amounts of PE equivalents. Experiment to experiment variation was observed in the overall time course and extent of activation, which appeared to be dependent on the length of time the T-cells had been in culture, but relative levels of activation induced by the various dimers were the same in different experiments.

Competitive Binding Assays—Competitive binding assays were performed as previously described (15, 27). Phycoerythrin-labeled, streptavidin-coupled MHC oligomers (SA-PE), prepared by binding biotinylated MHC monomers to streptavidin preparations (34), were used as a binding probe, with competition by unlabeled or fluorescein-labeled MHC-peptide oligomers. These SA-PE oligomers exhibit a strong fluorescence from the R-phycoerythrin conjugate and bind to T-cells in an antigen-specific manner (34). Various concentrations of MHC dimers and monomer were incubated with a constant amount of SA-PE oligomer ([MHC]= 4 μg/ml) and 5×10^4 HA1.7 T-cells in 96-well round-bottomed plates for 3 h at 37 °C, 7% CO_2. Cells were washed and fluorescence arising from bound SA-PE oligomer was measured by flow cytometry as described above.

RESULTS

MHC-Peptide Dimers—To investigate the orientation requirements of T-cell triggering, we prepared soluble dimers of the human class II MHC protein HLA-DR1, with a variety of different intermolecular orientations and conformational constraints. The oligomerization strategy involved specific cross-linking at the sulfhydryl moiety of a cysteine residue introduced either at the C terminus of the α-subunit immunoglobulin domain (α182), the C terminus of the β-subunit immunoglobulin domain (β190), or after the β-subunit connecting peptide region (β191–198), which immediately precedes the native transmembrane domain (Fig. 1*B*). Proteins carrying the introduced cysteine residues ($_{Cys}\alpha\beta$, $\alpha\beta_{Cys}$, or $\alpha\beta_{L\text{-}Cys}$, respectively) were produced by *in vitro* folding in the presence of antigenic (Ha) or endogenous (A2) peptides, using previously described protocols (27, 28). MHC-peptide complexes were dimerized either by using a direct disulfide bond between the introduced cysteine thiols (31), or using a sulfhydryl-reactive synthetic cross-linking reagent of varying length (Fig. 1*A*). The synthetic cross-linkers X3X, X9X, and X14X are based on a flexible peptide scaffold containing glycine, serine, and glutamic acid residues, and each carry an N-terminal fluorophore and two maleimidylcaproyl (X) groups, attached as amides to lysine residues. MHC-peptide dimers coupled by direct disul-

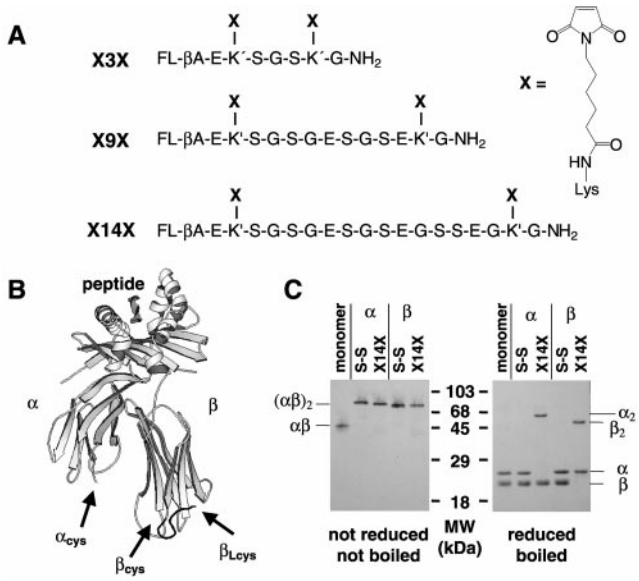

FIG. 1. **Cross-linking strategy for formation of dimeric MHC-peptide complexes.** *A,* synthetic peptide-based cross-linking reagents used to make dimeric MHC-peptide complexes. All cross-linkers carry a fluoresceinyl-β-alanine (*FL-βA*) at the N terminus, and two maleimide functional groups for coupling to HLA-DR1 cysteine residues. Estimated maleimide to maleimide distances for these cross-linkers in fully extended conformations are 50 Å (X3X), 70 Å (X9X), and 90 Å (X14X). (For comparison, a disulfide-linked bond would have a length of ~2 Å.) *B,* ribbon diagram of the HLA-DR1-peptide complex (67), showing the positions of introduced cysteine residues (*arrows*). *C,* analysis of MHC-peptide dimers by SDS-PAGE. Cross-linked MHC-peptide dimers form the expected disulfide or covalent bond through either the alpha (α) or beta (β) subunit. *Left panel*, nonreducing SDS-PAGE (12.5%) of purified MHC-peptide dimers with samples not boiled before loading; the αβ peptide complex stays associated in the absence of boiling (35), indicating that cross-linking did not interfere with peptide binding. *Right panel*, Reducing SDS-PAGE of boiled samples.

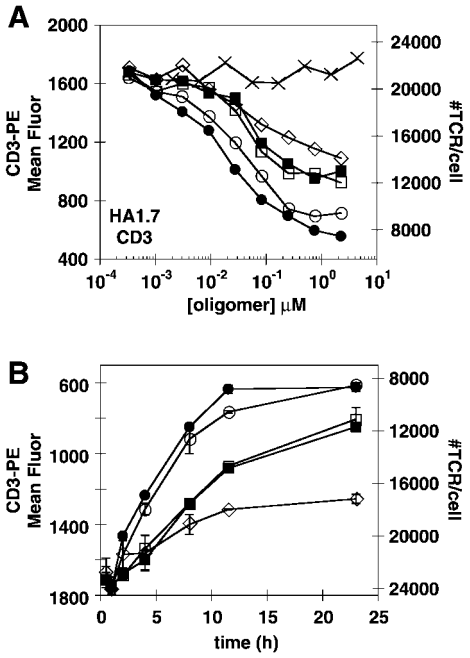

FIG. 2. **Receptor orientation is not crucial for CD4+ T-cell triggering.** *A,* dose dependence of HA1.7 T-cell triggering induced by MHC dimers as assayed by CD3/TCR down-regulation after 20 h incubation. *B,* time course of CD3 down-regulation induced with 0.16 μM of MHC dimer. Dimers linked with direct disulfide bonds through the α-subunit (*closed circles*) or β-subunit (*open circles*) induce similar levels of T-cell activation. Dimers coupled with the X14X cross-linker through the α-subunit (*closed squares*), β-subunit (*open squares*), or β-subunit containing the connecting peptide region (*open diamonds*) are less efficient at triggering activation than dimers linked through disulfide bonds. Monomeric MHC-peptide complexes (×) do not trigger T-cell activation. The *left axis* indicates mean PE-fluorescence measured by flow cytometry for antibody against the CD3ε subunit, the *right axis* the corresponding number of TCR remaining on the cell surface. Data are representative of several experiments performed on HA1.7 T-cells.

fide bonds (S-S) or by the synthetic cross-linkers, through either the α- or β-subunit, each had the desired covalent linkage (Fig. 1*C, right panel*). Each of the dimers retained the ability to tightly bind peptide antigen, as demonstrated by resistance to SDS-induced chain dissociation (35) (Fig. 1*C, left panel*), and exhibited the expected apparent molecular weight with no tendency to aggregate, as determined by gel filtration chromatography (see below).

Effects of Receptor Orientation on CD4+ T-cell Triggering—The orientation dependence for T-cell activation was investigated by comparing the dose-response curves of MHC dimers linked through the α- or β-subunit. MHC dimers were tested for their ability to trigger T-cell activation processes in HA1.7, a well characterized human T-cell clone (33) specific for an antigenic peptide derived from influenza virus hemagglutinin (Ha) as presented by the class II MHC protein HLA-DR1 (29). T-cells down-regulate engaged TCR (CD3), as part of the activation process (36, 37). The down-modulation of TCR in response to the soluble MHC dimers was measured by flow cytometry using a PE-labeled antibody against the TCR CD3ε subunit. Disulfide-linked dimers of MHC proteins, complexed with Ha peptide and cross-linked through either the α- or β-subunit ($_{Cys}\alpha\beta$ S-S and $\alpha\beta_{Cys}$ S-S), induced TCR down-regulation in a dose-dependent manner (Fig. 2*A, open* and *closed circles*). Dimers coupled with a long, flexible cross-linker ($_{Cys}\alpha\beta$ X14X and $\alpha\beta_{Cys}$ X14X, *open* and *closed squares*), or dimers carrying the additional flexible connecting peptide linker on the β-subunit ($\alpha\beta_{L\text{-}Cys}$ X14X, *open diamonds*) also induced CD3 down-regulation. The level of activation induced by dimers linked through either the α- or β-subunit was similar for complementary pairs of disulfide-bonded dimers, and for complementary pairs of dimers coupled through the peptide-based cross-linkers. As previously observed (15, 27), neither MHC-peptide monomers (Fig. 2*A,* ×), nor oligomers carrying the non-antigenic, endogenous peptide A2 (data not shown), induced significant T-cell triggering in the concentration range tested. Down-regulation of CD3 induced by the MHC dimers was observed as early as 2 h and continued to increase over time, with similar time courses for complementary dimers linked through the α- or β-subunits (Fig. 2*B*). Collectively, these data demonstrate that receptor orientation is not crucial for signaling, as in each case dimers linked through the α- or β-subunit induced T-cell activation to an equivalent extent.

Similar responses were observed for other measures of T-cell activation processes, including up-regulation of the early T-cell activation marker CD69 (38) (Fig. 3*A*), up-regulation of the low-affinity IL-2 receptor α-subunit (CD25) (39) (Fig. 3*B*), and up-regulation of transferrin receptor (CD71) (40) (Fig. 3*C*). In these assays, dimers with a range of cross-linker lengths were used. For each T-cell activation marker studied, MHC dimers coupled through the α- or β-subunit with direct disulfide bonds (S-S) induced the most potent response. As the dimer cross-linker length was increased, an incremental decrease in the extent of T-cell activation was observed. Dimers coupled through MHC-peptide complexes carrying the additional flexible connecting peptide regions on the β-subunit (LX14X) exhibited the least potent activation. These data indicate a systematic activation dependence on linker length, with only a slight dependence on inter-molecular orientation.

Effects of Orientation and Proximity on the Activation of a Polyclonal T-cell Line—To address whether the observed acti-

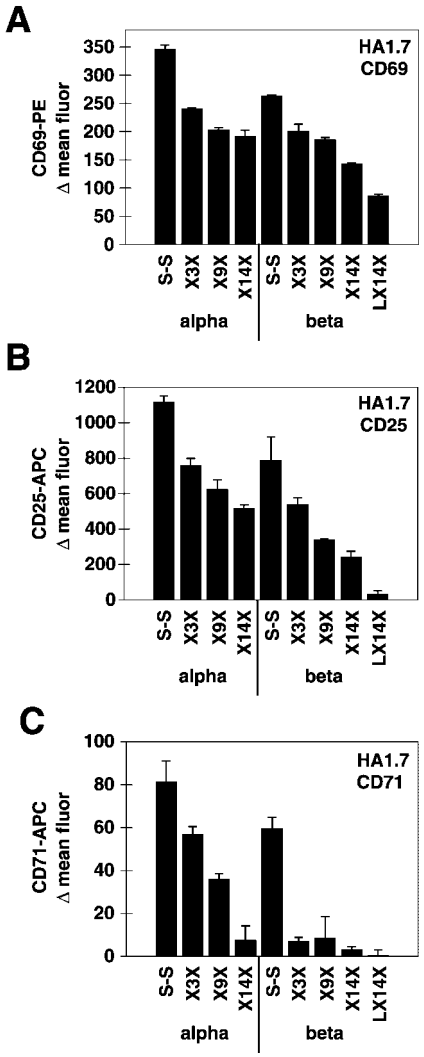

FIG. 3. **CD4$^+$ T-cell triggering is dependent on cross-linker length.** *Bars* indicate levels of T-cell activation induced by incubating 0.16 μM MHC dimer with the HA1.7 T-cell clone as measured by: *A,* CD69 up-regulation after 12 h incubation; *B,* CD25 (IL-2R) up-regulation after 36 h incubation; and *C,* CD71 (transferrin receptor) up-regulation after 60 h incubation. Disulfide-bonded dimers (S-S) linked through the α- or β-subunit provided the most potent stimulus. Increasing cross-linker length resulted in decreasing levels of T-cell triggering for dimers linked through either the α- or β-subunit. *Error bars* represent replicates performed during the same experiment, and similar trends are observed when experiments are performed on different days.

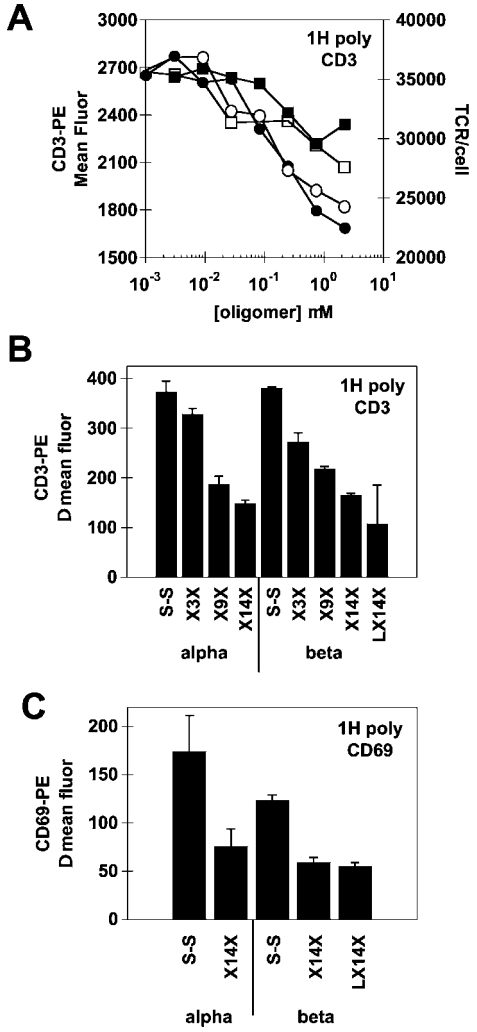

FIG. 4. **Effects of orientation and proximity on the activation of the 1H polyclonal T-cell line.** *A,* dose-response curves of CD3 down-regulation. *B* and *C,* levels of CD3 down-regulation (*B*) and CD69 up-regulation (*C*) induced by 0.22 μM MHC dimers. For the dose-response curves (*A*), CD3 levels were measured after 6 h incubation. For the *bar graphs* (*B* and *C*), CD3 and CD69 levels were measured after 12 h incubation.

vation responses were due to idiosyncratic aspects of the long-term T-cell clone HA1.7, we repeated the activation experiments using a polyclonal T-cell line raised from the peripheral blood of a DR1$^+$ individual (designated 1H). The 1H polyclonal T-cell line exhibited CD3 down-regulation in response to MHC dimers, with dose-response curves for disulfide-linked and X14X-linked MHC dimers (Fig. 4*A*) that were similar to those observed for the HA1.7 T-cell clone. Activation of CD3 down-regulation (Fig. 4*B*) and CD69 up-regulation (Fig. 4*C*) in the 1H polyclonal line were relatively independent of intermolecular orientation, but were dependent on cross-linker length, with the shortest cross-links (S-S) exhibiting the most potent signal, as observed for the HA1.7 clone.

Correlation of Hydrodynamic Radii and T-cell Activation—The observed dependence of T-cell triggering on cross-linker length was investigated in more detail. To evaluate the actual intermolecular spacing in the intact oligomers, an apparent hydrodynamic radius was characterized for each of the various dimers by gel filtration chromatography (Fig. 5*A*). For the $_{Cys}\alpha\beta$ dimer series, a systematic dependence of apparent hydrodynamic radius (R_S) on cross-linker length was observed (Table I), with dimers coupled through short disulfide cross-links (S-S) exhibiting the most compact conformation. Similar hydrodynamic behavior was observed for the $\alpha\beta_{Cys}$ dimer series (Table I). Dimers linked through the β-subunit exhibited slightly but systematically larger apparent hydrodynamic radii than the corresponding dimers linked through the α-subunit. Plots of the apparent hydrodynamic radius *versus* the T-cell response induced by the MHC dimers exhibited a striking linear dependence. This dependence was observed in the HA1.7 T-cell clone for CD3 down-regulation (Fig. 5*B*), CD69 up-regulation (Fig. 5*C*), and CD25 up-regulation (Fig. 5*D*), and was also evident in the 1H polyclonal line (not shown). Thus, T-cell activation is more efficiently triggered by more compact dimers, for each of the responses studied.

MHC Dimer Binding to T-cells—The observed dependence of T-cell triggering on linker length could possibly be due to decreased binding for the dimers coupled through longer cross-links. To address this possibility the relative binding of the MHC dimers was measured, using a competitive binding assay in which unlabeled dimers compete for the T-cell surface with streptavidin-linked, phycoerythrin-labeled (SA-PE) MHC oli-

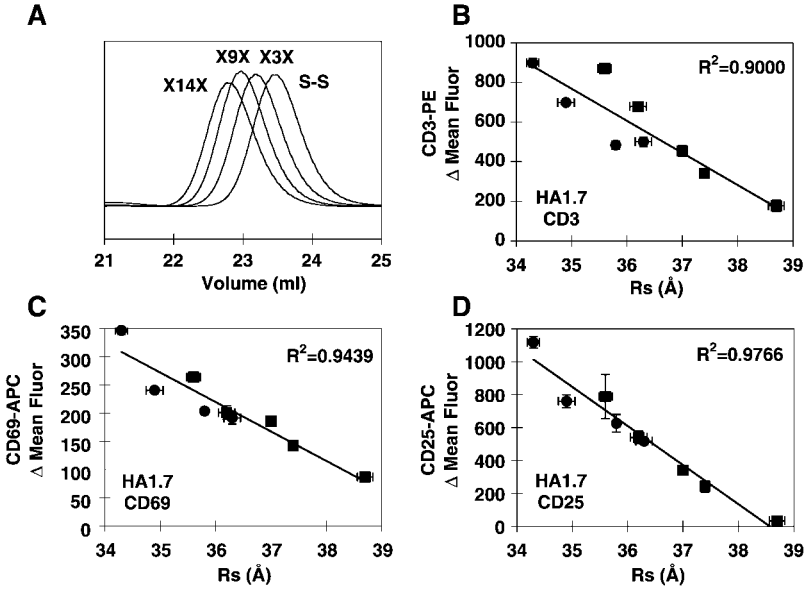

FIG. 5. **Correlation of T-cell activation with apparent hydrodynamic radii (R_S).** *A,* gel filtration chromatography of MHC dimers. Apparent molecular weights from gel filtration, with actual molecular masses in parentheses: S-S, 94 ± 1 kDa (89,843); X3X, 99 ± 1 kDa (91,380); X9X, 107 ± 0.4 kDa (91,926); X14X, 112 ± 1 kDa (92,345). Confidence intervals ($\pm\sigma$) reflect the standard deviation from the mean of replicate samples in separate experiments. Apparent hydrodynamic radii extracted from these data are shown in Table I. *B, C,* and *D,* correlation of apparent hydrodynamic radii (R_S) with CD3 down-regulation (*B*), CD69 up-regulation (*C*), and CD25 up-regulation (*D*) for the HA1.7 T-cell clone. The linear relationship observed for each activation marker demonstrates a dependence of T-cell triggering on dimer compactness. The correlation coefficient (R^2) is shown in each panel. Activation data for CD69 and CD25 were taken from Fig. 3. Data for CD3 were obtained under similar conditions.

TABLE I
Hydrodynamic properties of MHC dimers as determined by gel filtration

Complex	Cross-link	R_s[a]
		Å
$_{Cys}\alpha\beta$	S-S	34.3 ± 0.11
$_{Cys}\alpha\beta$	X3X	34.9 ± 0.15
$_{Cys}\alpha\beta$	X9X	35.8 ± 0.06
$_{Cys}\alpha\beta$	X14X	36.3 ± 0.15
$\alpha\beta_{Cys}$	S-S	35.6 ± 0.12
$\alpha\beta_{Cys}$	X3X	36.2 ± 0.15
$\alpha\beta_{Cys}$	X9X	37.0 ± 0.06
$\alpha\beta_{Cys}$	X14X	37.4 ± 0.06
$\alpha\beta_{L\text{-}Cys}$	X14X	38.7 ± 0.14

[a] Stokes radius estimated from gel filtration data as described (32).

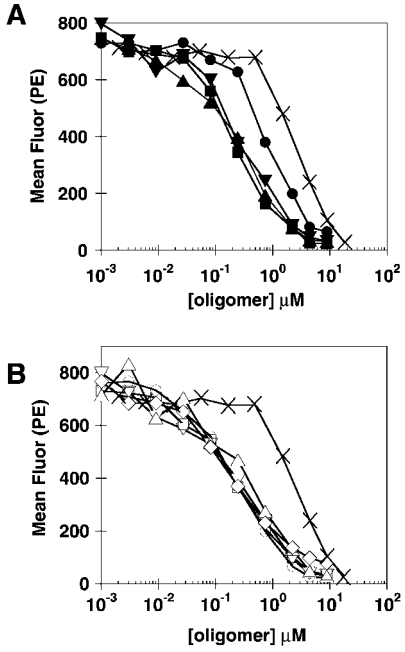

FIG. 6. **MHC-peptide dimers bind similarly to T-cells.** Competitive binding assay of: *A,* α-subunit; and *B,* β-subunit linked dimers. Varying concentrations of MHC monomers and dimers were used to compete with phycoerythrin-labeled MHC oligomers for binding to the HA1.7 T-cell clone: dimers linked through the α-subunit (*closed symbols*) or β-subunit (*open symbols*), with the X3X (*inverted triangles*), X9X (*triangles*), X14X (*squares*) cross-linkers; β-linked dimers containing the connecting peptide linker (*open diamonds*); and monomers (×). Most dimers competed similarly for binding with a half-maximal inhibitory concentration of ~0.3 μM. α-Subunit dimers linked through direct disulfide bonds (*closed circles*) competed slightly worse for binding than the other dimers with a half-maximal inhibitory concentration of ~0.8 μM. All dimers competed better than MHC monomers, which had a half-maximal inhibition of ~2.5 μM. Data are representative of several experiments.

gomers (34). MHC dimers linked through the α-subunit with the X3X, X9X, and X14X cross-linkers each exhibited essentially identical competition curves (Fig. 6*A*), with half-maximal inhibitions of ~0.3 μM. Dimers linked though the α-subunit with a direct disulfide bond (S-S) exhibited slightly weaker binding, with a half-maximal inhibition of ~0.8 μM (Fig. 6*A, filled circles*). MHC dimers linked through the β-subunit with the X3X, X9X, and X14X cross-linkers or direct disulfide bonds (S-S) bound similarly (IC_{50} ~0.3 μM) (Fig. 6*B*). Overall, the MHC dimers competed for binding more efficiently than monomeric MHC-peptide complexes, which exhibited an IC_{50} of ~2.5 μM (Fig. 6*A*, ×), as previously observed (15, 27). Since the competition curves for all of the MHC dimers are similar, the decreased activation exhibited by MHC dimers coupled through longer cross-linkers cannot be attributed to a reduction in binding, and must be due to a decreased ability to trigger T-cells once engaged to the receptor.

DISCUSSION

T-cell Triggering Is Not Dependent on Receptor Orientation—We have shown previously that $CD4^+$ T-cells can be activated with defined chemically coupled oligomers of MHC-peptide complexes (15, 27), extending earlier studies using other types of soluble MHC oligomers (14, 41–44) or TCR cross-linking agents (6, 7, 20–22, 45). It was determined that an MHC dimer was the minimal oligomer valency sufficient to trigger several characteristic T-cell activation processes (14, 15). In this report, we evaluated the constraints on inter-molecular orientation and spacing within an activating MHC dimer. We hypothesized that if T-cells were triggered by a mechanism that relied on formation of a particular arrangement (or rearrangement) of TCR in the membrane, then activation would be sensitive to the MHC spacing and orientation within an activating dimer. However, if T-cells were triggered by a generalized co-localization mechanism, activation would be less dependent on the receptor orientation. This approach follows one used previously to evaluate similar questions in the erythropoietin receptor, in which a strong dependence on re-

ceptor orientation was observed (46, 47). In the MHC/TCR system, we did not observe a strong dependence on receptor orientation for initiation of T-cell signaling. For each of several different cross-linkers, conformationally constrained MHC dimers coupled through either the α- or the β-subunit induced relatively similar levels of T-cell activation, indicating that MHC orientation within the dimers was not critical for activation. Similar results were observed for a polyclonal culture of primary T-cells in addition to a long-term T-cell clone, and for several markers of T-cell activation.

Since the first structural studies of class II MHC proteins, there has been much speculation about the potential physiological relevance of a class II MHC dimer observed in several crystal structures (1, 48–51). In that dimer, the MHC molecules are arranged in a roughly parallel manner with juxtaposed peptide-binding sites, and with α-subunit C termini that are substantially closer then the β-subunit termini (13 *versus* 39 Å). This crystallographic orientation could be adopted by any of the ${}_{\mathrm{Cys}}\alpha\beta$-linked dimers, and possibly by the $\alpha\beta_{\mathrm{Cys}}$ dimers coupled through longer cross-links, but not by the disulfide-linked dimer $\alpha\beta_{\mathrm{Cys}}$ S-S. We observed that the $\alpha\beta_{\mathrm{Cys}}$ S-S dimer was able to induce T-cell activation, and in fact it was among the most potent dimers tested herein. Thus, formation of the crystallographic MHC dimer is not required for efficient T-cell triggering. Similarly, any T-cell triggering mechanism that requires a particular receptor orientation can be excluded, as the ${}_{\mathrm{Cys}}\alpha\beta$ S-S and $\alpha\beta_{\mathrm{Cys}}$ S-S dimers cannot both adopt the same configuration without substantial unfolding, yet both trigger T-cell activation with similar efficiency. These considerations suggest that T-cell activation by MHC oligomers proceeds through a mechanism of generalized receptor co-localization, and not through formation of a particular activating receptor configuration.

T-cell Triggering Correlates with Receptor Proximity—T-cell triggering was dependent on the MHC cross-linker length, with the most potent stimulus provided by MHC dimers linked through a short disulfide bond. The degree of activation decreased monotonically as the length of the linker was increased (Figs. 3 and 4), although the binding remained constant (Fig. 6). These results indicate that inter-receptor proximity is important in triggering T-cell activation processes. Cross-linking strategies that constrained the MHC molecules to be closest together in the most compact dimers were the most effective in activating T-cells, while MHC molecules coupled through longer cross-linkers were less effective. This implies that for induction of T-cell activation by soluble MHC oligomers, TCR complexes need to be brought closely together.

The earliest biochemical marker of T-cell activation is phosphorylation of TCR cytoplasmic domains by membrane-associated Src family tyrosine kinases (5). It is possible that clustering of receptor subunits by MHC oligomers could trigger such phosphorylation events simply through a mass action mechanism. In this scheme, TCR clustering would serve to increase the local concentrations of receptor cytoplasmic domains and tyrosine kinases, tipping the balance of kinases and phosphatases toward phosphorylation of receptor-associated signaling proteins. For example, a fraction of the total cellular tyrosine kinase Lck is found associated with the cytoplasmic tail of CD4, a co-receptor for class II molecules (52), and TCR clustering could facilitate receptor phosphorylation by CD4-associated Lck. However, some T-cells can be activated in the absence of CD4 (44), indicating a role for CD4-independent signaling processes. We have proposed another possible mechanism based on mass action (53), in which an increase in the local TCR concentration induces a conformational change in the TCR ζ-subunit cytoplasmic domain that allows phosphorylation by local tyrosine kinases. In another possible mechanism, TCR clustering could lead to phosphorylation through changes in the local receptor environment. Relative to the bulk membrane around a resting TCR, the membrane regions around clustered TCR might be enriched or depleted in particular lipids (54) and/or proteins (55) involved in the signaling mechanism, leading to initiation of signaling processes around clustered TCR. We have observed that multivalent engagement of TCR by MHC oligomers is accompanied by cytoskeletal rearrangements and is inhibited by disruption of lipid rafts (34), suggesting that receptors can experience substantial alterations in their membrane environment after clustering. Although these and other potential triggering mechanisms remain to be definitively evaluated in the MHC/TCR system, the results presented herein establish that any plausible model would have to account for a lack of dependence on receptor orientation, but a substantial dependence on receptor proximity.

T-cell Activation by Antigen-presenting Cells—Our results suggest that on a cellular level, the inter-receptor distances required for T-cell activation by soluble MHC oligomers are very small. It is not likely that specific MHC-peptide complexes would be found in such proximity on the surface of an antigen-presenting cell, where MHC proteins are bound to a wide spectrum of specific and nonspecific peptides and distributed across the cell membrane. If T-cell activation induced by antigen presenting cells proceeds similarly to oligomer-induced activation, an active process may be required to cluster the infrequent specific MHC-peptide complexes on the cell surface to a density sufficient for TCR oligomerization (26, 56). Some evidence has been presented in support of the existence of MHC oligomers on the surface of antigen presenting cells prior to encounter with a T-cell (51, 57–60). More recently, activated B cells have been shown to cluster MHC-peptide complexes into lipid rafts (61), and dendritic cells have been shown to transport MHC-rich vesicles from endocytic compartments to the cell surface, forming semi-stable patches rich in MHC-peptide complexes (62). In addition, processes involving the T-cell could be responsible for this active clustering of cell-surface components. Antigen-independent interactions of molecules from both the antigen presenting cell and T-cell contribute to efficient T-cell activation, in a process known as costimulation (reviewed in Ref. 63). MHC/TCR binding with simultaneous ligation of costimulatory molecules has been shown to stimulate an active, cytoskeletal rearrangement of the cell surface molecules involved in T-cell signaling, and appears to drive receptor accumulation at the T-cell antigen-presenting cell interface (64). Costimulation-dependent events that lead to TCR localization at the cell-cell interface might play an important role in lowering the TCR density required for cellular activation (65). Indeed, saturating T-cell responses to MHC-peptide complexes incorporated into planar bilayers in the absence of costimulation have been observed when the average inter-receptor distance is ~200 Å or less (66), similar to the MHC-to-MHC distances for the cross-links used in this study. By contrast, in the presence of costimulation the required density of MHC peptide complexes incorporated into supported bilayers has been reported to be ~60 molecules per μm^2, corresponding to an average distance of ~1800 Å between T-cell receptors (26). It remains to be definitively established whether cytoskeletal reorganizations induced by costimulation serve only to localize TCR to a density required for efficient T-cell activation, or whether they contribute to other events in the overall T-cell activation process.

Conclusion—In summary, we have demonstrated that T-cell activation by soluble MHC oligomers is not sensitive to inter-receptor orientation, but does depend on receptor proximity.

These results suggest that the triggering mechanism involves a ligand-induced oligomerization of TCR, and not a molecular rearrangement of a TCR receptor oligomer. Furthermore, these results are consistent with a transmembrane signaling mechanism that relies on co-localization of receptor cytoplasmic domains, and not on formation of a particular TCR dimer in an activating conformation.

Acknowledgments—We thank Glen Paradis and the MIT Flow Cytometry Core Facility for expert help in flow cytometry, Bader Yassine-Diab for obtaining clinical samples, and Jennifer Zarutskie for assistance with hydrodynamic calculations.

REFERENCES

1. Germain, R. N. (1994) *Cell* **76,** 287–299
2. Hennecke, J., and Wiley, D. C. (2001) *Cell* **104,** 1–4
3. Ullman, K. S., Northrop, J. P., Verweij, C. L., and Crabtree, G. R. (1990) *Annu. Rev. Immunol.* **8,** 421–452
4. Glimcher, L. H., and Singh, H. (1999) *Cell* **96,** 13–23
5. Qian, D., and Weiss, A. (1997) *Curr. Opin. Cell Biol.* **9,** 205–212
6. Bekoff, M., Kubo, R., and Grey, H. M. (1986) *J. Immunol.* **137,** 1411–1419
7. Yoon, S. T., Dianzani, U., Bottomly, K., and Janeway, C. A., Jr. (1994) *Immunity* **1,** 563–569
8. Cochran, J. R., Aivazian, D. A., Cameron, T. O., and Stern, L. J. (2001) *Trends Biochem. Sci.* **26,** 304–310
9. Delon, J., Gregoire, C., Malissen, B., Darche, S., Lemaitre, F., Kourilsky, P., Abastado, J. P., and Trautmann, A. (1998) *Immunity* **9,** 467–473
10. Chambers, C. A., and Allison, J. P. (1999) *Curr. Opin. Cell Biol.* **11,** 203–210
11. Bachmann, M. F., and Ohashi, P. S. (1999) *Immunol. Today* **20,** 568–576
12. Sousa, J., and Carneiro, J. (2000) *Eur. J. Immunol.* **30,** 3219–3227
13. Fox, B. S., Quill, H., Carlson, L., and Schwartz, R. H. (1987) *J. Immunol.* **138,** 3367–3374
14. Boniface, J. J., Rabinowitz, J. D., Wulfing, C., Hampl, J., Reich, Z., Altman, J. D., Kantor, R. M., Beeson, C., McConnell, H. M., and Davis, M. M. (1998) *Immunity* **9,** 459–466
15. Cochran, J. R., Cameron, T. O., and Stern, L. J. (2000) *Immunity* **12,** 241–250
16. Hubbard, S. R., and Till, J. H. (2000) *Annu. Rev. Biochem.* **69,** 373–398
17. Falke, J. J., Bass, R. B., Butler, S. L., Chervitz, S. A., and Danielson, M. A. (1997) *Annu. Rev. Cell Dev. Biol.* **13,** 457–512
18. Klemm, J. D., Schreiber, S. L., and Crabtree, G. R. (1998) *Annu. Rev. Immunol.* **16,** 569–592
19. Jiang, G., and Hunter, T. (1999) *Curr. Biol.* **9,** R568–571
20. Romeo, C., Amiot, M., and Seed, B. (1992) *Cell* **68,** 889–897
21. Letourneur, F., and Klausner, R. D. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8905–8909
22. Irving, B. A., and Weiss, A. (1991) *Cell* **64,** 891–901
23. San Jose, E., Sahuquillo, A. G., Bragado, R., and Alarcon, B. (1998) *Eur. J. Immunol.* **28,** 12–21
24. Fernandez-Miguel, G., Alarcon, B., Iglesias, A., Bluethmann, H., Alvarez-Mon, M., Sanz, E., and de la Hera, A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1547–1552
25. Exley, M., Wileman, T., Mueller, B., and Terhorst, C. (1995) *Mol. Immunol.* **32,** 829–839
26. Grakoui, A., Bromley, S. K., Sumen, C., Davis, M. M., Shaw, A. S., Allen, P. M., and Dustin, M. L. (1999) *Science* **285,** 221–227
27. Cochran, J. R., and Stern, L. J. (2000) *Chem. Biol.* **7,** 683–696
28. Frayser, M., Sato, A. K., Xu, L., and Stern, L. J. (1999) *Protein Expr. Purif.* **15,** 105–114
29. Roche, P. A., and Cresswell, P. (1990) *J. Immunol.* **144,** 1849–1856
30. Chicz, R. M., and Strominger, J. L. (1992) *Nature* **358,** 764–768
31. Bubis, J., and Khorana, H. G. (1990) *J. Biol. Chem.* **265,** 12995–12999
32. Zarutskie, J. A., Sato, A. K., Rushe, M. M., Chan, I. C., Lomakin, A., Benedek, G. B., and Stern, L. J. (1999) *Biochemistry* **38,** 5878–5887
33. Lamb, J. R., Eckels, D. D., Lake, P., Woody, J. N., and Green, N. (1982) *Nature* **300,** 66–69
34. Cameron, T. O., Cochran, J. R., Yassine-Diab, B., Sekaly, R.-P., and Stern, L. J. (2001) *J. Immunol.* **166,** 741–745
35. Stern, L. J., and Wiley, D. C. (1992) *Cell* **68,** 465–477
36. Itoh, Y., Hemmer, B., Martin, R., and Germain, R. N. (1999) *J. Immunol.* **162,** 2073–2080
37. Valitutti, S., Muller, S., Cella, M., Padovan, E., and Lanzavecchia, A. (1995) *Nature* **375,** 148–151
38. Testi, R., D'Ambrosio, D., De Maria, R., and Santoni, A. (1994) *Immunol. Today* **15,** 479–483
39. Waldmann, T. A. (1989) *Annu. Rev. Biochem.* **58,** 875–911
40. Ponka, P., and Lok, C. N. (1999) *Int. J. Biochem. Cell Biol.* **31,** 1111–1137
41. Casares, S., Zong, C. S., Radu, D. L., Miller, A., Bona, C. A., and Brumeanu, T. D. (1999) *J. Exp. Med.* **190,** 543–554
42. Abastado, J. P., Lone, Y. C., Casrouge, A., Boulot, G., and Kourilsky, P. (1995) *J. Exp. Med.* **182,** 439–447
43. Appel, H., Gauthier, L., Pyrdol, J., and Wucherpfennig, K. W. (2000) *J. Biol. Chem.* **275,** 312–321
44. Hamad, A. R., O'Herrin, S. M., Lebowitz, M. S., Srikrishnan, A., Bieler, J., Schneck, J., and Pardoll, D. (1998) *J. Exp. Med.* **188,** 1633–1640
45. Spencer, D. M., Wandless, T. J., Schreiber, S. L., and Crabtree, G. R. (1993) *Science* **262,** 1019–1024
46. Syed, R. S., Reid, S. W., Li, C., Cheetham, J. C., Aoki, K. H., Liu, B., Zhan, H., Osslund, T. D., Chirino, A. J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B. A., Matthews, D. J., Wendoloski, J. J., Egrie, J., and Stroud, R. M. (1998) *Nature* **395,** 511–516
47. Livnah, O., Johnson, D. L., Stura, E. A., Farrell, F. X., Barbone, F. P., You, Y., Liu, K. D., Goldsmith, M. A., He, W., Krause, C. D., Pestka, S., Jolliffe, L. K., and Wilson, I. A. (1998) *Nat. Struct. Biol.* **5,** 993–1004
48. Brown, J. H., Jardetzky, T. S., Gorga, J. C., Stern, L. J., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1993) *Nature* **364,** 33–39
49. Fields, B. A., and Mariuzza, R. A. (1996) *Immunol. Today* **17,** 330–336
50. Schafer, P. H., Pierce, S. K., and Jardetzky, T. S. (1995) *Semin. Immunol.* **7,** 389–398
51. Triantafilou, K., Triantafilou, M., and Fernandez, N. (2000) *Crit. Rev. Immunol.* **20,** 359–373
52. Janeway, C. A., Jr. (1992) *Annu. Rev. Immunol.* **10,** 645–674
53. Aivazian, D., and Stern, L. J. (2000) *Nat. Struct. Biol.* **7,** 1023–1026
54. Langlet, C., Bernard, A. M., Drevot, P., and He, H. T. (2000) *Curr. Opin. Immunol.* **12,** 250–255
55. van der Merwe, P. A., Davis, S. J., Shaw, A. S., and Dustin, M. L. (2000) *Semin. Immunol.* **12,** 5–21
56. Reay, P. A., Matsui, K., Haase, K., Wulfing, C., Chien, Y. H., and Davis, M. M. (2000) *J. Immunol.* **164,** 5626–5634
57. Schafer, P. H., Malapati, S., Hanfelt, K. K., and Pierce, S. K. (1998) *J. Immunol.* **161,** 2307–2316
58. Roucard, C., Garban, F., Mooney, N. A., Charron, D. J., and Ericson, M. L. (1996) *J. Biol. Chem.* **271,** 13993–14000
59. Cherry, R. J., and Fernandez, N. (1998) *J. Cell Biol.* **140,** 71–79
60. Triantafilou, K., Triantafilou, M., Wilson, K. M., and Fernandez, N. (2000) *Hum. Immunol.* **61,** 585–598
61. Anderson, H. A., Hiltbold, E. M., and Roche, P. A. (2000) *Nat. Immunol.* **1,** 156–162
62. Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M., and Mellman, I. (2000) *Science* **288,** 522–527
63. Chambers, C. A. (2001) *Trends Immunol.* **22,** 217–223
64. Wulfing, C., and Davis, M. M. (1998) *Science* **282,** 2266–2269
65. Viola, A., and Lanzavecchia, A. (1996) *Science* **273,** 104–106
66. Watts, T. H. (1988) *J. Immunol.* **141,** 3708–3714
67. Stern, L. J., Brown, J. H., Jardetzky, T. S., Gorga, J. C., Urban, R. G., Strominger, J. L., and Wiley, D. C. (1994) *Nature* **368,** 215–221