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Ag-specific immune tolerance in clinical organ transplantation is currently an unrealized but critical goal of transplant biology. The specificity and avidity of multimerized MHC-peptide complexes suggests their potential ability to modulate T cell sensitization and effector functions. In this study, we examined the ability of MHC-peptide dimers to modulate T cell function both in vitro and in vivo. Soluble MHC dimers induced modulation of surface TCR expression and inhibited T cell cytolytic activity at nanomolar concentrations in vitro. Furthermore, engagement of TCR by soluble dimers resulted in phosphorylation of the TCR ζ-chain and recruitment and phosphorylation of ζ-associated protein-70 to the signaling complex, the latter of which increased upon dimer cross-linking. Significantly, Ag-specific inhibition of an alloreactive TCR-transgenic T cell population in vivo resulted in consequent outgrowth of an allogeneic tumor. The prolonged Ag-specific suppression of expansion and/or effector function of cognate T cells in vivo suggests that soluble MHC dimers may be a means of inducing sustained Ag-specific T cell unresponsiveness in vivo. The Journal of Immunology, 2001, 167: 2555–2560.

Materials and Methods

Mice

BALB/c (H-2\textsuperscript{b}) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Recombination-activating gene (RAG)-2-deficient mice (H-2\textsuperscript{b}), obtained as a gift from Dr. C. Simon (University of Chicago, Chicago, IL), were bred in house. The 2C TCR-Tg (H-2\textsuperscript{b}) mice were originally obtained from Dr. D. Loh (Washington University School of Medicine, St. Louis, MO) and intercrossed with RAG-2\textsuperscript{-/-} mice to obtain 2C × RAG-2\textsuperscript{-/-} mice (2C-RAG-knockout (KO) mice).

Abbreviations used in this paper: Tg, transgenic; ZAP-70, ζ-associated protein-70; RAG, recombination-activating gene; KO, knockout; MCMV, murine CMV; MFI, mean fluorescence intensity.
Reagents
Anti-CD69-FITC, goat anti-mouse IgG1-PE (Southern Biotechnology Associates, Birmingham, AL), and hamster anti-mouse-TCR PE H57 (anti-CD3; BD PharmMingen, San Diego, CA) were used in FACS (BD Biosciences, Mountain View, CA) analyses. A mAb to TCR c-chain (H146-968) (17) and anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY) were used, respectively, in immunoprecipitations and Western bloting. Rat anti-mouse IgG1 (Southern Biotechnology Associates) was used to cross-link the MHC-peptide dimers. Rat anti-hamster Ab (Southern Biotechnology Associates) was used to cross-link hamster antimerine CD3, 145-2C11. The latter was produced and purified in our laboratory. Peptides were synthesized using F-moc solid-phase technology by the Molecular Genetics Facility at the University of Georgia (Athens, GA). The K\(^{\text{b}}\) binding peptides were SIYRYYGL (SIY) and SIYFYYSV (DQK)(8). The H-2K\(^{\text{d}}\) dimer was engineered as previously described (18). The divalent H-2L\(^{\text{d}}\) protein was constructed, expressed, and purified using previously published methods (19). Specifically, the BALB/c H-2L\(^{\text{d}}\) cDNA from pl.444 (20) was amplified using primers 5'-AGATATATGC-ACGCGTCGCAGATGGGGGCGATGGCTCC and 3'-TCTCGAGGTTTCGATGGGCTGAGGGGTACG (12). The fragment was digested with MluI and XhoI and inserted into the same sites of pXlg (18). The sequence was verified. The resulting plasmid was cotransfected by electroporation with a human \(\beta_{2}\)-microglobulin expression plasmid into 35SSL plasma- cytoma cells. A clone that secreted relatively high levels of protein, as determined by ELISA as specific for H-2L\(^{\text{d}}\) or the IgG1 portion of the molecule, was grown in hybridoma serum-free medium (Life Technologies, Rockville, MD), and protein was purified from supernatants by affinity chromatography to the V region of the Ig portion.

**In vitro activation of T cells**
Splenocytes were prepared from spleens of 2C-RAG-KO mice. After maceration, RBCs were lysed. Debris was then excluded by filtering the suspension through sterile Nixex mesh (Terex, Elmford, NY). BALB/c splenocytes were prepared and irradiated to serve as APCs to present the octapeptide, p2Ca, derived from p2Ca, with no staining observed with a noncognate peptide (20). Sensitivity of peptide-loaded dimers for cognate TCR, as well as statistical analysis, was performed using CellQuest software (BD Biosciences). Staining with anti-human IgG1-PE was then added for a final wash and then assayed on a FACScan and analyzed using CellQuest software (BD Biosciences). Staining with anti-human IgG1-PE was then added for a final wash and then assayed on a FACScan and analyzed using CellQuest software (BD Biosciences). Two measurements were taken at 90° to each other, and the square root of their product was calculated to give an estimate of mean tumor diameter. The measuring was performed8.

**Results**

**Sensitivity of class I MHC dimers for cognate TCR**
Peptide-loaded MHC dimers supported by a scaffold of whole Ig are specific for T cells bearing cognate ligand (19). To analyze the sensitivity of peptide-loaded class I dimers for cognate TCR, primed T cells (day 5) were stained with various concentrations of dimers. The L\(^{\text{d}}\) dimer loaded with peptide cognate for 2C TCR, P1.LHTR, showed a 300-fold greater mean fluorescence intensity (MFI) above background when 2 ng (0.4 nM) final concentration was added to 3 \times 10\(^{5}\) cells (Fig. 1A). In contrast, a 3-log higher concentration of L\(^{\text{d}}\) dimer with noncognate peptide did not stain the cells above background. High doses of L\(^{\text{d}}\) dimer, 40 nM, were associated with lower MFI, as is often seen with mAb. This is likely due to weaker, monomeric interactions between a single arm of an MHC dimer and TCR, as is commonly seen with excess dimeric reagent. Similar staining patterns were observed with syngeneic MHC (K\(^{\text{b}}\)) dimer loaded with the strong agonist peptide, SIYRYYGL (SIY) (16), with no staining observed with a noncognate peptide, SIINFEKL (OVA; data not shown).

**In vivo suppression assay**
Spleen cells from 2C-RAG-KO mice were stimulated and harvested at day 6 as described above. Cells were washed three times in PBS and administered directly to the retro-orbital blood sinus of RAG\(^{-/-}\) mice (10\(^{5}\) cells/animal). The following day (day 0), PBS-washed cells from the highly transfectable variant of the murine mastocytoma P815 (H-2\(^{\text{d}}\); referred to hereafter as P1.HTR) were administered to the left flank s.c. (1 \times 10\(^{5}\)animal), and dimer (50 \(\mu\)g/dose) or PBS was administered i.p. on alternate days for a total of four injections. Animals were palpated for tumor beginning on day 7, and growth was quantified with a Vernier caliper by measuring two perpendicular axes. Two measurements were taken at 90° to each other, and the square root of their product was calculated to give an estimate of mean tumor diameter. The measuring was performed 8.

**MHC dimers modulate TCR expression**
As a first approach toward examining the functional effects of soluble MHC dimers on T cell function, TCR expression was assayed on preactivated 2C T cells cultured with various concentrations of peptide-loaded L\(^{\text{d}}\) dimers in vitro for 20 h (Fig. 1B). There was an order of magnitude reduction in MFI of the 2C T cells at dimer concentrations of 20 nM. Incubation of the Tg T cells with as little as 2 nM peptide induced an ~50% reduction in TCR expression (Fig. 1B, right panel). In contrast, no evidence of modulation of TCR expression was observed using MHC dimers loaded with noncognate peptide, MCMV (Fig. 1B, left panel).

**Cytolysis assay**
Target cells, T2-L\(^{\text{d}}\) or T2-K\(^{\text{m}}\), were loaded with \(^{35}\text{Cr}\) at 37°C for 1 h followed by three washes. Cells were then incubated with 10 nM peptide at room temperature for 2 h, counted, and added to 96-well plates (5000/ well). Effectors were incubated in the presence of dimer at room temperature for 1 h at the concentrations indicated and then added to the targets in triplicate wells at the ratios indicated. Plates were incubated at 37°C (4 h) and harvested on a 1470 Wizard automatic gamma counter (PerkinElmer Wallac, Gaithersburg, MD). Results were calculated as percentage of cyto- toxicity using the following equation: (experimental release – spontaneous release)/(maximum release – spontaneous release) \times 100. Maximum release refers to the amount of \(^{51}\text{Cr}\) released from target cells alone resulting from lysis by 10% Triton X-100, and spontaneous release refers to the amount of \(^{35}\text{Cr}\) released from untreated cells alone.
Similar down-regulation of TCR was observed using K\textsuperscript{b} dimer loaded with SIY but not with K\textsuperscript{b} loaded with OVA peptide (data not shown).

**MHC dimers induce early signaling events**

The fact that dimers induce TCR down-regulation (Fig. 1) indicates that their inhibition of CTL cytolytic activity may involve active functional events and not merely receptor/ligand blockade. Indeed, incubation of the TCR-Tg T cells with 20 nM of QL9-loaded L\textsuperscript{d} dimer induced a 10-fold increase in the expression of the early activation marker, CD69 (Fig. 2A). The control dimer, MCMV-loaded L\textsuperscript{d}, had no effect on CD69 expression. To obtain a direct assessment of the early signaling events mediated by the QL9-loaded dimer, the cells were stimulated with the dimer loaded with the cognate peptide (2 min) and analyzed for early biochemical changes by probing anti-\(\zeta\)-chain immunoprecipitates, separated on SDS PAGE, with a phosphotyrosine-specific mAb. As seen in Fig. 2B, QL9-loaded L\textsuperscript{d} dimer alone induced phosphorylation of TCR \(\zeta\)-chain as evidenced by an increase in the p23:p21 ratio. When the dimer was cross-linked, the p23:p21 ratio showed little change, whereas the level of coprecipitated ZAP-70 increased 3-fold (Fig. 2, B and C). These results were consistently different from those produced by the non-cross-linked vs cross-linked 2C11, in which case p23:p21 ratios increased 3-fold upon cross-linking and ZAP-70 expression increases were greater (5-fold vs 3-fold; Fig. 2, B and C). Thus, MHC dimer alone does indeed trigger early signal-transduction events. Both naïve and previously activated 2C cells produced the same phosphorylation patterns, and soluble dimer with bulk peptide, with or without cross-linking, consistently gave no signal above the unstimulated control.

**MHC dimers inhibit cytolytic activity in vitro**

Dimers loaded with a defined peptide allow for specific targeting to Ag-reactive CTL and quantitative analysis of the inhibitory activity. The effects of L\textsuperscript{a} and K\textsuperscript{b} dimers loaded with various peptides on cytolytic activity mediated by 2C cells in vitro was compared using target cells bearing an allogeneic peptide-MHC combination, dEV-8-K\textsuperscript{b}\textsuperscript{m3} or p2Ca-Ld (Fig. 3). L\textsuperscript{d} dimers loaded with QL9 inhibited CTL activity over the entire range tested. The IC\textsubscript{50} was \(\sim 8\) nM at an E:T of 10:1 (Fig. 3, left panel). In contrast, MCMV-bearing L\textsuperscript{d} did not inhibit target cell lysis even at a final concentration of 100 nM (Fig. 3, left panel). Syngeneic dimer, K\textsuperscript{b}, loaded with SIY inhibited lysis with an IC\textsubscript{50} of \(\sim 20\) nM at an E:T of 3:1, whereas OVA-loaded K\textsuperscript{b} did not effect CTL activity at any concentration tested. Dimers (L\textsuperscript{a} and K\textsuperscript{b}) with bulk undefined peptide showed the same ineffectiveness as inhibiting lysis (data not shown) as dimer with defined, noncognate peptide (MCMV and OVA; Fig. 3). The suppressive effect of the MHC-peptide dimers on CTL activity was observed at concentrations well below the concentration necessary to visualize dimer binding by staining. These results suggest that the threshold concentration for modulating T cell activity is lower than can be observed by flow cytometry, suggestive of a mechanism beyond steric hindrance that may involve signaling events discussed above.

**Prolonged suppression of Ag-driven CTL responses in vivo**

The suppression of cytolytic activity by the MHC dimer in vitro combined with the signal observed biochemically suggests that Ag-driven CTL responses in vivo may be suppressed by signals induced by MHC dimer. To study the effects of the dimers in vivo, an adoptive transfer model was developed. Resting 2C T cells were transferred (i.v.) to RAG2\textsuperscript{−/−} recipients followed by s.c. administration of P815 tumor cells that express the H-2L\textsuperscript{d} alloantigen naturally bearing the peptide derived from \(\alpha\)-ketoglutarate dehydrogenase, p2Ca (21). Previous studies have shown that the transfer of 2C T cells in RAG-deficient animals results in tumor rejection (22). In these studies, we used the highly transflectable variant of P815, P1.HTR, which forms a solid tumor when administered s.c. and continually expresses the target Ag for at least 69 days in vivo.

Simultaneous administration of the allogeneic QL9-loaded L\textsuperscript{d} dimer significantly inhibited the rejection of the tumor by 2C, allowing for robust tumor growth (Fig. 4, left panel). The rate of tumor growth in these animals resembled the unabated rate of growth of tumor in animals that did not receive an adoptive transfer of 2C CTL. Surprisingly, MCMV-loaded allogeneic L\textsuperscript{d} dimer mediated suppression equivalent to that mediated by QL9-loaded L\textsuperscript{d}. Suppression of tumor rejection was similarly observed in mice treated with the syngeneic SIY-loaded K\textsuperscript{b} (Fig. 4, right panel). Bulk peptide-containing K\textsuperscript{b} consistently mediated an intermediate level of suppression (\(\sim 70\%\) of that mediated by SIY-loaded K\textsuperscript{b}). In marked contrast, tumor rejection by the 2C T cells was unaffected in mice treated with vesicular stomatitis virus-loaded K\textsuperscript{b} (noncognate for 2C T cells; data not shown). These results demonstrate that class I MHC dimers are effective at protecting Ag-bearing tissue by inhibiting previously activated CTL in vivo.
Discussion
The present study was undertaken to test whether MHC dimers presented in a soluble form on a full Ig scaffold can regulate Ag-specific responses of effector CTL in vivo. In this study, we demonstrate that peptide-loaded MHC dimers are specific and effective inhibitors of cell-mediated immunity in vitro and in vivo.

Their potential for in vivo modulation was first indicated by the blockade of CTL activity in vitro at nanomolar concentrations. The inhibition of the cytolytic activity by MHC dimer in vitro was similar to the blocking observed by the anti-clonotypic 2C TCR-specific mAb 1B2 at the highest concentration. The simplest explanation of the inhibitory activity by the peptide-MHC dimers is competitive inhibition of 2C-specific Ag recognition of the target cells by soluble MHC. A total of 1 nM of dimer is theoretically sufficient to give a soluble MHC:cell surface-bound TCR ratio of >200:1. However, additional studies suggested that an active mechanism, including potentially inhibitory signaling events and TCR down-regulation, may contribute to the diminished cytolytic activity in this 4-h assay. First, 2C-mediated CTL activity was not blocked with a soluble 2C TCR dimer that binds the MHC of target cells (data not shown). Second, the cognate dimers mediated 2C TCR down-regulation upon engagement in vitro (Fig. 1B). Finally, TCR ζ-chain phosphorylation and ZAP-70 recruitment and phosphorylation were induced as a consequence of TCR engagement. These results lead to the hypothesis that dimer-mediated suppression of activated CTL may be the net result of mechanisms of suppression similar to those mediated by 2C11–TCR down-modulation and the induction of an apoptotic signal to activated T cells (23, 24). This putative inhibitory signaling outcome is in contrast to a finding in a recent report in which an MHC multimer was used for in vivo activation and enhancement of effector function (25). However, there are several differences in experimental design, including the route of administration (i.p. vs i.v.) and the dosage (alternate days at 50 μg vs a daily dose of 130 μg). To distinguish

![Diagram](http://www.jimmunol.org/)

**FIGURE 2.** MHC dimer induces CD69 expression and transduction of early signaling events. A, The 2C cells activated as described (see Fig. 1 legend) were incubated on day 5 with 20 nM QL9 or MCMV (20 h at 37°C) followed by staining with anti-CD69 (rat anti-mouse CD69-FITC). Results were similar in three independent experiments. B, Naive purified 2C splenic cells were stimulated for 2 min at 37°C with dimer or 2C11 incubated in the presence or absence of cross-linking Ab followed by immunoprecipitation of TCR ζ-chain with H146-968 and blotting with anti-phosphotyrosine mAb 4G10.
between these mechanisms in vivo, the model system will need to be substantially modified so that sufficient numbers of cells can be recovered for ex vivo analyses.

It is striking that, in the absence of cross-linking, there was a 3-fold greater p23:p21 ratio between peptide-MHC dimer and 2C11. This greater degree of phosphorylation of the CD3/H9256-chain induced by dimer may reflect the different way each reagent binds TCR. In contrast to 2C11 binding of TCR, the physiologic ligation of TCR by MHC dimer may include CD8 binding to the a3 domain of the MHC and, hence, LCK recruitment to the signaling complex, as indicated by recent reports using tetramers in cross-linking TCR (26). However, the 3-fold greater increase in the amount of phosphorylated ZAP-70 upon cross-linking does provide evidence that multimerizing the dimer does result in a stronger stimulus than dimer alone. This physiologic induction of early biochemical signals suggests that MHC dimers will be useful tools for quantitative studies of TCR engagement with various peptide-MHC combinations. In contrast to APC, studies with MHC dimers can be conducted in the absence of other surface molecules, allowing quantitative assessments of the contribution of MHC alone. The relative contribution of CD8 coligation, for example, can be studied by comparing its role using a variety of ligands: allogeneic, agonist, altered peptide, and positively selecting.

These studies do not rule out a role for costimulatory molecules in the effects of the dimer in vivo. It is possible that the effector cells may be engaging TCR in the absence of CD28 coligation, which could result in a lack of Bcl-xL up-regulation necessary to maintain long-term T cell survival (27).

It is remarkable that MCMV-loaded dimer suppressed the effector activity of 2C cells as well as QL9-loaded dimer. No in vitro result predicted this activity. This is in contrast to bulk peptide-loaded syngeneic Kb, which consistently mediated less suppression (~30%) than Kb loaded with cognate SIY peptide. Although neither bulk peptide-loaded Kb nor MCMV-loaded Ld could be detected in binding 2C, in inducing changes in CD69 or TCR expression or in inducing ζ-chain phosphorylation, they each mediated suppression of 2C effector function in vivo. The role of APC-mediated dimer clustering will have to be studied in vitro to begin to understand the mechanism by which each mediates suppression. The efficient suppression mediated by MCMV-loaded Ld could be explained by a degree of allore cognition of 2C TCR for Ld that is not detectable in vitro assays. In a similar manner, within the bulk-loaded Kb, there may exist conformers that resemble agonist peptide Kb whose recognition by 2C TCR is below the limits of detection in vitro assays but avid enough for mediating a signal in vivo.

In summary, peptide-loaded dimers offer a novel approach to the active induction of immunosuppression. Regardless of the mechanism, MHC dimers may be effective in promoting stable peripheral tolerance for Ag-specific T cells. In cases of organ transplantation, a small number of different allogeneic MHC conformers compatible with the graft may be useful early on in promoting graft survival and perhaps also inducing linked suppression. In cases of autoimmunity in which a peptide is implicated, dimers may be useful in suppressing effector functions in an Ag-specific manner.
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