

# Allelic Variation of MHC Structure Alters Peptide Ligands to Induce Atypical Partial Agonistic CD8<sup>+</sup> T Cell Function

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## Abstract

T cell receptors recognize small changes in peptide ligands leading to different T cell responses. Here, we analyzed a panel of HLA-A2-Tax11-19 reactive T cell clones to examine how small allelic variations of MHC molecules could alter the functional outcome of antigen recognition. Similar to the effects induced by antigenic altered peptide ligands, weak or partial agonistic T cell functions were identified in individual T cell clones with the recognition of MHC-altered peptide ligands (MAPLs). Interestingly, one subtype of HLA-A2 molecules induced an unusual type of partial agonistic function; proliferation without cytotoxicity. Modeling of crystallographic data indicated that polymorphic amino acids in the HLA-A2 peptide binding groove, especially the D-pocket, were responsible for this partial agonism. Reciprocal mutations of the Tax peptide side chain engaging the D-pocket indeed restored the agonist functions of the MHC-peptide complex. Whereas early intracellular signaling events were not efficiently induced by these MAPLs, phosphorylated c-Jun slowly accumulated with sustained long-term expression. These data indicate that MAPLs can induce atypical partial agonistic T cell function through structural and biochemical mechanisms similar to altered peptide ligands.

Key words: cytotoxic T lymphocyte • HLA-A2 subtypes • cytotoxicity • proliferation • MHC-altered peptide ligand

## Introduction

The TCR does not act in a binary fashion as either an “on” (activated) or “off” (unresponsive) switch, but instead can be differently engaged by the MHC-peptide complex to elicit a variety of functional outcomes (1). Depending on the capacity to elicit T cell responses, TCR ligands can be classified as an agonist, partial agonist, or antagonist. The agonistic ligands include full agonists, which are highly immunogenic and elicit the full array of T cell effector functions, and weak agonists, which elicit the full array of T cell effector functions but at decreased levels and only when significantly greater amounts of peptide antigen are used. Partial agonists are defined to selectively induce some effector functions while failing to induce others. Antagonist

peptides, when presented in combination with native, immunogenic peptide, can inhibit effector function.

The molecular basis of TCR recognition of MHC-peptide complexes has been elucidated by the crystallographic analysis of a number of MHC-peptide complexes and more recently trimolecular complexes, including the TCR-Tax11-19-HLA-A2 (A\*0201) complex. H-bonds between conserved MHC side chains and polar backbone atoms of the peptide termini provide a general peptide binding capacity, whereas more limited peptide sequence selectivity is introduced by the binding of a few peptide “anchor” side chains in pockets formed by polymorphic MHC side chains (2–5). Although the terminal anchor side chains of antigenic peptides are bound similarly in the MHC structure, the main chains and side chain conformations can be very different in the center of the binding site. The crystal structure of TCR-Tax11-19-HLA-A2 trimolecular complex shows that the TCR is oriented diagonally across the MHC-peptide complex surface. This binding mode allows the flat TCR surface to interact with the pep-

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tide by fitting between the highest points of the MHC helices (6). The only exception is a deep pocket located at the center of TCR, which is shaped by the CDR3 loops of both TCR  $\alpha$  and  $\beta$  chains. This pocket is occupied by Y5 of the Tax peptide (6). The total contact surface of the MHC–peptide complex with TCR is relatively small, approximately a third of which is contributed by peptide ( $\sim 326 \text{ \AA}^2$  of  $998 \text{ \AA}^2$ ). Interestingly, structural comparison among the A6–TCR and four different peptides including agonist, partial agonists, and antagonists–HLA–A2 complexes, failed to show a correlation between the types of minor structural adjustments at the interface and the functional outcomes (7).

The kinetic analyses investigating TCR binding to MHC–peptide complexes greatly contributed to our understanding of the underlying mechanism for the discrimination of ligands by the TCR. It has been generally observed that less potent ligands have a lower affinity and a faster dissociation rate (8, 9). The kinetic proofreading model based on these analyses predicts that partial or antagonist ligands cannot engage the TCR for the time required to activate all the downstream signaling pathways due to low affinity and fast dissociation (10). It was also demonstrated that the shorter off time of TCR engagement with the MHC–altered peptide complex allows for the recruitment of different signaling complexes associated with immune receptor tyrosine-based activation motifs of the TCR  $\zeta$  chain (11). However, there are some exceptions to this model (12, 13), and it is currently not possible to measure TCR affinities and dissociation rates under physiological conditions (the interaction between membrane bound forms of TCR and MHC–peptide molecule).

Although altered TCR ligands represent a complex between the TCR and MHC–peptide complexes, partial agonist and antagonist of TCR–associated functions have been examined predominantly with alterations of the peptide antigen. The structural data of TCR recognition of Tax11–19 presented by HLA–A\*0201 allowed us to analyze how subtle changes in MHC structure could potentially alter the peptide ligand, resulting in partial T cell agonist function of the MHC–peptide complex.

Here, we demonstrate that amino acid changes in HLA–A2 subtypes induce major functional changes of T cell recognition of peptide antigen, analogous to effects seen with amino acid changes in peptide itself. Interestingly, one subtype of HLA–A2 molecule elicited proliferative activity without cytotoxicity from T cell clones when presenting peptide. This functional phenotype does not follow the hierarchy of T cell responses in relation to the previously well established activation thresholds (14, 15). From the analysis of structural interaction between TCR and MHC–peptide complex, this specific partial agonistic T cell function was closely related with an amino acid change of MHC molecule located in the peptide-binding groove. Thus, it appears that changes in the MHC peptide-binding groove induced alterations on the bound peptide that could be recognized by the TCR. Reciprocal alterations of the Tax11–19 peptide side chain predicted to engage the MHC

pocket allowed for restoration of the agonist functional activity of the MHC–peptide complex. Our signaling data suggest that this partial agonistic function comes from inefficient proximal activation processes, which are directly related with cytotoxicity, but cumulative intermediate signals lead to proliferation.

## Materials and Methods

**Peptides.** Tax11–19 peptide (LLFGYPVYV) was synthesized (Bio-Synthesis) and was  $>93\%$  pure as determined by HPLC. Tax11–19 peptide analogues with a single amino acid substitution at position 3 were synthesized by Chiron Mimotopes at a 1-mg scale on pins and provided by Dr. Wucherpfennig (Dana-Farber Cancer Institute, Boston, MA).

**Cell Lines.** EBV-transformed B cell lines expressing HLA–A\*0201 (KS.B) or A\*0205 (DAH) were generated by incubation of PBMCs in the presence of supernatant from EBV-producing cell line B95.8 and  $1 \mu\text{g/ml}$  cyclosporin A. EBV-transformed B cell lines expressing HLA–A\*0203 (FUN) or A\*0206 (CLA) and mouse B cell lymphoma P815 transfected with HLA–A\*0202 were characterized previously and provided by Dr. Sette (Epimune, San Diego, CA; reference 16). These lymphoblastoid cell lines (LCLs)\* were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and used as APCs or target cells.

**Human CD8<sup>+</sup> T Cell Clones Reactive to Tax11–19.** A panel of Tax11–19–reactive T cell clones were generated by the single cell sorting and expansion of HLA–A\*0201–Tax11–19 tetramer binding CD8<sup>+</sup> T cells obtained from an HLA–A\*0201 expressing patient with typical HTLV-I associated myelopathy as described previously (17). KS2E11.7 T cell clone was produced from PBMCs of another HTLV-1–infected patient by repeated antigenic stimulation and limiting dilution method as described previously (18). The different clonal origin of these T cell clones has been defined by their different TCR  $\alpha/\beta$  chain usages and CDR3 region sequences (17, 19). T cell clones were expanded by the stimulation with PHA in the presence of allogeneic irradiated (5,000 rad) PBMCs in complete medium (RPMI 1640 supplemented with 10% human serum [Omega], 4 mM glutamine, 100 U/ml penicillin, 100  $\mu\text{g/ml}$  streptomycin, and 10 mM Hepes; reference 17), and were used in functional assays 9–14 d after PHA stimulation.

**Cytotoxicity Assay.** Cytotoxicity was measured by a standard 4-h  $^51\text{Cr}$  release assay as described previously (17). Delayed cytotoxicity was examined by counting of viable target cells after coculture. Target cells were first labeled with carboxyl fluorescein succinimidyl ester (CFSE; Molecular Probes) in PBS at a final concentration of  $1 \mu\text{M}$  for 5 min and pulsed with  $10 \mu\text{M}$  of peptides. Peptide-pulsed target cells were cocultured with effector T cells for the indicated times. Apoptotic or dead cells were stained with annexin V–PE apoptosis detection kit I (BD Biosciences) according to the manufacturer's instructions. The number of viable target cells was analyzed on a flow cytometer (FACScan™, Becton Dickinson) in CFSE-labeled target cells with the exclusion of annexin V and 7-AAD–stained cells by acquisition of cells for a constant time. Each sample was counted three times and the average number of cells was calculated.

\*Abbreviations used in this paper: APL, altered peptide ligand; CFSE, carboxyl fluorescein succinimidyl ester; LAT, linker for activation of T cells; LCL, lymphoblastoid cell line; MAPK, mitogen-activated protein kinases; MAPL, MHC–altered peptide ligand.

**Proliferation Assay.** Proliferation was assessed by [ $^3\text{H}$ ]thymidine incorporation assay. For the preparation of APCs, LCLs were irradiated with 5,000 rad, pulsed with the indicated concentrations of peptide for 2 h at 37°C, and washed twice in complete medium to remove free peptide.  $2 \times 10^4$  APCs and  $10^5$  CD8 $^+$  T cell clones were added to each well of 96-well round-bottom plates in a final volume of 200  $\mu\text{l}$ , and were cultured for 72 h, including pulse with 1  $\mu\text{Ci}$ /well [ $^3\text{H}$ ]thymidine for the last 18 h. Cells were harvested on a cell harvester (Tomtec) and incorporation of [ $^3\text{H}$ ]thymidine was determined using liquid scintillation counter (model 1205 Betaplate counter; LKB-Wallac).

**Cytokine Assay.** Cloned CD8 $^+$  T cells were stimulated with LCLs prepulsed with Tax11-19 peptide, as described in *Proliferation Assay*. After 48 h of incubation, supernatants were collected and tested for the presence of cytokines. IFN- $\gamma$  and IL-4 were quantified by capture ELISAs as described previously (20). Cytokine contents were calculated from a standard curve. The detection limits were 100 pg/ml for IFN- $\gamma$  and 10 pg/ml for IL-4.

**Immunoprecipitation and Western Blot.** TP60 T cells were incubated on ice with LCLs prepulsed with peptides, and warmed to 37°C for 5 min. The cells were lysed for 15 min on ice by the addition of cold  $2 \times$  lysis buffer (final concentration 1% Nonidet P-40, 150 mM NaCl, 25 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM sodium orthovanadate, 10  $\mu\text{g}/\text{ml}$  leupeptin, and 10  $\mu\text{g}/\text{ml}$  aprotinin). After clarification by centrifugation, detergent extracts were incubated with agarose-conjugated antiphosphotyrosine antibodies (Santa Cruz Biotechnology, Inc.) or anti-TCR  $\zeta$  chain antibody (Santa Cruz Biotechnology, Inc.) plus 25  $\mu\text{l}$  of protein A/G-agarose, as indicated, for at least 2 h at 4°C. Immunoprecipitates were separated by SDS-PAGE on 6–15% acrylamide gradient gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were probed with antibodies against phosphotyrosine (a gift from T. Roberts, Dana-Farber Cancer Institute, Boston, MA), a linker for activation of T cells (LAT), or PLC- $\gamma$ 1 (Santa Cruz Biotechnology, Inc.), followed by horseradish peroxidase-coupled secondary Ab (Amersham Biosciences), and developed by ECL<sup>TM</sup> Western blotting detection reagents (Amersham Biosciences) according to the manufacturer's instructions.

**Intracellular Staining of pMAPKs and p-c-Jun.** LCLs were labeled with CFSE and pulsed with peptides as described in *Cytotoxicity Assay*. T cell clones were cocultured with peptide-pulsed LCLs for indicated times. The cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized with permeabilization buffer (PBS containing 0.1% saponin, 0.1% sodium azide, and 1% FBS). Phospho-mitogen-activated protein kinases (MAPKs) and c-Jun were detected by sequentially incubating the cells with mouse anti-phospho-JNK mAb (G-7; Santa Cruz Biotechnology, Inc.), mouse anti-phospho-p44/42 MAPK mAb (E10; Cell Signaling Technology), rabbit anti-phospho-p38 Ab (Cell Signaling Technology), or mouse anti-phospho-c-Jun mAb (KM-1; Santa Cruz Biotechnology, Inc.), followed by biotinylated anti-mouse IgG (Vector Laboratories) or biotinylated anti-rabbit IgG (Jackson ImmunoResearch Laboratories), and finally by streptavidin-PE. Each of these incubation steps was for 30 min at room temperature with reagents diluted in permeabilization buffer and was followed by two washes in the same buffer. The level of phospho-MAPKs and c-Jun in T cells was analyzed on a flow cytometer (FACScan<sup>TM</sup>; Becton Dickinson) by excluding CFSE-labeled APCs.

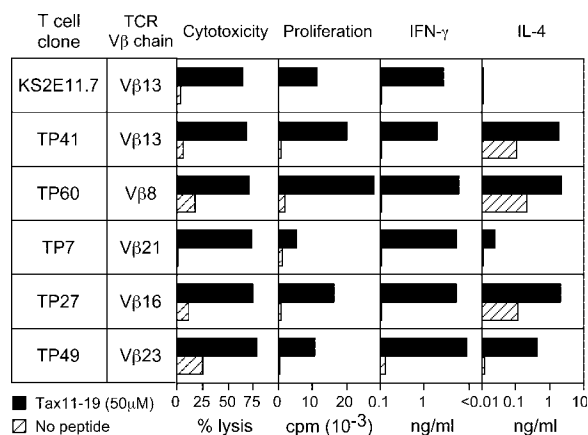
**Measurement of Calcium Flux.** T cell clones were loaded with 2 mg/ml Indo-1, AM (Molecular Probes) ester for 45 min at 37°C in complete medium. After washing with serum-free media, the

cells were mixed with peptide-pulsed LCLs at a cell ratio of 2:1 in a serum-free media. After a brief spin, the changes in FL4/FL5 ratio of the cells at 37°C were analyzed for 10 min using FACSVantage<sup>TM</sup> (Becton Dickinson). The baseline FL4/FL5 ratio was obtained by running Indo-1-labeled T cells alone for 1 min.

## Results

**Functional Characteristics of Tax11-19 Reactive CD8 $^+$  T Cell Clones.** Using an HLA-A2/Tax11-19 tetramer for the selection and expansion of antigen-specific CD8 $^+$  T cells, we generated a diverse clonal population of CD8 $^+$  T cells reactive to Tax11-19 from the peripheral blood of an HLA-A\*0201-expressing patient with HTLV-1-associated myelopathy (17). Five representative T cell clones established by this method and one T cell clone established previously by repeated antigenic stimulation were used in this work (18). The functional activities of these clones on the stimulation with cognate MHC/Ag (HLA-A\*0201-Tax11-19) were examined and summarized in Fig. 1. As expected, all of the T cell clones exhibited a high degree of cytotoxic activity accompanied by secretion of  $>1$  ng/ml of IFN- $\gamma$  in an antigen-specific fashion, whereas variable amounts of proliferation and IL-4 secretion were observed among different T cell clones (Fig. 1).

**The Same Antigenic Peptide Presented by APCs with Different HLA-A2 Subtypes Behaves Like an Altered Peptide Ligand (APL).** T cells can respond differently to recognition of variant peptides presented by the same MHC molecule (1, 21–23). As TCRs recognize the bimolecular complex composed of antigenic peptide and MHC molecule, we hypothesized that the same antigenic peptide presented on a variant MHC molecule could induce a partial agonist T



**Figure 1.** Functional characteristics of Tax11-19-specific T cell clones on the recognition of cognate MHC/peptide ligand and their TCR V $\beta$  chain usage. EBV-transformed B cells expressing HLA-A\*0201 incubated either with or without 50  $\mu\text{M}$  Tax11-19 peptide were used as target cells or APCs. The  $^{51}\text{Cr}$  release assay was performed using 10:1 E/T ratio for the cytotoxic activity. Proliferative activity was assessed by [ $^3\text{H}$ ]thymidine incorporation. IFN- $\gamma$  and IL-4 were measured from culture supernatants by capture ELISA after 48 h of incubation and the detection limits were 100 and 10 pg/ml for IFN- $\gamma$  and IL-4, respectively. TCR V $\beta$  chain usage was determined by indirect immunofluorescence staining using specific mAb.

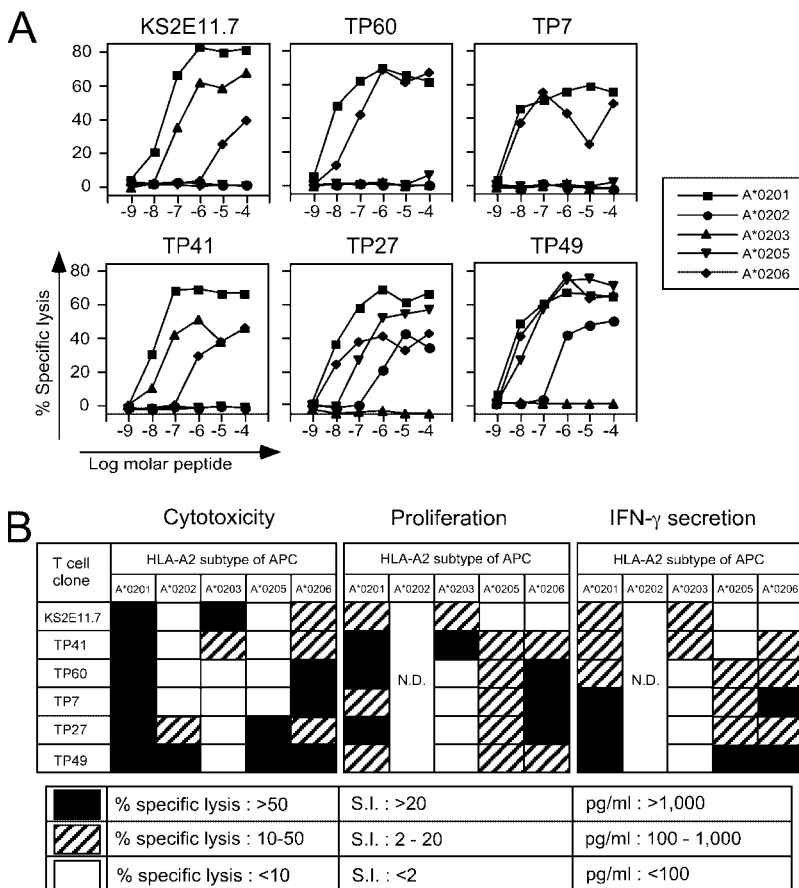
**Table I.** Summary of Amino Acid Sequences at Polymorphic HLA-A2 Positions

| HLA-A2 subtype | Amino acid sequence at a specific position |    |    |           |     |     |
|----------------|--|----|----|-----------|-----|-----|
|                | α1 domain                                  |    |    | α2 domain |     |     |
|                | 9  | 43 | 95 | 149       | 152 | 156 |
| A*0201         | F  | Q  | V  | A         | V   | L   |
| A*0202         | F  | R  | L  | A         | V   | W   |
| A*0203         | F  | Q  | V  | T         | E   | W   |
| A*0205         | Y  | R  | L  | A         | V   | W   |
| A*0206         | Y  | Q  | V  | A         | V   | L   |

cell response. To test this possibility, we examined the functional responses of Tax11-19 reactive CD8<sup>+</sup> T cell clones after antigen stimulation using LCLs expressing different subtypes of HLA-A2 as follows: A\*0201, A\*0202, A\*0203, A\*0205, and A\*0206. These HLA-A2 subtypes have one to four amino acid differences at specific positions, which are summarized in Table I.

The peptide-antigen dose-response curves measuring cytotoxic activity of six T cell clones against LCLs expressing

different HLA-A2 subtypes showed a diverse pattern. As shown in Fig. 2 A, all of the T cell clones elicited a similar dose-response cytotoxic activity against LCLs expressing HLA-A\*0201, with peptide concentrations inducing half maximal response ranging from 3 nM to 27 nM. LCLs expressing HLA-A\*0206, which differs by only one amino acid from A\*0201, were also lysed in a similar dose-response manner by all T cell clones, except for TP41 and KS2E11.7, which needed 10–100 times more peptide for the same level of cytolysis. Interestingly, the cytolysis pattern of LCLs expressing other HLA-A2 subtypes, which differ by three or four amino acids, was quite different depending on the individual T cell clones. HLA-A\*0202 and A\*0205 loaded with Tax11-19 peptide induced cytotoxic activity from the TP27 and TP49 T cell clones, but not from other T cell clones. In contrast, LCLs expressing the HLA-A\*0203 subtype were lysed only by the TP41 and KS2E11.7 T cell clones, requiring a 10-fold higher dose of peptide compared with A\*0201 (Fig. 2 A). Fig. 2 B summarizes the functional responsiveness including cytotoxicity, proliferation, and IFN-γ secretion of T cell clones when they were stimulated by peptide complexed with different subtypes of HLA-A2. Overall, A\*0203 subtype did not elicit any functional responsiveness in four T cell clones, whereas A\*0206 induced cytotoxic activities with or without other functions in all of the T cell clones. Unexpectedly, the A\*0205 subtype induced proliferation with



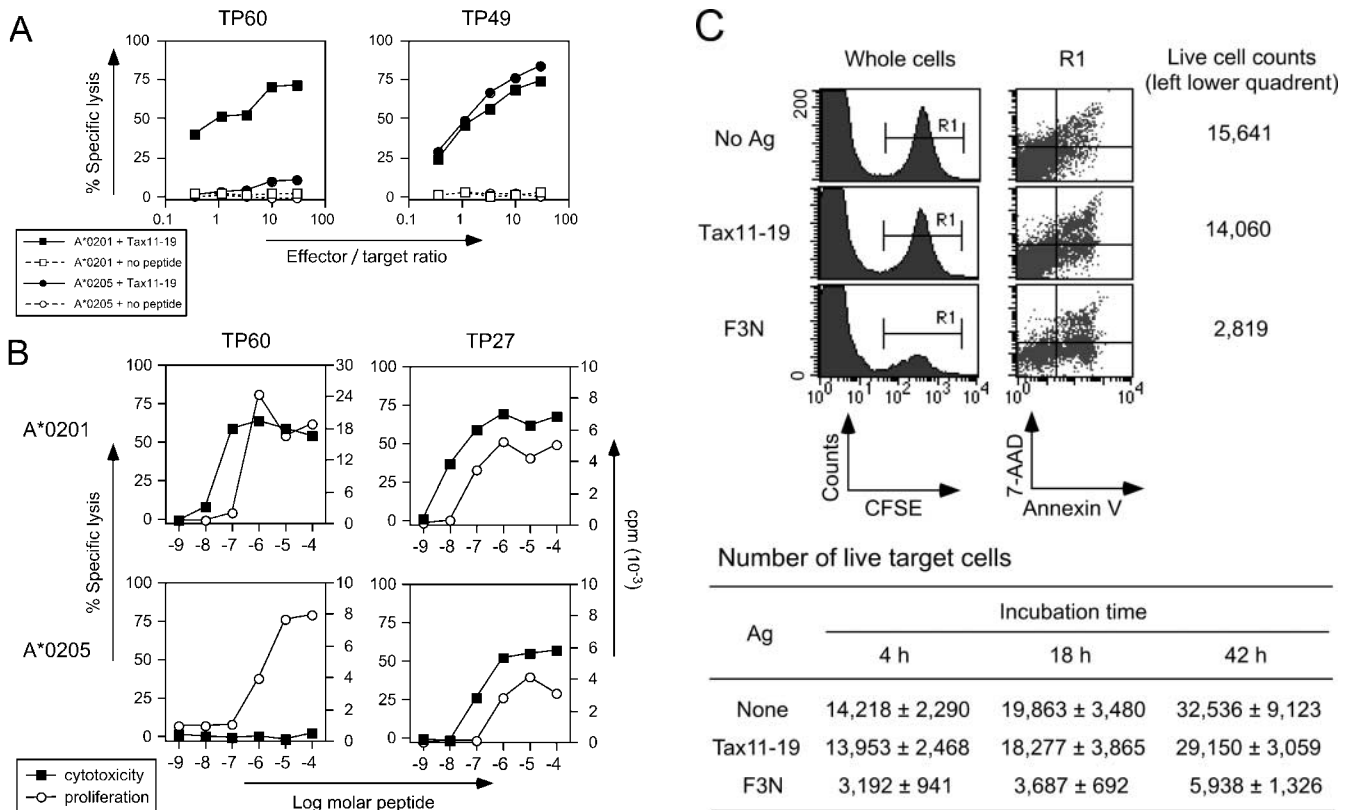
**Figure 2.** Tax11-19-HLA-A\*0201 reactive T cell clones were differentially restricted by HLA-A2 subtypes. (A) Peptide dose-response cytotoxic activities of T cell clones. LCLs were pulsed for 2 h at different peptide concentrations and used as targets in a <sup>51</sup>Cr release assay. The E/T ratio was 10:1 and spontaneous release of <sup>51</sup>Cr was <20%. The lysis of peptide nonpulsed cells was <5%, and SEM of triplicate wells were <10%. (B) Summary of CD8<sup>+</sup> T cell clone functions with Tax11-19 presentation by different HLA-A2 subtype molecules. LCLs expressing different HLA-A2 subtypes were loaded with 50 μM Tax11-19 and used as target cells or APCs. Cytotoxic activity was tested by <sup>51</sup>Cr release assay at an E/T ratio of 10:1. IFN-γ was measured in culture supernatants by ELISA after 48 h of incubation and the detection limit was 100 pg/ml. Proliferation was determined by 18 h [<sup>3</sup>H]thymidine incorporation assay at the end of a 72-h culture and expressed as S.I. (stimulation index). N.D., not determined.

or without IFN- $\gamma$  secretion in three out of four T cell clones that did not show cytotoxic activity to this HLA-A2 subtype. Thus, different functional responses of T cell clones to the same antigenic peptide presented by MHC class I molecules, which differ at only a few amino acids, mimic those induced by different antigenic peptides (full agonist, weak agonist, and partial agonist) presented on the same MHC class I molecule.

It was possible that these observations could be partially explained by differences in the binding affinity of the Tax11-19 peptide to each of the HLA-A2 subtypes. However, Tax11-19 peptide has similar binding affinity to HLA-A\*0201, A\*0202, and A\*0205 (24). In addition, the identical dose-response curves for cytotoxic activity of TP49 between A\*0201 and A\*0206, and <1 log difference between A\*0201 and A\*0203 for TP41 and KS2E11.7 suggest that Tax11-19 peptide binds well to these HLA-A2 subtypes (Fig. 2 A).

*Dissociation of T Cell Function with Stimulation by Tax11-19–HLA-A\*0205.* Many papers have demonstrated that cytotoxic activity can be easily induced in CTLs with weak

stimuli, whereas stronger stimuli are needed to induce proliferation or cytokine secretion of T cells (14, 15, 25). However, three T cell clones proliferated in response to Tax11-19 presented by the HLA-A\*0205 subtype even though they did not exhibit any cytotoxic activity (Fig. 2 B). To further explore the selective cytotoxic activity in this panel of T cell clones, we reevaluated their cytotoxic activity using increasing E/T ratios and antigen dose. A representative noncytotoxic T cell clone (TP60) did not lyse LCL target cells expressing HLA-A\*0205 regardless of increasing E/T ratio or antigen dose, whereas representative cytotoxic clones (TP49 and TP27) showed typical responses to increasing E/T ratio and antigen dose (Fig. 3, A and B). The TP60 T cell clone exhibited a brisk proliferative response to Tax11-19 presented by HLA-A\*0205 in an antigen dose-dependent manner, in contrast with their total lack of cytotoxic activity (Fig. 3 B). In these experiments, cytotoxicity was assessed after a 4-h incubation, whereas proliferation was measured after a 3-d incubation. Some peptide ligands have been shown to induce cytotoxicity at late time points, mainly by a Fas-mediated mechanism with



**Figure 3.** Tax11-19 presented by HLA-A\*0205 did not induce cytotoxic activity from a subset of T cell clones, even at a high E/T ratio, high antigenic peptide concentrations, and delayed time points. (A) Specific lysis of  $^{51}\text{Cr}$ -labeled LCLs expressing either HLA-A\*0201 or A\*0205 pulsed with 50  $\mu\text{M}$  Tax11-19 by representative T cell clones was examined at various E/T ratios. (B) Cytotoxic and proliferative activity of T cell clones in response to various concentration of Tax11-19 presented by HLA-A\*0201 (top) or A\*0205 (bottom) were determined at a 10:1 E/T ratio by  $^{51}\text{Cr}$  release assay and [ $^3\text{H}$ ]thymidine incorporation assay. Cytotoxicity (closed squares) is shown on the left y-axis and proliferation (open circles) on the right y-axis. (C) Delayed cytotoxicity was examined by counting the viable target cells at late time points as described in Materials and Methods. One representative staining profile obtained after an 18-h incubation is shown. Results from three independent experiments are summarized in the bottom panel, displaying average cell number  $\pm$  SD at each time points. There was no statistical significance ( $P > 0.05$ ) in viable target cell numbers between antigen-unpulsed and Tax11-19-pulsed conditions at any time points, as determined by Student's  $t$  test.

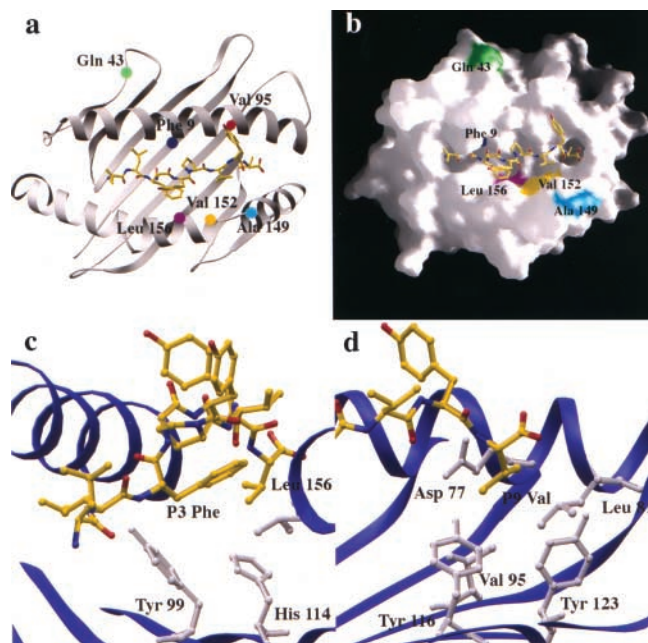
an inability to induce early perforin/granzyme-mediated cytotoxicity (26). LCLs used in our paper express Fas on their surface (unpublished data). Therefore, we examined whether HLA-A\*0205-presenting Tax11-19 peptide could induce delayed cytotoxic activity from T cell clones. Due to the high background in a traditional chromium release assay at late time points, we used live target cell counts to measure delayed cytotoxicity (Fig. 3 C). The results obtained using this approach were comparable to results with the chromium release assay at the 4-h time point when Tax11-19 presented by HLA-A\*0205 did not induce target cell loss. In contrast, the F3N peptide presented by HLA-A\*0205 induced a 78% decrease in viable target cell number, which was also detected in chromium release assay (see Fig. 5). After longer incubation with effector T cells up to 42 h, no statistically significant differences in viable target cell numbers were detected between antigen-unpulsed and Tax11-19-pulsed target conditions (Fig. 3 C). In these analyses, differential target cell division affecting the final viable cell number could be excluded because the CFSE dilution pattern of target cells was similar between these two conditions. These data indicate that HLA-A\*0205 presentation of Tax11-19 peptide does not induce cytotoxic activity from certain T cell clones at either early or late time points in contrast to its ability to induce late proliferative activity. One possible explanation for this novel functional dissociation might be the engagement of inhibitory NK receptors (27). However, we could not observe any blocking effects by anti-HLA B, C, or G antibodies, and T cell clones were not stained with a panel of antikerler inhibitory receptor, anti-CD94, and anti-CD85 antibodies (unpublished data). Thus, the engagement of NK receptors was unlikely to be responsible for this functional dissociation.

**Polymorphic Amino Acids at the Peptide-binding Site of HLA-A2 Molecules Can Affect Functional Responsiveness of T Cell Clone.** The different functional activities of T cells activated by peptide presentation with different HLA-A2 subtypes may have occurred either from differences on the MHC residues in direct contact with the TCR or alternatively from the alterations of the peptide structure buried in the MHC molecule. To address this issue, we examined which of the polymorphic amino acid residues associated with allelic variation of HLA-A2 were most likely to have a direct effect on TCR recognition inducing functional dissociation. This work was based on the structural data of the trimolecular complex (A6 TCR, HLA-A\*0201, and Tax11-19 peptide) resolved by X-ray crystallography (6, 28).

As shown in Fig. 4 (a and b), the amino acid residue at position 9 of HLA-A2 is located in the B-pocket of groove. However, this position alone is unlikely to affect the TCR recognition of Tax11-19 because all of the T cell clones showed similar recognition pattern to both A\*0201 and A\*0206, which have identical amino acid sequence except for this position. The amino acid residue at position 43 of HLA-A2 is located in a loop connecting  $\beta$  sheets, not in the vicinity of peptide or TCR (Fig. 4, a and b), so this position is unlikely to have an effect on TCR recognition.

The amino acid residue 95 is situated at the base of the groove (F-pocket), but is not in direct Van der Waals contact with the peptide (Fig. 4, a and d). A polymorphism at this position could have an indirect effect on bound peptide structure, although the conservative Val to Leu substitution at this position is unlikely to have a dramatic effect on the conformation of the bound peptide. Finally, residue 156 is within the Van der Waals radius of the P3 side chain of the Tax peptide (Fig. 4 c), so the substitution of Leu to a bulky Trp is more likely to affect the structure of the peptide bound in the MHC molecule and, thus, indirectly affect TCR recognition. This analysis demonstrates that polymorphic amino acids in the peptide binding site of HLA-A2 molecule, particularly residue 156, are likely to affect functional activity of T cell clones.

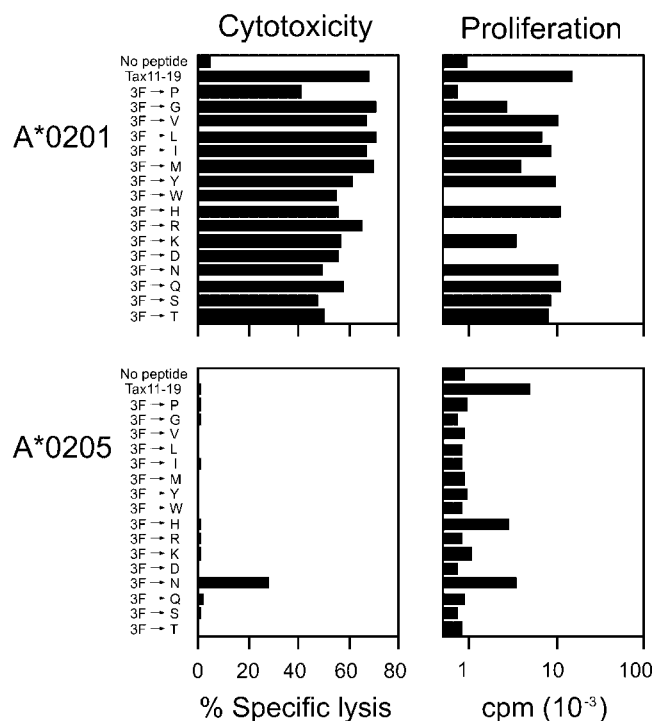
**Single Amino Acid Substitution at the Corresponding Position of Tax11-19 Can Revert the Altered MHC to Agonistic MHC.** Our analysis suggested that amino acid changes at the peptide-binding site of the MHC pocket can convert the agonistic MHC-peptide ligand to partial agonistic MHC-peptide ligand in some T cell clones. If this is the case, it should be possible to identify single amino acid substitutions on the antigenic peptide that bind the relevant HLA-A2 D-pocket and revert the partial agonistic MHC-peptide ligand to an agonistic MHC-peptide



**Figure 4.** Location of HLA-A2 subtype polymorphic residues. (a and b) The HLA-A2-Tax11-19 complex viewed from above the peptide binding groove. The positions of the HLA-A2 subtype polymorphic residues shown in Table I are marked on the MHC. Residue 9, dark blue; residue 43, green; residue 95, red; residue 149, cyan; residue 152, yellow; and residue 156, magenta. The peptide is shown in yellow. (c and d) The HLA-A2-Tax11-19 complex viewed from within the peptide-binding groove. The peptide is shown in yellow and the MHC is in blue. (c) The location of polymorphic residue 156 in the D-pocket. (d) The location of polymorphic residue 95 in the F-pocket. a, c, and d were drawn using RIBBONS (44); b was generated with Grasp (45).

ligand. This was examined by investigating the responsiveness of the T cell clone to the Tax peptide with a single amino acid substitution at P3 position, which engages the D-pocket of HLA-A2 molecule. Remarkably, a Phe to Asn substitution at P3 induced cytotoxic activity against the A\*0205 target cells by TP60 with maintenance of proliferative activity (Fig. 5). TP7 also showed the same but weak functional restoration of cytotoxicity against A\*0205 target cells when Phe was substituted with Met (unpublished data). The requirement for different amino acid substitutes for the restoration of T cell function may be related to different TCR structures between these two T cell clones as revealed by different V $\beta$  chain usage (Fig. 1) and different CDR3 region sequences (19). This result demonstrates that depending on individual TCR fine specificity, changing the P3 position of Tax11-19 peptide engaging the D-pocket, which was altered by the Leu to Trp change, resulted in a gain of function which reverted a partial agonistic MHC-peptide complex to an agonistic MHC-peptide complex.

*Induction of Weak Early Signaling Events but Sustained c-Jun Phosphorylation by MHC-altered Peptide Ligand (MAPL).* We further investigated this novel functional dissociation by analyzing the intracellular signaling pathways activated upon stimulation. Previous papers indicate that partial ago-

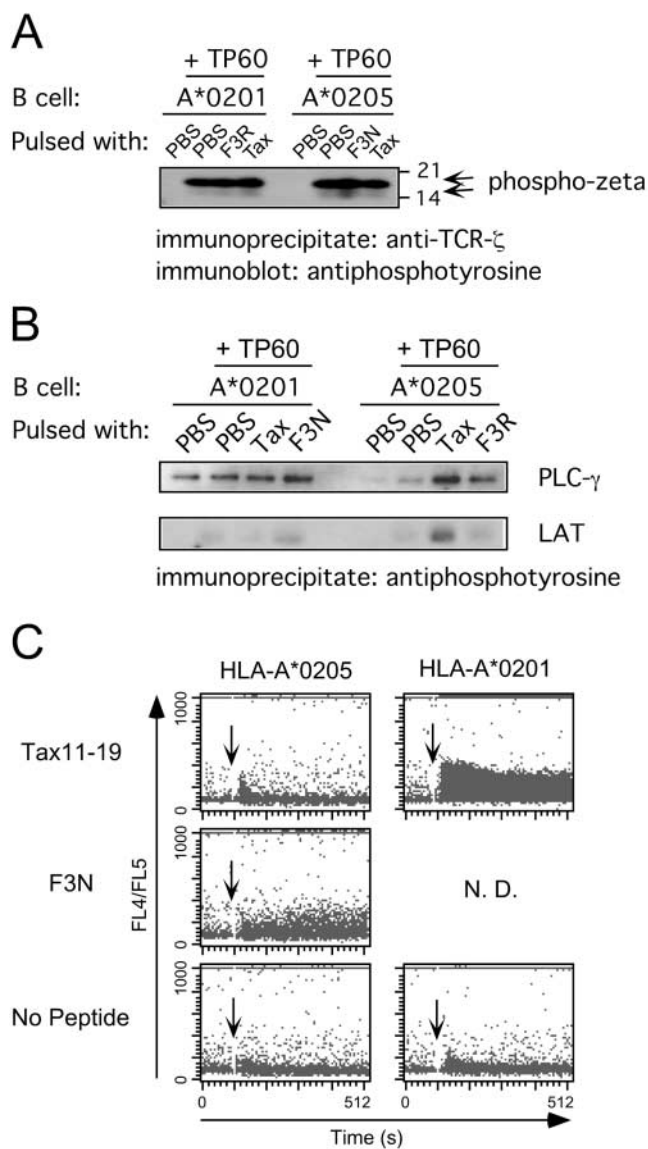


**Figure 5.** Cytotoxic and proliferative responses of TP60 to Tax11-19 peptide analogues with single amino acid substitutions at P3 position presented by HLA-A\*0201 or A\*0205. LCLs expressing HLA-A\*0201 or A\*0205 were pulsed with 10  $\mu$ M Tax11-19 peptide analogues and were used as target cells or APCs. Cytotoxicity was measured by a  $^{51}\text{Cr}$  release assay. The E/T ratio was 10:1. Proliferative activity of T cell clones was measured by [ $^3\text{H}$ ]thymidine incorporation assay.

nists can trigger only a subset of early T cell signals (29–31). Therefore, first we examined phosphorylation status of TCR- $\zeta$  chain after stimulation with different ligands. TP60 cells incubated with either unpulsed KS.B or DAH cells showed some level of constitutively phosphorylated TCR- $\zeta$ , possibly due to the basal activation status of cloned T cells. There was an increase in TCR- $\zeta$  phosphorylation after incubation with the agonistic ligands, Tax11-19–HLA-A\*0201 or F3N/HLA-A\*0205. However, incubation of TP60 cells with DAH cells pulsed with Tax11-19 did not significantly enhance the intensity of TCR- $\zeta$  phosphorylation (Fig. 6 A). In these experiments, we could not detect discrete bands of phosphorylated TCR- $\zeta$  chain even with the strong agonistic stimulation, which has been described previously in human as well as murine T cells (15, 29). This difference might come from the use of different antibodies and/or different experimental approaches.

We further traced downstream signaling pathways. Consistent with the phosphorylated TCR- $\zeta$  chain profile, the appearance of the phosphorylated form of ZAP-70 was not detected after stimulation with Tax11-19–HLA-A\*0205 (unpublished data). Phosphorylated LAT was also barely detectable under this stimulatory condition (Fig. 6 B). Exploring one of the major downstream signaling pathways after recruitment of the adaptor molecules, LAT and SLP-76, we found weakly phosphorylated PLC- $\gamma$ 1 accompanied by a very weak and transient pattern of  $\text{Ca}^{2+}$  flux after stimulation with Tax11-19–HLA-A\*0205 (Fig. 6, B and C). As predicted by the functional assays, F3N/HLA-A\*0205 induced activation of TCR- $\zeta$ , LAT, and PLC- $\gamma$ 1 albeit to a lesser extent than the strong agonistic ligand (Tax11-19–HLA-A\*0201; Fig. 6, A and B). Another ligand, F3R/HLA-A\*0201, induces a classical form of partial agonism, cytotoxicity without proliferation, from TP60 T cells (Fig. 5). Interestingly, this ligand induced greater activation of all the signaling pathways we examined as compared to Tax11-19–HLA-A\*0205 but less than F3N/HLA-A\*0205 (Fig. 6, A and B, and unpublished data).

Another major signaling pathway in T cell activation is the Ras–MAPK pathway (32). When we examined phosphorylated MAP kinases, including ERK1/2, p38 MAPK, and JNK, we could not detect the activation of these kinase pathways after stimulation with Tax11-19–HLA-A\*0205 (Fig. 7). However, a high amount of phosphorylated c-Jun (described previously as one of the signaling intermediates; reference 33) was detected for a prolonged time period, up to 42 h after stimulation with Tax11-19–HLA-A\*0205 with delayed kinetics even though upstream active kinase, pJNK, could not be detected at any time points (Fig. 7). In contrast, a higher amount of p-c-Jun was induced initially but reverted to background levels within 18 h after stimulation with a classical form of APL, F3R/HLA-A\*0201 (Fig. 7). In total, these data demonstrate that Tax11-19–HLA-A\*0205 cannot trigger TP60 TCR strongly enough to induce early signaling cascades, but can induce the accumulation of signaling intermediates by trickle through the weak early activation cascade.



**Figure 6.** Activation of early signaling molecules and  $\text{Ca}^{2+}$  flux are not efficiently induced in TP60 T cells by Tax11-19-HLA-A\*0205. (A) Anti-TCR  $\zeta$  immunoprecipitates were prepared from lysates made from  $10^7$  KS.B (HLA-A\*0201) cells alone,  $10^7$  DAH (HLA-A\*0205) cells alone, or from  $2 \times 10^7$  TP60 cells stimulated for 5 min with KS.B/PBS, KS.B/F3R, KS.B/Tax11-19, DAH/PBS, DAH/F3N, or DAH/Tax11-19. Tyrosyl-phosphorylated  $\zeta$  chain was detected by immunoblotting with antiphosphotyrosine antibody and the proteins were visualized by ECL. (B) Antiphosphotyrosine immunoprecipitates were prepared from lysates from  $10^7$  DAH cells alone,  $10^7$  KS.B cells alone, or from  $2 \times 10^7$  TP60 cells stimulated for 5 min with DAH/PBS, DAH/Tax11-19, DAH/F3N, KS.B/PBS, KS.B/Tax11-19, or KS.B/F3R. PLC- $\gamma$  (top) or LAT (bottom) was detected by immunoblotting and the proteins were visualized by ECL. (C) TP60 T cells were stimulated with  $10 \mu\text{M}$  of each peptide presented by KS.B or DAH and  $[\text{Ca}^{++}]_i$  increase in T cells was monitored. Arrows indicate the addition of peptide-pulsed APCs. One representative experiment of four is shown. N.D., not determined.

## Discussion

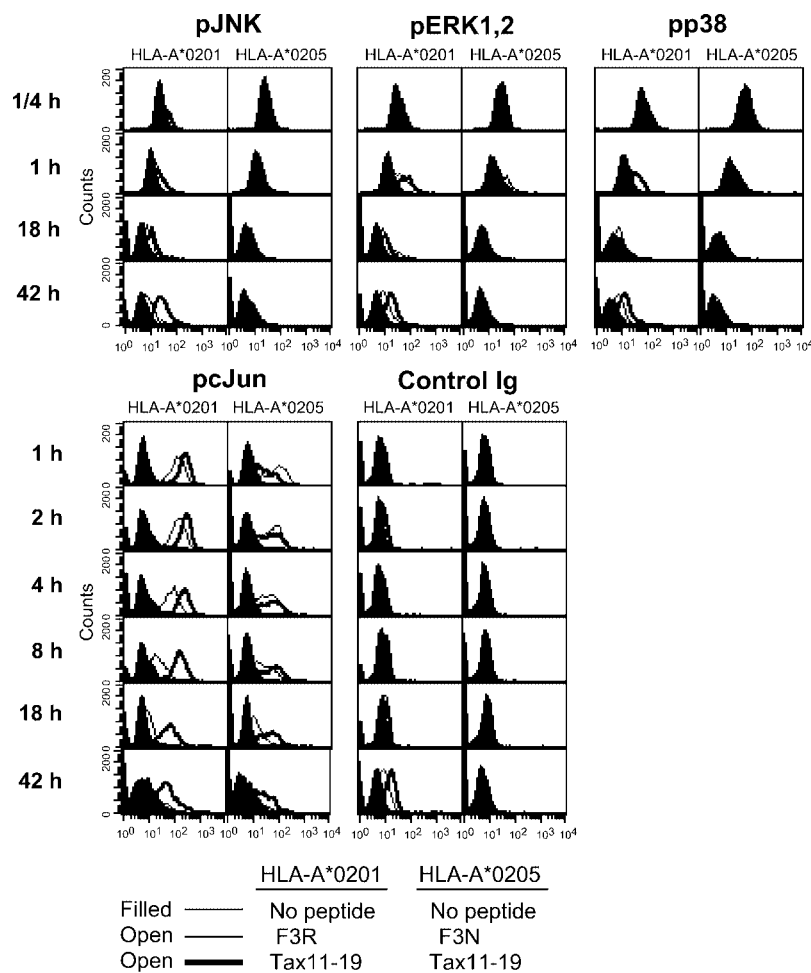
The same antigenic peptide presented by different MHC subtypes with subtle differences in MHC sequence induced partial agonism of T cell function, including an atypical

functional dissociation. This may have occurred either from differences in the solvent-exposed MHC residues in direct contact with the TCR or alternatively from the alterations of the peptide structure buried in the MHC molecule. Crystallographic analysis of the peptide binding to HLA-A2 revealed that whereas the terminal anchor side chains of antigenic peptides were bound similarly in the MHC structure, the conformations can be very different in the center of the binding site. This has led to the conclusion that the peptide itself dominates the antigenic identity of the MHC-peptide complex through a unique sequence-dependent conformation that is imposed by the relatively fixed MHC structure (2). As APLs have been identified as partial T cell agonists, it might be predicted that subtle variations of MHC sequence inducing changes in the peptide conformation available to the TCR, might also induce partial agonism for T cell functions. Examination of the crystal structure of the trimolecular Tax11-19-HLA-A\*0201-A6 TCR complex, in combination with our functional studies, reveals that changes in predominantly the D- and possibly F-pockets found with different HLA-A2 subtype are associated with partial agonist function of the MHC-peptide complex. Thus, we demonstrate that MAPLs can induce partial agonism of T cell function similar to APLs.

It has been shown that each MHC pocket can contribute to T cell recognition of peptides in an alloresponse (34, 35). Thus, a subpopulation of cytotoxic T cells can recognize all MHC subtypes capable of binding an antigenic peptide, whereas others are subtype specific. These published data are consistent with the differences among T cell clones in recognizing the Tax11-19 peptide in the context of different HLA-A2 subtypes we described here. The ability of MHC class II molecules to change the nature of the antigenic response has been suggested previously (36). Germain and colleagues demonstrated that a peptide presented on a mutant MHC class II molecule will induce a response that is different from the response induced with the native MHC molecule (36). In this model, the response of a Th1 type murine T cell clone to mutant  $\text{E}\alpha\text{E}\beta^k$  molecules revealed that peptide inhibited the IL-2 response to an allostimulatory mutant form of the MHC class II molecule. Although the mutated MHC molecule induced an alloreactive response that was altered by the antigen peptide, it is not clear if a similar mechanism as observed in the present paper was operative. Similarly, Doherty et al., also investigating MHC class II restricted responses, demonstrated that multiple MHC alleles restricted T cell responses in a degenerative fashion (37). Our investigation demonstrates that the nature of the functional response to viral peptides is similarly altered by changes in the peptide-binding pockets of MHC molecules.

Hierarchies have been observed among partial antigenic peptide agonists for inducing different T cell functions. Examination of MHC class I restricted APLs has revealed that  $\text{CD8}^+$  T cell cytotoxicity has been perhaps the most sensitive indicator of T cell function, with induction of cell cycle and proliferation requiring high strengths of signals (14, 15). Thus, it was surprising that alterations of peptide struc-





**Figure 7.** Phospho-MAPKs are not induced but phospho-c-Jun is slowly induced and sustained in TP60 T cells after stimulation with Tax11-19–HLA-A\*0205. TP60 T cells were cocultured with LCLs prepulsed with 10  $\mu$ M of indicated peptides. After incubation for the indicated times, cells were fixed and intracellularly stained for phospho-MAPKs and c-Jun. Expression levels of phospho-MAPKs and c-Jun were analyzed in T cells with the exclusion of LCLs as described in Materials and Methods. One representative experiment of four is shown.

ture imposed by a few amino acid changes in the MHC could result in the complete loss of cytotoxic function, yet retain proliferation. These MAPLs also differentially affected cytokine secretion. The loss of cytotoxicity imposed by A\*0205 presentation of Tax11-19 could not be secondary to particular characteristics of the APC because single amino acid mutations of the Tax11-19 peptide at P3, which is predicted to engage the D-pocket and to induce conformational changes in the peptide ligand, could restore cytotoxic function.

Cytotoxicity requires some of the early signaling events leading to microtubule reorganization for granule exocytosis, whereas proliferation depends on gene expression for the secretion of growth factors and their receptors (38). However, the specific intracellular signaling events required for these functional activities are still poorly understood. Our investigation reveals that MAPL induces weaker or negligible early signaling events, including TCR- $\zeta$  chain phosphorylation, LAT phosphorylation, and calcium flux as compared with the common type of APL signaling events. In contrast, MAPL provoked sustained phosphorylation of c-Jun, which was not observed with APL stimulation. If these signaling events are directly linked to the functional activity of T cells, it is highly possi-

ble that even though Tax11-19–HLA-A\*0205 ligand cannot trigger TP60 TCRs strongly enough to overcome the threshold for cytotoxicity, which depends mainly on the early signaling events such as calcium flux or PKC activity (39–41), it can engage TCRs for a long enough time to generate sustained signaling intermediates critical for proliferative activity. This explanation is consistent with revised kinetic proofreading model for T cell activation recently proposed by Rosette et al. (33). According to this model, low-affinity TCR ligands that cannot efficiently induce early activation events allow late T cell responses by the accumulated signals that “trickle through” the early activation cascade.

In summary, we demonstrate that changes in the MHC pocket associated with alterations of peptide structure change the hierarchy of T cell effector function. Reciprocal amino acid changes in the antigenic peptide engaging the MHC pocket results in a gain of function mutation. These data provide evidence for a role of the MHC molecule in providing a scaffolding that subtly changes T cell effector function depending on the peptide conformation in the MHC groove. Thus, MHC restriction should not be regarded as simply a binary event, resulting in a positive or negative response; instead, subtle variations found among

MHC alleles can induce a new level of diversity in functional immune responses. What are the potential immunologic consequences of MAPL? It has been shown that co-presentation of naturally occurring APLs by the same APC can induce antagonism of T cell function (42, 43). As each MHC class I molecule has two allelic forms expressed on the surface, we postulate that in some instances, the same peptide presented in the context of two different MHC molecules may be recognized as MAPLs, resulting in either different functional outcomes or direct antagonism of T cell function.

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