A Partially Modified Retro-Inverso Pseudopeptide Modulates the Cytokine Profile of CTL Specific for an Influenza Virus Epitope

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A Partially Modified Retro-Inverso Pseudopeptide Modulates the Cytokine Profile of CTL Specific for an Influenza Virus Epitope

Marina Ostankovitch,* Gilles Guichard,† Francine Connan,* Sylviane Muller,† Aude Chaboissier,* Johan Hoebeke,† Jeannine Choppin,* Jean-Paul Briand,† and Jean-Gérard Guillet*

There is considerable evidence that peptides corresponding to MHC class I-restricted epitopes can be used as immunogens or immunomodulators. Pseudopeptides containing isosteric replacements of the amide bond provide more stable analogues, which may even have enhanced biologic activity. But there have been very few studies on the use of pseudopeptides to initiate or modulate the cellular immune response. This study describes the immunogenicity of a partially modified retro-inverso pseudopeptide of an influenza virus epitope and shows that this pseudopeptide modulates the cytokine profile expressed by CD8+ CTL generated from primed precursors. Moreover, the pseudopeptide is much more efficient at low concentration than the wild-type epitope to stimulate IFN-γ secretion by CD8+ T effector cells. These results are analyzed with reference to changes in the conformation of the MHC molecule/peptide complex deduced from molecular modeling. The findings support the idea that partially modified retro-inverso analogues can be used as altered peptide ligands to enhance the stimulation of natural epitope-specific CTL and to modify their functional properties. Hence, pseudopeptide ligands might be promising tools for use in immunotherapy. The Journal of Immunology, 1998, 161: 200–208.

CD8+ CTL play a major role in the clearance of viruses by recognizing viral peptides bound to MHC class I molecules via the TCR. The peptides in the MHC class I/peptide complex are in an extended conformation and are buried in the MHC binding site, so that only a few side chains of the peptide are accessible for recognition by the TCR (1, 2). T cells are activated after TCR aggregation, but signaling via the TCR may also require conformational changes (3). The TCR are responsible for specific Ag recognition, while the multivalent invariant CD3 chains are involved in transducing activation signals (4). Recent studies indicate that the engagement of the TCR by analogues of immunogenic peptides containing single amino acid substitutions can lead to T cell antagonism or partial activation, and suggest that a variety of signals can be induced, depending on the degree of phosphorylation of the CD3 chains (5).

Recent advances have greatly facilitated the use of peptides corresponding to MHC class I-restricted epitopes as immunogens or immunomodulators. But peptide-based immunotherapy is limited by the great susceptibility of peptides to proteolysis. Pseudopeptides that contain isosteric replacements of the amide bond have been used extensively in pharmacology to provide more stable analogues, some with biologic activity (6). This approach has also led to the discovery of compounds that are potent antagonists or partial agonists. It has been demonstrated that retro-inverso peptidomimetics and reduced peptide bond pseudopeptides can have antigenic and immunogenic properties (7–11) and may be useful in the development of synthetic vaccines. In contrast to research on humoral immune response, there have been very few studies on the use of pseudopeptides to initiate or modulate cellular immune responses (12).

We have shown that partially modified retro-inverso (PMRI)3 pseudopeptides derived from the M58–66 epitope of the influenza virus matrix protein bind well to the HLA-A2 molecule (13). The present study documents the immunogenicity of such a pseudopeptide, PMRI 58–59, and compares it with that of the wild-type peptide. PMRI 58–59-specific CTL effectors were generated from CD8+ T cell-primed precursors, and the profiles of gene coding for cytokines expressed by the CD8+ CTL lines were studied. We have also analyzed the ex vivo secretion of IFN-γ by CD8+ effector T cells from PBMC stimulated with each of the three peptides using a highly sensitive enzyme-linked immunospot (ELISPOT) assay. The results are discussed with reference to changes in the conformation of the MHC/peptide complex deduced from molecular modeling. Our analysis of the pseudopeptide-induced cellular immune response supports the idea that pseudopeptides can be used as altered peptide ligands to modify the functional quality of CTL and to enhance the stimulation of natural

3 Abbreviations used in this paper: PMRI, partially modified retro-inverso; ELISPOT, enzyme-linked immunospot.
Table I. Analogues of peptide M58–66 from the influenza A virus matrix

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tr>
<td>M58–66</td>
<td>H2N-(\text{Leu-Gly-Phe-Val-Phe-Thr-Leu-OH})</td>
</tr>
<tr>
<td>[Leu59]M58–66</td>
<td>H2N-(\text{Leu-Gly-Phe-Val-Phe-Thr-Leu-OH})</td>
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*Abbreviation: PMRI 58–59.*

epitope-specific CTL. This type of approach may be useful for CTL-based immunotherapy.

**Materials and Methods**

**PBMC of an HLA-A2 healthy donor**

A leukapheresis sample was taken from a healthy HLA-A2 subject, who had given his informed consent, at the Cochin Hospital blood center (Paris, France). The PBMC were separated out on a gradient of Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden).

**Viruses**

Influenza virus strains A/Bangkok (A/Ban) and B/Singapore (B/Sin) were grown in the allantoic cavity of embryonated chicken eggs by C. Hannoun (Institut Pasteur, Paris, France).

**Synthetic peptides**

Peptide M58–66 (GILGFVFTL) was derived from amino acid sequences of the influenza A virus matrix, and NP383–391 (SRYWAIRTR) from the virus nucleoprotein. Peptide Pol476–484 (ILKEPVHGV) was derived from the polymerase of HIV. These peptides were synthesized by Neosystem (Strasbourg, France).

The PMRI analogue derived from the wild-type epitope M58–66 contained a leucine at residue 59, and the peptide bond between residues 58 and 59 changed to CO-NH instead of NH-CO (Table I). It ([Gly58-\(\Phi(NH-CO)\)-Leu59] M58–66) was synthesized by conventional methods in solution. The retro-inverso modification was obtained by replacing Gly58 with the corresponding gem-diaminoalkyl residue, and Leu59 by the malonate derivative. Details of the synthesis have been described (13). This compound was characterized by 500 MHz \(^1\)H-NMR (nuclear magnetic resonance) spectroscopy, fast atom bombardment spectrometry (FAB-MS (m/z); 966.5 [M\(^+\)H\(^+\)]), and analytic HPLC using a linear gradient of A: 0.1% aq. trifluoroacetic acid, and B: MeCN (HPLC t\(_R\) = 11.92, 20–80% B in 20 min). The PMRI 58–59 was more than 95% pure, as assessed by analytic HPLC. The peptide containing leucine at position 59 ([Leu59] M58–66) was considered to be the parent peptide (Table I). This peptide was synthesized by solid-phase methodology using Fmoc chemistry. Protease digestion assay using the parent peptide and the PMRI 58–59 incubated in fresh mouse serum (diluted 1/2) and analyzed by HPLC showed that the t\(_{1/2}\) of PMRI 58–59 was twofold higher (23 min) than that of the parent peptide (11 min).

**Assembly and stability of peptide/HLA-A2 complexes**

The capacity of peptides to promote assembly of HLA-A2 molecules was tested as previously described (14). Aliquots of T2 cells (8 \(\times\) 10\(^3\)) were lysed in 64 \(\mu\)l Tris-buffered saline, pH 7.5, containing 1% Nonidet P-40 plus protease inhibitors. The cells were lysed in Eppendorf microfuge tubes with different concentrations of exogenous peptide, and incubated at 20°C for 2 h, and then at 4°C for 23 h. The stability of the MHC complexes was assessed by incubation at 37°C for 24 h. Stable HLA-A2 complexes were detected by incubation with the HLA-A2-specific mAb BB7.2 (American Type Culture Collection, Manassas, VA) overnight at 4°C (for assembly analyses) or for 1 h at 37°C (for stability analyses). The wells of microtiter plates were coated with Ig BB7.2 (1 \(\mu\)g in 100 \(\mu\)l PBS). HLA-A2 complexes were then revealed with anti-\(\beta_2\)-microglobulin Ig M28 coupled to alkaline phosphatase, and enzyme activity was measured at 360/460 nm.


A CTL line specific for influenza A virus was generated from the PBMC of the HLA-A2 donor (15), and the PBMC were stimulated in vitro weekly with autologous irradiated cells infected with 10 hemagglutination units of influenza A/Ban virus per 10\(^6\) cells. Effector cells were tested for their ability to recognize peptides M58–66 and PMRI 58–59 in a cytotoxic assay after two stimulations. Three CTL lines were generated, one with peptides M58–66, one with [Leu59] M58–66, and one with PMRI 58–59 using the protocol previously described with some modifications (16). Unfractionated PBMC (4 \(\times\) 10\(^6\) cells/well) were seeded in 24-well plates with tetanus toxoid (1 \(\mu\)g/ml) and 1 \(\mu\)M peptide. IL-7 (Pepro Tech, London, U.K.) was added on day 3 or 4 (20 IU/ml). Replicates were stimulated with irradiated peptide-pulsed PBMC on day 8. The PBMC (10\(^10\) ml) were pulsed separately with each peptide (1 \(\mu\)M) for 1.5 h. The pulsed PBMC were then diluted to 10\(^6\) ml. One milliliter of each replicate supernatant was removed and replaced with 1 ml complete medium containing 10\(^9\) pulsed PBMC. One day later, 1 ml was again removed from each replicate and replaced with complete medium containing IL-2 (10 IU/ml) and IL-7 (20 IU/ml). This was repeated 4 days later, then every week. The CTL lines were tested for their ability to recognize peptides M58–66, [Leu59] M58–66, PMRI 58–59, or influenza A virus in a cytotoxic assay.
Cytolytic activity was detected by a standard 4-h 51Cr assay using autologous EBV-transformed lymphoblastoid cells as targets. These cells were labeled with 100 μCi sodium chromate (51Cr, 10 mCi/ml; Dupont/NEN, Boston, MA). They were infected with influenza virus (15), or incubated for 1 h with 5 μm peptide (M58–66, [Leu59]M58–66, or PMRI 58–59), and dispensed at 3000 or 5000 cells/well. Spontaneous release never exceeded 25% of the maximum 51Cr uptake. The values shown are the means of duplicate samples. The percentage of lysis was determined as follows: % release = 100 × (experimental release – spontaneous release)/(maxi-
mum release – spontaneous release).

Cytokine analysis

Analysis of cytokine mRNA. The expression of genes coding for cytokines by the M58–66, [Leu59]M58–66, and PMRI 58–59-specific CTL lines was assessed by semiquantitative reverse-transcriptase PCR in two separate experiments. For each of these experiments, total RNA and cDNA were prepared. One week after the third stimulation, lymphocytes (10^9) derived from each of the three CTL lines were stimulated by 10^6 PBMC in 1 ml complete medium. The three CTL lines were stimulated under four conditions. The PBMC were pulsed with 1 μM peptide M58–66, [Leu59]M58–66, or PMRI 58–59, or left unpulsed for 1 h and 30 min and washed. The lymphocytes from the three CTL lines were stimulated for 4 h and collected by centrifugation, and RNA was extracted by the RNazol technique (Bioprobe, Montreuil, France). Preliminary results obtained with stimulation for 2, 4, 6, and 12 h showed that a 4-h stimulation gave the optimal profile for studying the expression of the cytokine genes tested. The RNA was reverse transcribed with the reverse-transcribease Super-
script II (Life Technologies, Paisleys, Scotland). cDNA synthesis was carried out in a total volume of 20 μl. The cDNA was diluted fivefold with water, and the concentration was measured by competitive PCR with a plasmid (pQ/B2) (a gift from D. Shire, Sanofi Recherche, Labège, France) containing the β-actin gene. The PCR reactions were performed with 1 μl cDNA, 0.4 μM sense and anti-
sense β-actin primers, and serial dilutions of the plasmid pQ/B2. The PCRs were done in a final volume of 25 μl in 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, and 2 U AmpliTaq DNA polymerase (Perkin-
Elmer, Roche Molecular Systems, Branchburg, NJ). Each tube went through an initial 5-min denaturation at 94°C, followed by 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, followed by a 10-min 72°C final elongation in a regulated thermostat (Perkin-Elmer 9600). The quantity of cDNA was evaluated from the equivalent point observed after β-actin amplification. The expression of the genes coding for IL-2, IL-4, IL-10, IFN-γ, and TNF-α was assessed in the same conditions (final vol-
ume of 25 μl in 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 mM sense and antisense primers, and 2 U AmpliTaq DNA polymerase) with a quantity of cDNA equivalent to 30,000 copies of β-ac-
tin. Thirty cycle reactions were used for exponential amplifications. The cDNA were amplified in two or three separate experiments. The primers surrounding splice sites have been described (17) and are given in Table II.

Amplified fragments were separated on agarose gels using 10 μl of the PCR products, and densitometric measurements following ethidium bro-
mide staining were used.

ELISPOT assay for single cell IFN-γ release. Secretion of IFN-γ by Ag-specific effectors from PBMC was detected after separation activation with each of the three peptides, M58–66, [Leu59]M58–66, and PMRI 58–59, as previously described (18). Ninety-six-well surfactant-free mixed-cellulosic ester membrane (MAHA S45; Millipore, Bedford, MA) was coated with 4 μg/ml of the mouse anti-human IFN-γ mAb (Genzyme, Cambridge, MA) overnight at 4°C. Plates were washed three times with PBS and blocked with complete medium for 2 h at 37°C. PBMC of the donor were added in 100 μl/well to the precoated plates. Input cell numbers were 5 × 10^5/well. Peptides were added to a final concentration of 10 μg/ml to 100 μg/ml. Plates were incubated for 4 h at 37°C, and the assay was arrested by shaking off the contents and washing five times with PBS. A total of 100 μl of a 1 μg/ml polyclonal rabbit anti-human IFN-γ Ab (Genzyme) was next added. After an overnight incubation, plates were washed five times with PBS/0.05% Tween-20, and 100 μl of 1 μg/ml of a goat anti-rabbit biotin (Boehringer Mannheim, Mannheim, Germany) was added to the wells, and the plates were incubated at 37°C for an additional 2 h. The plates were washed five times with PBS/0.05%Tween-20, and 1/6000 dilution of streptavidin alkaline phosphatase conjugate (Sigma, St. Louis, MO) was added to the wells, and the plates were incubated at 37°C for an additional 1 h. The plates were washed again five times, and 100 μl of chromogenic alkaline phosphatase substrate (Bio-Rad, Hercules, CA), di-
luted 1/25 with deionized water, was added. After 30 min, the colorimetric reaction was stopped by washing with tap water, and plates were air dried. Spots were counted under magnification of ×20 with a stereomicroscope. Only large spots with fuzzy borders were scored as spot-forming cells. Responses with peptides were compared with those obtained with medium alone.

Molecular modeling

The starting coordinates were taken from the crystal structure of HLA-A2 complexed with M58–66 at a resolution of 2.5 Å, and deposited in the Protein Databank (Brookhaven, New York, NY) with the entry 1HHI (19). Using the Biopolymer module (Molecular Simulations, San Diego, CA), hydrogen atoms were added in the capping mode for a pH of 7.4. A subset with molecular properties encompassing residues 1 to 180 of the HLA-A2 α-chain and residues 1 to 9 of the peptide was created and used for further modeling (HLA2IM). The HLA2[Leu59]M58–66 complex was built by replacing the Ile60 of HLA2IM with a Leu residue in the same module (HLA2LEU). The HLA2–PMRI 58–59 complex was built from HLA2LEU by changing the C residue to a NH–CO bond (HLA2PMRI). The three structures were then relaxed by a steepest descent minimization (DISCOVER module of Molecular Simulations) of 1000 steps, taking into account the charges. The RMS deviation and the value of bonded energies confirm that the three structures were relaxed to a comparative degree.

To explore the conformational space of the peptides in the binding pocket, a combination of molecular dynamics and minimization starting from the minimized structures, was used. The nine residues of the peptide were allowed to move, while the rest of the structure was fixed. Since the N-terminal and C-terminal residues of the peptide are known to be essential for anchoring, these residues were tethered with a force constant of 100 kcal/mol/A2. The procedure used was a simple form of simulated annealing: 20 ps of dynamics at 900 K was initiated; the conformation with minimal total energy was selected for a further round of 20 ps of dynamics at 300 K. The conformation with minimal total energy was again selected and minimized using the steepest descent algorithm until the derivative was <0.5 kcal/mol/A, and the conjugate gradient algorithm until the derivative was <0.1 kcal/mol/A. All calculations were performed with DISCOVER in vacuo using a 10 Å nonbond cutoff, a 1 fs integration time, and a di-

electric constant of 1. The total energy was plotted vs time to analyze the resulting peptide conformations.

Results

Stability of the peptide/HLA-A2 complexes

Peptides PMRI 58–59, [Leu59]M58–66, and M58–66 (Table I) were compared to determine the influence of the retro-inverso modification on the stability of HLA-A2 molecules. The assembly of peptide/HLA-A2 complexes was assessed by incubation at 4°C, and their stability by incubation at 37°C (Fig. 1). The three peptides had equivalent abilities to promote the assembly of HLA-A2 molecules at 4°C, but the stabilities of the three HLA-A2 complexes formed differed. The [Leu59]M58–66 peptide was the most efficient stabilizer, while peptide PMRI 58–59 stabilized HLA molecules to a degree intermediate between peptides [Leu59]M58–66 and M58–66.

Table II. Primers used in this study

<table>
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<th>Fragment Size</th>
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<tbody>
<tr>
<td>β-actin 5'</td>
<td>237</td>
</tr>
<tr>
<td>β-actin 3'</td>
<td>345</td>
</tr>
<tr>
<td>IL-2 5'</td>
<td>375</td>
</tr>
<tr>
<td>IL-2 3'</td>
<td>385</td>
</tr>
<tr>
<td>IL-4 5'</td>
<td>462</td>
</tr>
<tr>
<td>IL-4 3'</td>
<td>525</td>
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<tr>
<td>IL-10 5'</td>
<td>325</td>
</tr>
<tr>
<td>IL-10 3'</td>
<td>375</td>
</tr>
<tr>
<td>IFN-γ 5'</td>
<td>501</td>
</tr>
<tr>
<td>IFN-γ 3'</td>
<td>555</td>
</tr>
<tr>
<td>TNF-α 5'</td>
<td>702</td>
</tr>
<tr>
<td>TNF-α 3'</td>
<td>755</td>
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</table>
Recognition of PMRI 58–59 by a virus-specific CTL line

A CTL line specific for influenza A virus was generated from the PBMC of the HLA-A2 healthy donor and tested for its ability to recognize peptides M58–66 and PMRI 58–59 in a cytotoxic assay. The virus-specific CTL line lysed target cells that had been incubated with the PMRI 58–59 analogue, or the wild-type peptide M58–66 to the same degree (Fig. 2).

Recognition of influenza virus A-infected cells by a CTL line generated with PMRI 58–59

CTL lines obtained from the PBMC of the healthy donor by stimulation with peptides were tested for their ability to recognize naturally processed epitopes on infected cells. The CTL line generated with PMRI 58–59 efficiently lysed virus A-infected cells. This ability was similar to that of the CTL line stimulated with peptide M58–66 (Fig. 3).


The relative capacities of these three peptides to induce CTL were assessed. CTL lines were generated from the PBMC of the healthy subject by stimulation with the peptides (10^{-7} M). All of the three CTL lines recognized PMRI 58–59, M58–66, and [Leu59]M58–66 in the cytotoxic assay, and gave equivalent lyses (Fig. 4). Their cytotoxic activities were similar, even when different concentrations of peptide were used to generate the CTL lines (10^{-9}–10^{-10} M), or to pulse target cells (10^{-6}–10^{-14} M) (data not shown).

Cytokine analysis

Analysis of genes coding for cytokines. The stimulatory capacity of each peptide was assessed by measuring, in two separate experiments, the expression of genes coding for cytokines (IL-2,
IL-4, IL-10, IFN-\(\gamma\), and TNF-\(\alpha\)) in a semiquantitative reverse-transcriptase PCR assay. The profiles of the cytokine genes expressed by the three CTL lines were determined by measuring the cytokine mRNA, in CTL lines stimulated for 4 h with PBMC pulsed with the PMRI 58–59 analogue, the wild-type peptide M58–66, or the parent peptide [Leu\(^{59}\)]M58–66. The background

**FIGURE 5.** Cytokine profiles of M58–66-, [Leu\(^{59}\)]M58–66-, and PMRI 58–59-specific CTL lines. Cytokine mRNA were detected by stimulating the CTL lines for 4 h with the PBMC pulsed with peptide or unpulsed (control). The cDNA samples were prepared, quantified, and amplified, as described in Materials and Methods. The cDNA were amplified in two or three separate experiments, and representative results are shown. a, PCR products on an ethidium bromide-stained agarose gel. M58–66 (A, B, C, and D), [Leu\(^{59}\)]M58–66 (F, G, H, and I), and PMRI 58–59 (J, K, and L) CTL lines were stimulated with peptides M58–66 (A, F, and J), [Leu\(^{59}\)]M58–66 (B, G, and K), and PMRI 58–59, or unstimulated (D, I, and M). b, Visualization of PCR products by densitometry. Background staining intensity was measured in a lane containing no PCR product and subtracted from the test intensities. Results shown are for M58–66 CTL in A, for [Leu\(^{59}\)]M58–66 CTL in B, and for PMRI 58–59 CTL in C.
levels of cytokine gene expression were measured using unpulsed PBMC. The 12 cDNA samples were first quantified by amplifying the actin gene (data not shown). The cDNA were amplified in two or three experiments for studying the expression of cytokine genes. The results were consistent from experiment to experiment, and representative results are shown in Figure 5.

The CTL line generated with M58–66 expressed genes coding for IL-2, IL-4, and IFN-γ after presentation of the homologous peptide. The CTL line stimulated with [Leu59]M58–66 produced smaller amounts of IL-2 and IL-4 mRNA and more IFN-γ mRNA. Stimulation with PMRI 58–59 resulted in slightly more IL-2, IL-4, and IFN-γ mRNA as did the wild-type peptide. Moreover, the retro-inverso analogue induced expression of the gene coding for TNF-α, while stimulation with M58–66 did not.

The CTL line generated with [Leu59]M58–66 produced IFN-γ mRNA, a small amount of IL-2 mRNA, but no IL-4 mRNA. The amounts of mRNA produced by this CTL line after stimulation with each of the three peptides appeared to be similar.

The CTL line obtained with PMRI 58–59 responded to peptide M58–66 by the expression of genes coding for IL-2 and IFN-γ, but IL-4 mRNA was not detected. It also responded to [Leu59]M58–66 with a similar profile. Stimulation with PMRI 58–59 resulted in more IL-2 and IFN-γ mRNA than did stimulation with M58–66 or [Leu59]M58–66; it also stimulated the production of a small amount of TNF-α mRNA. The CTL lines generated with peptides [Leu59]M58–66 and PMRI 58–59 produced only very small amounts of IL-4 mRNA after stimulation with any of the three peptides.

**ELISPOT assay for single cell IFN-γ release.** As it has recently been shown, M58–66-specific CD8+ T cells from donor without active influenza infection display effector function within 6 h of Ag contact (18). This effector function can be detected from unstimulated PBMC by a highly sensitive ELISPOT assay for single cell IFN-γ secretion. We have exploited this enhanced sensitivity to compare the efficiency of PMRI 58–59, [Leu59]M58–66, and M58–66 peptides to stimulate the secretion of IFN-γ by unstimulated M58–66-specific CD8+ effector cells. Responses with different concentrations of peptides PMRI 58–59, [Leu59]M58–66, and M58–66 (10⁻⁶–10⁻¹⁰ M) were compared with those obtained with the medium alone. Figure 6 shows that PMRI 58–59 was much more potent to activate specific effector cells for the secretion of IFN-γ than [Leu59]M58–66 and M58–66, since the concentration threshold for a significant response with PMRI 58–59 was 100-fold lower than with peptides [Leu59]M58–66 or M58–66. These results were consistent from two different experiments, and similar results were also observed when experiments were performed after 24 h of Ag contact.

**Molecular modeling**

Although the minimization procedure used resulted in a great decrease in non-bond energy in the peptides (Table III), the overall structures of the three peptides were very similar, with only minimal changes in the peptide main chain and reorientations of the side chains that are not involved in peptide-HLA interactions. The main difference between the structures is in the hydrogen-bonding pattern. In the crystal structure of HLA-A2 complexed with M58–66, the carbonyl oxygen at the P1-P2 peptide bond is hydrogen bonded to the hydroxyl group of the conserved Tyr159, while the amide nitrogen is H bonded to the carboxylate of Glu63 (Fig. 7A). These two hydrogen bonds are not present in the modeled structure of PMRI 58–59 because of the retro-inverso modification between P1 and P2 (Fig. 7C). The Leu59 modification had no effect on these hydrogen bonds (Fig. 7B).

![FIGURE 6. Ex vivo secretion of IFN-γ by Ag-specific effectors from PBMC. Secretion of IFN-γ was detected after separate activation with each of the three peptides, M58–66, [Leu59]M58–66, and PMRI 58–59, as described in Materials and Methods. Input cell numbers were 5.10⁵/well. Responses with different concentrations of peptides PMRI 58–59, [Leu59]M58–66, and M58–66 (10⁻⁶–10⁻ⁱ⁰ M) were compared with those obtained with the medium alone. These results have been calculated from duplicates and are representative of two different experiments. Results are shown for 6 h of Ag contact.](http://www.jimmunol.org/)

The carboxylate of Glu63 is hydrogen bonded to the NHε of Lys66 in the structure of HLA-A2 cocryrstallized with M58–66. Removal of the hydrogen bond between the amide nitrogen at P2 and Glu63 results in reorientation of this salt bridge. There are shifts in the Nε of Lys66 (1.05 Å) and in the Cδ of Glu63 (0.66 Å), whereas the oxygen atom Oδ1 of Glu63 is displaced by 1.83 Å and Oδ2 by 1.14 Å. The Ile-Leu substitution results in a shift of 0.34 Å for the Nε of Lys66, of 0.58 Å for the Cδ, 1.42 Å for the Oδ1, and 0.78 Å for the Oδ2 of Glu63.

**Discussion**

The retro-inverso transformation has been used to convert biologically active peptides into more stable compounds in the course of designing peptide drugs (20). The potential application of such structures was described recently to generate high titers of cross-reactive Abs (8, 10), but retro-inverso analogues have been seldom

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<tr>
<td>Nonbonded energy (kcal/mol)</td>
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<td>61.7</td>
<td>112</td>
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<tr>
<td>Residue M59 nonbonded energy</td>
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<td>10.8</td>
<td>-12.9</td>
<td>-19.4</td>
<td>-0.2</td>
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*The energy values before are for the relaxed structures, calculated in Table III. Those after were calculated after a simulation-annealing protocol.*

![Image](http://www.jimmunol.org/)
FIGURE 7. Hydrogen bonds between the HLA-A2 molecule and residue 59 of the peptides. The presented conformations are those found after minimization and molecular dynamics treatments described in Materials and Methods. The binding pocket is in magenta. The atoms of the residues involved are shown as sticks in the standard colors (carbon, green; nitrogen, blue; oxygen, red; and hydrogen, white). The hydrogen bonds are yellow lines. A, HLA-A2/IM58–66 complex: a network of four hydrogen bonds linking Ile\textsuperscript{59} of the peptide with the binding pocket. B, HLA-A2-[Leu\textsuperscript{59}]IM58–66 showing the same network as in A. The Glu\textsuperscript{63} and Lys\textsuperscript{66} of the HLA molecule are slightly reoriented. C, HLA-A2/RPMI, two of the hydrogen bonds have disappeared. The Glu\textsuperscript{63} and Lys\textsuperscript{66} residues of the HLA molecule have moved even more to the surface of the complex than in B.
used in the design of more potent stimulators of cellular immune responses (12). Nonnatural peptides that are not subject to proteolytic degradation and have more appropriate pharmacokinetics could well be most attractive materials for immunotherapy. This work, therefore, investigates the physicochemical properties and cellular immunogenicity of a partially modified retro-inverso analogue of the influenza matrix-derived peptide, M58–66, which has a leucine at position 59 and a modified bond between residues 58 and 59. A major problem limiting the use of peptides in immunotherapy or in vaccine development is their in vivo instability. Backbone modifications, such as changes in the amide bond, can sometimes result in analogues endowed with improved biologic activity (6). The partially modified retro-inverso analogue PMRI 58–59 used in this study is significantly more resistant to proteolysis than the corresponding parent peptide in a protease digestion assay (see Materials and Methods).

The PMRI 58–59 compound has a greater capacity to maintain HLA-A2 molecule assembly than the wild-type peptide M58–66. However, according to previous results showing that the peptide M58–66, with a leucine at position 59, forms more stable complexes with HLA-A2 molecule (21), we found that peptide [Leu10]M58–66 is the most potent stabilizer. Molecular models of the retro-inverso analogue suggest that two hydrogen bonds are lost in the structure of the pseudopeptide as a result of the retro-inverso modification between P1 and P2. The lower binding energy due to the removal of the two hydrogen bonds is not crucial, as it does not prevent the binding of PMRI 58–59 to HLA-A2; however, it might be responsible for the finding that the $t_{1/2}$ of the HLA-A2/PMRI 58–59 complex is shorter than that of the HLA-A2/parent peptide [Leu10]M58–66 complex. The complex formed by the pseudopeptide and the HLA-A2 molecule is more stable than that obtained with the cognate wild-type epitope. Nonbonded energy calculation of amino acid 59 in its environment (cutoff: 10Å) is unfavorable for peptide M58–66 (+10.8 kcal/mol), favorable for peptide [Leu10]M58–66 (−19.4 kcal/mol), and intermediate for peptide PMRI 58–66 (−5.5 kcal/mol) (Table III). Although these values are calculated in vacuo, they explain at least qualitatively the relative stabilities of the three peptides. The nonbonded energy of the anchoring residue 59 is better correlated with the observed experimental stability of the complex than with the overall nonbonded energy of the peptide (Table III).

The retro-inverso analogue is recognized by virus-specific CTL lines, and influenza-infected cells are lysed by PMRI 58–59-specific CTL. The CTL lines generated by peptides PMRI 58–59, [Leu10]M58–66, and M58–66 all had equivalent lysis activities, even when different concentrations of peptide were used to generate them. This shows that neither the retro-inverso modification nor the leucine substitution makes the modified peptide more efficient than the wild-type peptide M58–66 in generating CTL lines in vitro. Parker et al. found that a peptide having leucine substitution was not more active than M58–66 in a cytolytic assay (21). In our work, it is noteworthy that the CD8+ CTL effectors have been generated from HLA-A2 precursors that probably have been primed in vivo by influenza virus.

Moreover, the three peptides induce different profiles of expression of genes coding for cytokines, and in an ELISPOT assay, we showed that PMRI 58–59 ex vivo activated CD8+ effector T cells for the secretion of IFN-γ at a 100-fold lower concentration than the wild-type epitope. The differences in the profiles obtained after stimulation of the CTL line with PMRI 58–59, or [Leu10]M58–66 and that obtained with the wild-type epitope suggest that the PMRI 58–59 and [Leu10]M58–66 peptides modulate the expression of genes coding for cytokines and/or stimulate different T lymphocyte populations. As discussed above, molecular modeling suggests that two hydrogen bonds are missing in the structure of the retro-inverso analogue. The shifts in the Gly163-Lys66 salt bridge reflect remodeling in vacuo. The loosening of the hydrogen bond network could open this salt bridge to water molecules, resulting in the projection of the solvated Gly163 and Lys66 ions toward the surface. Although Tyr159, which loses a hydrogen bond with the PMRI 58–59 peptide, does not move after molecular dynamic calculations, it cannot be excluded that this residue also becomes accessible to solvation molecules. This hypothesis is favored by the observation that the Gly1(O)-Tyr159 hydrogen bond is more stable than the hydrogen bonds with Gly1(N), as calculated previously (22). These changes could slightly alter the topology of the upper surface of the binding groove when the partially modified retro-inverso analogue is bound.

The pseudopeptide PMRI 58–59 appears to induce the synthesis of TNF-α mRNA, which is not produced in response to the parent peptide. Moreover, this modified peptide is much more potent to stimulate effector cells for the production of IFN-γ than the wild-type epitope. These findings may have important implications for peptide-based immunotherapy, in which it is desirable to modulate the quality of immune responses. Retro-inverso analogues may be more potent inducers of protective immune responses against viruses or tumors. The production of TNF-α and IFN-γ is of particular interest, since these cytokines are involved in the response to viral infections and in the eradication of tumors (23, 24).

Thus, more stable peptide/MHC complexes and/or changes in their conformation can alter TCR signaling, leading to a different recruitment of protein kinases involved in downstream signaling. It has recently been shown that modulation of the T cell responses with altered peptide ligands can be correlated with a lack of recruitment and/or activation of src kinases (5, 25, 26). The differences in signaling produced by these ligands could be qualitative or quantitative. The stimulatory activity of ligands can be positively correlated with higher affinities for the TCR, slower dissociation rates, and the number of TCR molecules triggered (27). Lyons et al. recently reported that antagonist ligands bind to TCR with lower affinities and dissociate faster than do agonists (28). But, it has also been suggested that, upon ligand binding, both receptor cross-linking and a change in conformation must occur for successful activation to ensue (29). Subtle changes in the ligand, and hence in the TCR contact site, may change the composition of molecules involved in these interactions, like the number of TCR/CD3 complexes, coreceptors, or adhesion and/or costimulation molecules. Slight changes in the TCR conformation induced by a peptide/MHC complex define the quality of the response, and thus trigger either an antagonist or an agonist signal (30).

In conclusion, this study is the first describing that pseudopeptides can selectively modulate the cellular CD8+ immune responses. The design of such altered peptide ligands by isosteric replacement of the amide bond in immunogenic peptides might be a promising immunotherapeutic approach to the treatment of allergies, autoimmune diseases, or cancers.

References