Primming of Autoreactive CD8\(^+\) T Cells Is Inhibited by Immunogenic Peptides Which Are Competitive for Major Histocompatibility Complex Class I Binding

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In the present study, we investigated if priming of autoreactive CD8\(^+\) T cells would be inhibited by competitive peptides for major histocompatibility complex (MHC) class I binding. We used a mouse model of vitiligo which is induced by immunization of Kb-binding tyrosinase-related protein 2 (TRP2)-180 peptide. Competitive peptides for Kb binding inhibited IFN-\(\gamma\) production and proliferation of TRP2-180-specific CD8\(^+\) T cells upon ex vivo peptide restimulation, while other MHC class I-binding peptides did not. In mice, the capability of inhibition was influenced by T-cell immunogenicity of the competitive peptides. The competitive peptide with a high T-cell immunogenicity efficiently inhibited priming of TRP2-180-specific CD8\(^+\) T cells in vivo, whereas the competitive peptide with a low T-cell immunogenicity did not. Taken together, the inhibition of priming of autoreactive CD8\(^+\) T cells depends on not only competition of peptides for MHC class I binding but also competitive peptide-specific CD8\(^+\) T cells, suggesting that clonal expansion of autoreactive T cells would be affected by expansion of competitive peptide-specific T cells. This result provides new insights into the development of competitive peptides-based therapy for the treatment of autoimmune diseases.

INTRODUCTION

Immune responses against self-antigens caused by failure of self-tolerance develop autoimmune diseases which affect approximately 7~9% of the general population (1). However, current treatments of autoimmune diseases are not curative and induce general immunosuppression which is associated with serious infection and other adverse outcomes (2). Therefore, it is necessary to develop new therapeutics in order to selectively suppress autoreactive immune cells, including B cells, CD4\(^+\) T cells, and CD8\(^+\) T cells.

Unlike B cells, T cells recognize epitope peptides presented by major histocompatibility complex (MHC) molecules. Binding of T-cell epitope peptides to MHC is determined by hydrophobic or charge interactions between the residue of peptides and the cleft of MHC molecules (3,4). Thus, peptides presented on MHC molecules can be replaced extracellularly by other peptides depending on their affinity to MHC molecules (5,6). If peptides with high affinity to MHC molecules are present extracellularly in excess, they would occupy the cleft of MHC molecules and replace preexisting peptides on MHC molecules. This concept might be exploited for the treatment of autoimmune diseases. Autoimmune responses induced by self peptide-specific T cells could be inhibited by the addition of competitive peptides that replace...
self peptides on MHC molecules and inhibit presentation of the self peptide on antigen presenting cells (APCs).

In the present study, we investigated if priming of autoreactive CD8⁺ T cells would be inhibited by competitive peptides for MHC class I binding. We used a mouse model of vitiligo which is induced by immunization of Kᵇ-binding tyrosinase-related protein 2 (TRP2)-180 peptide. Previously, we established a novel mouse model of vitiligo by immunization with TRP2-180 peptide in combination with lipopolysaccharides (LPS) and CpG oligodeoxynucleotides (CpG ODN) (7). This mouse model of vitiligo was characterized by progressive depigmentation of the skin due to selective loss of melanocytes. In addition, the extent of skin depigmentation correlated with the frequency of TRP2-180-specific CD8⁺ effector T cells in the spleen. Therefore, we used this mouse model of vitiligo to analyze how efficiently the competitive peptides for MHC class I binding inhibit priming and activation of melanocyte-specific autoreactive CD8⁺ T cells.

In this study, competitive peptides for Kᵇ binding inhibited IFN-γ production and proliferation of TRP2-180-specific CD8⁺ T cells upon ex vivo peptide restimulation. Interestingly, the capability of inhibition was influenced by T-cell immunogenicity of the competitive peptides in vivo. The competitive peptide with a high T-cell immunogenicity efficiently inhibited priming of TRP2-180-specific CD8⁺ T cells in vivo, whereas the competitive peptide with a low T-cell immunogenicity did not. Taken together, the inhibition of the priming of autoreactive CD8⁺ T cells depends on not only competition of peptides for MHC class I binding but also competitive peptide-specific CD8⁺ T cells. Thus, highly T cell-immunogenic peptides which are competitive for MHC binding might be useful for inhibiting priming of autoreactive T cells for the treatment of autoimmune diseases.

MATERIALS AND METHODS

Animals and peptides
Male C57BL/6 mice were kept in accordance with the institutional guidelines, and used at the age of 4–5 weeks for peptide immunization. Peptides were purchased from Peptron (Daejeon, Korea) and dissolved in 5% DMSO phosphate-buffered solution (PBS).

Measurement of relative Kb binding affinity of peptides
Splenocytes of naive C57BL/6 were incubated with different combinations of peptides, including SIINFEKL at 0.5 μM and various concentrations of additional peptides (0.5, 5, or 50 μM) as indicated, at 37°C for 4 hrs. After incubation, splenocytes were washed and fixed by 4% paraformaldehyde. The density of SIINFEKL-binding Kᵇ molecules on lymphocytes was analyzed by flow cytometry using 25-D1.16 monoclonal antibody (8) that reacts with SIINFEKL-binding Kᵇ molecule. The percentage of decreased density of SIINFEKL-binding Kᵇ molecules by additional peptides was calculated as follows: [100−mean fluorescence index (MFI) of SIINFEKL-binding Kᵇ in SIINFEKL at 0.5 μM + additional peptides at 5 μM/MFI of SIINFEKL-binding Kᵇ in SIINFEKL at 0.5 μM × 100].

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Inhibition of IFN-γ production by competitive peptides in vitro
C57BL/6 mice were subcutaneously immunized twice at 1-week intervals in the footpad with TRP2-180 (50 μg), LPS (5 μg), and CpG ODN (5 μg). One week after the second immunization, splenocytes were harvested and stimulated with different combinations of peptides, including TRP2-180 at 0.1 μM and various doses of additional peptides (0, 0.1, 1, or 10 μM). TRP2-180-specific IFN-γ production was analyzed by flow cytometry after intracellular cytokine staining (ICS) as described in the section of 'Intracellular Cytokine Staining'.

Generation of TRP2-180-specific CD8⁺ T cell lines
CD45.2 C57BL/6 mice were subcutaneously immunized 3 times at 1-week intervals in the hind footpad with TRP2-180 (50 μg), LPS (5 μg) and CpG ODN (5 μg). One week after the last immunization, splenocytes were harvested, and splen-ic CD8⁺ T cells were sorted using anti-CD8 microbeads (Miltenyi Biotec, Auburn, CA). These sorted CD8⁺ T cells were restimulated in vitro 3 times at 2-week intervals with TRP2-180 peptide (10 μg/ml) in the presence of IL-2 (20 IU/ml) and CD45.1 splenocytes as APCs. The purity of TRP2-180-specific CD8⁺ T cells was evaluated by TRP2-180-specific IFN-γ production and was >98%. Two weeks after the last stimulation, CD8⁺ T cells were used for proliferation analysis.

Inhibition of T cells proliferation by competitive peptides in vitro
CD45.2⁺, TRP2-180-specific CD8⁺ T cell line (0.2 × 10⁶ cells) labeled with CFSE at 10 μM (Invitrogen Ltd, Paisley, UK) was cocultured with CD45.1⁺ splenocytes (1 × 10⁶ cells) pulsed with different combinations of peptides, including TRP2-180.
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Inhibition of priming of TRP-180-specific CD8+ T cells in vivo
C57BL/6 mice were subcutaneously immunized in the footpad with TRP-180 (5 nmole), LPS (5 μg), and CpG ODN (5 μg). One day after the immunization, additional competitive peptides (50 nmole) were injected into the same footpad without adjuvants twice at 1-day intervals. One week after the 2nd immunization, splenocytes were harvested and stimulated with TRP-180 (50 nmole) and brefladin A (GolgiPlug, BD Biosciences) was added 1 hr later. After another 5 hrs of incubation at 37°C, cells were stained with ethidium monoazide (Sigma-Aldrich, St. Louis, MO, USA). After washing, the cells were washed with anti-CD3-pacific blue, anti-CD4-APC-Cy7, anti-CD8-PE-Cy7, and anti-CD44-PE (all from BD Biosciences), permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), and stained with anti-IFN-γ-APC (BD Biosciences). Stained cells were analyzed on an LSR flow cytometer (BD Biosciences).

Statistical analysis
Data were presented as the mean ± SEM. All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad software, La Jolla, CA, USA). A two-tailed Student’s T-test was performed to examine statistical significance for differences between the groups.

RESULTS
Relative affinity of peptides for Kβ binding
In the present study, Kβ-binding TRP-180 peptide was used as a self peptide to induce melanocyte-specific CD8+ T cells and develop vitiligo (Table 1) (7,9). For competition with TRP-180 for Kβ binding, we included vaccinia virus A11R D8-205 (VV A11R-198) (10), lymphocytic choriomeningitis virus GP 118-125 (LCMV GP-118) (10), and β-actin 219-227 (β-actin-219) (11) which bind to Kβ. We also included Japanese encephalitis virus NS4B 215-223 (JEV NS4B-215) (12) which bind to Dβ, and Epstein-Barr virus BMLF1 280-288 (EBV BMLF1-280) (13) which bind to human leukocyte antigen (HLA)-A0201, as non-competitive peptides for Kβ binding.

First, we evaluated relative Kβ-binding affinity of the peptide by a competition assay using SIINFEKL peptide. In this assay, splenocytes were pulsed with SIINFEKL peptide in combination with competitive peptides, then the density of SIINFEKL-binding Kβ molecules was quantified by flow cytometry using antibody specific to SIINFEKL-binding Kβ molecule. Without competitive peptides, SIINFEKL peptide pulsing increased the density of SIINFEKL-binding Kβ molecule in a...
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Table I. Characteristics of peptides

| Peptide name | Sequence     | MHC restriction | References |
|--------------|--------------|-----------------|------------|
| TRP2-180     | SVYDFFVWL    | H-2 K<sup>b</sup> | (7, 9)     |
| VV A11R-198  | AIVNYANL     | H-2 K<sup>b</sup> | (10)       |
| LCMV GP-118  | ISHNFCNL     | H-2 K<sup>b</sup> | (10)       |
| β-actin-219  | VALDFEQEM    | H-2 D<sup>b</sup> | (11)       |
| JEV NS4B-215 | SAVWNSTA     | H-2 D<sup>b</sup> | (12)       |
| EBV BMLF1-280| GLCTLVAML    | HLA-A0201       | (13)       |

Figure 1. Relative affinity of T cell epitope peptides for K<sup>b</sup> binding. Splenocytes of naive C57BL/6 mice were incubated with peptides. Peptide-pulsed splenocytes were fixed with 4% paraformaldehyde, and the density of SIINFEKL-binding Kb molecules on lymphocytes was analyzed by flow cytometry using antibody specific to SIINFEKL-binding Kb molecule (clone 25-D1.16). (A) A dashed line represents the mean fluorescence intensity (MFI) of 25-D1.16-stained lymphocytes pulsed with the indicated concentration of SIINFEKL without additional peptides. Each solid line represents MFI of 25-D1.16-stained lymphocytes pulsed with SIINFEKL at 0.5 μM in combination with the indicated concentration of additional peptide. Each dot represents the mean ± SEM from 3 independent experiments. (B) The percentage of decreased MFI of SIINFEKL-binding Kb molecules by additional peptides (SIINFEKL at 0.5 μM and additional peptide at 5 μM) was calculated as follows: \[100 - \frac{\text{MFI of SIINFEKL-binding Kb in SIINFEKL at 0.5 μM}}{\text{MFI of SIINFEKL-binding Kb in SIINFEKL at 0.5 μM}} \times 100\]. Each bar graph represents the mean ± SEM from 3 independent experiments.

Inhibition of TRP2-180-specific IFN-γ production by competitive peptides in vitro
To evaluate the inhibition of TRP2-180-specific IFN-γ production by competitive peptides, splenocytes of TRP2-180-immunized mice were stimulated ex vivo with TRP2-180 peptide alone or in combination with competitive peptides, and IFN-γ ICS was performed. IFN-γ production was observed in CD4<sup>+</sup>, antigen-experienced CD8<sup>+</sup> T cells. Antigen-specific IFN-γ production of TRP2-180-specific T cells in a dose-dependent manner, while JEV NS4B-215 and EBV BMLF1-280 did not (Fig. 2A and B).

Inhibition of TRP2-180-specific T-cell proliferation by competitive peptides in vitro
To examine the inhibition of T cells proliferation by competitive peptides in vitro, a TRP2-180-specific T-cell line was established, and their proliferation responding to TRP2-180 peptide was evaluated by CFSE labeling. Proliferation of the T-cell line was increased by stimulation with TRP2-180 peptide (Fig. 3A). VV A11R-198, LCMV GP-118, and β-actin-219 inhibited TRP2-180-specific proliferation of T cells, while JEV NS4B-215 and EBV BMLF1-280 did not (Fig. 3B). The percentage of proliferation inhibition was calculated and presented for each peptide (Fig. 3C).

Inhibition of TRP2-180-specific T-cell priming by competitive peptides in vivo
Next, we determined if K<sup>b</sup>-binding competitive peptides would inhibit priming of TRP2-180-specific T cells in vivo. C57BL/6 mice were subcutaneously immunized in the footpad with TRP2-180, LPS, and CpG ODN. One day after the immunization, doses 10-times higher than competitive peptides were injected into the same footpad without adjuvants.
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Figure 2. Inhibition of TRP2-180-specific IFN-γ production of CD8+ T cells by competitive peptides for Kb binding in vitro. C57BL/6 mice were immunized with TRP2-180, and splenocytes were harvested and stimulated ex vivo with TRP2-180 peptide alone or in combination with competitive peptides. Then, IFN-γ ICS was performed and analyzed by flow cytometry. (A) The splenocytes were stimulated ex vivo with 0.1 μM of TRP2-180 peptide alone or in combination with competitive peptides at 10 μM. Representative FACS dot plots for IFN-γ and CD44 are shown in the gate of the CD8+ T cell population. (B) The splenocytes were stimulated ex vivo with TRP2-180 peptide at 0.1 μM alone or in combination with the indicated concentration of competitive peptides, and the percentages of TRP2-180-specific IFN-γ+ cells in the CD8+ T-cell population are presented as bar graphs. Each bar graph represents the mean ± SEM from 6 independent experiments (***p < 0.01).

Figure 3. Inhibition of TRP2-180-specific CD8+ T-cell proliferation by competitive peptides for Kb binding in vitro. A TRP2-180-specific T-cell line (CD45.2) labeled with CFSE at 10 μM was cocultured with splenocytes (CD45.1) pulsed with TRP2-180 peptide alone or in combination with competitive peptides. Four days after coculture, cocultured cells were stained with anti-CD45.2-APC-H7 and analyzed by flow cytometry. (A) CFSE-labeled T-cell line was cocultured with splenocytes pulsed with the indicated concentration of TRP2-180. The fluorescence intensity of CFSE was analyzed in the gate of the CD45.2+ T-cell population. (B) CFSE-labeled T-cell line was cocultured with splenocytes pulsed with TRP2-180 at 0.1 μM in combination with additional peptides at 10 μM. The fluorescence intensity of CFSE was analyzed in the gate of the CD45.2+ T-cell population. (C) The percentage of proliferation inhibition by additional peptide was calculated as follows: $100 - \% \text{ of proliferation in absence of TRP2-180}(\% \text{ of proliferation in TRP2-180 at } 0.1 \mu M - \% \text{ of proliferation in the absence of TRP2-180} \times 100)$, and presented by bar graphs.
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Figure 4. Inhibition of TRP2-180-specific CD8⁺ T-cell priming by competitive peptides in vivo. C57BL/6 mice were subcutaneously immunized in the footpad with TRP2-180 (5 nmole), LPS, and CpG ODN. One day after the immunization, competitive peptides at 50 nmole were injected into the same footpad without adjuvants twice at 1-day intervals. One week after TRP2-180 immunization, splenocytes were harvested. (A) TRP2-180-specific IFN-γ⁺ CD8⁺ T cells were analyzed by ICS and flow cytometry in each group. (B) Competitive peptide-specific IFN-γ⁺ CD8⁺ T cells were analyzed by ICS and flow cytometry in each group. Each bar graph represents the mean ± SEM from 3 independent experiments with 3 mice per group (*p<0.05).

Competitive peptides do not inhibit disease progression in a mouse model of vitiligo
C57BL/6 mice were subcutaneously immunized in the footpad with TRP2-180, LPS, and CpG ODN twice at 1-week intervals. One week after the second immunization, TRP2-180 with the same adjuvants was injected into the tail dermis for disease induction twice at 1-week intervals. Competitive peptides were injected into the tail dermis 1 day after the third and fourth TRP2-180 immunization without adjuvants twice at 1-day intervals. Five weeks after the last TRP2-180 immunization, splenocytes were harvested and TRP2-180-specific IFN-γ⁺ CD8⁺ T cells were analyzed by ICS and flow cytometry. Competitive peptide-specific IFN-γ⁺ CD8⁺ T cells were also analyzed. In this case, no peptide decreased the frequency of TRP2-180-specific IFN-γ⁺ CD8⁺ T cells, while the 2 Kᵇ-binding peptides (VV A11R-198 and LCMV GP-118) were immunogenic (Fig. 5A and B). As expected from the fact that no peptide decreased the frequency of TRP2-180-specific IFN-γ⁺ CD8⁺ T cells, disease progression evaluated by the percentage of depigmented skin lesions in a tail was not prevented by any peptide (Fig. 5C). VV A11R-198 showed a tendency to decrease the area of depigmented lesions in a tail, without statistical significance (Fig. 5C).

DISCUSSION
In the present study, we employed competitive peptides for Kᵇ binding to inhibit activation of TRP2-180-specific CD8⁺ T cells, melanocyte-specific autoreactive CD8⁺ T cells. The competitive peptides displayed a higher Kᵇ-binding affinity than TRP2-180 peptide, an autoimmunogenic peptide (Fig. 1A and B) and inhibited IFN-γ production and proliferation of
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Figure 5. Competitive peptides do not prevent progression of depigmented skin lesions in a mouse model of vitiligo. C57BL/6 mice were subcutaneously immunized in the footpad with TRP2-180 (5 nmole), LPS, and CpG ODN twice at 1-week intervals. One week after the second immunization, TRP2-180 (5 nmole) with the same adjuvants was injected into the tail dermis for the disease induction twice at 1-week intervals. Fifty nmole competitive peptides were injected into the tail dermis 1 day after third and fourth TRP2-180 immunization without adjuvants twice at 1-day intervals. Five weeks after the last TRP2-180 immunization, splenocytes were harvested. (A) TRP2-180-specific IFN-γ+ CD8+ T cells were analyzed by ICS and flow cytometry in each group. (B) Competitive peptide-specific IFN-γ+ CD8+ T cells were analyzed by ICS and flow cytometry in each group. (C) Disease progression was evaluated by the percentage of depigmented skin lesions in the tail in each group. Each bar graph represents the mean±SEM from 3 independent experiments with 3 mice per group (n.s., not significant).

TRP2-180-specific CD8+ T cells in vitro, while non-Kb-binding peptides did not (Fig. 2 and 3). The data demonstrate that the inhibition of TRP2-180-specific CD8+ T-cell activation in vitro depends on competition of peptides for MHC binding.

When we tested if Kb-binding competitive peptides inhibit priming of TRP2-180-specific CD8+ T cells, only VV A11R-198 significantly inhibited the priming (Fig. 4A), while β-actin-219 did not (Fig. 4A). Interestingly, the ability of Kb-binding peptides to inhibit TRP2-180-specific T-cell priming was associated with T-cell immunogenicity of each peptide. In fact, VV A11R-198 showed the highest immunogenicity to T cells (Fig. 4B), and β-actin-219 did not show any immunogenicity (Fig. 4B). These results suggest that clonal expansion of autoreactive T cells might be affected by expansion of competitive peptide-specific T cells. These findings are reminiscent of clonal competition in immunodominance and immune hierarchy in CD8+ T-cell responses (14). T cells primed by highly immunogenic peptides compete with autoreactive T cells for resources required for T-cell survival and proliferation (15). Therefore, we should consider not only competition of therapeutic peptides for MHC class I binding but also T-cell immunogenicity of therapeutic peptides in competitive peptides-based therapy for the treatment of autoimmune diseases.

Although VV A11R-198 inhibited the priming of TRP2-180-specific CD8+ T cells (Fig. 4A), it did not inhibit the boosting of them (Fig. 5A), and thus disease progression was not prevented by VV A11R-198 in a mouse model of vitiligo (Fig. 5C). The results demonstrate that VV A11R-198 could inhibit priming of naive T cells in vitro but could not inhibit reactivation of memory T cells in the tail skin. Since memory T cells rapidly proliferated and efficiently differentiated to effector T cells compared with naive T cells (16,17), our results could be explained by the difference between memory T cells...
and naive T cells. If competitive therapeutic peptides also target memory T cells, autoreactive memory T cells might be inhibited by competitive peptides. In this context, T-cell epitope peptides from influenza A virus or cytomegalovirus will be candidates for competitive peptides-based therapy because the majority of human population have a memory T-cell pool against these viruses (18,19).

In summary, competitive peptides for K\(^\beta\) binding inhibited IFN-\(\gamma\) production and proliferation of TRP2-180-specific CD8\(^+\) T cell in vitro. In mice, inhibition of TRP2-180-specific CD8\(^+\) T-cell priming required competitive peptides with a high immunogenicity for T cells. Although competitive peptides did not prevent the progression of the depigmented skin lesion in a mouse model of vitiligo, our results provide new insights into the development of competitive peptides-based therapy for the treatment of autoimmune diseases.

ACKNOWLEDGEMENTS

This work was supported by National Research Foundation grants funded by the Korea government (Ministry of Education, Science and Technology) (2012-M3C1A1-048860).

CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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