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Brief Report

Modifications in Immune Response Patterns Induced by Kynurenine and One-Residue-Substituted T Cell Epitopes in SARS-CoV-2-Specific Human T Cells

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Abstract: Peptide p176-190, derived from the SARS-CoV-2 spike protein, is one of the major T cell epitopes that elicits the HLA-DR-restricted IL-8 response of human $CD4^+$ T cells. Using PBMCs from a healthy individual primed with an S-protein-based SARS-CoV-2 vaccine, we established a $CD4^+$ T cell line (TM45) and cloned T cells (TM45.2) specific for the peptide. We showed that (i) co-incubation with kynurenine leads to increased IL-8; (ii) T cells incubated in the absence of kynurenine recovered the original levels of cytokine production; and (iii) peptide p176-190 substituted at 176 Leucine for neutral hydrophilic serine completely abolished the cytokine responses of TM45.2 cells, thereby suggesting that 176 L is the first anchor residue for binding to HLA-DR. These observations collectively indicate that (i) enhanced IL-8 responses can be induced by kynurenine, which is produced under infectious conditions in COVID-19; (ii) the response is not a permanent change in the T cell phenotype; and (iii) IL-8 responses associated with harmful neutrophil extracellular traps can be abrogated by a single amino acid substitution of the viral antigens. These findings may shed light on a novel strategy for designing vaccines for viral infections that are accompanied by increased kynurenine production.

Keywords: $CD4^+$ T cells; COVID-19; IL-8; kynurenine; HLA-DR

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1. Introduction

Coronavirus disease 2019 (COVID-19) is an illness caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1,2]. Neutrophils are known as indicators of the severity of respiratory symptoms and poor prognosis in COVID-19 [3–5]. In severe cases of COVID-19, interleukin (IL)-8-induced neutrophilic inflammation and similar factors trigger disseminated intravascular coagulation (DIC) through neutrophil extracellular traps (NETs) [6–8]. DIC induced by NET formation is an important cause of acute respiratory distress syndrome (ARDS) in patients with COVID-19, leading to a fatal prognosis [9–11]. Previous studies have demonstrated that IL-8 produced under T-helper (Th) 17-polarizing conditions is upregulated in the presence of aryl hydrocarbon receptor (AHR) activation [12]. In fact, certain viral infections such as coronaviruses and Zika virus are reported to induce kynurenine production, which activates AHR [13–17].

IL-8 acts as a neutrophil chemotactic factor and is secreted by various cells, such as macrophages, airway smooth muscle cells, epithelial cells, and vascular endothelial cells [18]. IL-8 is also produced by Th1 and Th17 cells, making it one of the cytokines involved in neutrophilic inflammation [19]. We previously showed that (i) peptide p176-190 derived from the SARS-CoV-2 spike protein is presented to T cells in the context of HLA-DR to cluster of differentiation (CD) 4^+ T cells; (ii) in COVID-19, $CD4^+$ T cells, upon antigen presentation, produce IL-8, triggering neutrophilic inflammation; and (iii) istradefylline (an

adenosine A2a receptor antagonist) and ropinirole (a dopamine D2-like receptor agonist) are known to suppress neutrophilic inflammation and inhibit COVID-19 spike protein-derived peptide-specific IL-8 responses [19].

Peptide p176-190, derived from the SARS-CoV-2 spike protein, is one of the major T cell epitopes that elicit the human leukocyte antigen (HLA)-DR-restricted IL-8 response of human $CD4^+$ T cells [6]. In this study, using peripheral blood mononuclear cells (PBMCs) of a healthy individual primed with an S-protein-based SARS-CoV-2 vaccine, we established a $CD4^+$ T cell line (TM45) and cloned T cells (TM45.2) specific for peptide p176-190. Moreover, we analyzed changes in the immune response patterns of kynurenine and one-residue-substituted T cell epitopes.

2. Materials and Methods

2.1. Preparation of PBMCs

Peripheral blood was obtained from three healthy individuals with a history of COVID-19 vaccination using protocols approved by the Saitama Medical University Ethics Committee (#787-III). Blood was centrifuged for 10 min at 450× *g* and separated into blood cells and plasma. After adding RPMI1640 (Sigma-Aldrich, St. Louis, MO, USA) to the blood cells, the samples were overlaid onto a Ficoll-Paque PLUS (GE Healthcare, Buckinghamshire, England, UK) and centrifuged for another 40 min at 450× *g*. PBMCs were recovered from the topmost layer of Ficoll-Paque.

2.2. Peptides

We synthesized the 15-mer peptide p176-190 derived from the SARS-CoV-2 spike protein, as described previously [19], which is expected to bind to HLA class II allelic products frequently observed in the Japanese population [19,20]. In addition, we synthesized two analog peptides with a single residue substitution. L176S refers to peptide p176-190 with a substitution of hydrophobic leucine at position 176 for neutral hydrophilic serine, whereas M177S indicates peptide p176-190 with a substitution of hydrophobic methionine at position 177 for neutral hydrophilic serine. These 15-mer peptides, as shown in Table 1, were synthesized in Eurofins (Tokyo, Japan). These peptides did not contain any point mutation sites observed in the alpha, beta, gamma, or delta strains of the virus.

Table 1. The 15-mer peptides derived from the SARS-CoV-2 spike protein and two analogue peptides carrying one residue substitution.

Peptide	Amino Acid Sequence
Wild type (position: p176-190)	LMDLEGKQGNFKNLR
L176S	SMDLEGKQGNFKNLR
M177S	LSDLEGKQGNFKNLR

2.3. Human T Cell Clones

Antigen-specific T cell lines were established as previously described [21]. PBMCs from three individuals were used to establish T cell lines independently, without mixing them to avoid mixed lymphocyte reactions. Briefly, spike proteins of SARS-CoV-2-specific T cell lines were established from PBMCs of healthy individuals with a history of COVID-19 vaccination by stimulating PBMCs (1×10^5/well) with 1 μM peptide p176-190 in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) medium containing 10% human serum, 1% L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin (R10H medium) in 96-well flat-bottom culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA), at 37 °C in a humidified atmosphere containing 5% CO_2. After 9 days, irradiated autologous PBMCs pulsed with the peptide p176-190, human rIL-2 (50 U/mL; PEPROTECH, Cranbury, NJ, USA), and human rIL-4 (10 ng/mL; Primmune Inc., Hyogo, Japan) were added to the culture wells carrying T cell blasts and maintained for 7 additional days. A human $CD4^+$ T cell line (TM45) specific for a 15-mer peptide, p176-190-SARS-CoV-2, was used throughout

the study. Cloning was performed using Terasaki plates (Nunc, Roskilde, Denmark), and limiting dilution was at one cell per well. One human $CD4^+$ T cell clone (TM45.2), specific for peptide p176-190, was used throughout the study.

2.4. Assessment of T Cell Responses

The peptide-induced responses of the TM45 T cell line were induced in the presence of 1×10^6/well irradiated (30 Gy) autologous PBMC prepulsed with the peptide, human rIL-2 (50 U/mL), with or without kynurenine (Sigma-Aldrich, St. Louis, MO, USA) in R10H medium in 24-well flat-bottom culture plates. After 7 days, TM45 cells (1×10^4/well) were incubated in 96-well flat-bottom culture plates in the presence of irradiated autologous PBMC (1×10^5/well) and 5 μM peptide, using R10H medium. A portion of the TM45 cells was maintained for an additional 7 days. After 4 days, supernatants were collected for enzyme-linked immunosorbent assays (ELISAs).

Peptide-induced responses of the T cell clone TM45.2 cells were assayed by culturing the T cells (1×10^4/well) in 48-well flat-bottom culture plates in the presence of irradiated autologous PBMCs and 5 μM each of peptide (peptides p176-190, L176S, and M177S) in R10H medium. After four days, the supernatant was collected for ELISAs.

2.5. Cytokine ELISAs

The concentrations of IL-8, IL-5, interferon (IFN) γ, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in the culture supernatant fluids were measured using specific ELISA kits (DuoSet Kit, R&D, Minneapolis, MN, USA). Values below the lower limit of detection (15.6 pg/mL) were set to 0. Cytokine cross-reactivity was not observed within the detection range of these kits. If necessary, the samples were diluted appropriately so that the measurements fell within the appropriate detection range for each cytokine.

2.6. Statistical Analysis

Differences between the two groups were analyzed using an unpaired Student's *t*-test. Differences between three or more groups were analyzed using a one-way analysis of variance (ANOVA) with Tukey's post hoc test. All calculations were performed using the KaleidaGraph software program version 4.5.2 (Synergy Software, Reading, PA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Kynurenine Increases the Production of IL-8 Induced by Peptide Stimulation

We previously reported that T cells from COVID-19 patients exhibited an HLA-DR-restricted IL-8 response to spike protein-derived peptides [19]. In this study, we established a Th0 line (TM45) and cloned Th0 cells (TM45.2) specific for spike protein-derived peptides from PBMCs of healthy individuals with a history of COVID-19 vaccination. When TM45 cells were incubated with 5 μM peptide p176-190 for 4 days, 3055 pg/mL IFNγ and 2996 pg/mL IL-5 were detected (not shown). This indicates that TM45 cells are a peptide p176-190-specific Th0 line. When TM45 cells were incubated with kynurenine for seven days, the IL-8 response was enhanced in a dose-dependent manner (Figure 1A). The viability of TM45 cells co-cultured with 100 μM kynurenine was >90% (trypan blue stain). In contrast, there was no increase in GM-CSF production in TM45 cells (Figure 1B). IFNγ, and IL-5 did not show a dose-dependent increase with kynurenine co-culture (not shown). As shown in Figure 1C, when TM45 cells co-cultured with kynurenine for 7 days were subsequently cultured without kynurenine, the cytokine production recovered to its original levels.

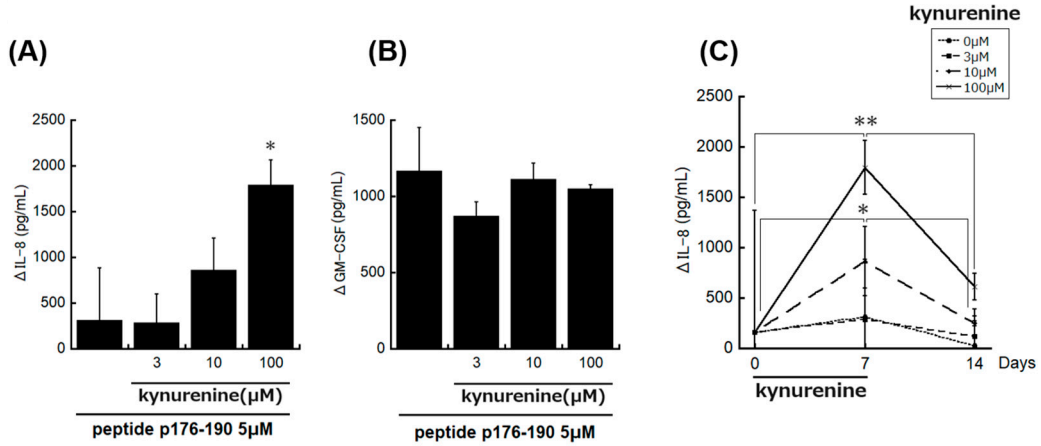


Figure 1. TM45 cells were incubated in the presence of irradiated (30 Gy) autologous PBMCs prepulsed with the peptide p176-190 and human rIL-2 (50 U/mL), with or without kynurenine. After 7 days, TM45 cells (1×10^4/well) were cultured in 96-well flat-bottom culture plates in the presence of irradiated autologous PBMCs (1×10^5/well) and 5 μM peptide p176-190 in R10H medium. Four days later, the culture supernatant fluid was collected for IL-8 (**A**) and GM-CSF (**B**) ELISAs (n = 6). Values obtained by subtracting the IL-8 concentration in the absence of peptide (Δpg/mL) are shown. * $p < 0.05$, compared to ΔIL-8 with peptide p176-190 in the absence of kynurenine. (**C**) TM45 cells were incubated in the presence of irradiated (30 Gy) autologous PBMCs prepulsed with the peptide p176-190, human rIL-2 (50 U/mL), with or without kynurenine. After 7 days, TM45 cells (1×10^4/well) were cultured in 96-well flat-bottom culture plates in the presence of irradiated autologous PBMCs (1×10^5/well) and 5 μM peptide p176-190 using R10H medium. A portion of TM45 cells was maintained for an additional 7 days. Four days later, culture supernatant fluid was collected for an IL-8 ELISA (n = 6). Values obtained by subtracting the IL-8 concentration in the absence of the peptide (Δpg/mL) are shown. Data are expressed as the mean ± standard deviation (SD) and were compared using a one-way analysis of variance and Tukey's post hoc test. * $p < 0.05$ and ** $p < 0.01$ compared to Day 0 or Day 14.

3.2. L176S and M177S Completely Abolish the Cytokine Response of TM45.2 Cells

Next, using TM45.2 cells (a p176-190-specific Th0 clone), we observed T cell responses against wild-type p176-190 and analog peptides. As shown in Figure 2A, an HLA-DR-binding peptide has a pronounced counterclockwise twist within the groove, which is characteristic of a type II polyproline helix [22,23]. As shown in Figure 2B, the side chains project from the peptide backbone approximately every 130°. The peptide's first anchor (position 1) protrudes within the HLA peptide-binding groove at the first pocket, forming hydrophobic interactions, and remains unrecognizable to the T cell receptor (TCR). Adjacent position 2 interacts with TCR, but does not bind to HLA [23,24]. We then co-cultured p176-190, L176S, and M177S with TM45.2 cells for four days. L176S and M177S completely abolished IFN-γ production in TM45.2 cells (Figure 2C).

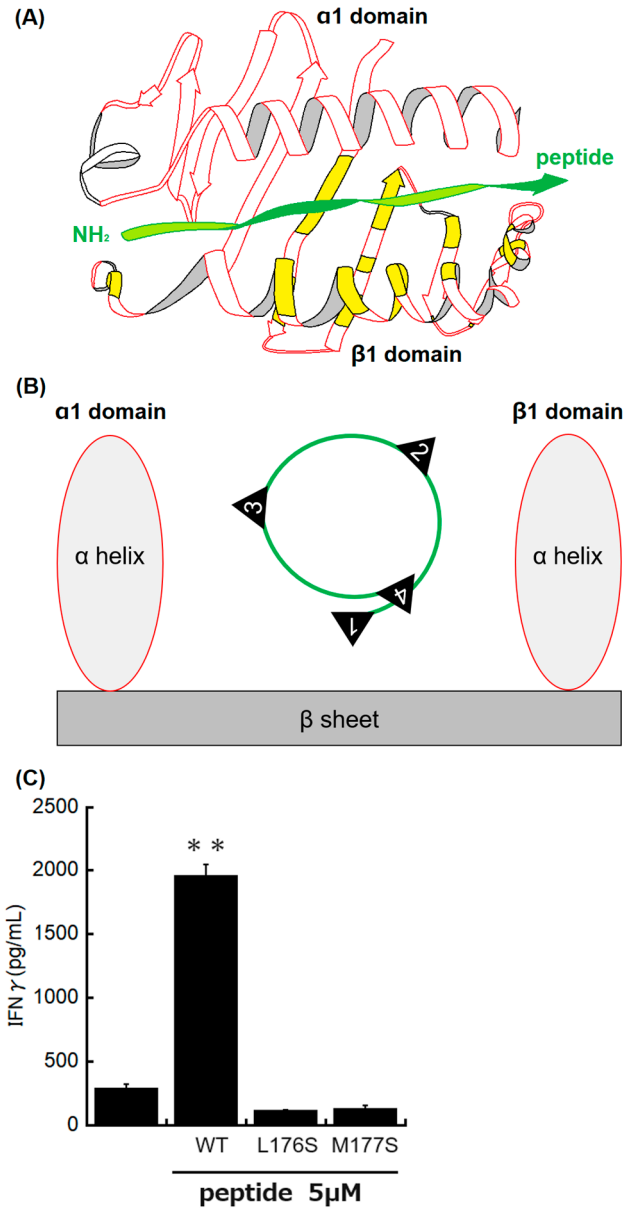


Figure 2. (**A**) An overhead view of the HLA class II-peptide complex from the perspective of the TCR. Peptides rotate 130 degrees counterclockwise with each advancement of one residue. Yellow painted parts of the beta 1 domain indicate polymorphic residues. This is an original illustration. (**B**) A schematic diagram of the HLA class II-peptide complex viewed from the N-terminus of the binding peptide along the longitudinal axis (displaying only the first 4 residues). This is an original illustration. (**C**) TM45.2 cells were cultured in 96-well flat-bottom culture plates in the presence of irradiated autologous PBMCs and three types of peptides using R10H medium. "WT" represents the peptide p176-190. L176S refers to peptide p176-190 with a substitution of leucine at position 176 replaced by the neutral hydrophilic serine, while M177S indicates peptide p176-190 with a substitution of methionine at position 177 replaced by the neutral hydrophilic serine. Four days later, culture supernatant fluid was collected for an IFNγ ELISA (n = 6). Data are expressed as the mean ± SD and were compared by a one-way analysis of variance and Tukey's post hoc test. ** $p < 0.01$ compared to the culture without peptide.

4. Discussion

When TM45 cells were incubated with kynurenine for 7 days, they produced IL-8 in a dose-dependent manner; however, there was no increase in the production of GM-CSF

from TM45 cells. This suggests that the oversecretion induced by kynurenine is specific to the IL-8 response. Co-incubation with kynurenine increased the IL-8 levels in TM45 cells. When incubated in the absence of kynurenine, the original levels of cytokine production were recovered. This indicates that the IL-8 response could be induced by kynurenine produced under the infectious conditions of COVID-19, which might not be a permanent change in the T cell phenotype.

Kynurenine is produced from tryptophan by indoleamine 2,3-dioxygenase (IDO). IDO is expressed in various cells, including immune cells and mucosal tissues of the digestive tract [25]. In the immune system, IDO is expressed in antigen-presenting cells such as monocytes, macrophages, and dendritic cells. The activity of IDO is minimal under basal conditions, but its expression increases with inflammatory cytokines, such as IFNγ [26]. The blood concentration of kynurenine in humans infected with COVID-19 has been reported to be around 10–15 μM [27]. To obtain sufficient kynurenine (>100 μM) to trigger the hypersecretion of IL-8, T cells may need to make contact with other cells to form a microenvironment where a high kynurenine concentration can be achieved, as observed during T cell-antigen-presenting cell (APC) interactions at immunological synapses [19]. Indeed, in antigen-presenting cells during inflammation, the increased expression of IDO has been observed [25], which attests to the plausibility within the human body.

The first anchor of HLA-DR-binding peptides is invariably a hydrophobic residue. L176S is an analog peptide with a substitution of hydrophobic 176 Leucine with neutral hydrophilic serine. Additionally, the residue on the N-terminal side of the first anchor does not play a role in binding with HLA or in interactions with TCR [22,24]. Therefore, 176 L is considered to be the first anchor to bind HLA-DR. The first anchor is inevitable for binding to HLA; hence, L176S completely loses its ability to bind with HLA. In addition, 177 Methionine (position 2) is a residue recognized solely by TCR; therefore, it is conceivable that M177S has completely lost its ability to bind to TCR. These analog peptides did not elicit any response in TM45.2 cells. This suggests that the suppression of the IL-8 response in COVID-19 infection does not necessarily require the loss of whole T cell epitopes, as it could be lost due to a single amino acid substitution in the viral antigen. In particular, based on numerous previous studies, the loss of binding affinity for HLA-DR by substituting the first anchor with a hydrophilic residue appears to be a universal phenomenon [28,29]. Furthermore, a single-residue substitution should be able to eliminate potentially harmful T cell epitopes, while preserving beneficial B-cell epitopes.

5. Conclusions

Our observations collectively indicate that (i) enhanced IL-8 responses can be induced by kynurenine, which is produced under infectious conditions in COVID-19; (ii) the response is not a permanent change in the T cell phenotype; and (iii) IL-8 responses associated with harmful neutrophil extracellular traps can be abrogated by a single amino acid substitution of the viral antigens. These findings may shed light on a novel strategy for designing vaccines for viral infections, such as coronaviruses and Zika virus, which are known to be accompanied by increased kynurenine production. Future research directions regarding the role of kynurenine and vaccine development may include the use of compounds that inhibit AHR signaling downstream of kynurenine. The association between HLA-DR and neutrophilic inflammation needs to be tested in haplotypes frequently observed in the Japanese population.

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Institutional Review Board Statement: The protocol for this research project was approved by the Saitama Medical University Ethics Committee (#787-III, March 2023) and conforms to the provisions of the Declaration of Helsinki.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is unavailable due to privacy or ethical restrictions.

Conflicts of Interest: The author Sho Matsushita is employed by the company iMmno, Inc. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; interleukin, IL; DIC, disseminated intravascular coagulation; NETs, neutrophil extracellular traps; ARDS, acute respiratory distress syndrome; Th, T-helper; AHR, aryl hydrocarbon receptor; CD, cluster of differentiation; HLA, human leukocyte antigen; PBMCs, peripheral blood mononuclear cells; ELISA, enzyme-linked immunosorbent assays; IFN, interferon; GM-CSF, granulocyte-macrophage colony-stimulating factor; TCR, T cell receptor; IDO, indoleamine 2,3-dioxygenase; APC, antigen-presenting cells; SD, standard deviation; n, number of repeated experiments.

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