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Original Article

Comprehensive Analysis of Epstein-Barr Virus LMP2A-Specific CD8⁺ and CD4⁺ T Cell Responses Restricted to Each HLA Class I and II Allotype Within an Individual

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ABSTRACT

Latent membrane protein 2A (LMP2A), a latent Ag commonly expressed in Epstein-Barr virus (EBV)-infected host cells, is a target for adoptive T cell therapy in EBV-associated malignancies. To define whether individual human leukocyte antigen (HLA) allotypes are used preferentially in EBV-specific T lymphocyte responses, LMP2A-specific CD8⁺ and CD4⁺ T cell responses in 50 healthy donors were analyzed by ELISPOT assay using artificial Ag-presenting cells expressing a single allotype. CD8⁺ T cell responses were significantly higher than CD4⁺ T cell responses. CD8⁺ T cell responses were ranked from highest to lowest in the order HLA-A, HLA-B, and HLA-C loci, and CD4⁺ T cell responses were ranked in the order HLA-DR, HLA-DP, and HLA-DQ loci. Among the 32 HLA class I and 56 HLA class II allotypes, 6 HLA-A, 7 HLA-B, 5 HLA-C, 10 HLA-DR, 2 HLA-DQ, and 2 HLA-DP allotypes showed T cell responses higher than 50 spot-forming cells (SFCs)/5×10^5 CD8⁺ or CD4⁺ T cells. Twenty-nine donors (58%) showed a high T cell response to at least one allotype of HLA class I or class II, and 4 donors (8%) had a high response to both HLA class I and class II allotypes. Interestingly, we observed an inverse correlation between the proportion of LMP2A-specific T cell responses and the frequency of HLA class I and II allotypes. These data demonstrate the allele dominance of LMP2A-specific T cell responses among HLA allotypes and their intra-individual dominance in response to only a few allotypes in an individual, which may provide useful information for genetic, pathogenic, and immunotherapeutic approaches to EBV-associated diseases.

Keywords: HLA class I and II; Epstein-Barr virus; LMP2A; CD8⁺ and CD4⁺ T cells; Dominant allotype; Intra-individual dominance

INTRODUCTION

Most of the human population has been infected with Epstein-Barr virus (EBV), also known as human gamma-herpes virus 4. EBV infects naïve B cells in oropharyngeal lymphoid tissues and is maintained through a balance of lytic and latent infections (1,2). EBV is associated with

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
aAPC, artificial Ag-presenting cell; BCR, B cell receptors; CMV, cytomegalovirus; DC, dendritic cell; EBV, Epstein-Barr virus; IM, infectious mononucleosis; IRB, Institutional Review Board; LMP1, latent membrane protein 1; LMP2A, latent membrane protein 2A; SFC, spot-forming cell.

Author Contributions
Conceptualization: Jo HA, Hyun YS, Kim TG; Formal analysis: Jo HA, Kim TG; Investigation: Jo HA, Hyun SJ, Hyun YS, Lee YH; Resources: Kim SM, Beak IC, Sohn HJ; Writing - original draft: Jo HA, Kim TG; Writing - review & editing: Jo HA, Hyun YS, Kim TG.

human malignancies, including Burkitt's lymphoma, Nasopharyngeal carcinoma, Hodgkin's lymphoma, and lymphoproliferative disorders, in immunocompromised individuals (3). EBV latency-II Ags such as Epstein-Barr nuclear Ag-1, latent membrane protein 1 (LMP1), and latent membrane protein 2A (LMP2A), are expressed in malignant cells (4,5). LMP2A mimics the activated B cell receptors (BCR) and inhibits normal signal transduction. The N-terminal domain of LMP2A has similarities to the signaling domain of the BCR complex (3,6,7).

EBV-specific T cell responses suppress viral replication during latent infection in healthy individuals (8). As LMP2A potentially constitutes a major target Ag for immunotherapy of EBV-associated malignancies (9-11), many researchers have aimed to identify T cell epitopes located on this protein (12,13). In patients with infectious mononucleosis (IM), EBV Ag-specific T cells are dominated by $CD8^+$ T cells, which recognize peptides presented by MHC class I molecules (14,15). $CD4^+$ T cells are required for expansion and memory development in $CD8^+$ T lymphocytes. In particular, EBV-specific $CD4^+$ T cells can directly recognize EBV-infected B cells, such as EBV-transformed lymphoblastoid cell lines which express HLA class II molecules (16-18). Despite the important role played by $CD4^+$ T cells, research has focused on $CD8^+$ T cell responses, and the $CD4^+$ T cell response has not been fully investigated (19). In our previous study, $CD8^+$ and $CD4^+$ T cells responding to LMP1 and LMP2A, respectively, were measured in mRNA-transfected dendritic cells (DCs). As a result, we observed that the LMP1-specific $CD4^+$ T cell response was significantly higher than the LMP2A-specific $CD4^+$ T cell response (5).

HLA-A, HLA-B, and HLA-C are classical MHC class I proteins that present peptides to $CD8^+$ T cells, while HLA-DR, HLA-DQ, and HLA-DP are classical MHC class II proteins that present Ags to $CD4^+$ T cells. These classical MHC class II proteins are heterodimers composed of α and β chains, encoded by A and B genes. HLA class II molecules are expressed in professional Ag-presenting cells, such as DCs and B cells. HLA class I molecules present peptide fragments induced by proteolysis of intracellular proteins (endogenous pathway), and HLA class II molecules present peptide fragments sampled from extracellular proteins (extrinsic pathway) (20). HLA molecules are characterized by extremely high polymorphism, with each HLA having allele-specific polymorphic residues in the peptide-binding pocket. These residues generate different peptide-binding patterns for various HLA molecules (21,22), allowing for high-affinity binding to allele-specific binding motifs (23). Therefore, HLA molecules represent a distinctive peptide-binding repertoire, and individuals with different HLA genotypes can present specific peptides that induce differential immune responses (24,25).

As individuals can express 6 HLA class I allotypes, and more than six functional heterodimer HLA class II allotypes (in those who are heterozygous), a panel of K562-based artificial Ag-presenting cells (aAPCs) expressing a single HLA allotype has been established to facilitate measurement of T cell responses restricted by a single HLA allotype (26,27). Previous studies using these aAPCs expressing a single allotype revealed HLA-allele dominance in cytomegalovirus pp65 Ag-specific $CD8^+$ and $CD4^+$ T cell responses (26,27). Herein, we analyzed $CD8^+$ and $CD4^+$ T cell responses specific to the EBV LMP2A Ag according to HLA class I and class II at the single-allotype level. Allele dominance was observed in each individual, even in LMP2A-specific T cell responses, which were challenging to measure. These results suggest that it will be necessary to transfer third-party donor T cells that are reactive to the matched allele during adoptive immunotherapy treatments targeting EBV-associated tumors (5). This study also provides basic immunobiological information to explain the immunogenetic results showing that the development of EBV-associated tumors is associated with specific HLA alleles.

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MATERIALS AND METHODS

Human Blood Samples

This study was approved by the Institutional Review Board (IRB) of the Catholic University of Korea (MC20SESI0117). PBMCs were collected from 50 healthy adult Korean donors who provided written informed consent. The participants consisted of 6 females and 44 males with an average age of 26.02±3.9 years. PBMCs were purified by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Chicago, IL, USA). PBMCs were cryopreserved in RPMI 1640 medium (Lonza, Basel, Switzerland) containing 10% dimethyl sulfoxide (Mylan, Canonsburg, PA, USA) and 40% heat-inactivated FBS (Invitrogen, Waltham, MA, USA). HLA alleles were typed for HLA-A, HLA-B, HLA-C, HLA-DRA1, HLA-DRB1, HLA-DQA1, HLA-DQB1, HLA-DPA1, and HLA-DPB1 loci by the Catholic Hematopoietic Stem Cell Bank (Seoul, Korea). The list of HLA class I and II genotypes of the 50 donors used in this study is presented in **Supplementary Tables 1** and **2**.

Isolation of $CD8^+$ and $CD4^+$ T cells

Cryopreserved PBMC were thawed, and $CD8^+$ and $CD4^+$ T cells were isolated using magnetic beads (AutoMACS Pro; Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of $CD8^+$ and $CD4^+$ T cells was confirmed using flow cytometry, and these T cells were cryopreserved again until use. Prior to use in subsequent experiments, survival rate was confirmed to be ≥90% using trypan blue.

Establishment of single HLA class I and II allotype-expressing aAPC

A total of 32 HLA class I allotypes and 56 HLA class II allotypes-specific aAPC panels were used in this study (26,27). Seven additional HLA class II alleles were also generated as previously described (27). Briefly, cDNA was isolated from lymphoblastoid cell lines encoding each allele of HLA class I and II (740902.50; Macherey Nagel, RT300M; Enzynomics, Hanam, Korea). HLA alleles were transduced into 1×10^4 CD80, CD83, and CD137L-expressing K562 cells at a MOI=20 (27). Cells were stained with anti-HLA class I (APC; G46-2.6; AB_395934; BD Biosciences, Piscataway, NJ, USA), HLA-DR (APC; G46-6; RRID: AB_1727527; BD Biosciences), HLA-DQ (APC; Tü169; RRID: AB_2738963, Tü39; RRID: AB_395940; BD Biosciences), and HLA-DP (PE; B7/21; H1586; Leinco Technologies, Fenton, MO, USA). After transduction of the allele-specific lentiviruses, positive aAPCs were isolated using flow cytometry-guided sorting (FACSAria Fusion; BD Biosciences). aAPCs expressing single HLA class I and II were cultured in RPMI 1640 medium (Lonza) containing 10% heat-inactivated FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycins (Lonza).

Genes encoding α and β chains can form four heterodimers at an HLA allele in double-heterozygous individuals. Based on haplotype analysis, the most likely combination of each of the four HLA-DQ and HLA-DP combinations was determined (28). As the DRA locus is almost monomorphic, HLA-DR was used with DRA*01:01 and DRB1 alleles.

IFN-γ ELISPOT assay

IFN-γ producing $CD8^+$ and $CD4^+$ T cells against LMP2A were quantified by ELISPOT assay, as previously described (26,27,29). LMP2A was cloned from the B95.8 type EBV strain. LMP2A tagged with T2A and GFP was cloned into the PiggyBac vector and transfected into the panel of aAPCs using the Amaxa® Cell Line Nucleofector® Kit V (Lonza). Briefly, 5×10^4 aAPCs and aAPCs stably expressing LMP2A were transfected or untransfected with HLA class I allotype.

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The aAPCs were then incubated with 5×10^5 $CD8^+$ T cells for 20 h at 37°C. The aAPCs expressing a single HLA class II allotype were incubated with a peptide pool in serum-free RPMI1640 medium for 4 h. To measure $CD4^+$ T cell responses, we utilized a peptide pool of 15 amino acid peptides spanning 11 amino acid overlaps (60 nM concentration; JPT Peptide Technologies, Berlin, Germany). The Ag-pulsed aAPCs were washed with fresh serum-free RPMI1640 medium using a centrifuge. After washing, 5×10^4 Ag-pulsed aAPCs were incubated with 5×10^5 $CD4^+$ T cells for 20 h at 37°C. As controls, untransfected or unpulsed HLA-matched aAPCs and aAPCs that did not express HLA were co-cultured with $CD8^+$ and $CD4^+$ T cells. As previously described, the magnitude of the HLA-restricted T cell response was calculated as (26,27,29):

{(Response to aAPCs-LMP2A Expressing a Single HLA Allotype)
− (Response to aAPCs Expressing Ssingle HLA Allotype)}
− {(Response to aAPCs-LMP2A)
− (Response to aAPCs)}.

ELISPOT assay mean and SD background values for $CD8^+$ T cell responses to aAPCs expressing single HLA allotypes, aAPCs-LMP2A, and aAPCs only, were 139.3 SFCs/$5x10^5$ cells (SD, 253.9 SFCs/$5x10^5$ cells), 22.8 SFCs/$5x10^5$ cells (SD, 49 SFCs/$5x10^5$ cells), and 21.1 SFCs/$5x10^5$ cells (SD, 47.5 SFCs/$5x10^5$ cells), respectively. When evaluating HLA class II allotypes, background values for $CD4^+$ T cell responses to aAPCs expressing single HLA allotype, aAPCs-LMP2A, and aAPCs only, were 38.9 SFCs/5×10^5 cells (SD, 69 SFCs/5×10^5 cells), 1.8 SFCs/5×10^5 cells (SD, 4.9 SFCs/5×10^5 cells), and 0.9 SFCs/5×10^5 cells (SD, 2.5 SFCs/5×10^5 cells), respectively. Although some aAPCs expressing a single HLA allotype without an Ag may show a high background value depending on the individual, the Ag-specific response was determined by subtracting the background value from the value in the presence of the Ag. Since the value in the presence of the Ag was always higher than the background value, the Ag-specific response value did not appear negative. The number of SFCs was determined using an AID ELISPOT Reader System (AID Diagnostika GmbH, Straßberg, Germany).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 software. Statistical significance was determined using one-way ANOVA, unpaired *t*-test (with a two-tailed test of significance) and Pearson's correlation analysis. Results of a single experiment with 50 healthy donors were obtained. Data are presented as mean ± SD or SEM with p-values represented by <0.05, <0.01, <0.001, and <0.0001.

RESULTS

Population analysis of EBV LMP2A-specific $CD8^+$ T cells according to HLA class I loci and allotypes

LMP2A-specific $CD8^+$ T cell responses in 50 healthy donors were analyzed for each HLA class I restriction locus and allotype. The magnitudes of total HLA class I and HLA locus responses were calculated as the sum of 6 and 2 allotypes, respectively (**Fig. 1A**). The sum of $CD8^+$ T cell responses in all HLA class I allotypes had a mean of 98.2 SFCs/5×10^5 $CD8^+$ T cells, and HLA-A, HLA-B, and HLA-C had means of 41.8, 30, and 26.4 SFCs, respectively.

Among the seven HLA-A allotypes, A*02:06 had the highest response, being significantly higher than that of A*11:01, A*02:01, A*24:02, and A*02:07 (**Fig. 1B**). HLA-A allotypes in

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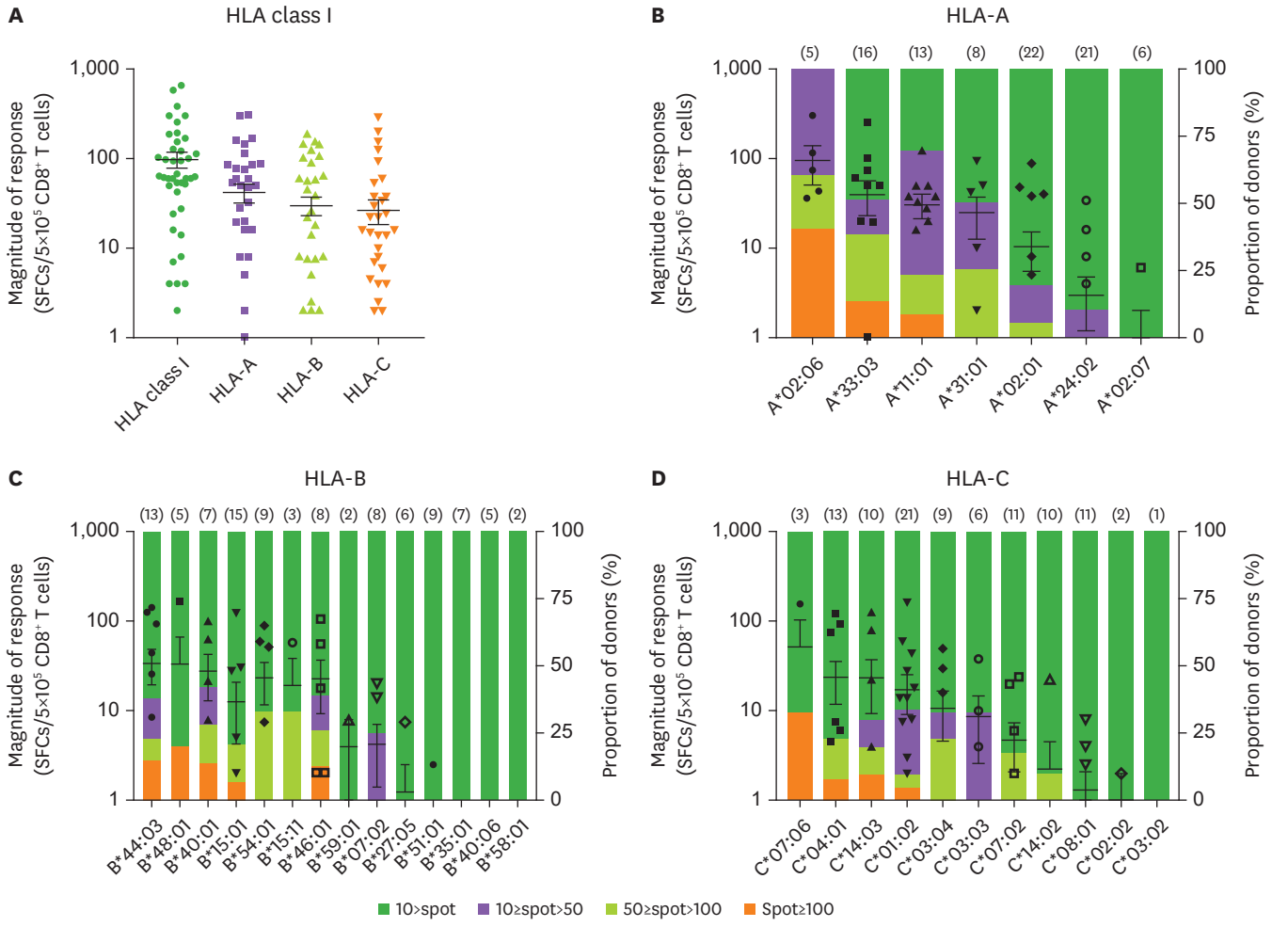

Figure 1. CD8⁺ T cell responses to EBV LMP2A according to HLA class I loci and allotypes.
(A) Distribution of LMP2A-specific CD8⁺ T cell responses according to each locus of the HLA-A, HLA-B, and HLA-C in healthy donors (n=50). (B-D) Each dot presents the magnitude of response according to indicated allotypes of HLA-A, HLA-B, and HLA-C, respectively. The relative proportion of donors according to the strengths of CD8⁺ T cell responses is presented in a stacked bar graph. The number of donors per allotype is shown in parentheses. Error bars present mean±SEM, statistical analysis was performed using one-way ANOVA. Because negative numbers cannot be shown on a logarithmic Y-axis, negative values and some down vertical bars are not displayed.

which at least one donor showed a T cell response of more than 50 SFCs/5×10⁵ CD8⁺ T cells, included A*02:06, A*33:03, A*11:01, A*31:01, and A*02:01. Among the 14 HLA-B allotypes, B*44:03, B*48:01, B*40:01, B*15:01, B*54:01, B*15:11, and B*46:01 induced T cell response of more than 50 SFCs/5×10⁵ CD8⁺ T cells (**Fig. 1C**). No T cell responses were observed for B*35:01, B*40:06, and B*58:01. Among the 11 HLA-C allotypes, C*07:06, C*04:01, C*14:03, C*01:02, and C*03:04 induced a T cell response of more than 50 SFCs/5×10⁵ CD8⁺ T cells (**Fig. 1D**). No T cell responses were observed for HLA-C*03:02. The distribution of CD8⁺ T cell immune responses to the LMP2A Ag differs depending on the HLA class I allotype, suggesting that the LMP2A Ag-presenting ability of each allotype is different.

Population analysis of EBV LMP2A-specific CD4⁺ T cells according to HLA class II loci and allotypes

LMP2A-specific CD4⁺ T cell responses were analyzed for each HLA class II restriction locus and allotype. The sum of CD4⁺ T cell responses in all HLA class II allotypes in an individual had a mean of 40.8 SFCs per 5×10⁵ CD4⁺ T cells. The T cell response to HLA class II was

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significantly lower than those observed against HLA class I (one-way ANOVA, p=0.0087). The mean responses to HLA-DR, HLA-DQ, and HLA-DP were 26.3, 6.3, and 8.2 SFCs, respectively (**Fig. 2A**). HLA-DR showed the highest response among the HLA class II loci. The response of the HLA-DR locus was significantly higher than that of HLA-DQ and HLA-DP (one-way ANOVA, p=0.0003 and p=0.0013). Unlike HLA class I responses, $CD4^+$ T cell immune responses to LMP2A Ag differed by locus, and more allele responses in the HLA-DR locus demonstrate that this locus plays a more important role in the Ag-presenting abilities of $CD4^+$ T cells than other loci.

Among the 23 HLA-DR allotypes, those in which at least one donor showed more than 50 SFCs/5×10^5 $CD4^+$ T cells included DRB1*10:01, DRB1*04:03, DRB1*03:01, DRB1*04:04, DRB1*01:01, DRB1*09:01, DRB1*08:03, DRB1*14:05, DRB1*13:02, and DRB1*04:05 (**Fig. 2B**).

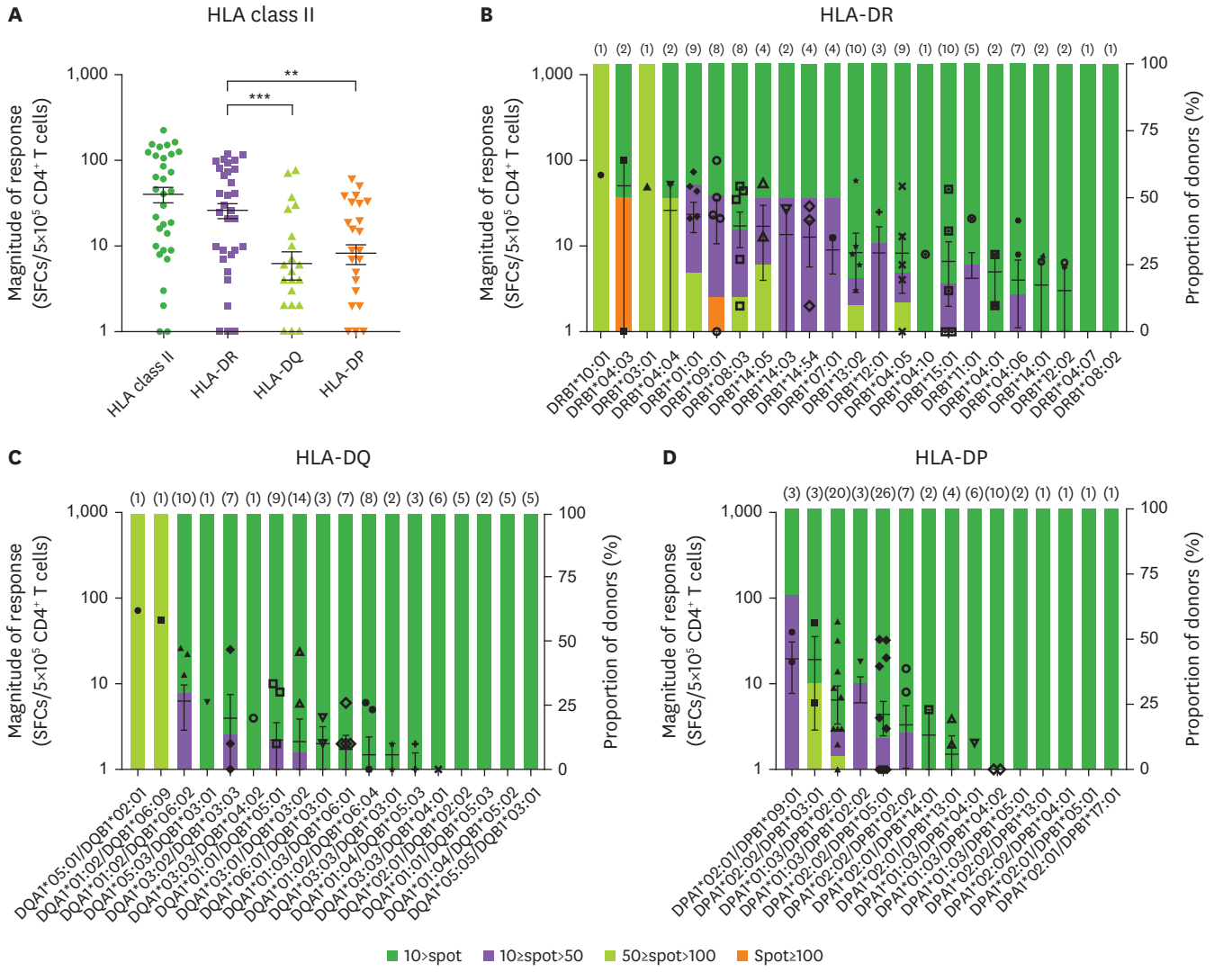

Figure 2. $CD4^+$ T cell responses to EBV LMP2A according to HLA class II loci and allotypes.
(A) Distribution of LMP2A-specific $CD4^+$ T cell responses according to each locus of the HLA-DR, HLA-DQ, and HLA-DP in healthy donors (n=50). (B-D) Each dot presents the magnitude of response according to indicated allotypes of HLA-DR, HLA-DQ, and HLA-DP, respectively. The relative proportion of donors according to the strengths of $CD4^+$ T cell responses is presented in a stacked bar graph. The number of donors per allotype is shown in parentheses. Error bars present mean±SEM, statistical analysis was performed using one-way ANOVA. Because negative numbers cannot be shown on a logarithmic Y-axis, negative values and some down vertical bars are not displayed.
**p<0.01, ***p<0.001.

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No T cell responses were observed for DRB1*04:07 and DRB1*08:02. Among the 18 HLA-DQ allotypes, DQA1*05:01/DQB1*02:01 and DQA1*01:02/DQB1*06:09 induced a T cell response of more than 50 SFCs/5×10^5 CD4$^+$ T cells (**Fig. 2C**). No T cell responses were observed for DQA1*02:01/DQB1*02:02, DQA1*01:01/DQB1*05:03, DQA1*01:04/DQB1*05:02, and DQA1*05:05/DQB1*03:01. Among the 15 HLA-DP allotypes, DPA1*02:02/DPB1*03:01, and DPA1*01:03/DPB1*02:01 induced T cell responses of more than 50 SFCs/5×10^5 CD4$^+$ T cells (**Fig. 2D**). No T cell responses were observed for DPA1*01:03/DPB1*05:01, DPA1*02:02/DPB1*13:01, DPA1*02:02/DPB1*04:01, DPA1*02:01/DPB1*05:01, and DPA1*02:01/DPB1*17:01.

Intra-individual dominance of LMP2A-specific CD8$^+$ T cell responses to HLA class I allotypes

Of the 50 donors, 23 (46%) had CD8$^+$ T cell responses of more than 50 SFCs/5×10^5 CD8$^+$ T cells in at least one allotype (**Fig. 3A**). Seventeen donors (34%) had T cell responses of >50 SFCs/5×10^5 CD8$^+$ T cells in only 1 allotype. Two donors had T cell responses of >50 SFCs/5×10^5 CD8$^+$ T cells in 2 allotypes, two donors had T cell responses of >50 SFCs/5×10^5 CD8$^+$ T cells in three allotypes, and 2 donors had T cell responses of >50 SFCs/5×10^5 CD8$^+$ T cells in 5 allotypes. Of the 50 donors, 27 had weak or no responses.

To elucidate allele dominance from a demographic point of view, the LMP2A-specific CD8$^+$ T cell responses by allotype of each donor were classified in the highest response order, and then the distribution of each group was analyzed (**Fig. 3B**). The highest responses (mean of 68.6) were significantly higher than the second (mean of 26.4, one-way ANOVA, $p<0.0001$), third (mean of 12.8, one-way ANOVA, $p<0.0001$), and fourth (mean of 8.7, one-way ANOVA, $p<0.0001$) responses for other allotypes within an individual.

Among the 17 allotypes that showed CD8$^+$ T cell responses of more than 50 SFCs/5×10^5 CD8$^+$ T cells in at least 3 or more donors, the probability of intra-individual dominance was analyzed based on the order of the highest responses in a specific allotype (**Fig. 3C**). Fourteen allotypes showed the first intra-individual dominance and three allotypes did not. In particular, although HLA-A*02:01 and HLA-B*44:03 were present with high frequencies of 26% and 40%, respectively, among the subjects of this study, the probability of the first intra-individual dominance was relatively low at less than 10%. These results demonstrated the dominance of specific HLA class I allotypes within individuals, and the probability of a dominant CD8$^+$ T cell response varied.

Intra-individual dominance of LMP2A-specific CD4$^+$ T cell responses according to HLA class II allotypes

Of the 50 donors, 10 (20%) had CD4$^+$ T cell responses of more than 50 SFCs/5×10^5 CD4$^+$ T cells in at least one allotype (**Fig. 4A**). Six donors (12%) had T cell responses of >50 SFCs/5×10^5 CD4$^+$ T cells in only 1 allotype. Three donors had T cell responses of >50 SFCs/5×10^5 CD4$^+$ T cells in 2 allotypes, and 1 donor had T cell responses of >50 SFCs/5×10^5 CD4$^+$ T cells in 3 allotypes. Forty donors had weak or no responses.

To elucidate allele dominance from a demographic point of view, the LMP2A-specific CD4$^+$ T cell responses by allotype of each donors were classified in the highest order and then the distribution of each group was analyzed (**Fig. 4B**). The highest responses (mean of 30.8) were significantly higher than the second (mean of 17.7, one-way ANOVA, $p=0.0328$), third (mean of 8.2, one-way ANOVA, $p<0.0001$), and fourth (mean of 4.9, one-way ANOVA, $p<0.0001$) responses for other allotypes within an individual.

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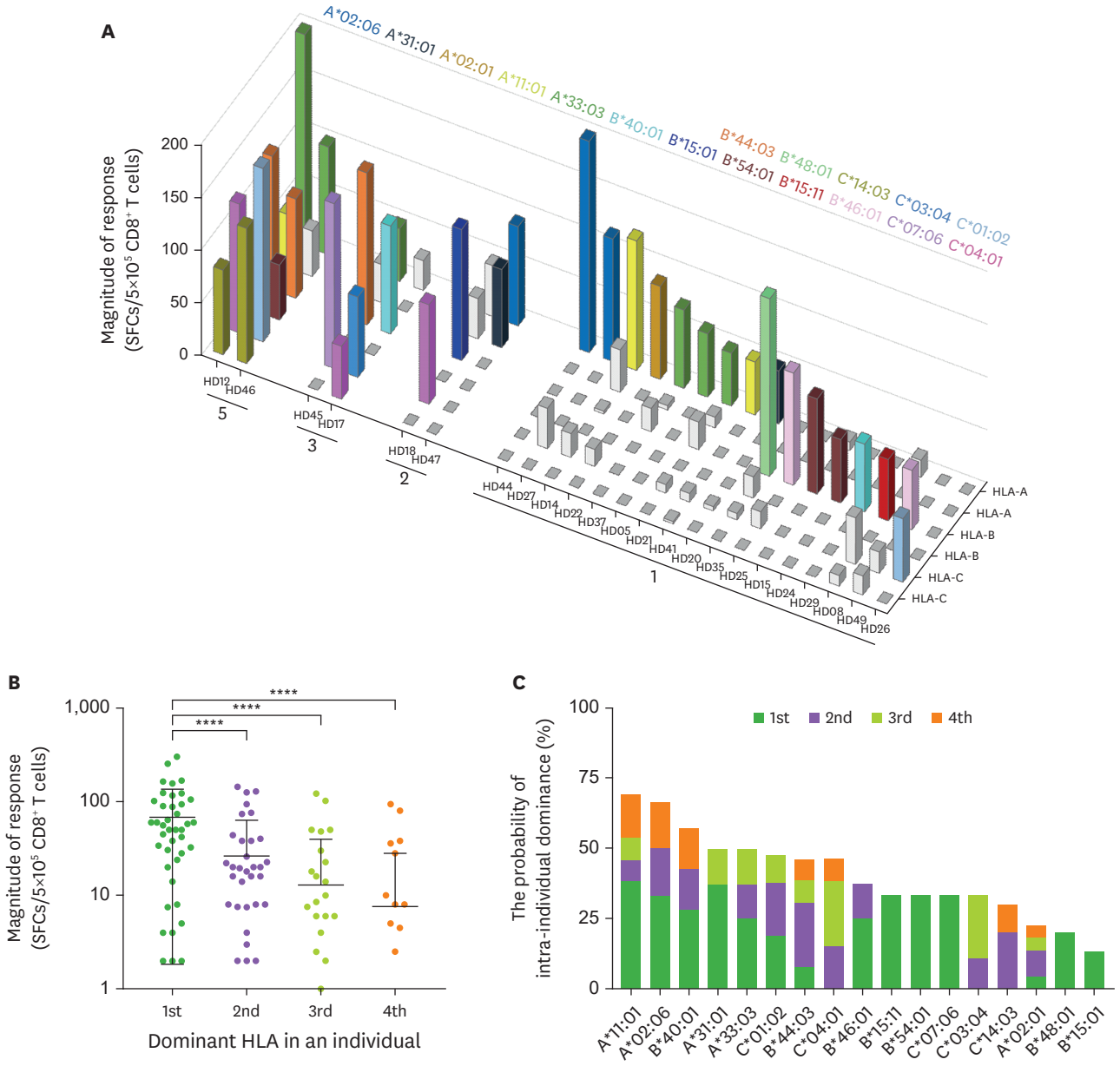

Figure 3. LMP2A-specific CD8⁺ T cell responses according to HLA class I allotypes within individuals.
(A) LMP2A-specific CD8⁺ T cell responses in 23 healthy donors with T cell response of 50 SFCs/5×10⁵ CD8⁺ T cells or more. The color bar indicated allotypes with CD8⁺ T cell responses of more than 50 SFCs/5×10⁵ CD8⁺ T cells. (B) LMP2A-specific CD8⁺ T cell responses by allotypes of each individual in 50 donors were listed in the highest order and then the distribution of each group was analyzed. The magnitude of response value less than 1 is not presented. Error bars present mean±SD, statistical analysis was performed using one-way ANOVA. (C) The probability of intra-individual dominance is the first, second, and third highest response by a specific allotype in an individual. The alleles were included in cases that showed CD8⁺ T cell responses of more than 50 SFCs/5×10⁵ CD8⁺ T cells from at least 3 or more donors.
****p<0.0001.

Among the 8 allotypes showing CD4⁺ T cell responses of more than 50 SFCs/5×10⁵ CD4⁺ T cells in at least 3 or more donors, the probability of intra-individual dominance was analyzed based on the order of the highest responses in a specific allotype of an individual (**Fig. 4C**). Six allotypes showed first intra-individual dominance and 2 allotypes did not show first intra-individual dominance. DRB1*04:05 was the lowest among the HLA class II allotypes

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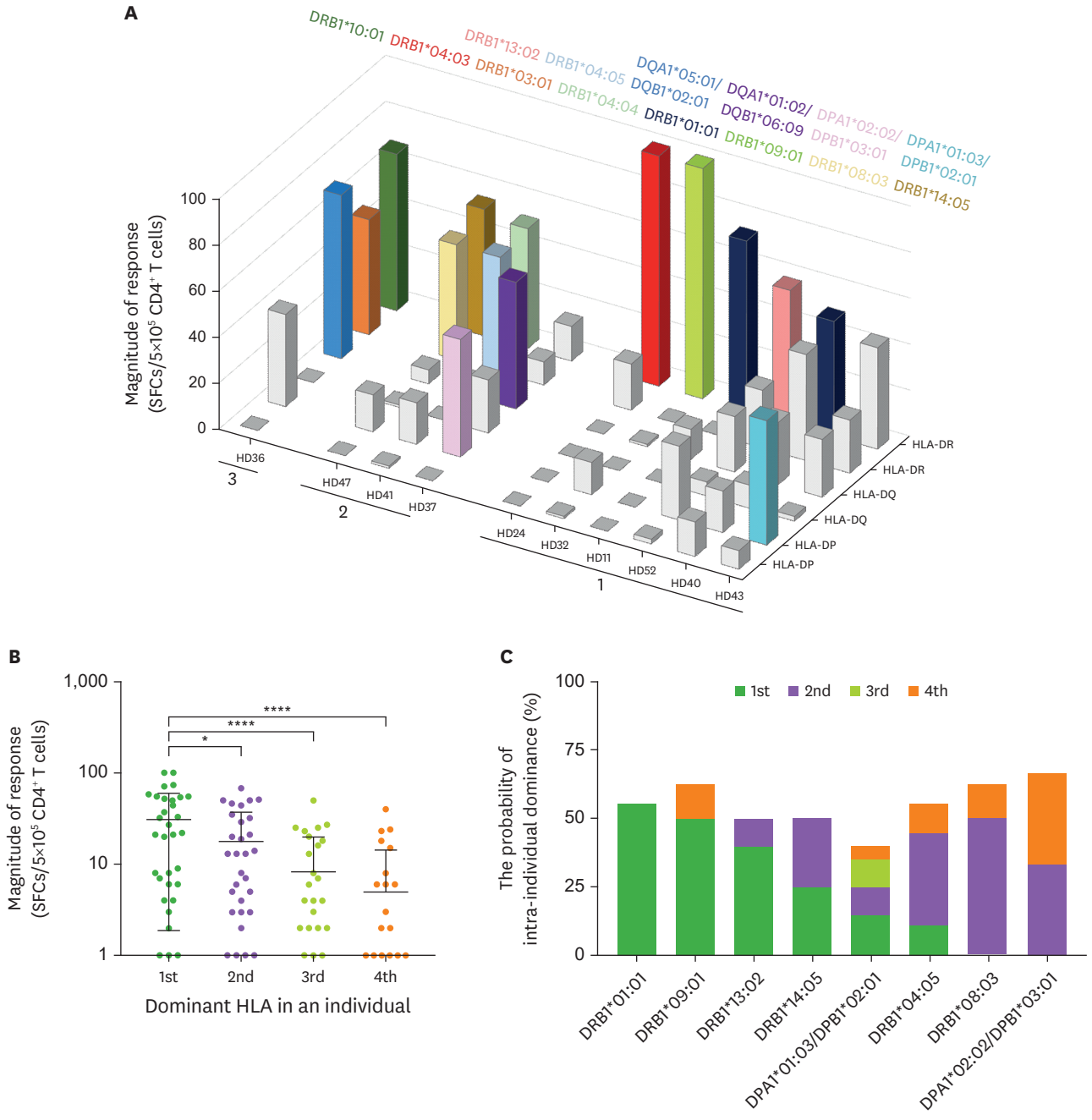

Figure 4. LMP2A-specific CD4⁺ T cell responses according to HLA class II allotypes within individuals.
(A) LMP2A-specific CD4⁺ T cell responses in 10 healthy donors with T cell response of 50 SFCs/5×10⁵ CD4⁺ T cells or more. The color bar indicated allotypes with CD4⁺ T cell responses of more than 50 SFCs/5×10⁵ CD4⁺ T cells. (B) LMP2A-specific CD4⁺ T cell responses by allotypes of each individual in 50 donors were listed in the highest order and then the distribution of each group was analyzed. The magnitude of response value less than 1 is not presented. Error bars present mean±SD, statistical analysis was performed using one-way ANOVA. (C) The probability of intra-individual dominance is the first, second, and third highest response by a specific allotype in an individual. The alleles were included in cases that showed CD4⁺ T cell responses of more than 50 SFCs/5×10⁵ CD4⁺ T cells from at least 3 or more donors.
*p<0.05, ****p<0.0001.

with the first intra-individual dominance. These results also demonstrate the dominance of specific HLA class II allotypes within individuals, and the probability of a dominant CD4⁺ T cell response varies.

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Comparison of LMP2A-specific CD8⁺ and CD4⁺ T cell responses according to HLA allotypes within individuals

We further compared and analyzed the association between CD8⁺ T cell responses to HLA class I and CD4⁺ T cell responses to HLA class II within individuals. The proportion of donors who responded only to HLA class I was 38%, whereas the proportion of donor who responded only to HLA class II was 12%. The proportion of donors who responded to both HLA class I and II was 8%, 42% of the donors did not respond to either HLA class (**Fig. 5A and B**). Overall, among the total HLA allotypes in an individual, one HLA class I allotype showed a dominant T cell response to LMP2A. Because epitopes of Ags on CD8⁺ and CD4⁺ T cells are frequently shared, and CD4⁺ T cells are known to help CD8⁺ T cells in response, the relationship between CD8⁺ and CD4⁺ T cells can be predicted. However, no significant association was observed

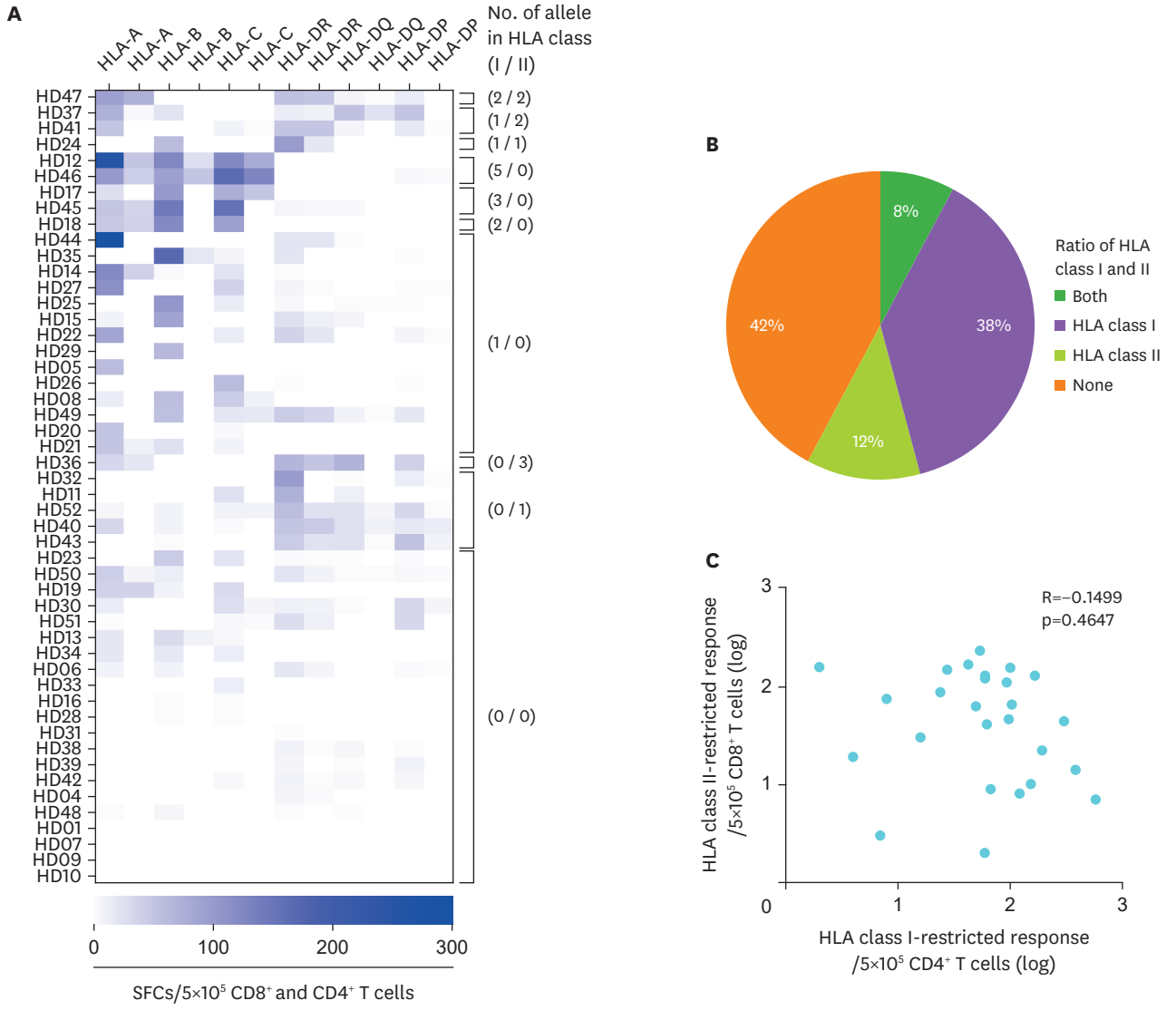

Figure 5. Comparison between CD8⁺ and CD4⁺ T cell responses within an individual.
(A) LMP2A-specific CD8⁺ and CD4⁺ T cell responses restricted by HLA class I and II allotypes were indicated as a heatmap. The number of allotypes of HLA class I and II in which Ag-specific T cell responses showed more than 50 SFCs/5×10⁵ T cells are indicated in parentheses. (B) The proportion of CD8⁺ and CD4⁺ T cell responses of more than 50 SFCs/5×10⁵ T cells by HLA class I and II allotypes. (C) The correlation between LMP2A-specific CD8⁺ and CD4⁺ T cell responses was analyzed. Statistical analysis was performed using Pearson's correlation analysis.

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between HLA classes I and II in T cell responses to LMP2A (Pearson's correlation, p=0.4647; **Fig. 5C**). Further studies are required to confirm these results.

Correlation analysis of LMP2A-specific CD8⁺ and CD4⁺ T cell responses with HLA allele frequencies

We compared T cell responses and frequencies of HLA class I and II alleles for population analysis. Seventeen HLA class I and 14 HLA class II alleles were investigated, excluding allotypes that did not show a T cell response (**Fig. 6**). The less frequent HLA class I and II alleles showed a proportion of CD8⁺ and CD4⁺ T cell responses of 50 SFCs/5×10^5 T cells or higher. Overall, the proportion of T cell responses of more than 50 SFCs/5×10^5 T cells was inversely correlated with the frequency of HLA class I and II alleles (Pearson's correlation, p=0.0359 and p=0.0175, respectively). The fact that LMP2A Ag-specific T cell responses are low in most people with a high frequency of HLA class I and class II alleles is thought to be helpful for the immunological understanding of EBV latent infection in humans.

DISCUSSION

In our previous study using DCs, there was a correlation between the mRNA and peptide Ag types when measuring CD4⁺ T cell responses, indicating that both types of Ags can be used. However, there were differences between mRNA and peptides in measuring CD8⁺ T cell responses; therefore, we had to select a more appropriate antigenic form (5). Therefore, to measure CD8⁺ T cell responses, it is presumed that generating Ags in cells by electroporating mRNA rather than peptides is more natural. In contrast, peptides are more suitable for measuring CD4⁺ T cell responses. We did not define 50 SFCs/5×10^5 cells as the cut-off for a positive response, but rather as a tentative setting for data analysis. To determine the cutoff value to confirm a positive response to the EBV Ag, collaborative studies conducted by several research groups will be necessary.

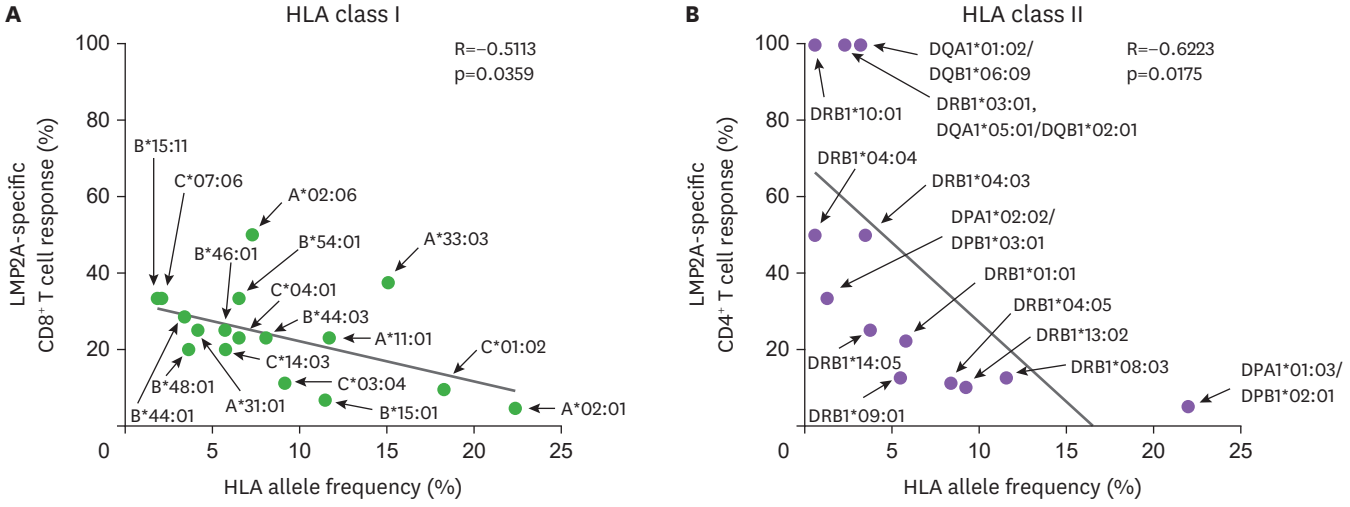

Figure 6. Association of allele frequency with the proportion of LMP2A-specific CD8⁺ and CD4⁺ T cell responses by HLA class I and II allotypes.
(A, B) Correlation analysis between the frequency of each HLA class I and II allotypes and the proportion of CD8⁺ and CD4⁺ T cell responses of 50 SFCs/5×10^5 T cells or more. HLA-A, 7 HLA-B, and 5 HLA-C allotypes in HLA class I, and 10 HLA-DR, 2 HLA-DQ, and 2 HLA-DP allotypes in HLA class II showed T cell responses of more than 50 SFCs/5×10^5 T cells to LMP2A. Statistical analysis was performed using Pearson's correlation analysis. Best-fit values were analyzed by demining linear regression with 95% confidence intervals (solid line).

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When $CD8^+$ and $CD4^+$ T cell responses were measured using DCs transfected with LMP2A mRNA (5), $CD8^+$ T cell responses were higher than $CD4^+$ T cell responses, and similar results were observed in this study (**Figs. 1A** and **2A**). Among the donors with T cell responses of more than 50 SFCs/5×10^5 T cells, only 1 HLA class I allotype was observed to dominate within the individual in most cases across HLA class I and II allotypes, suggesting that $CD8^+$ T cells are predominantly responsive to LMP2A (**Fig. 5**). There was no significant correlation between HLA class I-restricted $CD8^+$ T cell responses and HLA class II-restricted $CD4^+$ T cell responses in individuals with LMP2A (**Fig. 5C**) (5). In contrast, there was significant correlation between HLA class I allotypes and HLA class II allotype-restricted $CD8^+$ and $CD4^+$ T cell responses to cytomegalovirus (CMV) pp65 in individuals (27). CMV pp65 is a molecule constituting the envelope structure of the virus and is known to cause a strong T cell response; however, LMP2A is a molecule belonging to the EBV latency II Ag and is expressed only in the latent state, so it induces a weaker T cell response (**Figs. 1** and **2**). Therefore, it is possible that the weaker T cell responses to the LMP2A Ag did not reach a significant level in this study with a limited number of samples.

Evaluation of the relative contribution of each HLA class I locus to the LMP2A-specific $CD8^+$ T cell response revealed that the magnitude of $CD8^+$ T cell responses was ranked from highest to lowest in the order of HLA-A, HLA-B, and HLA-C loci (**Fig. 1A**). This result was similar to the previously reported magnitude of $CD8^+$ T cell responses to CMV pp65 (26). The low response of HLA-C is thought to be due to its lower cell surface expression relative to HLA-A and HLA-B (30). Conversely, the response to $CD8^+$ T cells in the HIV-1 infected South African population is restricted to HLA-B (31). The relative contribution of the HLA class II locus to LMP2A was also similar to that of CMV pp65 and dengue virus, with response ranking from highest to lowest in the order of HLA-DR, HLA-DP, and HLA-DQ (**Fig. 2A**) (27,32). Since the α and β chains of HLA-DQ have higher polymorphism than those of HLA-DR and HLA-DP, this may contribute to a lower probability of having an immune response (33,34). Furthermore, the low response of dengue virus-specific $CD4^+$ T cells to HLA-DQ has been shown to be influenced by the lower expression levels of HLA-DQ (32,35,36). Since there are four possible combinations of HLA-DQ and DP with polymorphism within an individual, we determined and applied the most likely combinations of α and β chains based on haplotype analysis (28).

We evaluated whether HLA class I or HLA class II allotypes were preferentially used in the LMP2A-specific immune response (**Figs. 1** and **2**). HLA class I and class II allotypes showing dominant $CD8^+$ and $CD4^+$ T cell responses against LMP2A are different from those against the CMV pp65 Ag (26,27). In this study, we measured the response to the LMP2A whole Ag; however, additional research should be conducted to determine which epitopes are recognized by T cells reacting with a specific allotype. In addition, LMP2A-specific T cells were measured only by ELISPOT for IFN-γ secretion. To better characterize immunological properties, the polyfunctionality of T cells should also be investigated by measuring the secretion of other cytokines (37-39).

As shown in **Figs. 1** and **2**, when the dominant allele was co-expressed in one individual, T cell responses restricted by these alleles did not occur simultaneously and mainly occurred in one allele (**Figs. 3** and **4**). Allele dominance has been reported in individuals with a positive response to pp65 (26,27). A possible mechanism that could explain allele dominance may be the selection of clones with various affinities for a particular epitope. In the early stages of CMV infection, the repertoire of T cells specific to the presented peptide varies, but in the late stages, the repertoire of high-affinity T cells decreases, and a few low-affinity T

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cell clones become dominant (40,41). When human $CD8^+$ T cell responded to EBV from primary infection through to the persistent carrier state, there were marked differences in the epitope-specific composition of the T cell populations between the 2 stages of infection. The primary response is dominated by lytic epitope specificity, which then decreases. In contrast, specificities of the LMP2A epitope restricted by HLA-A*02:01 are less abundant in the primary phase but often then increase in the $CD8^+$ T cells pool, and latent epitope responses remain $CD45RA^-RO^+$ with a greater tendency to acquire CCR7 (42).

Interestingly, an inverse correlation was observed between the frequency of HLA class I and II alleles and the proportion of LMP2A-specific $CD8^+$ and $CD4^+$ T cell responses (**Fig. 6**). However, allele frequencies and $CD8^+$ T cell responses to CMV pp65 are correlated, whereas $CD4^+$ T cell responses are inversely correlated (26,27). As the entire LMP2A Ag was used in this study, it will be necessary to carry out further studies in the future to identify the specific epitope for each allotype. Two selective explanations have been proposed: heterozygous and rare allele advantages (43). The heterozygous advantage hypothesis asserts that a heterozygous host can present more antigenic peptides from the pathogen than a homozygous host. In contrast, the rare allele advantage hypothesis asserts that hosts with rare alleles are more likely to survive infectious diseases because they are more likely to respond to pathogens. Populations in which a particular HLA type is common may be prone to severe disease, endemic persistence, or frequent pathogen emergence (44). These advantages may be related to the evolution of MHC (43,45). In our analysis, we assumed that the negative allele did not functionally present the LMP2A Ag, and we analyzed alleles with more than 50 SFCs/5×10^5 T cells responses, demonstrating a significant inverse correlation. When all alleles were analyzed, there was no significant correlation; therefore, it is necessary to investigate and revalidate more numbers in the future.

In summary, the magnitude of $CD8^+$ and $CD4^+$ T cell responses differed according to HLA class I and II loci, and only a few allotypes within an individual showed allele dominance to LMP2A. The specific T cell response to LMP2A was higher in $CD8^+$ T cells than in $CD4^+$ T cells, which is consistent with previous results. These data demonstrate the allele dominance of LMP2A-specific T cell responses among HLA allotypes and their intra-individual dominance in response to only a few allotypes in an individual and also provide basic immunological data to explain the association of the development of EBV-associated tumors with specific HLA allotypes. This technique to measure the T cell plaque for a single HLA molecule can be used to pre-investigate HLA restriction of EBV-CTL lines in a third-party CTL bank using partially HLA-matched virus-specific CTLs (46,47).

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Genotypes of HLA class I allotypes in 50 healthy Korean donors

Click here to view

Supplementary Table 2

Genotypes of HLA class II allotypes in 50 healthy Korean donors

Click here to view

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