Preferential HLA Usage in the Influenza Virus-Specific CTL Response

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To study whether individual HLA class I alleles are used preferentially or equally in human virus-specific CTL responses, the contribution of individual HLA-A and -B alleles to the human influenza virus-specific CTL response was investigated. To this end, PBMC were obtained from three groups of HLA-A and -B identical blood donors and stimulated with influenza virus. In the virus-specific CD8+ T cell population, the proportion of IFN-γ- and TNF-α-producing cells, restricted by individual HLA-A and -B alleles, was determined using virus-infected C1R cells expressing a single HLA-A or -B allele for restimulation of these cells. In HLA-B*2705- and HLA-B*3501-positive individuals, these alleles were preferentially used in the influenza A virus-specific CTL response, while the contribution of HLA-B*0801 and HLA-A*0101 was minor in these donors. The magnitude of the HLA-B*0801-restricted response was even lower in the presence of HLA-B*2705. C1R cells expressing HLA-B*2705, HLA-A*0101, or HLA-A*0201 were preferentially lysed by virus-specific CD8+ T cells. In contrast, the CTL response to influenza B virus was mainly directed toward HLA-B*0801-restricted epitopes. Thus, the preferential use of HLA alleles depended on the virus studied. The Journal of Immunology, 2004, 172: 4435–4443.

Cytotoxic T lymphocytes have been shown to play an important role in the control and clearance of virus infections, including those caused by influenza virus (1, 2). Although a large number of peptides are generated during processing of viral proteins in infected cells, only some of these peptides are ultimately presented by MHC class I molecules and recognized by specific CTL. This limited recognition of a small number of dominant CTL epitopes has been termed immunodominance (3–5). Usually immunodominance is defined using a number of synthetic dominant CTL epitopes. Thus, it might be expected that if CTL responses were analyzed by individual HLA-A and -B alleles, the majority of CTL would recognize influenza A virus-infected targets in a HLA-A*0201-restricted fashion. Although the use of synthetic peptides provides some information on HLA usage in CTL response, this approach has limitations and does not account for the full repertoire of epitopes presented by infected cells.

In the present study, the contribution of individual HLA-A and -B alleles to the CTL response against a model viral pathogen was investigated. To this end, influenza virus-specific memory CTL response was studied, using C1R cells expressing a single HLA-A or -B allele and influenza virus-stimulated PBMC obtained from three groups of healthy blood donors with defined HLA class I haplotypes. Between groups, the individuals shared two or three of the four HLA-A and -B alleles. Intracellular IFN-γ and TNF-α staining was used for the quantification of virus-specific CTL response by a single HLA-A or -B allele, and the lytic capacity of these cells was determined in classical 51Cr release assays.

We found that individual HLA alleles were not used to the same extent and that in each group of HLA-typed individuals a hierarchy existed between HLA alleles. Especially HLA-B*2705 and HLA-B*3501 were found to be preferred alleles in the influenza A virus-specific CTL response. The preferential HLA usage was found to depend on the type of influenza virus (A or B) studied.

Materials and Methods

Cells

PBMC obtained from 13 healthy blood donors (Table I) were isolated between 1999 and 2002 using Lymphoprep (Nycomed, Oslo, Norway) gradient centrifugation and cryopreserved at −135°C. Three groups of donors, aged between 35 and 50 years of age, were selected according to serological homology within the A locus and B locus of HLA class I molecules.
In vitro stimulation of PBMC with influenza virus

Stimulation of PBMC with influenza virus was performed, as described previously (15). Cells were resuspended at 1 x 10^6 cells/ml in R10F and infected for 1 h at 37°C with Resvir-9 or B/Harbin/7/94, at a multiplicity of infection (MOI) of 3. Next, the cells were washed once and resuspended in RPMI 1640 medium supplemented with 10% human AB serum, 2 mM glutamine, 100 μg/ml streptomycin, 100 IU/ml penicillin, and 20 μM 2-ME (R10F), and added to uninfected PBMC at a ratio of 1:1 in a 25-cm² culture flask. After 2 days, rIL-2 (final concentration 50 U/ml; Chiron B.V., The Netherlands) was added and the cells were incubated for another 6 days at 37°C and evaluated for cytotoxic T cell activity in a 51Cr release assay or intracellular cytokine staining (ICS) assay (see below).

Isolation of CD8⁺ T cells

CD8⁺ T cells were isolated from the effector cell populations by magnetic sorting, using a CD8⁺ cell selection kit (Dynal Biotech GmbH). First, the cells were washed once in PBS supplemented with 2.0% FCS (P2F) and finally resuspended in P2F at a concentration of 1 x 10⁶/ml. Capture beads were added to the cell suspension at a bead to CD8⁺ T cell ratio of 8:1. Following a 30-min incubation at 4°C, the bead/ cell complex was washed six times with 5.0 ml of P2F. The beads, together with the attached cells, were reconstituted in 200 μl of RPMI 1640 medium with 1.0% FCS. To detach the cells from the beads, 20 μl of DETACHaBEAD (Dynal Biotech GmbH) was added and incubated for 1 h at 20°C. The released cells were isolated, washed once in R10F, and used as effector cells in 51Cr release assays (22).

Preparation of target cells for ⁵¹Cr release assay

HLA-A- and -B-matched BLCL, C1R, and C1R cells transfected with various HLA genes were used as target cells in ⁵¹Cr release assays. All cells were infected with Resvir-9 at a MOI of 3 in RPMI 1640 medium, containing 0.1% BSA, 2 mM glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin (R10B). Following a 1-h incubation at 37°C, the cells were washed once in R10F and incubated in R10F for 16 h at 37°C. The following day, 10⁶ cells were washed once in R01B medium and incubated at 1 h for 75 μCi of Na₂¹⁵CrO₄. The cells were then washed three times in R10F and used as target cells. Uninfected cells of the HLA-matched BLCL, C1R, and HLA-transfected C1R cell line were included to determine nonspecific lysis of target cells.

The Ag presentation capacity of HLA-transfected C1R cells was compared with cells of two BLCL. To this end, the minimal peptide concentration was determined for which 50% of the target cells were killed (EC₅₀) by epitope-specific CTL clones. After a 1-h incubation with Na₂¹⁵CrO₄, the cells were washed twice in R10F and distributed in 96-well V-bottom plates. Next, the cells were incubated with a 10-fold serial dilution of the peptides, washed once in R10F, and used as target cells in a ⁵¹Cr release assay. The HLA-A2, -A3, -B27, and -B35-restricted influenza A virus-specific CTL clones were previously described (23–25), while the HLA-A1- and -B8-restricted influenza A virus-specific CTL clones were established, as described previously (25).

Intracellular cytokine staining

Influenza virus-stimulated PBMC were also used for ICS assay. One hundred thousand PBMC were incubated in 100 μl of R10F containing GolgiStop (moneisin; BD PharMingen, Alphen a/d Rijn, The Netherlands) and GolPlugi (brefeldin; BD PharMingen). In addition, 2 x 10⁶ influenza virus-infected (Resvir-9, MOI = 3 or B/Harbin/7/94, MOI = 1) and uninfected HLA-matched BLCL cells and C1R cells with or without HLA transgene were also incubated in 100 μl of R10F containing Golplugi and GolgiStop and used as stimulator cells. After 30 min at 37°C, the stimulating cells were added to the PBMC for 6 h at 37°C. Next, the cells were washed, stained, and fixed, as previously described (26), using FITC-conjugated anti-CD8 (Dakocytomation, Glostrup, Denmark), PerCP-conjugated anti-CD3 (BD Biosciences, Alphen a/d Rijn, The Netherlands), PE-conjugated anti-IP-10 (BD PharMingen), and allophycocyanin-conjugated anti-TNF-α (BD Biosciences) mAb. At least 2000 gated CD3⁺/CD8⁺ T cells were acquired using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo software (BD Biosciences) and are expressed as the percentage of cytokine-positive cells following stimulation with influenza virus-infected cells versus the percentage of cytokine-positive cells after stimulation with uninfected cells. To determine the percentage of allele-specific cytokine-positive T cells, the proportion of cytokine-positive cells following stimulation with C1R control cells is subtracted from the
The percentage of cytokine-positive cells following stimulation with HLA-transfected C1R cells.

**ELISPOT assay**

Virus-specific CD8\(^+\) T cells and their HLA restriction element were also quantified in ELISPOT assays, which were performed as previously described (15). Before stimulation with uninfected and virus-infected C1R cells expressing single HLA class I molecules, for the induction of IFN-\(\gamma\) production, CD8\(^+\) CD16\(^{-}\) cells were isolated from PBMC using Dynabeads.

**\(^{51}\)Cr release assay**

\(^{51}\)Cr release assays were performed, as described previously (15). Influenza A virus-specific CTL clones were added to 5 \(\times\) \(10^5\) \(^{51}\)Cr-labeled target cells at E:T ratio of 10:1 or 5:1. Isolated CD8\(^+\) T cells, obtained from influenza A virus-stimulated PBMC cultures, were also used as effector cells at E:T ratios of 10:1 to 1:25:1. After 4 h at 37°C, the culture supernatants were harvested (Skatron Instruments, Sterling, VA) and radioactivity was measured by gamma counting. The percentage of specific lysis was calculated with the following formula: ((experimental release - spontaneous release)/(maximum release - spontaneous release)) \(\times\) 100.

The percentage of influenza A virus-specific lysis was calculated from the percentage of lysis of infected minus the percentage of lysis of uninfected cells of at least three wells.

**Flow cytometry**

Following isolation of CD8\(^+\) T cells, the purity of the CD8\(^+\) T cell population was tested. Approximately 10\(^5\) cells were washed once in PBS, and incubated in 50 \(\mu\)L of PBS containing anti-CD8, anti-CD4 (Dynabeads, One Lambda) mAb, respectively. C1R-B35 cells were incubated with FITC-conjugated streptavidin (Dynabeads). The level of HLA expression on C1R and C1R cells with various HLA transgenes was determined by flow cytometry using saturating levels of anti-HLA-ABC Abs (BD PharMingen). All Ab-staining procedures were performed in P2F at 4°C. At least 10,000 cells were acquired with a FACSCalibur flow cytometer and analyzed with CellQuestPro.

**Statistical analysis**

To identify statistical differences between IFN-\(\gamma\) responses restricted by individual HLA-A or -B alleles, the Student t test was performed. To investigate preferential HLA-A or -B usage in the three groups of donors, a Friedman test was performed, comparing the response to individual alleles with a theoretical random contribution of the four HLA-A and -B alleles. Differences in ratio IFN-\(\gamma\)- and TNF-\(\alpha\)-positive cells were analyzed with a univariate ANOVA post hoc analysis. Ratio of IFN-\(\gamma\)/TNF-\(\alpha\)-positive cells was determined if both proportions of cytokine-positive cells were more than 1.0%. Values of p < 0.05 were considered statistically significant.

**Results**

**Validation of HLA-transfected C1R cells**

Before using C1R cells expressing HLA transgenes as APCs, their HLA expression and Ag-presenting capacity were tested. As shown in Fig. 1A, all C1R cells transgenic for individual HLA genes exhibited HLA class I expression after incubation with an Ab specific for all HLA-A, -B, and -C alleles. The expression was in the same order of magnitude as in normal BLCL cells (solid lines, Fig. 1A). Control C1R cells also exhibited surface expression of HLA class I molecules to a limited extent as a result of endogenous HLA-Cw4 expression (17, 18). This expression was at least 10-fold lower than in the HLA-transfected C1R cells.

**IFN-\(\gamma\) responses in CD8\(^+\) T cells restricted by individual HLA-A and -B alleles**

The proportion of IFN-\(\gamma\)- and CD8\(^+\) T cells in influenza A virus-specific PBMC cultures, which were restimulated with HLA-matched BLCL or C1R cells expressing individual HLA-A and -B alleles. An example of such an analysis is shown in Fig. 2.

Using this procedure, PBMC of all (n = 13) donors were tested. In donors of group I (HLA-A1, HLA-A2, HLA-B8, HLA-B35), the proportion of CD8\(^+\) T cells producing IFN-\(\gamma\) after stimulation with HLA-matched BLCL cells was 46% (Fig. 3A). Stimulation with HLA-transfected C1R cells showed that on average 16.4% of these cells were restricted by HLA-B*3501. HLA-A*0201-, HLA-B*0801-, and HLA-A*0101-restricted CD8\(^+\) T cells accounted for 10.2, 9.2, and 5.5% on average, respectively. No significant differences, summarized in Table II, were observed between the frequencies of virus-specific CTL restricted by single HLA-A or -B alleles in these donors.

In donors of group II (HLA-A1, HLA-A2, HLA-B8, HLA-B27), on average 41% of the CD8\(^+\) T cells were IFN-\(\gamma\)- after stimulation with HLA-matched BLCL cells. The majority of these cells were specific for CTL epitopes presented in the context of HLA-B*2705 with an average of 23.8% of IFN-\(\gamma\)- CD8\(^+\) T cells in these donors (Fig. 3A). This HLA-B*2705-restricted response differed significantly with the percentages of IFN-\(\gamma\)- CD8\(^+\) T cells found after stimulation with C1R-A2 (10.5%, p = 0.008), C1R-A1 (2.4%, p = 0.002), and C1R-B8 (2.9%, p = 0.002). The proportion of IFN-\(\gamma\)-cell responses after stimulation with C1R-A2 (10.5%) also differed from the responses to C1R-A1 (p = 0.016) and C1R-B8 (p = 0.02).

In donors of group III (HLA-A1, HLA-A3, HLA-B8, HLA-B35), the proportion of CD8\(^+\) T cells producing IFN-\(\gamma\)- after stimulation with HLA-matched BLCL cells was on average 32% (Fig. 3A). Most of the influenza A virus-specific CD8\(^+\) T cells were specific for CTL epitopes presented by HLA-B*3501 (15.9% IFN-\(\gamma\)-cells), followed by those specific for C1R-A3 (10.0%), C1R-B8 (7.8%), and C1R-A1 (3.5%) restricted epitopes. The average percentage of IFN-\(\gamma\)- restricted CD8\(^+\) T cells in all HLA-A*0101-restricted CD8\(^+\) T cells was significantly lower than the proportion of cells restricted HLA-A*0301 (p = 0.007).
We also compared the magnitude of the responses restricted by shared alleles between donors of different groups. The HLA-B*0801-restricted response was significantly lower in donors of group II (2.9%) than in those of group III (7.8%, 

\[ p / H11005 0.04 \)] and to a lesser extent in those of group I (9.1%, 

\[ p / H11005 0.1 \]). The HLA-A*0101-restricted response in group I (5.5%) did not significantly differ from that observed in groups II (2.4%) and III (3.5%). Also, no differences were found between groups in the proportion of IFN-\( \gamma / H9253 \) CD8\(^+ \) T cells restricted by HLA-A*0201 (10.2 vs 10.5%) or HLA-B*3501 (16.4 vs 15.9%).

To demonstrate preferential HLA usage in individual donors, pie charts were constructed (Fig. 3B), illustrating the IFN-\( \gamma / H9253 \) responses for each HLA allele (shades of gray) within the CD8\(^+ \) T cell population (entire circle). Not all CD8\(^+ \) T cells produced IFN-\( \gamma / H9253 \) upon stimulation with virus-infected stimulator cells (open part). In donors of group I, an HLA-A*0201-restricted response was always found, which was dominant in donors 1 and 2. HLA-B*3501 was found to be an immunodominant allele in two other donors (3 and 4). Subdominant HLA-B*0801-restricted responses were found in all donors, while the HLA-A*0101-restricted response was detected in 3 of 4 donors. Preferential HLA usage in this group of donors was not demonstrated (\( p / H11005 0.165 \)).

In all four donors of group II, a dominant HLA-B*2705-restricted IFN-\( \gamma / H9253 \) response was observed in addition to the HLA-A*0201-restricted IFN-\( \gamma / H9253 \) response. The HLA-A*0101- and HLA-B*0801-restricted IFN-\( \gamma / H9253 \) responses only contributed, to a limited extent, to the influenza A virus-specific CTL response in these donors. The preferred usage of certain HLA class I alleles in these donors was statistically significant (\( p / H11005 0.011 \)).

In group III, the most dominant response was restricted by HLA-B*3501 in donors 1, 2, 4, and 5, while in donor 3 this response was only minor (Fig. 3B). An HLA-A*0301-restricted response was observed in all five donors and was found immunodominant in three donors. Also, the HLA-A*0101- and -B*0801-restricted responses were detected in all donors, but were found to be subdominant. Again, the preferred recognition of certain alleles in these donors was statistically significant (\( p / H11005 0.005 \)).
**Discussion**

The contribution of individual HLA-A and -B alleles in influenza virus-specific CTL responses was determined in groups of HLA class I-matched donors. It was shown that influenza virus-stimulated PBMC of HLA-A- and -B-matched donors preferentially recognized certain HLA alleles, which depended on the type of virus studied. Furthermore, it was demonstrated that cytokine production profiles of CD8+ CTL depended on their HLA class I restriction elements.

Before investigation of CTL responses restricted by individual HLA-A and -B alleles, the use of HLA-transfected C1R cells as APC was validated. The level of expression of HLA molecules was similar in the different C1R cells as measured with saturating amounts of Ab. These cells were also capable of processing and presenting influenza virus-specific CTL epitopes to a similar extent as EBV-transformed BLCL. In addition, only small differences in susceptibility of the individual C1R cells for infection with influenza A virus were found (between 55 and 80% infected), with the exception of C1R-B35. This, however, did not interfere with our ICS assay, because an excess number of stimulator cells was used to stimulate influenza virus-specific CTL in the in vitro stimulated PBMC cultures. In contrast to the use of BLCL matched for a single HLA allele, the use of C1R expressing single HLA alleles prevents possible competition for processing/presentation between overlapping epitopes (27). In EBV-transformed BLCL cells or C1R cells, competition for available HLA-A or -B alleles between epitopes from influenza virus and EBV may occur. For example, an immunodominant HLA-B*0801-restricted CTL epitope of EBV (EBNA3A325–333) could interfere with the presentation of HLA-B*0801-restricted influenza virus epitopes. However, influenza A virus-infected C1R-B8 cells were readily killed by HLA-B*0801-restricted influenza A virus-specific

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**FIGURE 2.** IFN-γ expression in CD8+ T cells after stimulation of PBMC with influenza virus. PBMC expanded after stimulation with influenza A virus were restimulated with HLA-matched BLCL. C1R, and HLA-transfected C1R cells infected with influenza A virus (Resvir-9) at a ratio of 1:2 for 6 h in the presence of monensin and brefeldin. Stimulation with uninfected cells was used as negative controls. Virus-specific CTL restricted by individual alleles were visualized after staining with different fluorochrome-conjugated mAb specific for CD8, CD3, and IFN-γ. The dot plots show an example of the expression of IFN-γ in T cells following stimulation of PBMC of donor 1 (group I) with infected and uninfected cells of an HLA-matched BLCL and C1R control cells and infected C1R cells expressing HLA-A1, HLA-A2, HLA-B8, or HLA-B35. The proportion of IFN-γ+ cells in the CD3+CD8+ T cell fraction is indicated.

The sum of the IFN-γ+ CD8+ T cells observed after stimulation with four different C1R cell lines expressing the four HLA-A and -B alleles was compared with the number of IFN-γ+ CD8+ T cells observed after stimulation with HLA-matched BLCL cells. As shown in Fig. 3B (bar charts), the sum of the responses restricted by individual alleles ($\square$) almost accounted for the total influenza A virus-specific response ($\bigtriangledown$) in most cases. In 11 of 13 donors, the difference was less than 10% and only in donor 4 of group II and donor 3 of group III the differences exceeded 10%.

**HLA-A- and -B-restricted lysis of target cells by CD8+ CTL**

The contribution of individual HLA-A and -B alleles in the recognition of infected target cells by influenza A virus-specific CTL was also studied in $\text{^{51}Cr}$ release assays, using infected and uninfected HLA-transfected C1R cells and HLA-matched BLCL cells (Fig. 4). The CD8+ effector cell populations obtained from donors in group I recognized HLA-matched BLCL, C1R-A1, and C1R-A2 cells to a similar extent (average percentage of 65%). The average percentages of specific lysis of C1R-B8 and C1R-B35 were 40 and 30%, respectively.

Influenza A virus-specific CD8+ T cells obtained from donors in group II lysed influenza virus-infected C1R-B27 cells efficiently (average percentage of 70%). The lysis of virus-infected C1R-A2 and C1R-A1 ranked second and third, respectively. Finally, the lysis of infected C1R-B8 cells was lower than the percentage lysis of C1R-B27 ($p = 0.001$) and C1R-A2 ($p = 0.02$).

CD8+ effector cells obtained from donors in group III did not exhibit a clear preferred HLA usage in the recognition of their target cells.

**HLA-A- and -B-restricted IFN-γ responses specific for influenza B virus**

The IFN-γ response restricted by HLA-A and -B alleles was determined in PBMC of donors of group II (HLA-A1, HLA-A2, HLA-B8, and HLA-B27) stimulated with influenza B virus, to investigate whether HLA-B*0801 was also the preferred allele in response to another type of influenza virus. On average, 41% IFN-γ+ CD8+ T cells were detected after restimulation with influenza B virus-infected HLA-matched BLCL cells (Fig. 5). The majority of these cells recognized their epitopes in the context of HLA-B*0801 (22.7%), followed by the HLA-B*2705 (7.4%), HLA-A*0101 (2.4%), and HLA-A*0201-restricted (2.3%) responses. The proportion of HLA-B*0801-restricted IFN-γ+ CD8+ T cells was significantly greater than the proportion of C1R-A1 ($p = 0.05$)- or C1R-A2 ($p = 0.05$)-restricted IFN-γ+ CD8+ T cells. The HLA-A*0101- and HLA-A*0201-restricted IFN-γ responses were also significantly smaller than the HLA-B*2705-restricted response ($p = 0.017$ and $p = 0.002$, respectively).

**IFN-γ and TNF-α expression in influenza A virus-specific CTL restricted by individual HLA-A and -B alleles**

The expression of TNF-α was determined as a third functional parameter after restimulation of influenza A virus-stimulated PBMC with HLA-matched BLCL and HLA-transfected C1R cells. The ratio between percentage of IFN-γ+ and percentage of TNF-α+ cells within the CD8+ T cell fraction for each individual for the different HLA-transfected C1R cells and HLA-matched BLCL cells was calculated (data not shown). The ratio of IFN-γ+ and TNF-α+ cells following stimulation with HLA-matched BLCL cells is on average 1.1, indicating that most virus-specific cells produce both cytokines upon restimulation. However, stimulation with HLA-transfected C1R cells resulted in ratios starting from 1.2 for C1R-B27 to 1.8 for C1R-A2, C1R-A3, C1R-B8, and C1R-B35, while stimulation with C1R-A1 cells resulted in an average ratio of 0.7. This indicates that some HLA-A*0101-restricted influenza A virus-specific CTL produce TNF-α, but not IFN-γ. This ratio was significantly different from the average ratio of C1R-A2 ($p = 0.011$), C1R-B8 ($p < 0.001$), and C1R-B35 ($p = 0.001$).
indicating that competition between epitopes of these two viruses does not constitute a significant problem.

Using the HLA-transfected C1R cell lines, it was shown that the influenza A virus-specific CTL response in these donors measured by intracellular IFN-γ staining was dominated by HLA-B*2705- and HLA-B*3501-restricted CTL in groups II and I/III, respectively. Based on previous findings with synthetic peptides (15), it was anticipated that the HLA-A*0201-restricted response, presenting the immunodominant epitope M158–66, would be recognized preferentially. Therefore, the observed hierarchy of virus-specific responses indicates that other yet unidentified HLA-B*2705- and HLA-B*3501-restricted epitopes exist. Recently, an immunodominant HLA-B*3501-restricted epitope (NP418–426) was identified (25), which may have contributed to the preferred usage of HLA-B*3501 in the CTL response. In addition to the dominance of HLA-B27 and HLA-B35, a large proportion of T cells was specific for epitopes presented by HLA-A*0201 and HLA-A*0301. Most likely the presentation of the immunodominant epitope M158–66 has contributed to the dominance of the HLA-A*0201-restricted response. The large proportion of HLA-A*0301-restricted CTL indicates that more unknown immunodominant epitopes exist, because the HLA-A*0301-restricted NP265–273 epitope has been shown to be subdominant in response to influenza A viruses (15). Indeed, a novel HLA-A3-restricted CTL epitope was identified recently, which could contribute to the influenza A virus-specific CTL response (28). Finally, the IFN-γ response specific for epitopes presented in the context of HLA-A*0101 and HLA-B*0801 contributed little to the overall influenza A virus-specific CTL response, which is in agreement with previous work (29). The small contribution of these alleles to the overall CTL response could be caused by the presence of alleles HLA-B*2705 or HLA-B*3501 presenting immunodominant epitopes. This hypothesis

![Figure 3](image-url)
Table II. Preferential HLA usage in the influenza A virus-specific CTL response

| Group | Preferential HLA Usage | p Value | Allelic Preference | p Value |
|-------|------------------------|---------|--------------------|---------|
| I     | No                     | 0.165   | ND                 | ND      |
| II    | Yes                    | 0.011*  | A2 > A1*           | 0.016   |
|       |                        |         | A2 > B8            | 0.02    |
|       |                        |         | B27 > A1           | 0.008   |
|       |                        |         | B27 > A2           | 0.002   |
|       |                        |         | B27 > B8           | 0.002   |
| III   | Yes                    | 0.005   | A3 > A1            | 0.007   |

*The preferential HLA usage was determined in donors of groups I to III, using the percentage of IFN-γ+ CD8+ T cells restricted by single HLA-A or -B alleles.
*The average percentage of virus-specific CTL restricted by individual HLA-A or -B alleles, as measured by intracellular IFN-γ staining, was compared.
ND, no significant differences detected.
*Values of P < 0.05 are considered statistically significant.
*A2 > A1 indicates that the percentage of virus-specific HLA-A*0201-restricted CTL is higher than the percentage restricted by HLA-A*0101.

The preferential HLA usage was supported by a high proportion of IFN-γ+ HLA-B*0801-restricted cells in an HLA-A*0101, HLA-B*0801 homozygous donor (data not shown). In addition, the HLA-B*0801-restricted response was lower in donors of group II (HLA-B27+) than in donors in the two other groups (HLA-B35+ donors), which is in agreement with the poor recognition of the HLA-B*0801-restricted NP380–388 epitope in HLA-B27+ individuals (15, 27). These data also indicate that more immunodominant high affinity HLA-B*0801-restricted epitopes are not existing for influenza A virus.

Changes in epitope specificity of the virus-specific CTL response as a result of differences in HLA expression profiles were previously reported in mice infected with influenza A virus (3, 11, 30). In these studies, the expression of an H-2Kb allele (either transgenic or through breeding with C57BL mice) resulted in a reduced H-2Kb-restricted response.

A possible skewing of the CTL response in favor of certain HLA restrictions during in vitro stimulation of PBMC in the presence of rIL-2 was excluded, because it was found in a previous study that the hierarchy of peptide-specific CTL responses measured ex vivo in PBMC correlated with the hierarchy of lytic activity measured after in vitro expansion of virus-specific CTL using the same stimulation protocol (15). Furthermore, ELISPOT assays were conducted with CD8+ CD16+ cells isolated from PBMC of several donors from groups I and II to determine preferential HLA usage ex vivo. In the donors of group II, the same hierarchy of preferred HLA usage was observed ex vivo, as was observed after in vitro expansion of virus-specific CTL. In group I, without preferential HLA usage after in vitro expansion of virus-specific cells, ex vivo no-preferred HLA usage was also demonstrated (data not shown).

In a previous study (15), we reported a lower influenza A virus-specific immune response in HLA-A*0201-negative donors (group III). Also in the current study, the average number of IFN-γ+ cells following restimulation with HLA-matched BLCL is lower in HLA-A*0201-negative donors than in HLA-A*0201-positive donors, although this difference was not statistically significant (p = 0.28). This is partly explained by the exclusion of two HLA-A*0201-negative donors with an HLA-B*3503 instead of an HLA-B*3501 genotype.
Using influenza B virus for the stimulation of PBMC, we demonstrated that the preferential HLA usage is dependent on the virus studied. In contrast to the influenza A virus-specific CTL response, the HLA-B*0801-restricted response specific for influenza B virus was highly immunodominant, followed by HLA-B*2705. The HLA-A*0101- and HLA-A*0201-restricted responses were shown to contribute little to the overall influenza B virus-specific response. It is difficult to correlate these responses to known (immunodominant) epitopes because only four influenza B virus CTL epitopes are known (31, 32), of which three are presented in the context of HLA-B8 (32). These data indicate that the available epitope repertoire determines the outcome of the CTL response and preferred HLA usage.

In contrast to other studies addressing the preferential use of HLA molecules in CTL responses, we used virus-infected human cells expressing a single HLA allele, which accounts for the full repertoire of CTL epitopes presented by these HLA molecules. The preferential usage of certain alleles in the virus-specific CTL response has also been reported for EBV-specific CTL responses. These studies demonstrated that certain HLA class I alleles were dominantly recognized, such as HLA-B8, HLA-A11, or HLA-B44, while HLA-A1 was not (7, 9). For HIV-specific CTL responses, it was shown with synthetic peptides that the HLA-A2-restricted response hardly contributed to the overall HIV-specific response (12, 14).

Most of these studies used IFN-γ production to identify epitope-specific CTL. However, our data suggest that CTL differ in their ability to produce cytokines depending on the epitopes recognized and/or the HLA molecules presenting these epitopes. Influenza A virus-specific HLA-A*0101-restricted CTL produced less IFN-γ and more TNF-α than CTL restricted by other HLA molecules. Therefore, some caution should be exercised in interpreting frequencies of CTL based on IFN-γ production alone. The preferred HLA usage, as demonstrated by quantification of virus-specific IFN-γ-CD8+ T cells and the lytic activity, of these cells differed, which also could be attributed to functional differences of HLA-A1- and HLA-B35-restricted CTL in particular. To our knowledge, this is the first study to identify differences in cytokine production in CD8+ CTL effector cell populations. Previous studies identified functional differences between EBV-, CMV-, and HIV-specific CTL, based on perforin and surface marker staining (33, 34). These studies also showed reduced killing of two HIV tetramer-positive cell populations in comparison with a CMV tetramer-positive population. Our data provide evidence also that acute viral infections, like influenza virus, induce functionally different CD8+ T-cell populations. At present, it is unclear what the underlying mechanism is for differential cytokine expression in virus-specific CTL and how epitope specificity and the HLA molecules control this. Additional studies are required to further characterize these functional differences in CTL function and to investigate the implications of differential cytokine expression.

Thus, collectively, the present study has shown in donors of well-defined HLA genotypes that: 1) in response to virus infection, CTL responses are induced that use certain HLA molecules preferentially, depending on the available repertoire of CTL epitopes; 2) CTL exhibit differential cytokine expression depending on their epitope specificity and/or HLA restriction.

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