Preferential HLA Usage in the Influenza Virus-Specific CTL Response[1]

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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 peptides representing known CTL epitopes, without taking into account the full repertoire of potential epitopes. Little information is available on the complete repertoire of CTL epitopes of viral pathogens and the corresponding HLA alleles presenting these epitopes to specific CTL. Using transient HLA expression systems, HLA-A11, HLA-B8, and HLA-B44 were identified as the preferred restriction elements in the CTL response to EBV (6–9). These studies also demonstrated that the HLA-A1-restricted EBV-specific CTL response is of minor importance.

In most studies addressing the contribution of individual HLA molecules in CTL responses, synthetic peptides representing known CTL epitopes have been used (10–15). It has been shown recently that the magnitude and specificity of influenza A virus-specific CTL response, using nine peptide analogues of known influenza A virus CTL epitopes, are related to the MHC class I

haplotype of individuals (15). In this study, it was demonstrated that the magnitude of the influenza A virus-specific CTL response was highest in HLA-A*0201-positive individuals. This association was thought to be related to recognition of an HLA-A*0201-restricted immunodominant epitope (M1$_{58-66}$) from the matrix protein. 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 restricted by a single HLA-A or -B allele, and the lytic capacity of these cells was determined in classical ^{51}Cr 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.

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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

Table I. *HLA-A- and -B genotype of donors*

Group	HLA-A and -B Genotype	No. of Donors
I	A*0101, A*0201, B*0801, B*3501	4
II	A*0101, A*0201, B*0801, B*2705	4
III	A*0101, A*0301, B*0801, B*3501	5

(Table I). Genetic subtyping was performed in the laboratory for Histocompatibility and Immunogenetics at the Sanquin Bloodbank Rotterdam using a commercial typing system (GenoVision, Vienna, Austria). All donors had serum Abs against one or more influenza A virus strains, measured by hemagglutination inhibition assay, indicative of one or more exposures in the past. Also, three of the four donors tested for influenza B virus-specific CTL immunity (group II) had influenza B virus-specific Abs.

Of each group of HLA-A- and -B-matched donors, EBV-transformed B lymphoblastoid cell lines (BLCL)[3] were produced, as described previously (16). BLCL were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 μg/ml streptomycin, and 100 IU/ml penicillin (R10F).

Hmy2-C1R (C1R) is a human BLCL with low expression of endogenous HLA-Cw4 (17, 18), but no expression of HLA-A or -B alleles. Control C1R cells and those transfected with individual HLA-A or -B genes were cultured in R10F. The transfected C1R cells were kindly provided by several investigators: HLA-A1-transfected C1R cell line by P. Cresswell (Section of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT), HLA-A2.1-transfected C1R cell line by P. Romero (Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, Lausanne, Switzerland), HLA-A3-transfected C1R cell line by W. Biddison (Molecular Immunology Section, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD), HLA-B*2705-transfected C1R cell line by J. Lopez de Castro (Centro de Biologia Molecular Severo Ochoa, Universidad Autonoma de Madrid, Facultad de Ciencias, Madrid, Spain), and HLA-B*3501-transfected C1R cell line by M. Takiguchi (Division of Viral Immunology, Kumamoto University, Kumamoto, Japan). Finally, a HLA-B*0801-transfected C1R cell line was established, as described previously. (35). Although the genotype of the majority of these HLA-transfected C1R cell lines is known, the cell lines will be depicted by their serotype throughout the manuscript (for example, C1R-B27). Because C1R-A1 cells gradually lose the expression of HLA-A1 on their surface, an enrichment procedure was performed every 2–3 wk using anti-HLA-A1,36 mAbs (BIH0331; One Lambda, Canoga Park, CA) together with DNA-linked anti-mouse Ig beads (Dynal Biotech GmbH, Hamburg, Germany), according to manufacturer's instructions, to ensure a high percentage of HLA-A1+ C1R cells (minimal of 80% HLA-A1+ cells).

Serology

Plasma samples were stored at −20°C and tested for the presence of influenza A virus (H3N2)- and influenza B virus-specific Abs in a hemagglutination inhibition assay according to standard methods (19, 20) using turkey erythrocytes and four hemagglutinating units of virus. The sera were tested for Abs against 11 vaccine strains of subtype H3N2 used since 1968 and 11 influenza B virus strains (from 1979 till 2001). Ferret sera raised against the test Ags were used as positive controls.

Influenza viruses and peptides

Sucrose gradient-purified influenza viruses Resvir-9 and B/Harbin/7/94 (kindly provided by CSL, Parkville, Victoria, Australia) were used for infection of cells. Resvir-9 is a reassortant virus between strains A/Puerto Rico/8/34 (H1N1) and A/Nanchang/933/95 (H3N2), containing the nucleoprotein (NP), hemagglutinin, and neuraminidase of A/Nanchang/933/95. The infectious virus titers of the virus preparations were determined in cell culture using Madin-Darby canine kidney cells as indicator cells, as described previously (21). Peptide analogues of influenza A virus CTL epitopes were manufactured, HPLC purified, and analyzed by mass spectrometry (Eurogentec, Seraing, Belgium). The peptide analogues correspond to the HLA-A1-restricted NP_{44-52}, HLA-A*0201-restricted $M1_{58-66}$, HLA-A3-restricted $NP_{265-273}$, HLA-B*0801-restricted $NP_{380-388}$, HLA-B*2705-restricted $NP_{383-391}$, and HLA-B*3501-restricted

$NP_{418-426}$ epitope. The peptides were dissolved in DMSO at 5.0 mg/ml, diluted to 100 μM in RPMI 1640, and stored at −20°C.

In vitro stimulation of PBMC with influenza virus

Stimulation of PBMC with influenza virus was performed, as described previously (15). Cells were resuspended at 1×10^6 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 (R10H), and added to uninfected PBMC at a ratio of 1:1 in a 25-cm^2 culture flask. After 2 days, rIL-2 (final concentration 50 U/ml; Chiron B.V., Amsterdam, The Netherlands) was added and the cells were incubated for another 6 days at 37°C and used as effector cells in a ^{51}Cr 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 1×10^7/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 beads/cells were 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 ^{51}Cr release assays (22).

Preparation of target cells for ^{51}Cr release assay

HLA-A- and -B-matched BLCL, C1R, and C1R cells transfected with various HLA genes were used as target cells in ^{51}Cr 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 (R0.1B). Following a 1-h infection at 37°C, the cells were washed once in R10F and incubated in R10F for 16 h at 37°C. The following day, 10^6 cells were washed once in R0.1B medium and incubated for 1 h at 37°C with 75 μCi of $Na_2[^{51}Cr]O_4$. 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 (EC_{50}) by epitope-specific CTL clones. After a 1-h incubation with $Na_2[^{51}Cr]O_4$, 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 in R10F for 1 h at 37°C, washed once in R10F, and used as target cells in a ^{51}Cr 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 (monensin; BD PharMingen, Alphen a/d Rijn, The Netherlands) and GolgiPlug (brefeldin; BD PharMingen). In addition, 2×10^5 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 GolgiPlug and GolgiStop and used as stimulator cells. After 30 min at 37°C, the stimulator 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-IFN-γ (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 CellQuestPro (BD Biosciences) and are expressed as the percentage of cytokine-positive cells following stimulation with influenza virus-infected cells minus 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

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[3] Abbreviations used in this paper: BLCL, B lymphoblastoid cell line; ICS, intracellular cytokine staining; MOI, multiplicity of infection; NP, nucleoprotein.for publication.

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-γ 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 × 10^3 ^{51}Cr-labeled target cells at an E:T cell 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)) × 100. The percentage of influenza A virus-specific lysis was calculated from the percentage of lysis of infected cells 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 P2F, and incubated in 50 μl of P2F containing anti-CD8, anti-CD4 (Dakocytomation), and anti-CD3 mAb for 30 min at 4°C. Next, the cells were washed once in P2F, and at least 10^4 events were acquired using a FACSCalibur flow cytometer. The CD8$^+$ T cell purity of the isolated CD8$^+$ T cells was generally >90%.

Before ^{51}Cr release and ICS assays, target cells were tested for the expression of transfected HLA genes. C1R-A1 and C1R-A3 cells were incubated with biotin-conjugated anti-HLA-A1/36 and anti-HLA-A3 (BIH0269; One Lambda) mAb, respectively, followed by FITC-conjugated streptavidin (Dakocytomation). C1R-A2, C1R-B8, and C1R-B27 cells were incubated with FITC-conjugated anti-HLA-A2,28 (FH0037; One Lambda), anti-HLA-B8 (FH0536A; One Lambda), or anti-HLA-B27 (B27F50X; One Lambda) mAb, respectively. Finally, C1R-B35 were incubated with culture supernatant of hybridoma 4D12 (ATCC HB-178; American Type Culture Collection, Manassas, VA), and subsequently stained with FITC-conjugated anti-mouse Ig Fab (Dakocytomation). 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-γ responses restricted by individual HLA-A and -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-γ^+ cells and TNF-α^+ cells were analyzed with a univariate ANOVA post hoc analysis. Ratio of IFN-γ/TNF-α-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. The expres-

sion of HLA-A or -B molecules by the respective C1R cells was further confirmed using allele-specific Ab preparations (data not shown). Furthermore, the HLA expression was not reduced after infection of these cells with influenza A viruses (data not shown).

The concentration of peptide analogous of CTL epitopes required to give 50% of the maximum lysis (epitope concentration (EC)$_{50}$ values) of target cells by corresponding CTL clones was used as a measure for Ag-presenting capacity of C1R cells. As shown in Fig. 1B, the EC$_{50}$ values ranged from 10^{-7} M for the HLA-B*0801-restricted epitope (NP$_{380-388}$) to 10^{-10} M for the HLA-B*3501-restricted epitope (NP$_{418-426}$). More importantly, the EC$_{50}$ values for the HLA-transfected C1R cells were similar or slightly lower than the EC$_{50}$ values measured with HLA-matched BLCL cells, indicating that functional presentation of peptides is comparable.

Using HLA-transfected C1R cells infected with influenza A virus, it was demonstrated in a ^{51}Cr release assay that the natural CTL epitope was liberated from viral proteins and presented to specific CTL clones (Fig. 1C). In addition, the respective CTL clones only recognized HLA-transfected C1R target cells expressing the matching HLA molecule.

IFN-γ responses in CD8$^+$ T cells restricted by individual HLA-A and -B alleles

The contribution of individual HLA-A and -B alleles to the influenza A virus-specific CTL response was determined by measuring the proportion of IFN-γ^+ 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-γ 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-γ^+ 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-γ^+ CD8$^+$ T cells in these donors (Fig. 3A). This HLA-B*2705-restricted response differed significantly with the percentages of IFN-γ^+ 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-γ^+ cells 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-γ 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-γ^+ 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-γ^+ cells of HLA-A*0101-restricted CD8$^+$ T cells was significantly lower than the proportion of cells of restricted HLA-A*0301 ($p = 0.007$).
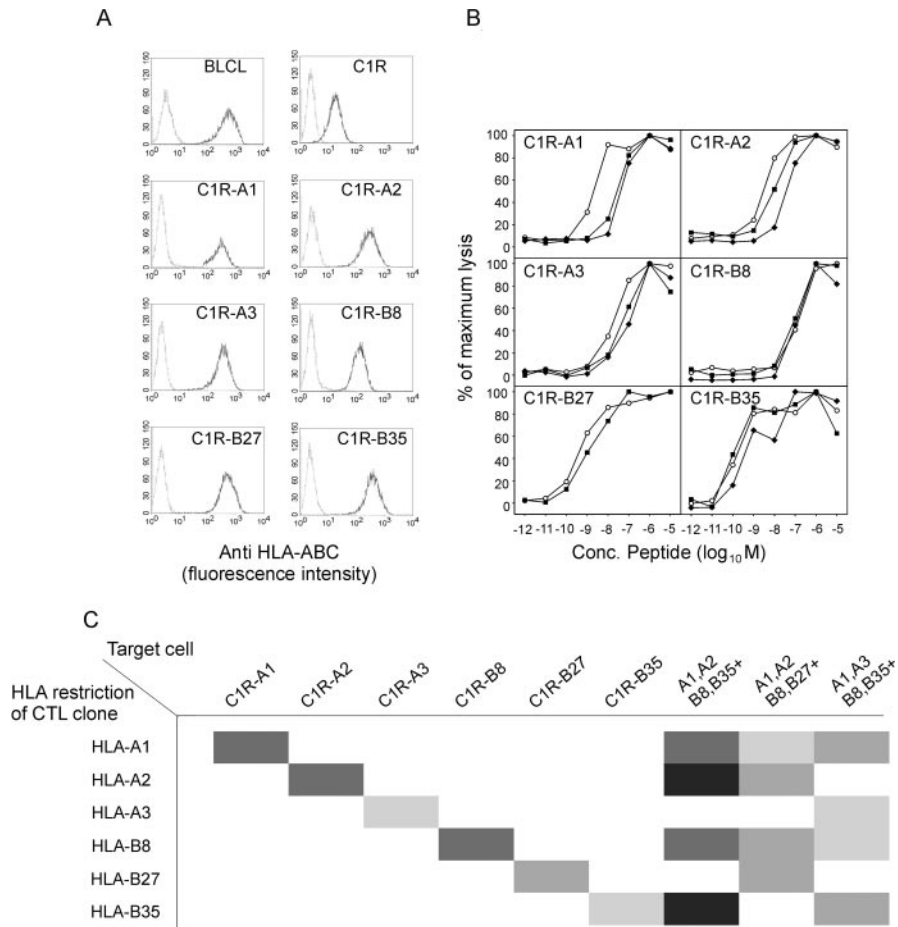
FIGURE 1. Expression of foreign HLA-A or -B alleles on C1R cells. *A*, HLA expression on BLCL, C1R, and HLA-transfected C1R cells. HLA expression was measured with saturating levels of anti-HLA-ABC mAb. The level of HLA expression (fluorescence intensity, solid line) is shown for cells of BLCL, C1R, and HLA-transfected C1R cell lines. Unstained cells are shown as dotted lines in the figures. *B*, HLA-transfected C1R cell lines stimulate influenza A virus-specific CTL clones. HLA-transfected C1R cells (open symbols) and two HLA-matched BLCL (closed symbols) were used as target cells in a ^{51}Cr release assay after a 1-h incubation with 10-fold serial dilutions of peptide analogous of epitopes. C1R-A1 cells were incubated with the NP_{44-52} epitope; C1R-A2 cells with the $M1_{58-66}$ epitope; C1R-A3 cells with the $NP_{265-273}$ epitope; C1R-B8 cells with the $NP_{380-388}$ epitope; C1R-B27 cells with $NP_{383-391}$ epitope; and finally, C1R-B35 cells with the $NP_{418-426}$ epitope and used as target cells for CTL clones specific for the respective epitopes (E:T ratio of 10:1). *C*, HLA-restricted recognition of influenza A virus-infected HLA-transfected C1R cells by virus-specific CTL. HLA-transfected C1R cells, C1R control cells, and three different BLCL cells were infected with Resvir-9 (MOI = 3) and used as target cells in a ^{51}Cr release assay. Influenza A virus-specific CTL clones (see above) were used as effector cells at an E:T ratio of 10:1. The results are given as the percentage of lysis of each target cell minus the lysis of virus-infected C1R cells. HLA-genotyped BLCL were included as controls to confirm the HLA restriction of the influenza A virus-specific CTL clones. The percentage of lysis, represented by different shades of gray, is (darkest to lightest) (100–80%; 80–60%; 60–40%; 40–20%; 20–0%).

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%, $p = 0.04$) and to a lesser extent in those of group I (9.1%, $p = 0.1$). 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-γ^{+} 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. 3*B*), illustrating the IFN-γ responses for each HLA allele (shades of gray) within the CD8^{+} T cell population (entire circle). Not all CD8^{+} T cells produced IFN-γ 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 ($p = 0.165$).

In all four donors of group II, a dominant HLA-B*2705-restricted IFN-γ response was observed in addition to the HLA-A*0201-restricted IFN-γ response. The HLA-A*0101- and HLA-B*0801-restricted IFN-γ 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 ($p = 0.011$).

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. 3*B*). 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 ($p = 0.005$).
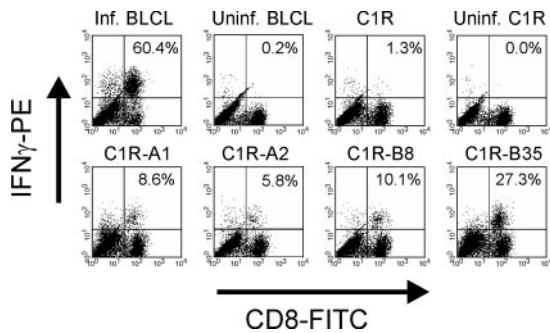
FIGURE 2. IFN-γ expression in CD8$^+$ T cells after stimulation of PBMC with influenza virus. PBMC expanded after stimulation with influenza A virus were restimulated with HLA-matched BLCL, C1R, and HLA-transfected C1R cells infected with influenza A virus (Resvir-9) at a ratio of 1:2 for 6 h in the 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. 3*B* (bar charts), the sum of the responses restricted by individual alleles (□) almost accounted for the total influenza A virus-specific response (■) 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 ^{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*2705 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 in-

fluenza 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 ($p = 0.001$).

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 A 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 (EBNA3A$_{325–333}$) 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
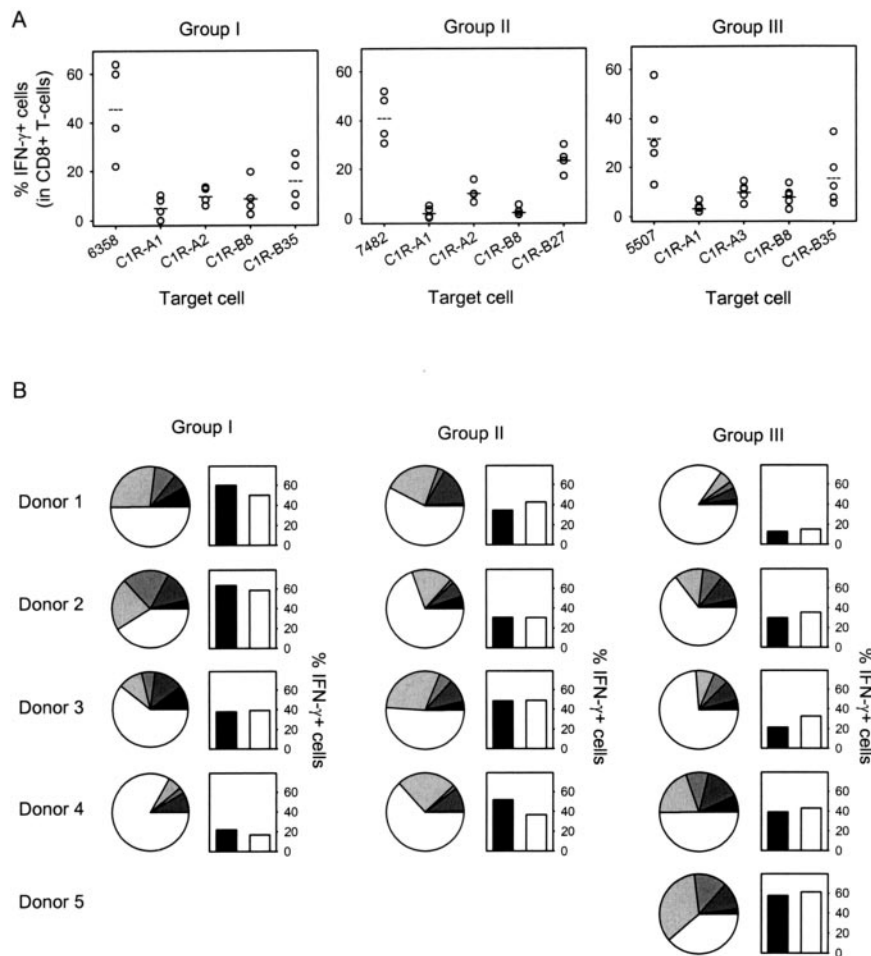
FIGURE 3. Influenza A virus-specific IFN-γ responses restricted by individual HLA-A and -B alleles in three groups of HLA-A- and -B-matched donors. *A*, Virus-specific effector cells expanded from PBMC were restimulated with influenza A virus-infected and uninfected HLA-matched BLCL, C1R, and HLA-transfected C1R cells at a ratio of 1:2 for 6 h in the presence of monensin and brefeldin. Virus-specific CTL were visualized after staining for CD8, CD3, and IFN-γ. The proportion of IFN-γ⁺ cells in the CD3⁺ CD8⁺ T cell fraction after stimulation with infected HLA-matched BLCL cells (6358, 7482, and 5507) or C1R cells expressing individual HLA-A or -B alleles is shown, as indicated. The proportion of influenza A virus-specific IFN-γ⁺ CD8⁺ T cells was calculated by subtracting the percentage of IFN-γ⁺ CD8⁺ T cells following stimulation with uninfected cells and virus-infected C1R control cells from the percentage of IFN-γ⁺ CD8⁺ T cells obtained after stimulation with virus-infected HLA-transfected C1R cells. *B*, The contribution of HLA-A and -B alleles in the influenza A virus-specific CTL response. The different parts in the pie charts represent the proportion of CD8⁺ T cells restricted by individual HLA alleles (shades of gray), starting with HLA-A*0101 and going counterclockwise via HLA-A*0201 in groups I and II or HLA-A*0301 in group III, and HLA-B*0801 to HLA-B*2705 in group II and HLA-B*3501 in groups I/III. The proportion of unresponsive CD8⁺ T cells is also shown (white area). The bar charts indicate the percentage of influenza A virus-specific IFN-γ⁺ CD8⁺ cells after stimulation with HLA-matched BLCL cells (■) or the sum of the percentage of IFN-γ⁺ CD8⁺ T cells after stimulation with C1R cells transfected with individual HLA-A and -B alleles (□). The average proportion of IFN-γ⁺ CD8⁺ T cells of all donors in groups I-III is shown in *A* by horizontal bars (−).

CTL, 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 M1$_{58-66}$, 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 (NP$_{418-426}$) 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 M1$_{58-66}$ 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 NP$_{265-273}$ 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

Table II. *Preferential HLA usage in the influenza A virus-specific CTL response*

Group	Preferential HLA Usage[a]		Allelic Preference[b]	
		p Value		p Value
I	No	0.165	ND[c]	ND
II	Yes	0.011[d]	A2 > A1[e]	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

[a] 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 and -B alleles.
[b] The average percentage of virus-specific CTL restricted by individual HLA-A or -B alleles, as measured by intracellular IFN-γ staining, was compared.
[c] ND, no significant differences detected.
[d] Values of $P < 0.05$ are considered statistically significant.
[e] A2 > A1 indicates that the percentage of virus-specific HLA-A*0201-restricted CTL is higher than the percentage restricted by HLA-A*0101.

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 re-

FIGURE 5. Influenza B virus-specific IFN-γ responses restricted by individual HLA-A and -B alleles in donors of group II. Effector cells were restimulated with influenza B virus-infected (MOI = 1) and uninfected HLA-matched BLCL cells, C1R cells, and HLA-transfected C1R cells at a ratio of 1:2 for 6 h in the presence of monensin and brefeldin. Influenza B virus-specific CTL were visualized after staining for CD8, CD3, and IFN-γ. The data represent the proportion of influenza B virus-specific IFN-γ^+ cells in the CD3$^+$ CD8$^+$ T cell fraction after stimulation with HLA-matched BLCL cells (7482) and C1R cells expressing individual HLA alleles, as indicated. In addition, the sum of the proportion of IFN-γ^+ CD8$^+$ T cells restricted by individual HLA-A and -B alleles is depicted for each individual. The average proportion of IFN-γ^+ CD8$^+$ T cells of all donors in group II is shown by a horizontal bar ($-$). The symbols represent the four different donors from this group.

sponse was lower in donors of group II (HLA-B27$^+$ donors) 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 NP$_{380-388}$ 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-2K^b allele (either transgenic or through breeding with C57BL mice) resulted in a reduced H-2^k-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.
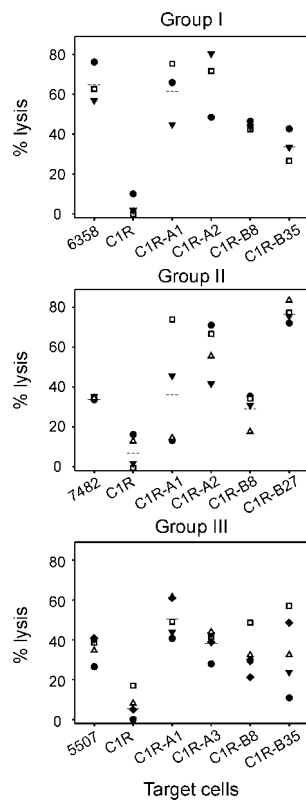

FIGURE 4. Lytic activity of CTL restricted by individual HLA-A and -B alleles. CD8$^+$ cells were isolated from influenza A virus-stimulated PBMC and used as effector cells in a ^{51}Cr release assay. The lytic activity of virus-specific CTL is calculated as the percentage of lysis of influenza A virus-infected HLA-matched BLCL (6358, 7482, 5507), C1R, and HLA-transfected C1R cells minus the lysis of uninfected cells, at an E:T cell ratio of 5:1 for HLA-matched BLCL cells and 10:1 for C1R and HLA-transfected C1R cells. Each graph represents a group of HLA-A and -B identical donors, and each donor is depicted by a different symbol. Donor 4 in group I was excluded because not sufficient cells were recovered to perform the assay. The average percentage of lysis of target cells by effector cells of the respective donors is shown ($-$).
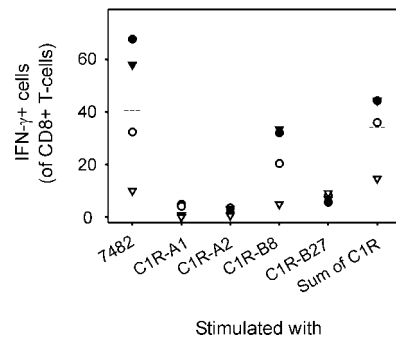
Using influenza B virus for the stimulation of PBMC, we demonstrated that the preferential HLA usage is dependent on the virus studied. In contrast to the influenza A virus-specific CTL response, the HLA-B*0801-restricted response specific for influenza B virus was highly immunodominant, followed by 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 data 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$^+$ CTL 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.

Acknowledgments

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