Stable Isotope Tagging of Epitopes
A HIGHLY SELECTIVE STRATEGY FOR THE IDENTIFICATION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I-ASSOCIATED PEPTIDES INDUCED UPON VIRAL INFECTION★

Identification of peptides presented in major histocompatibility complex (MHC) class I molecules after viral infection is of strategic importance for vaccine development. Until recently, mass spectrometric identification of virus-induced peptides was based on comparative analysis of peptide pools isolated from uninfected and virus-infected cells. Here we report on a powerful strategy aiming at the rapid, unambiguous identification of naturally processed MHC class I-associated peptides, which are induced by viral infection. The methodology, stable isotope tagging of epitopes (SITE), is based on metabolic labeling of endogenously synthesized proteins during infection. This is accomplished by culturing virus-infected cells with stable isotope-labeled amino acids that are expected to be anchor residues (i.e. residues of the peptide that have amino acid side chains that bind into pockets lining the peptide-binding groove of the MHC class I molecule) for the human leukocyte antigen allele of interest. Subsequently these cells are mixed with an equal number of non-infected cells, which are cultured in normal medium. Finally peptides are acid-eluted from immunoprecipitated MHC molecules and subjected to two-dimensional nanoscale LC-MS analysis. Virus-induced peptides are identified through computer-assisted detection of characteristic, binomially distributed ratios of labeled and unlabeled molecules. Using this approach we identified novel measles virus and respiratory syncytial virus epitopes as well as infection-induced self-peptides in several cell types, showing that SITE is a unique and versatile method for unequivocal identification of disease-related MHC class I epitopes.

The adaptive immune response to virus infections is characterized by the generation of CD8+ cytotoxic T lymphocytes (CTLs),1 which recognize peptides presented by MHC class I molecules of infected cells. The MHC class I molecules display peptides from proteins expressed inside the cell that are usually generated in the classical proteasomal pathway. Under physiological conditions these peptides are derived from self-proteins. During viral infection, the MHC class I display of peptides is affected due to changes in host protein expression and as a result of the production of viral proteins (1, 2). Identification and quantification of such altered MHC-peptide expression is a goal as part of a new field of research referred to as immunoproteomics and is of strategic importance for vaccine development (1, 3–5). The analytical challenge lies in the discrimination between the few disease-related peptides among a majority of nondisease-related peptides (6). Several strategies have been developed to explore changes in the MHC-associated peptide display on antigen-presenting cells. These analyses are generally based on the isolation of the MHC-peptide complexes, acid elution of the bound peptides from the MHC molecules followed by analysis using LC and MS as pioneered by Hunt et al. (7) and adapted for various purposes by other groups (4, 8–11). We successfully used the mass spectrometric method to identify novel MHC class I-restricted viral epitopes by subtractive analysis of peptide pools isolated from virus-infected and control cells (9). This comparative method, however, needs meticulous parallel sample processing of the infected and control peptide batches, and differences in peptide recovery as well as chromatographic retention and stability between uninfected and infected peptide samples cannot be excluded. This has limited the use of subtractive analysis and might have led to the failure to identify viral epitopes (8, 10).

The importance of CD8+ T cells in the recovery from respiratory infections such as measles virus (MV) or respiratory syncytial virus (RSV) is generally acknowledged (12, 13). Al-

1 The abbreviations used are: CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; HLA, human leukocyte antigen; DC, dendritic cell; iDC, immature dendritic cell; MHC, major histocompatibility complex; m.o.i., multiplicity of infection; MV, measles virus; PBMC, peripheral blood mononuclear cell; PTM, post-translational modification; RSV, respiratory syncytial virus; RSV-F, RSV fusion protein; RSV-NS, RSV nonstructural protein; SCX, strong cation exchange; SITE, stable isotope tagging of epitopes; RRf, relative response factor; nanoscale LC-MS, nanoscale reversed phase LC and MS.
though healthy children clear these infections in 7–21 days, children with disorders in the CTL response may suffer from complications after MV (13, 14) or RSV infection (15). Interestingly findings in humans as well as in animal models suggested that CD8+ T cells may confer protective immunity to RSV infection but may also induce enhanced pulmonary pathology (16–18). Hence the ability to mount a CD8+ T cell response is considered a major condition for protective immunity to MV or RSV infection but is also associated with increased pulmonary disease in the case of RSV. As only limited knowledge is available about the target epitopes for these viruses expressed in frequently occurring HLA types such as HLA-A*0201 and HLA-B7 (13, 19), further elucidation of the naturally processed and MHC class I-presented viral epitopes may benefit vaccine development as well as analysis of the CTL response.

Here we set out to develop a new strategy named stable isotope tagging of epitopes (SITE) for the facilitated identification of virus-induced, MHC class I-presented peptides by mass spectrometry. Protein expression in infected cells was performed in the presence of stable isotope-labeled amino acids, which enabled detection of virus-induced peptides by LC-MS. The analysis was done on one single peptide sample derived from a mixture of infected and uninfected cells. We applied SITE to detect virally induced epitopes for the common HLA class I alleles A*0201 and B7. In addition to a renewed survey of HLA-A*0201-presented MV-derived epitopes as detected by comparative analysis earlier (9), we used the technique to identify and semiquantify RSV epitopes and RSV-induced self-epitopes for HLA-A*0201 and HLA-B7. The application of SITE resulted in the identification and semiquantification of several novel naturally processed viral epitopes and virus-induced self-epitopes, which might add to our understanding of the induction and maintenance of antiviral immunity.

**EXPERIMENTAL PROCEDURES**

**Cell Culturing and Measles Virus Infection—** An HLA-A*0201 homozygous Epstein-Barr virus-transformed B-lymphoblastoid cell line was cultured in complete RPMI 1640 medium (Invitrogen) supplemented with 5% heat-inactivated fetal bovine serum (FBS, HyClone) in a humidified atmosphere with 5% CO2 at 37 °C to a total number of 3·10^6 cells. One-half of this batch was harvested and infected with plaque-purified MV (Edmonston B strain) at a multiplicity of infection (moi) of 0.5 for 2 h. These cells were subsequently incubated for 40 h in RPMI 1640 medium without L-leucine and L-methionine (Invitrogen), which was supplemented to obtain standard concentrations of L-leucine and L-methionine with 50% 5,5,5-[13C3]leucine (Cambridge Isotope Laboratories), 50% unlabeled L-leucine, 50% methyl-[1,2,3,4,5]methionine-L-[13C7]methyl-L-[13C7]leucine, 50% unlabeled L-methionine (all from Sigma), and 5% FBS. RPMI 1640 medium containing 100% of the unlabeled amino acids and 5% FBS was used for the uninfected cells. Both control and infected batches were harvested, washed three times with PBS, and equally mixed (i.e. 1.5·10^6 cells from each batch) mixed. The cells were pelletted, snap frozen, and stored at −70 °C until peptide isolation and analysis.

**Cell Culturing and RSV Infection—** Peripheral blood mononuclear cells (PBMCs) from an HLA-A*0201, -B7, -Cw7, -DQ1, -DR2 homozygous donor were isolated after leukapheresis by centrifugation to waste. After 4 min, the trapping column was loaded with 10 µl and stored at −70 °C until analysis.

**SITE: Stable Isotope Tagging of Epitopes**
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A

Self-epitopes, non-infection related

Viral epitopes

| Culture medium | 50 atom% labeled (heavy) medium | Self-protein | light and heavy self-peptides |
|----------------|--------------------------------|-------------|-----------------------------|
| Control        | Unlabeled (light) medium       | Control     |                             |
| Infected       | Unlabeled (light) medium       | Infected    |                             |
|                | Unlabeled (light) medium       |             |                             |
|                | Unlabeled (light) medium       |             |                             |

MHC class I processing and presentation

1:1 pooling of cell batches

Isolation of MHC class I-associated peptides

SCX fractionation

SCX fractionation (26 individual fractions)

Nanoscale LC-MS analysis of the SCX fractions

Searching for infection-induced peptides

Search algorithm

Allocation of tagged peptides

Sequence analysis of targeted peptides

B

unaltered self-peptide

2-fold upregulated self-peptide

5-fold upregulated self-peptide

20-fold upregulated self-peptide

100-fold upregulated self-peptide

unaltered self-peptide

2-fold upregulated self-peptide

5-fold upregulated self-peptide

20-fold upregulated self-peptide

100-fold upregulated self-peptide
switched in series with an SCX column (polysulfoethyl aspartamide, 12-cm length × 200-μm internal diameter, packed in house), and the peptides were eluted to the SCX column by injection of a 1-μl plug of 50% acetonitrile in 0.1 M acetic acid. Finally a linear salt gradient was run to 500 mM KCl in 35% acetonitrile at a column flow rate of 2 μl/min. A total number of 26 fractions (2 min each) were collected and dried using a vacuum centrifuge. The fractions were stored at −70 °C until analysis.

**Nanoscale Reversed Phase LC and MS (Nanoscale LC-MS)—** The individual SCX fractions were reconstituted in 20 μl of 5% formic acid and 5% dimethyl sulfoxide, fortified with two standard peptides angiotensin-III and oxytocin, each at a concentration level of 200 amol/μl, and analyzed using a nanoscale LC-MS system exactly as described earlier (27). The system comprises an AquITM C18 trapping column (15-mm length × 100-μm internal diameter, 5-μm particle size, 200-Å pore size, Phenomenex®) and an Aqua C18 analytical column (25-cm length × 50-μm internal diameter, 5-μm particle size, 125-Å pore size). The analytical column was butt-connected to a gold-coated spray tip (15-mm length × 25-μm internal diameter) manually tapered to 5-μm internal diameter. A 5-μl portion of the SCX fraction was loaded onto the trapping column at a flow rate of 3 μl/min while directing the column effluent to waste. The trapping column was then switched in line with the analytical column, and a linear gradient to 60% acetonitrile with 0.1 M acetic acid was run at a column flow rate of 125 nl/min. A home-built sheathless electrospray interface was used mounted on a Q-TOF Ultima API mass spectrometer (Waters). The mass spectrometer was operated in V-mode at a resolving power of 10,000. Full mass scan spectra were acquired in continuum mode at a scan speed of 1 s/scan. Candidate epitopes were identified by targeted CID MS/MS analyses using identical chromatographic conditions and on the same Q-TOF Ultima API mass spectrometer operated at optimized collision energy.

**Automated Data Processing—** All instrument LC-MS data files were converted to the NetCDF file format and subsequently imported into the MATLAB® technical computing environment (The MathWorks, Inc.). Essential mass spectral information (defined by the data arrays “mass_values” and “intensity_values”) was extracted and used for the search algorithm. First simulated isotope patterns were calculated based on (i) the stable isotope labels used, (ii) the natural occurrence of these stable isotopes, (iii) the presumed maximum number of labeled amino acids incorporated in the epitope, (iv) the experimental design, and (v) the charge state of the ions involved. Each individually simulated isotope pattern was mathematically moved along the mass axis of the MS spectrum. Matching isotope clusters were selected for MS/MS analysis in which the unlabeled isomer within an isotope cluster was chosen as the parent ion. The resolution values for quadrupole 1 (i.e. low mass resolution value and high mass resolution value) were set to 10 to prevent including the first labeled isomer in the CID spectrum. Analyses using a larger selection window were indicated in the CID spectra where appropriate.

**Identification of Candidate Epitopes—** The acquired MS/MS spectra were analyzed using MassLynx version 4.0 SP1 software and processed by background subtraction (first polynomial order below 45% curve), spectrum smoothing (Savitzky Golay, two smooths at ±6 channels), and deconvolution (MaxEnt3, using automatic peak width determination). The resulting MS/MS spectra were manually interpreted to compose a sequence tag (28). This tag was submitted to the on-line search algorithm MASCOT at the matrixscience.com website for peptide identification utilizing the National Center for Biotechnology Information non-redundant (NCBI) protein database (versions between 20040113 and 20041201) within the taxonomy parameter Homo sapiens and viruses. No enzyme specificity was selected. The peptide and MS/MS tolerances were set to ±0.2 and ±0.3 Da, respectively, for the high abundance candidates. For the low abundance epitopes these tolerances were ±0.4 and ±0.8 Da, respectively. Final validation of each of the peptide sequences was based on the following: (i) equal molecular masses of the natural epitope and the proposed sequence, (ii) matching the total number of labeled amino acids (i.e. Leu, Met, or Val) contained within the epitope with that in the proposed peptide sequence (this number was deduced from the mass spectral isotope distribution in the MS spectra), (iii) mutual comparison of the MS/MS spectra of the natural processed epitope and its synthetic analogue, and (iv) comparison of the chromatographic retention times of the epitope and its synthetic analogue applying similar instrumental conditions.

**Semiquantification of Epitopes—** Relative response factors (RRf) were calculated by the intensity-amout ratio of the synthetic analogue of the identified epitope and divided by the mean of the intensity-amout ratios of the standard peptides angiotensin-III and oxytocin. These RRf values were subsequently used for the semiquantification of the numbers of natural epitopes present in the pooled cell batch. The expression level of each epitope was determined for viral and self-peptides as follows.

- **Viral** epitopes were only generated in the infected cell batch. The expression level was therefore calculated as the quotient of the number of epitope molecules and the number of infected cells. 
- **Up-regulated self**-peptides might originate from both the infected and control cells. The degree of up-regulation of the self-peptides on the infected cells was derived from a curve defining the relation between the intensity ratio M/[M + Δ] (Fig. 1B) versus the degree of up-regulation for a particular epitope.

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**Fig. 1.** Schematic representation of the experimental design for the allocation and identification of virally induced MHC class I-associated peptides using the SITE strategy. A, the antigen-presenting cells are grown in normal culture medium. One half of the cell batch is virus-infected in culture medium fortified with 50% of the labeled (i.e. heavy) and 50% of the unlabeled (i.e. light) amino acids, known as the HLA-specific anchor residues. These amino acids are incorporated in the endogenously synthesized proteins at a 50% probability, visualized by the half-filled circles (white = incorporation of a light amino acid; black = incorporation of a heavy amino acid). Here the identification of peptides containing two heavy amino acids is shown. After infection, the non-infected control cell batch, cultured in unlabeled light medium, is pooled with an equal number of the infected cells. By pooling the cell batches, the binomial isomer distributions of non-induced HLA-presented self-epitopes are affected (left panel), whereas the heavy/light distributions of peptides exclusively produced during viral infection remain unaltered (right panel) as these peptides are not present in the control batch. HLA-associated peptides are then isolated by immunoaffinity purification and acid elution and finally fractionated into 26 fractions by SCX chromatography. Each fraction is analyzed by nanoscale LC-MS analysis on a high resolution mass spectrometer. A search algorithm is used to allocate candidate MV- or RSV-associated epitopes, which are identified by targeted nanoscale LC-MS/MS analysis. B, theoretical isotope distributions of self-peptides containing two heavy amino acids from pooled uninfected and virus-infected cells grown in 100% light and 50% light, 50% heavy medium, respectively. The distributions are shown for an unaltered expression of the self-peptides and for a 2-, 5-, 20-, and 100-fold up-regulated expression after virus infection.
Expression of the earlier described ‘stress’ epitope ILDKKVEKV originating from the self-protein heat shock protein 90β-(570–578) (Hsp90β-(570–578)) was detected in this study on HLA-A*0201 of MV-infected B cells. However, it was not significantly up-regulated, confirming earlier reports on the variedly increased expression of this epitope after MV infection (37).

| HLA allele | Host cell | Peptide sequence | MH⁺ calc | MH⁺ exp | Abundance | Source protein | NCBI accession number | Function |
|------------|-----------|------------------|----------|----------|-----------|----------------|-----------------------|----------|
| MV Viral   | A*0201 B cell | KLIHHLALN         | 1022.6   | 1022.8   | 2         | Nonstructural-1 protein | P04544                | Possibly role in RNA synthesis |
|           | A*0201 B cell | KLIHHLALN         | 1022.6   | 1022.7   | 0.1       | Nonstructural-1 protein | P04544                | Possibly role in RNA synthesis |
| Self      | A*0201 B cell | TLIDLPGRTRV       | 1197.7   | 1197.7   | 25 (6)    | Interferon-regulated resistance GTP-binding protein (MxA) | P20591                | Inhibition of RNA virus replication |
|           | A*0201 B cell | SLIGHTQTL         | 981.6    | 981.8    | 30 (4)    | Dual specificity protein phosphatase 5 | Q16690                | Antagonize members of the MAP kinase superfamily |
| RSV Viral | A*0201 DC   | KLIHHLALN         | 1022.6   | 1022.8   | 2         | Nonstructural-1 protein | P04544                | Possibly role in RNA synthesis |
|           | A*0201 Epithelial cell | KLIHHLALN      | 1022.6   | 1022.7   | 0.1       | Nonstructural-1 protein | P04544                | Possibly role in RNA synthesis |
|           | A*0201 B7 DC | KARSTPVTM         | 972.6    | 972.7    | 5         | Fusion protein | P03420                | Fusion of viral envelope with host cell |
| Self      | A*0201 DC   | FIIQGLRSV         | 1032.6   | 1032.7   | 10 (2)    | HLA class II DQ1 α-chain HLA-DQA1*05; HLA-DQ*06 | P01906 P01907         | Peptide presentation to CD4+ T cells |
|           | A*0201 DC   | IQHDLIFSL         | 1085.6   | 1085.7   | 1 (0.3)   | Hypoxia-induced factor-1α (HIF-1α) | Q16665                | Transcriptional regulator of several genes |
|           | A*0201 DC   | TLIDLPGRTRV       | 1197.7   | 1197.8   | 15 (1)    | Interferon-regulated resistance GTP-binding protein (MxA) | P20591                | Inhibition of RNA virus replication |

* The values represent the copy numbers of the peptides on the infected cells and on the non-infected cells (in parentheses).
| HLA allele | Host cell | Peptide sequence | MH<sup>c</sup><sub>calc</sub> | MH<sup>c</sup><sub>exp</sub> | Abundance<sup>a</sup> | Source protein | NCBI accession number | Function |
|------------|-----------|------------------|----------------|----------------|----------------|----------------|---------------------|----------|
| B7         | DC        | APLRPAAPGQTPM    | 1288.7         | 1288.9         | 0.2 (0.01)    | Proline-rich nuclear receptor coactivator-1 (B4-2 protein) | Q12796   | Activator of gene transcription |
| B7         | DC        | AVHPSSGVAL       | 850.5          | 850.6          | 2 (0)         | Interferon-inducible protein-15K (IFI-15K) | NM_005101 | NK cell proliferation and non-MHC-restricted cytotoxicity |
| B7         | DC        | KPYPLHRIKL       | 1264.8         | 1265.0         | 3 (0.5)       | Asparaginyl endopeptidase (AEP) | Q99538   | Processing of proteins for MHC class II antigen presentation in the lysosomal or endosomal system |
| B7         | DC        | SPAEPSVYATL      | 1134.6         | 1134.6         | 2 (0.5)       | Leukocyte immunoglobulin-like receptor 5 (LIR-5) | Q8NHJ6   | Modulation of CD4<sup>+</sup> T cell function |

**HLA leader sequences**

| HLA allele | Host cell | Peptide sequence | MH<sup>c</sup><sub>calc</sub> | MH<sup>c</sup><sub>exp</sub> | Abundance<sup>a</sup> | Source protein | NCBI accession number | Function |
|------------|-----------|------------------|----------------|----------------|----------------|----------------|---------------------|----------|
| B7         | DC        | APRTLVLLL       | 995.7          | 995.7          | 35 (3)        | HLA class I A*2 precursor | P01892   | Peptide presentation to CD8<sup>+</sup> T cells |
| B7         | DC        | APRTVLLL        | 882.6          | 882.7          | 1 (0.1)       | HLA class I B*7 precursor | P01889   | Peptide presentation to CD8<sup>+</sup> T cells |
| B7         | DC        | APRTVLLLSAA     | 1153.7         | 1153.9         | 20 (1)        | HLA class I B*7 precursor | P01889   | Peptide presentation to CD8<sup>+</sup> T cells |
| B7         | DC        | APRTVLLLSAAA    | 1224.8         | 1224.9         | 2 (0.1)       | HLA class I B*7 precursor | P01889   | Peptide presentation to CD8<sup>+</sup> T cells |
| B7         | DC        | LVMAPRTVL       | 999.6          | 999.7          | 10 (1)        | HLA class I B*7 precursor | P01889   | Peptide presentation to CD8<sup>+</sup> T cells |
| B7         | DC        | RVMAPRALL       | 1026.6         | 1026.8         | 50 (3)        | HLA class I Cw*7 precursor | P10321   | Peptide presentation to CD8<sup>+</sup> T cells |
| B7         | DC        | APRALLLLL       | 979.7          | 979.8          | 100 (7)       | HLA class I Cw*7 precursor | P10321   | Peptide presentation to CD8<sup>+</sup> T cells |
Peptide Synthesis—Synthetic peptides were prepared by Fmoc (N-(9-fluorenylethyl)carbonyl) solid phase synthesis using a SYRO II simultaneous multiple peptide synthesizer (MultiSyntech GmbH, Witten, Germany).

RESULTS AND DISCUSSION

The general strategy of SITE is shown in Fig. 1A. A control batch of cells is cultured in normal “light” medium, and another is grown in modified medium after viral infection and containing 50% light and 50% heavy HLA-binding anchor amino acids using the stable isotopes of carbon, hydrogen, or nitrogen. This allows all self- and viral proteins synthesized in the virus-infected batch to incorporate these “heavy” residues at a 50% probability, resulting in a binomial distribution pattern, before being processed in the MHC class I pathway. Equal numbers of cells from both batches are pooled prior to isolation of the MHC-associated peptides. By pooling the cell batches, the binomial isomer distributions of stably expressed self-epitopes are affected. However, the heavy/light distributions of peptides originating from proteins exclusively produced during viral infection remain unaltered as these peptides are not present in the control batch. After elution of the peptides from the HLA class I molecules, SCX chromatography is used in an off-line mode of operation to highly fractionate the sample. A portion of each fraction is then subjected to nanoscale LC-MS analysis to search for virally induced peptides (27). This allocation is based on the computer-assisted recognition of the binomially distributed mass spectral isotope patterns of peptides containing one or more labeled amino acid residues. Sequence information of the selected epitopes is obtained by targeted nanoscale LC-MS/MS analysis on the remaining portion of the SCX fraction.

SITE allowed the identification of known and novel viral epitopes and virus-induced self-peptides in the MHC class I-associated peptide display of HLA-A*0201 homozygous B cells after MV infection (Table I). During the 40-h infection period, the culture medium of the MV-infected cell batch was enriched with 50% of both $L[^{13}C_1,^2H_3]$-leucine and $L[^{13}C_1,^2H_2]$-methionine (mass increments of 3 and 4 Da, respectively, as compared with their unlabeled analogues), known as HLA-A*0201-binding anchors. Fig. 2A illustrates a typical mass spectral isotope distribution of a candidate infection-related peptide showing ions at $M + 3$ and $M + 6$ Da, which indicates that the epitope contains two leucine residues. Targeted MS/MS analysis revealed the amino acid sequence as GLASFILTI, which originates from the nucleocapsid protein of MV. This peptide has not been reported earlier as a naturally processed and presented MV epitope. In addition to GLASFILTI, two other known MV epitopes were allocated and identified with the SITE strategy, including the highly abundant KLWESPOQEI (9). Furthermore SITE also revealed a highly up-regulated MHC class I-presented self-peptide, TLIDLPGITRV (Fig. 3), that stems from the γ-interferon-induced protein MxA. This protein has a direct antiviral function against RNA viruses (29). Interestingly up-regulated expression of the same MxA-derived epitope was also detected on RSV-infected DCs (see below), reflecting a shared component in the host antiviral response.

Next SITE was applied to identify epitopes induced by RSV for which MHC class I epitope expression is largely elusive. Epitope analyses after RSV infection were performed in HLA-A*0201 homozygous epithelial cells and in monocyte-derived
dendritic cells homozygous for HLA-A*0201 and HLA-B7. The RSV-infected cells were now cultured in a medium containing 50% each of the heavy amino acids L-[13C6]leucine, L-[13C5,15N1]methionine, and an additional labeled amino acid L-[13C5,15N1]valine. Based on the analysis of the MV epitopes (H9004/H11005/H1100130 or H110014 Da), we anticipated that an equal and higher mass increment (H9004/H11005/H110016 Da) for all labeled amino acids would be beneficial for the recognition process. The additional anchor residue was applied to ensure a maximal chance of incorporation of at least one labeled amino acid.

For HLA-A*0201, which is the most frequently occurring Caucasian HLA type, the RSV epitope KLIHLTNAL was identified (Fig. 4A), originating from the N terminus of the RSV nonstructural (NS)-1 protein. This peptide was detected both on RSV-infected epithelial cells and dendritic cells. DCs are professional antigen-presenting cells and are uniquely capa-
Fig. 4. MS/MS spectra of the RSV-originating epitopes. A, KLIHLTNAL (doubly charged precursor ion \( m/z \) 511.9 Da) presented on epithelial cells. Product ions labeled with an asterisk are background ion signals that do not originate from KLIHLTNAL (see supplemental data). B, KARSTPVTL (doubly charged precursor ion \( m/z \) 486.9 Da) presented on DCs and their respective synthetic analogues.
ble of priming naive CD8$^+$ T cell responses against epitopes they expose. Primed RSV-specific CD8$^+$ T cells, in turn, migrate to the respiratory tract and recognize RSV epitopes displayed on HLA of infected epithelial cells, which are the primary target for RSV infection. Therefore, expression of the HLA-A*0201-restricted epitope KLIHLTNAL on DCs and on epithelial cells allows priming of CD8$^+$ T cells that can exert effector function after recognition of cognate antigen on RSV-infected epithelial cells. Furthermore HLA-B7 molecules on DCs contained the viral epitope KARSTPVTL (Fig. 4B), which originates from the RSV fusion protein (RSV-F).

Both KARSTPVTL and KLIHLTNAL are the first naturally processed and presented epitopes to be described for RSV. Interestingly CD8$^+$ T cell responses in children who had just recovered from severe RSV infection were reported only for the RSV-F and RSV-NS proteins and not for the seven other RSV proteins that were investigated (30). Hence epitope origin of naturally processed and presented viral peptides on infected DCs markedly coincides with the primary infant CD8$^+$ T cell targets. We therefore suggest that epitope expression on infected DCs in young children favors the induction of predominant RSV-NS- and RSV-F-specific CD8$^+$ T cell responses.

In earlier studies based on CD8$^+$ T cell reactivity against a panel of overlapping peptides covering the nucleoprotein of RSV, NPKASLLSL was characterized as an HLA-B7 CD8$^+$ T cell epitope in adults (19). Natural processing and presentation of this epitope, however, was not detected in our experiments, implying that 40 h-infected DCs do not express this epitope above the detection limit (~5 copies/cell) of our LC-MS system. All of the peptides that were expressed exclusively after infection and half of the up-regulated peptides could be identified, depending on the expression level and the quality of the MS/MS spectrum.

In total 14 up-regulated self-epitopes were identified after RSV infection of DCs (Table I). Interestingly seven of 11 up-regulated self-epitopes presented on HLA-B7 stem from the signal peptides of the HLA-A, -B or -C $\alpha$-chain, including several length variants. Although the expression of peptides from HLA-derived signal sequences on HLA-A*0201 and HLA-B7 under physiological conditions was reported earlier (32–34), the increased expression of peptides derived from all three class Ia signal sequences as well as the array of length variants seem specifically induced by the RSV infection of DCs. The immunologic consequence of the dominant expression of these signal sequences is not clear; however, enhanced processing and presentation of autoantigens during an infection may prime T cells for self-peptides, resulting in autoimmunity (35–37).

Information on the identity of CTL epitopes is a major asset in identifying targets for vaccine development but may also be applied for the development of diagnostic tools to investigate the host-immune response after vaccination (38). The use of prediction algorithms to this end was shown not to be helpful for screening epitopes in proteins unknown as T cell targets (39). Similarly the proteome of most viruses is too large to evaluate the complete T cell response by using banks of overlapping peptides. In addition, these strategies do not allow detection of post-translational modifications (PTMs). It is estimated that 50–90% of all human proteins are post-translationally modified (40). PTMs may therefore result in important changes in the immunogenicity of MHC-associated peptides (41–43). Detection of naturally presented peptides in MHC class I by SITE enables the recognition of any possible PTM of the epitopes introduced during the infection in contrast to the identification of disease-related peptides using epitope prediction algorithms. Our experiments, however, did not reveal any PTM of MHC class I epitopes following either MV or RSV infection.

In the last decade, metabolic labeling strategies have become increasingly important in quantitative proteomics (31). Recently we successfully used stable isotopes for the identification of pathogen-derived epitopes presented in MHC class II molecules (22). Until now, no such method has been described for the identification of virally induced MHC class I-expressed epitopes. The strong advantage of SITE is that the information regarding whether a peptide is associated with the infection or not is embedded in one single sample. This enables a high degree of fractionation (~25 individual fractions) in two-dimensional separation systems for increased separation efficiency. In addition, a single sample strategy is advantageous over approaches based on subtractive data analysis of two samples, necessitating identical and reproducible sample processing to prevent artificial differences (9) as changes in e.g. peptide recovery or chromatographic retention stability can be misinterpreted as altered peptide expression. This also holds for a method based on stable isotope labeling of the MHC-associated peptides after peptide isolation (11). Furthermore as this method is based on the determination of intensity ratios within mass spectral doubles, viral epitopes or completely up- or down-regulated self-peptides are represented as single spectral peaks, which may hamper the recognition of these peptides in complex samples.

In addition to the qualitative recognition of virally induced peptides, SITE allows the absolute quantification of the epitope expression. For up-regulated self-peptides, the epitope quantities were revealed by mathematical comparison of the experimental and theoretical isotope distribution patterns indicating the degree of up-regulation of self-peptides (Fig. 1B). This method of epitope quantification precludes artificial variances as it is not affected by variations in the peptide isolation procedure or chromatographic or mass spectrometric stability because the stable isotope labels are incorporated during the viral infection. The degree of up-regulation is therefore anchored at the moment the control and infected cell batches are pooled. The MV epitope KLWESPQEI e.g. (reported earlier at an expression level of

**SITE: Stable Isotope Tagging of Epitopes**
Able at http://www.mcponline.org) contains supplemental material.

Applied to this end, expression levels of individual RSV epitopes showed marked differences demonstrated by the HLA-B7-specific RSV epitope KARST-PVTL, which was expressed 2.5 times higher than the HLA-A*0201-specific RSV epitope KLIHLTNAL on infected DCs. Moreover expression levels appeared to be influenced by the cell type as illustrated by the epitope KLIHLTNAL, which was expressed 20 times higher on DCs as compared with epithelial cells.

In conclusion, application of SITE elucidates the expression of virally induced epitopes and induced self-epitopes on HLA class I molecules of several cell types. Applied to this end, SITE will attribute to development of antiviral or vaccine strategies that make use of the MHC class I route.

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SITE: Stable Isotope Tagging of Epitopes

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