HLA-B27, but Not HLA-B7, Immunodominance to Influenza Is ERAP Dependent

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HLA-B27, but Not HLA-B7, Immunodominance to Influenza Is ERAP Dependent

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Endoplasmic reticulum–associated aminopeptidase-1 (ERAP1) plays a critical role in the processing of peptides prior to binding to MHC class I molecules. In this article, we show for the first time, to our knowledge, that the HLA-B27 immunodominant influenza nucleoprotein (NP) 383–391 epitope is made as an N-terminally extended 14-mer before it is trimmed by ERAP. In the absence of ERAP, there is a significant reduction in the CTL response to the B27/NP383–391 epitope in influenza A (flu)–infected B27/ERAP−/− mice. With the use of tetramer staining, the number of naive CD8+ T cells expressing TCR Vβ8.1 in B27/ERAP−/− transgenic mice is significantly lower than that seen in B27/ERAP+/+ mice. HLA-B27 surface expression in naive and flu-infected B27/ERAP−/− mice is also lower than the expression seen for the same allele in naive and flu-infected B27/ERAP+/+ mice. In contrast, surface expression of HLA-B7 was unaffected by the absence of ERAP in B7/ERAP−/− transgenic mice. The B7-restricted NP418–426 CTL response in flu-infected B7/ERAP−/− and B7/ERAP+/+ mice was also similar. These results provide, to our knowledge, the first in vivo demonstration of ERAP functionally influencing host immune response in an HLA allele–specific manner. This principle has relevance to diseases such as ankylosing spondylitis, in which HLA-B27 and ERAP jointly contribute to disease predisposition.

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absence of ERAP. As identifying antigenic peptides is difficult in the clinical setting, these double Tg mice (i.e., B27/ERAP$^{-/-}$ and B7/ERAP$^{-/-}$) would serve as an informative model on the mechanisms of host immune responses following an infectious challenge that may entail interaction of HLA and ERAP. As the immunodominant (ImD) epitopes for various human HLA alleles for influenza virus are well known [i.e., nucleoprotein (NP) 383–391 for HLA-B27$^a$ and NP418–426 for HLA-B7$^a$ individuals], we used flu as a model to investigate the effects of HLA-B27 and ERAP$^{-/-}$ in vivo.

Studies with ERAP-deficient mice have shown reduced cell surface expression of MHC I molecules, but not MHC II (10, 11). These mice show no differences in the profile of CD4 and CD8 compared with mice with intact ERAP. The peptide repertoire generated in ERAP-deficient mice following a viral infection differed from that in ERAP$^{+/+}$ mice. Following infection, the CTL response to OVA OVA$^{257–264}$, LCMV NP$^{396–404}$, mouse CMV YL9, and flu compared with mice with intact ERAP. The peptide repertoire generated in ERAP-deficient mice following a viral infection differed reduced B27/NP383-specific naive variable by the University Health Network Research Committee.

were housed in the specific pathogen–free animal facility at Toronto Western GGAGTTTGGTTTTATGGAGGGTTG-3

HF10 epitope of T. gondii study demonstrated that there were no CTL responses to the ImD involvement of ERAP in the generation of this epitope (15). Expansion of HF10-specific CD8+ T cells was shown to be impaired in ERAP$^{-/-}$ mice, indicating the in

ERAP-deficient and DKO mice were made to generate ERAP Generation and identification of HLA Tg ERAP

The mAbs and detection reagents used for flow cytometry and their specificities and sources are as follows: ME1 (specificities: HLA-B7, -B27, -Bw22, and -B14) and B7.1 (specificity: HLA-B7) were from the American Type Culture Collection (Manassas, VA); CD4-PE (0.5 mg/ml, diluted 1:100), CD3-PerCP (0.5 mg/ml, diluted 1:150), and CD8a-FTTC (0.5 mg/ml, diluted 1:150) were from BD Pharmingen (San Diego, CA). The anti-TCR Vβ mAbs were from BD Pharmingen (0.5 mg/ml, diluted 1:100) (San Diego, CA). FITC-conjugated F(ab')2 goat anti-mouse IgG (Fc-specific, 0.5 mg/ml, diluted 1:150) and FITC-conjugated F(ab')2 goat anti-rat IgG (Fc-specific, 0.5 mg/ml, diluted 1:150) were from Accurate Chemical and Scientific (Westbury, NY).

Influenza A/X31 (H3N2) infection and IFN-γ ELISPOT assays

Mice between 7 and 10 wk of age were infected as described (7). Briefly, Tg mice were transiently anesthetized with methoxyflurane (Metofane; Pitman-Moore) and infected intranasally (i.n.) with 20 hemagglutinating units (HAUs) of influenza A/X31 (H3N2) (SPAFAS, North Franklin, CT) in 30 µl PBS. At 11 d post flu infection, IFN-γ ELISPOT assays were used to determine the frequency of peptide-specific IFN-γ–producing cells in spleens of flu-infected mice. Both HLA-B27$^a$ human CTL and Tg B27$^b$ mouse CTL recognize flu NP383–391, whereas the CTL response in HLA-B7$^a$ humans and Tg B7$^b$ mice is directed primarily to flu NP418–426 (7). HLA-B27$^a$ mice may also respond to NP$^{383–391}$. In non-Tg wild-type (WT) C57BL/6J mice, the anti-flu CTL response is directed at the NP366–374 in the context of H2-D$^b$. Each of these respective peptides at prespecified concentrations was used as described. All peptides were synthesized and purchased from Bio Basic (Markham, ON, Canada).

Tetramer staining and enrichment of Ag-specific CD8$^+$ T cells

Cell suspensions from spleen and lymph nodes (LNs) were enriched for T cells using Pan T Cell Isolation Kit II mouse as directed by the manufacturer’s protocol (Miltenyi Biotec, San Diego, CA). The details of cell preparation and staining have been described (7). Briefly, spleen and LN were washed and were used as a positive control, and lysed with ACK lysis buffer. Cells were washed and counted before single-cell suspensions (∼10$^6$ cells) were labeled and passed over a magnetized LS column (Miltenyi Biotec). Columns were washed and the bound cells were eluted and stained with T cell markers. Tetramers specific for NP366–374 (1.2 mg/ml, diluted 1:100), NP383–391 (1.2 mg/ml, diluted 1:100), and NP418–426 (1.3 mg/ml, diluted 1:100) were synthesized and purified by National Institutes of Health (Atlanta, GA). Cells were washed and fixed with 1% paraformaldehyde before being analyzed with an LSR II cytometer (Becton Dickinson, Mississauga, ON, Canada). Data analysis was performed using CellQuest and FlowJo software programs (BD Immunocytometry Systems, San Jose, CA).

Peptide immunization

The peptide immunization protocol used has been described (7). Naive HLA-Tg mice were immunized s.c. with CpG (10 µg/ml, 50 µg per mouse in 1 × PBS) 2 d prior to peptide immunization. Two days later, the mice were stratified into three different groups: one group of mice was coimmunized s.c. with synthetic NP418–426 (10 µg/ml, 75 µg per mouse) and NP383–391 (10 µg/ml, 75 µg per mouse) peptides in IFA, or IFA alone as control; the second group was immunized with an N-terminally extended 14-mer version of NP383–391 (10 µg/ml, 75 µg per mouse) (TLEIERSRYWAIRTR) and NP418–426 (10 µg/ml, 75 µg per mouse) (SVORMLPF) and the last group was immunized with an N-terminally extended 18-mer of NP383–391 (10 µg/ml, 75 µg per mouse) (TLEIERSRYWAIRTR) and NP418–426 (10 µg/ml, 75 µg per mouse) (TPFTSVYRNLPFDFRTTM). At 11 d post immunization, spleen cells were removed and tested by ELISPOT.

Body weight loss and H&E staining

Following flu infection, mice were weighed on a daily basis for 12 d. On day 12, flu-infected HLA Tg mice, along with allele- and age-matched naive counterparts, were sacrificed; the lungs were excised and fixed in 10% formalin. The fixed lung samples were embedded and stained with H&E dyes. Pictures were generated using a Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U2 camera and NIS-Elements BR 3.1 acquisition software. This microscope was equipped with a 10× objective (numerical aperture 0.3) and 20× objective (numerical aperture 0.45). The lung slides were scored in a blinded fashion by Dr. Cathy Streuver (University of Toronto).

Cytokine analysis

Serum was prepared according to directions provided by Eve Technologies (Calgary, AB, Canada). Briefly, naive and flu-infected mice were sacrificed, and blood was immediately removed by cardiac puncture. Blood was allowed to clot for 2 h before being spun at 10,000 rpm for 10 min at 4°C. Supernatants were
aliquoted and stored at −20°C before analysis. Lung tissue homogenates were prepared as described (18). Lung tissues were homogenized in PBS-based buffer (20 mM Tris-Cl, pH 7.5; 1% Triton X; 0.05% SDS; 5 mg/ml deoxycholic acid; 50 mM NaCl; and 1 mM PMSF) containing various protease inhibitors (Roche, Germany). Lung homogenates were filtered (0.22 μm), spun at 12,000 rpm for 4 min, and stored at −20°C before analysis by Eve Technologies. The mouse cytokine array 32-plex panel by Eve Technologies was used for this investigation.

Statistical analysis

Data were analyzed by two-way ANOVA (two variables: naive versus flu infected and ERAP+/+ versus ERAP−/−) in GraphPad Prism 5.0, GraphPad Software, La Jolla, CA) with a Bonferroni posttest correction. All values are expressed as mean (± SEM), p < 0.05 (adjusted p) was considered significant. The Student t test was also performed as indicated.

Results

Characterization of novel B7/ERAP−/− and B27/ERAP−/− mice

ERAP-deficient mice were identified by PCR and flow cytometric analysis, as described (9). Consistent with previous findings, we saw no significant differences in the percentage of CD4+ and CD8+ T cells in LN (Fig. 1A) or spleen (not shown) between the ERAP+/+ and ERAP−/− HLA Tg mice. Non-Tg WT mice had

- **FIGURE 1.** Characterization of HLA Tg ERAP−/− mice. (A) LNs from different Tg mice were stained for CD3 and CD8. The bar graph shows the percentage of CD3+CD8+ T cells in different Tg mice. Data are shown as mean ± SEM from n = 6 mice per group and are pooled from three independent experiments. *p < 0.05 versus Tg mice using the Student t test. (B) The expression level of HLA-B7 (as detected by BB7.1 mAb) and HLA-B27 (as detected by ME1 mAb) in single and double HLA Tg mice. Representative plots of flow cytometric analyses for different mice are shown. This experiment was repeated three times with n = 6 mice per group. Significance was assessed using two-way ANOVA.

- **FIGURE 2.** ELISPOT analysis of the CD8+ CTL response to flu infections in single (A and C) and double (B and D) HLA Tg ERAP+/+ and ERAP−/− mice. Mice were infected with flu i.n., as described in Materials and Methods. After 11 d post infection, the CTL response was tested by IFN-γ ELISPOT for various flu peptides. Data are shown as mean ± SEM of n = 6 mice per group and are pooled from six independent experiments. Significance was assessed using two-way ANOVA. ***p < 0.0001 versus NP383–391 CTL response seen in Tg B27 mice.
the highest level of CD8+ T cells compared with HLA Tg ERAP−/− mice. As expected, DKO mice had the lowest CD8+ T percentage, confirming previous results (7). The absence of ERAP did not significantly alter the cell surface expression of HLA-B7 (Fig. 1B, left panel). In contrast, the expression of HLA-B27, as detected by ME1 Ab, was significantly \( p = 0.0032 \) for mean fluorescence intensity (MFI) reduced in B27/ERAP−/− mice (Fig. 1B, right panel) in comparison with B27 Tg mice.

### Reduced B27/NP383–391 CD8+ T cell response in flu-infected B27/ERAP−/− Tg mice

Previous studies of ERAP−/− mice did not address the “interaction” of MHC-I, either endogenous or Tg, with ERAP, whereas our current mouse constructs allow a specific analysis of possible roles of HLA alleles and ERAP on CD8+ T cell responses following influenza infection. We infected B7/ERAP−/− and B27/ERAP−/− Tg mice, along with the corresponding ERAP+/+ HLA Tg controls, with flu, as described (7) (Fig. 2). Spleen cells were examined by IFN-γ ELISPOT assay 11 d post i.n. flu infection. B7/ERAP−/− and B7/ERAP+/+ mice both showed strong CTL responses to the B7-restricted NP418–426 ImD epitope (Fig. 2A, 2B). There was no significant difference in the B7/NP418–426 CTL response between these two mouse strains. Controls indicated no CTL response in the presence of irrelevant peptide or in the absence of peptide in all mice. In contrast, B27/ERAP−/− mice unexpectedly showed reduced CTL responses to the B27-restricted NP383–391 flu epitope compared with CTL responses seen in B27/ERAP+/+ mice (Fig. 2C, 2D). The B27/NP383–391 CTL response was significantly \( p < 0.0001 \) reduced in the B27/ERAP−/− Tg mice. The PB2.702–710 CTL response appeared higher \( p = 0.0681 \) in B27/ERAP−/− Tg mice than in B27/ ERAP+/+ mice. Overall, the CTL response to other subdominant B27 flu epitopes did not reach significance above the background in both ERAP+/+ and ERAP−/− B27+ mice (Fig. 2C, 2D).

### Reduced viral clearance in B27/ERAP−/− Tg mice

The ELISPOT results above prompted us to assess the overall response to flu in these mice (Fig. 3). Others have used fluctuations in the body weight following a viral infection as a viral clearance index (19, 20). We used the same index following flu infection of ERAP+/+ and ERAP−/− HLA Tg mice (Fig. 3A, 3B). We followed the changes in body weight on a daily basis for 12 d post infection. There were significant \( p < 0.05 \) differences in the body weight between the B27/ERAP−/− and B27 Tg mice 5–9 d post flu infection.

FIGURE 3. Differences in the profile of HLA Tg ERAP+/+ and ERAP−/− Tg mice following flu infection, as examined by body weight baseline change, cytokine analysis, and H&E staining of lung sections. (A and B) Percent change in baseline body weights of B27 and B27/ERAP−/− (A) and B7 and B7/ERAP−/− (B) Tg mice. Mice were infected i.n. with 20 HAU of influenza A/X31(H3N2), and the body weight change was tracked for 12 d post flu infection. Data are shown as mean ± SEM of \( n = 6 \) mice per group and are pooled from three independent experiments. (C) Cytokine profiles of proinflammatory cytokines in naive and infected (day 11 post infection) Tg mice. Lungs were homogenized and examined for the expression of different cytokines. Data are shown as mean ± SEM of \( n = 6 \) mice per group and are pooled from three independent experiments. (D) Histopathological states characteristic of naive and flu-infected Tg lung sections. Lung sections were stained with H&E (original magnification, ×10). Bar graph indicates the presence or absence of edema in different Tg mice (right panel). Histopathology scores of lung sections were assigned in a blinded fashion by a lung pathologist. Data are shown as mean ± SEM of \( n = 6 \) mice per group. Significance was assessed using two-way ANOVA. *\( p < 0.05 \), **\( p < 0.001 \) as indicated.
fection (Fig. 3A). However, such differences were observed nei-
ther in the initial stages of flu infection (i.e., between days 1 and 5)
nor in the latter parts of recovery (i.e., between day 11 and day 12)
(Fig. 3A). Both B27/ERAP<sup>−/−</sup> and B27 Tg mice recovered from
flu infection and ended up with a similar profile of body weight
change 12 d post flu infection (Fig. 3A). Such significant differ-
ences in the baseline body weight were not observed in the B7/
ERAP<sup>−/−</sup> and the B7 Tg mice (Fig. 3B). Although the body
weight profiles of flu-infected B7/ERAP<sup>−/−</sup> and the B7 Tg mice
were similar, it is noteworthy that the B7/ERAP<sup>−/−</sup> Tg mice,
compared with B7 Tg mice, seemingly (but not significantly) lost
less weight overall during the course of infection (Fig. 3B).

**Flu-infected B27/ERAP<sup>−/−</sup> Tg mice have reduced levels of
inflammatory cytokines and increased edema**

Differences seen in body weight 5–9 d post flu infection indicative
of differential viral clearance between the B27/ERAP<sup>−/−</sup> and B27
Tg mice suggest that ERAP influences the adaptive immune re-
sponse in a time-dependent manner. To investigate whether any
difference exists in the level of inflammatory cytokines produced
in ERAP<sup>+/+</sup> and ERAP<sup>−/−</sup> Tg mice, we determined the cytokine
profile in serum and lung homogenate of naive and flu-infected
mice (Fig. 3C) (18). As expected, the cytokine level of flu-infected
animals was higher than that of its naive controls, confirming
previous results (21, 22). There was a significant (p < 0.001)
difference in the levels of IL-1α, IL-6, and MIP-1α produced in the
lung of B27/ERAP<sup>+/+</sup> and B27/ERAP<sup>−/−</sup> Tg mice (Fig. 3C). A
similar trend of IL-6 and MIP-1α, but not IL-1α, production was
observed in infected serum samples of B27/ERAP<sup>+/+</sup> and B27/
ERAP<sup>−/−</sup> mice. The effect of ERAP on differential expression of
IL-6 and MIP-1α in the lung seen in B27 Tg mice was not seen in
B7 Tg mice. Next, to determine whether any differences were
present in the pathological state of the target organ, we stained
naive and flu-infected fixed lung sections with H&E (Fig. 3D).
The slides were subsequently scored by a lung pathologist in a
blinded fashion. Overall, as evident in Fig. 3D, edema was in-
creased in the lungs of flu-infected ERAP<sup>+/+</sup> and ERAP<sup>−/−</sup> Tg
mice, compared with naive mice, regardless of mouse strain. The
alveolar membranes showed significant damage following flu in-
fection. The total edema score for infected B27/ERAP<sup>−/−</sup> was
significantly (p < 0.001) higher than that of B27/ERAP<sup>+/+</sup> Tg
mice (Fig. 3D, bar graph). No differences in the edema score were
observed between the B7 and B7/ERAP<sup>−/−</sup> Tg mice (Fig. 3D).

**Reduced number of B27/NP383–391 CD8<sup>+</sup> T cells in B27/
ERAP<sup>−/−</sup> Tg mice**

We recently showed that double Tg B7/B27 mice (i.e., ERAP-intact
mice) have a reduced number of naive B27/NP383–391 CD8<sup>+</sup>
T cells, compared with single Tg B27 mice (7). Differences in the
naive B27/NP383–391 CD8<sup>+</sup> T cell number accounted for the
decreased NP383–391 CTL response following flu infection in
B7/B27 Tg mice. To investigate whether the difference in the B27/
Generation and presentation of the B27/NP383–391 flu epitope are ERAP dependent

ERAP is involved in the generation and trimming of different peptides for recognition by CTLs (1). To directly address the role of ERAP in NP383–391 and NP416–426 peptide generation, we immunized HLA Tg ERAP+/+ and ERAP−/− mice with canonical B27 and B7 flu epitopes and examined the CTL response by IFN-γ ELISPOT assay, as described (Fig. 6A, 6B) (7). The B27/NP383 CTL response in B27/ERAP−/− mice was significantly lower (p < 0.001) than the CTL response in B27 Tg mice (Fig. 6A). No significant differences were found in the CTL response to the NP418–426 flu epitope in B7 Tg and B7/ERAP−/− mice (Fig. 6B). Because ERAP has been shown to trim some N-terminally extended peptides in ER in a sequential manner, we immunized the HLA Tg mice with N-terminally extended modifications of B7 and B27 flu epitopes and examined the CTL response by IFN-γ ELISPOT assay (Fig. 6C, 6D). Surprisingly, we observed a significant difference in the generation of the B27/NP383–391 flu epitope in B27/ERAP−/− and ERAP−/− mice compared with B27 Tg mice. Unlike Vβ8.1 expression, there was no drastic change in the overall expression of TCR Vβ6−expressing CD8+ T cells in naive (compare Fig. 5C, 5D) and flu-infected (not shown) B7 Tg and B7/ERAP−/− mice. Overall, significant differences observed in the geometric MFI for Vβ8.1 expression in naive B27 Tg and B27/ERAP−/− mice (Fig. 5E) and the lack of differences present in the geometric MFI for Vβ6 expression in naive B7 Tg and B7/ERAP−/− mice (Fig. 5F) confirm the above results. There were no major deviations in the expression of other TCR Vβ markers between the naive and flu-infected ERAP+/+ (not shown) and ERAP−/− B7 or B27 Tg mice (Fig. 5G, 5H).

Deletion of Vβ8.1* CD8+ T cells in B27/ERAP−/− Tg mice

We have previously shown that TCR Vβ6*CD8+ T cells recognize the NP418–426 flu epitope in B7+ human and B27 Tg mice (7). The recognition of the NP383–391 flu epitope in both B27+ humans and B27 Tg mice is dependent on TCR Vβ8.1-expressing CD8+ T cells. Our recent published data showed that coexpression of B7 with B27 led to negative selection of B27/NP383–391 flu-specific naive Vβ8.1* expressing CD8+ T cells in B7/B27 Tg mice (7). In this article, we identify a significant difference (p < 0.001) in the expression of Vβ8.1 CD8+ T cells in naive (compare Fig. 5A and 5B) and flu-infected (not shown) B27/ERAP−/− Tg mice compared with B27 Tg mice. Unlike Vβ8.1 expression, there was no drastic change in the overall expression of TCR Vβ6−expressing CD8+ T cells in naive (compare Fig. 5C, 5D) and flu-infected (not shown) B7 Tg and B7/ERAP−/− mice. Overall, significant differences observed in the geometric MFI for Vβ8.1 expression in naive B27 Tg and B27/ERAP−/− mice (Fig. 5E) and the lack of differences present in the geometric MFI for Vβ6 expression in naive B7 Tg and B7/ERAP−/− mice (Fig. 5F) confirm the above results. There were no major deviations in the expression of other TCR Vβ markers between the naive and flu-infected ERAP+/+ (not shown) and ERAP−/− B7 or B27 Tg mice (Fig. 5G, 5H).

FIGURE 5. Analysis of TCR Vβ repertoire of ERAP+/+ and ERAP−/− HLA Tg mice. Spleen cells from naive mice were stained for the expression of CD3, CD8, and various TCR Vβs. By gating first on the CD3+CD8+ T cells (not shown), the relative abundance of cells expressing each TCR Vβ was examined. Representative graphs showing the relative abundance of (A) naive B27 and (B) B27/ERAP−/− cells expressing TCR Vβ8.1. Representative graphs showing the relative abundance of (C) naive B7 and (D) B7/ERAP−/− cells expressing TCR Vβ6. (E and F) Bar graphs of geometric MFI of (E) Vβ8.1 and (F) Vβ6 expression as seen in different HLA Tg mice. (G and H) TCR Vβ expression in naive and flu-infected HLA (G) B27/ERAP−/− and (H) B7/ERAP−/− Tg mice. The values were normalized as described in Ref. 7. Data are shown as mean ± SEM of n = 6 mice per group and are pooled from three independent experiments. Significance was assessed using two-way ANOVA. **p < 0.001.
Following 14-mer NP383 peptide immunization, there were no CTL responses to the NP383–391 flu epitope in B27/ERAP2 mice, whereas the CTL response in B27 Tg mice to the same epitope was significantly ($p$, 0.0001) higher (Fig. 6C). Immunization with the 18-mer version of the NP383–391 flu epitope did not result in any CTL responses in any HLA Tg mice regardless of ERAP status (data not shown). Immunization with the 14-mer version of the NP418–426 flu epitope demonstrated no significant differences in the CTL response between B7 Tg and B7/ERAP2/2 mice (Fig. 6D). As was the case with the 18-mer version of NP383–391 vaccination, immunization with the 18-mer version of NP418–426 flu epitope demonstrated no significant differences in the CTL response between B7 Tg and B7/ERAP2/2 mice (Fig. 6D). As was the case with the 18-mer version of NP383–391 vaccination, immunization with the 18-mer version of NP418–426 was not associated with a detectable CTL response in any mouse strain (date not shown). The non-Tg WT/NP366–374 flu epitope was included as a negative control for our peptide immunization experiments.

**Discussion**

The results presented in this article signify the importance of HLA-B27 and ERAP collaboration in peptide generation and presentation. This is the first report, to our knowledge, showing that the absence of ERAP has led to partial deletion of allele-specific naive CD8+ T cells. Our results complement a number of previous findings with ERAP2/2 mice while adding new knowledge about the role of ERAP in adaptive immune response. These results suggest that the function of ERAP is dependent on its MHC-I context: the presence or absence of ERAP in combination with HLA-B7 had no effects on host immune responses, whereas the absence of ERAP significantly altered the immune response to infection when coexpressed with HLA-B27. These in vivo studies complement the recent genetics studies (3) that have implicated class I allele–specific interactions with ERAP not only in AS but also in psoriasis and Behçet’s disease (23). Discovery of pathogenic peptide epitopes has been very difficult in these diseases, and the current study provides a proof-of-principle demonstration of specific ERAP–MHC I “interactions” influencing host response to infection.

ERAP has been shown to influence the overall peptide repertoire available for presentation (13, 14, 24). Our findings demonstrate that generation and presentation of the B27-restricted NP383–391 flu epitope is critically dependent on ERAP. Absence of ERAP in B27/ERAP2/2 mice leads to significant reduction in the CTL response to the NP383–391 epitope following flu infection and peptide vaccination. Our peptide immunization studies suggest that the NP383 epitope is most likely generated as an N-terminally extended 14-mer that is subsequently trimmed in the ER by ERAP before being loaded into the MHC I peptide-binding groove. ERAP has been shown to cleave polypeptides at specific sites expressing leucine (L), methionine (M), phenylalanine (F), and tyrosine (Y) in the amino acid sequence (25, 26). The natural 14-mer sequence of NP383–391 (i.e., TLELR SRYWAIRTR) contains two leucines upstream of NP383–391 at positions T1 and 13, which provide potential cleavage sites for ERAP. Absence of an NP383-specific CTL response following 14-mer and 18-mer NP383–391 peptide immunization in B27/ERAP2/2 Tg mice indicates that this epitope is indeed trimmed by ERAP. In contrast, TAP has been shown not to transport peptides containing proline.
(P) at position 2 within its sequence (27). The presence of proline at position 2 of NP418–426 (i.e., VQRNLFFDRTTIM) strongly suggests that this epitope is initially generated as an N-mer before being transported into the ER by TAP. Lack of B7/NP418 CTL response difference between the B7 Tg and B7/ERAP−/− mice following flu infection and peptide immunization speaks against a specific role for ERAP in the final generation of the B7 epitope. The absence of ERAP’s preferred cleavage amino acids upstream of the NP418–426 flu sequence seems to confirm this. It is worth noting that immunization with exogenous peptide precursors does not always guarantee ER trimming. Aminopeptidases located at the cell surface or in the endosome (e.g., IRAP) can also contribute to the overall peptide trimming. Given that the only difference between the B27/ERAP and the B27/ERAP−/− Tg mice is absence of ERAP expression, these other possibilities were ruled out as contributing factors. Our B7 findings confirm previous observations with ERAP−/− mice (11, 12). These studies showed there was no significant difference in the CTL number and CTL response to flu NP366–374, PA224–233, NS2114–121, PB1F262–70, and PB2198–206 epitopes. It is known that ERAP can influence the CTL response to one epitope, but not another originating from the same virus. Previously, Niedermann’s group (11) showed that absence of ERAP expression leads to diminished LCMV CTL responses to ImD NP396–404, but not to ImD GP33–41. Our findings are, to our knowledge, the first to show that the B27-restricted flu CTL responses, but not the B7-restricted flu CTL response, is influenced by ERAP. Further future investigations are needed to determine whether ERAP plays an integral role in the final generation and presentation of the NP418–426 flu epitope.

ERAP, when coexpressed with HLA-B27, plays multiple critical functions in host immunity. First, as discussed above, it trims the 14-mer version of the Imd flu NP383–391 epitope to the appropriate length before it is presented by HLA-B27. Second, ERAP may figure critically in determining the T cell repertoire in B27/ERAP−/− mice. It is notable that the reduction in T cells reactive with NP383–391 is detected in naive as well as flu-infected mice. During thymic selection in B27 Tg mice, ERAP may contribute to deletion of self-reactive Ags, some of which might mimic flu NP383–391 epitopes, thus allowing positive selection of those thymocytes capable of recognizing the NP383–391 flu epitope later on in life. When ERAP is absent, as is the case in B27/ERAP−/− mice, deletion of self-reactive T cells recognizing Ags that mimic the flu NP383–391 epitope does not take place, leading to eventual partial negative selection of B27/NP383-specific thymocytes expressing TCR Vβ8.1+. Partial deletion of a specific T cell population may occur for different reasons. We have recently shown that coexpression of HLA-A27 with HLA-B27 in double Tg B7/B27 mice (i.e., ERAP-intact) leads to partial negative selection of B27/NP383-reactive T cells (7). Thus, both MHC I allelic coexpression and ERAP-mediated peptide trimming contribute in an allele-specific manner to negative selection of T cells. Last, surface expression of HLA-B27, but not HLA-B7, is significantly reduced when ERAP is absent. Our B27 results are consistent with the published results of others showing reduced endogenous MHC class I expression in ERAP−/− mice. These results suggest that ERAP normally stabilizes the B27 H chain (HC) by presenting it with appropriate B27-specific peptides during assembly of MHC I. When ERAP is absent, such peptide presentation to newly synthesized B27 molecules is impaired. This result may contribute to two of the proposed mechanisms whereby HLA-B27 contributes to disease pathogenesis, namely, misfolding of the HC of B27 within the ER, resulting in an unfolded protein response (28) phenomenon; and homodimerization of the B27 HC, leading to altered interaction with NK cells (29, 30). These two phenomena associated with B27 may lead to slower transport of a diminished number of mature B27 molecules to the cell surface. Taken together, partial negative selection of B27/NP383-specific CD8+ T cells and reduced B27 surface expression in B27/ERAP−/− mice account for both the reduced number of NP383–391 CD8+ T cells and the reduced NP383–391 CTL response following flu infection and peptide immunization in these mice.

To date, this is, to our knowledge, the first report directly linking ERAP as a determining factor in T cell repertoire generation during thymic development. ERAP appears not to influence presentation of other B7-restricted subdominant epitopes (Fig. 2). Increased weight loss 5–9 d post flu infection in B27/ERAP−/− Tg mice is reflective of partial impaired viral clearance, likely secondary to lower numbers of NP383–391-specific CD8+ T cells. Increased edema in the lung in the same mice, reflecting enhanced direct lung injury, recapitulated this trend. Lower levels of proinflammatory cytokines in the lung of B27/ERAP−/− Tg mice are indicative of impaired local response to the virus. Blinded scoring of the lung pathological state revealed lower numbers of acute and chronic inflammatory infiltrates in B27/ERAP−/− mice compared with B27/ERAP+/+ Tg mice, accounting for the lower inflammatory cytokine levels in B27/ERAP−/− Tg mice. We have previously shown and confirmed in this article that the predominant CTL response in B27 Tg mice is directed at NP383–391. In addition to this specific NP383 CTL response, it is possible other B27-specific flu epitopes, not investigated in this study, contribute to the overall viral clearance. Slight increases in the level of CTL responses to other subdominant B27 flu epitopes (e.g., PB2702–710, Fig. 2D) in B27/ERAP−/− Tg mice seem to partially make up for the reduced B27/NP383–391 CTL response, aiding in overall viral clearance.

We propose that in the absence of ERAP there is a relative inability to trim the N-terminally extended NP383–391 peptide and that this accounts for the reduced number of B27/NP383-specific CD8+Vβ8.1+ T cells seen in naive and flu-infected B27/ERAP−/− mice. Use of a well-controlled in vivo animal model can enhance our ability to resolve the cohesive ERAP-B27 “interaction” in disease pathogenesis. Such a system will shed more light on the mechanisms behind the genetic basis of AS.

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Disclosures

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References


