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

Ali Akram, Aifeng Lin, Eric Gracey, Catherine J. Streutker and Robert D. Inman

J Immunol 2014; 192:5520-5528; Prepublished online 16 May 2014;
doi: 10.4049/jimmunol.1400343
http://www.jimmunol.org/content/192/12/5520

References This article cites 30 articles, 8 of which you can access for free at:
http://www.jimmunol.org/content/192/12/5520.full#ref-list-1

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
HLA-B27, but Not HLA-B7, Immunodominance to Influenza Is ERAP Dependent

Ali Akram,*†‡ Afifeng Lin,* Eric Gracey,*†‡ Catherine J. Streutker,§ and Robert D. Inman*†‡

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 Vb8.1 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. The Journal of Immunology, 2014, 192: 5520–5528.

The immune system faces immunogenic challenges on a daily basis. Following a viral infection, the host immune response is directed to prompt clearance of the virus. Elements of innate immunity act rapidly to contain the spread of the virus while providing elements of adaptive immunity sufficient time to mount a specific and sustained immune response. The adaptive immune response depends on the ability of CTL to recognize, via TCR, antigens from infected cells. Antigenic viral peptides are subsequently bound by MHC I and transported to the endoplasmic reticulum aminopeptidase (ERAP) to peptides of 8–20 aa in length. Terminally extended peptides are further trimmed in the ER by endoplasmic reticulum aminopeptidase (ERAP) to generate peptides of 8–10 aa in length, which are appropriate for MHC I binding. These peptides are subsequently bound by MHC I and transported to the cell surface, where they are recognized by CTL. Recent genome-wide association studies have emphasized the role of ERAP in host immune response to infection. ERAP-deficient mice have provided strong indirect support for the concept that processing and presentation of arthritogenic epitopes likely play a central role in the pathogenesis of AS. The codominant expression of multiple human class I alleles contributing to immunodominance has hindered detailed immune response analysis of clinical samples. A recent insight highlighting allele-specific events in AS comes from a new study showing that whereas B27 confers susceptibility to AS, B7 confers protection. These advances in the genetic basis of AS have provided strong indirect support for the concept that processing and presentation of arthritogenic epitopes likely play a central role in the pathogenesis of AS. However, the identity of such arthritogenic epitopes remains unknown at this time. These findings emphasize the need for a controlled experimental system in which the role of ERAP and different MHC I alleles can be systematically addressed. To overcome this, we have developed single and double HLA transgenic (Tg) (HLA^b^ Tg) mice. The endogenous mouse MHC I genes were deleted [i.e., H-2K^−/− and H-2D^−/−, double knockout (DKO)], and selected human HLA genes, specifically HLA-B7 and HLA-B27, were introduced into these DKO mice. Following challenge with influenza virus, the HLA Tg mouse immune response was comparable to that of humans expressing similar MHC alleles. These findings indicated that the Tg mice can be informative in dissecting genetic control of human immune response to infection. Concurrently, there has been interest in addressing the role of ERAP in host immune response to infection. ERAP-deficient mice have been used to study the effects of ERAP on generation and presentation of immunogenic peptides following lymphocytic choriomeningitis virus (LCMV), mouse CMV, influenza A (flu), and Toxoplasma gondii infections. We used these ERAP^−/− mice and crossed them with our HLA-B7 and HLA-B27 Tg mice (on a DKO background) to create HLA-Tg mice in the
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 an 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* and NP418–426 for HLA-B7* 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 OVA257–264, LCMV NP396–404, mouse CMV YL9, and flu NP366–374, PA224–233, NS214–121 and PB2198–206 epitopes was reduced, whereas that of LCMV GP33–41 and histocompatibility gene SVL9 epitopes increased (11–14). In addition to viral infection, one study demonstrated that there were no CTL responses to the ImD HFL10 epitope of T. gondii in ERAP−/− mice, indicating the involvement of ERAP in the generation of this epitope (15). Expansion of HFL10-specific CD8+ T cells was shown to be impaired in ERAP−/− mice, rendering these mice more susceptible to toxoplasmosis.

In this article, we show for the first time, to our knowledge, that the generation and presentation of the ImD B27-restricted flu peptide NP383–391 is ERAP dependent. This B27/NP383 epitope is likely made as an extended 14-mer that is subsequently trimmed by ERAP. Furthermore, we show B27/ERAP−/− mice to have reduced B27/NP383-specific naive variable β (Vβ8)1.1–expressing CD8+ T cells in comparison with their ERAP-intact counterparts. Surface expression of HLA-B27 in naive and infected B27/ERAP−/− mice was also significantly reduced. HLA-B27/ERAP−/− Tg mice had increased edema and low levels of inflammatory cytokines. These events were not paralleled in the HLA-B7 mice: no differences were observed in the B7 surface expression or in the number of B7/NP418-specific naive Vβ6-expressing CD8+ T cells in B7/ERAP−/− compared with their B7/ERAP+/+ counterparts. These results indicate an important cohesive relationship played by HLA-B27 and ERAP in host immunity.

Materials and Methods

**Generation and identification of HLA Tg ERAP−/− mice**

The generation of single Tg HLA-B27/DKO and HLA-B7/DKO has been described (8, 16). The generation of ERAP-deficient mice has also been described (9). These mice were a generous gift from Dr. N. Shastri (University of California, Berkeley, Berkeley, CA). Appropriate crosses of C57BL/6J mice, the anti-flu CTL response is directed at the NP366–376, and PB1.571–579 flu epitopes (17). 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^d. Each of these respective peptides at prespecified concentrations was used as described. All peptides were synthesized and purchased from Bio Basic (Markham, ON, Canada).

**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) (TELEIRSRYWIRTR) and NP418–426 (10 µg/mL, 75 µg per mouse) (SVORMLPFDRRTIM); and the last group was immunized with the N-terminally extended 18-mer of NP383–391 (10 µg/mL, 75 µg per mouse) (TELEIRSRYWIRTR) and NP418–426 (10 µg/mL, 75 µg per mouse) (V2P2S3YMLPFPFRTIM). At 11 d postimmunization, 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 hematoxylin and eosin by guest on April 16, 2017 http://www.jimmunol.org/ Downloaded from

**Flow cytometry analysis**

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 BB7.1 (specificity: HLA-B7*) were from the American Type Culture Collection (Manassas, VA); CD4-PE (0.5 mg/ml, diluted 1:150), 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 V8 mAbs were from BD Pharmingen (0.5 mg/ml, diluted 1:100) (San Diego, CA). FITC-conjugated Fab' goat anti-mouse IgG (Fc-specific, 0.5 mg/ml, diluted 1:150) and FITC-conjugated Fab' 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) (SPAFA, 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 infected mice. Both HLA-B27* human CTL and Tg B27 mouse CTL recognize flu NP383–391, whereas the CTL response in HLA-B7* humans and Tg B7 mice is directed primarily to flu NP418–426 (7). HLA-B27 mouse may also respond to NS1.87–95, PB2.702–710, PB2.368–376, and PB1.571–579 flu epitopes (17). In non-Tg wild-type (WT) C57BL/6J mice, the anti-flu CTL response is directed at the NP366–374. 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 using a plunger, filtered, and lysed with ACK lysis buffer. Cells were washed and counted before single-cell suspensions (≤10^7 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 by National Institutes of Health (Atlanta, GA). Cells were washed and fixed with 1% paraformaldehyde before being analyzed with an LSR II cytometer (Bechtro Dickinson, Mississauga, ON, Canada). Data analysis was performed using CellQuest and FlowJo software programs (BD Immucytometry Systems, San Jose, CA).

**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 1000 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+/+ (7) 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 postinfection, 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 postinfection. There were significant (p < 0.01) differences in the body weight between the B27/ERAP−/− and B27 Tg mice 5–9 d post–flu infection.
fection (Fig. 3A). However, such differences were observed neither 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$^{−/−}$ 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 differences in the baseline body weight were not observed in the B7/ERAP$^{−/−}$ and the B7 Tg mice (Fig. 3B). Although the body weight profiles of flu-infected B7/ERAP$^{−/−}$ and the B7 Tg mice were similar, it is noteworthy that the B7/ERAP$^{−/−}$ 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$^{−/−}$ 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$^{−/−}$ and B27 Tg mice suggest that ERAP influences the adaptive immune response in a time-dependent manner. To investigate whether any difference exists in the level of inflammatory cytokines produced in ERAP$^{+/+}$ and ERAP$^{−/−}$ 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$^{+/+}$ and B27/ERAP$^{−/−}$ 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$^{+/+}$ and B27/ERAP$^{−/−}$ 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 increased in the lungs of flu-infected ERAP$^{+/+}$ and ERAP$^{−/−}$ Tg mice, compared with naive mice, regardless of mouse strain. The alveolar membranes showed significant damage following flu infection. The total edema score for infected B27/ERAP$^{−/−}$ was significantly ($p < 0.001$) higher than that of B27/ERAP$^{+/+}$ Tg mice (Fig. 3D, bar graph). No differences in the edema score were observed between the B7 and B7/ERAP$^{−/−}$ Tg mice (Fig. 3D).

**Reduced number of B27/NP383–391 CD8$^+$ T cells in B27/ERAP$^{−/−}$ 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$^+$ T cells, compared with single Tg B27 mice (7). Differences in the naive B27/NP383–391 CD8$^+$ 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/ERAP$^{−/−}$
NP383–391 CTL response (compare Fig. 2C with 2D) seen between the B27/ERAP+/− and B27 Tg mice is due to a reduced number of B27/NP383–391 naive CD8+ T cells in B27/ERAP+/− Tg mice, we stained T cell–enriched splenocytes with flu NP383–391 tetramer, as described in Materials and Methods (Fig. 4A). The percentage of CD3+CD8+NP383+Tet+ T cells in naive and flu-infected B27/ERAP+/− Tg mice is lower (i.e., 0.601% and 1.31%) than our previous results for naive and flu-infected B27 Tg mice (i.e., 1.15% and 6.82%) (7), respectively (Fig. 4A). These percentage differences reflect the variance seen in the actual numbers of B27/np383-specific CTL. The number of naive and flu-infected B27/np383–391 CD8+ T cells is significantly (p < 0.0001) lower in B27/ERAP+/− Tg mice compared with B27 Tg mice (Fig. 4B, 4C). Tetramer staining with the NP418–426 flu epitope did not reveal any major differences in the CD3+CD8+NP418+Tet+ T cell number in naive and flu-infected B7 and B7/ERAP+/− Tg mice (Fig. 4B, 4C). We used the non-Tg WT/np366–374 flu tetramer as a negative control to show the specificity of our tetramer staining (Fig. 4B, 4C). We used the non-Tg WT/np366–374 flu tetramer as a negative control to show the specificity of our tetramer staining (Fig. 4B, 4C). We used the non-Tg WT/np366–374 flu tetramer as a negative control to show the specificity of our tetramer staining (Fig. 4B, 4C).

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 B7 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/ERAP2/2 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 NP383–391 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 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 11 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., VQRNLFPDRTTIM) 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 NP383–391 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, 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 naïve 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-B7 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 B27-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., PB2.702–710, 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 naïve 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.

Acknowledgments

We thank N. Shastri for providing the ERAP-deficient mice and Dr. Florence Tsui for critically reviewing this manuscript.

Disclosures

The authors have no financial conflicts of interest.

References

5528 HLA-B27 FLU RESPONSE IS ERAP DEPENDENT


