The Human Leukocyte Antigen (HLA)-B27 Peptidome in Vivo, in Spondyloarthritis-susceptible HLA-B27 Transgenic Rats and the Effect of Erap1 Deletion*

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HLA-B27 is a class I major histocompatibility (MHC-I) allele that confers susceptibility to the rheumatic disease ankylosing spondylitis (AS) by an unknown mechanism. ERAP1 is an aminopeptidase that trims peptides in the endoplasmic reticulum for binding to MHC-I molecules. ERAP1 shows genetic epistasis with HLA-B27 in conferring susceptibility to AS. Male HLA-B27 transgenic rats develop arthritis and serve as an animal model of AS, whereas female B27 transgenic rats remain healthy. We used large scale quantitative mass spectrometry to identify over 15,000 unique HLA-B27 peptide ligands, isolated after immunoaffinity purification of the B27 molecules from the spleens of HLA-B27 transgenic rats. Heterozygous deletion of Erap1, which reduced the Erap1 level to less than half, had no qualitative or quantitative effects on the B27 peptidome. Homozygous deletion of Erap1 affected approximately one-third of the B27 peptidome but left most of the B27 peptidome unchanged, suggesting the possibility that some of the HLA-B27 immunopeptidome is not processed in the presence of Erap1. Deletion of Erap1 was permissive for the AS-like phenotype, increased mean peptide length and increased the frequency of C-terminal hydrophobic residues and of N-terminal Ala, Ser, or Lys. The presence of Erap1 increased the frequency of C-terminal Lys and Arg, of Glu and Asp at intermediate residues, and of N-terminal Gly. Several peptides of potential interest in AS pathogenesis, previously identified in human cell lines, were isolated. However, rats susceptible to arthritis had B27 peptidomes similar to those of non-susceptible rats, and no peptides were found to be uniquely associated with arthritis. Whether specific B27-bound peptides are required for AS pathogenesis remains to be determined. Data are available via ProteomeXchange with identifier PXD005502. Molecular & Cellular Proteomics 16:10.1074/mcp.M116.066241, 642–662, 2017.

The class Ia MHC allele HLA-B27 (B27)1 is highly associated with the rheumatic disease ankylosing spondylitis (AS) (1, 2). The molecular basis for this association remains unexplained, and there are currently at least four hypotheses that attempt to explain it. Two of these hypotheses involve misfolding of the HLA-B27 heavy chain, with formation of disulfide-linked heavy chain homodimers or higher oligomers. A free cysteine at position 67 in the B pocket of the peptide-binding groove is a characteristic feature of B27, which enhances formation of disulfide-linked dimers of its heavy chains in vitro and in vivo (3, 4). B27 heavy chain misfolding within the endoplasmic reticulum of HLA-B27 transgenic rat leukocytes has been shown to activate the unfolded protein response, with increased IL-23 production (5). B27 heavy chain homodimers expressed on cell surfaces have been shown to activate innate immune receptors, particularly the NK receptor KIR3DL2, to trigger IL-17-related inflammatory responses (2). The third hypothesis, the arthritogenic peptide hypothesis, suggests that B27 presents specific peptides, presumably to CD8+ T cells, that induce pathogenic adaptive immune responses. Although this is the most straightforward hypothesis, to date no specific peptide or responding T cell has been convincingly implicated (1, 2, 6). Moreover, the

1 The abbreviations used are: B27, HLA-B27; AS, ankylosing spondylitis; SNP, single nucleotide polymorphism; ZFN, zinc finger nuclease; ER, endoplasmic reticulum; ERAP1 and ERAP2 (human) and Erap1 (murine), ER aminopeptidase associated with antigen processing.
arthritides and spondylitis that develops spontaneously in HLA-B27 transgenic rats (7) occur even in rats lacking CD8 and CD8+ T cell responses (8). The most recent hypothesis, based on evidence that MHC molecules, including HLA-B27, help shape the intestinal microbiota, places B27 effects on gut microbiota as an intermediary in disease predisposition (9). Whatever the molecular role of B27, evidence from human and animal studies suggests that it involves abnormalities in antigen-presenting cells (10–12).

B27-associated disorders respond dramatically to biological agents targeting TNF-α. Moreover, recent evidence, including therapeutic studies in patients with AS, has strongly implicated a central role for the IL-23/IL-17 cytokine pathway in the pathogenesis of spondyloarthritis (13–18). It is therefore likely that B27 interacts with the TNF-α and/or IL-23/IL-17 pathways in eliciting spondyloarthritis.

Genome-wide association studies based on single nucleotide polymorphisms have revealed well over 100 non-MHC genes or genetic regions that influence susceptibility to AS with odds ratios of up to 2 (19–21). Among these loci are four genes encoding aminopeptidases, ERAP1, ERAP2, LNPEP (insulin-regulated aminopeptidase or placental leucyl/cystinyl aminopeptidase), and NPEPPS (puromycin-sensitive aminopeptidase) (19). The most robust of these aminopeptidase associations is with ERAP1, the primary enzyme that trims peptides within the endoplasmic reticulum to generate optimal ligands for MHC class I molecules (22–26). Peptides degraded in the cytosol are transported into the ER by the transporter associated with antigen processing (TAP), which preferentially transports peptides of ~9–16 residues into the ER (27). Of these, peptides longer than ~9–10 amino acids are trimmed in the ER, predominantly by ERAP1, which efficiently trims peptides from lengths of up to ~16 residues down to lengths of at least 8 residues. ERAP1 trims peptides with hydrophobic C termini more efficiently than those with charged C termini (28). It therefore preferentially leaves behind charged C-terminal peptides as potential HLA ligands. For practical purposes, this means positively charged C termini, because none of the HLA allomorphs binds peptides with negatively charged C termini (29). These properties of ERAP1 have been named the molecular ruler (28). ERAP1 also prefers trimming peptides with basic middle residues (30). The structural basis for these observations can be inferred from the ERAP1 crystal structure (31–33). In humans, ERAP2 also contributes to peptide trimming, but it is independent of peptide substrate size and seems to have less influence than ERAP1 on the final HLA-I peptidome (34–36). Rodents lack Erap2 altogether (34), which makes rodent systems preferred models for the analysis of the effect of ERAP1 on the MHC peptidome, without interference from ERAP2. Erap1 knock-out mice (alternatively called Erap in the mouse) show a dramatic alteration in their MHC-bound peptide repertoires, with partial loss of MHC-I expression, altered antigen presentation, binding of longer than normal peptides, and marked reciprocal cytolytic T cell alloreactivity between Erap1−/− and wild type mice (37–43).

Intense interest has focused on the combined functional significance of HLA-B27 and ERAP1 alleles associated with AS (1, 2, 5, 23, 24). At least six single nucleotide polymorphisms (SNP) of ERAP1 show association with AS (44, 45). The overall association has been reported to be with SNP haplotypes (45) or, in one publication, a combinatorial effect of both haplotypes (24, 46, 47). An association with one ERAP1 SNP was found among the ~80% of AS patients who carry HLA-B27 (44, 48), and also in HLA-B27-negative patients with the much more weakly associated allele HLA-B*40:01, but not with those with any of the other four HLA alleles independently associated with AS (1).

The allelic (SNP) differences in ERAP1 that affect susceptibility to AS show functional differences in aminopeptidase activity (44, 49, 50). This was most comprehensively shown in the context of HLA-B27, in which the peptidome of one B27 subtype (B27:04) was analyzed in cell lines carrying ERAP1 haplotypes showing either increased or decreased susceptibility to AS (36, 49, 51). The protective ERAP1 haplotype showed lower activity and less efficient peptide trimming and was associated with peptide epitopes conferring lower molecular stability to the B27 molecules. The haplotype associated with AS showed more efficient peptide trimming, higher B27 stability, and destruction of potentially antigenic long peptides. However, experiments with pairs of ERAP1 alleles cloned from AS patients and healthy controls and transfected into cells lacking endogenous ERAP1 seemed to show the opposite result, with ERAP1 allelic pairs from AS patients showing insufficient trimming and less surface expression of properly folded B27 molecules (46).

Both the quantity and quality of peptides available in the ER to bind to HLA-B27 influence the biology of B27 (52), and these in turn are strongly affected by the action of ERAP1. Whatever the mechanism of HLA-B27 in the pathogenesis of AS, it is likely that the peptide supply plays an important role. Insight into this process can potentially be obtained through characterization of the peptides bound to HLA-B27 (the peptidome). HLA-bound peptides can be identified after immunoaffinity purification of the HLA molecules, followed by capillary chromatography and tandem mass spectrometry analysis of the recovered peptides (53–56). Indeed, the HLA-B27 peptidome has been characterized in several large-scale studies by recovering the HLA molecules of cultured cell lines, resulting in identification of a large number of peptides of 9 amino acids and longer (36, 49, 57–64).

Here, we report the first determination of the HLA-B27 peptidome formed in vivo, with the analysis of very large B27 peptidomes recovered from the spleens of spondyloarthritides-prone rats transgenic for HLA-B2705 and human (β)-2-microglobulin (β2m) (7). We compared the peptidomes of rats with and without spondyloarthritis, and in addition, we determined
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EXPERIMENTAL PROCEDURES

Transgenic HLA-B27 Rats and Genomic Deletion of Erap1—The transgenic rat lines 21-3, 283-2, 120-4, and Dazl-deficient (Dazl<sup>def</sup>) have been described previously (7, 65, 66) and are summarized in Table I. A zinc finger nuclease (ZFN) reagent (CompoZ<sup>R</sup> Knockout ZFN Kit) purchased from Sigma-Aldrich was used to target rat Erap1, using standard ZFN methodology (67). The binding site for this ZFN construct lies in exon 2 (first coding exon), centered on the AGAGAA 6-base recognition site for the Foil nuclease portion of the construct (supplemental Fig. S1). Fertilized Lewis rat eggs were produced as described previously (68). The ZFN mRNA was microinjected into both the pronuclei and the cytoplasm, and the eggs were then implanted in pseudopregnant Sprague-Dawley females, as described previously (68). Genomic DNA from tail biopsies of the offspring was subjected to PCR amplification of a 326-bp sequence described previously (68). Genomic DNA from tail biopsies of the offspring implanted in pseudopregnant Sprague-Dawley females, as described previously (68). The ZFN mRNA was microinjected into both the pronuclei and the cytoplasm, and the eggs were then implanted in pseudopregnant Sprague-Dawley females, as described previously (68). Genomic DNA from tail biopsies of the offspring was subjected to PCR amplification of a 326-bp sequence described previously (68). Genomic DNA from tail biopsies of the offspring implanted in pseudopregnant Sprague-Dawley females, as described previously (68).

Experimental Design and Statistical Rationale—The transgenic rat lines 21-3, 283-2, 120-4, and Dazl-deficient (Dazl<sup>def</sup>) were used for the HLA peptidomics analysis. As controls for these peptidomes, wild type Lewis rats and transgenic HLA-B<sup>T</sup> rats were used, because these do not express any human HLA or express a completely different HLA (B<sup>T</sup>) whose ligands can be easily distinguished from the B27-bound peptides. The HLA-B27 peptidomes were recovered from the spleens of four animals from each of the respective genotypes and were analyzed separately as biological replicates. Dazl-deficient old male and female rats, both of which were free from the disease phenotype, were used as controls for the Erap1-positive male rats displaying the AS-like phenotype. The Erap1-KO and the DAZL-low and were analyzed separately as biological replicates. Dazl-deficient rats displayed the AS-like phenotype. The Erap1-KO and the DAZL-low and were analyzed separately as biological replicates.

Western Blotting, Flow Cytometry, and Quantitative PCR—These were carried out as described previously (66, 68, 73). Quantitative RT-PCR of rat spleen cDNA was carried out with primer pairs from four regions spanning the rat Erap1 coding sequence (supplemental Table S1).

Monoclonal Antibody (mAb) Production—B1.23.2 is a high affinity mouse IgG2a mAb with specificity for HLA-B and -C and some HLA-A molecules, primarily β2m-associated, which does not react with rat MHC even in the presence of human β2m (74–76). The hybridoma cell line producing B1.23.2 was grown in a 5% CO<sub>2</sub>-humidified incubator, in CELLine CL 350 bioreactors (Sartorius, Gottingen, Germany) containing filtered DMEM supplemented with 10% FBS, 1% L-glutamine, 1% sodium pyruvate, and 100 units/ml penicillin/streptomycin. The conditioned media containing the mAbs were separated from the cell compartment, and the cells were reseeded for another cycle of 9 days. The antibody was purified by affinity chromatography on protein A-Sepharose (Thermo Fisher Scientific, Waltham, MA) columns and eluted with 100 mM acetic acid.

Affinity Purification of the HLA Molecules and Analysis of the Bound Peptides—Rat spleens were resected after sacrifice with CO<sub>2</sub> and immediately frozen in liquid N<sub>2</sub>. The spleens were kept at –80 °C until thawed in lysis buffer. Solubilization was carried out on ice in lysis buffer containing PBS, 0.25% sodium deoxycholate, 0.2 mM iodoacetamide, 1 mM EDTA, 1% octyl-β-D-glucopyranoside, 1:200 protease inhibitor mixture (Sigma-Aldrich, P8340), and 1 mM PMSF, as described in Ref. 77. The splenic extracts were clarified by centrifugation for 60 min at 4 °C and 48,000 × g. The HLA molecules were immunoaffinity-purified using the B1.23.2 mAb covalently linked to AminoLink-agarose resin (Thermo Fisher Scientific) as described previously (78). The HLA molecules with their bound peptides were eluted with 1% TFA, which induces dissociation of the HLA-β2m-peptide complexes. The released peptides were separated from the HLA heavy subunit, the β2m, and from other bound proteins using disposable reversed-phase MicroTip C18 columns (Harvard Apparatus, Holliston, MA) and eluted in 30% and acetonitrile and 0.1% TFA, as described previously (77). The peptides were analyzed by a capillary HPLC on pulled capillaries of 0.075-mm inner diameter and about 20 cm long (79) packed with C<sub>18</sub> reversed-phase 3.5-μm beads (Reprosil-C18-Aqua, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany).

Chromatography was performed with the UltiMate 3000 RSLC nano-capillary UHPLC system (Thermo Fisher Scientific), which was coupled by electrospray to tandem mass spectrometry on Q-Exactive-Plus (Thermo Fisher Scientific) using the same parameters as in Ref. 80. The HLA peptides were eluted with a linear gradient over 2 h from 5 to 28% acetonitrile with 0.1% formic acid at a flow rate of 0.15 μl/min. Data were acquired using a data-dependent “top 10” method, fragmenting the peptides by higher energy collisional dissociation (HCD). The full scan MS spectra were acquired at a resolution of 70,000 at 200 m/z with a target value of 3 × 10<sup>6</sup> ions. Ions were accumulated to AGC target value of 10<sup>6</sup> with maximum injection time of 100 ms. No fragmentation was performed for peptides with unsignd precursor ion charge states or charge states of four and above. The peptide match option was set to Preferred. Normalized collision energy was set to 25%, and MS/MS resolution was 17,500 at 200 m/z. Fragmented masses were dynamically excluded from further selection for fragmentation for 20 s.

Data Analysis—Peptides were identified and quantified using the MaxQuant software tool (70) version 1.5.0.25 with the Andromeda search engine (71) using the rat section of the UniProt/Swiss-Prot database (release 2014_11, containing 27,311 entries). Peptides were identified in the database assuming no specific enzyme proteolysis. Methylene oxidation and N-acetylation were accepted as variable modifications. The peptide precursors and fragment mass tolerances were set at 6 and 20 ppm, respectively. The minimal peptide length was set to eight amino acid residues. The false discovery rate (FDR) was set, separately, for 0.01 or 0.05 for MHC peptides. The rate of identifications were about 21% of the MS-MS spectra for the 5% FDR and about 11% for the 1% FDR. The LC-MS-MS data files of separate sets of peptidomes immunoaffinity purified and analyzed separately by LC-MS-MS were analyzed independently by the MaxQuant software to reduce misidentifications by the “match between runs” sub-routine. MaxQuant quantifies the relative signal intensities of the peptides using their LC-MS peak volumes. Graphical and statistical analysis of the results was performed with Perseus (72). Assignment of HLA scores to the different identified peptides were done by NetMHC, which ranks the peptides according to their fitness to one of the HLA allomorphs, with peptides ranking below 2% relative to 400,000 different peptides in the NetMHC database considered as intermediate affinity ligands of the particular HLA (81, 82). Additionally, peptides were assigned with B27’s scores according to the BIMAS MHC peptide database (www-bimas.cit.nih.gov) (83). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (84) (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD005502.
RESULTS

The rats in this study were F1 offspring of the 21-3 and 283-2 transgenic lines, carrying HLA-B27:05 and human β2-microglobulin (Table I). The female HLA-B27/hβ2m (21-3 × 283-2)F1 rats remain completely healthy. In the males, epididymo-orchitis starts between 1 and 2 months of age, with 100% prevalence, whereas spondyloarthritis first occurs between 3.5 and 7 months of age, with ~70% prevalence (7, 8, 66). A transgene construct that confers reduced expression of the gene Dazl renders males oligospermic, and the (21-3 × 283-2)F1 males carrying the Dazl knock-down transgene (Dazl<sup>−</sup>) do not develop a disease phenotype (66). The 120-4 line transgenic for HLA-B7 and hβ2m (65) and wild type Lewis rats served as controls.

Construction of Knockout Erap1 in HLA-B27 Transgenic Rats by Genomic Mutation—One of 34 viable pups derived from fertilized ova microinjected with the ZFN construct, described under “Experimental Procedures,” was heterozygous for a 2-bp deletion within the targeted AGGAGA sequence of Erap1 (supplemental Fig. S2). This deletion introduced numerous stop codons into the Erap1 coding sequence. A homozygous line was established from this founder. Immunoblotting of splenic extracts demonstrated lack of immunoreactive Erap1 protein in the homozygous mutant and decreased protein level in the heterozygous mutant (Fig. 1A). Quantitative PCR of cDNA derived from splenic mRNA at four sites along the Erap1-coding sequence was 0.157 ± 0.035 in the homozygous knockout and 0.515 ± 0.057 in the heterozygous knockout, as a fraction of the wild type expression (supplemental Table S1). The mutant Erap1 allele was crossed into the 21-3 and 283-2 lines (expressing HLA-B27 and hβ2m), and the offspring were bred to produce (21-3 × 283-2)F1 Erap1 hetero- and homozygous knock-out progeny (i.e. containing 0, 1 or 2 mutant Erap1 alleles). Genotypes were determined by restriction digestion of PCR products of genomic DNA, as shown in supplemental Fig. S2, E and F. Surface expression of folded HLA-B27, human β2m, and HLA-B27 heavy chain dimers in peripheral blood mononuclear cells in (21-3 × 283-2)F1 rats, which were heterozygous or homozygous for the mutant Erap1 allele, was indistinguishable by flow cytometry from that in (21-3 × 283-2)F1 rats with wild type Erap1 (Fig. 1B).

Homozygous Erap1 Deletion Does Not Prevent Development of Spondyloarthritis in the B27/hβ2m Transgenic Rats—As indicated in Tables I and II, male F1 rats of the cross between the 21-3 and 283-2 lines develop spondyloarthritis, with arthritis first appearing typically between 120 and 180 days of age. The (21-3 × 283-2)F1 rats with the three different Erap1 genotypes were observed for a minimum of 6 months, and the phenotypes are summarized in Table II. Both arthritis (Fig. 1C) and spondylitis (Fig. 1, D and E) were observed in rats of all three Erap1 genotypes, and the frequencies, severities, and ages of onset showed no statistically significant differences among the genotypes. The frequency of arthritis in the Erap1 wild type and heterozygous rats was similar to the ~70% frequency that we have previously observed in (21-3 × 2832–2)F1 male rats (7, 8, 66), whereas the frequency of arthritis in the Erap1 knock-out rats was lower. The frequency of tail spondylitis (visible bumps) was somewhat lower in all three groups than we have previously observed. Female (21-3 × 283-2)F1 rats do not develop arthritis or spondylitis (Table I), and none was seen in the females heterozygous or homozygous for the Erap1 mutation. Genomic deletion of functional Erap1 thus had at most a mild impact the basic disease phenotype.

Comprehensive HLA-B27 Peptidomes Identified from Transgenic Rat Spleens—Transgenic rat spleens are good sources for comparative analyses, because large and complex HLA peptidomes could be detected by capillary LC-MS-MS analysis of immunoaffinity-purified B27 molecules from individual spleens (a composite number of 31,304 peptide were identified), including 22,552 peptides of 8–15 amino acids in length that were absent from the non-transgenic Lewis rat and the HLA-B7 transgenic rat samples (Fig. 2 and supplemental Table S3). FDR was set to 5% to increase the numbers of identified true ligands of the HLA-B27, because the use of 1%
FDR resulted in identification of fewer peptides but caused loss of many B27 ligands (Fig. 2 and supplemental Fig. S3). As expected, large fractions of the identified peptides were unlikely to be true ligands of the transgenic HLA-B27 and B7. To filter out these contaminating peptides and ligands of the rats’ MHC, the identified peptides were tagged as ligands of HLA-B27, B7, or the rat MHC based on their established consensus motifs. The mAb (B1.23.2) used for the immunoaffinity

**Fig. 1. Erap1 knock-out rats.** A, Erap1 protein expression in rat splenocytes. Protein extracts of spleens from rats of the indicated genotypes were probed with a rabbit anti-ERAP1 monoclonal antibody (Abcam 124669) and an antibody to GAPDH as a protein-loading control. B, flow cytometry. Peripheral blood mononuclear cells from rats of the indicated genotypes were stained with monoclonal antibodies of the indicated specificities. Folded HLA-B27, B1.23.2; human β2m, BBM.1; HLA-B27 heavy chain homodimers, HD6. C, arthritis in both proximal hind paws in a 151-day-old (21-3 × 283-2)F1 Erap1−/− male rat (arrows). D, normal rat tail vertebral joint. AF, annulus fibrosis; BM, bone marrow; C, cartilage end plate; CT, connective tissue; E, enthesis; LL, longitudinal ligament; NP, nucleus pulposus. E, inflamed tail vertebral joint in a 204-day-old (21-3 × 283-2)F1 Erap1−/− male rat, with asterisks marking inflammation at the sites labeled in D.

| Phenotype of Erap1−/− and Erap1+/− rats compared with wild type 21-3 × 283-2 |
|-------------------------------------------------------|
| Wild type | Erap1 heterozygous KO | Erap1 homozygous KO |
|---|---|---|
| Arthritis prevalence/total | 4/6 | 5/7 | 6/14 |
| Arthritis age onset (days) | 141 ± 10 | 150 ± 40 | 140 ± 23 |
| Arthritis severity (6 maximum) | 4.5 ± 1.0 | 4.3 ± 1.7 | 3.8 ± 1.5 |
| Spondylitis prevalence/total | 1/6 | 2/7 | 3/14 |
| Spondylitis age onset (days) | ND | 170 ± 18 | 185 ± 15 |

All comparisons are p > 0.05. ND, not determined, +/− indicates standard deviation.
purification does not bind efficiently the endogenous rat MHC molecules, and the consensus binding motifs of both the rats’ MHC and the B7 are very different from that of B27. Consequently, 10,839 of the identified peptide sequences conformed to the consensus sequences of HLA-B27 according to NetMHC using a threshold for inclusion of weak binders. NetMHC defines peptides as possible ligands of a particular HLA/MHC allele if they rank below 2% in their affinity of all peptides in the NetMHC database (81, 82). As many as 14,654 of the peptides were defined as B27 ligands with scores larger than 100 according to BIMAS (83). In addition, of the same list of identified peptides, a total of 15,054 peptides were assumed to be possible HLA-B27 ligands, using the criteria of being 8–15 amino acids long, containing Arg or Gln at the P2 position and containing aromatic, long aliphatic, or basic residues at their C terminus (29). Almost all of the peptides meeting these criteria were 9 amino acids or longer, and only very few 8-mers could be observed (supplemental Table S3 and Fig. 3). Some of the peptides contained consensus motifs of peptides that bind rat MHC-I molecules, and these presumed contaminants were easily distinguished because their sequence motifs are very different from those of HLA-B27 and HLA-B7 (29, 85). The B27 peptidomes of the different rat groups were relatively similar in the repertoires of peptides and in their LC-MS intensities (selected examples are shown in Fig. 4, A–C). Pearson correlation coefficients as high as 0.89 were observed between the peptidomes of the different individual rats with identical genetic background (Fig. 4D).

Knockout of Erap1 Affected About One-third of the HLA-B27 Peptidome—As many as 4840 peptides of the 8–15-amino-acid-long peptides differed significantly between the combined genotype groups of wild type and heterozygous Erap1 knock-out rats, with both differing to a similar extent from the Erap1 homozygous knock-out rats (example in Fig. 4B and supplemental Table S3). The peptidomes of the wild type and the Erap1 heterozygote rats (23,745 peptides of the “Erap1 project”) were so similar that they showed interspersed clustering within the hierarchical clustering of the MS intensities of the 8–15-amino acid-long peptides (16,446 peptides, Fig. 5). Thus, even though the levels of Erap1 were about half in the heterozygote rats relative to the wild type rats, the effect of the reduced level of Erap1 on the HLA peptidome was minimal.

Minimal Effect of Heterozygous Erap1 Knockout on the B27 Peptidome—The large HLA peptidomes collected from the rat spleens were very similar in the Erap1 wild type and heterozygous Erap1 knock-out rats, with both differing to a similar extent from the Erap1 homozygous knock-out rats (example in Fig. 4B and supplemental Table S3). The peptidomes of the wild type and the Erap1 heterozygote rats (23,745 peptides of the “Erap1 project”) were so similar that they showed interspersed clustering within the hierarchical clustering of the MS intensities of the 8–15-amino acid-long peptides (16,446 peptides, Fig. 5). Thus, even though the levels of Erap1 were about half in the heterozygote rats relative to the wild type rats, the effect of the reduced level of Erap1 on the HLA peptidome was minimal.

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**FIG. 2.** Scheme of the Erap1 and Dazl peptidome analysis and selection of the likely ligands. The data contain two sets of experiments that were based on separate immunoaffinity purifications of the B27 molecules and analyses of their bound peptidomes by LC-MS-MS (labeled Erap1 and Dazl experiments). The search was performed with 1 or 5% FDR, with about twice as many peptides identified when 5% FDR was used. The datasets of the two experiments were not clustered together to avoid erroneous clustering caused merely by the separate analyses. The numbers of identified peptides after each filtration step are shown. The last filtration step indicates the numbers of peptides conforming to calculated affinity by NetMHC of less than 1000 nM, having a BIMAS score of above 100 or containing Arg or Gln at their P2.
The most dramatic effect of Erap1 was on the lengths of the B27-associated peptides within the subset, the abundance of which was affected by the presence or absence of Erap1. Erap1 deletion affected about a third of the B27 peptidome, and its overall effect on the entire B27 peptidome was relatively modest (Fig. 8A and supplemental Table S3). The presence of Erap1, as either homozygous wild type or heterozygous Erap1 knockout, induced a higher abundance of significantly shorter peptides (Fig. 8B). In addition, longer peptides were observed among the group of B27 peptides that were either more abundantly or uniquely purified from the spleens of the homozygous Erap1 knock-out rats (Figs. 4C and 8B and supplemental Table S3). Many 11–14-amino acid-long peptides were observed in the HLA peptidome of Erap1 knock-out but not in the wild type rats (examples in Fig. 7). A clear change was apparent in the mean lengths of the subset of peptide populations affected by knockout of Erap1, from mostly 9 amino acids in the wild type to 10–12 amino acids in the knockout. These longer peptides were not N-terminally extended peptides of the same core sequences but were mostly unique peptides present only in the knock-out rats. Importantly, hardly any 8-amino-acid peptides were observed in either wild-type or knock-out B27 peptidomes (Figs. 3 and 8 and supplemental Table S3).

Erap1 Modulates the Sequence Motifs of the Subset of Affected Peptides, in Addition to Its Effect on Their Lengths—The majority of B27 peptides was not affected by the knock-out of Erap1, and therefore this knockout induced little modulation in the overall consensus binding motif of B27 in the transgenic rat spleens (Fig. 9, A and B). However, the sequence motifs of the subset HLA peptides that were affected in their levels were clearly different between the Erap1 knock-out and wild type rats (Fig. 9, C and D). The sequence motifs of the HLA-B27-bound peptides most affected by the presence of Erap1 were those longer than 10 amino acids. Although the vast majority of B27-bound peptides contain Arg at P2, this proportion was even higher in the peptides longer than 10 amino acids, displayed by HLA-B27 in the Erap1-knock-out rats (Fig. 9C), compared with those in the rats carrying Erap1 (Fig. 9D). Gly was observed more commonly at the N termini (P1 position) in the peptides up-regulated in the Erap1 wild type and heterozygote rats, relative to the Erap1-knock-out rats, whereas Ala, Ser, and Lys were found more in the peptidomes up-regulated by the knockout of Erap1 (Fig. 9, C and D). In addition, the C termini of the peptides longer than 10 amino acids shifted significantly from Lys and Arg to Tyr, Leu, and Phe in the part of the peptidome up-regulated in the Erap1-knock-out rats (Fig. 9, C and D). According to NetMHC, the B27 peptides with Lys and Arg at their C termini bind B27:05 with somewhat lower affinity than do the peptides of the same length with Tyr, Leu, and Phe at their C termini. Thus, Erap1 reduces to some extent the affinity of the B27-bound 9–12-amino acid-long peptidome (Fig. 10). The absence of Erap1 also caused a significant increase in the presence and abundance of peptides with acidic amino acids (Glu and Asp) in their middle residues (Fig. 9C).

Minimal Influence of Age, Sex, or Disease State on the B27 Peptidome—As shown in Table I, only male HLA-B27/hβ2m (21-3 × 283-2)F1 rats develop spondyloarthritis, whereas the females and the Dazldef males remain healthy. The B27 males first develop epididymo-orchitis before 3 months of age, and arthritis typically does not appear until after 4 months of age. Male (21-3 × 283-2)F1 rats that carry the Dazldef transgene are azospermic, and they do not develop either epididymo-orchitis or spondyloarthritis (66). Comparison of the peptidomes from young and old, male and female, and Dazldef...
Fig. 4. Examples of correlation between signal intensities of the HLA-B27 peptidomes of spleens of rats with different Erap1 genotypes. Similar peptidomes were observed in two wild type Erap1 rats (A) and in wild type and heterozygote rats (B), whereas larger differences were observed between the peptidomes of wild type or heterozygote and the peptidomes of Erap1-knock-out rats (C). Sets of B27 peptidomes collected and processed separately were not analyzed for their correlations because their dissimilarities arose mainly because the separate immunoaffinity purifications and LC-MS-MS analyses were performed separately (the Erap1 experiment is displayed here, and the Dazl experiment is displayed in supplemental Fig. S4). The axes are LC-MS intensities on a log2 scale, and the peptides length is displayed as color with a color scale on the right of each scatter. In this typical example (C), the longer (shared and unique) peptides are more abundant in the Erap1-knockout relative to the wild type Erap1 rats. Peptides detected in only one of the two samples were assigned an arbitrary intensity of 18 (on a log2 scale) and are therefore displayed as vertical or horizontal lines of dots next to the axis. The Pearson correlations between the signal intensities of all the samples included in this analysis are displayed as a color scale in D. The peptidomes collected from similar genotype animals and processed in parallel had high correlations coefficients although the peptidomes from different genotype animals were much lower (D).
and Dazl-wild type (21-3 \times 283-2)F1 rats showed relatively minor differences, compared with the more dramatic effect of Erap1 deletion (Figs. 4D and 2 and supplemental Fig. S4 and supplemental Table S3). Moreover, deletion of Erap1 exerted a similar effect on the B27 peptidomes of rats with and without disease (males versus females and young versus old males). These findings in the groups of healthy rats indicate that the effects on the B27 peptidome were caused by Erap1 or its absence and not by the disease phenotype, nor by age nor sex.

**DISCUSSION**

**HLA-B27 Peptidome in Vivo**—As noted in the Introduction, a large catalogue of HLA-B27-bound peptides has been developed from numerous studies of human cell lines (36, 49, 57–64, 87), including post-translationally modified B27 peptides (87–91). However, to our knowledge, a large in vivo B27 peptidome has not been described nor a comparison of peptidomes between healthy subjects and subjects with spondyloarthritis. Such a study would be difficult to carry out in humans, because of the relatively small amounts of cells that can be obtained from individual subjects. It was therefore of interest for us to use large-scale quantitative mass spectrometry to determine the B27 peptidome in the spleens of spondyloarthritis-prone HLA-B27/h\(\beta\)2m transgenic rats. It was of further interest to assess the effect of changes in the levels of expression and of complete deletion of functional Erap1 protein on both the disease phenotype and the B27 peptidome.

**Erap1 Is Not Necessary for Spondyloarthritis in Rats**—The genetic linkage between ERAP1 and AS has been firmly established in humans (19, 20). Deletion of ERAP1 has been shown to affect the repertoire of peptides bound to B27 in human cell lines (92) and in transgenic mice (93) and to have large-scale effects on the MHC-I peptidome in mice (43). However, HLA-B27 transgenic mice do not develop spondyloarthritis (93, 94), and hence this study is the first to examine the effect of Erap1 deletion both on the B27 peptidome in vivo and on the spondyloarthritis phenotype. The results presented here show unequivocally that the Erap1 homozygous knock-out genotype, which altered approximately one-third of the B27 peptidome, is permissive for the disease phenotype in the B27/h\(\beta\)2m transgenic rats. Although not statistically significantly different, the prevalence and severity of arthritis in the Erap1 knock-out rats were somewhat lower than in rats expressing Erap1, and it is possible that a larger study might uncover subtle effects on disease prevalence, severity, or other features that this study was not powered to find (data presented in Table II).

**HLA-B27 Binds Peptides with Similar Motifs in Humans and Transgenic Rats**—The consensus sequence motif of the HLA-B27 peptidome analyzed in this study conforms closely to the known consensus of the B27:05 peptidome of human cells, consisting predominantly of peptides 9–12 amino acids in length and containing mostly Arg at the second amino acid and aromatic, aliphatic, basic, and hydrophobic amino acids at the C termini (29, 49, 57–64, 95). Higher levels of Lys and Arg have consistently been observed at the C termini of the B27:05 peptides relative to other B27 alleles (61, 95). In this study, the C termini of the peptides have similar levels of Phe, Leu, and Tyr, compared with Lys and Arg (Fig. 9). The observed increase in the level of Lys and Arg in the subset of peptides affected by the presence of Erap1 fits the data of Chang et al. (28), who showed that human Erap1 trims peptides with basic C termini inefficiently thus leaving more of them as available ligands for B27. However, even in this subset, Phe, Leu, and Tyr were present at the C termini of the majority of B27 peptides (Fig. 9).

**Large HLA Peptidomes, Such as Determined in This Study, Include Many Contaminating Peptides, Yet Some of These Are Possibly Non-canonical Ligands**—HLA peptidomes collected from tissue samples include many obviously contaminant peptides. The detection of contaminating peptides in such a large-scale study is partially due to the exquisite sensitivity of modern mass spectrometers as well as to the mild conditions used for purification, which aims to prevent loss of less tightly bound true ligands. Most of the contaminating peptides can...
be distinguished from the true HLA peptide ligands by their clear deviation from the consensus binding motif of the alleles under study and by their absence from the majority of the B27 samples and/or presence in the control samples (in this study, HLA-B7 transgenic or non-transgenic control Lewis rats). Another typical feature of many contaminating peptides is the presence of a series of extended length peptides, many of which are derived from plasma proteins. The source proteins of these peptides co-purify with the HLA during the affinity purification, and the degradation of even a small fraction of them contaminates the HLA peptide pools, resulting in peptides with typically extended-end degradation products. We included the entire unfiltered list of identified peptides in supplemental Table S3, with the likely true HLA-B27 ligands tagged as such, and the likely contaminants left untagged, but we cannot exclude the possibility that some of untagged peptides are in fact true ligands or vice versa.

Distinguishing between potential contaminants and non-canonical true ligand peptides is indeed a daunting task. In this study, from a starting list of 31,304 identifications, the first filtration removed peptides detected either in Lewis or HLA-B7 rat spleens, resulting in 25,836 peptides. The second

**Fig. 6. Heat map showing clustering of the peptides into groups according to their signal intensities.** There are 1712 peptides elevated in the wild type and heterozygous Erap1 knock-out rats and 3128 peptides with elevated levels in the Erap1 knock-out rats.
filtration by size reduced the list to 25,552 peptides of 8–15 amino acids. Further filtrations to peptides that contain Arg or Gin at their second amino acid reduced the list to 15,054 peptides that receive a BIMAS score of above 100 reduced the list to 14,654. It is interesting to note that BIMAS gave B27 scores to significantly more peptides relative to NetMHC, which resulted in a list of likely B27 ligands of only 10,839 (Fig. 2). Thus, about 4000 peptides that are potential ligands of...
HLA-B27 according to BIMAS were excluded from the list by the more stringent filtration of NetMHC (81–83). NetMHC is currently the most effective tool for associating peptides with their presenting allomorph, but it is designed to select peptides more stringently to minimize selection of incorrect ligands, even though it might lose some non-canonical true ligands. The data described in this large-scale peptidome studies and the identification of many peptides that are very likely true B27 ligands but are not currently selected as such by NetMHC may help to expand the capabilities of such software tools to predict HLA ligands that do not conform completely to the canonical sequence rules for any given allele.

The entire list of identified peptides includes important non-canonical peptides that fit only partially the established HLA consensus sequence motif. Many of these non-canonical peptides are potentially more immunogenic, because they are longer than the canonical peptides. They “bulge out” in their middles (96) and provide a larger surface for interaction with the T cells receptors, and therefore they are potentially important members of the peptidome under study (97, 98). Many peptides, longer than 10 amino acids, were defined here as likely B27 ligands, mostly with Arg at the P2 position. Such longer peptides were more prevalent in the absence of Erap1 (Fig. 9). Binding of such B27 peptides is likely possible only by their bulging out in the middle (58, 61, 95, 99) and seems to rely on the anchor residues at P2 (mostly Arg) and at P9 (mostly Tyr, Phe, and Leu and to lesser extent Lys and Arg) (Fig. 9). It was surprising that NetMHC calculated relatively high affinities and good scores to a small group of peptides that either do not contain Arg or Gln at their P2 position or that have Asp or Glu at their C-terminal position (tagged with + in column “Non-canonical B27 peptides” in supplemental Table S3). Evaluation as to whether such non-
FIG. 9. Sequence logo representation of the relative abundance of different amino acids at different positions along the peptide lengths. The sets of logos in A and B display the distribution of amino acids in the sequences of peptides of 8–12 amino acids, affected by the presence or absence of Erap1 (as shown in Fig. 6 and tagged in supplemental Table S2) relative to all the peptides receiving a B27 score in NetMHC below 1000 nM. The sets of logos in C and D display the distribution of amino acids in the peptides up-regulated in the Erap1-KO and in the Erap1-Wt rats.
canonical peptides are true ligands of B27 is outside the scope of this study.

The monoclonal antibody MARB4 that binds to a small minority of surface-expressed HLA-B27 has been shown to identify B27 molecules that are associated with peptides of up to 33 amino acids (62). Subsequent experiments, similar to ones reported here but using MARB4 to isolate B27 molecules and their associated peptides, would be of interest in trying to expand the identified B27 peptidome.

**Erap1 Has Only a Minor Effect in Modulating the Consensus Motif of the Entire HLA-B27 Peptidome, Because It Trims Only a Subset of the Peptides**—The trimming of the peptide pool loaded onto MHC-I molecules by ERAP1 is evident from the data and is well documented in many publications (39, 43, 49, 92, 100–106). One clear observation in this study was the absence of an effect of Erap1 on the majority of peptides presented by B27 (Figs. 7–9). In collaboration with Lopez de Castro and co-workers (102), we have recently reported a similar effect of Erap1 on only subpopulations of the HLA-B51 peptidome in cultured cells. In that study, this differential affinity of Erap1 was attributed to peptides containing either Pro or Ala at the P2 position. Furthermore, a similar finding was recently reported in mice, in which the surface-expressed MHC-Ia peptidomes eluted from bone marrow-derived dendritic cells of wild type or Erap1-knock-out mice showed 74% overlap and therefore suggested that Erap1 (Eraap) plays a role in processing a subset of some, but not all, peptides presented by MHC-I (43). It has previously been suggested that the TAP1/2 heterodimer preferentially transports peptides of 8–16 amino acids and longer, with a preference for hydrophobic C termini (27, 43, 107, 108). If this is correct, it might be asked which enzyme(s) trim(s) the majority of 9–10-amino acid-long peptides that are presented by MHC-I but are not affected in their amounts in the absence of Erap1 in rodents (which lack Eraap2). An alternative possibility is that some or most of the peptides are loaded in cellular compartments from which Erap1 is absent. Such compartmentalization has previously been suggested (109). Consistent with this hypothesis, disease-associated HLA-B27 subtypes are more prone to localize in vesicles within the ER than either the disease-unassociated subtype B2706 or HLA-B7 (110, 111).

**ERAP1 has been shown in many studies to trim peptides to the preferred length of HLA ligands, i.e. 8–9 amino acids (28, 38, 112, 113). Because ERAP1 is more effective in binding and degrading longer peptides, peptides with hydrophobic C termini (28), and peptides with basic middles (114), it increases the availability of shorter peptides with basic C termini and acidic middles for binding to the B27, which still binds these peptides, albeit at lower affinity (Fig. 10). Our results confirm this observation by demonstrating that the presence of Erap1 causes destruction of potential ligands of B27 that are 9 amino acids and longer (Figs. 4C and 7 and [supplemental Table S3](#)). In addition, Erap1 shifts the pool of B27 peptides from hydrophobic to basic C termini (Fig. 9 and [supplemental Table S3](#)), which have lower affinity for B27 according to NetMHC (Fig. 10). Thus, by shifting the C termini to Lys and Arg, which are less favored as B27 ligands, the presence of Erap1 reduces the fitness of both short and long peptides to the B27 motif.

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**Fig. 10. Effect of the last amino acid on the affinity calculated by NetMHC of the HLA B2705 peptides.** Erap1 shifts a subset of the peptidome from hydrophobic to basic C termini, and this way lowers their calculated affinities to B27.
Selectivity of Erap1 Can Be Inferred from the Subset of the Peptidome That It Affects—The large size and presumed comprehensiveness of the peptidomes characterized in this study, in both the presence and absence of Erap1, enable us to better define the putative cleavage site sequence preferences for Erap1. Compared with the Erap1-knock-out peptidome, the N termini within the subset of peptides affected in their levels by the presence of Erap1 showed a relatively high abundance of Gly at P1. This observation suggests that Erap1 is less efficient in trimming peptides that have Gly (or Gly-Arg) at their N terminus, making them available in larger amounts for binding to HLA-B27. In contrast, Erap1 trims Ser, Lys, and Ala very well and thus reduces significantly their presence at the N termini of the B27-associated peptides. Therefore, although HLA-B27 prefers small amino acids, such as Gly, Ser, and Ala in the P1 position (36, 59, 103), Erap1 destroys many peptides with Ser, Ala, and Lys and shifts the part of the affected bound peptide to peptides with Gly at their N termini. These data suggest some modification to the Erap1 preference suggested by Hearn et al. (115), who suggested somewhat less efficient trimming of Ser than Gly (and did not test Ala) or (116) who did not check Gly. The possibility remains to be excluded that these differences are attributable to differences in specificity between rat Erap1 and human Erap1.

Do the More Active Erap1 Allotypes Trim Peptides to Eight Amino Acids and Reduce the Pool of Available Ligands for B27, Which Requires Nine or Longer Amino Acid Peptides?—Only 30 peptides of eight amino acids fitting the B27 motif were observed in any of the isolated B27 peptidomes, only ~0.2% of the total. We and others have previously found a low frequency of octamer peptides in the B27 peptidomes determined from human cell lines (58, 61, 95), and the frequency in this study is even lower than those previously reported. This may indicate that HLA-B27 does not bind 8-mers effectively, regardless of the effect of Erap1. However, in addition to the extent that Erap1 trims potential ligands down to 8 amino acids, it may also reduce the pool of available ligands for binding to HLA-B27, as well as other alleles that have a preference for 9-mer and longer peptides. This could negatively impact the folding and exit from the endoplasmic reticulum of HLA-B27 and alleles that have a preference for 9-mer and longer peptides. There is controversy as to whether the Erap1 alleles associated with AS tend to be stronger or weaker trimmers, and as to whether they are associated with greater or lesser B27 stability (46, 49, 117). However, 8-mers evidently bind poorly to B27, irrespective of whether there is an abundance of them made available by strong Erap1 activity or a dearth of them because of weak Erap1 activity. In either case, the result might be a peptide environment of low stability, either because of overtrimming of many potentially suitable peptides down to 8-mers or of relatively inadequate generation of 9+-mers. In apparent opposition to this hypothesis, one may argue that the shortage of peptides due to overtrimming by Erap1 cannot adequately explain the disease phenotype, because the non-associated subtypes B27:06 and B27:09 also do not bind 8-mers (61). However, these subtypes assemble much faster in the ER than the AS-associated subtypes B27:05, B27:04, and B27:02 (118), so presumably they find a suitable peptidome among the available 9+-mers more efficiently than do the disease-associated subtypes. Therefore, if Erap1 reduces the pool of 9-mers by trimming them down to 8-mers, this could have more of an effect on the slow-assembling disease-associated subtypes.

Age, Sex, and Disease State Have Relatively Little Effect on the B27 Peptidome—By far, the strongest influence on the subset of the B27 peptidomes observed in this study was the presence or absence of functional Erap1. Despite this, the homozygous Erap1 knockout genotype was clearly permissive for the expression of the disease phenotype in the B27/hβ2m transgenic male rats. In striking contrast, among the B27/hβ2m rats that expressed at least one wild type Erap1 allele, there were no major differences in B27 peptidomes regardless of whether the rats had spondyloarthritic or were healthy. Healthy females, Dazl-deficient healthy males, and males with spondyloarthritis of all ages exhibited similar B27 peptidomes in the presence of functional Erap1 (Figs. 4 and supplemental Fig. S4). In both humans and rats, there is a distinct window of age of onset of spondyloarthritis. Moreover, the inflammatory disease itself might be expected to have a pronounced effect on the B27 peptidome in the spleen. We therefore sought to compare the HLA-B27 peptidomes of rats of different ages, in addition to rats with and without established disease, thereby investigating the possibility that a difference in the peptidomes would correlate with the disease phenotype and potentially point to a particular molecular basis for spondyloarthritis. However, only relatively minor differences in the B27 peptidome could be attributed to age, sex, or disease susceptibility. In addition, no statistically significant differences were observed between the peptidomes presented in the spleens of the Erap1 homozygote and heterozygote rats, even though their levels of Erap1 expression differed ~2-fold. This observation seems contrary to the notion previously reported that less or more active ERAP1 variants contribute to disease induction or protection (44, 49, 50, 127). This may indicate that rat Erap1 is sufficiently active, such that, even when present in reduced amounts, it nevertheless trims all potential substrates available for trimming in its compartment.

It should be noted that all of the experiments were carried out on rats with the same HLA-B27 and hβ2m transgenes. Previous evidence suggests that both female and Dazl-deficient males are protected from spondyloarthritic because they do not develop epididymo-orchitis, which appears to be a prerequisite for spondyloarthritis in the (21-3 × 283-2) F1 males (66) and not because of any particular difference in their HLA-B27 biology. Similarly, if a particular aspect of the B27...
peptidome were responsible for the development of spondyloarthritis, it would be expected to be present before the onset of overt disease. Thus, any differences in the B27 peptidome between young pre-spondyloarthritis males and older males that have developed spondyloarthritis might be confined to perturbations from the disease itself. These differences could nonetheless potentially play a role in amplifying or perpetuating disease.

The differences in the B27 peptidomes between disease-susceptible and non-susceptible rats were small, whereas large differences in the B27 peptidomes between Erap1-knock-out and Erap1-sufficient rats had no strong effect on disease susceptibility. These contrasting results suggest that if either qualitative or quantitative differences in peptide binding to B27 can account for the difference between disease susceptibility and non-susceptibility, they will be difficult to identify by the large-scale sequencing approach taken here. A recent comparison in a human cell line of large B27 peptidomes from eight B27 subtypes, including the two subtypes known to have little or no association with AS, similarly failed to show any convincing qualitative difference between the associated and non-associated subtypes (61).

Nonetheless, we cannot completely exclude the possibility that spondyloarthritis is triggered by only one or a few peptides that are either absent or expressed in reduced amounts in non-susceptible versus susceptible individuals, whether in humans or rats. Moreover, the peptidomes studied here were isolated from spleen cells and not, for example, from cells in cartilage, bone, or enthesis, where the disease process takes place, nor from cells in the thymus, where T cell tolerance is induced. We thus cannot completely exclude the possibility that small quantitative or qualitative differences in the B27 peptidome present in a critical tissue at a critical time might be sufficient to trigger spondyloarthritis. Identifying peptides expressed in connective tissue would be technically difficult, even assuming that the appropriate tissue were known, because of the limited amount of HLA-B27 expressed and the potential for contamination from co-isolated B27 from leukocytes. An immunologic approach, in which a T cell readout was available to identify relevant peptides (119), combined with the methodology employed in this study, might have a greater chance of success.

Alternatively, or in addition, disease-relevant HLA-B27-bound peptides might be derived from bacterial products within the intestine. Recent evidence indicates that intestinal dysbiosis is strongly associated with spondyloarthritis, and as alluded to in the Introduction, HLA-B27 itself influences the composition of the intestinal microbiome in both rats and humans (120–122).

Both rat Erap1 and the absence thereof were permissive for spondyloarthritis in the B27/hJ2m rats studied here. It is possible that the pathogenesis of spondyloarthritis in these rats operates relatively independently of Erap1 function. Alternatively, it is conceivable that a different Erap1 allele, whether from rats or from a different species, or even another aminopeptidase, introduced as a transgene, might confer a significant degree of protection from the disease phenotype in the B27/hJ2m rats. Identification of such a protective gene would provide a potential in vivo model system for examining the interaction between B27 and Erap1 in the pathogenesis of spondyloarthritis.

Role of HLA-B27 in the Pathogenesis of Spondyloarthritis—To what degree does this study help confirm or refute any of the existing hypotheses regarding the pathogenetic role B27 or to formulate new ones? With regard to the “surface B27 homodimer hypothesis” (2), surface expression of B27 homodimers by the mAb HD6 in peripheral blood mononuclear cells was similar in rats with or without Erap1 (Fig 1B). Surface homodimer expression was not examined in other cell lineages. However, this limited finding, along with the occurrence of the disease phenotype in the Erap1 knockout rats, would not be inconsistent with the heavy chain homodimer hypothesis. This study did not examine formation of intracellular B27 heavy dimers or oligomers or triggering of the unfolded protein response, and hence it is not informative with regard to the “unfolded protein response” hypothesis (5). As noted above, the “arthritogenic peptide hypothesis” may be the most difficult to confirm by peptide analysis, because it would require identification of one or more specific peptides that correlate with disease onset, either through a peptide-specific immune response or through some other mechanism. For a similar reason, it may be the most difficult to refute through peptide analysis, both because of the sheer numbers of peptides involved and because of the tissue- and age-specific factors cited above. In this study, Erap1 knockout was permissive for the disease phenotype, but it only affected a third of the peptidome, thus leaving over a large number of potentially arthritogenic peptides.

Schittenhelm et al. (63) have recently identified 26 peptides, 9–13 amino acids in length, from a human cell line, which were isolated in higher abundance from AS-associated B27 subtypes than from the two non-associated subtypes, and thus they are considered candidate arthritogenic peptides. Eight of these peptides, ARTTIINEIQY, ARYVFQSENTF, GRFGYLEGQEY, HRAPPiGY, KRFQGPESVAY, QRWDEAFFRK, SRHHTPLSY, and TRYDLYHTF, were also identified in our study. Another peptide from their list, MRTIAALEY, matches the first 10 residues of the 13-mer MRTIAALEYYK isolated in our study (63).

Over 90 of the isolated peptides derived from HLA-B27 itself. Approximately half of these did not fit the B27-binding motif and appeared to be contaminants. The others showed features of authentic B27-bound peptides, including the sequence RRYMLENGKTLQR, which has previously been described as a self-peptide with homology to bacterial sequences (123), and the overlapping peptide LRRYLENGK. The first 9 amino acids of this latter peptide, LRRYLENGK, showed reactivity with B27-restricted cytolytic T cells from AS patients but not healthy B27+ controls (124). Further investi-
gation of these peptides in both humans and rats may help establish whether any of them is involved in the pathogenesis of AS.

Erap1 has also been genetically associated with the other two major HLA class I-associated diseases, psoriasis (125) and Behcet’s disease (126). In the latter case, epistasis was demonstrated between HLA-B51 and Erap1 risk alleles. Epistasis between Erap1 and HLA-B27 was previously shown for AS (44). More recently, weak association between five other HLA-B alleles and AS was identified or confirmed, and one of these, HLA-B*40:01, also showed epistasis with Erap1 (1). When specific residues in the HLA-B heavy chain sequence were analyzed, most of the association with AS could be explained by polymorphism affecting amino acid position 97, which lies at the floor of the peptide-binding groove, in the C/F pocket. Asn at position 97, which is found in the AS-associated HLA-B27 alleles, is highly associated with AS, and Thr, found in HLA-B51, is weakly associated, whereas Ser, found in HLA-B7 and -B8, and Val, found in HLA-B57, are protective (1). The amino acid at position 97 interacts with the side chain of the C-terminal amino acid of the peptide. In this study, the presence of Erap1 is associated with a higher frequency of peptides with basic C-terminal amino acids, whereas Ser, found in HLA-B7 and -B8, and Val, found in HLA-B57, are protective (1). The amino acid at position 97 continues to unfold. Arthritis Rheumatol. 68, 1057–1059

In conclusion, we have described the B27 peptidome from intact rat spleens. We have identified a larger set of peptides bound to HLA-B27:05 than has previously been described, and we have shown that genetic deletion of Erap1 affects approximately one-third of the peptidome with notable effects both on peptide sequence and length, and we have shown that Erap1 deletion is permissive for the disease phenotype. The data do not yet allow us to exclude any of the existing hypotheses for the role of B27 in spondyloarthritis, but further experiments based on these data may help differentiate between mechanisms arising from deficiencies in peptide loading or binding or conformational stability on the one hand, and specific peptides on the other hand.

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