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Cutting Edge

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Cutting Edge: Coding Single Nucleotide Polymorphisms of Endoplasmic Reticulum Aminopeptidase 1 Can Affect Antigenic Peptide Generation In Vitro by Influencing Basic Enzymatic Properties of the Enzyme

Irini Evnouchidou,* Ram P. Kamal,† Sergey S. Seregin,† Yoshikuni Goto,‡ Masafumi Tsujimoto,‡ Akira Hattori,§ Paraskevi V. Voulgari,¶ Alexandros A. Drosos,¶ Andrea Amalfitano,† Ian A. York,† and Efstratios Stratikos*

ER aminopeptidase 1 (ERAP1) customizes antigenic peptide precursors for MHC class I presentation and edits the antigenic peptide repertoire. Coding single nucleotide polymorphisms (SNPs) in ERAP1 were recently linked with predisposition to autoimmune disease, suggesting a link between pathogenesis of autoimmunity and ERAP1-mediated Ag processing. To investigate this possibility, we analyzed the effect that disease-linked SNPs have on Ag processing by ERAP1 in vitro. Michaelis–Menten analysis revealed that the presence of SNPs affects the Michaelis constant and turnover number of the enzyme. Strikingly, specific ERAP1 allele-substrate combinations deviate from standard Michaelis–Menten behavior, demonstrating substrate-inhibition kinetics; to our knowledge, this phenomenon has not been described for this enzyme. Cell-based Ag-presentation analysis was consistent with changes in the substrate inhibition constant K_i, further supporting that ERAP1 allelic composition may affect Ag processing in vivo. We propose that these phenomena should be taken into account when evaluating the possible link between Ag processing and autoimmunity. ***The Journal of Immunology*****, 2011, 186: 000–000.**

Antigenic peptides of the correct length for binding onto MHC class I molecules are generated intracellularly by complex proteolytic pathways. A key last step in this pathway is the trimming of N-terminally extended antigenic peptide precursors by intracellular aminopeptidases (1). The best characterized of these aminopeptidases is endoplasmic reticulum aminopeptidase 1 (ERAP1, also known as ERAAP) (2). ERAP1 trims antigenic peptide precursors down to the correct length of 8–10 aa so that they can bind onto nascent MHC class I molecules (3). ERAP1 is critical for the generation of many antigenic epitopes in vivo and can influence the generation of the antigenic peptide repertoire (4, 5). ERAP1 can also destroy antigenic epitopes by trimming them down to lengths too small for MHC binding (6). This complex landscape of ERAP1-mediated peptide customization has led to the characterization of this enzyme as an antigenic peptide editor (5).

ERAP1 has unusual enzymatic properties that fit with its biological role in Ag processing. It trims long peptides efficiently but spares smaller ones (7). Furthermore, ERAP1 can trim peptides with specificity for the whole length of the substrate, a property that may underlie its complex effects in antigenic peptide selection (8).

Recent genome-wide association studies indicated strong correlations between specific coding ERAP1 single nucleotide polymorphisms (SNPs) and predisposition to the inflammatory disease ankylosing spondylitis (AS) (9, 10). AS has a strong autoimmune component and a hereditary etiology. Ninety-five percent of AS patients carry the HLA-B27 MHC class I allele, and several B27-restricted epitopes have been hypothesized to contribute to the initiation or sustenance of the disease. However, only a small percentage of carriers of the B27 allele develops the disease, suggesting additional genetic and environmental components. The discovery of the link with ERAP1 SNPs suggests that ERAP1 may be one of these missing components (11). The important role of ERAP1 in Ag processing supports the hypothesis that Ag processing may contribute to disease pathogenesis.

*Protein Chemistry Laboratory, National Centre for Scientific Research Demokritos, Agia Paraskevi, Athens 15310, Greece; †Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824; ‡Faculty of Pharmaceutical Sciences, Teikyo-Heisei University, Ichihara, Chiba 290-0193, Japan; §Department of System Chemotherapy and Molecular Sciences, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo, Kyoto 606-8501, Japan; and ¶Rheumatology Clinic, Department of Internal Medicine, Medical School, University of Ioannina, 45110 Ioannina, Greece

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Address correspondence and reprint requests to Dr. Efstratios Stratikos, Protein Chemistry Laboratory, National Centre for Scientific Research Demokritos, Agia Paraskevi, Athens 15310, Greece. E-mail addresses: stratos@rrp.demokritos.gr or stratikos@gmail.com

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Abbreviations used in this article: AS, ankylosing spondylitis; ER, endoplasmic reticulum; ERAP1, endoplasmic reticulum aminopeptidase 1; k_{cat}, turnover number; K_i, inhibition constant; K_M, Michaelis constant; LAMC, L-leucine-7-amido-4-methyl coumarin; MM, Michaelis–Menten; PLC, peptide-loading complex; SNP, single nucleotide polymorphism.

Of the known ERAP1 SNPs, two have been repeatedly confirmed by nearly all population studies to confer strong susceptibility to the disease: rs30187 (K528R) and rs27044 (Q730E). The K528R variation was shown to reduce ERAP1 activity toward a model chromogenic dipeptide (12). However, the effects of these SNPs on the ability of ERAP1 to process antigenic peptides have not been evaluated.

In this study we constructed two ERAP1 variants that carry amino acid variations K528R and Q730E. These two variants, along with the ancestral ERAP1 sequence, define three possible ERAP1 allelic forms present in the human population. We produced the recombinant forms of these three alleles and studied their ability to process antigenic epitope precursors in vitro. We analyzed the trimming of three precursors to HLA-B27–restricted antigenic peptides linked to AS pathogenesis, as well as an unrelated HLA-A03 ligand. We also modified a previously established cell-based Ag-presentation assay to evaluate the effects of ERAP1 alleles in Ag processing in live cells. To our knowledge, this is the first study to demonstrate that ERAP1 allelic composition can alter basic enzymatic properties of the enzyme and affect antigenic peptide generation in vitro. We propose that these findings should be taken into account when interpreting ERAP1-influenced Ag presentation, as well as for the investigation of the possible link between ERAP1-mediated Ag processing and epitope-mediated autoimmunity.

Materials and Methods

ERAP1 variant construction

The ERAP1 variants were constructed on the previously described vector pTracerRFP-ERAP1-HA (6) using the QuikChange II XL site-directed mutagenesis kit from Agilent Technologies (Santa Clara, CA). The primers used were K528R mutation: 5′-GAACACTTGGACACTGCAGA**G**GGGTTT-TCCCCTAA-3′ (sense) and 5′-TTAGGGGAAAACCC**C**TCTG-CAGTG-TCCAAGTGTTC-3′ (antisense) and Q730E mutation: 5′-CGAATGCT-GCGGAGT**G**AA-CTACTACTCCTCG-3′ (sense) and 5′-CGAGGAGTA-GTAGTT**C**ACTCCGCAGCATTCG-3′ (antisense).

Protein expression and purification

The ERAP1 variants were produced using a variation of the 293F mammalian cell expression system described previously (13). The proteins were isolated 3 d posttransfection from the interior of the cells after lysis and were purified by affinity (anti-HA) and size-exclusion chromatography (S200). Batch-to-batch variation in sp. act. was <20%. All comparisons between alleles were made with enzyme preparations performed in parallel.

Enzymatic assays

All enzymatic assays were performed as previously described (8, 13). To ensure reliable measurement of initial trimming rates, reactions were limited to 10 min and were performed at 25°C. Substrate inhibition also was found to apply at 37°C (data not shown). Michaelis–Menten (MM) data were fit to the following equations: $y = k_{cat}^{*}x/(K_M + x)$ for classical MM kinetics and $y = k_{cat}^{*}x/(K_M + x^{*}[1 + x/K_i])$ for substrate-inhibition kinetics, where k_{cat} is the turnover number, K_M is the Michaelis constant, and K_i is the inhibition constant.

Cell-based Ag-presentation assay

A modified protocol of a previously described cell-based Ag-presentation assay was used (6). Briefly, HeLa-Kb-B27/47 cells were transiently transfected with 1 μg each ERAP1 allele-expressing pTracerCMV plasmids (RFP^+) and endoplasmic reticulum (ER) signal sequence/peptide plasmid (GFP^+). Forty-eight hours after transfection, cells were incubated with an HLA-B27 mAb, washed, and incubated with Cy5-tagged anti-mouse IgG Ab (Jackson Immunoresearch, West Grove, PA). GFP^+ cells were identified and analyzed for surface HLA-B27 expression levels by flow cytometry using a BD LSRII flow cytometer and FlowJo software (Tree Star, San Carlos, CA).

Results and Discussion

The recombinant versions of the ERAP1 allelic forms corresponding to the ancestral reference sequence NP_057526 and to the variations K528R and Q730E were overexpressed in 293F cells after transfection and purified by affinity and size-exclusion chromatography (Supplemental Fig. 1). Expression levels of all three variants were comparable, as judged by the yield of the isolation scheme (~0.5 μg purified enzyme/million cells). All three variants migrated as a monomer of ~110 kDa and were enzymatically active toward hydrolysis of the single amino acid substrate L-leucine-7-amido-4-methyl coumarin (LAMC) (Fig. 1, black bars). ERAP1 K528R was found to be ~4-fold less active in this assay, consistent with a previous study (12). All three alleles were able to trim antigenic peptide precursors of the nonamer antigenic peptides ATFPDTLTY (HLA-A03 ligand), KRFEGLTQR, KRVVINKDT, and SRHHAFSFR (HLA-B27 ligands) and to generate the corresponding mature antigenic peptide (Supplemental Fig. 2). We conclude that all alleles have the fundamental ability to generate antigenic peptides in vitro.

Chang et al. (7) reported that small peptides can activate ERAP1 cleavage of LAMC. Although the biological relevance of this property is not known, it has been hypothesized that it may relate to the enzyme's ability to process large peptides (7) (L.J. Stern, unpublished observations). To test whether allelic variation can affect this property, we measured LAMC trimming in the presence of the 8mer peptide SIINFEKL, a poor substrate for ERAP1 (3). In accordance with the previous study, we found that ERAP1-mediated hydrolysis of LAMC is enhanced in the presence of SIINFEKL. Surprisingly, the magnitude of activation was allele specific, with ERAP1 K528R being activated 4-fold, whereas ERAP1 Q730E was activated only 1.6-fold (Fig. 1, gray bars). Substrate activation seemed to ameliorate any differences in sp. act. toward LAMC hydrolysis among the three alleles: ERAP1 K528R was found to be the least active of the three but was activated the most; in contrast, ERAP1 Q730E was the most active and was activated the least. As a result, in the presence of an ERAP1 product (SIINFEKL), all three alleles had comparable trimming activity toward LAMC. Although the mechanistic and biological basis of ERAP1 activation is unknown, these results suggested possible mechanistic differences among the three alleles and demonstrated that LAMC trimming analysis of sp.

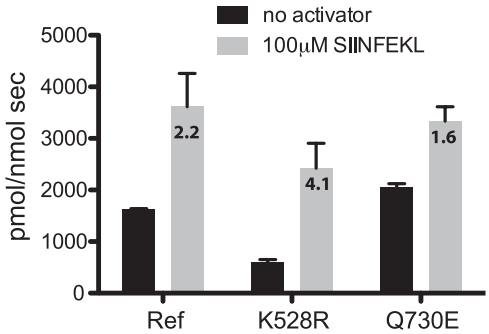

FIGURE 1. Trimming of the model fluorogenic substrate LAMC by ERAP1 alleles and activation by the nonsubstrate peptide SIINFEKL. Black bars, trimming of LAMC by ERAP1 alleles in the absence of activating peptides. Gray bars, trimming of LAMC by ERAP1 alleles in the presence of 100 μM SIINFEKL. Numbers in bars indicate the fold increase in sp. act. due to the presence of activating peptide.

act. may be misleading in interpreting allele-specific enzymatic differences.

To further investigate the effects of polymorphic variation in ERAP1, we analyzed the MM kinetics of peptide trimming (Fig. 2). We used four single-amino acid extended precursors to nonameric antigenic peptides because such precursors were shown to be the predominant species produced by the immunoproteasome (14). The experimental data were fit to a classical MM model (Fig. 2, solid lines) to allow the calculation of the K_M and k_{cat} parameters. Unexpectedly, in several cases the data could not be reliably fit to a classical MM model but could instead be more accurately explained by a more complex substrate-inhibition model (Fig. 2, dashed lines). This phenomenon was substrate and allele specific. For several cases (Fig. 2, *A2*, *B4*, *C3*, *C4*), the deviation from the simple MM model was highly statistically significant ($p < 0.0005$) and resulted in very robust fits for the substrate-inhibition model ($R^2 > 0.95$), allowing the accurate calculation of the substrate-inhibition constant K_i. The parameters calculated from the modeling are shown in Table I. The K_i values indicate significant affinity for the substrate leading to inhibition in some cases, with the stronger inhibition found for the LSRHHAFSFR precursor-ERAP1 Q730E combination for which the K_i was 45 ± 14 μM. It should be noted that this K_i is significantly lower than many substrate K_M values reported in the literature (7, 12).

Regardless of the presence of substrate inhibition, we detected significant changes in the K_M and k_{cat} values among alleles (Table I). These changes suggest possible alterations in the specificity of the enzyme that can lead to significant changes in processing rates, depending on substrate concentration. For example, for low substrate concentrations in which the k_{cat}/K_M is the relevant parameter, allele Q730E processed the LATFPDTLTY precursor 2-fold more slowly than did the other two alleles. In saturating conditions in which the k_{cat} value controls trimming rates, the same precursor was processed twice as quickly by the K528R allele. Similarly, at low substrate concentrations, the YKRFEGLTQR precursor was processed by the Q730E allele 3.5-fold faster than by the K528R allele. Finally, the LSRHHAFSFR epitope was processed 50% faster by the Q730E allele than by the reference allele at low substrate concentrations, but this trend was reversed at high concentrations as a result of substrate inhibition. ERAP1 processing rates were recently linked to antigenic peptide processing inside cells, and changes in these rates may influence the antigenic peptide repertoire (15).

To evaluate whether such changes in enzymatic properties are sufficient to affect Ag presentation in a cellular context, we modified a previously established cell-based Ag-presentation assay to account for ERAP1 allelic composition (6). We transfected HeLa-Kb-B27/47 cells with the ERAP1 alleles, along with a mini-gene that targets the peptide precursor ASRHHAFSFR to the ER. By following HLA-B27 surface presentation, we found that the K528R allele resulted in reduced surface presentation relative to the reference allele (Fig. 3). Presentation was further reduced with the Q730E allele. Although the magnitude of alterations in surface presentation was modest, this may be due to the presence of endogenous ERAP1 that reduces the effect of the transfected ERAP1 allele. Regardless of this limitation, allele-dependent effects were clearly visible. Furthermore, these results are consistent with the magnitude of K_i measured for these alleles (Table I), suggesting that allele-dependent substrate inhibition may be

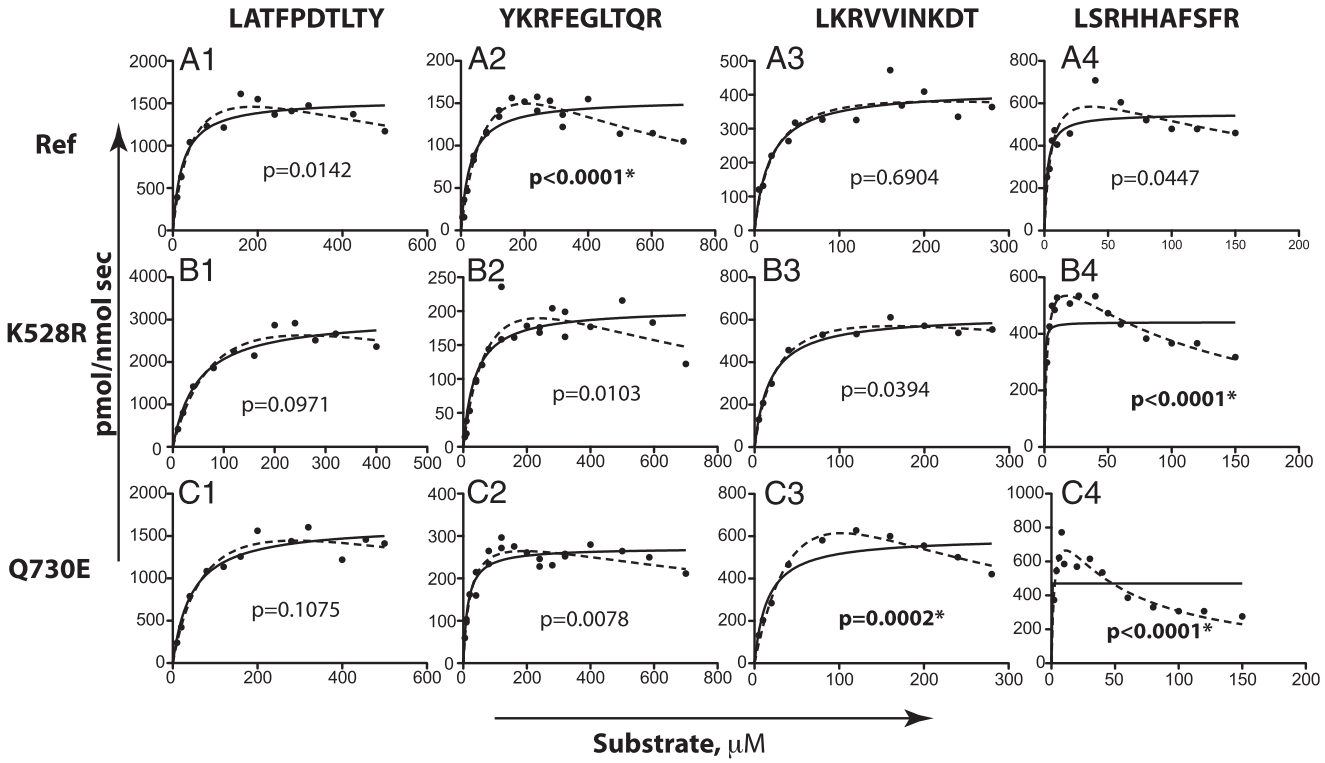

FIGURE 2. MM plots for the trimming of four antigenic peptide precursors (columns) by three ERAP1 allelic forms (rows). The data were fit to a standard MM model (solid line) and to a substrate-inhibition model (dashed line). The Student *t* test *p* value between the two fits is indicated within each plot. Smaller *p* values (*$p < 0.005$) indicate a very high probability that the substrate-inhibition model is the appropriate one for data fitting.

Table I. Kinetic parameters derived from fitting the MM kinetics data using the most appropriate model

Substrate	Allele	K_M (μM)	k_{cat} (s^{-1})	$k_{cat}/K_M \times 10^3$ (s^{-1} M^{-1})	K_i (μM)
LATFPDTLTY	Ref	23 ± 7	1.54 ± 0.09	67 ± 21	—
	528R	49 ± 13	3.09 ± 0.203	63 ± 17	—
	730E	45 ± 11	1.635 ± 0.10	36 ± 9	—
YKRFEGLTQR	Ref	30 ± 5[a]	0.304 ± 0.052[a]	10 ± 2[a]	394 ± 133[a]
	528R	37 ± 12	0.204 ± 0.015	5.5 ± 18	—
	730E	14 ± 3	0.272 ± 0.01	19 ± 4	—
LKRVVINKDT	Ref	18 ± 6	0.414 ± 0.029	23 ± 7	—
	528R	18 ± 3	0.622 ± 0.019	35 ± 6	—
	730E	80 ± 29[a]	1.57 ± 0.40[a]	20 ± 8[a]	132 ± 53[a]
LSRHHAFSFR	Ref	2.4 ± 0.9	0.550 ± 0.035	230 ± 80	—
	528R	2.3 ± 0.4[a]	0.679 ± 0.037[a]	300 ± 55[a]	127 ± 19[a]
	730E	2.8 ± 1.3[a]	0.997 ± 0.170[a]	360 ± 16[a]	45 ± 14[a]

Parameters were generated using the substrate-inhibition model only in the cases in which the data deviated from the classic MM model in a statistically significant fashion ($p < 0.005$).

[a]Derived from a substrate-inhibition model.

—, The K_i parameter is not applicable for data fit to a classical MM model.

an important parameter for regulating ERAP1-restricted presentation in vivo.

Two recently solved crystallographic structures of ERAP1 (PDB codes 2XDT and 3MDJ) allow the accurate mapping of these polymorphisms and provide hints toward understanding the structural basis behind these effects (Supplemental Fig. 3). In these two structures, ERAP1 is in a different conformation, leading to the exposure or occlusion of a postulated peptide-binding cavity to the solvent. This conformational change is hypothesized to be an important component of the ERAP1-trimming mechanism (L.J. Stern, personal communication). Both SNPs are >27 Å away from the catalytic site, so it is unlikely that they can affect catalysis directly. However, position 730 lies inside the cavity and could potentially make contact with the C-terminal moiety of substrates, affecting specificity. In contrast, position 528 lies on the outside of the cavity, in an interdomain junction that may affect conversion from one conformation to the other. Furthermore, the structural basis for the substrate-inhibition kinetics that we report in this article can be theorized based on the elongated peptide-binding site in ERAP1 (7, 8). Substrate inhibition can be the result of nonproductive binding of the substrate that competes with normal productive binding. In the case of ERAP1, a peptide substrate can potentially bind on its extended binding site, with its N terminus poised directly on the catalytic site for cleavage or possibly in a more distal, nonproductive orientation. Such a nonproductive orientation would be expected to preclude productive binding of another peptide. The peptide–substrate sequence will influence peptide–enzyme interactions, making this phenomenon more or less potent (larger or smaller K_i).

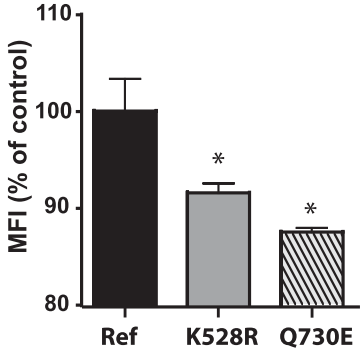

FIGURE 3. ERAP alleles differentially mediate Ag presentation in the HeLa_Kb-HLA-B27/ICP47 cell line. HeLa cells, stably expressing HLA-B27, as well as TAP1 ER-transporter blocker (ICP47), were transfected with HLA-B27–specific peptide precursor (ASRHHAFSFR) and one of three different ERAP alleles: Ref K528R, or Q730E. Surface expression of HLA-B27 was measured by flow cytometry (ME1 mAb). *$p < 0.05$.

A critical question is whether these fundamental enzymatic properties translate into changes in peptide processing in vivo. A critical parameter for evaluating the relevance of an MM analysis to in vivo function is the active concentration of substrate in the same cellular compartment as the enzyme. For substrate concentrations below the K_M, the k_{cat}/K_M ratio becomes the dominant parameter, whereas for substrate concentrations >2-fold the K_M, the k_{cat} value determines specificity. High substrate concentrations also make substrate inhibition important in determining trimming rates. Our cell-based Ag-presentation analysis results can be accounted for by the allele-specific effects on substrate inhibition, suggesting that, at least in some cases, ERAP1 may be operating under saturation kinetics inside the cell. Recently, ERAP1 was reported to be part of the peptide-loading complex (PLC) (meeting presentation, Dr. P.M. Kloetzel, 6th International Antigen Processing and Presentation Workshop, Cargese, Corsica, France, March 31, 2010). The macromolecular nature of the PLC may lead to local concentration phenomena, so that even small amounts of specific precursors may be at very high local concentrations in the vicinity of the PLC, causing the enzyme to operate at saturation levels and to be affected by substrate inhibition.

Substrate inhibition has been described for several enzymes. Some examples include tyrosine hydroxylase, acetylcholinesterase, phosphofructokinase, and DNA methyltransferase (reviewed in Ref. 16). It was proposed that substrate inhibition can serve as a regulatory mechanism to limit enzymatic turnover when large amounts of substrate are available and, therefore, regulate product production. Possible downregulation of ERAP1 function by increased amounts of high-affinity epitope precursors is an interesting and provocative

hypothesis, especially in view of the allele-specific effects described in this article. Carefully designed in vivo experiments that can account for antigenic peptide precursor delivery into the ER are necessary to test this hypothesis and evaluate substrate inhibition as a potential mechanism for ERAP1 activity regulation.

The genetic association between ERAP1 SNPs and AS provides a unique opportunity to gain novel insights into the mechanisms behind autoimmunity. In this study, we focused on the hypothesis that this link lies in the Ag-processing properties of ERAP1 that have recently attracted a significant amount of attention in the field. Although a distinct property of ERAP1 (or ARTS-1) in shedding the ectodomain of TNFR1 provides a separate possible link to AS pathogenesis, we did not analyze this property in the current study because it does not seem to be dependent on ERAP1's enzymatic activity, and it was recently demonstrated that ERAP1 SNPs do not affect serum cytokine receptor levels in AS patients (17, 18).

In conclusion, we demonstrated that ERAP1 epitope-processing activity can be modulated by allelic variants associated with altered susceptibility to AS. This finding supports the hypothesis that the pathogenesis of AS involves HLA-B27–mediated presentation of specific peptides (protective or pathogenic) and that risk for disease is associated with the abundance of such peptides. Altering the Ag-presentation pathway by changing ERAP1 activity would, in turn, alter the abundance of such peptides and would increase or reduce the risk for disease.

Disclosures

The authors have no financial conflicts of interest.

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