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The Behçet's disease risk variant HLA-B51/ ERAP1-Hap10 alters human CD8 T cell immunity

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Abstract

Objectives: The *ERAP1* haplotype *Hap10* encodes for a variant allotype of the ER-resident peptide-trimming aminopeptidase ERAP1 with low enzymatic activity. This haplotype recessively confers the highest risk for Behçet's diseases (BD) currently known, but only in carriers of *HLA-B*51*, the classical risk factor for the disease. The mechanistic implications and biological consequences of this epistatic relationship are unknown. Here, we aimed to determine its biological relevance and functional impact.

Methods: We genotyped and immune-phenotyped a cohort of 26 untreated Turkish BD subjects and 22 healthy donors (HD), generated CRISPR-Cas9 ERAP1 KOs from HLA-B51+ LCL, analyzed the HLA I-bound peptidome for peptide length differences, and assessed immunogenicity of genome-edited cells in CD8 T cell co-culture systems.

Results: Allele frequencies of *ERAP1-Hap10* were similar to previous studies. There were frequency shifts in between antigen-experienced and naïve CD8 T cell populations of carriers and non-carriers of *ERAP1-Hap10* in an *HLA-B*51* background. *ERAP1* KO cells showed peptidomes with longer peptides above 9mer and significant differences in their ability to stimulate alloreactive CD8 T cells compared with wild-type control cells.

Conclusions: We demonstrate that hypoactive ERAP1 changes immunogenicity to CD8 T cells, mediated by an HLA class I peptidome with under-trimmed peptides. Naïve/effector CD8 T cell shifts in affected carriers provide evidence of the biological relevance of ERAP1-Hap10/HLA-B51 at the cellular level and point to an *HLA-B*51*-restricted process. Our findings suggest that variant ERAP1-Hap10 partakes in BD pathogenesis by generating HLA-B51-restricted peptides, causing a change in immunodominance of the ensuing CD8 T cell response.

Keywords

ERAP1; HLA class I; Behçet's disease; HLA-B51; immunogenicity; T cells; Immunodominance; CD8

INTRODUCTION

Over the past decade, high-quality genome-wide association studies (GWAS) have identified several genes with potential impact on our understanding of mechanisms driving Behçet's disease (BD). These studies confirmed *HLA-B*51* as a major risk factor.[1, 2] In addition, they revealed other risk-conferring loci within and outside of the *HLA* region.[2, 3] Most of the latter, however, are shared with recurrent aphthous stomatitis (RAS), a common disease worldwide.[4] While RAS – which consists of oral ulcers only – is almost universally present in Behçet's disease, BD phenotypes are far more complex and must include additional manifestations such as skin lesions, genital ulcers, uveitis, and pathergy for the disease to be diagnosed.[5] Vision-threatening uveitis occurs in more than half of the

subjects with BD, and the disease can have significant morbidity and mortality through CNS and large vessel involvement, signifying a sharp contrast between RAS and BD in terms of its nature, severity, and burden.[6–8] This has dampened enthusiasm for some of the shared hits on BD and RAS GWAS as potential mechanistic research targets for BD. A notable exception to this relative lack of specificity is the discovery of epistasis between *HLA-B*51* and the *ERAP1-Hap10* variant of *ERAP1:* both gene variants together profoundly increase the risk for BD (11-fold) over that conferred by *HLA-B*51* alone (about 4-fold) in an interdependent relationship not shared with RAS.[9, 10]

ERAP1 encodes for endoplasmic reticulum aminopeptidase 1 (ERAP1) an ER-resident enzyme that trims peptides to a length of around 9 amino acids (9mer) which is ideal for loading into the tight binding groove of HLA class I molecules, such as HLA-B51. [11–13] These peptide-HLA complexes (p-HLA) then translocate to the cell surface where they are recognized by cognate T cell receptors (TCR) on CD8+ T lymphocytes for immune surveillance, which may or may not result in an immune-response, depending on the p-HLA complex seen by a specific TCR. Peptides presented via this “cytosolic”, or “HLA class I” pathway are either derived from intracellular proteins or are extracellular antigens introduced through intracellular infections, or are cross-presented by dendritic cells. It is apparent, that the risk-associated interdependence of two polymorphisms in genes of the HLA class I antigen presentation pathway strongly suggests its mechanistic relevance to BD. To date, however, it has remained unclear how this risk is mediated immunologically. In fact, any evidence for its possible impact on cellular immune-phenotypes or function is lacking. This has hampered the mechanistic understanding of BD pathogenesis in subjects affected by the variant. In a larger context, it has prevented progress in our understanding of possible mechanistic contributions of HLA-B51 to BD pathogenesis which has remained an enigma for almost 50 years.

To tackle this problem, and under consideration of knowledge gained from previous work which determined a low, almost absent, enzymatic activity of ERAP1-Hap10,[14–17] we hypothesized that a hypoactive ERAP1 leads to the loading of “under-trimmed”, longer peptides onto HLA class I resulting in an aberrant CD8 T cell response that partakes in driving the disease.

To address this question, we genotyped a cohort of untreated, active BD patients and healthy subjects, assessed potentially variant-dependent immune phenotypes in those subjects, and created an in vitro model system using CRISPR-Cas9 genome editing, which allowed us to determine the effect of low ERAP1 activity on peptide lengths in the HLA class I peptidome and its effects on immunogenicity as assessed through CD8 T cell effector function. The work presented here represents the results of our attempts to generate early evidence for the immunological significance of ERAP1-Hap10/HLA-B51 epistasis as a potentially disease-driving factor in BD.

METHODS

Recruitment of study subjects

28 diseased and 22 presumably healthy, age and sex-matched subjects were recruited at the Behçet's Disease Research Center at Istanbul University - Cerrahpasa in Istanbul, Turkey. All subjects fulfilled traditional ISG criteria for the diagnosis of BD, had active disease, and were without immunosuppressive treatment for at least 3 months prior to inclusion. Recruitment of these patients was random, but within "severe" BD phenotypes, *i.e.*, ocular and major vascular BD, to minimize diagnostic ambiguity. Two subjects were excluded from sample processing and analysis; one subject because of a BD incompatible ocular phenotype determined by uveitis ophthalmology, and another subject because of incomplete clinical information. Demographic features of the study populations are provided in Sup. Table 1. Patients or the public were not involved in the design, conduct, reporting, or dissemination plans for this research. The study was approved by the IRB Board of Istanbul University – Cerrahpasa and informed consent obtained from all participants.

Biological specimens

Peripheral blood obtained through venipuncture was processed for peripheral blood mononuclear cells (PBMC) through Ficoll gradient centrifugation as described,[18] cryopreserved,[19] and stored in liquid nitrogen until transport in N2 dewars to the US for subsequent cryostorage and experiments.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted per manufacturer's instructions (Qiagen Kit) from thawed PBMCs. ERAP1 exons (2/5/6/11/12/15) containing the haplotype defining missense SNPs previously described [10] and HLA-B exons (2/3) were amplified, and single-band products of the correct size, identified through agarose gel electrophoresis, sequenced by Psomagen (Rockville, Maryland). Gene sequence alignment and SNP analysis were done using gene blast (NCBI) and DNASTAR Lasergene 16 software. Forward and reverse sequences were aligned to test for concordance in exon sequence and all SNPs. Comparison with the previously published missense SNPs was done to predict coding haplotypes and zygosity. *HLA class I* typing was available from clinical care or obtained through anti-HLA B5 staining with confirmation through Sanger sequencing as described above. Additional information is provided in Sup. Table 2 and Sup. Fig. 1.

Flow cytometry

Flow cytometry was performed on thawed PBMC, cultured immortalized lymphoblastoid cell lines (LCL), or sorted CD8 responder T cells using our standard published protocols.[20] Cells were acquired using an LSR II.UV cytometer.

Genome editing and culture of cell lines

The LCL line GM23090 (Coriell), which carries *HLA-B*51*, *ERAP1Hap2/Hap7*, was edited using CRISPR-Cas9 exploiting the non-homologous end joining (NHEJ) pathway in a 2-step lentiviral transduction approach as described.[21] Briefly, Cas9 expressing HLA-B51+

LCL were generated through lentiviral transduction, cloned in limiting dilution, and then functionally vetted for stable Cas9 expression using the pXPR_011 GFP lentiviral system.[21] Clones with high Cas9 activity were selected for additional lentiviral transduction with a gRNA targeting *ERAP1* (KO condition) at exon 2, or non-sensical gRNA (WT control condition). Complete, previously published *HLA* typing results for LCL GM23090 as well as genotyping results for *ERAP1*, generated as described above, can be found in Sup. Tab. 3.[22]

Immunoprecipitation and mass spectrometry

LCL (at least 100×10^6/ LCL condition) were harvested and washed x 3, lysates were generated and immunoprecipitation was performed using anti-HLA-ABC antibody (clone W6/32, Biolegend) ligated to Dynabeads. HLA class I-associated peptides (HAPs) were eluted using 50mM Glycine at pH 2.8. For mass spectrometry, the peptide mixture was desalted on C18 SepPak columns and aliquots were loaded onto an EASY-Spray analytical column coupled to a Thermo Fisher Scientific Orbitrap Fusion Lumos Mass Spectrometer. The mass spectrometry raw data were deposited in MassIVE,[23] searched against the human reference proteome obtained from the human protein database Uniprot (June 2017), supplemented with a list of common contaminant proteins, using the search engine Byonic (v2.13.2). Peptide lengths were plotted. HLA-B51:01 binders were determined computationally using HLArestrictor with NetMHCpan V.2.4 applying a 2% rank and an IC50 of 500 for weak, and a 0.5% rank with an IC50 of 50 for strong binders. HLA-B51:01 binders selected by peptide length of the non-overlapping KO peptidome were subjected to microbial homology analyses as described by Luzka et al and in Sup. Tab 4.[24]

CD8 T cell purification, LCL cell irradiation and cell stimulation assays

Human CD8 T cells were isolated from cryopreserved PBMC through magnetic sorting (human CD8 isolation kit, STEMCELL, Catalog #17953) according to the manufacturer's instructions. LCL were co-cultured with allogeneic CD8 T cells. In long-term stimulation assays, LCL were irradiated to prevent outgrowth of T cells in the culture.

Intracellular Cytokine Staining and Degranulation Assay

Intracellular staining for IFN-gamma, granzyme B, and perforin was performed following surface staining for CD3 and CD8, fixation in 4% PFA and cell permeabilization with brefeldin as described.[25] Degranulation assays were performed separately, using a CD107a-APC antibody (BD Biosciences, catalogue# 560664, clone H4A3) at the manufacturer-recommended concentration, which was incubated with the cells over the entire stimulation period. After one hour of stimulation, monensin (BD Biosciences, catalogue # 554724) was added and cells incubated for another four hours as described, harvested, stained with titrated viability dye (fixable blue), CD3, CD8 antibodies and acquired using an LSRII.UV cytometer.[26]

ELISA

LCL/ CD8 cell coculture supernatants were harvested and IFN-gamma concentration was assessed with ELISA (Biolegend, Catalogue# 430107) as per manufacturer's instructions.

Raw data processing, computation and statistics

Compensation for flow cytometry experiments was performed at the time of sample acquisition on an LSR II using standard BD acquisition software. For targeted analyses, FCS files were imported into FlowJo and analyzed through gating on pertinent lineage markers (CD3, CD8) in bivariate plots followed by gating over fluorescence minus one (FMO) controls for the quantification of antigens expressed on a spectrum. Results for stimulated over unstimulated conditions were analyzed using Prism software after calculating the mean of intrinsic triplicates for each condition with t- and Mann-Whitney tests. A minimum of three independent experiments was performed for each readout. For unbiased analyses, FCS files were loaded into Cytobank and subjected to the CITRUS algorithm under application of a PAM model with an FDR 0.05 and visualized in CITRUS dimension reduction plots identifying differentially expressed cell populations classifying samples of the sample groups subjected to the analysis.

RESULTS

Genotypic profiles of diseased and healthy study subjects

In order to determine the allele frequencies of polymorphisms forming the *ERAP1-Hap10* haplotype in individual subjects of our cohort, we amplified the corresponding exons of genomic DNA at the *ERAP1* locus. Sanger sequencing of the amplified products revealed a frequency of *ERAP1-Hap10* $^{+/+}$ (homozygous) carriers (Fig. 1) in all subjects of 6.25% (3/48). 31.25% (15/48) were either heterozygous or homozygous for *ERAP1-Hap10*. Within the group of BD subjects, the frequency of heterozygotes was 30.77% (8/26) and 7.70% (2/26) for homozygous carriers of *ERAP1-Hap10*. BD subjects who carried *HLA-B*51* (73%) were heterozygous in 31.58% (6/19) and homozygous for *ERAP1–Hap10* in 5.26% (1/19) of cases.

A large (GWAS) of 1876 Turkish BD cases and 1761 controls had previously determined the frequency of homozygous *HLA-B*51* BD carriers of *ERAP1-Hap10* at 4.9% through imputation.[10] This is in line with our data (5.26%) obtained through direct Sanger sequencing of human genomic DNA. Our findings, therefore, confirm the relatively low frequency of homozygous carriers of *ERAP1-Hap10* in an *HLA-B*51* background in Turkish subjects with BD. They also indicate that heterozygotes are far more common.

A specific, highly differentiated CD8 T effector cell population distinguishes carriers of *ERAP1-Hap10* from non-carriers in an *HLA-B*51* background

To examine if and, if so, how the *ERAP1-Hap10* genotype affects the immune phenotypes of its carriers in an *HLA-B*51* background within the human T cell compartment, we employed an 11-color staining panel containing major T cell lineage and activation markers on genotyped PBMCs collected from the subjects of the cohort. Unbiased sample classification analysis of $CD45^+$ $CD3^+$ gated T cells using the cluster identification, characterization and regression (CITRUS) algorithm under application of a partition around medoids (PAM) clustering model determined $CD8^+CD57^+CD28^-CCR7^-$ cells as a major, significant discriminator between samples from carriers versus non-carriers of *ERAP1-Hap10* in *HLA-B*51*$^+$ subjects (Fig. 2 A–C). As $CD8^+CD57^+CD28^-CCR7^-$ cells represent

a matured, highly differentiated, typically oligoclonally expanded, cytotoxic phenotype of CD8 T cells,[27, 28] this finding strongly suggests a link of the risk genotype with oligoclonal expansions of HLA-I restricted human $CD8^+$ T cells that have effector function.

ERAP1-Hap10 shifts frequencies of antigen-experienced vs naïve CD8 T cell populations in carriers of *HLA-B*51*

Next, given the result of the CITRUS analysis for a specific cell population, we aimed to determine whether the carrier status of *ERAP1-Hap10* in *HLA-B*51*$^+$ subjects alters cell frequencies of antigen-experienced (memory, effector-memory, and TEMRA) vs antigen-inexperienced (naïve) CD8 T cells in general. We performed targeted flow cytometric analyses using CD3 (T cell), CD8 (CD8 T cell), CD45RA vs CCR7 and/ or CD27 vs CD28 (naïve vs effector/effector memory) to address this question. There were significant, reciprocal shifts in the frequencies of naïve vs effector-memory cells between *ERAP1-Hap10* carriers and non-carriers that were *HLA-B*51*$^+$ (Fig. 3 A). Effect sizes were large. For carriers and non-carriers of *ERAP1-Hap10* amongst *HLA-B*51*$^+$ subjects with BD, there were significant changes in frequencies of effector-memory $CD8^+$ cells with large effect sizes (Fig. 3 B). Changes in frequencies of naïve CD8 T cells in this group did not reach statistical significance, but still had moderate effect sizes and identical direction of effect as in the larger group comparisons for BD and healthy subjects combined, therefore likely representing an underpowered comparison.

When comparing subjects with BD vs HD in an *HLA-B*51* background but with *ERAP1 Hap 10* expression in BD and in the absence thereof in HD, inverse changes of differential expression of CD28 and CD27 in the CD8 T cell compartment in between these two groups were significant despite low n, and had very large effect sizes (Fig. 3 C). BD subjects who were carriers of the risk genotype also clearly had significantly different fractions of CD57 expressing CD8 T cell frequencies when compared to HLA-$B51^+$ healthy subjects (Fig. 3 D).

An important recent study experimentally assessed trimming activities for the ten most common ERAP1 allotypes, allowing trimming activity estimates for ERAP1 allotypes encoded by homozygous and compound heterozygous carriers of most *ERAP1* haplotypes. [15] Applying these estimates, we performed a data simulation excluding subjects whose trimming activity trended towards the mid-range, which increased effect sizes further (Sup. Fig. 3).

Combined, these results strongly suggest that the ERAP1-Hap10 allotype in the presence of HLA-B51 globally alters frequencies of antigen-experienced versus naïve CD8 T cells in the peripheral blood, likely reflecting significant migration of activated CD8+ T cells to inflamed tissues. Importantly, it also suggests that HLA-peptide recognition by cognate CD8 T cell receptors (TCR) may be controlled and modulated by allotypic ERAP1 in HLA-B51 restriction. The global modulation of naive/memory CD8+ T cell frequencies may reflect the fact that the hypomorphic *ERAP1-Hap10* has a global effect on peptide trimming and antigen presentation, thereby profoundly altering T cell receptor repertoire and T cell activation.

Loss of ERAP1 function results in longer peptides above 9mer

In order to assess for consequences of loss of ERAP1 function mechanistically, we knocked out *ERAP1* using a CRISPR-Cas9 approach in an LCL line derived from an *HLA-B*51*$^{+}$ human carrier (Sup. Table 3). As ERAP1-Hap10 has been shown to possess low enzymatic activity that resembles that of a functional KO,[14, 15] this represents a human in vitro model system that approximates ERAP1 activity in the risk variant. Following our hypothesis that the low enzymatic activity of ERAP1-Hap10 alters the HLA-B51-bound peptidome with a propensity for longer, *i.e.*, less efficiently trimmed peptides, we immunoprecipitated HLA I-peptide complexes from isogenic, HLA-B51^{+} ERAP1 competent (WT control) and ERAP1 KO LCL, performed peptide sequence identification by mass spectrometry, and compared peptide length frequencies of the respective HLA-class I peptidomes. Peptidomes contained overlapping and non-overlapping fractions in between WT and KO (Fig. 4 A). Peptide length frequencies (PLF) differed significantly (Fig. 4 B) between WT and KO. PLF of the KO peptidome peaked at 9mer increasing relatively and disproportionally to the KO up until 9mer, but then inverted with longer peptides becoming relatively more abundant in the KO than in the WT (Fig. 4 B). 9mer is the ideal peptide length for fit into the tight binding groove of most HLA class I molecules. As ERAP1 trims to around 9mer, our results indicate that absent ERAP1 impairs this process and leads to the loading of longer peptides onto HLA class I molecules.

To assess the relevance of this mechanism for HLA-B51 binding specifically, we computationally deconvoluted the non-overlapping KO (“under-trimmed”) and WT (“properly trimmed”) for predicted HLA-B51:01 binders and then re-analyzed peptide frequencies (Fig. 4 C). Again, WT control PLF was relatively outperformed by peptides derived from the KO above 9 mer, indicating that peptide under-trimming in the absence of ERAP1 leads to the loading of elongated peptides onto HLA-B51 and that dysfunctional ERAP1 alters the HLA-B51 bound peptidome. Therefore, low or absent ERAP1 activity has a pronounced effect on the length of HLA-presented peptides, likely affecting T cell recognition and modifying the antigenicity of selected peptides.

To determine whether long peptides generated in the absence of ERAP1 activity may resemble human pathogen sequences, we subjected HLA-B51:01 deconvoluted 10 and 11-mer peptides from the non-overlapping KO peptidome to a microbial homology analysis. [24] 14 out of 22 peptides displayed degrees of homology to linear microbial epitopes that had previously been determined to be immunogenic experimentally (Sup. Fig. 4).

Loss of ERAP1 function modulates CD8-mediated immunogenicity

Finally, we strived to assess whether the absence of functional ERAP1 in antigen-presenting cells would alter CD8 immune responses, given that changes in the HLA-class I bound peptidome are likely to change recognition by cognate TCR, *i.e.*, change immune-dominance and immunogenicity. To this end, we co-cultured ERAP1 KO and WT LCL with allogeneic human HD PBMC-derived CD8 T cells and assessed their responses through intracellular cytokine staining (ICS, Fig. 5 A, B, F, G), ELISA (Fig. 5 C), and proliferation of CFSE labeled cells (Fig. 5 D, E). The primary readout was IFN-gamma secretion from responder CD8 T cells in the coculture system as a surrogate for CD8 T cell-mediated

immunogenicity. The frequency of IFN-gamma producing CD8+ T cells was significantly different when co-cultured with KO vs WT control LCL (Fig. 5 A, B). Differences in the proliferation of IFN-gamma producing cells (Fig. 5 D, E) and IFN-gamma in the supernatant (Fig. 5 C), likewise, reached statistical significance with comparable, large effect sizes. As cytotoxic CD8 T cells possess an entire armamentarium of molecules mediating immunogenicity in addition to IFN-gamma, we also assessed the frequencies of granzyme B and perforin-producing CD8 T cells and, again, detected significant differences between KO and WT control-stimulated CD8 T cells (Fig. 5 F, G). Finally, we performed degranulation assays measuring CD107a expression on CD8 T cell membrane-fused and re-internalized granule membranes as a surrogate for cytotoxicity,[26, 29–31] which, likewise, showed significant differences between KO and WT effects (Sup. Fig. 4). These findings clearly indicate that diminished ERAP1 function changes immunogenicity and suggest that this is mediated through a change in the HLA-class I bound peptidome.[32, 33]

DISCUSSION

GWAS have identified immunogenetic risk factors for BD both within and outside of the *HLA* locus and recently unveiled an epistatic relationship between *HLA-B*51* and a haplotype of *ERAP1*, which encodes for the hypoactive enzyme allotype ERAP1-Hap10. This genotype confers the strongest and most BD-specific risk known to date. The overarching objective of our work was to initiate a process of understanding how this risk is mediated biologically. As both HLA-B51 and ERAP1 are molecular constituents of the endogenous antigen presentation pathway which presents peptides on HLA class I molecules to the TCR of CD8 T cells, we saw compelling rationale in hypothesizing that altered ERAP1 function would result in shaping the HLA-B51 peptidome to induce a change in CD8 T cell responses, *i.e.*, modulate immunogenicity and immunodominance.

At the gene level, we observed similar frequencies of the *ERAP1* variant encoding for *ERAP1-Hap10* in *HLA-B*51*$^{+}$ carriers in our small sample cohort as uncovered in large GWAS of BD and HD in the Turkish population by imputation and confirmed those by direct Sanger sequencing of gDNA. It had been unclear, however, if the risk genotype had any post-translational biological relevance – immune-phenotypically and/ or functionally – *i.e.*, whether there were any mechanistic, potentially disease-driving consequences at the cellular level. Here, we provide evidence that this is indeed the case by presenting data, which show that an altered HLA class I-bound peptidome, induced by reduced ERAP1 function, changes immunogenicity. Perhaps equally important, we also show that the variant carrier genotype has an effect on the immune-phenotype in humans, including in those that have fully expressed the disease. Expectedly, these alterations in immune-phenotype localize to the CD8 T cell compartment and affect highly differentiated, antigen-experienced CD8 T cell populations that are HLA class I restricted. Specifically, $CD57^{+}CD28^{-}CCR7^{-}CD8^{+}$ T cells which are known as an oligo-clonally expanded effector population that has undergone repetitive cycles of antigen stimulation in chronic inflammatory conditions, in particular in those due to viral infections,[27, 28] emerged as a population that distinguishes *ERAP1-Hap10* carriers from those that do not carry *ERAP1-Hap10*, in an *HLA-B*51* background – a finding that points to an HLA class I restricted, ERAP1-modulated process. This is further substantiated by additional results we obtained from targeted analyses of

antigen-experienced vs naive CD8 T cells, which indicated significant shifts of these subsets of CD8 T cells in an HLA-B51 background depending on ERAP1-Hap10 expression (Fig. 3).

Our results from microbial homology analyses show resemblance in a large fraction of long, HLA-B51:01 restricted peptides in the ERAP1 KO LCL conditions (Fig. 4C) with experimentally proven immunogenic epitopes of infections that are pathogenic in humans (Sup. Table 4). While these are interesting observations, the potential relevance of those findings should be interpreted with great caution when thinking in the context of a suggested causal link of these pathogens for BD. Their conceptual significance here is merely to demonstrate that long peptides, generated in the absence of ERAP1 activity, may contain epitopes with some degree of sequence homology to those that elicit powerful CD8 T cell responses necessary for fighting many of these infections.

While our data, and reasoning based on well-established immunological facts, strongly point to a pathogenic role for CD8 T cells at least in risk variant carriers, there has been scarce evidence for potentially disease-driving CD8 T cell-mediated immunity in BD per se so far.[34–36] This may be due to a general paucity of investigation directed to address this question specifically and a lack of appropriate tools to do so until recently. Several important previous studies, however, did provide clear proof of the presence of CD8 T cells at an important effector site in BD, *i.e.*, the anterior chamber of the eye in patients with uveitis due to BD.[37–39] Others have demonstrated their presence and transmigration in the cutaneous pathergy reaction, a highly BD-specific phenomenon, and in nodular skin and other lesions associated with BD.[40–44] Those data, most of which were generated well before *HLA-B*51-ERAP1* epistasis was discovered, underline the significance of the findings we present here and augment the notion that the variant may play an important part in inducing or maintaining disease in BD, including organ-threatening disease.

Previous work with human cells has significantly contributed to our understanding of the constituents and biophysical properties of the HLA-B51-bound peptidome but did not address the potential effects of such changes on effector function.[45–48] Early work by Shastri et al. showed alteration of CD8 immunogenicity in intersex adoptive transfer mouse models that induce a non-lethal rejection response and are, therefore, similar to our human mixed lymphocyte reaction system of LCL cocultured with allogeneic CD8 T cells. The CD8 response in the ERAAP deficient mice was diminished (but enhanced in an autologous system) upon adoptive transfer, which is very much in line with our findings in the human allogeneic MLR system.[32, 33] Combined, this implies aberrant immune function through loss of ERAP1/ERAAP: weakening of the physiologic allo-response to non-self, but induction of immunogenicity to self-derived intracellular proteins or those that have entered the HLA class I pathway through intracellular infection or cross-presentation.

The decades-long unsuccessful efforts to clearly link immune-phenotypes and mechanisms in BD to *HLA-B*51*, its traditionally most prominent genetic risk factor, has entered a new stage through the discovery of its epistatic relationship with *ERAP1*, which augments this risk profoundly. This has provided a strong conceptual rationale for the potential involvement of the endogenous antigen presentation pathway in this subset of BD. The

findings we present here support, extend and sharpen this assumption by showing that 1) absence of ERAP1 function clearly alters the immunogenicity to human CD8 T cells, likely induced by a propensity for longer “under-trimmed” peptides, and, 2) ERAP1-Hap10 – including in heterozygous subjects – induces shifts in antigen-experienced and naive CD8 T cell compartments in carriers of *HLA-B*51*, including in those afflicted with the disease. The data simulations shown in Sup. Fig. 3 further underpin that these shifts very likely depend on ERAP1 trimming activity per se and therefore apply across a large portion of heterozygote ERAP1-Hap10 carriers who, for the most part, fall into the low-trimming range according to the findings of a recent study.[15] Some caution interpreting these results is advised, however, given the small sample sizes after exclusion of mid-range trimmers and the understandably low number of peptides tested in that study, which may have left differences in substrate specificity unaccounted for which may alter trimming activity estimates.[49]

Alternate mechanisms mediating risk are conceivable and may include aberrancies of HLA assembly and folding in the ER, possibly with an associated stress response as postulated in *HLA-B*27* associated diseases. HLA recognition by NK cell receptors with sensitivity to HLA-bound peptides is another testable possibility awaiting exploration in the future.

The observation that ERAP1-Hap10 protects from HLA-B27^{+} ankylosing spondylitis and HLA-C06^{+} psoriasis also suggests HLA class I-restricted (tolerogenic) processes.[50, 51] However, the effects of ERAP1 KO in HLA-B27 transgenic rats seem more mechanistically diverse.[52–54] They may include a reduction of potentially ER stress-inducing unfolded HLA-B27 heavy chains and lower rates of disulfite-linked HLA-B27 that can bind to innate immune cell receptors.[54] Unique biophysical features of the HLA-B27 molecule, not shared with HLA-B51, make the conceptual extrapolation of these findings to HLA-B51 and ERAP1 in BD problematic, however, and leave HLA class I restriction as a potentially unifying theme.[55, 56]

Combined, our findings provide evidence for the immunological relevance of the *HLA-B*51/ERAP1-Hap10* risk variant in humans, including in those with BD. This strongly suggests the modulation, initiation, or termination of HLA-B51-restricted immune responses mediated by allotypic ERAP1-Hap10 in affected carriers through the aberrant generation and presentation of a finite, likely small, number of HLA-class I-restricted peptides as a potentially disease-driving process. Further understanding the fine-tuning of this process through the identification of pathogenic HLA class I-restricted peptides and their cognate TCR will enable the rational testing and design of compounds and genetic strategies that modulate ERAP1 activity as a therapeutic means, which may be targeted to patients carrying the risk genotype in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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KEY MESSAGES

What is already known about this subject?

- *ERAP1-Hap10* encodes for a hypoactive endoplasmic reticulum aminopeptidase (ERAP1) resembling a functional KO, and recessively confers the highest risk for Behçet's disease (BD) in the presence of *HLA-B*51* (epistasis).

What does this study add?

- *ERAP1-Hap10/HLA-B*51* skews frequencies and phenotypes of human antigen-experienced vs. naïve CD8 T cells in vivo, pointing to the biologic relevance of this variant and suggesting its importance in HLA-B51-restricted CD8 T cell activation.
- Knock-out of *ERAP1* – modeling hypofunctional *ERAP1-Hap10* – alters immunogenicity, mediated through an HLA class I bound peptidome, which is characterized by longer, *i.e.*, less trimmed peptides above 9mer.

How might this impact on clinical practice or future developments?

- The study provides rationale for the development of ERAP1 activity modulating therapy targeted to BD patient subsets defined by genotype as opposed to disease phenotype alone.
- The findings have relevance to understanding, risk stratifying, and treating other, clinically distinct HLA class I-associated diseases in whom epistasis between ERAP1 haplotypes and disease-associated HLA class I alleles has been shown to be linked to risk and protection, such as ankylosing spondylitis and psoriasis.

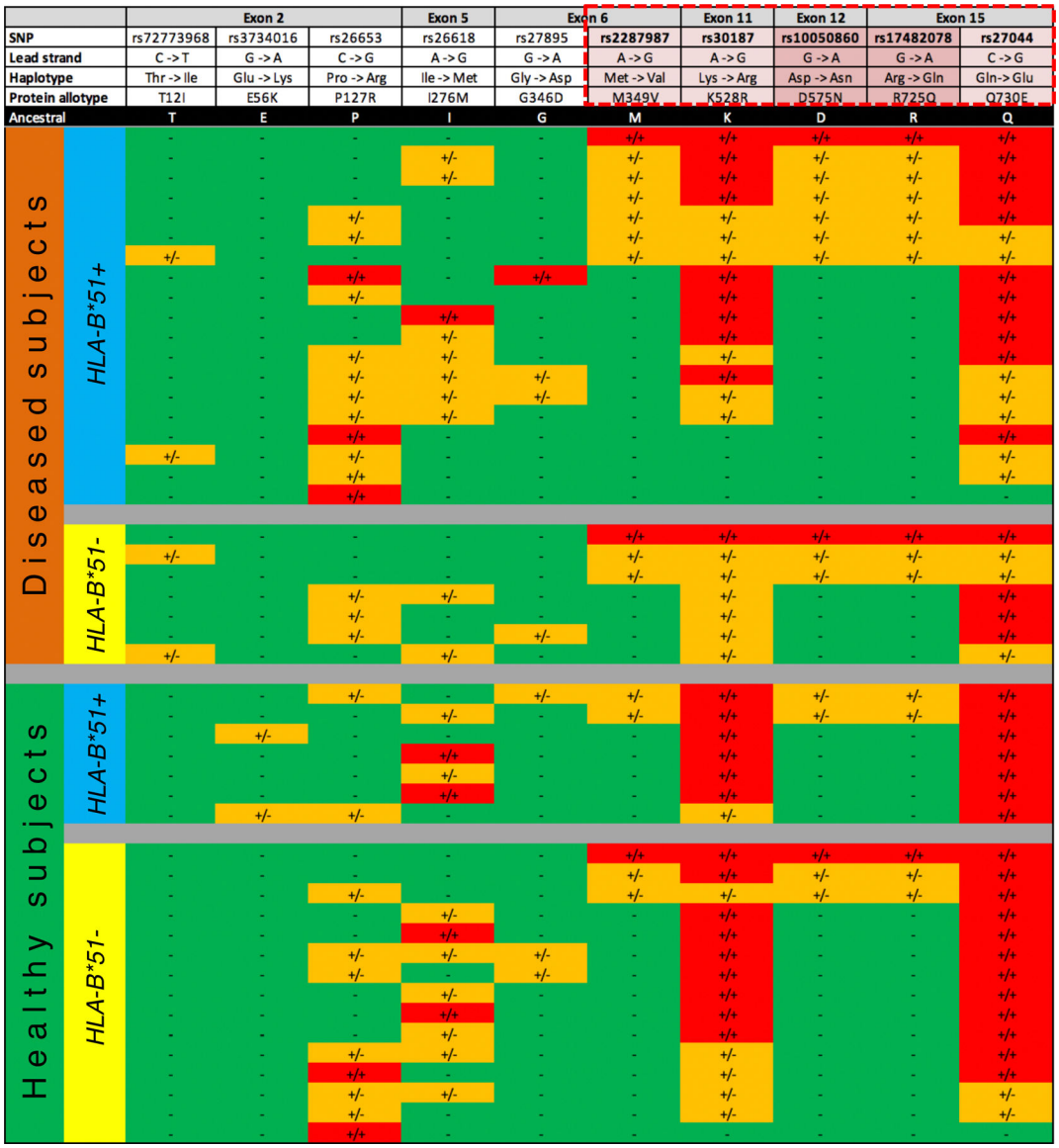


Fig. 1. Frequency distribution of *ERAP1-Hap10*

in 26 BD and 22 HD recruited in Turkey is consistent with the published allele frequencies determined through imputation in the Turkish population. Dotted red lines show the SNPs forming the core risk haplotype. +/+ = homozygous. −/+ = heterozygous. *ERAP1-Hap10* confers risk for BD in epistasis with *HLA-B*51*, but protects from ankylosing spondylitis and psoriasis.

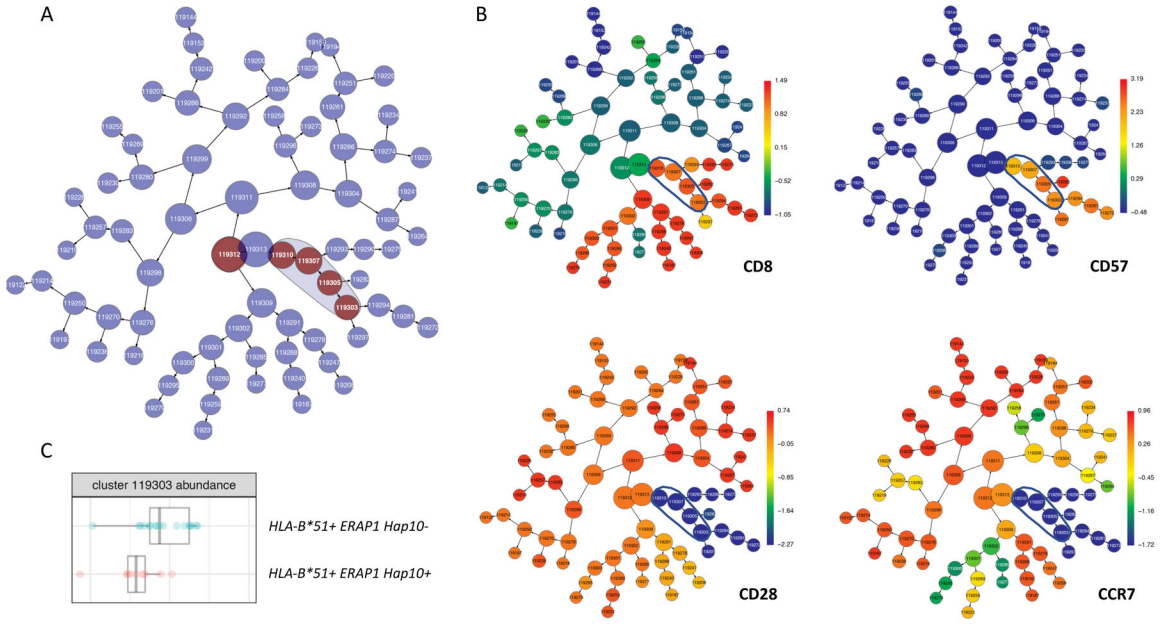
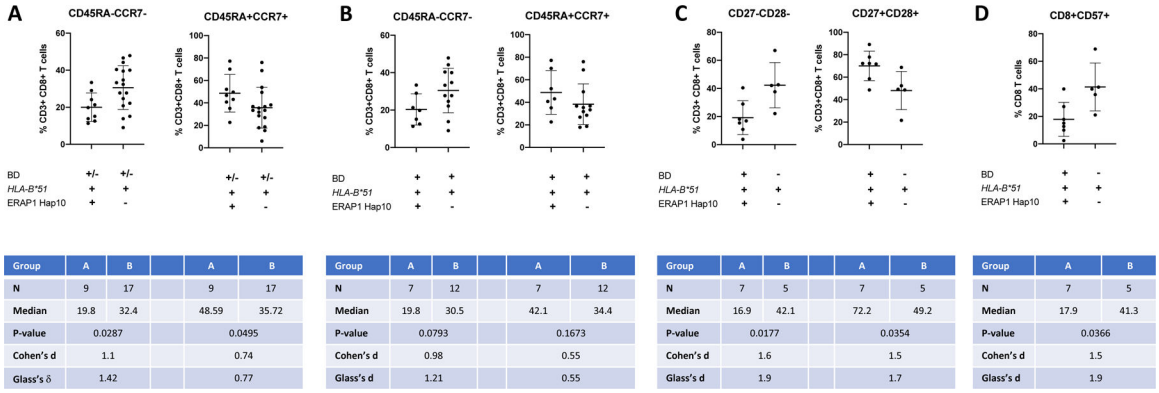


Fig. 2. CITRUS analysis T cell panel.

Data were pre-processed by gating on singlets, live CD45+CD3+Lymphocytes (T cells) prior to computation using the CITRUS algorithm. **A** = Feature plot indicates nodes which represent cell populations that discriminate with statistical significance in between *Group 1*: HLA-B-51+ERAP Hap10-subjects (n=17) and *Group 2:* HLAB-51+ERAP1-Hap10+ (n=9) **B** = Marker plots (one each for CD8, CD57, CD28, and CCR7) indicate the phenotypical identity of the nodes marked in A), *i.e.*, CD8+CD57+ T cells that have lost CCR7 and CD28 expression indicating maturation, oligoclonal expansion, terminal differentiation and high cytotoxicity. **C** = Box plot indicating abundance for a significant cluster on a log 10 scale. FDR set to 0.05. PAM model applied. PBMC stained for viability, CD45, CD3, CD4, CD8, CD57, CD45RA, PD1, CCR7, CD28, CD27. The complete analysis with abundance of all markers used is shown in Sup. Fig. 2.



Group	A	B		A	B
N	9	17		9	17
Median	19.8	32.4		48.59	35.72
P-value	0.0287			0.0495	
Cohen's d	1.1			0.74	
Glass's δ	1.42			0.77	

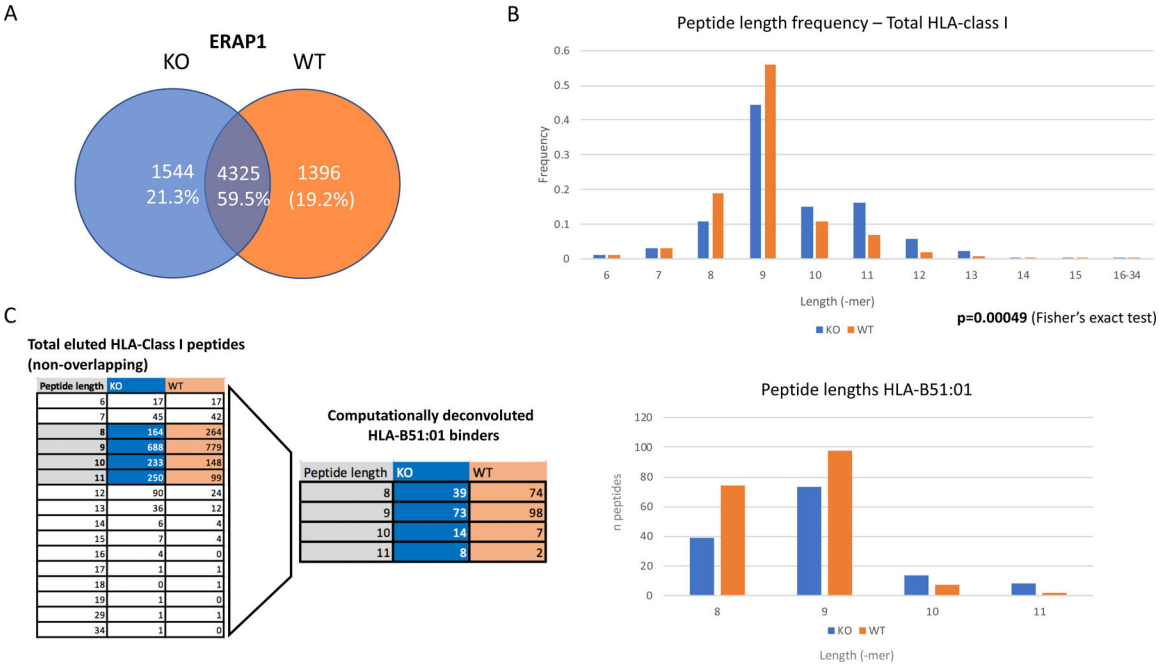
Group	A	B		A	B
N	7	12		7	12
Median	19.8	30.5		42.1	34.4
P-value	0.0793			0.1673	
Cohen's d	0.98			0.55	
Glass's d	1.21			0.55	

Group	A	B		A	B
N	7	5		7	5
Median	16.9	42.1		72.2	49.2
P-value	0.0177			0.0354	
Cohen's d	1.6			1.5	
Glass's d	1.9			1.7	

Group	A	B
N	7	5
Median	17.9	41.3
P-value	0.0366	
Cohen's d	1.5	
Glass's d	1.9	

Fig. 3. Inverse frequencies of CD8$^+$ naïve and effector-memory CD8 T cells

in between risk variant (*HLA-B*51*$^+$ and *ERAP1 Hap10*$^+$) carriers and non-carriers (*HLA-B*51*$^+$ *ERAP1-Hap10*$^-$) in **A** all donors regardless of disease status, and **B** within BD subjects, and **C, D** between *HLA-B*51*$^+$ *ERAP1 Hap10*$^+$ BD and *HLA-B*51*$^+$ *ERAP1 Hap10*$^-$ HD. Mann-Whitney U test. 2 different effect size measures are provided. Values >0.8 mark large, >0.5 medium and <0.5 small effect sizes for Cohen's, and Glass's estimates. These shifts in early - naïve (CD45RA$^+$CCR7$^+$ or CD27$^+$CD28$^+$) and late memory, highly aggressive antigen-experienced CD8$^+$ effector T cells (CD8$^+$CD57$^+$;CD27$^-$CD28$^-$; CD45RA-CCR7$^-$) suggest their antigen-specific, HLA-restricted activation and migration to diseased tissues. See Sup. Fig. 3 for data simulations that escalate trimming activity differences through the exclusion of mid-range trimmers across all genotypes.



Peptide length	KO	WT
6	17	17
7	45	42
8	164	264
9	688	779
10	233	148
11	250	99
12	90	24
13	36	12
14	6	4
15	7	4
16	4	0
17	1	1
18	0	1
19	1	0
29	1	1
34	1	0

Peptide length	KO	WT
8	39	74
9	73	98
10	14	7
11	8	2

Fig. 4. Shifts in peptide lengths frequencies

towards longer (less trimmed) peptides in the absence of ERAP1 at 9mer, the ideal peptide length for HLA-class I binding (**A, B**). This holds true after computational deconvolution for HLA-B51 binding peptides (**C**). Mass spectrometry of the HLA class I-bound peptides eluted from 1.25×10^8 LCL in each condition (genome-edited or not). One representative experiment out of 3 is shown. *Fisher's exact test. P=0.00049*. See Sup. Tab. 4 for microbial homology analyses of the 10 and 11-mer peptides from the non-overlapping peptidome deconvoluted for HLA-B51:01 in 4C.

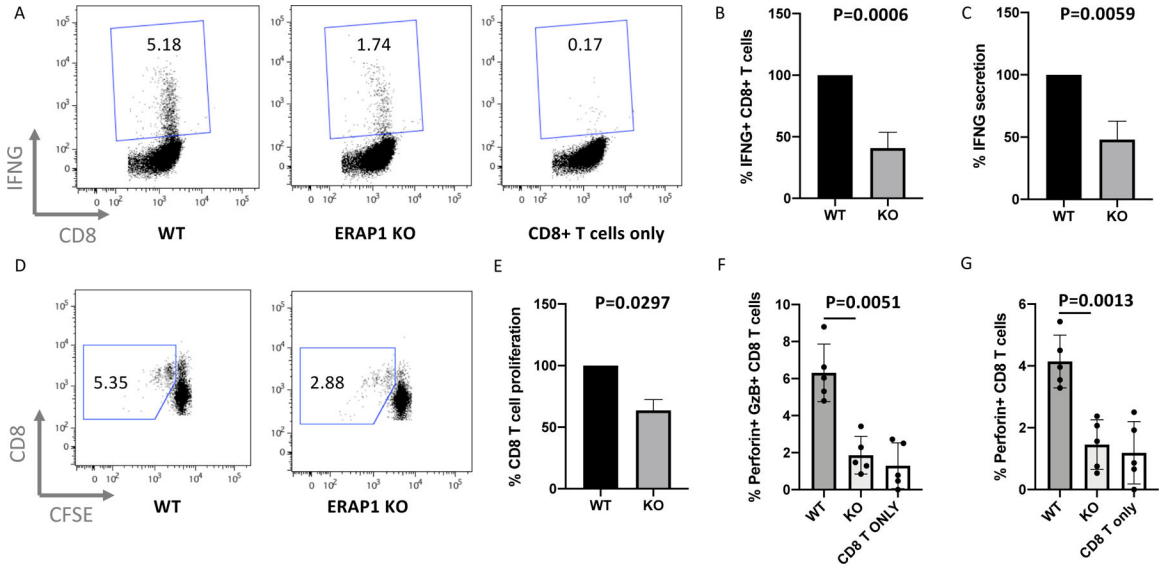


Fig. 5. Loss of ERAP1 function shifts CD8 T cell immunodominance.

ERAP1 KO significantly alters immunogenicity of LCL when cocultured with allogeneic human CD8 T cells, assessed here by IFN-gamma production at the single cell (ICS, **A, B**) and bulk (ELISA, **C**) levels. Other effector readouts underpin this finding: CD8 proliferation on CD3-gated PBMC (CFSE, **D, E**), perforin and granzyme B (**F, G**). CRISPR-Cas9 stable HLA-B51+ LCL were transduced with ERAP1-targeting gRNA (KO) or non-sensical gRNA (WT), and co-cultured with allogeneic human CD8 T cells in 1:4 ratio. In the long-term stimulations (**D–G**), LCL were irradiated with 6000 rad. 8 independent experiments. 6 (**A–E**) or 5 (**F,G**) different CD8 T cell donors, all in triplicates. Ratio-paired t-test. Normalized WT with gated examples (**A,B**; **D,E**). Raw data in (**F, G**) show distribution of data. See Sup. Fig. 4 for results of degranulation assays.