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Mice expressing human ERAP1 variants associated with ankylosing spondylitis have altered T-cell repertoires and NK cell functions, as well as increased *in utero* and perinatal mortality

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Abstract

Specific variants of endoplasmic reticulum-associated aminopeptidase 1 (*ERAP1*) identified by genome-wide association study modify the risk for developing ankylosing spondylitis. We previously confirmed that disease-associated ERAP1 variants have altered enzymatic abilities that can impact upon the production of pro-inflammatory cytokines from cells expressing the same ERAP1 variants. To determine if these ERAP1 variants also impacted immune responses *in vivo*, we generated two strains of transgenic mice expressing human *ERAP1* genes containing non-synonymous single-nucleotide polymorphisms associated with an increased (*ERAP1-High*) or decreased (*ERAP1-Low*) risk for developing autoimmune disease. After vaccination with foreign antigens, ERAP1-High mice generated unique populations of antigen-specific T-cell clones. The expression of ERAP1-High also reduced MHC-I expression on the surface of multiple cell types, demonstrating a global impact on the MHC-I peptidome. ERAP1 variants also affected the innate immune system, because NK cells from murine ERAP1 (mERAP1) knockout mice and ERAP1-High/mERAP1$^{-/-}$ mice had decreased surface expression of the activating receptor NKG2D on their NK and T cells, and NK cells derived from mERAP1$^{-/-}$ mice or ERAP1-Low mice demonstrated more active NK cell killing than NK cells derived from wild-type or ERAP1-High mice. Finally, these studies were conducted in female mice, as all male ERAP1-High mice died *in utero* or shortly after birth, making *ERAP1-High* one of the only dominant lethal autosomal genes known in mammals. Together, these results present the first direct evidence that human disease-associated ERAP1 variants can greatly alter survival, as well as antigen presentation, T-cell repertoire and NK cell responses *in vivo*.

Keywords: adaptive immunity, animal model, antigen presentation, dominant lethal allele, NKG2D

Introduction

Ankylosing spondylitis (AS) is an autoimmune disease that causes inflammatory back pain and a progressive spinal ankylosis that can eventually lead to fusion of all bones in the spine and pelvis (1). AS is one of the very few autoimmune diseases that are more common in men than women and is highly heritable (~90%) (2). Over 90% of AS patients have specific MHC-I alleles present (including *HLA-B*27:05*, *B*27:02*, *B*13:02*, *B*40:01*, *B*40:02*, *B*47:01*, *B*51:01* and *B*57:01*) (3, 4); however, only 5% of people harboring these MHC-I alleles develop the disease. As *HLA-B*27* alleles only contribute ~20% of the overall genetic risk for development of AS, other genes likely play a significant role in the genetic causation of AS (4). These findings justified subsequent genome-wide association study (GWAS), which identified specific polymorphisms in the endoplasmic reticulum-associated aminopeptidase 1 (*ERAP1*) gene that were also highly associated with AS risk. After *HLA-B*, *ERAP1* has the strongest association of any gene with AS, and epistatic gene–gene interactions between MHC-I and ERAP1 further increased the risk for developing AS (3, 5). Interestingly, gene–gene

interactions between *MHC-I* and *ERAP1* are also linked to other diseases in the same class as AS, including psoriasis (6) and Behçet disease (7), demonstrating that interactions between disease-linked *ERAP1* and *MHC-I* alleles likely have global impacts on the immune system.

The confirmation of gene–gene interactions between *ERAP1* and *HLA-B* in GWAS of AS has led to the hypothesis that alterations in antigen presentation may be the primary cause for AS. *In vivo*, HLA and ERAP1 proteins directly interact to facilitate MHC-I antigen presentation (8, 9). The proclivity by which ERAP1 trims peptides can also predetermine the selection of peptides presented on MHC-I molecules (10). The arthritogenic peptide hypothesis proposes that a unique peptide presented due to the presence of specific ERAP1 and MHC-I variants cause autoimmune arthritis by recruiting autoreactive CD8 T cells and/or NK cells to sites of inflammation in the spine and pelvis (11).

Even small changes in antigen presentation can have a large impact on the immune response. For instance, in mice, the life-saving CD8 T-cell response to *Toxoplasma gondii* requires a single peptide epitope that is produced only in the presence of murine ERAP1 (12). Indeed, our laboratory and others have demonstrated that in the absence of ERAP1, a completely different set of peptides may be loaded onto MHC-I molecules, dramatically altering the immunodominant T-cell repertoires present in ERAP1-deficient animals (12).

Having demonstrated that ERAP1 deficiency could completely alter the immunodominant peptidome, we next showed a similar effect was mediated by the presence of human disease-associated ERAP1 variants. For example, using *in vitro* biochemical assays, we found that single-nucleotide polymorphisms (SNPs) in ERAP1 affected their ability to process peptides, with each SNP trimming different peptides at different rates (13, 14). Furthermore, we demonstrated that the presence of all five AS-associated SNPs (associated with the highest risks for developing AS (an ERAP1 variant we refer to as ERAP1-High) in the ERAP1 protein had an additive effect, resulting in an ERAP1 protein having the fastest rate of peptide trimming, which likely resulted in over-trimming of peptides, and significant decreases in cell surface levels of MHC-I, including HLA-B*27 (13). It also appeared that ERAP1-High may have roles outside antigen presentation, as ERAP1-High-expressing cells stimulated with an adenovirus, secreted increased amounts of IL-1β compared to identically treated cells expressing ERAP1-Low (15). The converse was true for a human ERAP1 variant containing the five protective AS-risk SNPs (ERAP1-Low). ERAP1-Low had the slowest rate of peptide trimming, and this correlated with increased antigen presentation on the surface of cells expressing ERAP1-Low (13).

Although we confirmed that ERAP1 variants containing AS-associated SNPs trim peptides at different rates, and these changes correlate with surface expression of MHC-I, these results were obtained from biochemical assays and *ex vivo* based human tissue culture systems. To determine if AS-associated ERAP1 variants also influence immune responses *in vivo*, we created two new strains of transgenic mice, one strain ubiquitously expressing the ERAP1-High variant, and a second expressing the ERAP1-Low variant.

Methods

Animal procedures

All animal procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (http://iacuc.msu.edu/). All mice used in experiments were bred in house. Each mouse genotype was verified by PCR.

Polymerase chain reaction

For genotyping a ~0.05 mg biopsy was harvested from the tail of the animal and digested in tail buffer [50 mM Tris–HCl, pH 8, 50 mM KCl, 2.5 mM EDTA, 0.45% octylphenoxy poly(ethyleneoxy)ethanol, branched, 0.45% Tween 20 and 0.5 mg ml^{-1} proteinase K] overnight at 55°C. The mixture was spun and supernatant used directly for PCR reaction. The following primer sets were used for the following genotypes. For murine ERAP1 knockout ($mERAP1^{-/-}$) animals, ERAP1-KO-For (CTTTGCCCTCTGTGGTCATT) and mERAP1-KO-Rev (CAGAGGGGTCAGGAAGTCAA) were used to amplify a ~900 bp amplicon only in animals lacking murine ERAP1. For $mERAP1^{+}$ animals, mERAP1-WT-For (CACCTGCTTCCCAACTTCAT) and ERAP1-KO-Rev (CAAACTTAGAGACGTGAAAAGTGA) were used to amplify a 336 bp amplicon only in animals containing ERAP1. For ERAP1-High variant animals, ERAP1High-For (AAGTAAGCTTGGCATCACAA) and ERAP1High-Rev (GGTTATTAGGGGAAAACCCT) were used to amplify a 577 bp amplicon only in animals containing ERAP1-High. For ERAP1-Low variant animals, ERAP1Low-For (TTCATCACCAGCAAATCCA) and ERAP1Low-Rev (GGCGAGGAGTAGTAGTTC) were used to amplify a 501 bp amplicon only in animals containing ERAP1-Low variant. High and Low PCR reactions were carried out with 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 120 s. Other reactions were carried out with 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 120 s.

Reverse transcription–PCR

RNA was harvested from indicated tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) per the manufacturer's protocol. Following RNA isolation, reverse transcription was performed on 1 μg of total RNA using SuperScript III (Invitrogen, Grand Island, NY, USA) RT and random hexamers/oligo (dT) primers per the manufacturer's protocol. RT reactions were diluted to a total volume of 60 μl, and 2 μl was used as the template in the subsequent PCR reactions. Primers were designed using Primer Bank web-based software (http://pga.mgh.harvard.edu/primerbank/); a complete list of primers used is available upon request. Quantitative PCR (qPCR) was carried out on an ABI 7900HT Fast Real-Time PCR System as previously described (16).

Generation of transgenic mice

Zinc-finger nuclease (ZFN) mRNAs targeting ROSA26 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Plasmid donor DNA was constructed by cloning the ERAP1-High variant and ERAP1-Low variant sequences (Supplementary Figure 1, available at *International Immunology* Online) into

the pZDmRosa26 plasmid between flanking arms of homology to the murine ROSA26 locus. Plasmid donor DNA (final concentration 2.5 ng μl^{-1}) was mixed with ZFN mRNAs (final concentration of each ZFN mRNA was 2.0 ng μl^{-1}). The nucleic acid mixture was microinjected into zygotes obtained from the mating of superovulated WT (wild-type) female mice to WT male mice. Surviving zygotes were transferred to pseudopregnant B6D2F1 mice. C57BL/6J (stock number 000664) and B6D2F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animals were housed under specific pathogen-free conditions in static microisolator cages under a 0600–1800 light/dark cycle regimen with *ad libitum* access to water and Purina Lab Diet 5008 food. A total of 300 microinjections were performed for ERAP1-Low variant and 900 for ERAP1-High. Founder animals were identified by PCR as described above.

Isolation of lymphocytes from spleen and liver tissues

Spleen tissues were homogenized by passage through a 40-μm sieve. Red blood cells were removed from homogenate by incubating with 2 ml per spleen of ACK lysis buffer (Invitrogen, Carlsbad, CA, USA) at RT for 5 min. Splenocytes were subsequently washed two times with RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1× penicillin, streptomycin, fungizone (PSF), re-suspended and counted using the Countess automated cell counter (Invitrogen) as previously described (17).

NK cell assays

NK cells were isolated from fresh splenocytes with NK Cell Isolation Kit II (Miltenyi Biotech, San Diego, CA, USA) per the manufacturer's protocol. Isolated NK cells were re-suspended in complete RPMI (10% FBS, 1% Penicillin, 1% Streptomycin and 1× Amphotericin B) supplemented with 2 IU ml^{-1} rmIL-2. RMA-s cells (generous gift from Dr. Sungjin Kim) were stained with 1 μM CFSE (Thermo-Fisher, Waltham, MA, USA) as previously described (17). NK cells were then co-cultured at indicated E:T ratios with CFSE-stained RMA-s cells for 48 h at 37°C and 5% CO_2. Co-cultured cells were washed once in FACS and stained with propidium iodide 1 min before analysis on a BD LSR2 flow cytometer. Analysis was performed with FlowJo (Ashland, OR, USA). Percent cell lysis was calculated by gating on $CFSE^+$ cells and then calculating the % positive for propidium iodide staining.

Immunization and ELISPOT

Ad5-TA and Ad5-Gag were generated as previously described (17). Both Ad5 vectors were injected intramuscularly (i.m.) into the tibialis anterior of the right hindlimb of 6-week-old mice. A total of 1×10^{10} viral particles per mouse per vector in a volume of 30 μl PBS solution (pH 7.4) was injected. Fourteen days post-injection, spleens were harvested and splenocytes extracted, counted and plated in prepared ELISPOT plates as previously described (17); 96-Well Multiscreen high protein binding Immobilon-P membrane plates (Millipore, Billerica, MA, USA) were pretreated with ethanol, coated with mouse anti-IFN-γ capture antibody, incubated overnight and blocked with RPMI medium (with 10% FBS, 1% PSF) prior to the addition of 1.0×10^6 splenocytes per well. *Ex vivo* stimulation included the incubation of splenocytes in 100 μl of media alone (unstimulated), or media containing 2 μg per well of a single 15-mer peptide (as indicated in Fig. 4) for 18 h in a 37°C, 5% CO_2 incubator. Ready Set Go IFN-γ mouse ELISPOT kits were purchased from eBioscience (San Diego, CA, USA). Staining of plates was completed per the manufacturer's protocol. Spots were counted and photographed by an automated ELISPOT reader system (Cellular Technology, Cleveland, OH, USA).

Statistical analysis

Statistically significant differences were determined using a one-way ANOVA with a Student–Newman–Keuls *post hoc* test or by using two-tailed homoscedastic Student's *t*-tests (*P* value of <0.05 was deemed statistically significant). Graphs in this paper are presented as mean of the average ± SEM, unless otherwise specified. Statistical analyses were performed using GraphPad Prism (GraphPad Software).

Results

Generation of transgenic mice that express the ERAP1-High or the ERAP1-Low variant from the ROSA26 locus

To determine the physiological consequences of stable ERAP1-High expression on the immune system *in vivo*, we set out to generate transgenic mice that ubiquitously express ERAP1-High or ERAP1-Low (the protective control variant). *ERAP1-High* contains five disease-associated SNPs (*M*349V, *K*528R, *D*575N, *R*725Q, *Q*730E) and *ERAP1-Low* contains five protective SNPs (*V*349M, *R*528K, *N*575D, *Q*72R5, *E*730Q) (13, 15). The location of these SNPs on a space-filling model of the ERAP1-High protein is shown in Fig. 1(A), and their complete sequences are provided in Supplementary Figure 1 (available at *International Immunology* Online). To construct the respective mice, the ERAP1-High cDNA or the ERAP1-Low cDNA was subcloned into bacterial plasmids flanked by DNA homologous for the murine ROSA26 locus. These plasmids were co-injected into murine embryos along with ZFNs targeting double-stranded DNA breaks into the ROSA26 locus so that the *ERAP1* variants would be placed under transcriptional control of the ubiquitously expressed ROSA26 promoter after targeted recombination (Fig. 1B).

After one round of embryo microinjections using the ERAP1-Low cDNA construct (300 embryos microinjected resulting in 74 pups), we identified two founder animals that transmitted the *ERAP1-Low* transgene to offspring. In stark contrast, generating the ERAP1-High transgenic mouse strain was extremely difficult, as over 900 embryonic microinjections were required, resulting in only 89 pups, before a single ERAP1-High transgenic (female) founder mouse was identified.

To further verify expression of ERAP1-High and ERAP1-Low in the respective transgenic mouse strains, we performed quantitative RT–PCR. We confirmed that ERAP1-High mRNA was only expressed in mice transgenic for ERAP1-High (Fig. 1C), and that ERAP1-Low mRNA was only expressed in mice transgenic for ERAP1-Low

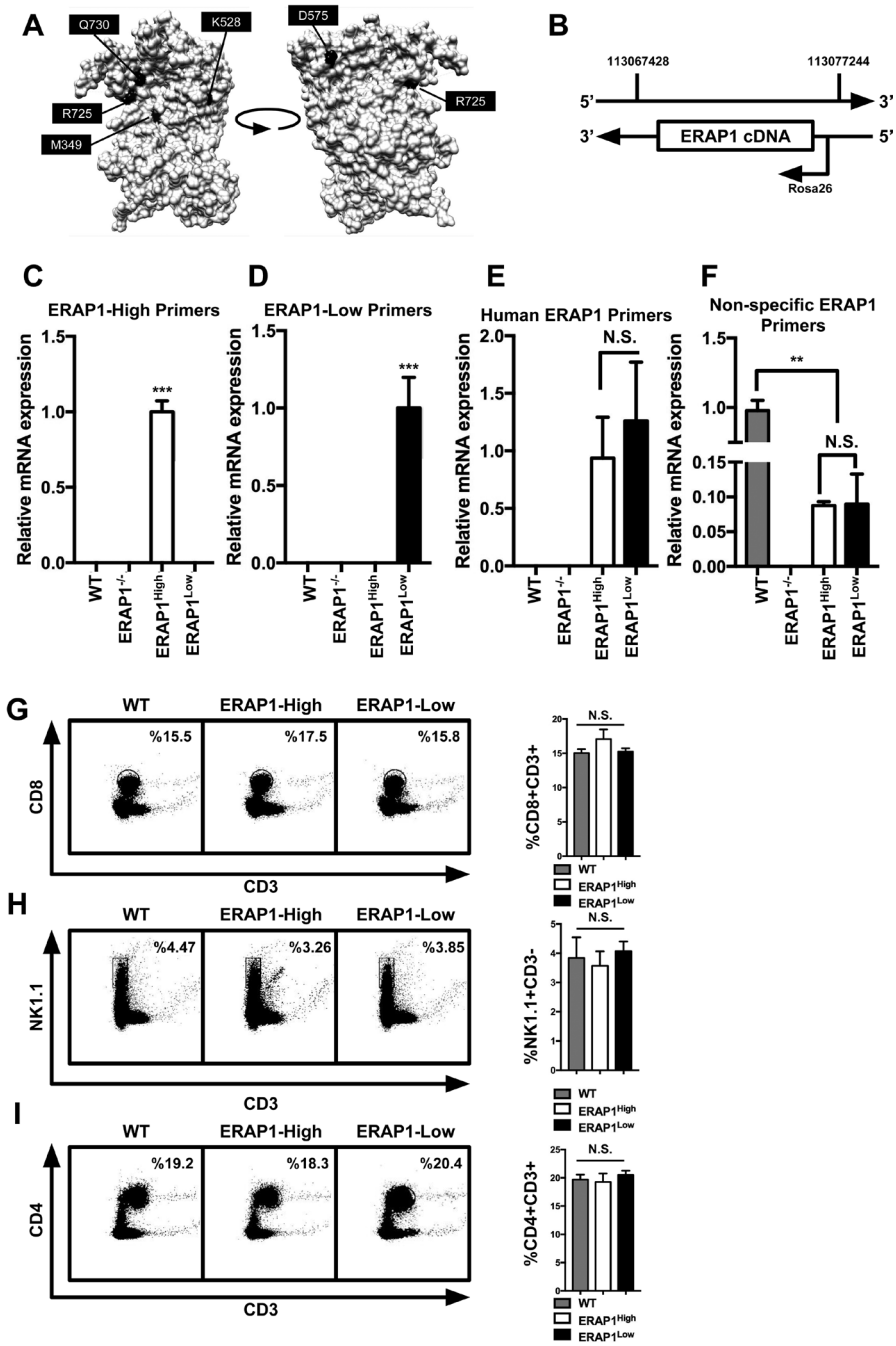

Fig. 1. Generation of ERAP1-High and ERAP1-Low mice. Five SNPs in ERAP1 were described to have an association with AS (51) and subsequently confirmed *in vitro* to have altered enzymatic activity and HLA-B27-restricted antigen presentation (13). We used a ZFN microinjection technique to generate a strain of mice expressing a human ERAP1 allele associated with increased risk for developing AS, and another with a decreased risk into the murine ROSA26 locus. (A) The location of the five SNPs on a space-filling model of human ERAP1. (B) Schematic showing the human ERAP1 gene inserted into the murine ROSA26 locus to be driven by the endogenous ROSA26 promoter on chromosome 6. (C) qPCR was used to confirm ERAP1-High expression using ERAP-High-specific primers. (D) qPCR was used to confirm ERAP1-Low expression using ERAP-Low-specific primers. (E) qPCR was used to compare relative expression of ERAP1-High and ERAP1-Low mRNA derived from liver tissue, using human ERAP1 primers and they were found to be equivalent. (F) qPCR was used to compare relative expression of ERAP1-High and ERAP1-Low mRNA with endogenous murine ERAP1 primers that overlapped a conserved region. ERAP1-High and ERAP1-Low mRNA was expressed many fold less than endogenous murine ERAP1. Flow cytometry was performed on splenocytes from the indicated genotypes and revealed an equivalent percent of (G) CD8 T cells, (H) NK cells and (I) CD4 T cells. Bars represent the relative mRNA expression normalized to GAPDH ± SEM. ***$P < 0.001$ compared to all other groups. **$P < 0.01$. The figure is representative of two separate experiments.

(Fig. 1D). We next compared the relative expression levels of ERAP1-High to ERAP1-Low mRNA using primers specific for human ERAP1 and confirmed that there were no significant differences in their expression levels when comparing the two strains of transgenic animals (Fig. 1E). Finally, to compare the expression level of the transgenic

human ERAP1 to the expression level of endogenous mERAP1, we crossed ERAP1-High or ERAP1-Low mice with mERAP1$^{-/-}$ mice and used a generic ERAP1 primer set that binds to regions conserved between the mERAP1, ERAP1-High and ERAP1-Low mRNAs. Expression of ERAP1-High and ERAP1-Low directed by the ROSA26 locus resulted in an approximately 10-fold lower level of expression than endogenous mERAP1 (Fig. 1F), a result that parallels prior studies using the ROSA26 locus in similar strategies (18, 19). We also performed each of these analyses on additional tissues derived from brain (ectoderm), liver, thymus (endoderm) and skeletal muscle tissues (mesoderm). Results from liver are shown (Fig. 1C–F) and did not differ significantly from other tissues (Supplementary Figure 2, available at *International Immunology* Online). Using flow cytometry we also confirmed that the two strains of mice were grossly immunologically intact, with equivalent percentage of CD4 T cells, CD8 T cells and NK cells in each strain (Fig. 1G–I).

Unlike ERAP1-Low, transmission of the ERAP1-High transgene from generation to generation had a remarkably low frequency, and after several generations, it became apparent to us that the ERAP1-High transgene was only being transmitted to female mice. To control for the small possibility that the ZFNs simultaneously damaged DNA on the X chromosome, female ERAP1-High transgenic mice were backcrossed five times onto the C57BL/6 background, but still no male carriers of the ERAP1-High transgene were identified. To formally confirm that the presence of ERAP1-High negatively impacted male viability, we crossed female ERAP1-High mice with male WT (C57BL/6) mice and recorded the number and genotypes of all offspring derived from these breedings (Fig. 2A). Of the 149 offspring derived from these crosses, no male ERAP1-High variant mice were identified to be among the surviving offspring (Fig. 2A). Importantly, this effect was unique to ERAP1-High, as the transmission of the ERAP1-Low transgene in mice followed simple Mendelian genetics (Fig. 2B). The loss of male ERAP1-High offspring significantly reduced the litter sizes derived from the breeding of female ERAP1-High mice (Fig. 2C). This decreased litter size was found to be due to a unique effect of ERAP1-High on only male offspring and was not due to a decrease in maternal fitness, as there was no impact on the numbers of female mice derived from these same crossings (Fig. 2D).

Our prior publications demonstrate that ERAP1 impacts NK cell activation and development (16) and that ERAP1-High can significantly alter the peptides presented on surface MHC-I molecules (17). Previous reports have also demonstrated that peptides presented on MHC-I can play a critical role in early fetal development and survival by inhibition of uterine NK cells and preventing premature spontaneous termination of the fetus (20). These findings suggest that the decreased fitness of male ERAP1-High

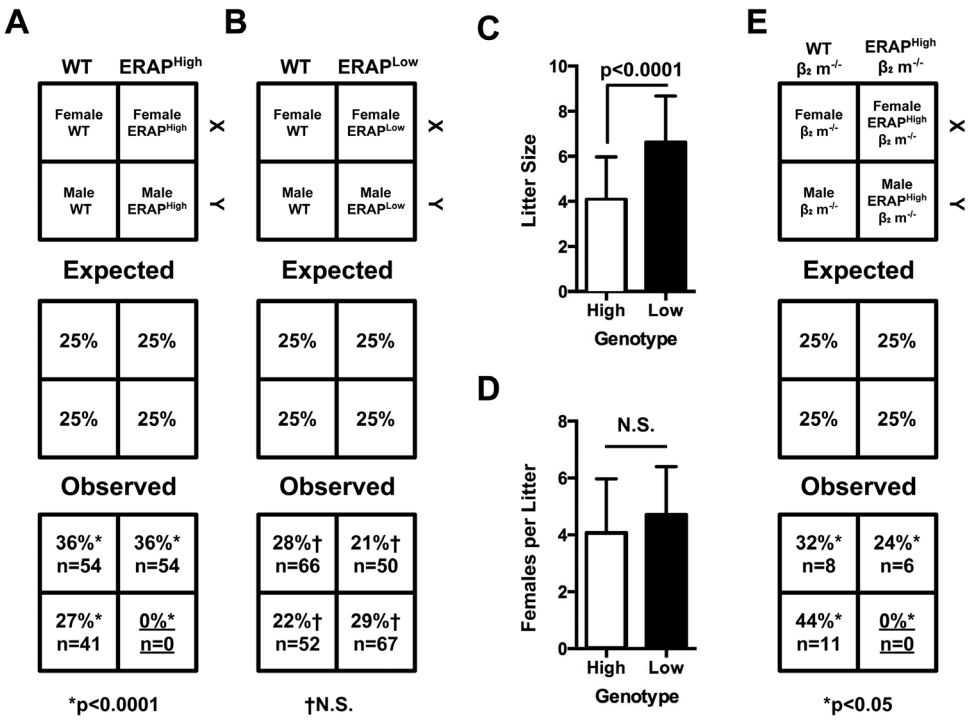

Fig. 2. ERAP1-High is lethal in male mice in an MHC-I-independent mechanism. (A) ERAP1High$^{+/-}$ females were crossed with WT males. The expected and observed results of 41 crosses are shown. No male mice with ERAP1-High were born. (B) A control cross was performed with ERAP1Low$^{+/-}$ females and WT males. The expected distribution of genotypes was observed. (C) The litter sizes of ERAP1-High mice were reduced, but the number of females per litter was unaffected. (D) To determine if ERAP1-High was lethal in males as a result of antigen presentation, ERAP1-High$^{+/-}$/β2m$^{-/-}$ females were crossed with β2m$^{-/-}$ males eight times. The removal of surface MHC-I by the cross failed to rescue the male ERAP1-High pups. *n* represents the number of offspring with the given genotype. Significance was determined by a chi-squared test with $P < 0.05$ deemed significant.

mice may be due to the presence of aberrant peptides loaded onto MHC-I. To determine if removing MHC-I expression on the surface of cells would rescue ERAP1-High male mice, we crossed ERAP1-High mice with homozygous β2-microglobulin knockout (β2m$^{-/-}$) mice. β2m$^{-/-}$ mice display no MHC-I on the surface of cells but generate a normal frequency of male pups after breeding. Of 25 offspring derived from these breedings, no male β2m$^{-/-}$/ERAP1-High$^{+/-}$ pups were ever detected in surviving offspring (Fig. 2E). This suggests that ERAP1-High is decreasing male fitness independent of the presence of MHC-I, or fetal antigen presentation. These results confirm that ERAP1-High has unique biological significance and suggest that ERAP1-High has a dominant gain-of-function mutation that causes *in utero* and perinatal lethality to male mice inheriting the transgene. As a consequence of this finding, all further studies described were performed using only female mice.

Expression of the ERAP1-High variant decreases surface levels of MHC-I in vivo

The sum of available peptides derived from endogenous protein breakdown positively correlates with the amount of MHC-I present on the cell surface (9, 21), which in turn shapes the development and function of T cells (21) and NK cells (22). Using an *in vitro* system, we previously demonstrated that ERAP1-High had increased enzymatic activity, over-trimmed peptides and decreased the surface expression of MHC-I on the surface of human cells (13). Therefore, we investigated whether or not ERAP1-High would have similar effects *in vivo*. We measured the surface level of murine MHC-I (H2-Kb) on a variety of immune cell types including CD8 T cells (Fig. 3A), NK cells (Fig. 3B) and CD4 T cells (Fig. 3C) derived from ERAP1-High and ERAP1-Low mice, with and without the addition of IFN-γ, which increases surface expression of MHC-I (23). While there were no significant differences detected at baseline, when cells were stimulated with IFN-γ,

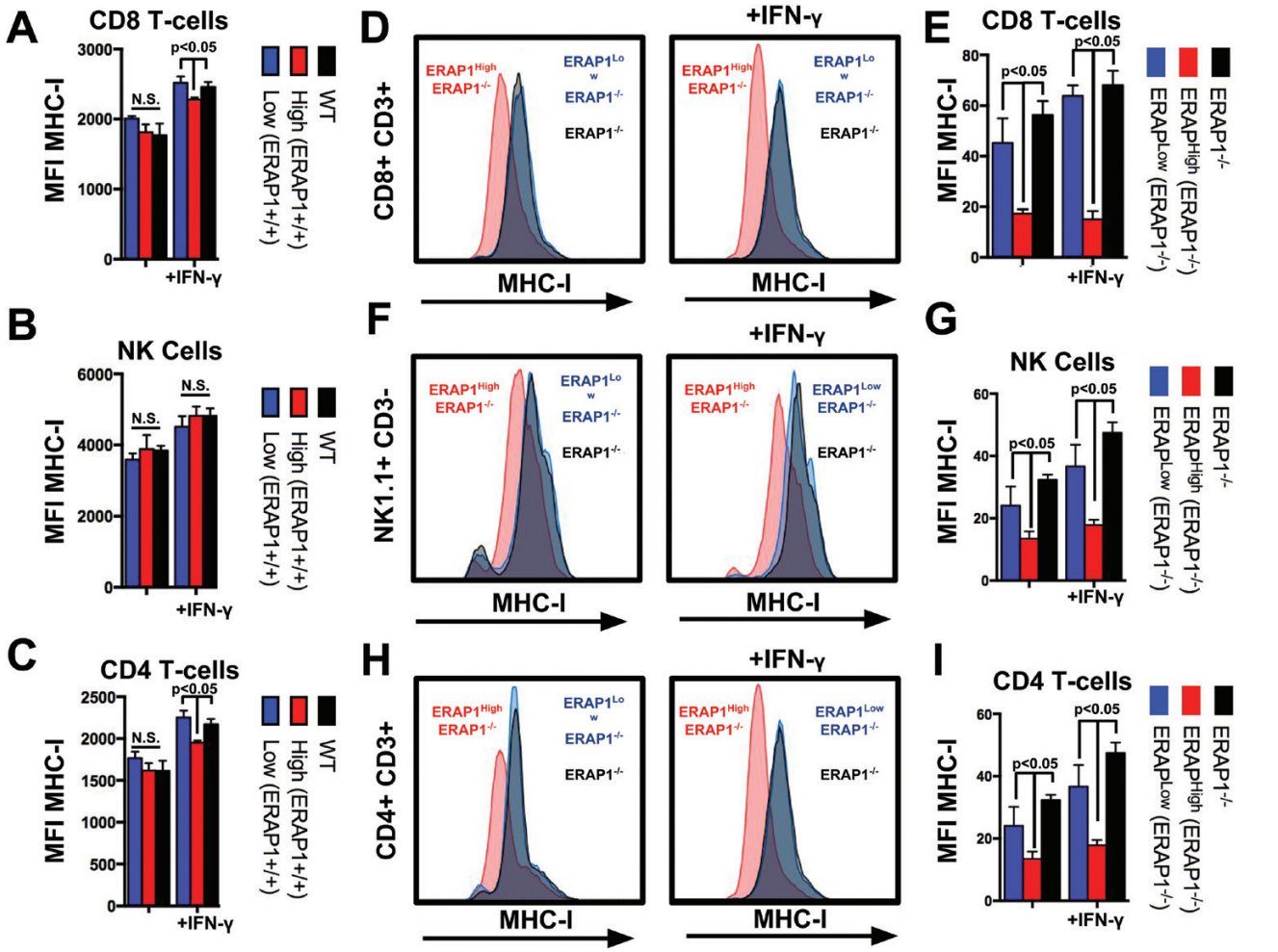

Fig. 3. ERAP1-High significantly reduces MHC-I on the surface of cells. Splenocytes from the indicated genotypes of mice were harvested as described in Methods. They were then activated with 0.1 μg ml^{-1} IFN-γ or 0 μg ml^{-1} IFN-γ for 2 h, stained and analyzed with flow cytometry. A subtle, but significant reduction in surface MHC-I, was observed in CD8^{+} T cells (A), NK cells (B), CD4^{+} T cell (C) derived from ERAP1-High mice when treated with IFN-γ compared to other groups. To remove the potential masking effect of endogenous mERAP1, ERAP1-High and ERAP1-Low mice were crossed with mERAP1$^{-/-}$ mice. On the mERAP1$^{-/-}$ background, ERAP1-High significantly reduced MHC-I surface expression in CD8^{+} T cells (D, E), NK cells (F, G), CD4^{+} T cells (H, I) with and without IFN-γ compared to ERAP1-Low or no mERAP.

CD4 T cells and CD8 T cells derived from ERAP1-High mice had a subtle, but significant decrease in their MHC-I surface expression levels (Fig. 3A and C). We hypothesized that differences mediated by ERAP1-High may be partially masked by endogenous mERAP1, especially because ERAP1-High was expressed at a lower level than mERAP1 (Fig. 1F). To determine if mERAP1 was masking the effect of ERAP1-High, we crossed ERAP1-High and ERAP1-Low mice onto the mERAP1$^{-/-}$ background and repeated the study. Without the interference of endogenous mERAP1, the presence of ERAP1-High correlated with significant decreases of MHC-I expression on CD8 T cells (Fig. 3D and E), NK cells (Fig. 3F and G) and CD4 T cells (Fig. 3H and I) compared to these same cells derived from similarly treated ERAP1-Low and control (mERAP1$^{-/-}$) mice. These changes demonstrate that ERAP1-High causes a net decrease in surface MHC-I.

The presence of ERAP1-High alters immunodominant T-cell adaptive immune responses in vivo

Immunodominance is a critical phenomenon in adaptive immunity and governs the effectiveness of adaptive immune responses against intracellular bacteria (12), viral infections (24) and cancer (25). Moreover, patterns of T-cell immunodominance have also been linked to risk for developing autoimmune diseases that are associated with ERAP1 polymorphisms (26, 27). We have previously shown that the presence of ERAP1 completely reshapes the immunodominant T-cell response to model antigens such as the *Clostridium difficile* toxin A (TA) (17). We therefore wished to determine if ERAP1-High and/or ERAP1-Low would similarly reshape the immune response to antigens such as TA. To identify peptides that may be differentially processed by ERAP1-High and ERAP1-Low, we performed a pilot study by immunizing mice with an adenovirus expressing TA (Ad-TA) and an adenovirus expressing HIV-Gag (Ad-Gag) as previously described (17). Fourteen days after immunization, splenocytes from the immunized animals were exposed to a 15-mer overlapping peptide library (peptides overlapping by five amino acids) that spanned the entire TA and Gag proteins, and the production of IFN-γ to each peptide was measured using IFN-γ ELISPOT. From the 208 peptides tested, 5 generated a strong T-cell response (Fig. 4A and B) in all strains of mice evaluated. Two of the peptides (ALTSYKIINGKHFYF and VNGSRYYFDTDTAIA) generated an equally strong response independent of the presence of ERAP1-High or ERAP1-Low and serve as an internal control. Three peptides (TGYTIINGKHFYFNT, EAMSQVTNSATIMMQ and YTSINGKHFYFNTDG) generated significantly reduced T-cell responses in ERAP1-High mice as compared to ERAP1-Low and WT control animals (Fig. 4A), demonstrating that ERAP1-High can selectively remove epitopes from the immunodominant T-cell response, even at a low expression level relative to mERAP1. These differences mediated by ERAP1-High also appear to be genetically dominant, as they occur in the presence of mERAP1, effectively 'overwriting' epitopes normally presented via MHC-I in WT mice (Fig. 4A). We repeated the studies in the absence of mERAP1 and found that the mere presence of ERAP1-High alone significantly altered the T-cell responses to a variety of peptides in the library (Supplementary Figures 3 and 4, available at *International Immunology* Online). One peptide (AAIHLCTINNDKYYF) had an unchanged, but strong response in all mice (Fig. 4D), and serves as a useful internal control. Similar to results on the mERAP$^{+/+}$ background, T-cell responses to several peptides were greatly diminished in ERAP1-High/mERAP1$^{-/-}$ mice (Fig. 4C), demonstrating again that the usual T-cell immune responses to these peptides are abrogated in the presence of ERAP1-High, and not ERAP1-Low. Surprisingly, several additional peptides now generated T-cell immune responses in splenocytes derived from the ERAP1-High/mERAP1$^{-/-}$ mice (Fig. 4E). Importantly, no such unique peptides were identified when splenocytes from ERAP1-Low mice were similarly evaluated, suggesting that ERAP1-Low shares some overlapping trimming specificity with murine ERAP1. Finally two peptides generated decreased T-cell responses in the presence of either ERAP1-High or ERAP1-Low (Fig. 4F). In our previous work, we demonstrated that these same peptides also failed to elicit T-cell responses in the presence of the mERAP1 protein (17) and were only immunogenic in the absence of mERAP1. Because they are removed from the immunodominant T-cell response by ERAP1-High, ERAP1-Low and mERAP1, they may belong to a set of peptides destroyed by many or all forms of ERAP1.

The presence of these same human ERAP1 variants did not significantly impact the humoral arm of the adaptive immune response of the mice, as an ELISA measuring total immunoglobulin responses to the TA and Gag peptides demonstrated no significant difference in the quantity or specificities of these antibody responses (data not shown).

Presence of ERAP1-High correlates with decreased NKG2D expression on CD8 T cells and NK cells

In addition to its usual role in fostering adaptive immune responses via antigen presentation, the presence or absence of antigen-loaded MHC-I molecules can also impact the development (22) and activation (28) of NK cells. We previously demonstrated that following innate stimulations, NK cells derived from mERAP1$^{-/-}$ mice had increased NK cell activation levels (CD69 expression) as compared to NK cells derived from WT mice (16). To determine if ERAP1-High or ERAP1-Low altered activation or phenotypes of NK cells at baseline (without immune stimulation), we harvested splenocytes from WT, mERAP1$^{-/-}$, ERAP1-High, ERAP1-Low, ERAP1-High/mERAP1$^{-/-}$ and ERAP1-Low/mERAP1$^{-/-}$ mice and phenotyped these cells with CD3, NK1.1, CD94, Ly49D, CD69 and CD107a fluorescent antibodies. CD3-NK1.1^{+} cells derived from all these strains of mice demonstrated similar expression levels of CD94, Ly49D, CD107a and CD69 (data not shown). Interestingly, mERAP1$^{-/-}$ mice lacked expression of NKG2D on their NK cells (Fig. 5A and B). ERAP1-High/mERAP1$^{-/-}$ mice also lacked NKG2D from the surface of their NK cells, although WT and ERAP1-Low/mERAP1$^{-/-}$ mice had normal surface expression levels of NKG2D (Fig. 5A and B). These surprising results demonstrate that deficiency of ERAP1 results in an almost complete loss of NKG2D expression on NK cells, and that the presence of ERAP1-Low (but not ERAP1-High) restores NKG2D surface expression to

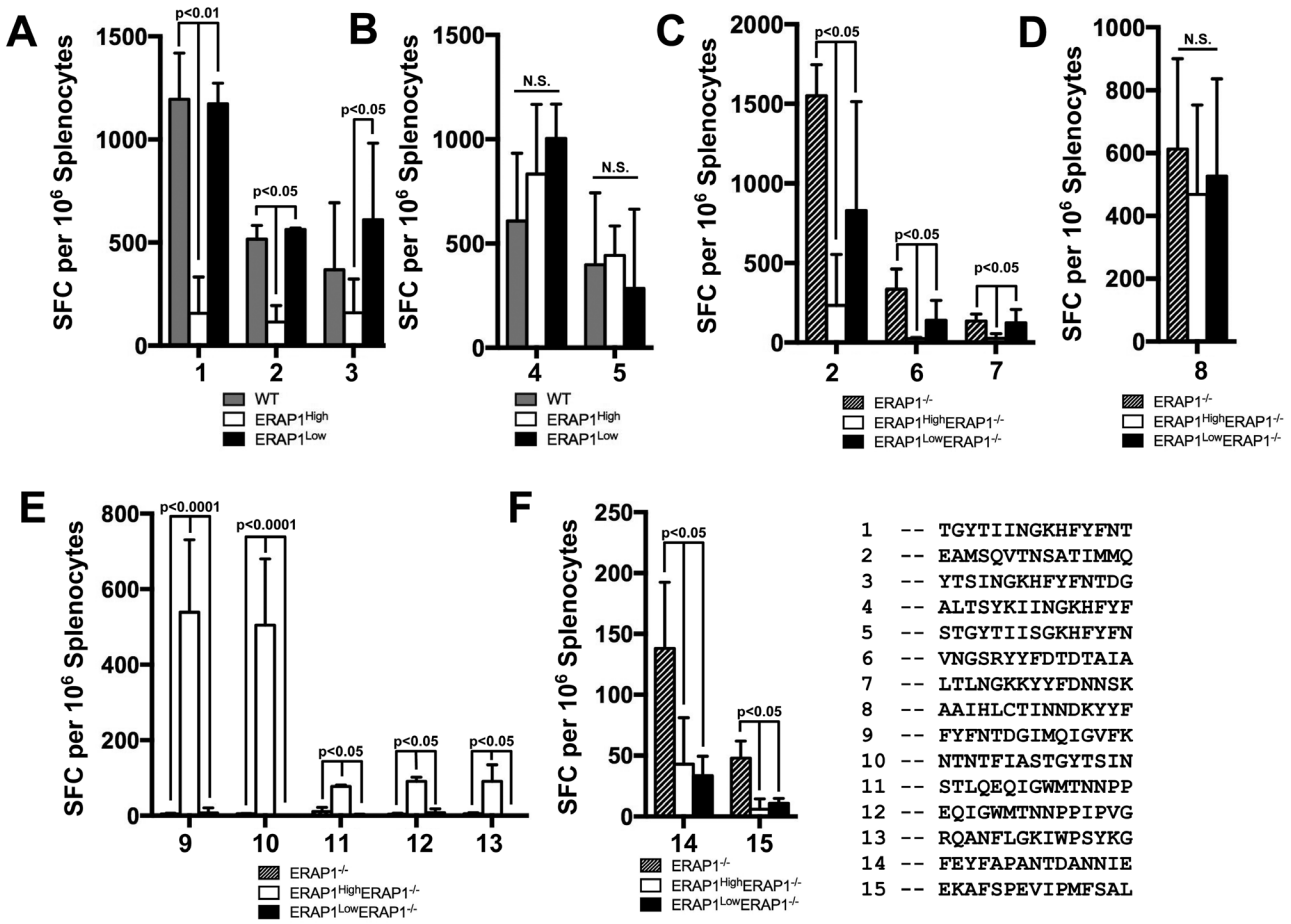

Fig. 4. ERAP1-High significantly alters the immunodominant profile to exogenous antigens. Mice were immunized i.m. in the tibialis anterior with Ad5-*Clostridium difficile*-TA and Ad5-HIV-Gag as described in Methods. At day 14, mice were sacrificed and splenocytes were harvested and stimulated with 2 μg per well of the listed 15-mer peptides. Splenocytes were then analyzed for IFN-γ production using IFN-γ ELISPOT. From the 84 and 121 peptide libraries, the peptides that generated a strong response (≥100 spots) were selected and repeated. (A) Peptides that were significantly different between ERAP1-High and ERAP1-Low. (B) Peptides that were strong responders but not significantly different between ERAP1-High and ERAP1-Low variant. All responding peptides that were different were reduced in ERAP1-High compared to ERAP1-Low variant and WT mice. To remove the effect of endogenous murine ERAP1, the experiment was repeated in mERAP1$^{-/-}$, ERAP1-High/mERAP1$^{-/-}$ and ERAP1-Low/mERAP1$^{-/-}$ mice. (C) Peptides that had significantly less T-cell response in ERAP1-High/ERAP1$^{-/-}$ compared to ERAP1-Low/ERAP1$^{-/-}$. (D) Peptides that were strong responders but not significantly different between ERAP1-High/ERAP1$^{-/-}$ and ERAP1-Low variant/ERAP1$^{-/-}$. (E) Peptides that only generated T-cell response in ERAP1-High/ERAP1$^{-/-}$. (F) Peptides that were significantly reduced by both ERAP1-High and ERAP-Low on the ERAP1$^{-/-}$ background. Bars represent the mean number of spot-forming cells per 10^6 splenocytes ± SEM. A *P* value <0.05 was statistically significant. The figure is representative of two separate experiments.

NK cells. ERAP1-High mice have normal levels of NKG2D expression when on the mERAP1$^{+/+}$ background (Fig. 5C).

NKG2D is a constitutively expressed activating receptor on CD8 T cells in mice (29). Similar to NK cells, CD8$^+$ CD3$^+$ cells derived from both mERAP1$^{-/-}$ and ERAP1-High/mERAP1$^{-/-}$ animals had no surface NKG2D expression (Fig. 5D and E), while NKG2D expression was preserved in ERAP1-High/mERAP1$^{+/+}$ mice (Fig. 5F).

mERAP1$^{-/-}$ and human ERAP1 variant-expressing mice have altered NK-mediated killing of MHC-I-deficient cells

We next determined if mERAP1 had a direct, intrinsic effect on NK cell killing capabilities, and if ERAP1-High and ERAP1-Low may alter this effect. Utilizing an *ex vivo* killing assay, NK cells derived from mERAP1$^{-/-}$ mice killed more RMA-s target cells (MHC-I deficient) than NK cells derived from WT mice (Fig. 6A). This is the first report demonstrating that ERAP1 intrinsically impacts NK cell killing functions. NK cells from WT, ERAP1-High and ERAP1-Low variant mice were similarly analyzed. NK cells from ERAP1-High mice had similar levels of RMA-s cell killing as NK cells derived from WT mice (Fig. 6B). Surprisingly, NK cells derived from ERAP1-Low mice killed significantly more target cells than NK cells derived from ERAP1-High mice, or WT mice (Fig. 6B).

As the differences in NKG2D and MHC-I expression levels mediated by ERAP1-High were observed only in the mERAP1$^{-/-}$ background, we repeated this experiment utilizing NK cells derived from ERAP1-High/ERAP1$^{-/-}$, and ERAP1-Low/ERAP1$^{-/-}$ mice. Again, the presence of ERAP1-Low in NK cells significantly increased their capacity to kill target cells, while the presence of ERAP1-High did not (Fig. 6C). Compared to ERAP1$^{-/-}$, ERAP1-Low/ERAP1$^{-/-}$ NK cells

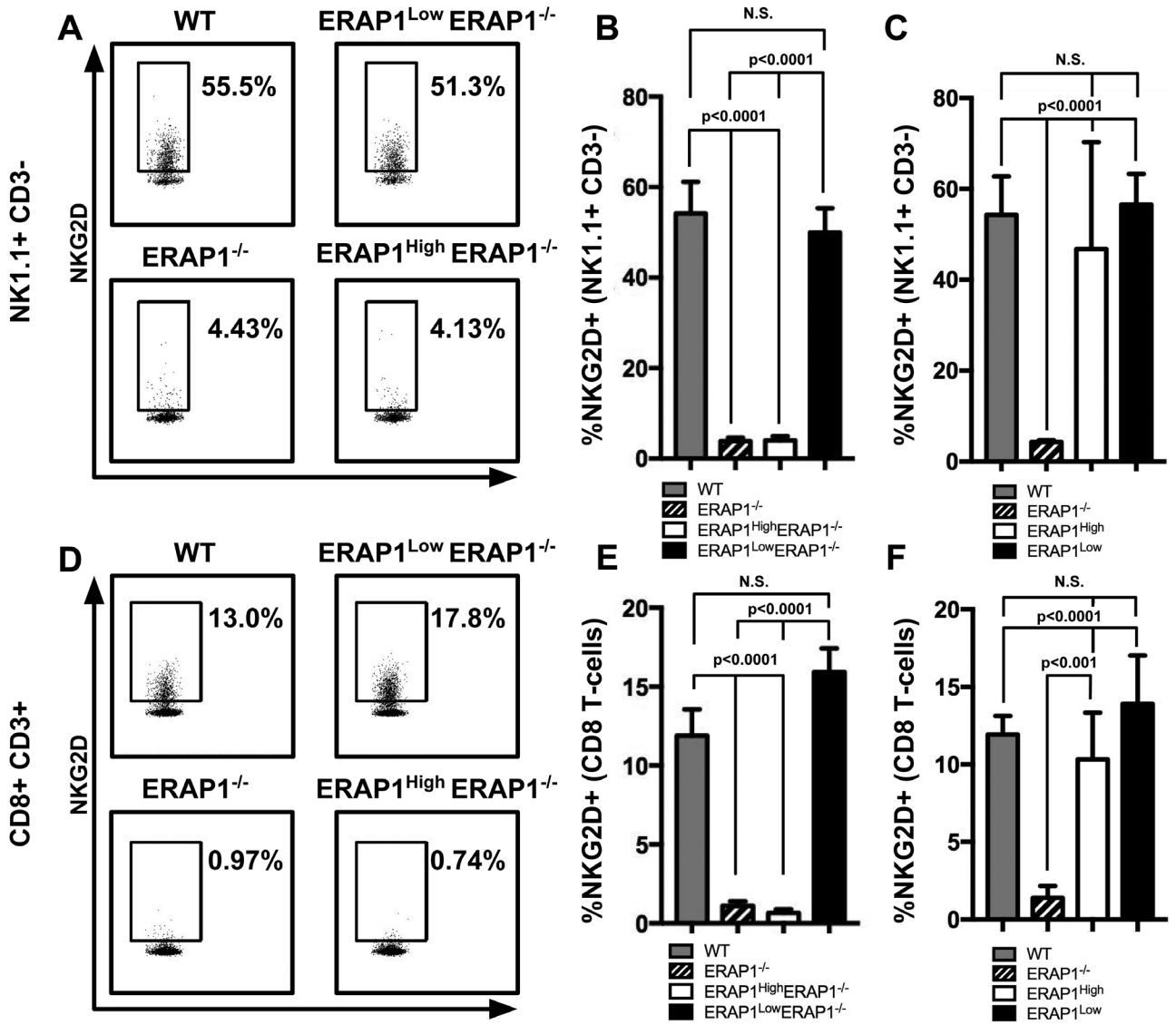

Fig. 5. ERAP regulates NKG2D expression on NK cells and CD8 T cells. NK cells were isolated from WT, ERAP1$^{-/-}$, ERAP1-High/ERAP1$^{-/-}$ and ERAP1-Low/ERAP1$^{-/-}$ mice and surface NKG2D was measured by flow cytometry. (A) Representative image of NKG2D on NK1.1^{+} CD3^{-} splenocytes from WT, ERAP1$^{-/-}$, ERAP1-High/ERAP1$^{-/-}$ and ERAP1-Low/ERAP1$^{-/-}$ mice. (B) %NKG2D on NK1.1^{+} CD3^{-} splenocytes demonstrates a complete loss of NKG2D on NK cells from ERAP1$^{-/-}$ and ERAP1-High ERAP1$^{-/-}$. (C) %NKG2D on NK1.1^{+} CD3^{-} splenocytes from WT, ERAP1$^{-/-}$, ERAP1-High and ERAP1-Low. (D) Representative image of NKG2D on CD8^{+} CD3^{+} splenocytes from WT, ERAP1$^{-/-}$, ERAP1-High/ERAP1$^{-/-}$ and ERAP1-Low/ERAP1$^{-/-}$ mice. (E) %NKG2D on CD8^{+} CD3^{+} splenocytes demonstrates a complete loss of NKG2D on NK cells from ERAP1$^{-/-}$ and ERAP1-High ERAP1$^{-/-}$. (F) %NKG2D on CD8^{+} CD3^{+} splenocytes from WT, ERAP1$^{-/-}$, ERAP1-High and ERAP1-Low. CD4^{+} CD3^{+} T cells were also examined in all genotypes and found to not express a significant amount of NKG2D in any genotype (data not shown). Representative of four repeats for A–D and two repeats for E and F. $n = 6$ for each group.

appeared to kill more target cells, suggesting that ERAP1-Low increases NK killing capacities via a mechanism independent of antigen presentation or NKG2D signaling.

Discussion

The correlation of the presence of specific HLA-B27 variants with AS susceptibility has been known for over 40 years. The association of these variants with AS remains one of the strongest associations in any complex genetic disorder, with an odds ratio of up to 170 (30). However, *HLA-B*27* contributes only ~20% to the overall genetic burden of AS (7), leaving ~70% of the heritable risk for AS unexplained (2). When GWAS were conducted to identify the remaining genetic risk factors, they identified *ERAP1* as the gene with the strongest association for AS after *HLA-B27* and found that the two genes interacted to further increase the odds for developing AS (3, 5). Unfortunately, GWAS can only correlate the presence or absence of SNPs with disease susceptibilities. Additional extensive studies are always required to ascribe biological significance to the genetic regions or specific gene variants identified in GWAS.

While we and others have utilized *in vitro* and *ex vivo* systems to identify biological impacts of AS-associated ERAP1 variants

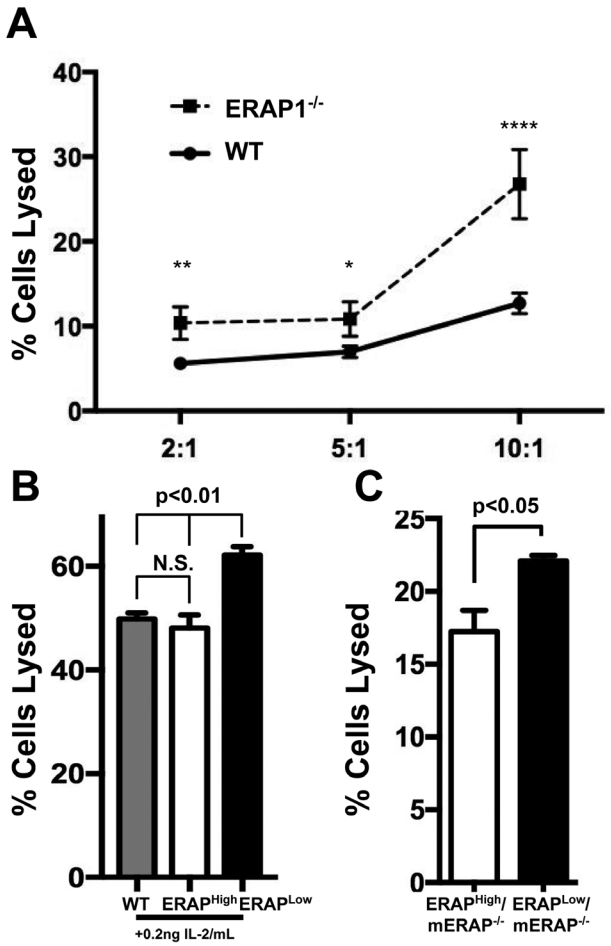

Fig. 6. ERAP1 regulates NK cell killing. NK cells were isolated from WT and ERAP1$^{-/-}$ mice and incubated with RMA-s target cells at the indicated effector to target (E:T) ratios for 48 h. (A) At all E:T ratios NK cells derived from ERAP1-deficient mice demonstrated increased killing. (B) To test the effect of disease-associated ERAP1 variants, NK cells were isolated from ERAP1-High, ERAP1-Low or WT mice and the NK killing assay performed as described in Methods. Surprisingly, ERAP1-Low had higher killing than ERAP1-High or mERAP1. (C) To determine if mERAP1 or IL-2 were impacting the effect of ERAP1-Low on NK cells, we performed the killing assay with NK cells derived from ERAP1-High/mERAP1$^{-/-}$ and ERAP1-Low/mERAP1$^{-/-}$ mice and observed that ERAP1-Low still mediated increased killing. Single, double and quadruple asterisks indicate *P* values of <0.05, <0.01 and <0.0001, respectively.

(13–15), here we demonstrate for the first time the systemic effects that AS-associated ERAP1 polymorphisms can have, using transgenic animal models. The work presented here is the first concrete proof that the expression of specific, AS-associated human ERAP1 variants can have significant impact on several aspects of mammalian biology, including *in utero* survival, and impact on the innate and adaptive immune systems.

Relative to impacts on the adaptive immune system, our results found that the immunodominant CD8^{+} T-cell repertoire to exogenous antigens in animals could be significantly altered dependent upon the presence or absence of specific, AS-associated ERAP1 variants. Furthermore, the changes in immunodominance mediated by the presence of the ERAP1-High variant were profound, and ERAP1-High had as much of an impact on the presentation of specific peptides as the differences between two HLA molecules (9, 31). Introduction of ERAP1-Low into the C57BL/6 mouse had almost no change on which peptides derived from an exogenous antigen became immunodominant (Fig. 5). The presence of ERAP1-High was also able to overwrite the impact that endogenous murine ERAP1 had on immune-dominant antigen selection, effectively ablating T-cell responses to specific peptides normally destined to generate an immunodominant T-cell response. We have previously shown that the ERAP1-High variant has an abnormally high enzymatic activity and is able to over-trim peptides, potentially destroying a unique set of peptide epitopes prior to their loading onto MHC-I. Therefore, it is likely that the presence of ERAP1-High in mice also removes otherwise immunodominant epitopes by destroying these peptides before they can be loaded onto MHC-I (13). Furthermore, this unique property of ERAP1-High appears to be globally impactful, since the presence of ERAP1-High correlates with a reduction in the overall surface levels of MHC-I on a variety of cell types *in vivo*, results consistent with our earlier *ex vivo* work (13). The presence of ERAP1-High also generated a unique set of immunodominant epitopes derived from exogenously provided antigens, furthering the potential impact this AS-associated variant may have on adaptive immune responses.

Abnormalities in the innate immune system, and in particular, NK cells, have been demonstrated in patients with AS (15, 16, 32, 33). Specifically, it has been shown that AS patients have higher numbers of circulating NK cells, and that NK cells from AS patients express a higher percent of CD56, and CD16 (32–34). The theory that NK cells may be involved in AS was strengthened by the observation that certain variants of HLA-B*27 form homodimers that can activate the inhibitory receptor KIR3DL1 on NK cells (35). Our prior *ex vivo* work demonstrated that ERAP1 can also impact NK cells, as NK cells from mERAP1$^{-/-}$ mice had higher frequencies of terminally matured NK cells, as well as higher frequencies of licensed NK cells and more active NK cells following TLR stimulation (16).

In this report, we confirmed that the presence or absence of mERAP1 can significantly impact NK cell killing, and further demonstrated that AS-associated polymorphisms in ERAP1 can also have an impact on these functionalities. To investigate how ERAP1 may be further altering the phenotype of NK cells, we measured the surface levels of several NK receptors and found only the levels of NKG2D were altered. Specifically, loss of *mERAP1* resulted in complete lack of NKG2D on the surface of NK cells and CD8 T cells (Fig. 5). The presence of ERAP1-Low restored the surface level of NKG2D to mERAP1$^{-/-}$ mice while the presence of ERAP1-High did not. This suggests that a loss-of-function mutation in ERAP1-High may impact the NKG2D pathway.

The discovery of a role for ERAP1 in the NKG2D pathway is significant because NKG2D itself has been directly implicated in autoimmune diseases in mice (36) and humans (37) and is also associated with AS via its ligand, MICA. MICA activates NK and CD8 T cells via its interaction with NKG2D, and polymorphisms in MICA are also associated with early-onset AS (38, 39). Interestingly, mice constitutively expressing MICA have decreased NKG2D surface levels on their NK cells (40),

and human NK cells stimulated for long periods with MICA also have decreased NKG2D surface levels (41). Therefore, it is possible that NKG2D may become over-stimulated in mERAP1$^{-/-}$ and ERAP1-High /mERAP1$^{-/-}$ mice because of the propensity of these mice to have exaggerated innate immune system responses to known innate immune system agonists (15, 16).

This would also support findings in AS patients, since the MICA polymorphism associated with early-onset AS mediates increased activation of NKG2D (39). However, these possibilities become somewhat clouded by our additional finding that the presence of ERAP1-Low increases NK-mediated killing. This surprising result suggests that ERAP1-Low increases NK-mediated killing of MHC-I deficient cells via a mechanism outside antigen presentation and NKG2D signaling. Future studies will be necessary to fully understand the impact that AS-associated ERAP1 variants have on NK cell biology.

Another unexpected result of this work was the observation that the presence of ERAP1-High is lethal to male mice. There are few dominant lethal alleles, and even fewer that selectively kill one sex (42). Furthermore, to our knowledge, there are no dominant lethal sex-linked alleles located on somatic chromosomes (42). It is interesting that we observed this sex-specific effect of ERAP1-High, as AS is one of the few autoimmune diseases more common in men than in women (1, 2). The mechanism behind this phenotype remains unclear. One potential explanation is that unique antigens derived from proteins on the Y chromosome are being differentially trimmed by ERAP1-High and therefore are only presented in ERAP1-High male fetuses (20). However, because the ERAP1-High male-lethal phenotype persisted despite extensive backcrossing onto a β2m$^{-/-}$ background, it seems that ERAP1-High acts as a dominant lethal allele independent of its role in antigen presentation. Possibly, the ERAP1-High protein acts in a dominant-negative fashion relative to a critical protein expressed from the murine X chromosome. Interestingly, direct interaction between ERAP1 and RBMX (RNA-binding motif gene, X chromosome) has been observed (43, 44). Possibly ERAP1-High is binding enough RBMX in male mice so as to significantly inhibit the RNA-splicing functions of RMBX, causing embryonic lethality.

The future study of these finding may help better understand the reported association of ERAP1 variants in preeclampsia and gestational disease (45). Alternatively, ERAP1-High may be toxic and indirectly influencing results obtained from ERAP1-High mice. From the data presented, this appears unlikely, as the fitness of ERAP1-High female mice was not impacted and their immune systems were grossly intact. Furthermore, in our previous work, we expressed ERAP1-High many fold greater in cells and observed no toxicity (13). However, it remains possible that ERAP1-High may have subtle toxic effects that are causing effects on immune cells. If this is the case in our model, it may also be the case in AS.

Beyond the role of ERAP1 and its variants in susceptibility to various diseases, this work is also important for the field of antigen presentation as a whole. Our results demonstrate that the presence or absence of specific ERAP1 variants can alter antigen presentation drastically *in vivo*. Much work has been done to predict which peptides will become immunodominant epitopes, especially for vaccine development, cancer immunotherapy and precision medicine (46–48). The majority of these efforts have focused exclusively on using specific MHC-I peptide binding and presentation models to attempt to predict the ultimate immunodominant CD8 T-cell responses to a given antigen (46, 47). Although these predictions work well in syngeneic animal models, they seem to have hit an upper range of accuracy (between 70 and 80%) when applied to predictions of human immunodominant immune responses (46, 49). These realities prompted the creation of multiple strains of HLA-humanized mice to help predict human immunodominant epitopes for vaccine development, but these mice also did not accurately predict which epitopes eventually became immunodominant when human studies were attempted (50). On the basis of the work presented here, we suggest that the plateau in human applications of these predictive models may be overcome by also considering the presence or absence of specific human ERAP1 alleles in the respective model systems.

In this work, the presence or absence of specific ERAP1 variants also appeared to be mediated by several mechanisms. Our results uncovered a mix of dominant and recessive phenotypes when the ERAP1-High or ERAP1-Low variants were expressed in mice. For example, the male-lethal phenotype (Fig. 2) and the destruction of various antigen-derived epitopes (Fig. 4A) appeared to be genetically dominant, as these phenotypes were observed in mice regardless of the presence of mERAP1. In contrast, ERAP1-High decreased the surface expression of MHC-I on the surface of cells (Fig. 3) and the loss of surface NKG2D (Fig. 6), only in the mERAP1$^{-/-}$ background, suggesting that these ERAP1-High dependent phenotypes are genetically recessive.

The presence or absence of the ERAP1-Low variant also mediated intriguing phenotypes in both dominant and recessive fashions. ERAP1-Low-dependent increases in the NK-mediated killing of MHC-I-deficient cells occurred regardless of the presence or absence of mERAP1. In contrast, the effect of ERAP1-Low on presented antigens was recessive, as it was only apparent with the removal of murine ERAP1 (Fig. 4F).

Overall, these studies demonstrate that human ERAP1 variants identified by GWAS as important, relative to AS and other autoimmune disease susceptibilities, can be demonstrated to have major impacts on antigen presentation, overall MHC-I abundance, T-cell immunodominance, NK cell functions *in vivo*, as well as overall male survival. The results here translate genetic associations made from GWAS (51) into a better understanding of the complex biological role these ERAP1 variants have within a living organism.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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