EXTENDED REPORT

Silencing or inhibition of endoplasmic reticulum aminopeptidase 1 (ERAP1) suppresses free heavy chain expression and Th17 responses in ankylosing spondylitis

Liyie Chen,1 Anna Ridley,1 Ariane Hammitzsch,1 Mohammad Hussein Al-Mossawi,1 Helen Bunting,1 Dimitris Georgiadis,2 Antoni Chan,3 Simon Kollnberger,1 Paul Bowness1

ABSTRACT

Objective Human leucocyte antigen (HLA)-B27 and endoplasmic reticulum aminopeptidase 1 (ERAP1) are strongly associated with ankylosing spondylitis (AS). ERAP1 is a key aminopeptidase in HLA class I presentation and can potentially alter surface expression of HLA-B27 free heavy chains (FHCs). We studied the effects of ERAP1 silencing/inhibition/variations on HLA-B27 FHC expression and Th17 responses in AS.

Methods Flow cytometry was used to measure surface expression of HLA class I in peripheral blood mononuclear cells (PBMCs) from patients with AS carrying different ERAP1 genotypes (rs2287987, rs30187 and rs27044) and in ERAP1-silenced/inhibited/mutated HLA-B27-expressing antigen presenting cells (APCs). ERAP1-silenced/inhibited APCs were cocultured with KIR3DL2/CD3ε-reporter cells or AS CD4+ T cells. Th17 responses of AS CD4+ T cells were measured by interleukin (IL)-17A ELISA and Th17 intracellular cytokine staining. FHC cell surface expression and Th17 responses were also measured in AS PBMCs following ERAP1 inhibition.

Results The AS-protective ERAP1 variants, K528R and Q730E, were associated with reduced surface FHC expression by monocytes from patients with AS and HLA-B27-expressing APCs. ERAP1 silencing or inhibition in APCs downregulated HLA-B27 FHC surface expression, reduced IL-2 production by KIR3DL2/CD3ε-reporter cells and suppressed the Th17 expansion and IL-17A secretion by AS CD4+ T cells. ERAP1 inhibition of AS PBMCs reduced HLA class I FHC surface expression by monocytes and B cells, and suppressed Th17 expansion.

Conclusions ERAP1 activity determines surface expression of HLA-B27 FHCs and potentially promotes Th17 responses in AS through binding of HLA-B27 FHCs to KIR3DL2. Our data suggest that ERAP1 inhibition has potential for AS treatment.

INTRODUCTION

Ankylosing spondylitis (AS) is the prototype of the spondyloarthritis (SpA), a group of closely related chronic inflammatory diseases sharing clinical symptoms and strong genetic association with the human leucocyte antigen (HLA)-B27. The mechanism by which HLA-B27 confers disease susceptibility remains unclear. The canonical function of HLA-B27 is to form heterotrimeric with β2-microglobulin (β2m) and antigenic peptides in the endoplasmic reticulum (ER), which then egress to the cell surface for CD8+ T cell recognition. However, lack of CD8+ T cells does not prevent disease in the HLA-B27–trangenic rat model of SpA, arguing against a primary role of CD8+ T cell activation by classical HLA-B27 in SpA.1–2 We and others have shown the presence of HLA-B27 β2m-free heavy chains (FHCs) on the surface of peripheral blood mononuclear cells (PBMCs) from patients with SpA and HLA-B27–trangenic rats.3–6 The biological function of HLA-B27 FHCs is supported by its superior binding affinity, in comparison to classical HLA-B27, to the immunoregulatory receptors killer cell immunoglobulin-like receptor 3DL2 (KIR3DL2) and leucocyte immunoglobulin-like receptor B2 (LILRB2).7–8 Importantly, binding of HLA-B27 FHCs to KIR3DL2 expressed by CD4+ T cells has been shown to promote the survival and proliferation of Th17 cells in AS.9–10

The strong genetic association of AS with ER aminopeptidase 1 (ERAP1) has been reported by multiple studies in different ethnic groups.11–17 Five AS-associated ERAP1 single nucleotide polymorphisms (SNPs) were found: rs30187 (C/T, K528R), rs27044 (G/C, Q730E), rs2287987 (T/C, M349V), rs10050860 (C/T, D575N), rs17482078 (C/T, R725Q) (risk alleles and their corresponding amino acids are underlined). ERAP1 locates in the ER and trims peptides to optimal length (usually 8–10 amino acids) before their binding to major histocompatibility complex (MHC) class I molecules. Strikingly, ERAP1 polymorphisms only affect AS risk in HLA-B27–positive individuals, implying that ERAP1 contributes to AS pathogenesis by altering HLA-B27 function.17–19 Indeed, ERAP1 silencing or polymorphisms has been shown to alter the length and sequence of HLA-B27-bound peptides.18–19 A recent study shows that AS-associated ERAP1 polymorphisms do not alter ER stress in patients with AS, arguing against the unfolded protein response theory.20 We hypothesised that ERAP1 might contribute to AS pathogenesis through altering cell surface HLA-B27 FHC expression.

To test this hypothesis, we studied the effect of ERAP1 silencing, inhibition and polymorphic...
variation on HLA-B27 FHC expression and Th17 function. Protective ERAP1 polymorphisms are associated with reduced HLA FHC expression in monocytes of patients with AS and HLA-B27-expressing antigen presenting cells (APCs). ERAP1 silencing or inhibition of APCs reduces HLA-B27 FHC expression, KIR3DL2 stimulation and Th17 responses. Finally, ERAP1 inhibition reduces HLA class I FHC expression and Th17 expansion in PBMCs from patients with AS.

MATERIALS AND METHOD
Patients with AS
Heparinated venous blood was obtained from 56 HLA-B27-positive patients with AS fulfilling the modified New York criteria. Patient demographics are shown (see online supplementary table S1). Patients were assessed for disease activity using Bath AS Disease Activity Index (BASDAI), functional capacity using Bath AS Functional Index (BASFI) and spinal mobility using Bath AS Metrology Index (BASMI).

Genotyping
DNA was prepared from peripheral blood cells using PureLink Genomic DNA Mini Kit (Life Technologies, UK). Three SNPs in the ERAP1 gene previously reported to be associated with AS, rs2287978 (T/C, M349V), rs30187 (T/C, K528R) and rs27044 (G/C, Q730E) were genotyped using functionally tested TaqMan Assays (Applied Biosystems, UK). Two additional AS-associated SNPs, rs10050860 (C/T, D575N) and rs17482078 (C/T, R725Q), were not genotyped in this study. However, we found that they are in strong link disequilibrium with rs2287978 (T/C, M349V) in a set of 60 patients with AS (rs10050860: r²=1, D'=1; rs17482078: r²=0.956, D'=1, data not shown). This strong linkage was also found to be present in the general population using a public SNPs link disequilibrium calculation tool (rs10050860: r²=0.92, D'=1; rs17482078: r²=0.959, D'=1, SNP Annotation and Proxy Search, Broad Institute of Massachusetts Institute of Technology and Harvard).

Cell lines
As described previously, HeLa.B27, C1R.B27 and mouse endoplasmic reticulum aminopeptidase associated with antigen processing (ERAAP) fibroblasts (ERAAP-/- fibroblasts (ERAAP-/- mFib.B27) were transfected to express HLA- HLA-B*27:05.18 21 See online supplementary methods for cell culturing conditions.

ERAP1 stable silencing and inhibition
As described previously, lentiviral ERAP1-shRNA was used to stably silence endogenous ERAP1 expression by HeLa.B27 and C1R.B27 cells.18 In the current study, DG013A was initially titrated at the concentration of 10 nM, 100 nM and 1000 nM. 1000 nM of DG013A was then used for ERAP1 inhibition.

Construction and transfection of shRNA-resistant ERAP1 plasmids
See online supplementary methods.

Isolation of PBMCs and CD4+ T cells
See online supplementary methods.

Flow cytometry
PBMCs were blocked with Fc receptor blocking reagent (Miltenyi Biotec), stained for HLA class I FHCs with the HC-10 (IgG2a) and classical HLA-B27 complexes with ME-1 (IgG1). An APC-conjugated antimouse IgG antibody (Santa Cruz, USA) was used to detect HC-10 and ME-1. Monocyte, B cell and T cell were stained by CD14-PE (Miltenyi Biotec), CD19-Pacific Blue and CD3- PerCP-Cy5.5 (BioLegend, USA), respectively. Dead cells were excluded using Fixable Viability Dye eFlour 780 (Ebioscience, UK). In order to ensure unbiased comparison of FHC expression, all PBMC samples were stained and analysed in a single batch.

HeLa.B27, C1R.B27 and mFib.B27 cells were stained by HC-10 and ME-1 antibodies followed by APC-conjugated antimouse IgG antibody. Dead cells were excluded using LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies). BD LSRRFortessa and Diva software were used. The latter converts channel value into fluorescence intensity using a logarithmic algorithm, therefore geometrical mean fluorescence intensity was used to quantify the intensity of HC-10 and ME-1 staining.

Intracellular cytokine staining of Th17/Th1 cells was carried out using BD Cytofix/Cytoperm kit (BD Bioscience, UK). Cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1 µg/mL Ionomycin (Sigma) for 4 h in the presence of Golgi STOP and Golgi plug. After surface staining using CD3-BV605, CD4-APC and CD8-BV510 antibodies (Biolegend), cells were fixed and permeabilised, stained with interleukin (IL)-17A-FITC (Ebioscience) and interferon (IFN)-γ-AF700 (Biolegend). Dead cells were excluded using Fixable Viability Dye eFlour 780 (Ebioscience).

KIR3DL2CD3e: reporter cell assay
The KIR3DL2CD3e reporter cells secreting IL-2 have been previously described.9 10 See online supplementary methods for details.

Coculture of CD4+ T cells with APCs
About 100 000 CD4+ T cells were cocultured with 5000 HeLa.B27/C1R.B27 cells in 50 µL/200 µL R10 supplemented with 1 pg/mL staphylococcal enterotoxin B (SEB) and 50 ng/mL IL-2. Supernatants were harvested on day 3 for IL-17A ELISA (Ebioscience) cells stained on day 6 for Th17.

ERAP1 inhibition of AS PBMCs
About 100 000 AS PBMCs were cultured overnight in 100 µL R10 with/without the addition of DG013A (1000 nM), stained for classical HLA-B27 and FHC surface expression. For Th17 responses, 500 000 AS PBMCs were cultured in 200 µL R10 with the addition of SEB (1 pg/mL), IL-2 (50 IU/mL) and DG013A (1000 nM) for 6 days. Supernatants were collected on day 3 for IL-17A ELISA, cells stained on day 6 for Th17. DG013A was replenished daily, SEB and IL-2 on day 3.

Statistics
Results are expressed as mean and SE of mean (figures 1, 4C, D and 5) or SD (other figures). The statistical significance of differences between means was assessed using Mann-Whitney test (figure 1), unpaired two-tailed t test (figures 2 and 4A, B), one way Analysis of variance (ANOVA) (figure 3) or paired two-tailed t test (figures 4C, D and 5). A p value <0.05 was considered statistically significant.

RESULTS
Protective ERAP1 variants are associated with reduced HLA class I FHC expression by AS monocytes
We first investigated the effect of ERAP1 polymorphisms on FHC expression in PBMCs from HLA-B27-positive patients with AS. Three AS-associated ERAP1 SNPs were studied: rs30187 (T/C, K528R), rs27044 (G/C, Q730E) and rs2287978 (T/C, M349V), rs30187 (T/C, K528R) and rs27044 (G/C, Q730E).
M349V). Risk alleles and their corresponding amino acids are underlined. No significant difference in the age, BASDAI, BASFI, BASMI was present between genotypical groups (see online supplementary table S1). Figure 1 shows that monocytes from patients carrying AS protective alleles of rs30187 or rs27044 (528R or 730E) express significantly lower absolute (HC-10) and relative (HC-10/ME-1 ratio) levels of HLA class I FHCs. A similar but non-significant trend was seen in B cells and T cells. No significant difference was found between patients carrying risk and protective alleles of rs2287987 (see online supplementary figure S1).

ERAP1 silencing or inhibition reduces HLA-B27 FHC expression

The K528R and Q730E ERAP1 variants, corresponding to protective alleles of rs30187 and rs27044 respectively, have been shown to reduce enzyme activity.17 18 22–24 We therefore explored the effects of ERAP1 silencing on HLA-B27 FHCs expression. Lentiviral ERAP1-shRNA was used to stably suppress the expression of endogenous ERAP1 by HeLa.B27 and C1R.B27 cells (85–90% suppression, see online supplementary figure S2). As shown in figure 2A–D, ERAP1 silencing led to a 30–40% reduction of HLA-B27 FHC expression by HeLa.B27 and C1R.B27 cells. Unlike the untransfected C1R cells, which express low levels of MHC class I molecules and bind only weakly to HC-10 antibody, untransfected HeLa cells could be stained by HC-10 (see online supplementary figure S3). In order to exclude the possibility that the reduced HC-10 staining following ERAP1-silencing in HeLa.B27 cells was due to down-regulation of other MHC class I, we silenced ERAP1 in untransfected HeLa. This did not decrease HC-10 staining (see online supplementary figure S4). In addition to ERAP1 silencing, we also examined the effect of ERAP1 inhibition on HLA-B27 FHC expression using a recently identified compound, DG013A.25 DG013A is a phosphinic pseudopeptide that binds to the catalytic site of ERAP1. It also inhibits ERAP2 and leucyl/cystinyl aminopeptidase (LNPEP) (both AS-associated).25 26 Figure 2E–H show that DG013A decreased FHC expression by HeLa.B27 and C1R.B27 cells in a dose-dependent manner, without reducing ME-1 levels. Moreover, ERAP1 silencing or inhibition also significantly reduced the staining by HD-6, an antibody raised against HLA-B27 homodimers (see online supplementary figure S5).10 27

Expression of wild type (WT)-ERAP1, but not AS protective variants, in ERAP1-silenced HeLa.B27 and ERAAP−/− mFib. B27 cells increases FHC expression

To further confirm the role of ERAP1 in determining HLA-B27 FHC expression, we next introduced WT-ERAP1 into ERAP1-silenced HeLa.B27 and ERAAP−/− mFib.B27 cells. SH RNA-resistant ERAP1 plasmids encoding WT and variants were constructed by introducing multiple synonymous mutations in the shRNA-targeting sequence (see online supplementary figure S6A). Online supplementary figure S6B shows that ERAP1 was successfully expressed in ERAP1-silenced HeLa.B27 cells using shRNA-resistant ERAP1 plasmids but not unmutated WT-ERAP1. Expression of WT-ERAP1 significantly upregulated...
the expression of HLA-B27 FHCs in ERAP1-silenced HeLa.B27 (figure 3A, B) and ERAAP−/− mFib.B27 cells (figure 3C, D) (see online supplementary figure S7 for representative stains). Transfection using WT-ERAP1 without synonymous mutations did not affect the expression of surface FHCs, further confirming that the increase of FHC expression is due to introduction of ERAP1 (see online supplementary figure S6C).

Figure 3 also shows that, in ERAP1-silenced HeLa.B27 and ERAAP−/− mFib.B27 cells, the K528R, Q730E and K528R/Q730E variants did not upregulate FHCs. The double variant was studied because of the genetic linkage between K528R and Q730E (r²=0.734, D'=1, SNP Annotation and Proxy Search). Notably, no significant difference in ERAP1 expression levels was observed between ERAP1 variants (figure 3A, C).
ERAP1 silencing or inhibition of HLA-B27-expressing APCs reduces ligation of KIR3DL2 by HLA-B27 FHCs and Th17 responses from AS CD4+ T cells

The best characterised function of HLA-B27 FHCs is their interaction with KIR3DL2. HLA-B27 FHCs bind to KIR3DL2 more strongly than classical HLA-B27 molecules, and stimulate the survival, proliferation and IL-17A production of KIR3DL2+ CD4+ T cells from patients with AS.9 We therefore asked if ERAP1 suppression/inhibition affects functional recognition of HLA-B27-expressing APCs by KIR3DL2. When HeLa.B27 or C1R.B27 cells were cocultured with KIR3DL2CD3ε reporter cells, ERAP1 silencing (figure 4A) or inhibition (figure 4B) reduced the activation of this receptor (reflected by IL-2 production). Blocking with either HC-10 or DX31 (specific for KIR3DL2) antibodies reduced IL-2 to basal levels, confirming that IL-2 secretion in this assay is due to the interaction of HLA-B27 FHCs and KIR3DL2.

We next investigated whether ERAP1 silencing or inhibition of APCs affected Th17 expansion when the APCs were cocultured with CD4+ T cells from patients with AS.4 We therefore asked if ERAP1 suppression/inhibition affects functional recognition of HLA-B27-expressing APCs by KIR3DL2. When HeLa.B27 or C1R.B27 cells were cocultured with KIR3DL2CD3ε reporter cells, ERAP1 silencing (figure 4A) or inhibition (figure 4B) reduced the activation of this receptor (reflected by IL-2 production). Blocking with either HC-10 or DX31 (specific for KIR3DL2) antibodies reduced IL-2 to basal levels, confirming that IL-2 secretion in this assay is due to the interaction of HLA-B27 FHCs and KIR3DL2.

ERAP1 inhibition reduces surface HLA class I FHCs and Th17 cell frequency of PBMCs in patients with AS

We lastly examined the potential of ERAP1 inhibition to modulate FHC expression and Th17 expansion in PBMCs from patients with AS. Figure 5A shows that overnight treatment of PBMCs using DG013A decreased HLA class I FHC expression by monocytes and B cells. No effect was observed on T cells (see online supplementary figure S8A). ERAP1 inhibition also decreased the Th17 expansion from AS PBMCs after 6 days (figure 5B), without significantly reducing the frequency of IFN-γ-producing Th1 cells (see online supplementary figure S8B).

**DISCUSSION**

In this study we show that ERAP1 plays an important role in determining the expression levels of cell surface HLA-B27 FHCs. Genetic silencing and chemical inhibition of ERAP1 led to similar reduction of HLA-B27 FHC expression, indicating that the effect is specific. By contrast, the introduction of wild-type ERAP1 into ERAP1-silenced or ERAAP-knockout cells increased FHC expression. Increased monocyte FHC expression in patients with AS has been described previously,4-6 but in these studies ERAP1 was not genotyped. We show for the first time that patients with AS carrying protective allelic variants of ERAP1, 528R and 730E, have reduced monocyte HLA class I FHC expression. Our result provides an immunological explanation for the low AS risk conferred by these two ERAP1 variants. The fact that the 528R and 730E variants, similar to ERAP1 silencing and inhibition, have downregulating effect on HLA-B27 FHCs suggests that they are effectively loss-of-function variants. This is supported by our finding that introduction into ERAP1-silenced or ERAP1−/− cell lines of these variants, singly or in combination, does not upregulate HLA-B27 FHC expression. This is also in accordance with previous in vitro studies using synthetic peptides and cell-based HLA-B27 peptidome studies.17 19 22-24 28 We did not observe a
significant effect for the 349V variant, noting that its genetic association with AS is much weaker. Notably, although all patients with AS in this study were HLA-B27-positive, the contribution of other HLA class I molecules to PBMC FHC expression could not be excluded. Our results differ in some aspects from the findings of Haroon et al., which found that 730E increased monocyte FHC levels in patients with AS and 528R had little effect. They also found that ERAP1 siRNA silencing of C1R.B27 cells did not alter cell surface FHC expression. In the current study we have achieved more efficient and stable ERAP1 silencing using shRNA, and studied the HeLa.B27 cells in addition to C1R.B27 cells. Moreover, in addition to monocytes of patients with AS, we showed the reduction of FHC expression by the 528R and 730E mutations in ERAP1-reconstituted cell lines. Finally, we also studied the effect of ERAP1 inhibition on FHC expression in cell lines and AS PBMCs.

We also show that ERAP1 silencing or inhibition of APCs has functional effects on KIR3DL2 stimulation and Th17 cell expansion. Silencing or inhibition of HeLa.B27 and C1R.B27 cells reduced recognition by a KIR3DL2 reporter cell line by approximately 30%, similar to the amount of reduction in FHC surface expression. When CD4+ T cells in patients with AS were cultured with APCs, ERAP1 silencing or inhibition reduced Th17 expansion and IL-17A production. These finding suggest that ERAP1 inhibition might have therapeutic benefit in AS.

Our findings might at first appear to be difficult to reconcile with our previous finding that ERAP1 silencing increases the length of peptides (11–13 polymers) bound to HLA-B27. However, these long peptides stabilised classical surface HLA-B27 molecules just as well as 9 nonamer HLA-B27 epitopes in T2 HLA-B27 stabilisation assays, suggesting that HLA-B27 complexes loaded with longer peptide ligands are in...
fact as stable as those carrying 9–10 polymer epitopes. These longer peptides are potentially optimal ligands for classical HLA-B27 complex formation, and are enriched when ERAP1 is silenced. Thus suppression of ERAP1 might increase the overall pool of high affinity peptide ligands available for binding to HLA-B27. This could explain our finding that surface HLA-B27 FHC expression is reduced by ERAP1 silencing or inhibition. Therefore, ERAP1 might contribute to disease by actively destroying HLA-B27-binding peptide epitopes. Indeed, WT-ERAP1 has been shown to more rapidly destroy multiple HLA-B27-destined peptides than loss-of-function ERAP1 variants.28 Recently published data has suggested that ERAP1 allotypes/haplotypes with abnormally high or low peptide trimming activity may predispose to AS by limiting the supply of optimal peptides to HLA-B27 and therefore adversely affecting its conformational stability.30 Alternatively, ERAP1 may contribute to a chaperone activity within the ER resulting in accelerated egress of HLA-B27 FHCs and classical HLA-B27 molecules. As shown in figure 2A–D, in addition to reducing HLA-B27 FHC surface expression, ERAP1 silencing of APCs has a trend, although not significant, to downregulate surface classical HLA-B27.

Notably DG013A is a potent inhibitor for ERAP2 and LNPEP as well as ERAP1.25 While we cannot exclude effects on these aminopeptidases, it is important to note that ERAP2 and LNPEP are also associated with AS.26 Any off-target effect could thus potentially be beneficial in AS treatment. Moreover, the reduction of HLA-B27 FHC levels by DG013A was not seen in 221.B27 cells (data not shown) and reduction of PBMC HLA class I FHC expression was not seen for two out of nine patients with AS (figure 5A). ERAP1 inhibitors with higher selectivity and/or potency are worth investigating. Overall, our data show that ERAP1 plays a critical role in determining levels of HLA-B27 FHC expression and Th17 responses in AS. ERAP1 silencing or inhibition reduces HLA-B27 FHC expression. Disease-protective ERAP1 alleles are associated with lower FHC expression levels in reconstituted cell lines and patients with AS. We also provide the first evidence that ERAP1 inhibition may suppress Th17 response in AS. Therefore, ERAP1 inhibition could potentially be used for therapy in AS.

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Contributors LC, SK and PB designed the study, MHA, HB, AC and PB recruited patients, LC, AR and AH performed experiments, DG provided key reagents, LC drafted the manuscript, PB finalised the manuscript.

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Competing interests None declared.

Patient consent Obtained.
REFERENCES


Figure S1  AS patient PBMC HLA class I free heavy chain expression is not affected by ERAP1 variant, M349V. HLA class I FHCs (HC-10 antibody) and classical HLA-B27 complexes (ME-1 antibody) surface expression by CD14+ monocyte, CD19+ B cell and CD3+ T cell from AS patient PBMCs are shown. HC-10 staining, ME-1 staining and HC-10/ME-1 are compared between AS patients with different genotypes of rs2287987 (TT, CT, CC). Risk allele and corresponding amino acid are underlined. Results are expressed as mean and standard error of mean, P value was determined by Mann-Whitney test.
Figure S2. Stable silencing of endogenous ERAP1 expression in HeLa.B27 (A) and C1R.B27 (B) cells using lentiviral ERAP1-shRNA. Protein band intensity was calculated using Image-J.
Figure S3  ME1 stains only HeLa.B27 and C1R.B27 cells, HC-10 stains HeLa and C1R but to a lesser extent compared to cells expressing B27.
Figure S4. ERAP1 silencing does not reduce MHC class I free heavy chain expression (HC-10) by HeLa cells (n=2).
Figure S5 ERAP1 silencing or inhibition reduces surface HD-6-reactive molecule by HeLa.B27 or C1R.B27 cells. Representative HD-6 staining of ctr-shRNA and ERAP1-shRNA-transduced HeLa.B27 cells (A) and C1R.B27 cells (C) are shown; corresponding summary is shown in (B) and (D). Representative stains of HeLa.B27 cells (E) and C1R.B27 cells (G) treated with 1000 nM DG013 are shown. Summaries of DG013A titration (0, 10, 100, 1000 nM) on HeLa.B27 cells and C1R.B27 cells are also shown in (F) and (H). All experiments were repeated three times. Results are expressed as mean and standard deviation. P value was determined using unpaired two-tailed t-test (* = P<0.05, ** = p<0.01).
Figure S6  Expression of ERAP1 in ERAP1-silenced cells using shRNA-resistant (SR) ERAP1 plasmid. (A) Mutation of ERAP1 shRNA binding site. (B) ERAP1 is expressed in ERAP1-silenced cells only by transfection using SR-ERAP1 variants, not un-mutated WT-ERAP1. (C) HLA-B27 free heavy chain expression (HC-10) is altered by transfection using SR-WT-ERAP1, but not WT-ERAP1.
Wild type ERAP1, but not the protective variants K528R or Q730E, increases cell surface HLA-B27 free heavy chain expression. Representative HC-10 and ME-1 stains of ERAP1-silenced HeLa.B27 cells (A) and ERAAP-/- mFib.B27 cells (B) transiently expressing WT-ERAP1 and protective ERAP1 variants (K528R, Q730E, K528R/Q730E).
Figure S8  Effect of ERAP1 inhibition on AS patient T cell HLA class I expression and PBMC IL-17A secretion.  (A) ERAP1 inhibition by DG013A does not affect HC-10 and ME-1 staining of CD3+ T cells from AS PBMCs.  (B) ERAP1 inhibition by DG013A does not affect the IL-17A secretion by AS PBMCs.
Cell lines

As described previously, HeLa.B27, C1R.B27 and mouse ERAP associated with antigen processing (ERAAP)-/- fibroblasts (ERAAP-/- mFib.B27) were transfected to express HLA- HLA-B*27:05. All three cell lines were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 0.1 mg/ml of streptomycin, and 100 units/ml of penicillin (R10).

Construction and transfection of shRNA-resistant ERAP1 plasmids

To reconstitute ERAP1 in silenced cells, multiple synonymous mutations within shRNA-targeting sequence were delivered to wild type (WT)-ERAP1 plasmid (118-139, from AAA-CGT-AGT-GAT-GGG-ACA-CCA-TTT to AAG-CGA-AGC-GAC-GGA-ACG-CCT-TTC). The WT-ERAP1 (accession number: BC030775) carries the AS risk alleles of rs30187 and rs27044. The shRNA-resistant WT-ERAP1 plasmid was then further mutated to encode AS-protective ERAP1 variants (K528R, Q730E, K528R/Q730E). All mutations were carried out using quickchange lightning multi-site-directed mutagenesis kit (Agilent, USA). These plasmids were transfected into ERAP1-silenced HeLa.B27 or ERAAP-/- mFib.B27 cells using GeneJuice following manufacturer’s instructions (Merck Millipore, Germany).

Isolation of peripheral blood mononuclear and CD4+ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque (Sigma, UK), then frozen and stored in liquid
nitrogen before staining. CD4+ T Cells were negatively isolated from PBMCs using a kit (Miltenyi Biotec, UK). The purity was 90-99% by flow cytometry.

**KIR3DL2CD3ε reporter cell assay**

Briefly, 200,000 Jurkat T cells transduced with a plasmid expressing the KIR3DL2CD3ε fusion protein were incubated for 2 days with 100,000 HeLa.B27/C1R.B27 in 50 μl/200 μl R10 with/without 50 μg/ml blocking antibodies (HC-10, DX31 and IgG2a). DX31 is a monoclonal IgG2a antibody for KIR3DL2. Supernatants were harvested for IL-2 enzyme-linked immunosorbent assay (ELISA) (EBiosciences).
Supplementary Table 1 AS patient demographics

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AS = Ankylosing Spondylitis; BASDAI = Bath Ankylosing Spondylitis Disease Activity Index; BASFI = Bath Ankylosing Spondylitis Functional Index; BASMI = Bath Ankylosing Spondylitis Metrology Index; none of the patients have been treated with TNF-α blockade (In some cases, score for BASDAI (n=4), BASFI (n=9) and BASMI (n=17) was not available).