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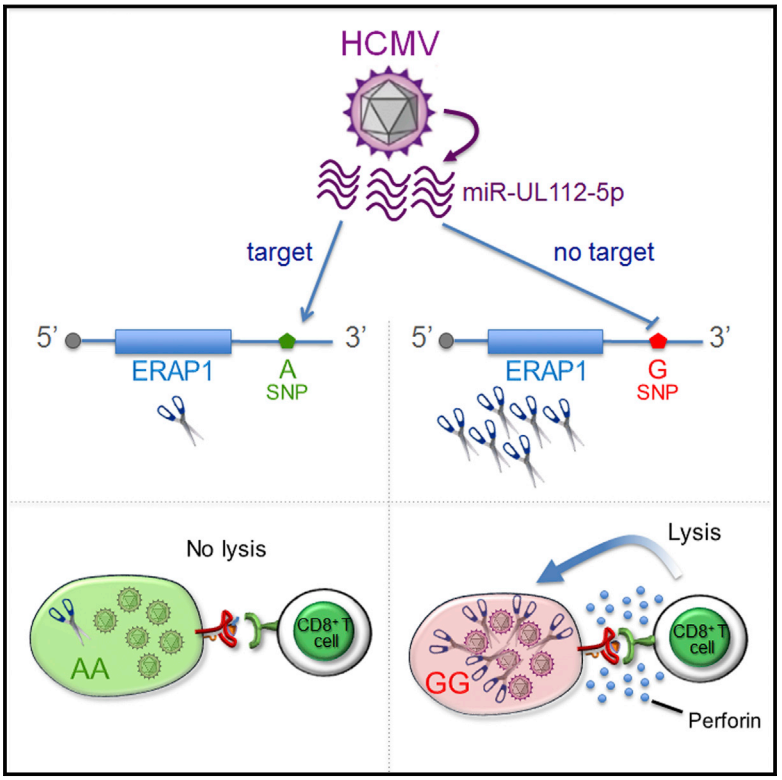
Identification of a Genetic Variation in ERAP1 Aminopeptidase that Prevents Human Cytomegalovirus miR-UL112-5p-Mediated Immunoevasion

Graphical Abstract



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In Brief

Romania et al. identify a single-nucleotide polymorphism in ERAP1 gene, which represents a resistance mechanism to HCMV miR-UL112-5p-based immune evasion strategy, with potential implications for individual susceptibility to viral infection and other diseases.

Highlights

- HCMV miR-112-5p targets ERAP1, preventing the presentation of viral epitopes to CTLs
- The rs17481334 variant G preserves ERAP1 from miR-UL112-5p-mediated degradation
- HCMV-infected GG cells are more efficiently lysed by HCMV-peptide-specific CTLs
- Decreased HCMV seropositivity is detected among multiple sclerosis GG patients

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Identification of a Genetic Variation in ERAP1 Aminopeptidase that Prevents Human Cytomegalovirus miR-UL112-5p-Mediated Immunoevasion

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SUMMARY

Herein, we demonstrate that HCMV miR-UL112-5p targets ERAP1, thereby inhibiting the processing and presentation of the HCMV pp65$_{495-503}$ peptide to specific CTLs. In addition, we show that the rs17481334 G variant, naturally occurring in the ERAP1 3′ UTR, preserves ERAP1 from miR-UL112-5p-mediated degradation. Specifically, HCMV miR-UL112-5p binds the 3′ UTR of ERAP1 A variant, but not the 3′ UTR of ERAP1 G variant, and, accordingly, ERAP1 expression is reduced both at RNA and protein levels only in human fibroblasts homozygous for the A variant. Consistently, HCMV-infected GG fibroblasts were more efficient in trimming viral antigens and being lysed by HCMV-peptide-specific CTLs. Notably, a significantly decreased HCMV seropositivity was detected among GG individuals suffering from multiple sclerosis, a disease model in which HCMV is negatively associated with adult-onset disorder. Overall, our results identify a resistance mechanism to HCMV miR-UL112-5p-based immune evasion strategy with potential implications for individual susceptibility to infection and other diseases.

INTRODUCTION

Human cytomegalovirus (HCMV) is a β-herpesvirus that causes widespread, persistent infection, and it is often lethal in subjects with impaired immunity (Stern-Ginossar et al., 2012). HCMV has evolved multiple strategies to interfere with innate and adaptive immune responses. Virtually, every single step within the MHC class I antigen processing and presentation pathway is targeted by viral proteins (van de Weijer et al., 2015), thus indicating that recognition of viral peptides by CD8$^+$ T cells is a key event in the elimination of HCMV-infected cells.

In addition to viral proteins, HCMV also deploys virally encoded microRNAs (miRNAs) to manipulate host immune responses. Currently, 24 mature miRNAs encoded by HCMV have been identified to target both viral and host genes involved in immune defense, chromatin remodeling, cell cycle regulation, apoptosis, signal transduction, and vesicular trafficking (Grey et al., 2007, 2010; Hook et al., 2014a, 2014b; Kim et al., 2015; Lee et al., 2012; Tirabassi et al., 2011). In particular, three viral

miRNAs have been found to affect components of the immune system: miR-UL112-1 targets *MICB*, a stress-induced ligand of the natural killer (NK) cell activating receptor NKG2D, which is crucial for NK cell-mediated killing of viral-infected cells (Stern-Ginossar et al., 2007); miR-UL148D targets the chemokine *CCL5*, which limits viral infection by recruiting immune cells to the site of infection (Kim et al., 2012); and miR-US4-1 targets *ERAP1*, a key component of the antigen processing that trims precursors into peptides of the correct length to bind MHC class I molecules, resulting in an impaired production of many viral epitopes and a reduced anti-viral $CD8^+$ T cell response (Kim et al., 2011).

Studies in ERAP1-deficient mice have demonstrated that ERAP1 is required for presentation of several viral epitopes, and its absence affects anti-viral $CD8^+$ T cell responses (Blanchard et al., 2010; Firat et al., 2007; Saveanu et al., 2005; Yan et al., 2006). In humans, DNA sequence variation in *ERAP1* has been associated to genetic-risk effects in a number of immune-mediated diseases, including ankylosing spondylitis, psoriasis, Behçet's disease, and multiple sclerosis (MS) (Stratikos et al., 2014). Functional studies have established that these variants affect ERAP1 activity, resulting in changes in the repertoire of antigenic peptides (Reeves et al., 2014), which is critical for shaping innate and adaptive immune responses (Cifaldi et al., 2011, 2015; James et al., 2013). The importance of ERAP1 trimming for immune recognition of virus-infected cells is demonstrated by several findings, including the evolution of escape mutations impairing ERAP1 activity and resulting in decreased $CD8^+$ T cell response (Draenert et al., 2004; Tenzer et al., 2009).

Functional genetic variations in miRNA processing machinery, genes, and target sites, named miR-single-nucleotide polymorphisms (miR-SNPs), have attracted special attention due to their involvement in several types of cancer (Ryan et al., 2010). These variants can affect the transcription of target genes, pre-miRNA processing, and/or modulate miRNA-mRNA interactions by affecting miRNA binding to target sites. It is therefore possible to envisage a complex scenario where ERAP1 functional polymorphisms may be relevant to individual susceptibility to both HCMV infection and (directly or indirectly) autoimmunity, through gene-environment (exposure) interactions.

In this study, we demonstrate that HCMV miR-UL112-5p specifically targets ERAP1, thereby inhibiting the processing and presentation of the HCMV $pp65_{495\text{-}503}$ peptide to specific cytotoxic $CD8^+$ T cells. In addition, we found a naturally occurring variant in the 3′ UTR of the *ERAP1* gene (SNP rs17481334), which prevents ERAP1 targeting and degradation by HCMV miR-UL112-5p.

RESULTS

rs17481334 G Disrupts miR-UL112-5p Binding to ERAP1 mRNA 3′ UTR

Recent deep-sequencing analyses of small RNAs from HCMV-infected human fibroblast cells led to the identification of novel viral miRNA precursors and refinement of miRNA annotations (Meshesha et al., 2012; Stark et al., 2012). In particular, sequences obtained for miR-US4-1 (now miR-US4-5p) resulted in being shifted by 5 bp at the 5′ end (Figure S1A), thereby complicating the interpretation of the seed-mediated ERAP1 3′ UTR targeting described so far (Kim et al., 2011). Regardless of the correct sequence of miR-US4-1, ERAP1 protein decreased during infection with wild-type HCMV, whereas it remained unchanged in cells infected with a mutant HCMV strain carrying a defective processing of the miR-US4-1 primary transcript, suggesting a key role for that region of ERAP1 3′ UTR in viral-mediated immune evasion.

An A-to-G polymorphism mapping to the 3′ UTR of the ERAP1 mRNA (SNP rs17481334**)**, and corresponding to one of the mutated nucleotides involved in the study by Kim et al. (Figure S1A), was predicted to disrupt the consensus sequence for miR-US4-1 binding. Because rs17481334 did not match the sequence of mature miR-US4-5p, we looked for other HMCV miRNAs targeting the region encompassing this genetic variant. The RNAhybrid algorithm (Rehmsmeier et al., 2004) predicted the miR-UL112-5p to directly target ERAP1 3′ UTR in that position (Figure S1B). This interaction was validated in vitro using ERAP1 3′ UTR dual-luciferase assays in 293T cells ectopically expressing miR-UL112-5p or a mutated version of miR-UL112-5p with a single base substitution corresponding to the SNP (Figure S1B). A significant decrease in luciferase activity was observed for the cells expressing the rs17481334 A variant of ERAP1 3′ UTR and the wild-type miR-UL112-5p, as well as for those expressing the rs17481334 G variant and the mutated miR-UL112-5p (Figure 1A). These data suggest that miR-UL112-5p directly targeted ERAP1 in the consensus site and that this interaction is affected by the SNP.

To test whether this differential binding could modulate ERAP1 expression, endogenous ERAP1 mRNA and protein levels were measured in fibroblasts isolated from individuals homozygous for ERAP1 rs17481334 A or G alleles equally transfected with wild-type or mutated miR-UL112-5p (Figure S1C). In these experiments, miR-UL112-5p induced 57% downregulation of ERAP1 mRNA expression in fibroblasts from the rs17481334 AA individual, but had no effect in rs17481334 GG fibroblast cells. Conversely, the mutated miR-UL112-5p had no effect in AA cells, whereas it induced 40% downregulation of ERAP1 mRNA expression in rs17481334 GG cells (Figure 1B). Of note, ERAP1 mRNA was reduced at similar levels in fibroblasts from both individuals by a siRNA targeting a coding region of ERAP1 (siERAP1) (88% and 91% in rs17481334 AA and GG fibroblasts, respectively) (Figure 1B). Virtually identical results were obtained in immunoblot experiments under the same conditions (Figure 1C).

HCMV Infection Fails to Downregulate ERAP1 Expression in rs17481334 GG Fibroblasts

Fibroblasts from rs17481334 AA and GG homozygous individuals were infected with the HCMV laboratory strain AD169 at two different multiplicity of infections (MOI). At 3 days post-infection (dpi), fibroblasts were infected at a similar level, as evaluated by expression of viral immediate-early antigens (IE1 and IE2) and MHC class I downregulation (Figure 2A). HCMV-infected cells sorted for reduced MHC class I expression (Figure 2B; Figure S2) showed a reduced overall amount of ERAP1 protein reaching a maximal decrease of 60% in fibroblasts

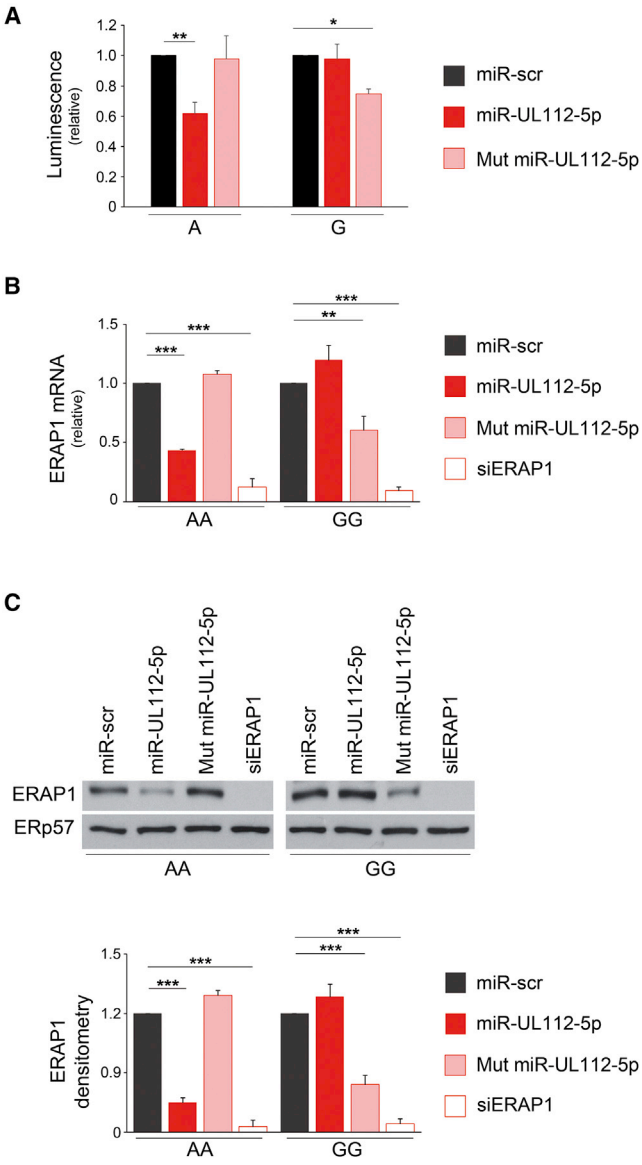

Figure 1. rs17481334 Variant G Abolishes HCMV miR-UL112-5p Binding to the 3′ UTR of ERAP1

(A) Luciferase assays of 293T cells transfected with dual-luciferase reporter vector bearing the rs17481334 A or G variant of ERAP1 3′ UTR and either a control scrambled sequence (miR-scr), miR-UL112-5p, or mutated miR-UL112-5p (Mut miR-UL112-5p). Data are representative of five independent experiments.

(B and C) Real-time qPCR (B) and representative immunoblotting analysis (C) of ERAP1 expression in fibroblasts with AA or GG genotypes, transfected with miR-scr, miR-UL112-5p, Mut miR-UL112-5p, or siERAP1. In (C), an ERp57 antibody (Ab) is used for normalization. Densitometric analysis of ERp57-normalized ERAP1 values of three independent experiments is shown below. Mean ± SD; *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

from AA individuals at MOI 5, as compared to uninfected cells (Figure 2C). Conversely, no changes of ERAP1 protein expression were detected in HCMV-infected fibroblasts from the GG individual (Figure 2C), suggesting that the presence of G at the rs17481334 3′ UTR site prevents ERAP1 downregulation during HCMV infection. Interestingly, the overall amount of ERAP1 protein remained unchanged at different time points in cells infected with a modified HCMV AD169 strain (HCMVΔUL112) deleted in the UL114 gene encompassing the miR-UL112-5p (Stern-Ginossar et al., 2007) (Figure 2D).

rs17481334 Affects the ERAP1-Dependent HCMV $pp65_{495-503}$ $CD8^+$ T Cell Epitope Presentation during HCMV Infection

To directly demonstrate the functional relevance of rs17481334 during HCMV infection, we tested the capability of fibroblasts from AA and GG homozygous individuals to present the HLA-A*0201-restricted immunodominant HCMV $pp65_{495-503}$ epitope, which is one of the well-known viral epitopes requiring ERAP1 activity for its *in vitro* generation (Urban et al., 2012). Thus, we isolated fibroblasts from AA and GG homozygous HLA-A*0201^+ individuals expressing similar levels of ERAP1 and cell surface HLA-A2 molecules, with a similar ability to process and present HCMV $pp65_{495-503}$ peptide to specific cytotoxic T lymphocytes (CTLs) (Figures S3A–S3G). Fibroblasts with these features were infected with wild-type HCMV or HCMVΔUL112 at MOI 1, and their susceptibility to HLA-A*0201-restricted/$pp65_{495-503}$-specific CTLs was evaluated at 3 dpi. Although fibroblasts from either genotype were similar in relation to IE1/2 expression and MHC class I downregulation (Figure 3A), cells from the GG individual infected with wild-type HCMV and expressing unchanged levels of ERAP1 (Figure S3H), were killed more efficiently than those from the AA individual, at all effector/target ratios tested (Figure 3B). This different susceptibility to CTL-mediated killing appeared to depend on miR-UL112-5p targeting of ERAP1, because it was not evident in fibroblast cells from AA and GG individuals infected with HCMVΔUL112 (Figure 3B; Figure S3H). Consistently, GG fibroblasts stably knocked down for ERAP1 were killed less efficiently than control cells when infected with wild-type HCMV (Figures 3C and 3D; Figure S4).

rs17481334 GG Genotype Associates with HCMV-Seronegative Profile in MS Patients

The potential impact of the rs17481334 polymorphism in host-virus interactions was studied by looking at HCMV serology data in relation to genotype, considering the GG homozygous combination the most likely to show biological effects. The G allele occurs at minor allele frequency (MAF) of 0.121 in Caucasians, with GG carriers representing 3% of the general population (ensembl.org). Inspection of genome-wide association study (GWAS) data from a Finnish general population previously studied in relation to HCMV seropositivity (Kuparinen et al., 2012) did not disclose any significant difference for rs17481334 GG homozygotes (data not shown). However, as shown in Figure 4, the same analysis performed in individuals suffering from MS, a condition where HCMV infection has been proposed to play a protective role (Sundqvist et al., 2014; Waubant et al., 2011), detected 5-fold decreased odds of HCMV seropositivity among GG patients compared to all other individuals ($p = 0.021$; odds ratio [OR] = 0.193 [95% confidence interval (CI), 0.041–0.913]).

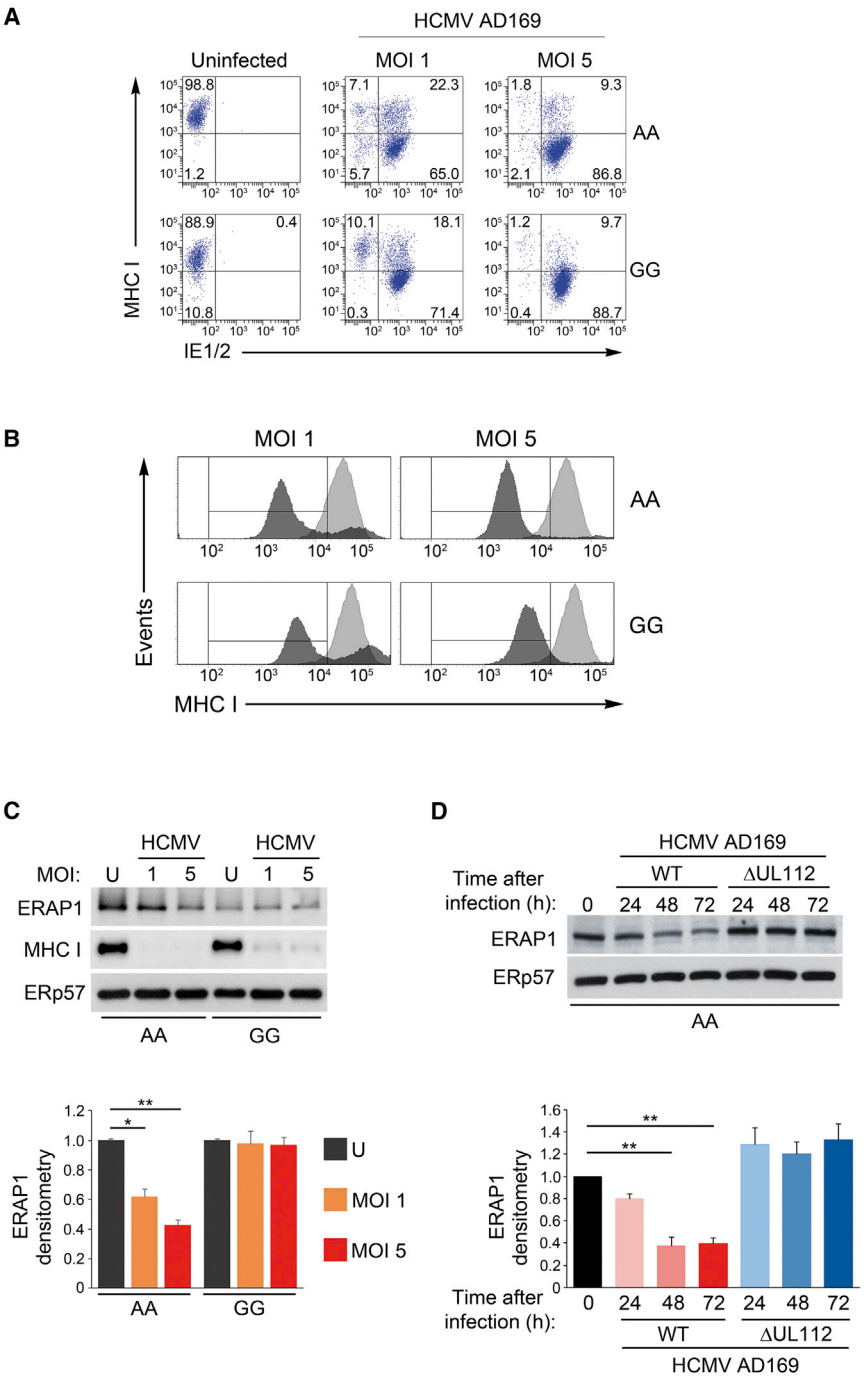

Figure 2. rs17481334 Controls ERAP1 Expression in HCMV-Infected Fibroblasts

(A) Representative flow-cytometric analysis of MHC class I cell surface expression and intracellular IE1/IE2 (IE1/2) viral protein expression in fibroblasts with AA or GG genotypes infected with HCMV AD169 strain at the indicated MOI, 3 days after infection. The percentage of cells in each quadrant is indicated.

(B) Gating strategy used to sort AD169-infected cells based on the viral-mediated MHC class I downregulation. MHC class I expression in uninfected and infected cells is shown in light and dark gray histograms, respectively.

(C) Representative immunoblotting analysis of ERAP1 and MHC class I expression in uninfected (U) or HCMV AD169-infected fibroblasts with AA or GG genotypes. Densitometric analysis of ERp57-normalized ERAP1 values relative to uninfected fibroblasts from three independent experiments is shown.

(D) Representative immunoblotting analysis of ERAP1 in fibroblasts with AA genotype, either uninfected (U) or infected with wild-type HCMV AD169 (WT) or HCMVΔUL112 (ΔUL112) at MOI of 5 at 24, 48, and 72 hr after infection. Densitometric analysis of ERp57-normalized ERAP1 values relative to uninfected fibroblasts from three independent experiments is shown. Mean ± SD; *$p < 0.05$, **$p < 0.001$.

DISCUSSION

In this study, we demonstrated that HCMV miR-UL112-5p targets ERAP1 and that the rs17481334 G variant naturally occurring in the 3′ UTR of the *ERAP1* gene preserves ERAP1 from miR-UL112-5p-mediated degradation. Accordingly, ERAP1 expression was reduced by miR-UL112-5p overexpression both at RNA and protein levels in human fibroblasts from AA individuals, but not in fibroblasts from GG individuals. Consistent with these results, AA fibroblasts infected with the wild-type HCMV, but not with the mutant miR-UL112-5p knockout virus, showed a lower amount of ERAP1 protein. HCMV-infected GG fibroblasts were more efficient in trimming viral antigens to generate immunogenic epitopes and more susceptible to elimination by HCMV-peptide-specific CTLs. Hence, these data demonstrate that the rs17481334 GG genotype hampers the HCMV miR-UL112-5p-based immunoevasion strategy.

HCMV has evolved to establish lifelong latent infections in immunocompetent individuals, with periodic and spontaneous reactivation. This asymptomatic coexistence is reflected by a prompt response of HCMV-specific T cells that dominate the memory compartment of exposed individuals, accounting for approximately 10% of total $CD8^+$ T cells (Sylwester et al., 2005). Conversely, HCMV infection in individuals with either an immature or compromised immune system (e.g., pre- or post-natal infants and transplant recipients, leukemia, or HIV-infected patients, respectively) can cause different diseases, the severity of which depends on the degree of immunosuppression (Halenius and Hengel, 2014).

For a virus to survive in an immunocompetent host, it is essential to avoid the presentation of viral epitopes to $CD8^+$ T cells

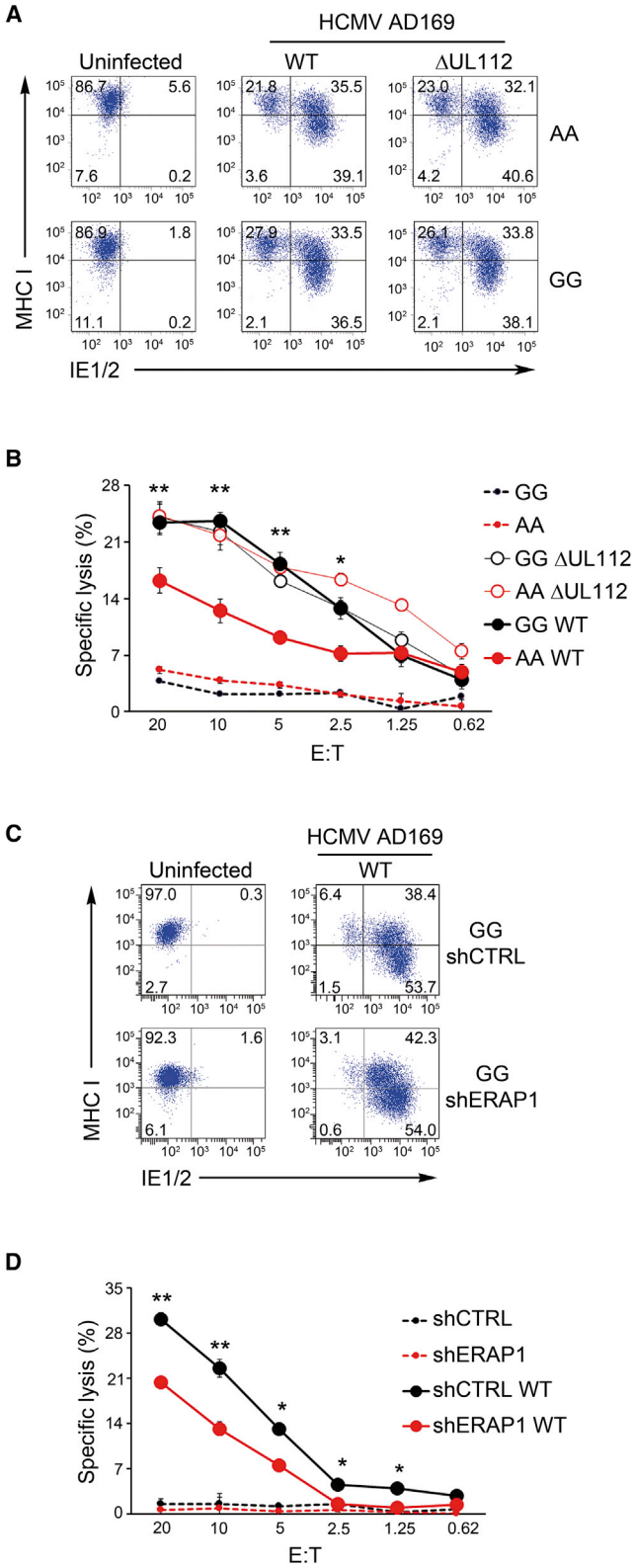

Figure 3. rs17481334 Affects pp65$_{495-503}$ Epitope Presentation

(A) Representative flow-cytometric analysis of cell surface MHC class I expression and intracellular IE1/2 viral protein expression of fibroblasts with AA or GG genotypes both uninfected (U) or infected at MOI 1 with wild-type HCMV AD169 (WT) or HCMVΔUL112 (ΔUL112), 3 days after infection. The percentage of cells in each quadrant is indicated.

(B) ^{51}Cr-release assay of HCMV-infected fibroblasts of (A) co-cultured with HLA-A2-restricted/pp65$_{495-503}$-specific CTLs at the indicated effector/target (E:T) ratio.

(C) Representative flow-cytometric analysis of cell surface MHC class I and intracellular IE1/2 viral protein expression of shERAP1- or shCTRL-transduced GG fibroblasts infected with wild-type HCMV at MOI 1, 3 days after infection. The percentage of cells in each quadrant is indicated.

(D) ^{51}Cr-release assay of HCMV-infected GG fibroblasts of (C) co-cultured with HLA-A2-restricted/pp65$_{495-503}$-specific CTLs at the indicated effector/target (E:T) ratio. All data are representative of three independent experiments. Mean ± SD; *p < 0.05, **p < 0.001.

(Halenius et al., 2015). In this context, HCMV has evolved several strategies to target key components in the MHC class I antigen-processing pathway, including ERAP1 (Kim et al., 2011). We attempted to explore this possibility with our pilot study of HCMV seropositivity in relation to ERAP1 rs17481334 genotype. Unsurprisingly, no significant finding could be detected at the level of the general (Finnish) population, although we observed a significant association when we investigated the relationship between GG genotype and HCMV serology using MS to "model" these interactions. Given the variant allele frequency and the size of the cohort examined, the obtained results, although limited to small number of individuals, were statistically significant. Although the true (if any) impact of GG genotype on HCMV infection, and eventually MS onset, can only be assessed via large-scale longitudinal epidemiological studies taking into account individual viral exposure, it is noteworthy that we observed a 5-fold decreased likelihood (OR = 0.193) of seropositivity among GG individuals with MS (that is, in a disease context where HCMV infection has been proposed to play a protective role) compared to other patients. Hence, although lacking mechanistic relation to causality, these results are intriguing because they suggest that important information may be gained by assessing multiple factors at once and open up novel lines of investigation, where the interplay between HCMV exposure, genotype-driven immune responses, and ultimately disease (autoimmunity/cancer) can be explored toward tailored preventive approaches. Consistently, Li et al. (2011) identified a novel link between HCMV infection and essential hypertension, in which miR-UL112 was independently associated with an increased risk of hypertension. Of note, ERAP1 is also known to play a role in the regulation of blood pressure through its involvement in the renin-angiotensin system (Hattori et al., 2000). Further studies are warranted to confirm and extend these findings, also in relation to the presence of multiple SNP-expression quantitative trait loci (eQTLs) at the ERAP1 locus, including rs17481334 where the G variant appears to associate with lower ERAP1 expression (https://gtexportal.org/).

In addition to the pp65$_{495-503}$ epitope, ERAP1 is known to trim many other immunogenic HCMV epitopes (Kim et al., 2011). Experiments in the ERAAP$^{-/-}$ mouse model have demonstrated that ERAP1 controls the magnitude of CD8^{+} T cell response during murine cytomegalovirus (mCMV) infection (Blanchard et al., 2010). Although neither all HCMV nor mCMV-derived epitopes require ERAP1 activity for their generation, recent mass spectrometry analyses have demonstrated that ERAP1 has a

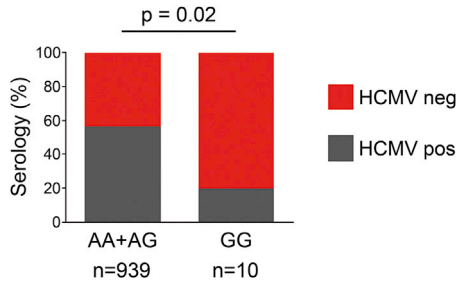

Figure 4. rs17481334 Genotype and HCMV Serology in MS Patients
Serological anti-HCMV Ab profiles are reported for MS patients stratified according to rs17481334 genotype.

significant influence on the overall peptidome (López de Castro et al., 2016; Nagarajan et al., 2016).

In conclusion, we demonstrate that a variant in the 3′ UTR of ERAP1, a key component of MHC class I antigen processing, impairs miR-UL112-5p-mediated mechanism of HCMV immunoevasion, with potential implications for the control of the viral infection itself and, eventually, indirect effects on the risk of HCMV-associated diseases.

EXPERIMENTAL PROCEDURES

3′ UTR Luciferase Assay

HEK293T cells were co-transfected with 1.6 μg of pEZX-MT01 vectors and 80 nmol/L miR-UL112-5p, mutated miR-UL112-5p (Mut miR-UL112-5p), or scrambled control (Ambion) using Lipofectamine 2000 (Invitrogen). After 48 hr, luciferase activity was measured with a LucPair Duo-Luciferase Assay Kit (Genecopoeia). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Transfection, Viral Infections, and Peptide Pulsing

Fibroblasts were transfected with 200 nM of either miR-scramble, miR-UL112-5p, Mut miR-UL112-5p (Ambion), or a siRNA targeting a coding region of ERAP1 (Sigma-Aldrich) (Table S1) with Lipofectamine 2000. As control, cells were transfected with the same amount of an oligo-FITC (Invitrogen) in order to assess the efficiency at 24 hr post-transfection by fluorescence-activated cell sorting (FACS) analysis. Fibroblasts were infected with HCMV strains at 80%–90% confluence in serum-free medium. After 2–3 hr of virus adsorption at 37°C, fresh growth medium was replaced (day 0). At 3 dpi, cells were harvested and analyzed. In some experiments, fibroblasts were infected either with a lentivirus containing pp65-GFP and sorted for GFP expression, or with a non-target shRNA control vector (SHC002), or the ERAP1 shRNA (TRCN0000060542) (Sigma-Aldrich) and selected with 2 μg/mL puromycin for 3 days. For peptide pulsing, cells were seeded in 96-well flat-bottom plates in triplicate and pulsed with 5 μg/mL $pp65_{495-503}$ peptide. After 2 hr, fresh growth medium was replaced.

Proliferation Assay and Chromium-Release Assay

For proliferation assays, infected or peptide-pulsed cells were seeded in 96-well flat-bottom plates in triplicate and incubated with HLA-A2-restricted/$pp65_{495-503}$-specific $CD8^+$ T cells. After 72-hr incubation at 37°C, cells were pulsed with ^{3}H-thymidine (1 μg Ci/well; specific activity, 6.7 Ci/mmol; PerkinElmer) and harvested after 18 hr. ^{3}H-thymidine incorporation was measured by standard procedures with Microbeta Trilux 1450 instrument (PerkinElmer). For cytotoxic activity, ^{51}Cr-labeled infected or peptide-pulsed target cells (5×10^3 per well) were mixed with HLA-A2-restricted/$pp65_{495-503}$-specific $CD8^+$ T cells in 96-well plates in triplicate at various ratios and incubated at 37°C. After 5 hr of incubation, supernatant was collected, and the ^{51}Cr release was measured by a TopCount NXT β detector (PerkinElmer). All experimental groups were analyzed in triplicate, and the specific lysis was determined as follows: 100 × (experimental release − spontaneous release)/(total release − spontaneous release). Spontaneous release lower than about 5% of the total release was observed in all assays.

MS Patient Cohort

Samples from 949 MS patients with ages between 16 and 70 years from the Epidemiological Investigation of Multiple Sclerosis (EIMS) were included in this study. EIMS is a nationwide population-based case-control study of incident cases of MS in Sweden (Hedström et al., 2011). All of the cases fulfilled the McDonald criteria for MS (Polman et al., 2005) and were examined and diagnosed by a neurologist at one of 30 recruiting centers in Sweden. Anti-HCMV serological status was determined in plasma using ELISA as described previously (Sundqvist et al., 2014). The HCMV antigen was prepared from embryonic fibroblasts infected with strain AD169 until complete cytopathic effect. Human plasma samples were analyzed in duplicates, at a dilution of 1/200. Alkaline phosphatase-conjugated, purified $F(ab')_2$ fragment goat anti-human IgG was used as conjugate (Jackson ImmunoResearch). The plates were read at 405 and 650 nm in a Multiskan FC reader (Thermo Fisher Scientific). Cutoff was calculated by adding 0.2 optical density (OD) units to the mean absorbance value of 160 runs of negative control samples. Plasma samples with OD values at cutoff were run in 2-fold dilutions (1/100–1/12,800) and then judged as positive or negative.

Ethics Statement

Ethical approval for generating and analyzing human fibroblasts from skin biopsies was obtained from the Institutional Review Board of the Hospital Bambino Gesù. Written informed consent was obtained from adult patients, or the guardians of patients who were children. The EIMS study has been approved by the Regional Ethical Review Board in Stockholm, Sweden (http://www.epn.se). Written informed consent from all study participants or their parents was obtained. Investigations were carried out according to guidelines from the Declaration of Helsinki.

Statistical Analysis

Data are presented as mean, and error bars indicate the SD. Digital images of western blots were analyzed by ImageJ (https://imagej.nih.gov/ij/index.html), and statistical significance was assessed by the two-way ANOVA test. χ^2 test was used to analyze the different distribution of rs17481334 genotype frequency in HCMV-positive and -negative Swedish MS cases.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.084.

AUTHOR CONTRIBUTIONS

D.F., F.L., and M.D. designed the study. D.F. supervised the work. P.R., L.C., B.P., N.S., V.D., O.M., G.L.P., E.G., and C.C. performed the experiments. R.C. and H.H. provided critical reagents. M.B., T.B., L.A., T.O., I.K., I.S., T.L., M.A.H., and M.D. performed analysis of multiple sclerosis. P.R., L.C., C.C., M.D., and D.F. analyzed and interpreted the data. P.R., M.D., F.L., and D.F. wrote the manuscript. A.S. and C.C. edited the manuscript.

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Cell Reports, Volume *20*

Supplemental Information

Identification of a Genetic Variation in ERAP1 Aminopeptidase that Prevents Human Cytomegalovirus miR-UL112-5p-Mediated Immunoevasion

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Supplemental Experimental Procedures

Cell lines and reagents

293T cells were obtained from the American Type Culture Collection (ATCC, CRL-3216) and cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Human cultured fibroblasts were obtained from skin biopsies of age-matched individuals who underwent punch skin biopsy for dermatological diseases. These cells were grown in DMEM-High glucose medium (4.5 g/L) supplemented with 10% FBS, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. A $CD8^+$ T cell line specific for the immunodominant HLA-A*0201-restricted HCMV $pp65_{495\text{-}503}$ epitope (NLVPMVATV) was produced by weekly restimulation of PBMC with autologous irradiated $pp65_{495\text{-}503}$-pulsed PBMC. HCMV $pp65_{495\text{-}503}$ epitope was produced by INBIOS with a >95% purity.

rs17481334 genotyping with RFLP

rs17481334 was genotyped employing a restriction fragment length polymorphism (RFLP) analysis. Genomic DNA served as template to amplify a 275 bp long product with primers specific for ERAP1-3'UTR (Table S1). The amplicon was subsequently subjected to restriction digestion with *Nla*III for 3 hours and restriction fragments resolved by agarose gel electrophoresis (1.5%). Amplicons derived from the G variant of rs17481334 remained uncut, while those derived from the A variant were cut once resulting in 178 bp and 97 bp long digestion products (data not shown).

Mutagenesis

For the luciferase assay, the 3' UTR of human ERAP1 isoform 2 inserted downstream of the firefly luciferase reporter gene in the pEZX-MT01 vector (Genecopoeia) was mutated by site-directed mutagenesis with the QuickChange II Site Directed Mutagenesis Kit (Stratagene). Mutagenesis was confirmed by DNA sequencing.

PCR Analysis

Total RNA was extracted using TRIzol Reagent (Thermo Fischer Scientific). First-strand cDNA was synthetized using the SuperScript IV Reverse transcriptase (Thermo Fischer Scientific). Primers for RT-PCR analysis are listed in Table S1. Reactions of qPCR were performed using pre-validated PrimeTime qPCR assays from IDT (Hs.PT.47.15073987 for ERAP1 and Hs.PT.47.1164609 for GAPDH). Relative gene expression was determined using the 2-ΔΔCt method with GAPDH as endogenous control.

HLA-A genotyping

Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen). Low- and high-resolution typing for HLA-A loci were performed by PCR sequence–specific oligonucleotides (PCR-SSO; Luminex) and PCR sequence–specific primers (PCR-SSP; Olerup), respectively.

Lentivirus and virus preparation

Lentiviral particles were generated in HEK293T cells by combining pLKO.1 plasmid containing shRNA sequences or a vector encoding viral tegument protein pp65 and GFP under the control of two different promoters, packaging plasmid pCMV-dR8.74, and envelope plasmid VSV-G/pMD2.G.

HCMV AD169 wild-type (ATCC-VR538) and deleted in UL114 gene (HCMVΔUL112) were prepared by infecting semi-confluent monolayers of primary human skin fibroblasts at a virus-to-cell ratio of 0.01,

and cultured until a marked cytopathic effect was seen. Stocks were prepared by 3 rounds of cell freezing and thawing, subjected to centrifugal clarification, and frozen at -80°C. Virus titer was measured by standard plaque assays on HFF cells. Stock solutions used in all experiments contained approximately $2x10^7$ PFU/ml.

Antibodies, flow cytometry and immunoblotting

The following monoclonal antibodies were used: AlexaFluor 488-conjugated anti-IE antigens (MAB810X) from Merck Millipore (Darmstadt, Germany); goat anti-mouse (GAM)-APC from Jackson Immunoresearch Laboratories (West Grove, PA); GAM-FITC from Cappel (Milan, Italy); W6/32 recognizing human fully-assembled MHC class I heavy chains; BB7.2 recognizing human HLA-A2 molecules; 6H9 recognizing denatured human ERAP1. ERAP1 antibody was kindly provided by P. van Endert. Rabbit polyclonal antibody ERp57 recognized human ERp57. Flow cytometry and cell sorting were performed on FACSCantoII and FACSDiva (BD Bioscences). For intracellular staining, cells were trypsinized and permeabilized in ice-cold phosphate-buffered saline (PBS)/ethanol 80%. Afterwards, cells were stained with specific antibodies. Whole-cell extracts, obtained using standard procedures, were quantified by the bicinchoninic acid assay (Pierce), resolved on 8% SDS-PAGE and electroblotted onto Hybond-C paper (Amersham Biosciences). Anti-ERp57 antibody was used as loading control.

Figure S1

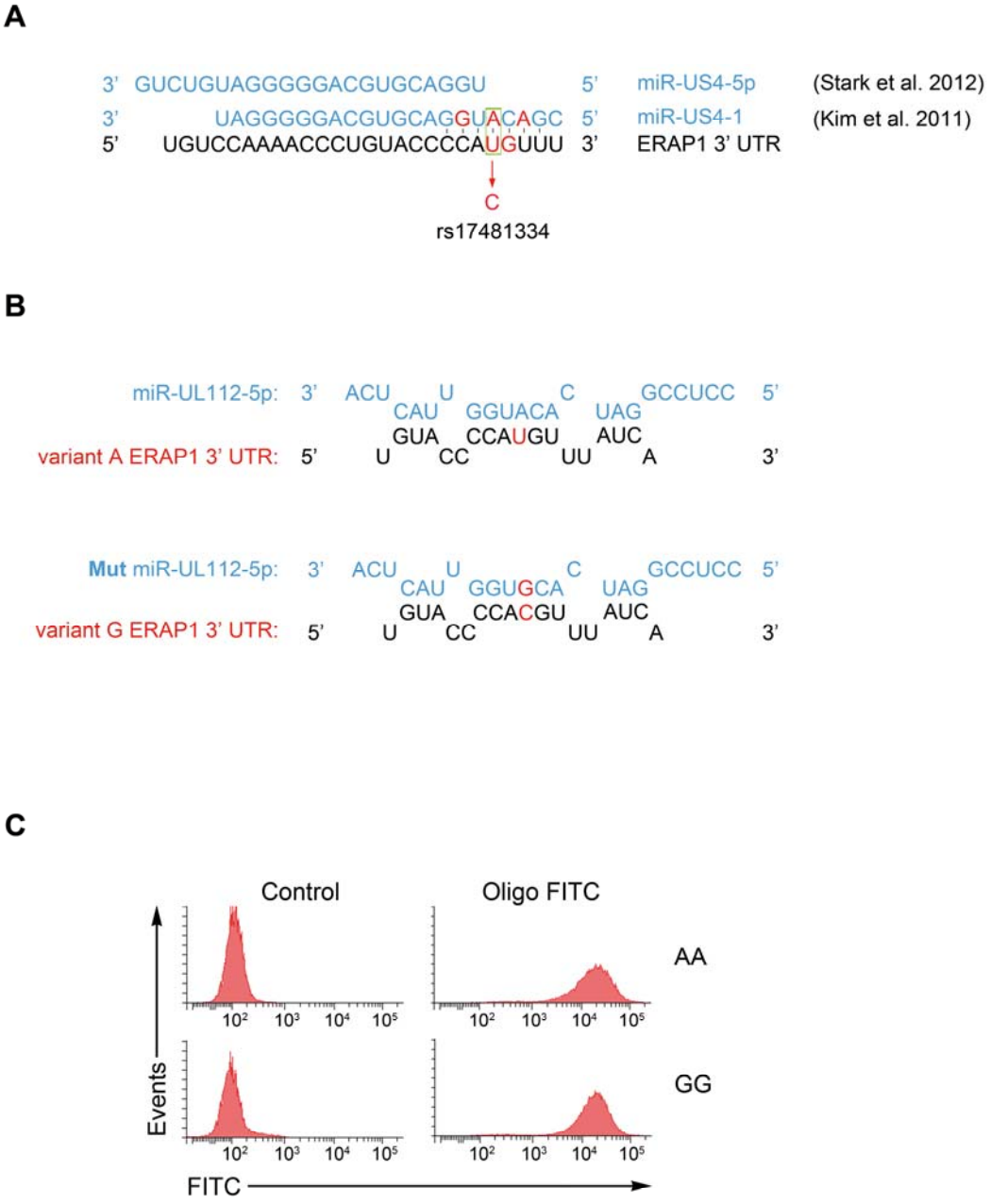

Figure S1, related to Figure 1

A, comparison between miR-US4-5p and miR-US4-1 sequences. Vertical lines between miR-US4-1 and the 3' UTR of ERAP1 indicate the expected seed region interaction site. The mutagenized nucleotides involved in the study by Kim et al., are indicated in red. rs17481334 position is indicated by the green square. **B**, schematic representation of the miR-UL112-5p binding site in the 3'UTR of ERAP1 variant A and the mutated miR-UL112-5p binding site in the 3'UTR of ERAP1 variant G. The mutagenized nucleotide and the rs17481334 are indicated in red. **C**, representative flow cytometric analysis of FITC expression in fibroblast cells with AA and GG genotype transfected with an oligo-FITC.

Figure S2

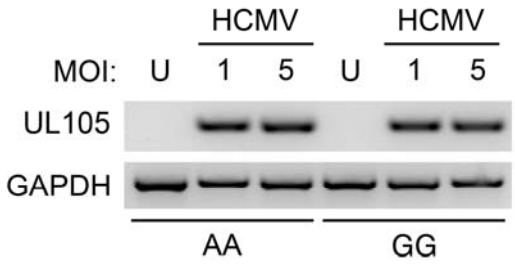

Figure S2, related to Figure 2

RT-PCR of UL105 and GAPDH in fibroblasts with AA or GG genotypes sorted for low MHC class I surface expression after 3 days of HCMV AD169 strain infection at the indicated MOI.

Figure S3

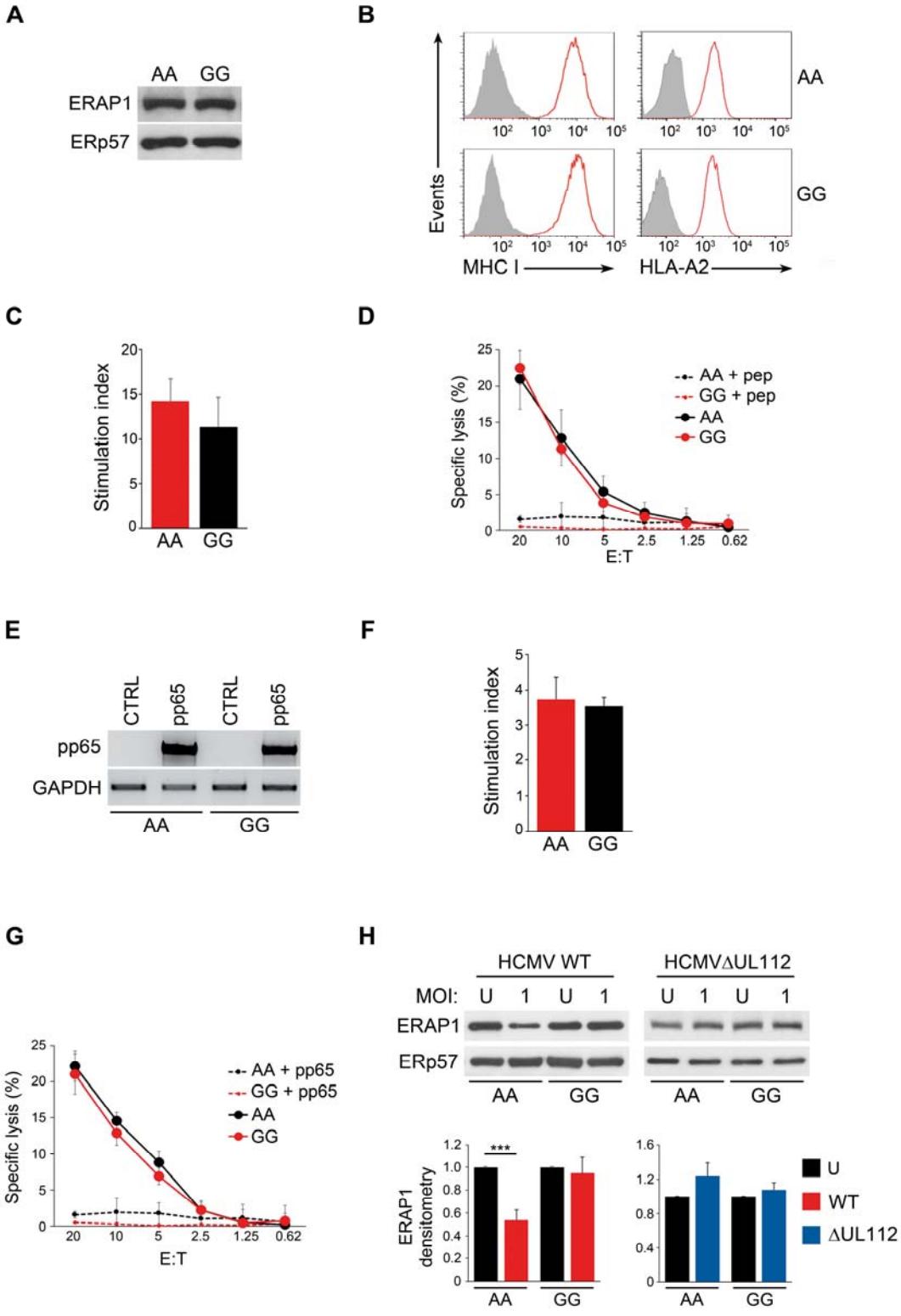
A
AA
GG
ERAP1
ERp57
B
AA
GG
Events
MHC I
HLA-A2
C
Stimulation index
AA
GG
D
Specific lysis (%)
AA + pep
GG + pep
AA
GG
E:T
E
CTRL
pp65
CTRL
pp65
pp65
GAPDH
AA
GG
F
Stimulation index
AA
GG
G
Specific lysis (%)
AA + pp65
GG + pp65
AA
GG
E:T
H
HCMV WT
HCMVΔUL112
MOI: U 1 U 1 U 1 U 1
ERAP1
ERp57
AA
GG
AA
GG
ERAP1 densitometry
***
AA
GG
AA
GG
U
WT
ΔUL112

Figure S3, related to Figure 3A and B

A, immunoblotting analysis of ERAP1 expression in fibroblast cells with AA or GG genotypes. ERp57 was used for normalization. A representative of three independent experiments is shown. **B**, flow cytometric analysis of MHC class I and HLA-A2 cell surface expression in the indicated fibroblasts using W6/32 and BB7.2 mAbs, respectively (red lines). Isotype-matched negative control mAbs are shown in light grey histograms. **C**, stimulation index of HLA-A2-restricted/pp65$_{495\text{-}503}$-specific CD8$^+$ T cells co-cultured with pp65$_{495\text{-}503}$-pulsed fibroblasts. **D**,^{51}Cr-release assay of pp65$_{495\text{-}503}$-pulsed co-cultured with HLA-A2-restricted/pp65$_{495\text{-}503}$-specific CD8$^+$ T cells at the indicated effector:target (E:T) ratio. **E,** RT-PCR of pp65 and GAPDH in fibroblasts with AA or GG genotypes infected with a lentivirus encoding HCMV pp65. **F**, stimulation index of HLA-A2-restricted/pp65$_{495\text{-}503}$-specific CTLs co-cultured with pp65-infected fibroblasts. **G**, ^{51}Cr-release assay of pp65-infected fibroblasts co-cultured with HLA-A2-restricted/pp65$_{495\text{-}503}$-specific CD8$^+$ T cells at the indicated effector:target (E:T) ratio. **H**, representative immunoblotting analysis of ERAP1 expression in fibroblasts infected with wild-type HCMV (WT) or HCMVΔUL112 at MOI 1, after 3 days of infection. ERp57 was used for normalization. Densitometric analysis of ERp57-normalized ERAP1 values relative to uninfected fibroblasts from three independent experiments is shown. Mean ± sd; *** $p<0.0001$.

Figure S4

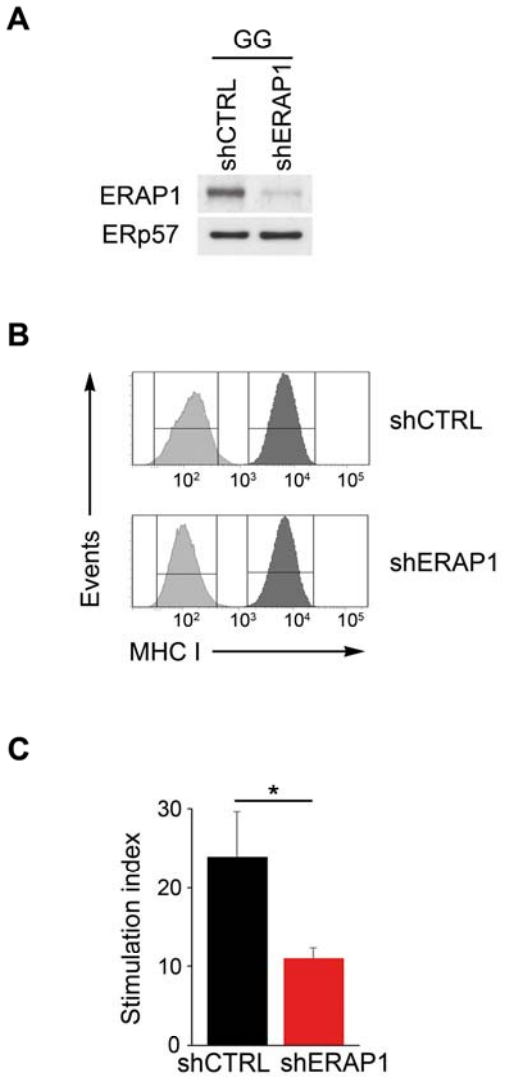

Figure S4, related to Figure 3C and D

A, immunoblotting analysis of ERAP1 expression in fibroblasts with GG genotype transduced with lentiviral vectors encoding either control shRNA (shCTRL) or ERAP1 shRNA (shERAP1). ERp57 was used for normalization. A representative of three independent experiments is shown. **B**, flow cytometric analysis of MHC class I expression in fibroblasts using W6/32 mAb (dark grey histogram). Isotype-matched negative control mAb is displayed in light grey histogram. **C**, stimulation index of HLA-A2-

restricted/pp65$_{495\text{-}503}$-specific CTLs co-cultured with shERAP1- or shCTRL-transduced GG fibroblasts infected with wild-type HCMV at MOI 1, 3 days after infection. Data are representative of three independent experiments.

Table S1

Primers used in this study, related to Fig 1, S2, S3 and methods

siERAP1	sense	GGGCAAGUCUCAUUAACAAdTdT
	antisense	UUGUUAAUGAGACUUGCCCdTdT
UL105 (RT-PCR)	Forward	CAAGAGGAGACCCACGACAT
	Reverse	GAAAAATGTCGCGGAAGGTA
pp65 (RT-PCR)	Forward	ATGGAGTCGCGCGGTCGCCG
	Reverse	TCAACCTCGGTGCTTTTTGG
GAPDH (RT-PCR)	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA
ERAP1[a] 3'-UTR (RFLP)	Forward	GCCCCTGGGCATGGAGCAAG
	Reverse	CCCAAGTGTTGGGTTCCCTGCC

[a] These primers were specific for the ERAP1 isoforms 2 and 3.