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

Graphical Abstract

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

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SUMMARY

Herein, we demonstrate that HCMV miR-UL112-5p targets ERAP1, thereby inhibiting the processing and presentation of the HCMV pp65495-503 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 MICA/B, 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_{495-500} 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 (Mesheha 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.
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 pp65495–503 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 pp65495–503 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 pp65495–503 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/pp65495–503-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]).
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.
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...
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 (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<sub>495-503</sub> peptide. After 2 hr, fresh growth medium was replaced. Supernatant was collected, and the yield 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 (Liedström 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')<sub>2</sub> fragment goat anti-human IgG was used as conjugate (Jackson ImmunoResearch). The plates were read at 405 and 650 nm in a Multiscan 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 ± standard error of the mean (SEM). 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<sup>2</sup> 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**

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