Dynamic Co-evolution of Host and Pathogen:
HCMV Downregulates the Prevalent Allele MICA*008 to Escape Elimination by NK Cells

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Supplemental Figures

Figure S1, associated with figure 1, US9 expression specifically reduces MICA surface expression in certain cell lines. (A) Intracellular localization of US9 demonstrated by confocal microscopy of US9 overexpression in RKO cells, stained with an anti-HIS tag antibody (green) and an anti-protein disulfide isomerase (PDI) antibody (ER marker; red). Nuclei are stained with DAPI (blue). The average correlation coefficient for colocalization between the two antibodies is indicated in the figure, calculated for >30 cells. (B) FACS staining for stress ligands (indicated in the figure) of the indicated cell lines transduced with an empty vector (EV; black histogram), US8 (blue histogram) or US9 (red histogram). Gray-filled histograms represent secondary antibody staining. Representative of two independent experiments.
Figure S2, associated with figure 2, US9 regulates MICA post-transcriptionally. (A) A single exposure of the same western blot as in figure 2A. (B) Western blot was performed on lysates obtained from the indicated cells expressing empty vector (EV), US8 or US9, using the indicated antibodies. Anti-GAPDH served as loading control. (C) Western blot was performed using anti-MICA antibody on lysates from RKO cells expressing EV, MICA*004-HA or MICA*008-HA and co-expressing EV, US7, US8 or US9 (listed as 7, 8 and 9 in the figure). Arrow indicates MICA form not susceptible to US9. Anti-PP2AC served as loading control.
Figure S3, associated with figure 3, US9 targets MICA*008 to proteasomal degradation. (A-B) RKO-MICA*008-HA cells co-transduced with empty vector (EV) (A) or US9 (B) were left untreated (N), or incubated for 8 hours with the translation inhibitor cycloheximide (CHX, 50 μg/ml), in combination with the appropriate mock-treatment or with the following inhibitors: leupeptin (LEU, 100 μg/ml), concanamycin A (CCM A 20 nM), epoxomicin (EPX, 2,4,8 μM), bortezomib (BTZ, 2,4,8 μM) or MG132 (4,8,16 μM). Following treatment, cells were lysed and blotted with anti-MICA. Anti-vinculin served as loading control. Arrows indicate MICA forms with and without carbohydrates (CH⁺⁻⁺⁻). (C-D) RKO cells transduced with MICA*008-HA and co-transduced with EV (C) or US9 (D) were left untreated (N), or incubated for 8 hours with CHX (50 μg/ml), in combination with mock-treatment (DMSO) or with epoxomicin (EPX, 2 μM). Cells were lysed and the lysates were untreated or digested with endoH or with PNGaseF (N, H and F, respectively). Blot was performed using anti-MICA. Anti-vinculin served as loading control. Arrows indicate MICA forms with and without carbohydrates (CH⁺⁻⁺⁻).
Figure S4, associated with figure 4, specific features of MICA*008 are required for US9-mediated downregulation, and a common variant of US9 is functional against MICA*008. (A) Anti-HA tag FACS staining of the same cells as in figure 4B, transduced with empty vector (EV; black histograms) or with US9 (red histograms). Grey-filled histograms represent secondary antibody staining. Representative of three independent experiments. (B) Western blot of the same cells as in figure 4B was performed with anti-MICA. Anti-GAPDH served as loading control. (C) Lysates from RKO cells transduced with the constructs described in figure 4A were treated with PNGase F and immunoblotted using anti-MICA. Anti-vinculin served as loading control. (D) Alignment of US9 sequences derived from Merlin (F5HC33; full length), AD169 (P09729; full length), HAN38 (ACT81817; truncated) and Towne (AAO22945; truncated) strains. Underlined letters mark the transmembrane region; red highlights mismatched amino acids. (E) FACS staining of RKO cells transduced with the indicated MICA allele and co-transduced with EV (black histogram) or with the 232AA variant of US9 (red histogram). Grey-filled histograms represent secondary antibody staining. Representative of 3 independent experiments.
Figure S5, associated with figure 5, US9 reduces MICA*008 surface expression during HCMV infection. (A) Quantitative RT-PCR for US9 relative expression. HFF of the indicated line were uninfected or infected at an MOI of 2-4 with AD169varL (WT) or ΔUS9. RNA was harvested at the indicated hours post infection (hpi). The figure shows the normalized relative expression from two independent experiments. A.U. = arbitrary units. Error bars represent SEM. (B) FACS staining for MICA of the indicated HFF, which were uninfected or infected at an MOI of 2 with TB40/E pp150-GFP (described in Sinzger et al., 2008), at 72 hpi. (C) FACS staining for additional stress ligands (indicated in the figure) of the indicated cell lines that were either uninfected or infected at an MOI of 2-4 with the indicated virus, 72 hours post-infection. Black histogram – uninfected cells, blue histogram – AD169varL, red histogram – ΔUS9. Grey-filled histograms represent staining with an isotype matched control. Representative of two independent experiments.
Figure S6, associated with figures 6 and 7, US9 and other viral factors act on MICA*008 during HCMV infection. (A-C) NK cells were untreated, incubated with an isotype-matched control mAb (12E7) or incubated with anti-NKG2D, and then incubated for 5 hours with FLS3 HFF, expressing either empty vector (EV; A), MICA*004 (B) or MICA*008 (C), which were uninfected (UI) or infected with AD169\textit{varL} (WT) or with ΔUS9 viruses for 72 hours at an MOI of 2-4. Errors bars represent SD. A student’s T-test was performed to evaluate significance. * = P<0.05. N.S. = non-significant. Representative of two independent experiments. (D) Confocal microscopy of FLS3 HFF expressing MICA*008 that were grown on glass slides and either uninfected or infected at an MOI of 2-4 with the indicated virus. Slides were stained with an anti-MICA antibody (green) and an anti-protein disulfide isomerase (PDI) antibody (ER marker; red). Nuclei are stained with DAPI (blue). The average correlation coefficient for colocalization between the two antibodies is indicated in the figure, calculated for >30 cells with detectable MICA staining.
| Cell Line | MICA Genotype      | MICA Expression          |
|-----------|--------------------|--------------------------|
| 293T      | MICA*008<sup>a</sup> | Expressed                |
| HeLa      | MICA*008<sup>b</sup> | Expressed                |
| MCC13     | MICA*008/*009:02   | Expressed                |
| HCT116    | MICA*001/*009:02<sup>b</sup> | Expressed                |
| A549      | MICA*001/*004      | Expressed                |
| RKO       | MICA*007:01        | Intracellular protein detected at very low levels |
| VH3       | MICA*008           | Expressed                |
| FLS1      | MICA*004/*009:01-*049 | Expressed                |
| FLS3      | MICA*002:01/*010:01-*069 | mRNA detected, protein not detected |

**Table S1, associated with figures 1-7, MICA Genotype and Expression.** Summary of MICA genotype and MICA expression in the cell lines used in this paper.

<sup>a</sup> McSharry et al., 2008

<sup>b</sup> Zhang et al., 2001
Supplemental Experimental Procedures

Cell culture

All cell lines and fibroblasts were kept in DMEM, except for MCC13 cells which were kept in RPMI. Media were supplemented with 10% FCS (Sigma-Aldrich) and with 1% each of pen-strep, sodium pyruvate, L-glutamine and non-essential amino acids (Biological Industries).

Primary and secondary antibodies used in this study

The following primary antibodies were used for flow cytometry: anti-MICA (clone 159227, R&D Systems), anti-MICB (clone 236511, R&D Systems), anti-ULBP1 (clone 170818, R&D Systems), anti-ULBP2/5/6 (clone 165903, R&D Systems), anti-ULBP3 (clone 166514, R&D Systems), anti-HIS tag (AD1.1.10, R&D systems), anti-HA tag (12CA5), anti-MHC class I (W6/32).

The following primary antibodies were used for immunofluorescence: anti-PDI (a kind gift from Dr. Hidde Ploegh, Whitehead Institute of Biomedical Research, MA, USA), anti-HIS tag (AD1.1.10, R&D systems): anti-MICA (clone 159227, R&D Systems).

The following secondary antibodies were used for flow cytometry and immunofluorescence: anti-mouse AlexaFluor 647, anti-mouse biotin, streptavidin-AlexaFluor 647 and anti-human APC, all purchased from Jackson Laboratories.

The following antibodies were used for blocking assays: anti-NKG2D (clone 149810, R&D Systems) and anti-CD99 (12E7, used as an isotype control, was a gift from A. Bernard, INSERM, France).

The following primary antibodies were used for western blotting: anti-HA tag (clone 3F10, Roche), anti-HIS tag (AD1.1.10, R&D systems), anti-MICA (clone 1.7AD (Zou et al., 2002), or clone EPR6568, Abcam), anti-PP2AC (rabbit polyclonal, Abcam) anti-GAPDH (clone 6C5, Santa Cruz) and anti-vinculin (clone EPR8185, Abcam).

The following secondary antibodies were used for western blotting: anti-mouse-HRP, anti-rat-HRP and anti-rabbit-HRP, all purchased from Jackson laboratories.

Primers and templates used for cloning and PCR mutagenesis

MICA*004, MICA*008 and MICB were amplified from cDNA and cloned into the lentiviral vector SIN18-pRLL-hEF1ap-E-GFP-WRPE by replacing the GFP with the desired sequence. US9 was amplified from cDNA derived from HCMV-infected cells (TB40/E strain) and cloned into the lentiviral vector pHAGE-DsRED(−)-eGFP(+) which also contains GFP.

The following primers were used for the initial cloning of US9, MICA*004, MICA*008 and MICB (all primers are listed 5' to 3'):
US9 FW – CCGCGGCCGCGCCGCCACCATGATCCTGTGGTCCCCGTC
US9 REV-HA tag – GGGATCCTCAATGATGATGATGATGATGATCGTCTTTTAGCCTCTTTCT
US9 REV-HIS tag - GGGATCCTCAATGATGATGATGATGATGATCGTCTTTTAGCCTCTTTCTCCC
MICA FW – CGGATCCGCGCCACCATGGGGCTGGGCC
MICA*004 REV – GGTGTACATTACAACGGACATAGAAAATAAA
MICA*008 REV – GGTGTACATTACAACGGACATAGAAAATAAA
MICB FW – CGGATCCGCGCCACCATGGGGCTGGGCC
MICB REV – GTTCACCTAGGGCCCTCAGTTGGA

For the introduction of an HA tag into the MICA N-terminus, the lentiviral constructs described above were used as templates for further rounds of PCR and cloning, using the following primers:

MICA-HA FW (used with the relevant MICA-rev primer) – TACCCATACGATGTTCCAGATTACGCTGAGCCCCACAGTCTTCGTTAT
MICA-HA REV (used with the MICA FW primer) – AGCGTAATCTGGAAACATCGTATGGGTAAGCAGCAGCTCCCGGAGGT

The two resulting PCR fragments were then unified using the MICA FW and MICA*004/*008 REV primers for MICA*004-HA and MICA*008-HA, respectively.

For introducing mutations into the MICA*004-HA lentiviral construct, it was used as template with the MICA FW primer and each of the following primers:

MICA*004-G-ins-HA –
GGTGTACATTACAACGGACATAGAAAATAATAATAACAAAAATAGCAGCAGCAGCAGCAAAC
AGCAGAAACATG
MICA*004-Stop-HA REV – GGTGTACATTACATAGAAAAATAATAATAACAAAAAT

For preparation of the doubly-mutated MICA*004-Dmut, the single mutant MICA*004-G-ins was used as template for the extracellular and transmembrane portions and MICA*004 was used as template for the intracytoplasmic tail. The following primers were used:

MICA*004-Dmut-HA REV (used with MICA FW) -
GCAGCTGATGTTTTCTTCTTCAAAACCGCAAACCGACATAGAAAAATAATAA
MICA*004-DMUT-HA FW (used with MICA*004 REV) –
TGTTGCGTTTGGTGAAGGAAAAACATCAGCTGC

The two resulting PCR fragments were then unified using the MICA FW and MICA*004 REV primers.

For preparation of the chimera MICA*008-ULBP3TM, MICA*008 was used as template for amplifying the extracellular portion, with the following primers:
MICA*008-ULBP3TM REV (used with MICA FW) -
TATGGCTTTGGGTTGAGCTAAACCATAATGACTCTGAAGCACCAG

ULBP3 was used as template for the transmembrane domain, with the following primers:
ULBP3TM FW - CTGGTGCTTAGAGTCAACGGACAGACATCCAGTATGGGTAGCTCTTCTCCTCCTCGGACCCAGGGAC
ULBP3TM REV - GGTGTACATCATGACCAGGAGGATGA

The two resulting PCR fragments were then unified using the MICA FW and ULBP3TM REV primers.

For introducing a C-terminus truncation into US9, the following primer was used: US9-truncated REV (used with the US9 FW primer) –
GGGATCCTTAAGCGTAATCTGGGAACATCGTATGGGTAGCTCTTCTCCTCGGACCCAGGGAC

For swapping MICB's transmembrane portion with that of MICA*008, MICB's extracellular portion was amplified from the MICB lentiviral construct using the MICB FW primer with the MICB-mut REV primer -
ACAGCAGAACATGGAATGTCTGGTCTGTCCGTTGACTCTGAAGC

The MICA*008 construct was used as template for amplifying the transmembrane portion using the MICA*008 REV primer and MICA*008-TM FW primer -
GCTTCAGAGTCAACGGACAGACCATGATTTCTGCTGT

The two resulting PCR fragments were then unified using the MICB FW and MICA*008 REV primers.

**ΔUS9 HCMV mutant generation**

The US9 deletion mutant (ΔUS9) was generated according to a previously published procedure (Wagner et al., 2002)

Briefly, a PCR fragment was generated using primers DeltaUS9-Kana1

CCCAACAGCAGCCCAAGGCGACGGGAGGAGGCGACGCCACGCTCATGCGGCTTCGCCAGGGAC

AATTCCAGAGTCTCGGTAC and DeltaUS9-Kana2
CTCCGCACACAGACGACGCGCCGGGCGGCTTCCTGCGGCCGGCCGCGGTGCCGGCGGCTTCATTACGCCAAGCTCC. The PCR fragment containing a kanamycin resistance gene was inserted into the parental BAC by homologous recombination in E. coli. Recombinant mutant virus was reconstituted from BAC DNA by Superfect (Qiagen) transfection into HCMV-permissive cells.

**Viral infection**

HFF were used to propagate all HCMV strains and virus stocks were titrated using a plaque assay and stored at -80°C. Infection with the various virus strains was carried out at a multiplicity of infection (MOI) of 2-4, in confluent fibroblasts. HCMV infection was enhanced by centrifugation at 800 g for 30 min. Mock-infected fibroblasts were incubated with medium only, and were plated at a lower density so they would be sub-confluent at the time point in which the infected cells were harvested for further analyses. Infection was verified by intracellular FACS staining with anti-CMV antibody (clone 8B1.2, Merck Millipore) at 24 hpi, and at least 85% of the cells were infected.

**qRT-PCR**

Total RNA was isolated by using the Quick RNA Miniprep kit (Zymo) according to the manufacturer’s instructions. Total RNA (0.25-2 μg) was reverse transcribed with mMLV Reverse Transcriptase (Invitrogen) and with 0.5 μg of poly(T) 3’ rapid amplification of complementary DNA ends (RACE) adaptor using the FirstChoice RLM-RACE kit (Ambion), according to the manufacturer's instructions. Quantitative PCR was used to measure mRNA expression as follows: cDNA was mixed with 150 μM of both the forward and reverse primers in a final volume of 5 μl and mixed with 5 μl of SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). hUBC and hHPRT were used as endogenous reference genes for PCR quantification. PCR was performed on QuantStudio12K Flex Real Time PCR System (Applied Biosystems).

The following primers were used (listed from 5’ to 3’):

- **hHPRT FW** – TGACACTGGCAAAAACAATGCA
- **hHPRT REV** – GGTCCTTTTTCACCAGCAAGCT
- **hUBC FW** – ATTTGGGTCGCGGTTCTTG
- **hUBC REV** – TGCCTTGACATTCTCGATGGT
- **MICA FW** – ATGGAACACAGCGGAATCA
- **MICA REV** – GCACTTTCCAGAGGGCAC
- **US9 FW** – AACGCCCTCAGACTTGGGAAC
- **US9 REV** – CTACCTGGACACCGAAGCTG
MICA genotyping

Genomic DNA was extracted from the relevant cell types using the AccuPrep genomic DNA extraction kit (Bioneer) according to the manufacturer's instructions. Exons 2-5 were amplified using the primers MICA-Genomic FW - CGTTCTTGTCCTTTGCCCCGTGTC and MICA Genomic REV – GATGCTGCCCCATTTCCCTTC, as previously described (Katsuyama et al., 1999). The resulting PCR products were then ligated into pGEM T-easy plasmids (Promega) and sent for sequencing. The results were then aligned to all known MICA alleles contained in the IMGT/HLA database (http://www.ebi.ac.uk/ipd/imgt/hla/) to determine which alleles fully matched the sequences. The results were verified by manually annotating the exons in the received sequences and aligning them again to the candidate alleles using global sequence alignment (emboss.bioinformatics.nl/). At least four separate sequencings were performed before a cell line was deemed to be homozygous.
**Supplemental References**

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