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Distinct Roles for Human Cytomegalovirus Immediate Early Proteins IE1 and IE2 in the transcriptional regulation of MICA and PVR/CD155 expression.

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1 **Up-regulation of NKG2D and DNAM-1 ligands in human cytomegalovirus infected cells:**
2 **role of the viral immediate early proteins IE1 and IE2 in MICA and PVR expression.**

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13

14 **Short title**

15 HCMV, IE proteins and upregulation of activating ligands

16

17

18 **Abstract**

19 NKG2D and DNAM-1 are two activating receptors expressed on cytotoxic lymphocytes, and
20 play an important role in the elimination of virally-infected cells. Their ligands (MICA/B and
21 ULBP1-6 for NKG2D, and Nectin-2 and PVR for DNAM-1) are often up-regulated upon cellular
22 stress, including viral infections. However, the molecular mechanisms driving their expression
23 are largely unknown, and most of these molecules have been described to be down-modulated
24 by human cytomegalovirus (HCMV) to avoid host immune surveillance. Here, we show that
25 laboratory and low-passage HCMV strains induced MICA, ULBP3 and PVR upregulation on
26 different cell types, suggesting that viral immunoevasion not always prevails. Ligands were still
27 up-regulated on HCMV-infected cells treated with phosphonophormic acid, an inhibitor of viral
28 DNA replication, indicating that some events in the early phases of infection are involved in
29 ligand increase. Our data show that the major immediate early (IE) proteins IE1 and IE2
30 differently contribute to MICA and PVR expression. MICA upregulation, at both transcriptional
31 and protein level, was mainly dependent on IE2, able to directly bind MICA promoter on a
32 specific consensus region that we identified. Both IE proteins were instead required for PVR
33 upregulation, with a mechanism independent from the DNA binding activity of IE2. We also
34 investigated the contribution of the DNA damage response (DDR) in ligand expression, since
35 this pathway is activated by HCMV and is implicated in ligand up-regulation. However, DDR
36 was not involved as ligands were still upregulated when ATM, ATR and DNA-PK kinases were
37 silenced in infected cells. Overall, our data suggest that IE-mediated activation of cellular genes
38 stimulating cytotoxic lymphocytes might be crucial for the anti-viral host response, and needs to
39 be better characterized in order to design more specific reagents.

40

41 **Author Summary**

42 Human cytomegalovirus (HCMV) is a β -herpesvirus whose infection is usually asymptomatic
43 and followed by the establishment of a life-long latency, controlled by the immune system.
44 However, primary infection or reactivation occurring in immunocompromised hosts, such as
45 AIDS or transplanted patients, or in the fetus, results in high levels of morbidity and mortality,
46 and birth defects. To date, HCMV is contrasted with anti-viral drugs that can produce toxic
47 effects or select mutant resistant strains. Therefore, agents able to potentiate anti-HCMV
48 immune responses could represent an alternative approach. NK and cytotoxic T lymphocytes
49 are crucial in controlling viral infections, and they use several receptor/ligand combinations, able
50 to turn on their anti-viral functions. NKG2D and DNAM-1 are important receptors recognizing
51 HCMV-infected cells, though their ligands are often targeted by the virus, that evolved many
52 strategies to resist cytotoxic lymphocyte attack. Here, we instead demonstrate that some of
53 these ligands can be upregulated on distinct cell types infected with different HCMV strains, and
54 identify some of the mechanisms involved. The findings show that the major viral immediate
55 early proteins, IE1 and IE2, enhance the expression of two ligands (MICA and PVR), opening
56 the way to develop new therapeutic approaches potentiating anti-HCMV immune responses.

57

58 **Introduction**

59 Human cytomegalovirus (HCMV) is a β -herpesvirus with a large dsDNA genome of
60 approximately 230 kbp, and containing more than 200 open reading frames (Murphy and
61 Shenk, *curr top microb immunol* 2008; Stern-Ginossar *N Science* 2012). Although the virus is
62 endemic within the human population, it does not usually cause clinically obvious disease upon
63 primary infection of healthy, immunocompetent individuals, but the immune response is unable
64 to clear the virus, which establishes a life-long latency (Sinclair J *JGV* 2006). Infection often
65 becomes clinically apparent, causing life-threatening diseases in immunocompromised
66 individuals, such as AIDS patients and organ transplant recipients, upon primary infection or
67 reactivation (Mocarski ES and Tan Courcelle C. 2001. In Knipe DM et al. (Eds), *Fields Virology*
68 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA). It is also the major viral cause of birth
69 defects, which can culminate in hearing and vision loss, along with various mental disabilities
70 (Schleiss MR. 2008. *Curr Treat Options Neurol*). Nearly all HCMV infections result in
71 widespread dissemination throughout the body, with diverse cell types supporting productive
72 viral infection, including fibroblasts, epithelial, endothelial and smooth muscle cells (Sinzger C
73 *JGV* 1995). In addition, the virus induces a plethora of immunomodulatory pathways to subvert
74 the host innate and adaptive immune responses (Mocarski ES *Trends Microbiol* 2002).
75 Although there have been numerous attempts to develop an effective HCMV vaccine, a
76 successful formulation has not yet been clinically approved. A limited number of anti-viral drugs
77 are available, but long-term treatment is frequently followed by toxic side effects and the
78 emergence of drug-resistant mutants (Khanna R *Trends Mol Med* 2006). Clearly, additional safe
79 therapeutic agents that limit HCMV replication are desirable, and thinking in terms of agents
80 able to potentiate anti-HCMV immune responses could be an alternative approach.

81 With this purpose, we investigated if some of the molecules able to activate cytotoxic
82 lymphocytes could be positively regulated following HCMV infection, favoring the recognition
83 and elimination of infected cells. In particular, we analyzed the expression and regulation of
84 NKG2D and DNAM-1 ligands. NKG2D and DNAM-1 (CD226) are two activating immune
85 receptors expressed by all cytotoxic lymphocytes, i.e, NK cells, CD8+ T cells and gamma-delta
86 T cells (Lanier Nat Immunol 2008). NKG2D delivers a potent activating signal and plays a
87 prominent role in the recognition and elimination of infected cells (Lanier Nat Immunol 2008;
88 Champsaur Immunol Rev 2010). Its ligands are the MHC-I-related molecules MICA, MICB, and
89 the ULBP proteins (ULBP1-6), whose expression is highly restricted in normal cells, but can be
90 rapidly up-regulated or induced upon a cellular stress, including a viral infection (Champsaur
91 Imm Rev 2010; Eagle RA 2007 Nat Rev Immunol). DNAM-1 has been shown to be fundamental
92 to NK cell-dependent anti-tumor immunity (Chan 2014; Gilfillan 2008; Iguchi-Manaka 2008;
93 Lakshmikanth 2009), and its role in viral infections is also starting to emerge (Cella 2010;
94 Nabekura 2014). It is a co-stimulatory Ig-like adhesion molecule and its ligands are PVR
95 (CD155) and Nectin-2 (CD112), belonging to the family of nectins and nectin-like proteins
96 (Takai Y 2008 Nat Rev Mol Cell Biol; Fuchs A 2006 Semin Cancer Biol). Upon engagement by
97 its ligands, DNAM-1 promotes leukocyte migration, activation, expansion and differentiation of T
98 cells, as well as effector responses of both NK and T cells (Takai Y 2008 Nat Rev Mol Cell Biol;
99 Fuchs A 2006 Semin Cancer Biol). Because NKG2D and DNAM-1 have an important role in
100 controlling both NK- and T-cell-mediated immunity, it is reasonable that these receptors/ligands
101 forced HCMV to evolve specific strategies of evasion (Lanier Nat Immunol 2008). In fact, much
102 of the work done on NKG2D/DNAM-1 ligands, including our, have been focused on the
103 immunoevasion strategies evolved by HCMV to inhibit their expression (Rossini G 2012

104 Mediators Inflamm; Wilkinson 2008). HCMV encodes indeed an impressive array of molecules
105 (UL16, UL141, UL142, US18 and US20, US9, miRNA-UL112) that suppress cell surface
106 expression of both NKG2D and DNAM-1 ligands. The outcome of these immune-evasion
107 strategies is an impaired recognition and elimination of HCMV-infected cells by NK cells and
108 other NKG2D+ and DNAM-1+ cells, likely dampening an adequate immune response (Rossini
109 G 2012 Mediators Inflamm; Wilkinson GWG 2008 J Clin Virol 41:206; per US9: Seidel E Cell
110 Reports 2015; Fielding CA, Plos path 2014). Despite these evidences, it is also known that
111 HCMV infection results in the induction of transcripts encoding activating ligands, in particular of
112 NKG2D (Welte SA EJI 2003; Zou Y JI 2005; Eagle RA Hum Imm 2006). Moreover, there are
113 evidences on the ability of viral immediate early (IE) proteins to up-regulate MICA, MICB and
114 ULBP2 ligands at the transcriptional or protein expression level (Venkataraman and Fielding).
115 Thus, there is the possibility that HCMV may at first positively regulate the expression of
116 activating ligands, allowing recognition of infected cells by cytotoxic lymphocytes and thus a
117 certain level of immune surveillance against the virus, at a time before late immunoevasion
118 genes exert their effects.

119 IE proteins play an important role in the very early phases of the infection, as they are the first
120 proteins to be expressed during HCMV lytic infection, before early (E) and then late (L) gene
121 products are expressed (Sinclair J JGV 2006). The most abundantly expressed proteins are the
122 72-kDa IE1 and the 86-kDa IE2 proteins (IE1 and IE2 from now on), together with the other IE
123 protein, known as IE55 (IE2-55). IE proteins are expressed in the absence of *de novo* protein
124 synthesis, are all produced from differentially spliced transcripts, and share the first 85
125 aminoacids, so that the N-terminal part is identical. However, the remaining sequences differ
126 and likely account for the divergent activities exhibited by each protein (Castillo JP Gene 2002;

127 Stinski MF Curr Top Microbiol Immunol 2008). IE1 and IE2 initiate the virus productive infection,
128 and they are absolutely critical for the temporal cascade of viral gene expression, as they are
129 known to transactivate E and L genes (Mocarski 2001, Stinski and Meier 2007), and either
130 positively or negatively autoregulate their own expression (Stinski MF, Isaacson MK, or Gibson
131 W Curr Top Microb Imm, 2008). Moreover, while IE1 alone is a relatively weak transactivator
132 and only affects a limited number of promoters that have been tested, IE2, considered to be the
133 most important regulatory protein codified by the virus, is a strong transcriptional activator of
134 cellular gene expression (Stinsky and Meier, Immediate-early viral gene regulation and function; in Human
135 Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Cambridge: Cambridge University Press; 2007) and seems to
136 operate at the promoter level by different mechanisms. It is able to bind DNA directly,
137 repressing its own promoter (Lang and Stamminger JV, 1993), and it binds to several cellular
138 transcription factors via protein-protein interactions, crucial for transcriptional activation of viral
139 and host genes, and for the regulation of vital cellular functions (Stinski MF Curr Top Microbiol
140 Immunol 2008).

141 Among the cellular pathways activated by IE proteins there is the DNA damage response (DDR)
142 (Castillo 2005, Xiaofei 2011), involved in cell-cycle checkpoint control, DNA replication, DNA
143 repair and apoptosis (Jackson SP, Nature 2009). DNA must be protected from damage
144 produced spontaneously during DNA replication or other physiological processes, as well as
145 after exposure to external stimuli and DNA-damaging agents. Therefore, to maintain genome
146 integrity and avoid mutated DNA duplication, cells respond with a complex series of cellular
147 stress-induced pathways to detect and repair DNA lesions. Likewise, infection by several
148 viruses, including herpesviruses, is sufficient to activate some or all of the DDR-mediated repair
149 pathways. Simplistically, this was perceived as recognition by the host cell of the incoming

150 genetic material as its own damaged DNA, but it is now considered to be, at least in part, an
151 anti-viral response aimed at combating the pathogen by posing a threat to viral genome integrity
152 and replication (Sinclair A, Expert Rev Mol Med 2004). On the other hand, DNA viruses create
153 nuclear environments beneficial to viral DNA replication through interactions with host proteins,
154 likely to rely on cellular repairing machineries to facilitate successful viral DNA replication
155 (Chaurushiya MS DNA Repair 2009; Xiaofei E Plos Pathogens 2011 and JV 2014). Therefore,
156 many viruses require DDR activation for a fully permissive infection (Turnell AS JGV 2012;
157 Xiaofei E Viruses 2014), and also HCMV induces a strong DDR and activation of multiple
158 markers of this pathway, including ATM, ATR and the downstream protein H2AX. The functional
159 relevance of DDR in HCMV replication is however unclear and controversial results have been
160 published (Shen YH 2004 Circ Res; Castillo JP 2005 J Virol; Gaspar M 2006 PNAS; Luo MH
161 2007 JV; Xiaofei E 2011 Plos Pathog; Xiaofei E JV 2014; Li R Cell Host Microbe 2011).
162 Noteworthy, DDR is also involved in NKG2D and DNAM-1 ligand expression. In fact, several
163 works including ours demonstrated that expression of MICA, MICB, ULBP1-3 and PVR is in part
164 dependent on the activation of the DDR and on ATM/ATR kinases (Gasser Nature 2005;
165 Cerboni Blood 2007; Soriani Blood 2009; Ardolino Blood 2011; Zingoni Frontiers Immunol
166 2012). However, it is unknown how HCMV modulates the expression of NKG2D and DNAM-1
167 ligands in relation to DDR activation. Our study aimed at investigating this aspect, in an attempt
168 to shed light into the involvement of virus-induced DDR pathway in ligand regulation. We also
169 extended our study on the role of IE proteins on NKG2D and, for the first time, DNAM-1 ligand
170 regulation, deepening the molecular mechanisms by which these viral proteins may act on
171 activating ligand expression. These aspects may shed light into some of the mechanisms and
172 molecules stimulating anti-viral immunity against HCMV.

173

174 **Results**

175

176 **1. Modulation of NKG2D and DNAM-1 ligand expression by different HCMV strains in**
177 **various cell types.**

178 Increased or *de novo* expression of T/NK cell activating ligands on the plasma membrane of
179 infected cells represents a crucial host immune defense mechanism to sense and react against
180 a wide variety of pathogens (Lanier LL, Nat Imm 2008; Ferrari de Andrade L, Immunology and
181 cell biology, 2014). It is known that HCMV regulates the expression of NKG2D and DNAM-1
182 ligands, but different and sometimes contradictory results have been reported so far.

183 Therefore, we first wanted to recapitulate the expression of NKG2D and DNAM-1 ligands on the
184 surface of HCMV-infected cells. We thus initially infected human primary foreskin fibroblasts
185 (HFF) with the laboratory strain AD169, and monitored by immunofluorescence and flow
186 cytometry for NKG2D and DNAM-1 ligand expression, at different days post-infection (dpi)
187 (Figure 1). Uninfected HFFs showed undetectable MICA, MICB, ULBP1 and ULBP4 cell surface
188 expression, but while no MICB, ULBP1 and ULBP4 molecules were detected at any time post-
189 infection on the plasma membrane of infected HFFs (Figure 1A and data not shown), MICA was
190 significantly induced by HCMV already at 1 dpi, with a peak at 3-4 dpi (Figure 1). Increased
191 levels of ULBP3 were also observed on the cell surface of infected fibroblasts, with maximal
192 expression around 3 dpi, while ULBP2 was down-modulated (Figure 1).

193 When the effect of HCMV infection on DNAM-1 ligands was investigated, a HCMV-mediated
194 upregulation of PVR, but not Nectin-2, was observed, with a significant increase of its cell
195 surface expression, particularly evident around 3 dpi (Figure 1).

196 Altogether, these results recapitulated but also extended previous findings and showed that

197 infection of HFFs with AD169 caused an increased cell surface expression of MICA, ULBP3 and
198 PVR activating ligands.

199 Since our general aim was at investigating the mechanisms underlying NKG2D and DNAM-1
200 ligand up-regulation, we focused on those molecules that on human primary fibroblasts were
201 either induced or increased by the virus, i.e., MICA, ULBP3 and PVR.

202 To verify that the HCMV-mediated increased expression of MICA, ULBP3 and PVR was not
203 confined to a unique cell type-viral strain combination, and since the highly-passaged strain
204 AD169 has a greater number of genetic differences compared to low-passage strains, including
205 a ~13-15 kb deletion (Cha TA JV 1996; Wilkinson GWG Med Microbiol Immunol 2015), we
206 extended our analysis to other viral isolates and cell types. In particular, we investigated ligand
207 modulation on: i) HFF cells infected with the low-passage strains VR-1814 and TR, and ii)
208 primary endothelial cells (HMVEC) and epithelial cells (ARPE-19) infected with the same strains
209 (Bronzini JV 2012). To this end, mock- and HCMV-infected cells were stained with mAbs
210 specific for MICA, ULBP3 and PVR, and analyzed by immunofluorescence and flow cytometry
211 (Fig. 2). Similarly to what observed on AD169-infected HFFs, we consistently and reproducibly
212 observed an induction of MICA expression on fibroblasts infected with VR-1814, particularly
213 evident at 2-3 dpi (Fig. 2A-B and data not shown). This result was obtained independently from
214 the used MOI (Fig. 2B). Induction of MICA expression was also observed on HFFs infected with
215 another low-passage strain, TR, though to a lower extent (Fig. 2C). However, this observation
216 was confirmed by confocal microscopy analysis, showing that an induced MICA expression was
217 clearly detectable on the cell surface of TR-infected fibroblasts (Fig. S1). When ULBP3 and
218 PVR ligands were examined on HFF cells infected with VR-1814 (Fig. 2A-B) or TR (Fig. 2C), we
219 always observed a statistically significant up-regulation of these molecules as well. Taken

220 together, these results demonstrated that at 3-4 dpi, MICA, ULBP3 and PVR were up-regulated
221 on the plasma membrane of infected primary fibroblasts in a strain-independent manner.
222 Next, we extended our investigation to other cell types and HMVECs and ARPE-19 cells were
223 infected with TR and VR-1814 strains (Figure 2C). MICA expression was either down-
224 modulated (on TR-infected HMVECs), or not affected in all other combinations (HMVECs
225 infected with VR-1814; ARPE-19 infected with TR or VR-1814), while ULBP3 and PVR were
226 always up-regulated, independently of the cell type and/or the viral strain used.
227 Thus, these results demonstrated that, despite few exceptions, HCMV positively regulated the
228 expression of MICA, ULBP3 and PVR activating ligands, with a pattern that generally overcame
229 cellular- or viral strain-related differences.

230

231 **2. Analysis of the role of the DNA damage response and of ATM, ATR and DNA-PK on** 232 **HCMV-induced ligand up-regulation and HCMV replication.**

233 Previously, it has been reported that HCMV manipulates the DDR pathway (Castillo JP JV 2005;
234 Luo MH JV 2007; Shen YH Circ Res 2004; Gaspar M and Shenk T PNAS 2006; Xiaofei E PLoS Pathogens 2011),
235 and that DDR activation can regulate NKG2D and DNAM-1 ligand expression (Gasser S Nature
236 2005; Cerboni Blood 2007; Soriani A Blood 2009; Ward J Plos Pathogens 2009; Richard J Blood 2010; Ardolino
237 Blood 2011; Soriani A JI 2014; Cerboni Frontiers 2014; Fionda C BMC Cancer 2015). Thus, we asked
238 whether DDR was involved in the up-regulation of ligands observed in HCMV-infected cells. To
239 this end, we used different genetic and pharmacological approaches (i.e., gene silencing, use of
240 cells lacking ATM, use of inhibitors), and monitored the effects of inactivating the DDR pathway
241 on MICA, ULBP3 and PVR ligand cell surface levels, as well as on HCMV IE protein expression
242 and viral productive replication (Fig. 3 and Fig. S2-6).

243 Initially, we verified that the virus-induced activation of the DDR pathway, by measuring the

244 levels of γ H2AX, the phosphorylated form of the histone variant H2AX, a well-known substrate
245 of DDR kinases, including ATM, ATR and DNA-PK (Burma S JBC 2001; Ward IM JBC 2001;
246 Park EJ Nucleic Ac Res 2003). To this end, γ H2AX levels were monitored by flow cytometry on
247 mock- or AD169-infected HFFs, at 3 dpi (Fig. S2). Although γ H2AX was present in uninfected
248 cells, its levels increased of approximately two-fold upon HCMV infection, demonstrating
249 activation of the DDR pathway in our experimental settings.

250 Next, we determined the contribution of the three main kinases of the DDR signaling pathway,
251 ATM, ATR and DNA-PK, on ligand expression, IE expression and viral replication. The
252 contribution of ATM was firstly investigated in fibroblasts derived from a patient affected by
253 ataxia-telangiectasia (AT^{-/-}) that do not express detectable levels of the ATM protein (data not
254 shown). Upon HCMV infection, the absence of ATM did not compromise MICA, ULBP3 and
255 PVR expression, as the virus still induced an increased expression of all the three ligands,
256 though with delayed kinetics compared to HFFs, with a peak of expression at 7 dpi (Fig. S3).
257 Moreover, both progeny virus production and IE expression were only partially affected by the
258 absence of ATM. In fact, compared to HFFs, the production of infectious virus, measured by
259 plaque assays, was reduced of approximately 1 to 2 log, from day 3 to day 7 post-infection
260 (data not shown), while the percentage of IE⁺ cells, analyzed by flow cytometry, was almost
261 overlapping with HFFs (data not shown). These results suggested that functional ATM, though
262 required for optimal infectious virus production (Xiaofei E, PLoS Pathogens 2011), is
263 dispensable for the HCMV-mediated up-regulation of MICA, ULBP3 and PVR expression.

264 Since there is a concern with using AT^{-/-} fibroblasts as a model because the prolonged absence
265 of a functional ATM in cells from AT patients may have resulted in secondary genetic and/or
266 biochemical changes that in turn could alter cellular environments and thereby influence HCMV

267 replication, we addressed the same issue by using specific siRNA to transiently deplete ATM
268 protein levels (siATM). To this end, HFFs were transfected with siATM or with a non-targeting
269 siRNA (siCtrl) 24 h prior to HCMV infection, and ligand expression, viral replication and gene
270 expression were monitored at 2 dpi (Fig. S4). Similarly to what we observed in AT-/- fibroblasts,
271 transient depletion of ATM did not affect MICA, ULBP3 and PVR expression induced by HCMV
272 infection (Fig. S4A), and it did not significantly compromise either the percentage of IE+ cells or
273 viral replication (Fig. S4C-D). Similar results were obtained with siRNA specific for ATR (Fig.
274 S5) or DNA-PK (Fig. S6).

275 Finally, we performed a triple gene silencing, by transfecting HFFs with the three siRNA specific
276 for ATM, ATR and DNA-PK (siDDR; Fig. 3). We observed a pattern similar to the one observed
277 by using single siRNA, with no significant modulation of MICA, ULBP3 and PVR (Fig. 3A),
278 despite the three siRNA had a strong inhibitory effect on the protein levels of their respective
279 target kinases (Fig. 3B). Similarly, activating ligands were still up-regulated in AD169-infected
280 HFFs treated with caffeine (data not shown), a well-known and broad spectrum inhibitor of the
281 DDR signaling pathway (Sarkaria JN, Canc Res, 1999; Block WD Nucleic acid Res, 2004). Of
282 note, in triple siRNA-transfected cells we observed a reproducible, though not statistically
283 significant, higher percentage of IE+ cells (Figure 4C) and viral titers (Fig. 3D). This could result
284 from the inhibition of the DDR-dependent cellular host response against HCMV, caused by the
285 triple silencing, which may allow an improved virus replication, less counteracted by DDR
286 kinases (Fig. 3C-D).

287 Altogether, these results suggest that DDR activation was not required for the HCMV-induced
288 up-regulation of MICA, ULP3 and PVR.

289

290 **3. The HCMV-induced MICA, ULBP3 and PVR expression depends from events occurring**
291 **prior to the onset of viral DNA replication.**

292 In order to identify the molecular mechanisms underlying ligand up-regulation in infected cells,
293 and since it has been suggested that IE proteins may positively regulate MICA/B expression
294 (Venkataraman JI 2007; Andresen JI 2007; Fielding Plos Pathogens 2014), we hypothesized
295 that some early events in the early stages of infection could be responsible for ligand increase.
296 To verify this hypothesis, HFFs were infected with HCMV and soon after virus adsorption,
297 phosphonoformic acid (PFA), a selective inhibitor of the viral DNA polymerase (Tymms AS JGV
298 1987), was added. Expression of ligands was then assessed at 3 dpi. As shown in Fig. 4, MICA,
299 ULBP3 and PVR levels were increased on the surface of infected cells even in the presence of
300 PFA, thus indicating that viral DNA replication and late gene expression are dispensable for
301 ligand up-regulation.

302

303 **4. HCMV infection increases MICA and PVR mRNA expression.**

304 The results obtained with PFA suggested that expression of viral IE and/or E genes was
305 necessary and sufficient for up-regulation of MICA, ULBP3 and PVR expression. Then, we
306 investigated whether the observed up-regulation of ligands was a consequence of a virus-
307 induced transcriptional activation. We focused on MICA and PVR, as prototype ligands of
308 NKG2D and DNAM-1, respectively, and their mRNA content was measured in infected HFFs by
309 real-time PCR at 6, 12, 24 and 48 hours post-infection (hpi). As shown in Fig. 5, MICA-specific
310 mRNA progressively increased by 48 hpi up to approximately 8-fold the amount present in
311 mock-cells at 48 hpi. Expression of PVR mRNA, whose kinetics in HCMV infected cells is
312 shown here for the first time, was increased as well upon infection, with a maximum of about

313 2.5-fold at 48 hpi.

314 These data indicate that the up-regulation of MICA and PVR cell surface levels by HCMV
315 infection is the outcome of a transcriptional activation of the corresponding genes.

316

317 **5. HCMV IE proteins up-regulate MICA and PVR expression and promoter activity.**

318 Given the observation that early steps of infection were crucial for ligand up-regulation, we
319 hypothesized that the main viral IE proteins – IE1 (IE1-72) and IE2 (IE2-86) - could have a role
320 in the modulation of MICA and PVR expression, since they are known as transcriptional
321 activators of both viral and cellular genes (Castillo and Kowalik, Gene 2002; Stinski and Petrik,
322 Curr. Top Microbiol. Immunol. 2008). Though IE proteins may positively regulate MICA/B
323 expression (Venkataraman JI 2007; Andresen JI 2007; Fielding Plos Pathogens 2014), the
324 molecular mechanisms mediating the modulation of MICA by IE proteins, as well as the
325 contribution of each IE protein, remain unknown. In addition, no information are available about
326 the effect of IE proteins on the expression of other activating ligands, including PVR.

327 To address this issue, we investigated whether IE1 and IE2 proteins regulate MICA and PVR
328 expression, by transducing HFFs with recombinant adenoviruses (AdV) encoding for IE1, IE2,
329 or for LacZ as a control. Transduction experiments were carried out with a single Adv or in
330 combination, at a total MOI of 4, and ligand expression was assessed at both mRNA and cell
331 surface expression level (Fig. 6). Statistical analysis of all Adv combinations for both ligands is
332 reported in Table S1 and S2. Transduction efficiency of AdV-IE1 or AdV-IE2 was similar, with a
333 percentage of IE+ cells on average of 61% and 68% at 3 dpi, respectively (data not shown).

334 Upon transduction, we observed a similar trend for modulation of both MICA mRNA and cell
335 surface protein expression, with a significant up-regulation induced by IE2 compared to not-

336 transduced or AdV-LacZ-transduced cells, while IE1 did not affect MICA levels (Fig. 6A-B).
337 Moreover, the combination of IE1 and IE2 expression did not induce a further increase in MICA
338 mRNA and protein expression compared to IE2 alone (Fig. 6A-B and Table S1). When PVR
339 was examined, its mRNA content was mostly up-regulated when both IE1 and IE2 were co-
340 expressed, while IE1 or IE2 alone had no effect (Fig. 6C). Accordingly, PVR cell surface levels
341 were mainly increased by the combined expression of the two proteins, while IE1 or IE2 alone
342 induced only a modest increase of the ligand (Fig. 6D and Table S2).
343 Taken together, these results demonstrated that the virus-induced MICA and PVR up-regulation
344 can be reproduced by the expression of the IE proteins only. However, their requirement is
345 ligand-specific, since IE2 alone is sufficient to stimulate both MICA mRNA and protein
346 expression, while co-expression of both IE1 and IE2 appear to be required for efficient PVR up-
347 regulation.

348

349 Next, to examine the possibility that the up-regulation of MICA and PVR mRNA and cell surface
350 levels induced by IE proteins involved the activation of ligand gene promoters, we performed
351 transient transfection assays by co-transfecting HFFs with pGL3-MICA (containing a 1 kb
352 fragment upstream from the *MICA* transcription start site) (Yadav D JI, 2009) or pGL2-PVR (-
353 571 bp fragment) (Solecki D JBC 1997) luciferase reporter plasmids, together with expression
354 vectors encoding the cDNAs of IE1 or IE2 proteins, used alone or in combination, or with the
355 empty control vector pSG5 (Klucher MCB 1993). When the effect of IE1 and IE2, alone or in
356 combination, was analyzed on the transcriptional activity of the *MICA* gene promoter, we
357 observed that, while IE1 did not induce a significant modulation, IE2 was able to transactivate
358 the promoter up to ~3-fold compared to the basal level obtained with the empty control vector

359 pSG5 (Fig. 7A). Co-transfection of IE2 with IE1 showed a further, though not significant, effect
360 on *MICA* promoter activity, as compared to IE2 alone.

361 Since among the two major IE proteins, IE2 seemed to exert the major transactivating effect on
362 the *MICA* gene promoter (Fig. 7A), we investigated further the mechanisms of this
363 transcriptional regulation, by addressing both the IE2 structural requirements and the interaction
364 with *MICA* gene promoter sequences. The IE2-55 protein is a splice variant of the IE2-86 gene
365 product, with a conserved N-terminus and a 155 aminoacid deletion between residues 365 and
366 519 in the C-terminus. The region that is deleted has been shown to be required for many IE2-
367 86 functions, including transcriptional activation and DNA binding (Malone et al., 1990, Pizzorno
368 et al., 1991, Chiou et al., 1993, Schwartz 1994; Petrik and Stinski, 2008). Consequently, unlike
369 IE2-86, IE2-55 fails to transactivate HCMV early promoters and to repress the Major IE
370 Promoter (MIEP) (Klucher et al., 1993). Thus, we used an IE2-55 expression vector as a tool to
371 investigate if the transcriptional activation and DNA binding properties of IE2-86, absent in IE2-
372 55, were important to transactivate the *MICA* gene promoter. To this end, the co-transfection
373 experiment with IE1 and IE2 proteins was performed replacing IE2-86 with IE2-55. Indeed, we
374 observed that expression of IE2-55 completely abolished the activating effect of IE2-86 (Fig.7B),
375 thus indicating that the C-terminus of IE2-86 is required for transactivation of *MICA* promoter.
376 Moreover, it has been reported that a zinc finger mutant of IE2-86 cannot bind to the *cis*-
377 repression signal in the MIEP (Jupp R JV 1993, *due lavori*). However, though this mutant
378 cannot bind to DNA, it retains the ability to transactivate E gene promoters by protein-protein
379 interactions (Jupp R JV 1993 67, 5595). We thus hypothesized that if the DNA binding ability of
380 wild-type IE2-86 was important for *MICA* promoter activation, this should have been lost by
381 substituting wild-type IE2-86 with its zinc finger mutant. Indeed, the co-transfection experiment

382 performed with IE1 and IE2-Zn mutant showed that the lack of the zinc finger hampered the
383 ability of IE2-86 to transactivate *MICA* promoter (Fig. 7C). Together, these results indicated that
384 the IE2 functional domains located primarily toward the C-terminal end of the protein are
385 required for transactivation of the *MICA* gene promoter.

386 Next, to gain more insights into the molecular mechanisms behind the regulation of *MICA*
387 promoter by IE proteins, we performed transient transfection assays with a shorter construct to
388 map the region(s) targeted by the viral proteins. To this end, we tested the pGL3 *MICA*
389 construct containing the luciferase gene driven by a 270 bp fragment upstream from the
390 transcriptional start site of the *MICA* promoter region (*MICA* -270), together with the
391 combination of IE1 and IE2, in light of the evidence that the effect of the two proteins did not
392 significantly differ from the one mediated by IE1 alone (Fig. 7A). The shorter *MICA* fragment
393 was indeed activated by IE1 and IE2 at similar levels compared to the longer *MICA* -1 kb region
394 (Fig. 8A), indicating that the responsive region to IE proteins was contained within the 270 bp
395 fragment.

396 The IE2-binding sites identified on different viral promoters, and on one cellular promoter as
397 well, have been shown to contain invariant CG residues at both ends of a 10-nucleotide
398 sequence (CG-N₁₀-CG), as reported in Fig. 8B (Waheed 1998;Lang, D. and Stamminger T JV
399 1993; Schwartz R. JV 1994; Arlt H JV 1994; Bresnahan WA, JBC 1998). A similar sequence,
400 endowed of two CG residues separated by 10 internal nucleotides, is present within the *MICA*
401 gene promoter, between residues -92 and -78, thus representing a potential IE2 binding site
402 (Fig. 8B). To investigate the contribution of this putative IE-binding site to the overall IE2-
403 dependent transactivation of *MICA* promoter, we changed by site-directed mutagenesis this
404 unique CG-N₁₀-CG motif into a AT-N₁₀-AT sequence within the context of the pGL3 *MICA* (-270)

405 construct (Fig. 8B). As shown in Fig. 8A, the IE2-dependent transactivation of *MICA* was
406 significantly reduced by the introduced mutations, thus supporting an involvement of the
407 putative IE2-binding site in the IE2-mediated regulation of *MICA* gene promoter.

408 Next, to address the capability of IE1/IE2 proteins to directly bind to *MICA* promoter, we
409 performed Chip assays in highly transfectable 293T cells, in which pGL3-*MICA* (-270) was co-
410 transfected with the IE1 and IE2 expression vectors, or with the empty vector as a control.
411 Using an anti-IE antigen (anti-IE) antibody and specific primers to amplify the promoter region
412 containing the putative IE2 binding site, we observed that the viral IE1/IE2 proteins were
413 recruited to *MICA* promoter. The interaction was not detectable with the empty vector pSG5 or
414 using normal rabbit serum as a negative control (Fig. 8C). Finally, we performed the same
415 experiments by transfecting cells with wild-type or the CG mutant form of the *MICA* promoter,
416 and compared the relative DNA enrichment upon immunoprecipitation. As reported in Fig. 8D,
417 disruption of the putative IE2-binding site of the *MICA* promoter reduced IE binding of about
418 ~60%, further demonstrating that this sequence is involved in the IE2-dependent transactivation
419 of *MICA* promoter.

420 Together, these results demonstrated the capability of IE2 to directly bind sequences within
421 *MICA* gene promoter, and that this binding is required for *MICA* transcriptional activation.

422

423 In relation to PVR regulation, to evaluate the role of IE proteins on its transcriptional activation,
424 we performed similar transient co-transfection assays with the pGL2-PVR construct containing a
425 571 bp fragment upstream from *PVR* transcriptional start site (Solecki D, JBC 1997) and vectors
426 expressing IE proteins. IE1 had a relevant effect on *PVR* promoter, activating its transcription up
427 to 10-fold over the control (Fig. 9A). In contrast, IE2 alone was ineffective in stimulating *PVR*

428 promoter activity. However, the combination of IE1 and IE2 induced a prominent activation of
429 the transcription that exceeded significantly that observed with IE1 alone (Fig. 9A). In contrast to
430 what observed for *MICA*, IE2-55 and the IE2-86 zinc finger mutant did not affect the overall
431 transactivation effect of the combination of IE1 and wild-type IE2-86 on *PVR* promoter activity
432 (Fig. 9B-C), suggesting a different regulation of IE-mediated *PVR* up-modulation compared to
433 what observed for *MICA*. Finally, we performed luciferase assays using progressive deletions of
434 the promoter (Solecki JBC 1997), trying to identify the region(s) regulated by IE proteins, by
435 transfecting both IE1 and IE2, as this was the more effective combination on *PVR* promoter
436 (Fig. 9A). The results showed that we could identify a 213 bp fragment upstream from the
437 transcriptional start site that was significantly less sensitive to IE proteins (Fig. 9C). The
438 significant drop in luciferase activity observed with the deletion of sequences between -281 and
439 -213 indicated that this fragment mediated most of the transactivating activity of IE1 and IE2
440 combination.

441 Taken together, the results included in this section indicate that the increase in cell surface
442 expression of *MICA* and *PVR* upon HCMV infection is mediated by transcriptional events
443 triggered by viral IE proteins.

444

445 **Discussion**

446 The molecular mechanisms driving the expression of NKG2D and DNAM-1 ligands remain
447 largely unknown and, in particular, very little is known concerning the mechanisms of their
448 induction in virus-infected cells. In this study, we have investigated the impact of HCMV
449 infection on the expression of NKG2D and DNAM-1 ligands, under a different perspective
450 compared to most of the work performed so far by many investigators, including us. In fact, the
451 majority of the studies on NK/T cell activating ligands in HCMV-infected cells have been
452 focused on the mechanisms evolved by the virus to escape recognition by cytotoxic
453 lymphocytes. The HCMV genome is indeed often described as a paradigm of viral immune
454 evasion, with several genes dedicated at eluding immune defenses (ref), including at down-
455 modulating both NKG2D and DNAM-1 ligands (ref). On the other hand, it seems likely that
456 NKG2D evolved and has been conserved to provide immunity against pathogens, and the
457 redundancy of its ligands and their extensive allelic polymorphism might be driven by selective
458 pressures of those pathogens that are evolving mechanisms to escape detection by immune
459 cells using NKG2D (ref). More recently, also DNAM-1 has been shown to be important in anti-
460 viral immunity (ref), including murine and human CMV infection (ref). Therefore, novel
461 therapeutic strategies to restore or enhance NKG2D- and DNAM-1-dependent activation of NK
462 or T cells may be beneficial at harnessing their anti-viral activity, and a better understanding of
463 the mechanisms regulating NKG2D and DNAM-1 ligand expression in infected cells is needed.
464 The results shown here demonstrated that, in most of the cell type-viral strain combinations we
465 investigated, MICA, ULBP3 and PVR ligands were up-regulated on the plasma membrane of
466 infected cells. In particular, we observed an increased MICA expression on infected HFFs,
467 independently from the strain used, while infection of endothelial or epithelial cells with low-

468 passage strains resulted in no up-regulation or down-regulation of cell surface levels,
469 suggesting that increased or *de novo* MICA expression may be restricted to certain cell types.
470 However, the evidence that MICA was induced on HFFs by both laboratory and low-passage
471 strains, suggests that the down-modulating activity exerted on MICA by UL142, US18 and US20
472 viral gene products (Chalupny NJ BBRC 2006; Ashiru O JV 2009; Fielding 2014) was not
473 adequate to prevent its cell surface expression. Of note, UL142 has been described to prevent
474 cell surface expression of ULBP3 as well (Bennett NJ JI 2010), but in our settings its expression
475 was always increased, independently from the cell type, the viral strain and the amount of virus
476 employed, consistent with previous findings (Rolle JI 2003). The discrepancies may be related
477 to different experimental settings, as well as to different cells and viruses employed. In fact, a
478 considerable polymorphism exists in the UL142 sequence among different strains, including
479 amino acid insertions (Chalupny NJ BBRC 2006), and it is thus possible that some variants of
480 UL142, US18 and/or US20 are less efficient at down-modulating MICA and ULBP3 expression
481 than others. At the same time, polymorphisms in both the coding and non-coding regions of
482 MICA and ULBP3 have been described, and this may also modulate NKG2D ligand expression
483 upon HCMV infection.

484 At present, there are few reports on PVR expression upon HCMV infection of fibroblasts and
485 myeloid dendritic cells (Tomasec P, Nature Immunol 2005, Prod'homme V, JGV 2010, Magri G,
486 Blood 2011). In relation to fibroblasts, despite it was shown that PVR was down-modulated by
487 the low-passage strain Merlin, we instead consistently and reproducibly observed a significant
488 up-regulation of cell surface PVR with both low-passage and laboratory strains, under all
489 experimental conditions tested. PVR was also up-regulated at the mRNA level in AD169-
490 infected HFFs. Though the discrepancies could be due to different experimental settings (e.g.,

491 MOI 1 instead of MOI 25; use of fibroblasts, as well as of epithelial and endothelial cells), our
492 results show for the first time that PVR can be upregulated upon HCMV infection, in different
493 cell types and with different viral strains, offering the immune system the opportunity to detect
494 and react against infected cells through the activating receptor DNAM-1. In fact, this receptor
495 has been suggested to play a relevant role in NK cell recognition of HCMV-infected myeloid
496 dendritic cells early during infection, whereas the effect of viral-mediated down-regulation of
497 DNAM-1 ligands prevails at later stages, thus also illustrating the importance of the kinetics of
498 immune evasion mechanisms (Magri G, Blood 2011). DNAM-1 is remarkable as it is expressed
499 by all NK cells and is an important activator of their effector functions (Martinet Immunol Cell
500 Biol 2014). However, the increased PVR expression caused by HCMV infection may have
501 ramifications that extend beyond the regulation of NK cell functions, as DNAM-1 is also
502 expressed by a big variety of leukocytes (activated T cells, NKT cells, a B cell subset, myeloid
503 cells, mast cells, megakaryocytes and platelets), thereby impacting on a wide range of
504 immunological responses (Bachelet., 2006; Bottino., 2003; Burns, 1985; Pende., 2006;
505 Reymond, 2004; Scott, 1989; Shibuya, 1996, 1999, 2003; Xu & Jin, 2010; Tahara-Hanaoka
506 2004; Takai 2008; Martinet Immunol Cell Biol 2014). In addition, PVR expression may impact
507 anti-viral immune responses mediated by two other receptors engaged by this ligand, Tactile
508 (CD96) (Fuchs JI 2004) and TIGIT (Staniesky N PNAS 2009).

509 To gain insights into the molecular mechanisms regulating activating ligand expression in
510 infected cells, we investigated the role of DDR, a pathway positively affecting the expression of
511 these molecules (Cerboni Rev Frontiers 2014) and activated by HCMV (Castillo JV 2005). We
512 observed an HCMV-induced DDR activation, as revealed by the increased histone H2AX
513 phosphorylation, but MICA, ULBP3 and PVR were still up-modulated in HCMV-infected HFFs in

514 which ATM, ATR, and/or DNA-PK were knocked-down, thus indicating that these kinases do not
515 play a role in activating ligand up-regulation in HCMV-infected cell, similarly to what has been
516 reported for murine NKG2D ligands in MCMV infection (Tokuyama Plos Pathogens 2011).

517 The observation that activating ligands were still expressed upon treatment of infected cells with
518 the viral inhibitor phosphonophormic acid suggested that viral IE proteins – which are
519 insensitive to inhibitors of viral DNA synthesis - were involved in these regulatory mechanisms.

520 HCMV IE proteins have been previously suggested to be implicated in the transcriptional
521 activation of MIC genes (Venkataraman, Andresen JI 2007), but several pieces of the puzzle
522 were missing, including the effect of the single versus the combination of the two IE, the
523 molecular mechanism(s) of such regulation, and the promoter region eventually responsive to
524 viral IE. In relation to PVR, no data have been instead reported on its HCMV-induced increase
525 and on the molecular mechanism(s) involved. Our results show that ectopic expression of IE1
526 and IE2 induced a significant increase of MICA and PVR, both at the mRNA and cell surface
527 expression level. In particular, IE2 emerged as the main transactivator of MICA promoter, with
528 the effect strictly dependent on its DNA binding activity, as it was lost in the presence of the IE2-
529 55 isoform and of the zinc finger mutant form of IE2-86. Accordingly, by ChIP and mutagenesis
530 approaches, we identified an IE2 consensus sequence in MICA promoter, critical for its
531 activation by this viral protein. Our study contributes to challenge the prevailing view that
532 activation of cellular genes by IE2 is due to protein-protein interactions. In fact, it was initially
533 thought that IE2 activates cellular promoters by interacting with basal transcription factors,
534 raising the notion that nucleotide-specific binding of IE2 to cellular promoters did not occur,
535 despite this appeared to be the predominant mode of regulation of HCMV promoters. However,
536 in 1998 Bresnahan and co-workers demonstrated that IE2 was capable to activate the cyclin E

537 promoter via a DNA-binding mechanism. In addition, they noticed that the IE2-binding sites
538 within the cyclin E promoter differed from those observed within HCMV promoters, with the 10-
539 internal nucleotides of the CG-N₁₀-CG motifs being GC-rich, rather than AT-rich (ref). Similarly,
540 the MICA IE2-binding site we have identified appears to be GC-rich, further supporting the idea
541 that IE2 is relatively sequence tolerant. It is also possible that IE2 DNA binding ability to cellular
542 promoters relies more on GC-rich than AT-rich motifs. However, since the cellular promoters
543 described so far to which IE2 directly binds are only two (cyclin E and MICA), this aspect needs
544 further investigations. Moreover, this is the first evidence that IE2 binds to and activates a
545 promoter of a cellular gene involved in the elimination of infected cells by cytotoxic lymphocytes,
546 and opens the possibility that other viruses may use their encoded proteins to directly induce
547 activating ligand expression.

548 In relation to PVR, our results demonstrate a different mechanism of its up-regulation. PVR
549 mRNA and protein expression were mostly increased by the co-expression of IE1 and IE2, an
550 observation further confirmed in transfection assays, in which IE1 was able to significantly
551 transactivate the promoter, but with a maximal activation obtained by the combination of the two
552 IE proteins. Experiments performed using progressive deletions of PVR promoter allowed us
553 the identification of a region between -281 bp and -213 bp mediating most of the transactivation
554 activity of IE1/IE2 combination. Though this fragment contains a potential IE2-responsive CG-
555 N₁₀-CG element (from -271 to -257: CG-CAGGCGCAGG-CG), direct DNA binding of IE1/IE2
556 proteins to PVR promoter does not occur, since i) the IE2-55 isoform and the zinc finger mutant
557 of IE2 retained the capability to activate PVR promoter, and ii) IE1 seems not to bind DNA
558 directly (Castillo and Kowalik 2002). Thus, a possible mechanism of PVR regulation is that in
559 the -281/-213 bp region could map the binding site(s) of other factor(s) recruited and/or

560 activated by IE proteins. However, PVR promoter regulation is largely unknown and further
561 investigations are needed to identify its transactivator(s).

562 One question that could arise is why a virus should increase the expression of molecules
563 involved in the elimination of infected cells. One possibility is that this could be a side effect of
564 the strong transactivating activity of HCMV IE proteins, and in particular of IE2. In fact, this
565 protein is crucial for a productive viral replication, so that the virus cannot avoid to express IE2
566 to survive (Castillo 2002). Moreover, it could be related to MICA gene polymorphism. In fact,
567 many different MICA genetic variants exist, with polymorphisms described both in the promoter
568 and coding regions. Of note, the IE2-consensus sequence we identified is conserved among
569 different allelic variants of MICA promoter (our unpublished observations and Cox ST Tissue
570 Antigens 2014; Jia Luo Human Immunol 2014). These observations suggest that, during the
571 virus-host coevolution, a positive selection of promoter sequences in MICA alleles carrying the
572 IE2 DNA binding site occurred, and that the host has made IE2 useful for its cellular gene
573 expression as well.

574 However, how can we reconcile the observed increase in the expression levels of MICA, ULBP3
575 and PVR upon HCMV infection, with the immunoevading strategies evolved by the virus to
576 target activating ligands? Our results suggest that there could be a temporal window in the early
577 phases of the infection, during which the expression of NKG2D and DNAM-1 ligands induced by
578 IE proteins precedes the expression of virus-encoded immunoevasion proteins. In this *window*
579 *of opportunity*, with elevated, functionally relevant levels of activating signals, the immune
580 surveillance against the viral infection could be sufficiently robust, allowing recognition of
581 infected cells by cytotoxic lymphocytes. Another option could be linked to tissue
582 compartmentalization of HCMV in human hosts. A hallmark of HCMV infections is dissemination

583 to a wide range of host tissues and cell types (Sinzger JV 1995,) and quantitative descriptions
584 of the virus have pointed out significant differences in the level of diversity between
585 compartments, e.g., with blood populations appearing more diverse than urine or intraocular
586 populations (Sowmya P J Med Virol 2009; Renzette N Plos Genetics 2013; Renzette N Curr
587 Opin Virol 2014). Although it is not clear yet neither the mechanism explaining HCMV
588 compartmentalization and intrahost genetic diversity nor their effects on clinical disease, one
589 possibility is that the generation of mutants may influence NK cell and/or T cell recognition,
590 depending on the compartment (Renzette N Curr Opin Virol 2014). Therefore, the balance
591 between expression and down-modulation of NK/T cell activating ligands, and thus between
592 immunorecognition and immunoevasion, may vary depending also on HCMV diversity and
593 tropism. We thus believe that a better understanding of the cell-intrinsic and -extrinsic
594 mechanisms regulating NKG2D and DNAM-1 ligands, and consequently affecting immune
595 responses mediated by these activating receptors expressed by cytotoxic lymphocytes is
596 needed to take full advantage of this potent immune pathway.

597

598 **Materials and methods**

599

600 **Antibodies and reagents**

601 The following monoclonal antibodies (mAbs) were used in flow cytometry: anti-MICA (M673)
602 and anti-ULBP4 (M475) from Amgen (Seattle, USA); anti-MICA (AMO-1) from BamOmaB
603 (Axxora, Gräfelfing, Germany); anti-MICB (MAB236511), anti-ULBP1 (MAB170818), anti-
604 ULBP2 (MAB165903), and anti-ULBP3 (MAB166510) from R&D Systems (Minneapolis, MN);
605 anti-Nectin-2 (CD112) from BD Biosciences (San Diego, CA); anti-PVR (SKII.4) kindly provided
606 by Dr M. Colonna (Washington University, St Louis, MO); Alexa fluor 488-conjugated anti-IE
607 antigens (MAB810X) and FITC-conjugated anti-phospho-histone H2AX (γ H2AX) (Ser139; clone
608 JBW301) from Merck Millipore (Darmstadt, Germany); mouse control IgG1 from Biolegend (San
609 Diego, CA); goat anti-mouse (GAM)-APC from Jackson Immunoresearch Laboratories (West
610 Grove, PA); GAM-FITC from Cappel (Milan, Italy); mouse control IgG₁-FITC from BD
611 Biosciences (San Diego, CA). The following antibodies were used in immunoblotting: anti-p85
612 subunit of PI-3 kinase from Merck Millipore (Darmstadt, Germany); anti-ATM (D2E2) from Cell
613 Signaling Technology (Danvers MA); anti-ATR (sc-1887) and anti-DNA-PK_{CS} (sc-5282) from
614 Santa Cruz (Dallas, TX).

615 Other reagents used were: gelatin, caffeine, methylcellulose, NU7026, phosphonoformic acid
616 (PFA) (Foscarnet) and crystal violet from Sigma Aldrich (St. Louis, MO); Lipofectamine 2000
617 from Invitrogen (Thermo Fisher Scientific, Waltham, MA), Dharmafect from Dharmacon (GE
618 Healthcare, Buckinghamshire, UK).

619

620 **Cells and culture conditions**

621 Primary human foreskin fibroblasts (HFF), the retinal epithelial cell line ARPE-19 and the human
622 embryo kidney 293T cells were purchased from the American Type Culture Collection (ATCC).
623 HFF and 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10%
624 fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100
625 µg/ml streptomycin sulfate, and ARPE-19 cells in a 1:1 mixture of DMEM and Ham's F-12
626 medium (Invitrogen) containing 10% FCS, 15 mM HEPES, 2 mM glutamine, 1 mM sodium
627 pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin sulfate. HFFs were used at passages
628 14 to 28. Human dermal microvascular endothelial cells (HMVECs) (CC-2543) were obtained
629 from Clonetics (San Diego, CA) and cultured in endothelial growth medium (EGM)
630 corresponding to endothelial basal medium (EBM) (Clonetics), containing 10% FCS, human
631 recombinant vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF),
632 human epidermal growth factor (hEGF), insulin growth factor 1 (IGF-1), hydrocortisone,
633 ascorbic acid, and heparin. Cells were seeded onto culture dishes coated with 0.2% gelatin.
634 Experiments were carried out with cells at passages 4 to 15. Fibroblasts derived from an Ataxia
635 Telangectasia Mutated patient and not expressing ATM protein (AT-/-), were kindly provided by
636 Drs. M. Fanciulli and T. Bruno (Regina Elena National Cancer Institute, Rome, Italy). They were
637 grown in DMEM containing 15% FCS and used at passages 5 to 8. All cells were maintained at
638 37°C in a 5% CO₂ atmosphere.

639

640 **HCMV preparations and infection conditions**

641 The HCMV AD169 strain (ATCC-VR538), was prepared by infecting semi-confluent layers of
642 HFF cells at a virus-to-cell ratio of 0.01, and cultured until a marked cytopathic effect was seen.
643 Stocks were then prepared after 3 rounds of cell freezing and thawing, subjected to centrifugal

644 clarification, and frozen at -80°C . Virus titers were measured by standard plaque assays on
645 HFF cells. Stock solutions used in all experiments contained approximately 2×10^7 PFU/ml.
646 Standard plaque assays were used also in different experiments to determine viral titers in the
647 supernatants harvested from infected cells.

648 HCMV TR was derived from an ocular specimen (Smith IL, Arch Ophthalmol 1998), and after a
649 few passages on fibroblasts, was cloned into a BAC (Murphy E PNAS 2003; Ryckman BJ JV
650 2006). Reconstitution of TR BAC in fibroblasts was performed as previously described (Bronzini
651 et al., J Virol 2012) by co-transfecting HFFs with the corresponding TR BAC and a plasmid
652 expressing HCMV pp71. Reconstituted infectious virus retained the ability to infect endothelial
653 and epithelial cells, as well as monocytes and macrophages (Ryckman BJ JV 2006; Bronzini et
654 al., 2012). HCMV VR1814 is a derivative of a clinical isolate recovered from a cervical swab of a
655 pregnant woman (Revello JGV 2001). This strain was propagated in HUVEC and titrated as
656 previously described (Caposio P, Antiviral Res 2007).

657 Cells were infected at about 80-90% confluence at a multiplicity of infection (MOI) of 1 PFU/cell,
658 unless otherwise specified, in their respective culture medium, without FCS. Virus adsorption
659 was carried out for 2 h (AD169, or TR) or 5 h (VR-1814) at 37°C , and the virus inoculum was
660 then discarded and replaced with fresh growth medium (day 0). Mock-infected control cultures
661 were exposed for the same amount of time to an equal volume of mock-infecting medium. At
662 various days post-infection (dpi), cells were harvested and analyzed. In some experiments,
663 phosphonoformic acid (PFA) was added after virus inoculation at a final concentration of 200
664 $\mu\text{g/ml}$, while caffeine was added 2 dpi at a final concentration of 10 mM.

665

666 **Adenovirus vectors and infections**

667 Recombinant adenoviruses (AdV) encoding HCMV IE86 (AdV-IE86) and *E. coli* β -galactosidase
668 (AdV-LacZ) have been previously described (Gariano et al., Plos Path 2012; Mercorelli B et al.,
669 AAC 2014), while AdV-IE72 was kindly provided by Dr. Timothy F. Kowalik (University of
670 Massachusetts Medical School, Worcester, USA) (Wilkinson, GW, and A. Akrigg. 1992. Nucleic
671 Acids Res.; Ahn, JH., and GS Hayward. 1997. J. Virol; Castillo, JP, AD Yurochko, and TF
672 Kowalik. 2000. J. Virol). Recombinant AdV stocks were generated, purified and titrated as
673 previously described (Gariano et al., Plos Path 2012; Mercorelli et al., AAC 2014; Castillo, JP,
674 AD Yurochko, and TF Kowalik. 2000. J. Virol).

675 For adenoviral transduction, HFFs were infected at about 80-90% confluence at an MOI of 4
676 pfu/cell in DMEM without FCS, for 2 h at 37°C. When the viral proteins were not expressed in
677 combination, the total MOI was equalized to 4 with AdV-LacZ. After 2 h, the virus inoculum was
678 discarded and replaced with fresh growth medium (day 0) and analyzed at the indicated dpi.
679 Mock-infected cells served as control cultures. Following infection, cultures were maintained in
680 growth medium and analyzed at the indicated dpi.

681

682 **Immunofluorescence and FACS analysis**

683 Mock-infected or infected cells were harvested at the indicated dpi and stained with mAbs
684 specific for MICA, MICB, ULBP1-4, PVR and Nectin-2, followed by GAM-APC or by GAM-FITC
685 (for experiments with PFA), and analyzed by flow cytometry on an FACSCalibur (Becton
686 Dickinson). The mean of fluorescence intensity (MFI) value of the isotype control antibody was
687 always subtracted from the MFI relative to each molecule. For intracellular staining of IE
688 antigens or phospho-histone H2AX (γ H2AX), cells were fixed in 1% formaldehyde, permeabilized
689 with 70% ethanol, and then incubated with Alexa fluor 488-conjugated anti-IE mAb (MAB810X)

690 or with FITC-conjugated anti- γ H2AX (JBW301), respectively.

691

692 **Immunoblot analysis**

693 Cells were lysed for 20 minutes at 4°C in a lysis buffer containing 0.2% Triton X-100, 0.3%
694 NP40, 1 mM EDTA, 50 mM Tris HCl pH 7.6, 150 mM NaCl, and protease inhibitors to obtain
695 whole-cell protein extracts.. Protein concentration was measured with the Bio-Rad Protein
696 Assay. Lysates (30-40 μ g) were resolved by SDS-PAGE and transferred to nitrocellulose
697 membranes (Merck Millipore). Membranes were blocked with 5% milk and probed with the
698 indicated antibodies. Immunoreactivity was revealed using an enhanced chemiluminescence kit
699 (Amersham).

700

701 **siRNA**

702 The ON-TARGETplus SMARTpool siRNA specific for ATM and ATR (siATM, siATR), and the
703 ON-TARGETplus non-targeting pool (siCtrl) were purchased from Dharmacon (Thermo Fisher
704 Scientific). siRNA specific for DNA-PKcs (sc-35200) (siDNA-PK) was from Santa Cruz. HFF
705 cells (70%-80% confluence) were transfected with 100-200 nM of siRNA using DharmaFECT
706 siRNA Transfection Reagent (Thermo Fisher Scientific), according to the manufacturer's
707 recommendations. One to two days after transfection, cells were infected with AD169, as
708 indicated in the figure legends. Cells and supernatants were harvested and analyzed at 2 or 3
709 dpi, as indicated.

710

711 **RNA isolation and real-time PCR**

712 Total RNA was extracted using TRI Reagent Solution (Life Technologies Inc., Grand Island,

713 NY), according to manufacturer's instructions, and 1 μ g of total RNA was used for cDNA first-
714 strand synthesis in a reaction volume of 25 μ l. Real-Time PCR was performed using the ABI
715 Prism 7900 Sequence Detection system (Applied Biosystems, Foster City, CA); cDNAs were
716 amplified in triplicate with primers for MICA (Hs00792195_m1), PVR (Hs00197846_m1), and
717 GAPDH (Hs03929097_g1), using specific TaqMan Gene Expression Assays (Applied
718 Biosystems, Foster City, CA). Relative expression of each gene versus GAPDH was calculated
719 according to the $2^{-\Delta\Delta C_t}$ method.

720

721 **Plasmids**

722 The pGL3-*MICA* promoter vector was previously described (Yadav DJ et al, JI 2009) and kindly
723 provided by Dr. J. Bui (University of California at San Diego, La Jolla, CA). To generate the
724 *MICA* -270 promoter plasmid, a fragment of 270 bp was obtained by cutting the 3.2 WT GFP
725 reporter (Dr. Skov, University of Copenhagen, Denmark) with the enzymes *KpnI* and *BglII*, and
726 cloned in pGL3-Basic luciferase vector (Promega Corp., Madison, WI) (Soriani JI 2014). Mutant
727 *MICA* -270-CG construct was generated using Quick Change Site-Directed Mutagenesis Kit
728 (Stratagene, La Jolla, CA) following the manufacturer's instructions. Primers were designed to
729 generate two CG mutations in the *MICA* promoter region of the pGL3 -270 bp *MICA*. Primer
730 sequences used were: -92 bp -**CGGTCGGGGGACCG** -78 bp; primers for mutagenesis: forward
731 5' -CCAGTTTCATTGGATGAGATGTCGGGGGACATGGCCAGGTGACTAAG-3'; reverse
732 5'-CTTAGTCACCTGGCCATGTCCCCCGACATCTCATCCAATGAAACTGG-3'. Inserted
733 mutations were verified by sequencing.

734 The pGL2-Basic luciferase vector containing -571 bp of the human *PVR* promoter and
735 progressive deletions (-470 bp, -343 bp, -281 bp and -213 bp) were kindly provided by Dr. G.
736 Bernhardt (Hannover Medical School, Hannover, Germany) (Solecki et al., 1997).

737 pSG5-IE72, pSG5-IE86, and pSG5-IE55 constructs contained the full-length cDNAs of the viral
738 IE proteins, cloned in the pSG5 vector (Stratagene) (Klucher et al., 1993). The IE86 cDNA
739 cloned in the pRSV vector, and the zinc finger mutant of IE86, with cysteines 428 and 434
740 mutated into serine residues (pRSV-IE86-Zn mut), were a generous gift of Prof. Jay Nelson and
741 were previously described (Jupp R et al, JV 1993).

742

743 **Transfection and luciferase assay**

744 In all transfection experiments, 3 µg of luciferase reporter, 0.25 µg of pRL-CMV-Renilla, and 2
745 µg of IE protein vectors or of pSG5 empty vector were co-transfected into 80-90% confluent
746 cells growing on a 10 cm² area using Lipofectamine 2000 (Invitrogen) according to the
747 manufacturer's protocols. In some experiments, the pSG5-IE86 vector was replaced by pSG5-
748 IE55, pRSV-IE86 or pRSV-IE86-Zn-mut, as indicated. 48 hours post-transfection, cells were
749 harvested and prepared for the luciferase assays, using the Dual-Luciferase Reporter Assay kit
750 and the Glomax Multi Detection System (Promega Corp., Madison, WI) following the
751 manufacturer's instructions. Relative luciferase activity was calculated by dividing the luciferase
752 activity of pGL3-MICA or pGL2-PVR reporter, co-transfected with pSG5, by the respective
753 pGL3- or pGL2-Basic, to remove the unspecific effect of IE proteins on the reporter vector. The
754 unspecific modulation of the reporter empty vector activity was probably due to a general
755 activation of the transcriptional machinery by IE proteins, and was more evident for IE1. A
756 similar correction allowed us to better appreciate the specific effect of the viral proteins on

757 ligand promoters.

758

759 **Chromatin immunoprecipitation assay (ChIP)**

760 293T cells were co-transfected with 5 µg of *MICA* -270 promoter plasmid, wild-type or mutated,
761 and pSG5-IE72 (10 µg) and pSG5-IE86 (10 µg), or pSG5 empty vector (20 µg), using
762 Lipofectamine 2000. After 48 hours, cells were cross-linked, harvested, and processed for ChIP
763 assays following the manufacturer's protocol of Magna ChIP AG chromatin immunoprecipitation
764 kit (Merck Millipore). Nuclei were isolated and chromatin was sonicated to generate 150-300 bp
765 fragments. Samples were immunoprecipitated overnight with 20 µl of a polyclonal rabbit anti-IE
766 antibody, that we generated and recognizing from 1 to 429 aa of HCMV IE2-86 protein, or with
767 an equivalent amount of a polyclonal rabbit serum used as control, and 20 µl of protein AG
768 magnetic beads. Immunoprecipitates were washed and digested with proteinase K for 2 hours
769 at 62°C. DNA was purified using spin columns and eluted in 50 µl of eluting reagent. 20 µl of
770 pre-cleared chromatin were processed as immunoprecipitated chromatin, for input DNA
771 controls. Purified DNA was quantified by real-time PCR, using the Power-SYBR Green mix
772 (Applied Biosystems, Foster City, CA). Primer sequences used were: *MICA* promoter forward
773 5'-AGGTCTCCAGCCCACTGGAATTTTCTC-3'; *MICA* promoter reverse 5'-
774 CGCCACCCTCTCAGCGGCTCAAGC-3'. PCRs were validated by the presence of a single
775 peak in the melt curve analysis, and amplification of a single specific product was further
776 confirmed by electrophoresis on agarose gel. Results are expressed as relative enrichment as
777 compared to the input. Negative control (polyclonal rabbit serum) values were subtracted from
778 the corresponding samples. Absolute quantifications were obtained by serial dilutions of the

779 input DNA samples. The analysis was performed using the SDS version 2.4 software (Applied
780 Biosystems).

781

782 **Confocal microscopy analysis**

783 For staining of cell surface MICA, HFFs were grown to semi-confluence on glass coverslips in
784 24-well plates and infected with AD169 and TR at a MOI of 1 PFU/cell for 2 h at 37°C. After 4
785 dpi, cells were washed with PBS, fixed in 1% paraformaldehyde for 15 min at room-temperature
786 (RT), blocked in 1% FCS diluted in PBS (20 min., RT), but not permeabilized, to not allow mAb
787 entry into the cells. Indirect immunofluorescence analysis was performed by incubating fixed
788 cells with the anti-MICA mAb AMO-1 (1:40) for 2 h at 37°C, followed by secondary antibody
789 incubation with CF594-conjugated rabbit anti-mouse IgG (Sigma) for 1 h at RT. Samples were
790 then visualized with an Olympus IX70 inverted laser scanning confocal microscope, and images
791 were captured using FluoView 300 software (Olympus Biosystems).

792

793 **Statistical analysis**

794 Statistical analysis of the data was performed using a paired Student *t*-test. A value of $p < 0.05$
795 was considered statistically significant.

796

797 **Figure legends**

798

799 **Figure 1. Evaluation of NKG2D and DNAM-1 ligand expression on AD169-infected**
800 **fibroblasts.** HFFs were infected with the HCMV laboratory strain AD169 (black line) at an MOI
801 of 1 PFU/cell or mock-infected (n.i., grey filled histograms) and harvested at different days post-
802 infection (dpi), as indicated. Ligand expression was evaluated by FACS analysis on cells
803 stained with mAbs specific for MICA, MICB, ULBP1-4, PVR and Nectin-2, followed by GAM-
804 APC. **A)** A representative experiment of at least four performed at 3 dpi is shown. Dashed lines
805 indicate mock-infected (n.i.) or infected cells stained with isotypic control IgG. **B)** The kinetics of
806 ligands with an increased expression upon HCMV infection is shown. Expression levels are
807 presented as mean of fluorescence intensity (MFI). MFI of isotypic control IgG was subtracted
808 from the MFI relative to each ligand. Values represent the mean of at least four independent
809 experiments \pm standard error (SE). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ with Student's *t*-test of n.i.
810 versus HCMV-infected cells.

811

812 **Figure 2. NKG2D and DNAM-1 ligands are up-regulated on different cell types by HCMV**
813 **low-passage strains VR-1814 and TR.** HFFs, HMVECs or ARPE-19 cells were not infected
814 (n.i.) or infected with the indicated HCMV low-passage strain. Cells were then harvested at 3 dpi
815 and ligand expression was evaluated by FACS analysis with mAbs specific for MICA, ULBP3
816 and PVR, or with an isotype control IgG, followed by GAM-APC. **A)** A representative experiment
817 of HFFs infected with the low-passage strain VR-1814, and with AD169 as a control, is shown.
818 **B)** HFFs were infected with VR-1814 at MOI of 1 and 5 PFU/cell, and the expression of MICA,
819 ULBP3, and PVR was assessed at 3 dpi. Expression levels are presented as the mean of three

820 independent experiments \pm SE. **C)** HFFs, HMVECs, and ARPE-19 were infected with TR or VR-
821 1814 at a MOI of 1 PFU/cell and the expression of MICA, ULBP3, and PVR was measured at 3
822 dpi. Expression levels are presented as the mean of at least three independent experiments \pm
823 SE. In panels B) and C) expression levels are presented as mean of fluorescence intensity
824 (MFI), calculated as in figure 1. * $p < 0.05$; ** $p < 0.01$ with Student's *t*-test of n.i. versus HCMV-
825 infected cells. The percentage of infected cells (measured by intracellular staining with the anti-
826 IE mAb) was as follows: HFF with VR1814 (MOI 1): $45 \pm 10\%$; HFF with TR: $96 \pm 0\%$; HMVEC
827 with TR: $76 \pm 8\%$; HMVEC with VR1814: $44 \pm 11\%$; ARPE-19 with TR: $77 \pm 6\%$; ARPE-19 with
828 VR1814: $48 \pm 6\%$.

829

830 **Figure 3. Triple silencing of ATM, ATR and DNA-PK does not affect MICA, ULBP3 and**
831 **PVR expression.** HFF were firstly transfected with siDNA-PK or with siCtrl. 24 h later, the same
832 cells were co-transfected with siATM and siATR, or with siCtrl. 24 h later, cells were either
833 uninfected or infected with HCMV (AD169) at MOI 1 PFU/cell, then, at 3 dpi cells and
834 supernatants were harvested and assayed for ligand expression, immunoblot analysis,
835 percentage of IE+ cells, and infectious virus production. **A)** Flow cytometry analysis of MICA,
836 ULBP3 and PVR expression, as described in figure 1. Vertical dotted lines indicate the center of
837 the peak for each ligand in not infected-siCtrl transfected cells. All panels derive from the same
838 experiment, representative of three. **B)** The levels of ATM, ATR and DNA-PK protein expression
839 were assayed by immunoblot analysis with antibodies specific for each molecule. p85 subunit of
840 PI-3K was used as a control of protein loading. **C)** The % of IE+ cells was analyzed by flow
841 cytometry on HCMV-infected cells by intracellular staining with a specific anti-IE mAb. **D)** Cell
842 culture supernatants were assayed for infectious virus production by plaque assay. ns: not

843 statistically significant difference with Student's t-test. siDDR: cells transfected with siATM,
844 siATR and siDNA-PK.

845

846 **Figure 4. Immediate early and early genes, but not late genes, are *per se* sufficient to**
847 **increase the expression of MICA, ULBP3 and PVR in infected cells.** HFFs were infected
848 with HCMV AD169 (MOI of 1 PFU/cell) or mock-infected, and then treated with 200 µg/ml of
849 phosphonoformic acid (PFA) immediately after infection. At 3 dpi, cells were harvested and
850 stained for MICA, ULBP3, PVR or isotype control IgG, followed by GAM-FITC. One
851 representative experiment out of three is shown.

852

853 **Figure 5. Up-regulation of MICA and PVR mRNA in HCMV-infected cells.** HFFs cells were
854 infected with HCMV AD169 at an MOI of 1 PFU/cell or mock-infected. At the indicated times
855 post-infection, total RNA was purified and reverse transcribed. cDNAs were amplified by real-
856 time PCR using primers specific for MICA, PVR, or GAPDH. Data, expressed as fold change
857 units, were normalized with GAPDH and referred to not infected cells considered as calibrators,
858 and set at 1. Values represent the mean of two independent experiments ± SE.

859

860 **Figure 6. Adenoviral-mediated overexpression of IE1 and IE2 proteins increases mRNA**
861 **and cell surface expression of MICA and PVR.** HFFs were transduced with adenoviral
862 vectors (AdV) expressing IE1, IE2, or LacZ as a control, alone or in combination, at a total MOI
863 of 4 PFU/cell. Cells were harvested at 48 h (for mRNA) or at 72 h (for cell surface expression)
864 post-transduction, and analyzed for ligand mRNA content and surface expression, respectively.
865 **A) and C)** Real-time PCR was performed with primers specific for MICA **(A)** and PVR **(C)**. Data,

866 expressed as fold change units \pm SE, were normalized with GAPDH and referred to not-
867 transduced cells (-), considered as calibrators and set at 1. Values derive from four independent
868 experiments. **B) and D)** Cell surface expression levels of MICA (**B)** and PVR (**D)** as measured
869 by FACS are presented as mean of fluorescence intensity (MFI), as described in figure 1.
870 Values represent the mean of three independent experiments \pm SE. ns: not significant; *
871 $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ with Student's t-test. See Tables S1 and S2 for a complete
872 statistical analysis.

873

874 **Figure 7. IE2 activates *MICA* promoter: role of IE2 DNA binding activity.** **A)** HFFs were
875 transiently transfected with pGL3-MICA (-1 Kb fragment) luciferase reporter plasmid, together
876 with expression vectors containing the cDNA of the indicated IE proteins, used alone or in
877 combination, or with the empty control vector pSG5. After 48 h, transfected cells were harvested
878 and protein extracts were used for luciferase assay. Luciferase activity was calculated as
879 described in Materials and Methods, and results are expressed as fold-induction compared to
880 the empty control vector. **B) and C)** IE2-86 was replaced by IE2-55 (**B)** or by a zinc finger
881 domain mutant IE2-86 (IE86 Zn mut) (**C**). IE2-86 Zn mut was cloned into pRSV vector, so in
882 panel C pRSV and pRSV-IE2-86 were used as controls. Data represent the mean value from at
883 least three independent experiments \pm SE. ns: not significant; * $p < 0.05$; ** $p < 0.01$ with Student's
884 t-test.

885

886 **Figure 8. Identification of an IE2 consensus site in *MICA* promoter.** **A)** HFFs were
887 transiently transfected with wild-type (wt) pGL3-MICA (-270 bp fragment) promoter luciferase
888 reporter vector, or with a mutated form (CG-mut), together with plasmid vectors containing the

889 cDNA of IE1 and IE2, or with the empty control vector pSG5. After 48 h, cells were harvested
890 and luciferase activity was calculated as described in figure 7. Data represent the mean values
891 from three independent experiments \pm SE. **B)** the CG-N₁₀-CG sequence identified on MICA
892 promoter, and its mutated form (CG-mut), are reported and compared with some of the IE2-
893 binding sites described on the HCMV MIEP, the 2.2 Kb early promoter and the cyclin E
894 promoter. **C)** 293T cells were co-transfected with wt pGL3-*MICA* (-270 bp fragment) promoter,
895 and plasmid vectors containing IE1 and IE2 cDNAs, or with pSG5. After 48 h, cells were
896 harvested and processed for the ChIP assay, as described in material and methods. Results
897 are shown as relative enrichment of samples immunoprecipitated with the anti-IE antibody (anti-
898 IE) in respect to IgG control. Data represent the mean values from three independent
899 experiments \pm SE. **D)** Both the wt and the mutant form of -270 bp *MICA* promoter were used in
900 ChIP experiments, and the relative enrichment was compared. Data are expressed as percent
901 of IE binding, with the relative enrichment of MICA -270 wt promoter set as 100%. Data
902 represent the mean value from three independent experiments \pm SE. * p<0.05; ** p<0.01; ***
903 p<0.001. MIEP: major immediate early promoter.

904

905 **Figure 9. Effect of IE1 and IE2 on the transcriptional activity of PVR gene promoter. A)**
906 HFFs were transiently transfected with pGL2-PVR (-571 bp fragment) promoter luciferase
907 reporter vector, together with plasmid vectors containing the cDNA of the indicated IE proteins,
908 used alone or in combination, or with the empty control vector pSG5. After 48 h, cells were
909 harvested and luciferase activity was calculated as reported in figure 7. **B) and C)** IE2-86 was
910 replaced by IE2-55 (**B)** or by a zinc finger domain mutant IE2-86 (IE2 Zn mut) (**C**), as described
911 in figure 7. **D)** HFFs were transiently transfected with wild-type pGL2-*PVR* (-571 bp fragment)

912 promoter luciferase reporter vector, or with 5'-deletions constructs (-470, -343, -281, and -213),
913 together with plasmid vectors containing IE1 and IE2 cDNA, or with pSG5. After 48 h, cells were
914 harvested and luciferase activity was calculated as reported in figure 7. Data represent the
915 mean value from at least four independent experiments \pm SE. ns: not significant; * $p < 0.05$; **
916 $p < 0.01$; *** $p < 0.001$ with Student's *t*-test.

917

918

919 **Supporting Information**

920

921 **Supplementary Figure 1. HCMV AD169 and TR strains induce expression of cell surface**
922 **MICA.** HFFs were grown to subconfluence and then mock-infected or infected with HCMV
923 AD169 and TR at an MOI of 1 PFU/cell, or mock infected. At 4 dpi, cells were fixed and
924 immunostained for MICA ligand, without permeabilization. Immunofluorescence experiments
925 were repeated three times, and representative results are presented. Magnification: 60X.

926 **Supplementary Figure 2. Activation of DDR pathway after HCMV infection.** HFFs were
927 infected with the HCMV (AD169) at a MOI of 1 PFU/cell or mock-infected (n.i.) and harvested at
928 3 dpi. Phospho-histone H2AX (γ H2AX) (Ser139) expression levels were evaluated by FACS
929 analysis on cells stained with a specific FITC-conjugated mAb. **A)** A representative experiment
930 of four performed at 3 dpi is shown. **B)** Data are presented as fold induction of γ H2AX MFI
931 values in HCMV-infected versus not infected (n.i.) cells, set at 1. Values represent the mean of
932 four independent experiments \pm SE. * $p < 0.05$ with Student's *t*-test of n.i. versus HCMV-infected
933 cells.

934

935 **Supplementary Figure 3. Absence of functional ATM does not prevent cell surface**
936 **expression of MICA, ULBP3 and PVR.** AT^{-/-} fibroblasts were mock-infected (n.i.) or infected
937 with HCMV AD169 at an MOI of 1 PFU/cell. At different dpi, cells were harvested and ligand
938 expression was analyzed as in figure 1. A representative experiment out of three is shown.

939

940 **Supplementary Figure 4. ATM silencing does not affect MICA, ULBP3 and PVR**
941 **expression.** HFFs were transiently transfected with siRNA specific for ATM (siATM) or with a
942 non-targeting siRNA (siCtrl). 24 h later, cells were either uninfected or infected with HCMV
943 AD169 at an MOI of 1 PFU/cell. At 2 dpi, cells and supernatants were harvested and assayed
944 for ligand expression, immunoblot analysis, percentage of IE⁺ cells, and infectious virus
945 production. **A)** Flow cytometry analysis of MICA, ULBP3 and PVR expression was performed as
946 described in figure 1. Vertical dotted lines indicate the center of the peak for each ligand in not
947 infected-siCtrl transfected cells (thin grey histograms). All panels derive from the same
948 experiment, representative of three. **B)** The levels of ATM protein expression were assayed by
949 immunoblot analysis with a specific antibody. Immunodetection of the p85 subunit of PI-3K was
950 used as a control of protein loading. **C)** The % of IE⁺ cells was analyzed by flow cytometry on
951 HCMV-infected cells by intracellular staining with a specific anti-IE mAb. **D)** Cell culture
952 supernatants were assayed for infectious virus production by plaque assay. ns: not statistically
953 significant difference with Student's t-test.

954

955 **Supplementary Figure 5. ATR silencing does not affect MICA, ULBP3 and PVR**
956 **expression.** HFFs were transfected with siRNA specific for ATR (siATR) or with a non-targeting
957 siRNA (siCtrl), infected and harvested as described in Fig. S4. **A)** Flow cytometry analysis of

958 MICA, ULBP3 and PVR expression was performed as described in figure 1. Vertical dotted lines
959 indicate the center of the peak for each ligand in not infected-siCtrl transfected cells (thin grey
960 histograms). All panels derive from the same experiment, representative of three. **B)** The levels
961 of ATR protein expression were assayed by immunoblot analysis with a specific antibody.
962 Immunodetection of the p85 subunit of PI-3K was used as a control of protein loading. **C)** The %
963 of IE+ cells was analyzed by flow cytometry on HCMV-infected cells by intracellular staining with
964 a specific anti-IE mAb. **D)** Cell culture supernatants were assayed for infectious virus production
965 by plaque assay. ns: not statistically significant difference with Student's t-test.

966

967 **Supplementary Figure 6. DNA-PK silencing does not affect MICA, ULBP3 and PVR**
968 **expression.** HFFs were transfected with siRNA specific for DNA-PK (siDNA-PK) or with a non-
969 targeting siRNA (siCtrl), infected and harvested as described in Fig. S4. **A)** Flow cytometry
970 analysis of MICA, ULBP3 and PVR expression as described in figure 1. Vertical dotted lines
971 indicate the center of the peak for each ligand in not infected-siCtrl transfected cells (thin grey
972 histograms). All panels derive from the same experiment, representative of three. **B)** The levels
973 of DNA-PK protein expression were assayed by immunoblot analysis with a specific antibody.
974 Immunodetection of the p85 subunit of PI-3K was used as a control of protein loading. **C)** The %
975 of IE+ cells was analyzed by flow cytometry on HCMV-infected cells by intracellular staining with
976 a specific anti-IE mAb. **D)** Cell culture supernatants were assayed for infectious virus production
977 by plaque assay. ns: not statistically significant difference with Student's t-test.

978

979

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984 adenovirus; Marco Colonna for the anti-PVR mAb SKII.4; Jack D. Bui for pGL-3 MICA promoter
985 vector, and Günter Bernhardt for PVR promoter progressive deletions. We wish to thank
986 members of the Santoni laboratory for discussions.

987

988 **Author Contributions**

989 Conceived and designed the experiments: BP, CF, VDO, MC, SL, GG, AS, CC. Performed the
990 experiments: BP, CF, AL, CC. Analyzed the data: BP, CF, MC, CC. Contributed
991 reagents/materials/analysis tools: CF, VDO, AL, AZ, MC, SL, GG, AS. Wrote the paper: BP, CF,
992 GG, AS, CC.

993

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1001 manuscript. **Nostro PRIN?**

1002 **Table S1.** Statistical analysis of the results obtained for MICA mRNA and cell surface
 1003 expression on HFF cells transduced with the indicated recombinant AdV.

MICA (mRNA)	-	LacZ	IE72	IE86	IE72+IE86
-	/	ns	ns	0.026	0.004
LacZ		/	ns	0.022	0.003
IE72			/	0.025	0.003
IE86				/	ns
IE72+IE86					/
MICA (cell surface)					
-	/	ns	ns	0.031	0.044
LacZ		/	ns	0.023	0.035
IE72			/	0.025	ns
IE86				/	ns
IE72+IE86					/

1004

1005

1006

Table S2. Statistical analysis of the results obtained for PVR mRNA and cell surface expression on HFF cells transduced with the indicated recombinant AdV.

VR (mRNA)	-	LacZ	IE72	IE86	IE72+IE86
-	/	ns	ns	ns	0.038
LacZ		/	ns	0.021	0.022
IE72			/	ns	0.025
IE86				/	0.019
IE72+IE86					/
PVR (cell surface)					
-	/	ns	0.0006	0.030	0.0008
LacZ		/	0.005	0.009	0.003
IE72			/	ns	0.003
IE86				/	0.024
IE72+IE86					/

1007

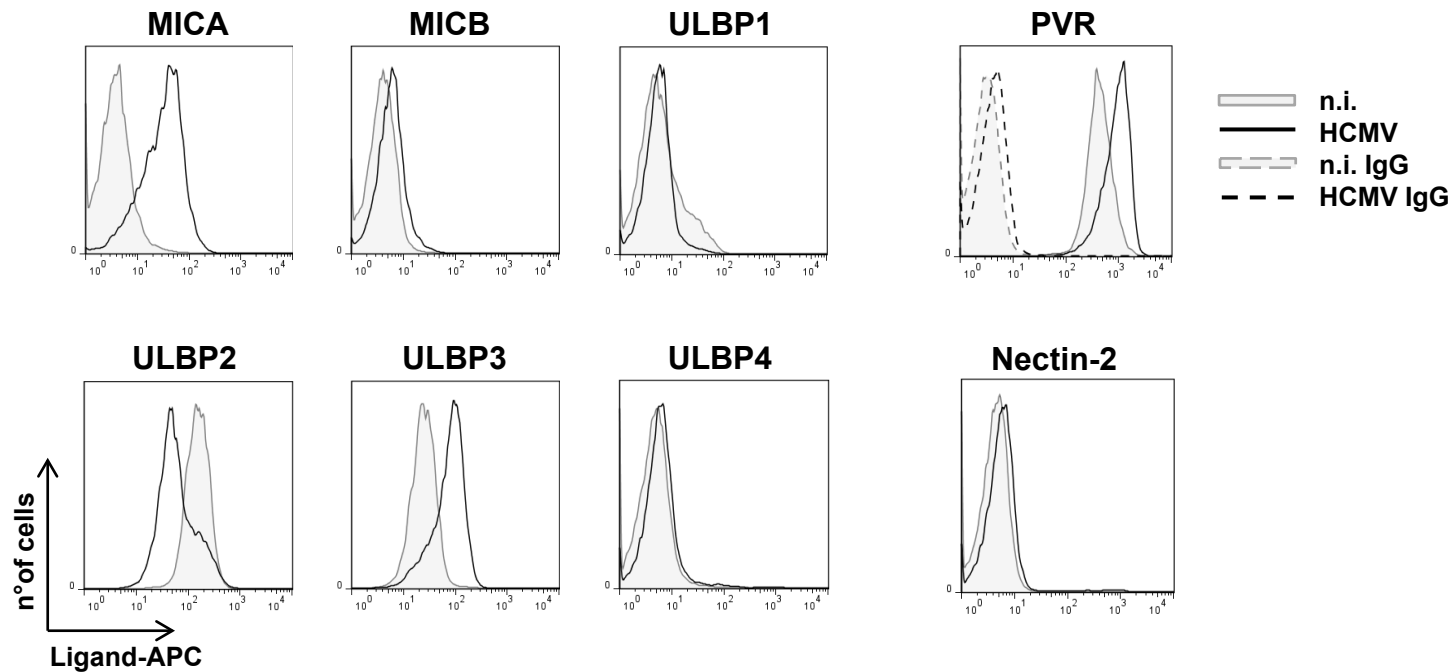
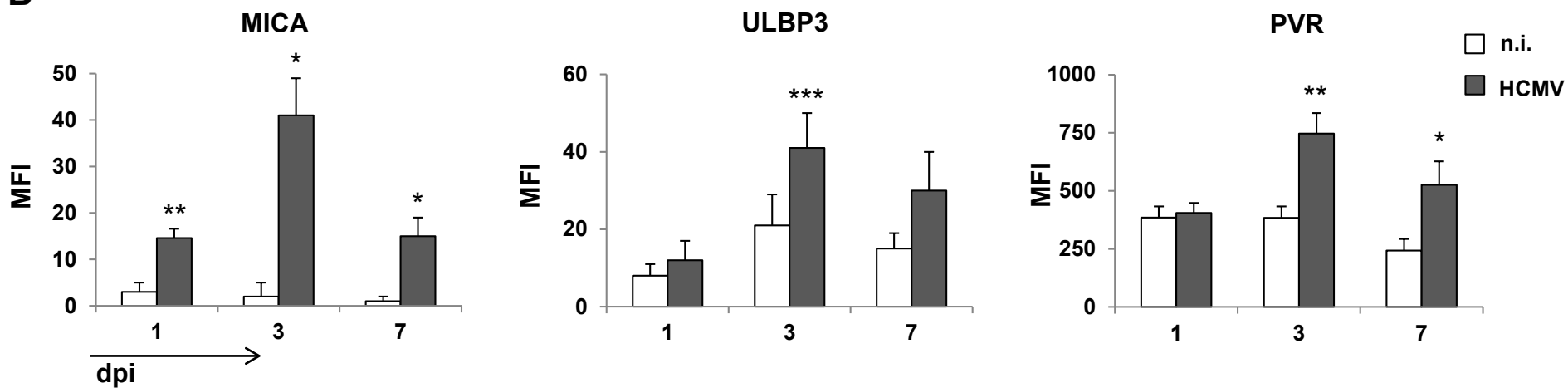
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Fig. 1

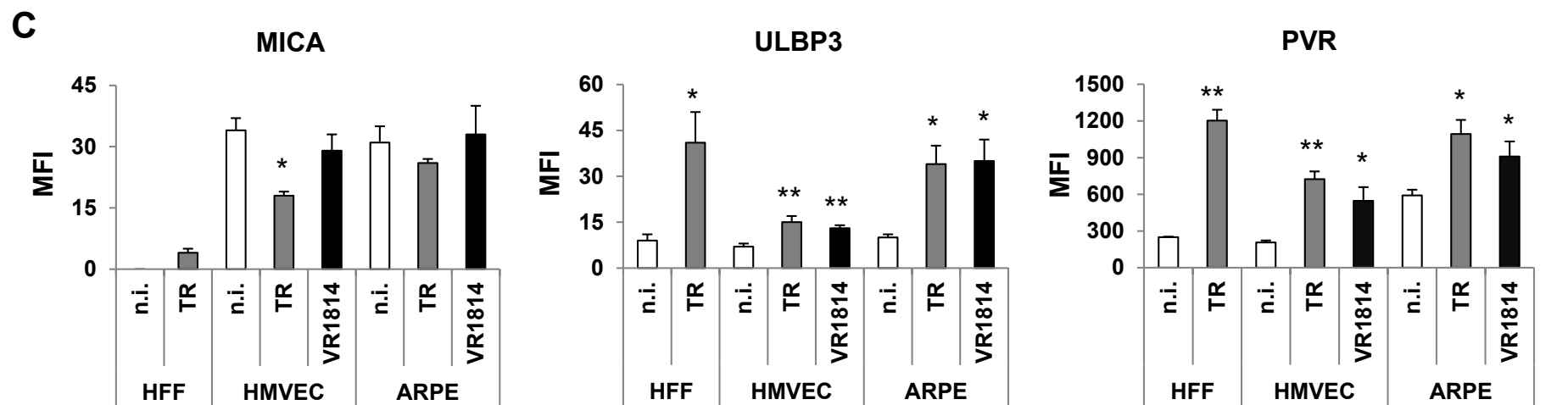
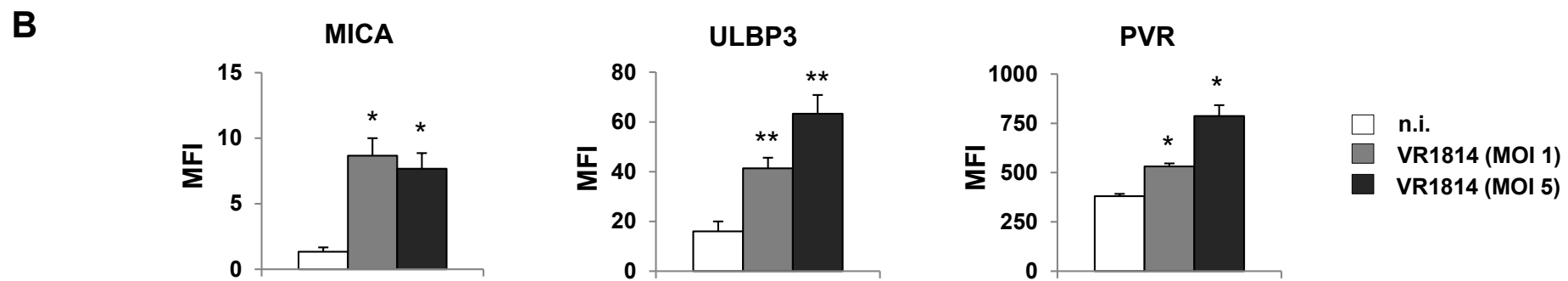
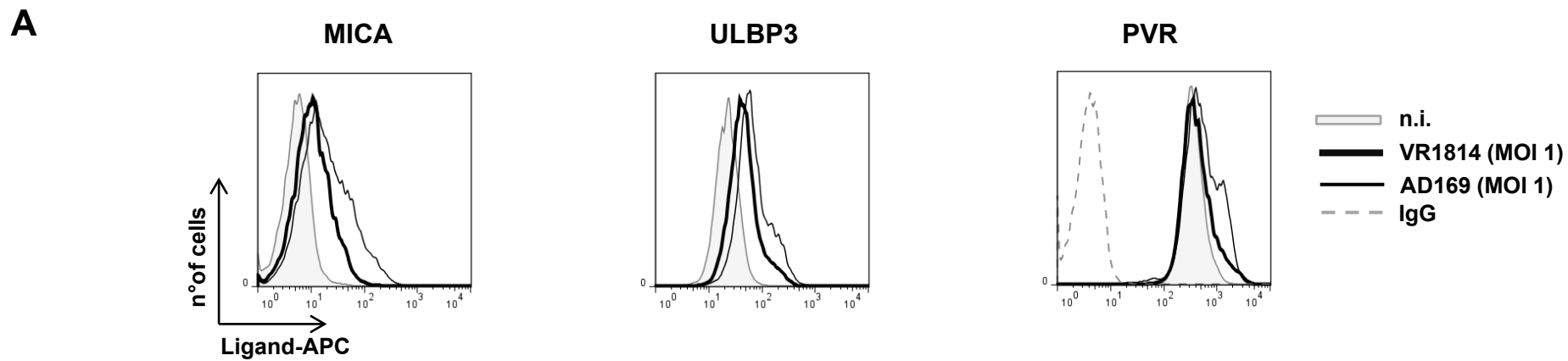


Fig. 2

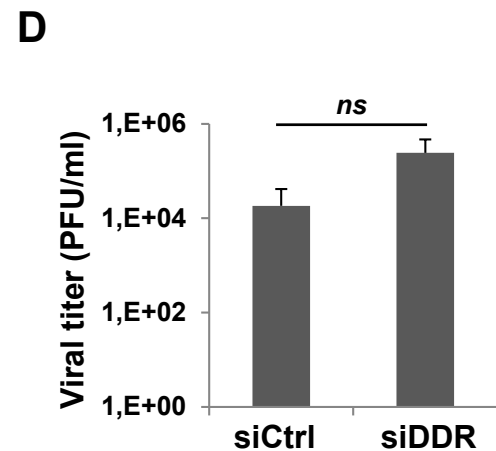
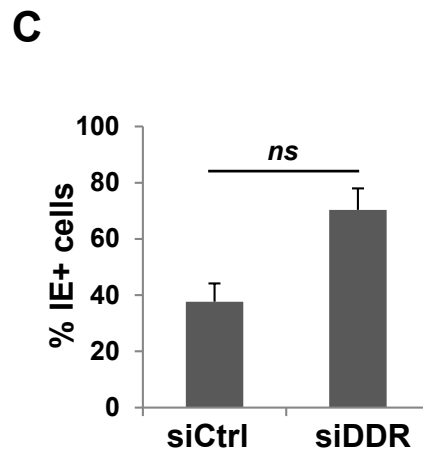
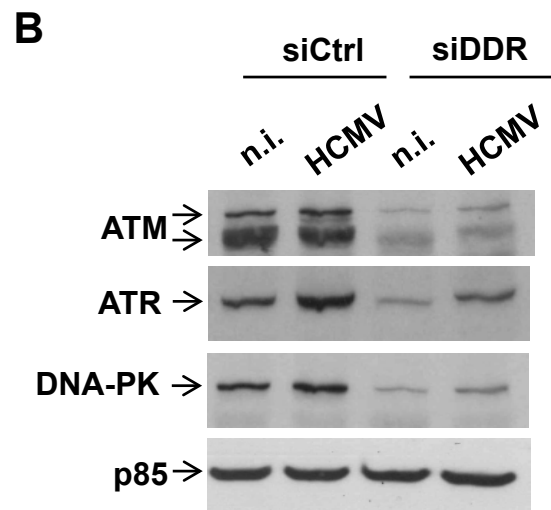
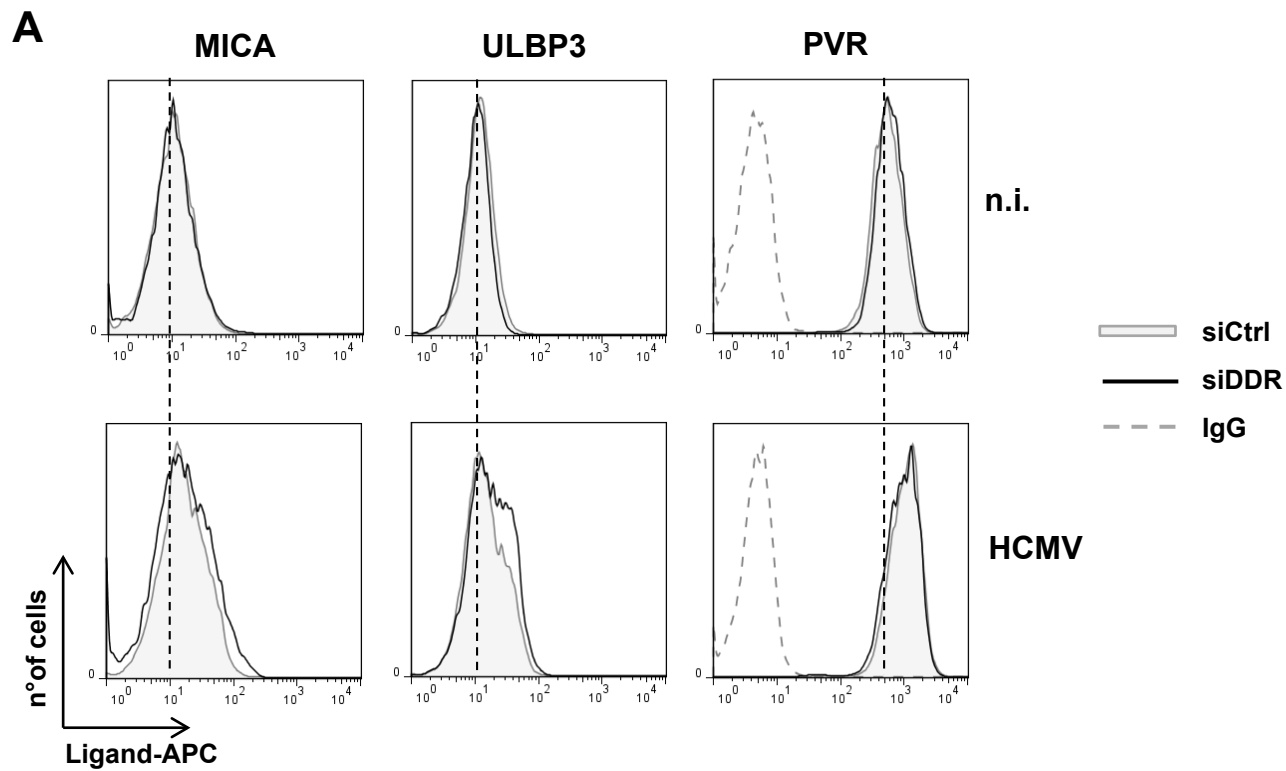


Fig. 3

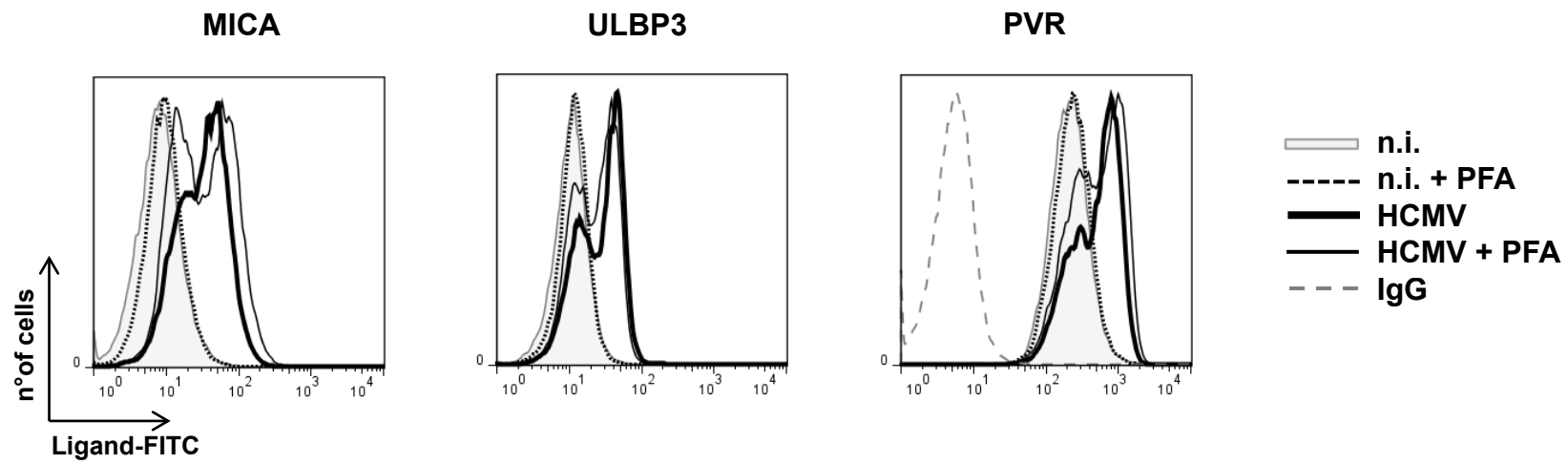


Fig. 4

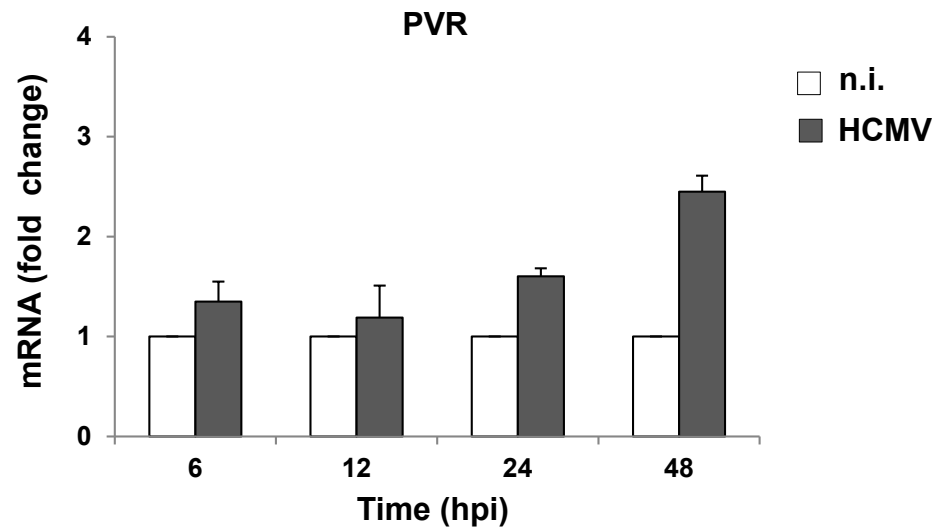
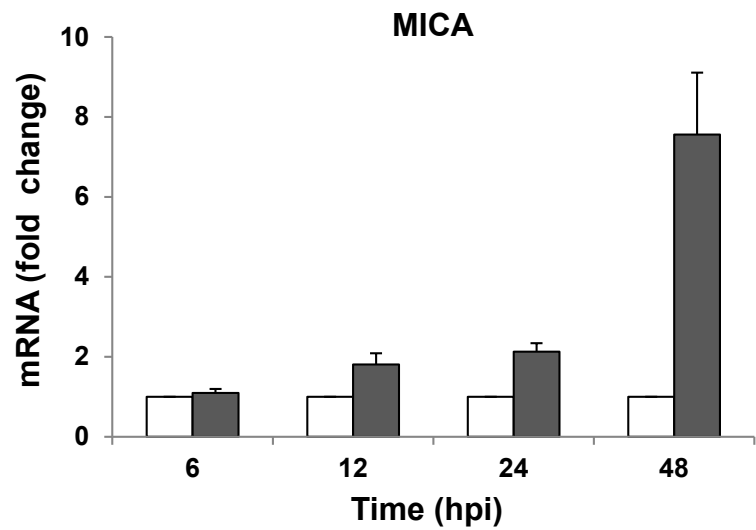


Fig. 5

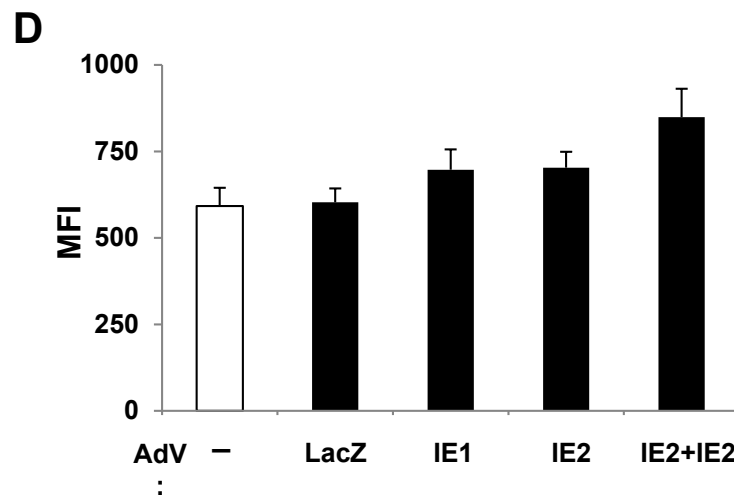
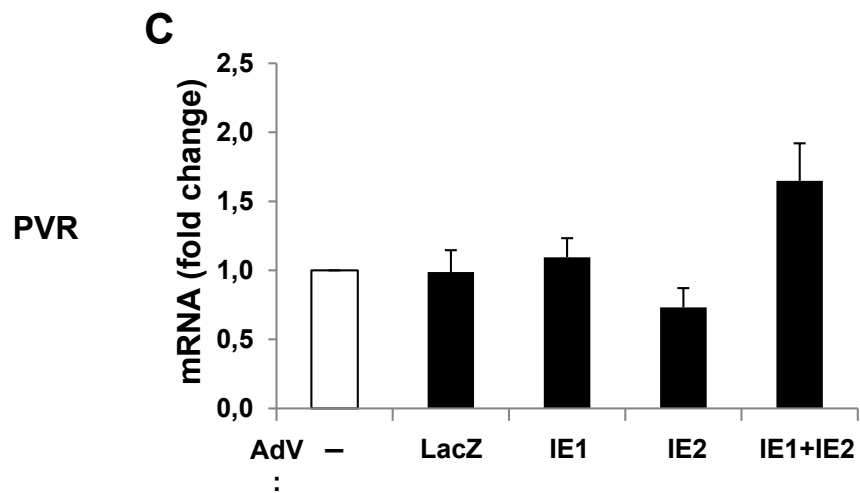
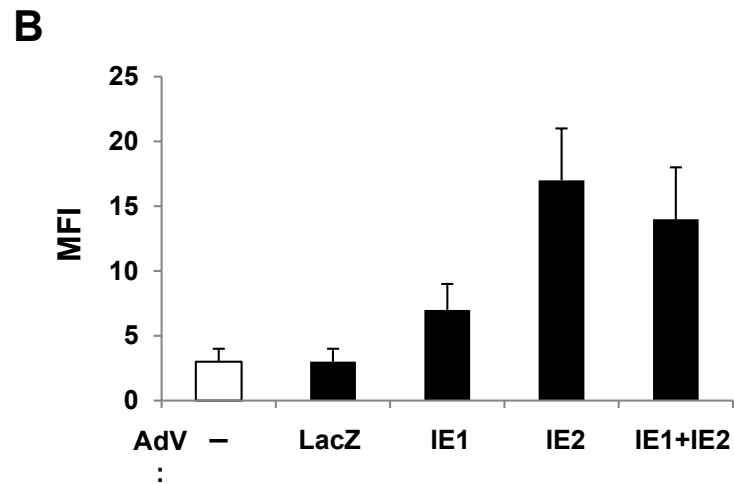
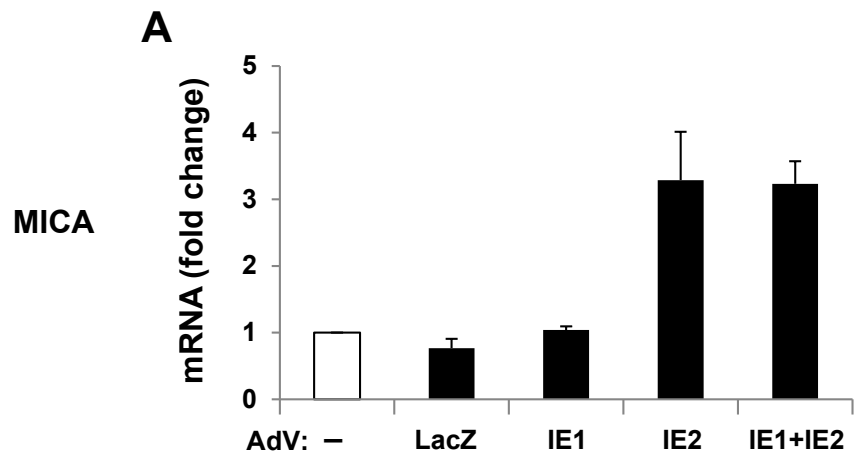


Fig. 6

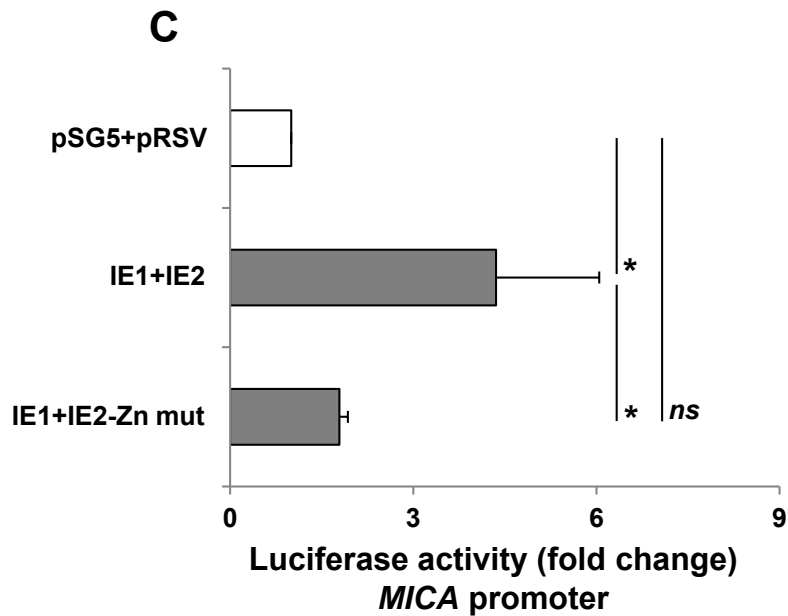
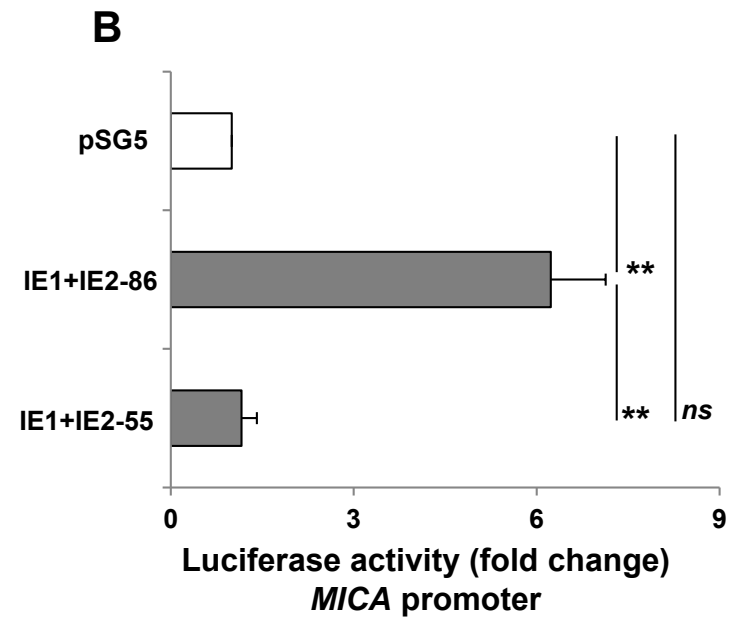
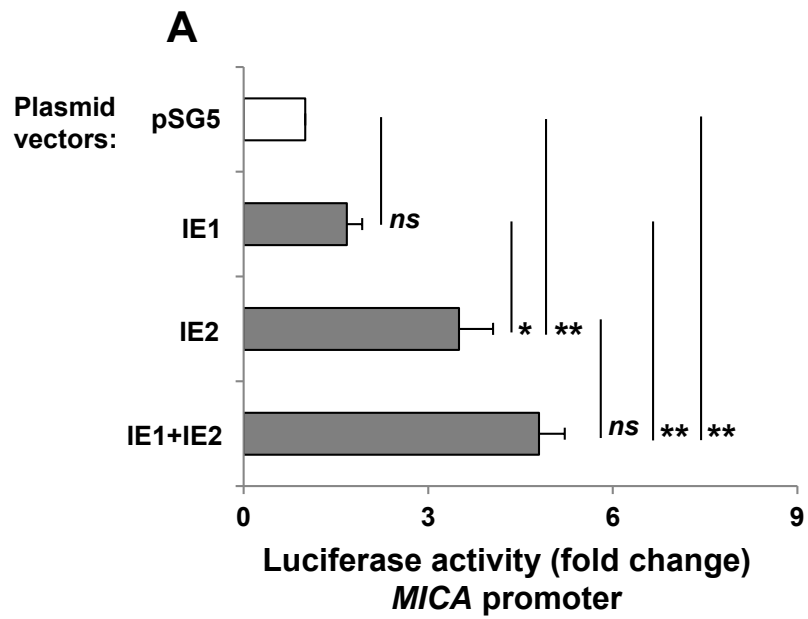
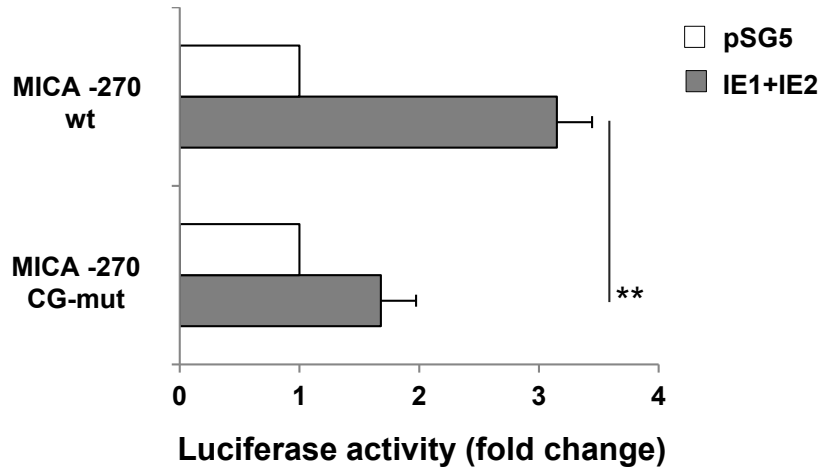
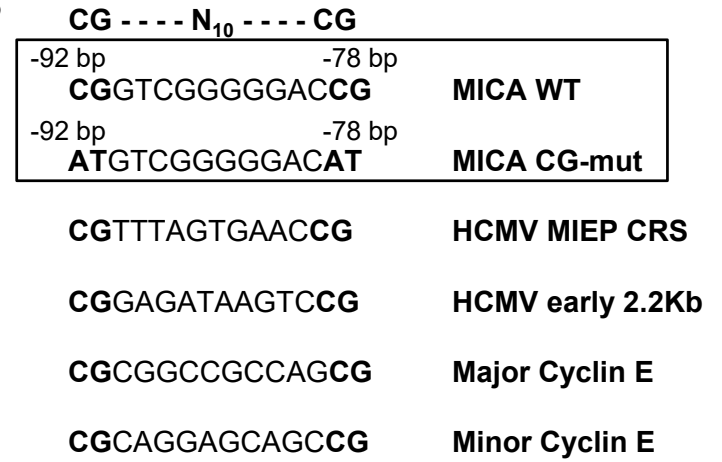
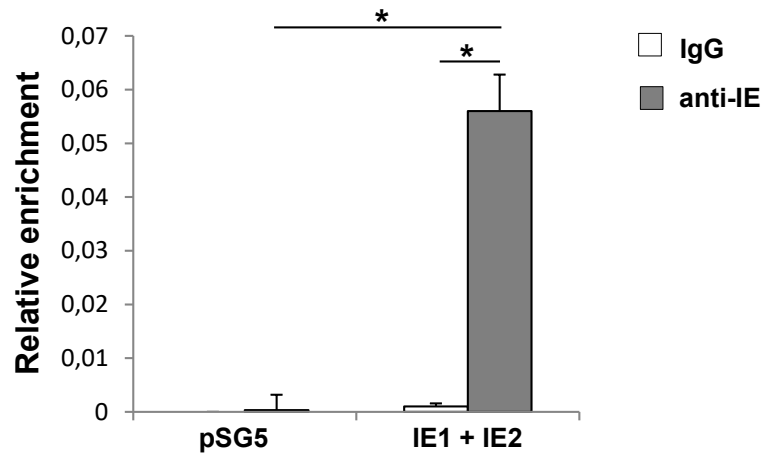
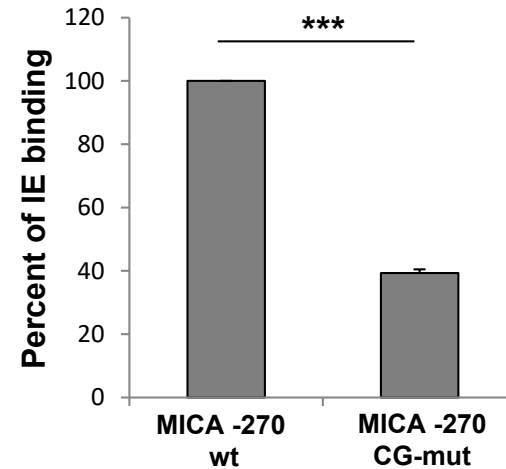


Fig. 7

A**B****C****D**

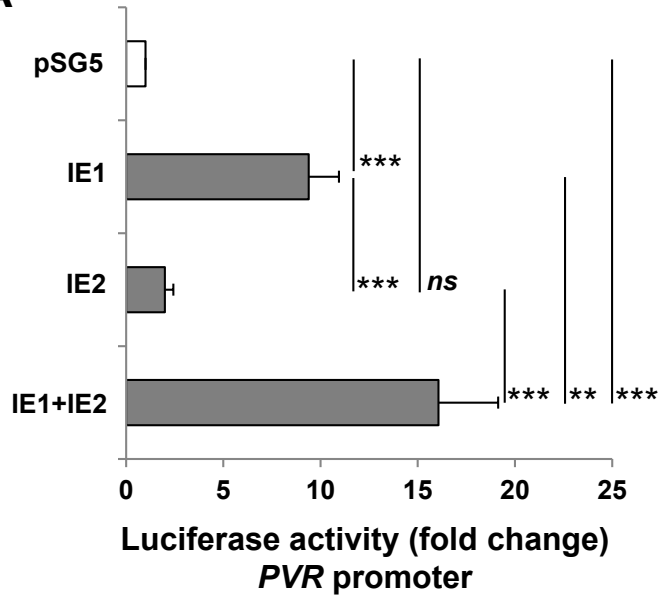
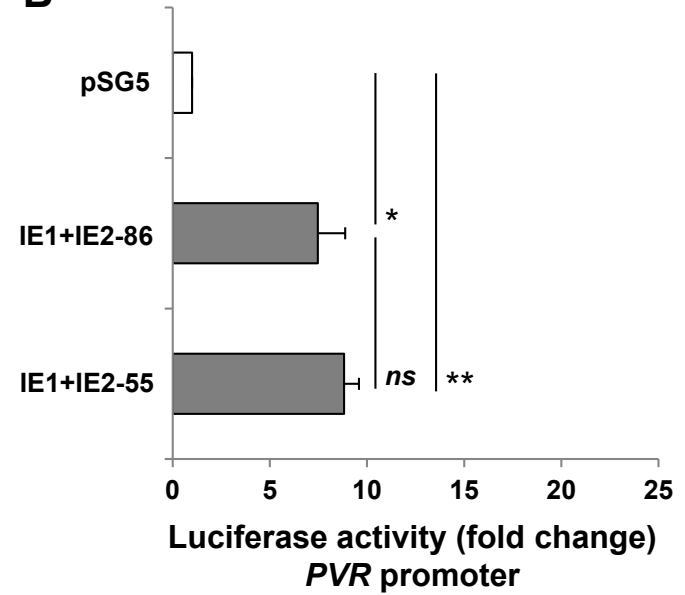
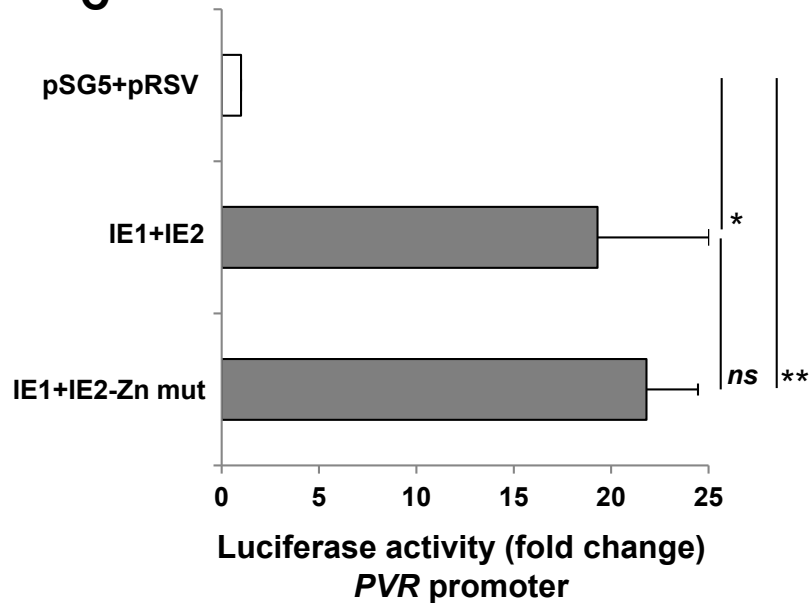
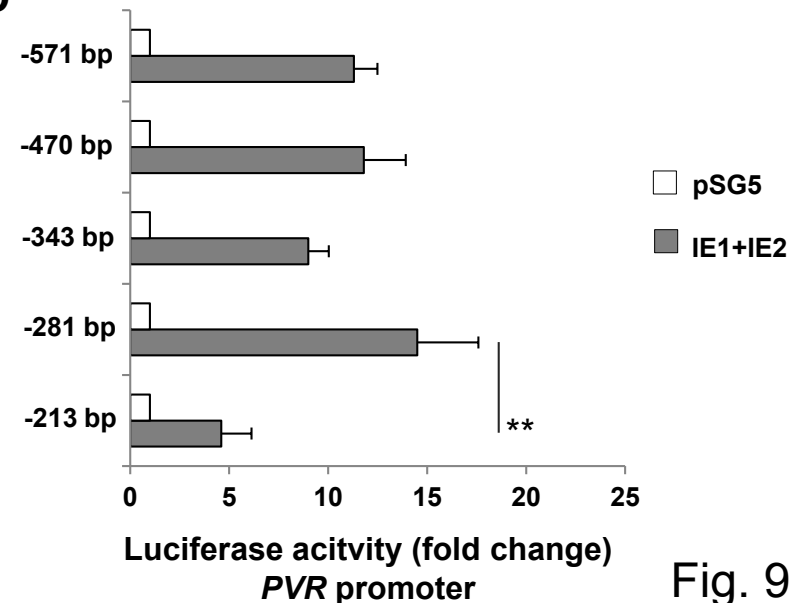
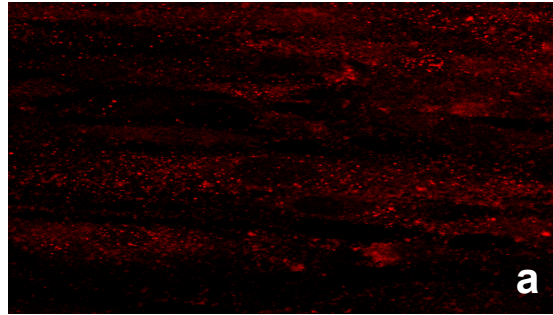
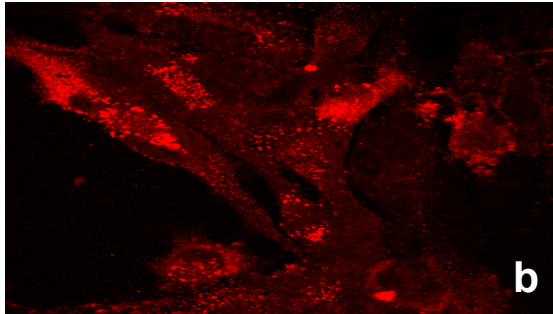
A**B****C****D**

Fig. 9

n.i.



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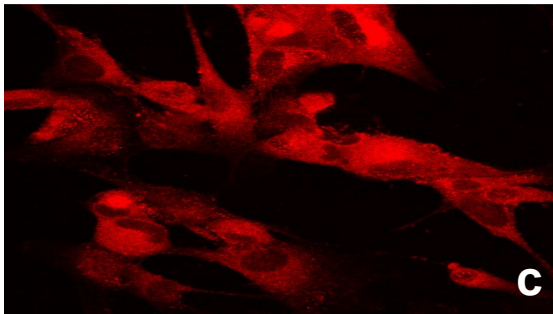


Fig. S1

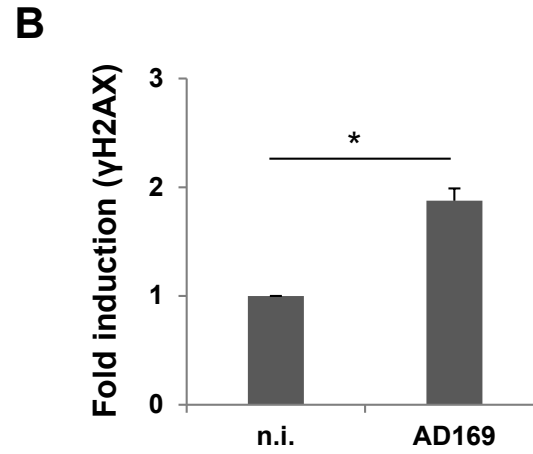
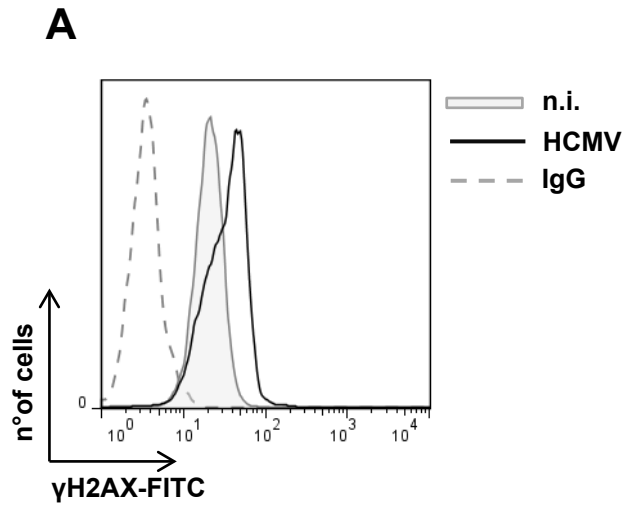


Fig. S2

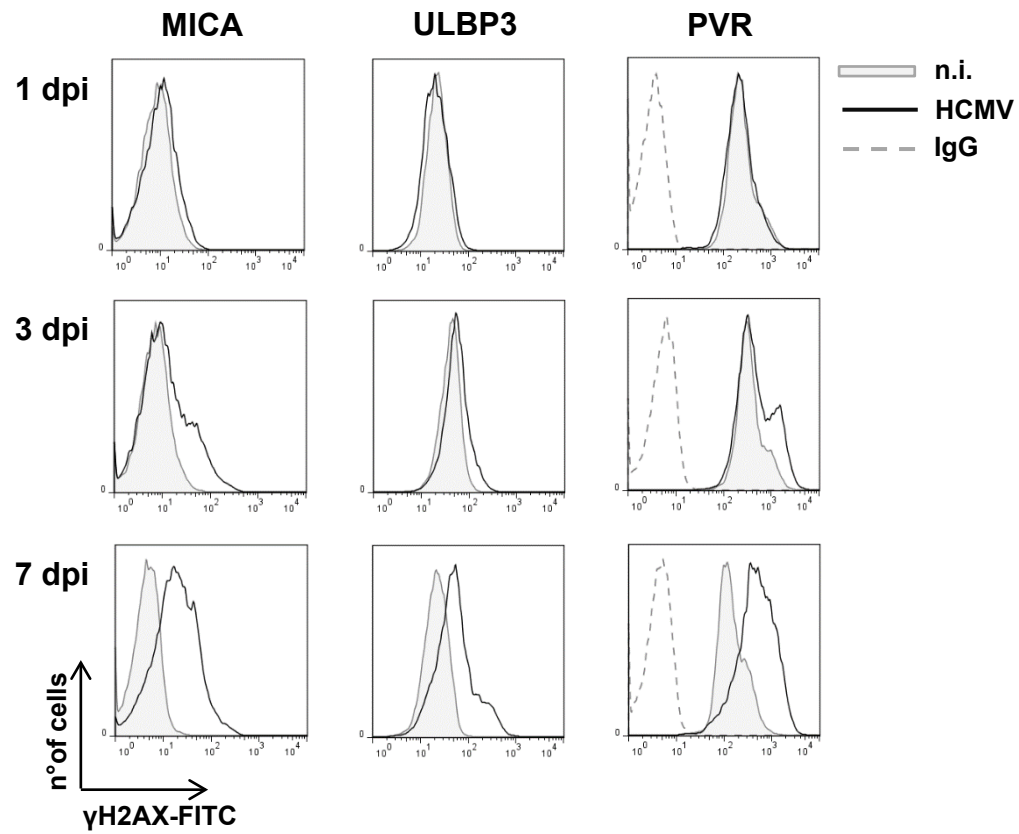


Fig. S3

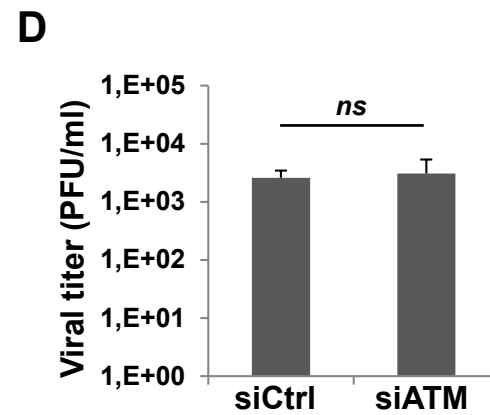
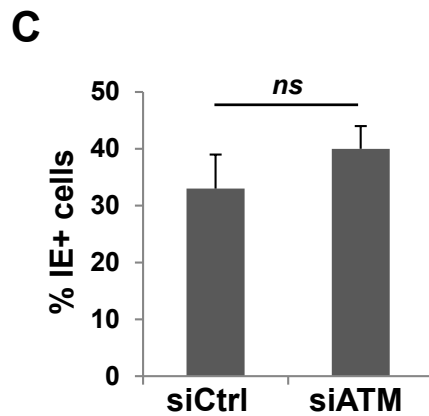
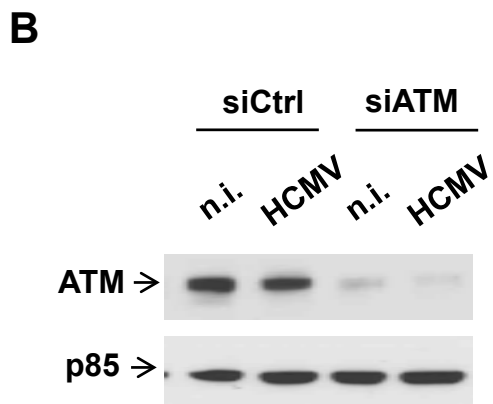
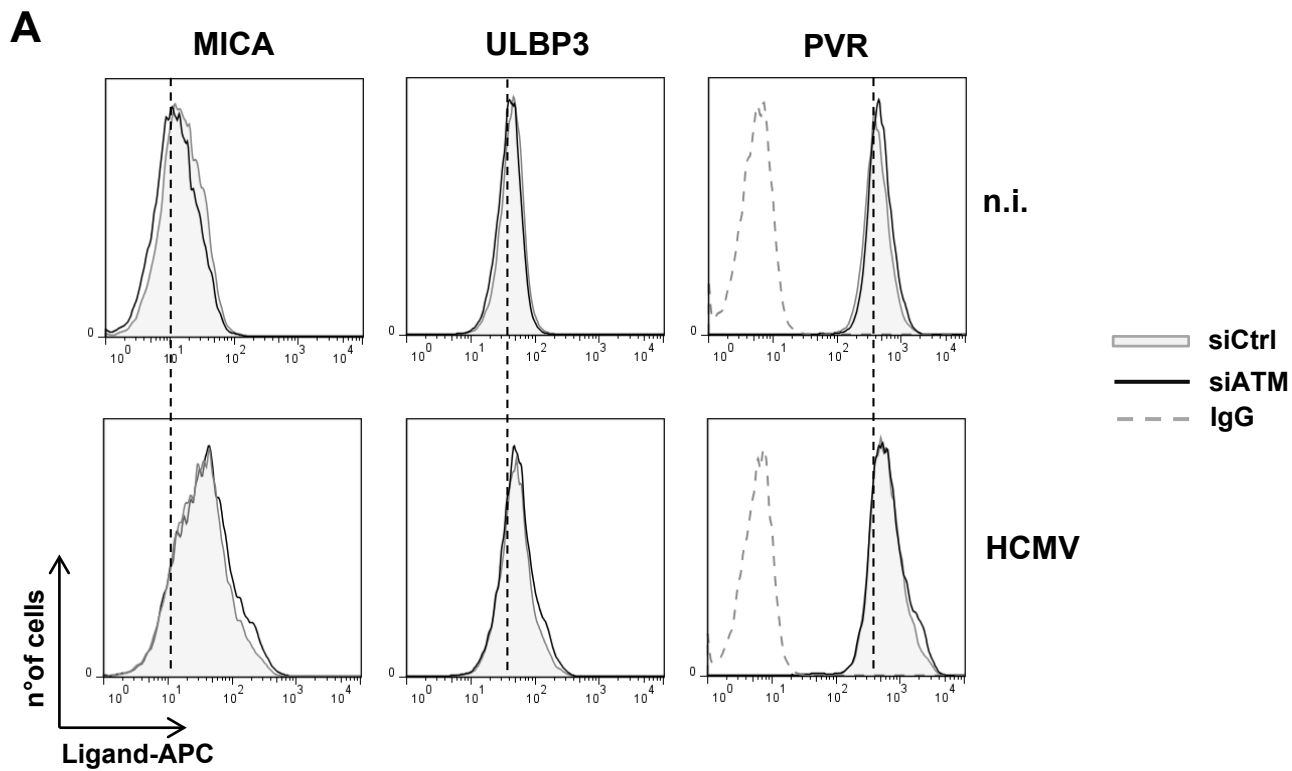


Fig. S4

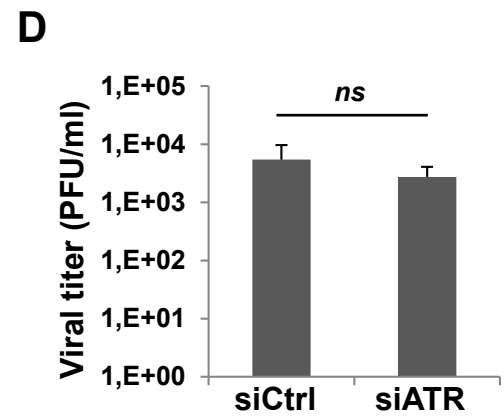
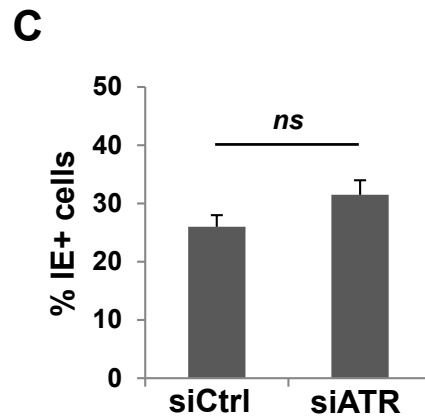
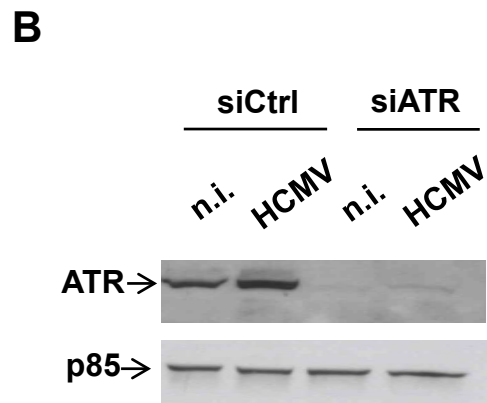
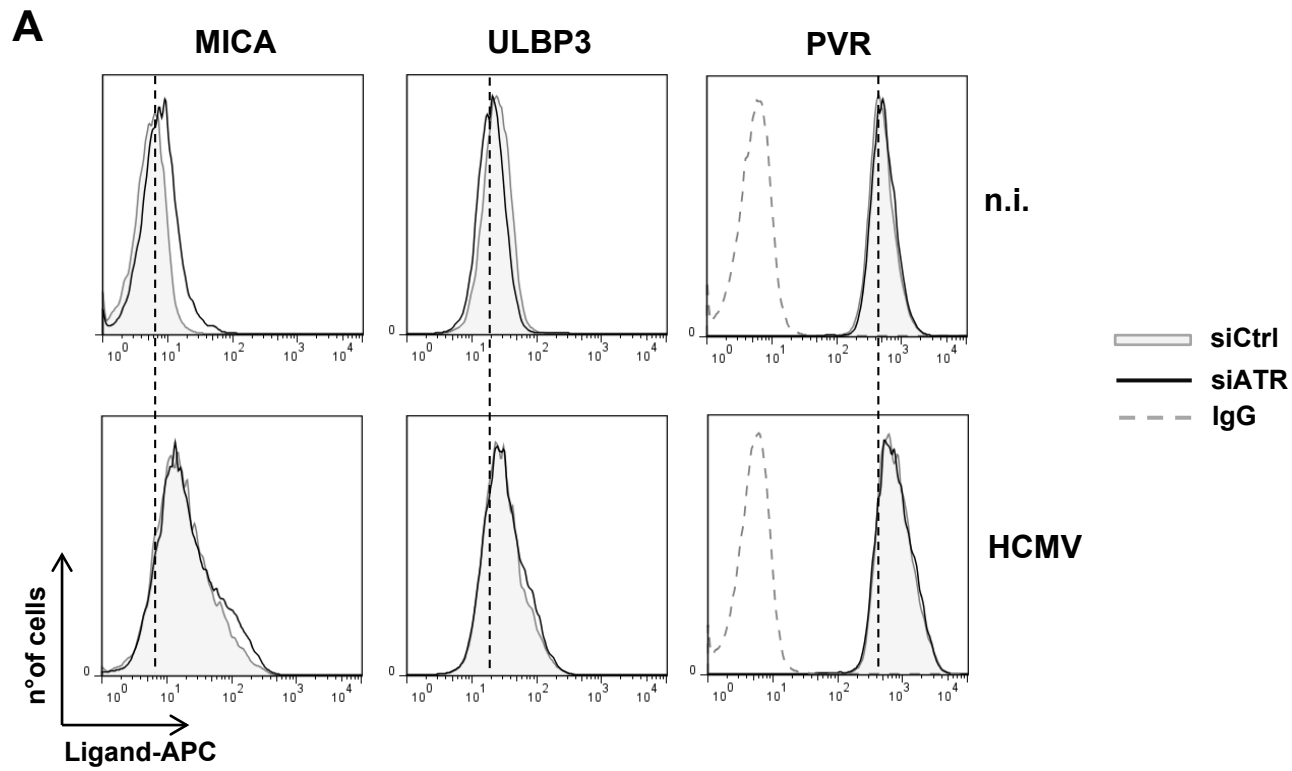


Fig. S5

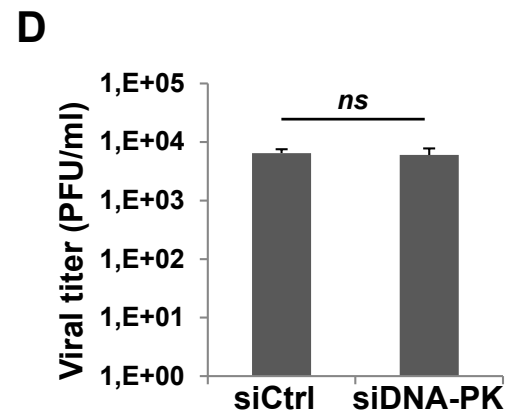
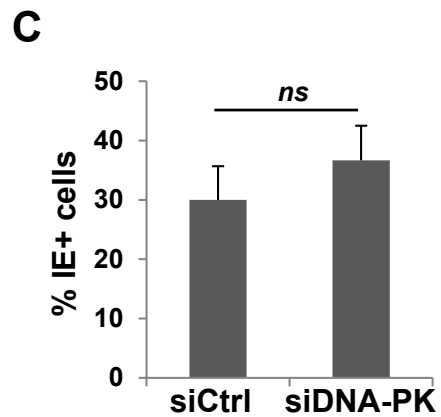
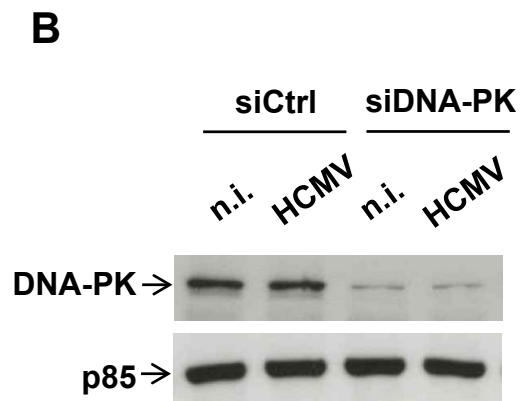
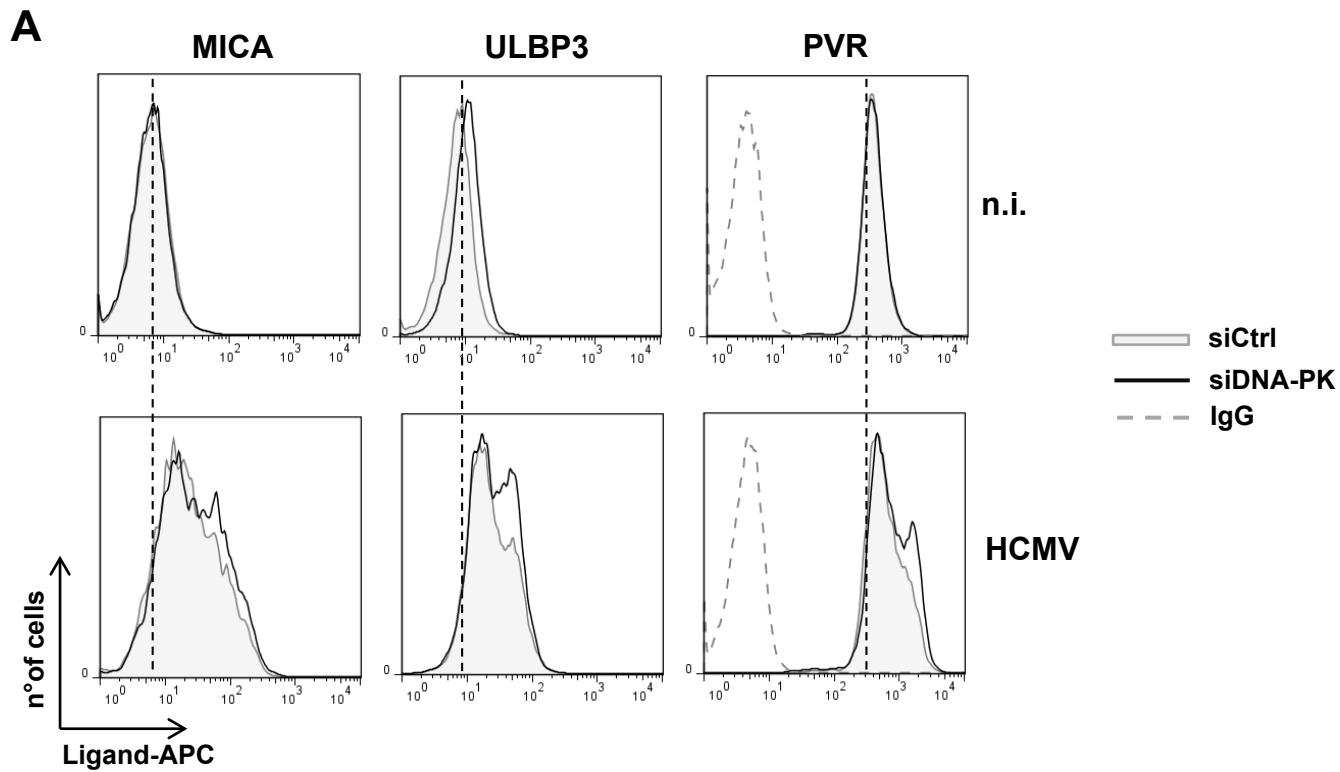


Fig. S6