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1 LIR1 expressing human Natural Killer cell subsets differentially recognize isolates of

2 human cytomegalovirus through the viral MHC Class I homolog UL18

3

4 Running Title: Differential responses of LIR1+ NK cell subsets to HCMV

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Abstract

Immune responses of Natural Killer (NK) cell are controlled by the balance between activating and inhibitory receptors, but the expression of these receptors varies between cells within an individual. Although NK cells are a component of the innate immune system, particular NK cell subsets expressing Ly49H are positively selected and increase in frequency in response to cytomegalovirus infection in mice. Recent evidence suggests that in humans certain NK subsets also have an increased frequency in the blood of HCMV infected individuals. However whether these subsets differ in their capacity of direct control of HCMV infected cells remains unclear. In this study we developed a novel *in vitro* assay to assess whether human NK cells subsets have differential abilities to inhibit HCMV growth and dissemination. NK cells expressing or lacking NKG2C did not display any differences when controlling viral dissemination. However, when *in vitro* expanded NK cells were used, cells expressing or lacking the inhibitory receptor Leukocyte Immunoglobulin-like receptor 1 (LIR1) were differentially able to control dissemination. Surprisingly, the ability of LIR1+ NK cells to control virus spread differed between HCMV viral strains, and this phenomenon was dependent on amino

33 acid sequences within the viral ligand UL18. Together, the results here outlined an *in*

34 *vitro* technique to compare the long-term immune responses of different human NK

35 cell subsets, and suggest, for the first time, phenotypically defined human NK cell

36 subsets may differentially recognise HCMV infected.

37 IMPORTANCE

38 HCMV infection is ubiquitous in most populations, it is not cleared by the host after
39 primary infection but persists for life. The innate and adaptive immune system
40 controls the spread of virus, of which Natural Killer (NK) cells play a pivotal role.
41 NK cells can respond to HCMV infection by rapid, short-term non-specific innate
42 responses, but evidence from murine studies suggested NK cells may display a
43 long-term, memory like responses to murine cytomegalovirus infection. In this study,
44 we developed a new assay that examines human NK cell subsets that have been
45 suggested to play a long-term memory-like response to HCMV infection. We show
46 that changes in a HCMV viral protein that interacts with an NK cell receptor can
47 change the ability of NK cell subsets to control HCMV while the acquisition odf
48 another receptor has no effect on virus control.

49

50 **Introduction**

51 Following primary human cytomegalovirus (HCMV) infection, lytic viral replication
52 is controlled by the host immune response, which includes humoral (1, 2), innate (3, 4)
53 and adaptive cellular immune responses (5-7). Despite this robust immune response
54 the virus is still able to establish latency in myeloid progenitor cells (8, 9). Virus can
55 reactivate when these cells differentiate to mature dendritic cells and as such the virus
56 is able to persist for the life-time of the host. Primary infection of healthy
57 immunocompetent individuals is most often asymptomatic, but the virus can cause
58 severe diseases in immunocompromised transplant patients, immunocompromised
59 patients with AIDS and the immune immature, particularly following in utero
60 infection (10-14).
61
62 Natural Killer (NK) cells are defined as a component of the innate immune system as
63 they do not undergo somatic DNA rearrangements in order to express highly diverse
64 antigen receptors in the same manner as B and T cells do (15). Instead NK cells
65 express a wide variety of both activating and/or inhibitory receptors that are able to
66 bind cellular ligands, some of which are normally expressed while others are induced

67 by infection or transformation (review in (16)). The balance between activating and
68 inhibitory signals determines if an NK cell is activated and exerts an effector function
69 or not. NK cells are implicated in control of herpesvirus infections, since individuals
70 with rare NK cell defects have been shown to have difficulty controlling multiple
71 different herpesvirus infections including HCMV(17, 18).
72
73 In order to avoid this NK-cell response, HCMV encodes multiple proteins that
74 modulate NK cell recognition of infected cells (19, 20). These NK evasion functions
75 act by preventing cellular ligands binding to activating NK cell receptors (UL16,
76 UL141, UL142, US18, US20, US9 (21-27) and miR-UL112 (21)), by expressing
77 proteins which engage inhibitory NK cell receptors (UL18 (28), UL40 (20, 29)) and
78 UL83 (30)), as well as modifying the structure of the immune synapse (UL135 (31)).
79
80 However NK cells are not homogeneous, instead numerous different NK cell subsets
81 exist within a given individual, since individual activating and inhibitory NK
82 receptors are independently expressed in varied combinations on different cells.
83 Murine studies have shown that the interaction between MCMV protein m157 and the

84 activating Ly49H receptor on murine NK cells leads to direct activation of NK cells

85 and the control of MCMV disease (32). In contrast, the only known example of direct

86 NK cell receptor binding with HCMV protein is the interaction of Leukocyte

87 Immunoglobulin-like receptor 1 (LIR1, now commonly known as LILRB1), an

88 inhibitory receptor that normally binds to human MHC class I molecules, with the

89 HCMV protein UL18, a viral homologue of cellular MHC Class I-like molecules (33,

90 34). Early work on the UL18 protein from HCMV strain AD169 suggested that it

91 could enhance cytotoxic killing by a NK cell line against a EBV-infected 293 cell line

92 target in chromium release assays (35), however these experiments did not consider

93 the level of expression of LIR1 on NK cells. Subsequently Prod'homme et al (28)

94 showed that the UL18 protein from HCMV strain AD169 actually lowered the

95 short-term cytotoxic responses of NK cells, but only if they expressed LIR1 (LIR1+),

96 leading to the conclusion that UL18 was an immunoregulatory protein that inhibited

97 NK cells from clearing HCMV lytically infected cells (28)

98

99 The activating C-type lectin receptor CD94/NKG2C, which normally binds to human

100 HLA-E, can also bind with HCMV UL18 protein but with 1000 fold weaker affinity

101 than LIR1 (36). NKG2C+ NK cells have been shown to be preferentially expanded in
102 HCMV-seropositive individuals (37-42). *In vitro* experiments demonstrated that
103 HCMV can induce expansion of CD94/NKG2C+ NK cells (43), and these cells show
104 enhanced cytotoxic responses against HCMV infected cells in the presence of
105 HCMV-specific antibodies (44). Except UL18, no other HCMV viral ligand has been
106 shown to bind directly with NKG2C/CD94. Moreover, although these reports support
107 a strong correlation between an increase in NKG2C+ NK cells and HCMV serostatus
108 (review in (16)), no report to date has determined whether the expression of NKG2C
109 on NK cells, in the absence of HCMV seropositive donor serum (44), leads to better
110 control of virus.
111
112 So far, the interpretation of the role of LIR1 and NKG2C receptors has focused
113 mainly on NK cell degranulation and cytotoxicity effector functions over short terms
114 (4 to 6 hours) co-culturing of NK cells with infected target cells. However NK cells
115 also secrete inflammatory cytokines, and can replicate after activation, and these can
116 also influence virus replication (16). Thus longer term assessment of anti-viral

117 activity of NK cells and particularly of NK cell sub-populations, would be a valuable
118 method to further understand the interaction of NK cells with HCMV infected cells.
119
120 We have recently developed and utilized such a viral dissemination assay to examine
121 the anti-viral activity of HCMV specific T cells (45). In this study, we have used a
122 VDA in conjunction with co-culture of different NK cell subsets in order to compare
123 their abilities to inhibit HCMV dissemination. *In vitro* expanded LIR1+ NK cells
124 controlled the spread of laboratory HCMV strain AD169 less effectively than
125 LIR1-non expressing (LIR1-) NK cells, in good agreement with the NK cell effector
126 function analyses by Prod'homme et al (28). However, when low passage/clinical
127 strains TB40/e and Merlin were used in the VDA, this was not the case. In fact,
128 LIR1+ NK cells displayed stronger control of virus spreading compare to LIR1- NK
129 cells, and this was observed in multiple different donors. Using the published crystal
130 structure of the LIR1-UL18/B_2m complex to inform the generation of specific
131 HCMV mutants, we identified three amino acids in the viral UL18 protein that were
132 responsible for this phenomenon. Furthermore, using NK cell subsets expressing
133 LIR1 and NKG2C, we demonstrated that LIR1, rather than NKG2C, plays the

134 dominant role in influencing the long-term responses of LIR1+ NK cells during

135 HCMV dissemination.

136 ## Material and Methods

137 **Donor sample collection and isolation**

138 Heparinized peripheral blood was collected from healthy donors. HCMV serostatus
139 was determined using an IgG enzyme-linked immunosorbent assay (Trinity Biotech,
140 Didcot, United Kingdom). Ten HCMV-seronegative and five HCMV-seropositive
141 donors were included in this study. Ethical approval involving donor peripheral blood
142 was obtained from the Addenbrookes National Health Service Hospital Trust
143 institutional review board (Cambridge Research Ethics Committee) for this study.
144 Informed written consent was obtained from all recipients in accordance with the
145 Declaration of Helsinki (LREC 97/092)

146

147 **Cell and viruses**

148 The Human Foreskin Fibroblast (HFF) cell line was obtained from a commercial
149 company (Invitrogen, Paisley, UK) and was cultured in EMEM (Life Technologies)
150 supplemented with 10% foetal calf serum (PAA, Linz, Austria), 100,000 IU/ml
151 penicillin (Life Technologies), and 100 mg/ml streptomycin (Life Technologies)
152 (MEM-10). The strains of HCMV used in these studies were AD169, AD169 with

153 UL18 ORF deleted (AD168-ΔUL18) (46), Merlin containing a GFP-UL32 fusion
154 protein (Merlin) (47), Merlin-GFP- UL32 with UL18 ORF deletion (Merlin-ΔUL18),
155 and TB40/e-GFP-UL32 (TB40/e, kind gift of Christian Sinzger). In addition the UL18
156 sequence modified virus strains (AD169-UL18Merlin and Merlin-UL18AD169) were
157 generated by recombineering as previously described (48) using primers listed in
158 Table 1. The method used to generate the mutant virus isolates does not lead to the
159 loss of US2-US6 HCMV genes.
160
161 **Sequencing of HCMV UL18 ORF**
162 DNA was extracted from HCMV infected fibroblasts using the DNeasy-Blood and
163 Tissue Kit (Qiagen). 30-cycle PCR was performed to amplify viral UL18 ORF using
164 forward primers shown in Table 1 and conditions as previously described (49).
165 Samples were sequenced by Source Bioscience Sequencing Team, Cambridge UK.
166
167 **Preparation of *in vitro* expanded NK cells**
168 Fresh peripheral venous blood was obtained by venipuncture, performed by a trained
169 phlebotomist. PBMC were isolated from fresh peripheral venous blood by Ficoll

170 Hypaque density gradient centrifugation (Axis-Shield, Oslo, Norway) as previously
171 described (50). NK cells were purified from PBMC, using EasySep-Human NK cell
172 enrichment kit (StemCell Technologies). *In vitro* expanded, activated NK cell lines
173 (referred to as *in vitro* expanded NK cells) were then generated from *ex-vivo* NK cells
174 by co-culturing with irradiated allogenic EBV transformed B cell lines and irradiated
175 autologous PBMC using methods previously described (24). The cell lines were
176 cultured in RPMI-10 (described earlier) with 25 IU/ml IL-2 (National Institute for
177 Biological Standards and Controls) replenished every 5 days.
178
179 **Preparation of sorted NK cell subsets**
180 *In vitro* expanded NK cell lines were stained with mouse anti-human CD56–pacific
181 blue (PB) or FITC (eBioscience UK), CD3–PerCP/Cy5.5 (Biolegend UK),
182 LIR1/CD85j–phycoerythrin (PE) (Biolegend UK), and NKG2C-Allophycocyanin
183 (APC)-antibodies (Biolegend UK). CD56+ CD3- NK cells were sorted into different
184 populations using a FACSJazz cell sorter running FACS DIVA software (Becton
185 Dickinson, UK).
186

187 **CD107a degranulation assays**

188 K562 ($1x10^6$) or HCMV infected fibroblasts (overnight infection with TB40/e,
189 MOI=5) were co-cultured with *in vitro* expanded NK cells at a ratio of 1:1 in 50µl
190 RPMI-10 and incubated at 37°C and 5% CO_2. Monensin (BioLegend) was added at
191 1:1000 dilution after 1 hour co-incubation and further incubated for 4 hours at 37°C
192 and 5% CO_2. Cells were washed in PBS before staining with anti-human CD56
193 (APC), CD3–FITC, and CD107a PerCP/Cy5.5 antibodies (Biolegend UK) before
194 being analysed by flow cytometry using FACSCalibur (BD) running CellQuest
195 software (BD). Results were analysed using FlowJo 7.6 (Tree Star Inc., OR, USA).

196

197 **CCL4 cytokines ELISAs**

198 *In vitro* expanded NK cells were co-cultured overnight with K562 or HCMV infected
199 fibroblasts (overnight infection with TB40/e, MOI=5) using the method described
200 earlier for CD107a assays, but without monensin. The co-cultures were left for 12
201 hours before the supernatant was harvested and the CCL4 cytokine concentration was
202 measured using an ELISA kit (R&D systems).

203

204 ***In vitro* viral dissemination assay**

205 The ability of NK cell subsets (sorted based on differences in specific receptor
206 expression) to control the spread of HCMV virus *in vitro* was measured. Allogeneic
207 human foreskin fibroblasts (HFF) cells were seeded in a 24-well flat-bottom culture
208 plates (LifeSciences, UK) to be 80-90% confluent when they were infected with virus
209 at an MOI of 0.1 overnight. Rested *in vitro* expanded NK cells were harvested,
210 washed, stained, sorted and resuspended in MEM-10 then added to the infected
211 fibroblasts at NK cell: Fibroblast ratios of 1.25, 0.625 or 0.3125:1 in 1 ml MEM-10
212 and incubated at 37°C and 5% CO2. Assessment of viral dissemination was
213 performed at 9 days post incubation. GFP expression (TB40/e and Merlin) was
214 detected by either fluorescent microscopy or flow cytometry. When HCMV AD169
215 was used, fibroblasts were stained intracellularly with anti-CMV IE-AlexaFluor488
216 antibody (Millipore) using Cell Fixation/ Permeabilization kit (An-Der-Grub
217 BioResearch). Fibroblasts were fixed with 2% paraformaldehyde-PBS solution and
218 analysed using flow cytometry as described earlier.

219 Viral spread in each well was determined as a percentage of control wells lacking NK
220 cells, using the equation:

221 ([Experimental % of infected cells - background % of HFF only control] / [% of

222 infected HFF control without NK cells - background % of HFF only control]) x100

223

224 **Phylogenetic tree**

225 BioEdit Sequencing Alignment Editor was used for sequence analysis. The

226 evolutionary phylogenetic trees were computed using Molecular Evolutionary

227 Genetics Analysis (MEGA).

228

229 **Statistics**

230 Statistical analysis was performed using GraphPad Prism version 4.00 for Windows

231 (GraphPad Software, San Diego, CA). Probabilities were calculated with one way or

232 two-way ANOVA paired Friedman test in the viral dissemination assay, assuming

233 not-repeated measures. Standard T-test analysis was used to analyse the cytokine

234 CCL4 release assay and CD107a degranulation assay. Results with $p<0.05$ were

235 considered as significant.

236

237 ## Results

238 **Control of different strains of HCMV dissemination by *in vitro* expanded**
239 **primary NK cell lines.**

240 We have previously developed an assay to measure T cell mediated anti-viral activity
241 based on the inhibition of HCMV dissemination through a permissive fibroblast
242 monolayer (51, 52). In addition a focal expression assay which is similar in concept to
243 our viral dissemination assay (VDA) has recently been described by others (52) and
244 used to examine anti-HCMV activity by NK cells (45). We wished to use our viral
245 dissemination assay (VDA) to study the antiviral activity of different NK cell subsets,
246 as defined by specific cell surface markers.

247

248 In order to validate this approach, we used the VDA against different strains of
249 HCMV to determine if *in vitro* expanded NK cells were able to control the spread of a
250 high passage laboratory adapted (AD169) and low passage isolates of HCMV TB40/e
251 and Merlin. The results clearly show that NK cells were able to prevent viral spread in
252 an effector: target ratio (E:T) dependent fashion. This was visualised by fluorescent
253 microscopy and quantified by flow cytometry, either by GFP if the virus expressed

254 GFP (Figure 1A) or by anti-HCMV IE antigen staining with a fluorescent antibody if

255 it didn't (Figure 1B).

256

257 High passage strains such as AD169 lack multiple immune evasion genes. This

258 renders AD169 infected cells more susceptible to NK cell mediated lysis as compared

259 to low passage HCMV isolates such as TB40/e or Merlin, in short-term cytotoxicity

260 assays (53, 54). In order to compare VDA results between different strains of virus,

261 data for each virus was normalized against its own positive (HCMV infected HFF

262 without NK cells) and negative (non-infected HFFs) controls. The positive control

263 represents the maximal spread of the virus over the course of the assay, while the

264 negative control is the background fluorescence .

265

266 Across multiple donors, it was clear that the virus spread of AD169 (n=4) was

267 significantly reduced compared to Merlin (n=9) and TB40/e (n=7), implying that

268 polyclonally activated NK cells were significantly more efficient at controlling the

269 spread of AD169 (Figure 1C). In addition, TB40/e was controlled less well than

270 Merlin at the highest E:T ratio of 1.25:1. A similar trend was evident at the lower

271 ratios, although this was not statistically significant (Figure 1C). Importantly, NK
272 cells still exerted some degree of control over Merlin as spread did not reach 100 %
273 for any of the E:T ratios tested (Figure 1C). We also performed VDAs using *in vitro*
274 expanded NK cells derived from either a HCMV seropositive or a seronegative donor
275 (Figure 1D). No major differences were seen between the polyclonal NK cells from
276 these donors, with cells from both being significantly more efficient at controlling the
277 spread of AD169 than either TB40/e or Merlin.
278
279 Taken together, these results demonstrate that, using this NK cell VDA, a
280 low-passage isolate of HCMV (Merlin) was resistant to NK cell mediated control,
281 while an isolate lacking NK immune evasion genes (AD169) was less resistant.
282 Despite low-passage strains being more resistant to NK cell control, polyclonal NK
283 cells did still exert some degree of control, indicating that this assay represented a
284 useful method to study the anti-viral properties of different subsets of NK cells, as
285 well as the ability of different HCMV isolates to affect NK cell recognition.
286
287 **Comparison of LIR1+ and LIR1- NK cell control of HCMV dissemination**

288 The VDA was able to identify differences in the ability of NK cells to control
289 different HCMV strains, we next wanted to use the assay to determine if certain NK
290 cell subsets, as defined by surface expression of particular phenotypic markers, were
291 more efficient at controlling HCMV infection. The HCMV MHC Class I homologue
292 UL18 (specifically from strain AD169) has already been shown to decrease direct NK
293 cell cytotoxic responses as it is able to bind the inhibitory receptor LIR1 present on
294 some NK cell subsets (28). As such, AD169 should be less well controlled by LIR1+
295 NK cells (which would be inhibited by HCMV UL18 protein expression) as
296 compared to LIR1- NK cells (which would not be inhibited by UL18 (28)).
297
298 Primary *in vitro* expanded NK cell lines were generated from different donors, NK
299 cells were sorted as CD3-CD56+ cells before sorting into subsets based on their LIR1
300 expression, achieving >95% purity after sorting (Figure 2A). To ensure that the
301 subsets maintained their effector functions, post-sorting cytotoxicity and cytokine
302 secretion against the classic K562 target cells was examined (55). NK cell
303 cytotoxicity was determined by CD107a degranulation assay and cytokine production
304 was assessed by measuring CCL4 production, one of the earliest inflammatory

305 cytokines produced following NK cell activation (56). While the NK cell lines were

306 capable of generating IFNγ against K562 target cells, the response was modest at

307 between 20-30 pg/ml. The CCL4 response was more substantial at 400pg/ml

308 and was also elicited by HCMV infected target cells, therefore we measured CCL4

309 for all the subsets as it provided a better dynamic range in order to determine if

310 cell sorting had caused differences in the sorted subsets.

311

312 Both LIR1+ and LIR1- subsets had a similar cytotoxic response and level of CCL4

313 secretion to the unsorted NK cell line from which they were derived (Figure 2B).

314 Thus NK cells maintain their cellular functions after sorting and also suggests that

315 engagement of anti-LIR1 antibodies did not cause inhibition of LIR1+ NK cell

316 effector functions.

317

318 The LIR1+ and LIR1- NK cell subsets were then used in a VDA against AD169

319 infected HFFs. As predicted, the results show that there was greater percentage of

320 viral spread in the presence of LIR1+ NK cells as compared to LIR1- NK cells

321 (Figure 2C). The VDA was also performed using HFFs infected with the low passage

322 HCMV strains TB40/e and Merlin. Surprisingly, different results were obtained.
323 Against these strains, LIR1+ NK cells demonstrated more efficient virus control
324 compared to the LIR1- NK cells (Figure 2D and E). The same results were observed
325 for three independent donor NK cell lines tested (Figure 2C-E). Thus differences
326 between HCMV strains can affect the ability of NK cell subsets to control virus
327 spread.
328

329 **Deletion of HCMV UL18 ORF abolishes the differential controls mediated by**
330 **LIR1+ and LIR1- NK cells**

331 We next investigated whether the above phenomenon (Figure 2C-E) was caused by
332 the viral UL18 protein. If it was due to the interaction of UL18 on HCMV infected
333 cells with LIR1 receptors on NK cells, the difference should be negated if a virus
334 deleted for UL18, or a blocking antibody to LIR1, were used in these assays. While
335 LIR1 blocking antibodies are available, they could deliver inhibitory signals through
336 LIR1, and we were concerned that the antibody concentrations was maintained over
337 the long period of this assay, as such we have determined the involvement of UL18
338 by using UL18 deletion mutants of both AD169 and Merlin.

339

340 VDA were conducted using HCMV AD169- ΔUL18 and Merlin-ΔUL18 viruses as

341 well as their parental strains. As before, LIR1+ NK cells were less able to control the

342 spread of AD169 compared to LIR1- NK cells; however when AD169-ΔUL18 was

343 used, this difference was eliminated (Figure 3A). In donor 410, the LIR1- NK cells

344 exhibit the same level of viral control for both AD169 and AD169-dUL18 virus

345 however, the major change occurs with LIR1+ NK cells, which exert better control

346 over AD169-dUL18 virus than AD169, in agreement with the other independent

347 donors. In donor 319, we noted a discrepancy, that both LIR1+ and LIR1- cells

348 exerted greater control when UL18 has been deleted in this experiment. However,

349 it seems to be donor specific (donor to donor variation) rather than virus specific

350 as it does not occur in other donors (405 for example), indicating that it is

351 unlikely to be due to additional changes in the AD169-dUL18 strain. Nevertheless,

352 although with donor 319 the AD169-dUL18 strain had a lower spread than

353 expected in the presence of both LIR1+ and LIR1- NK cells, the two subsets did

354 maintained the same pattern as the other donors (i.e. there were no significant

355 differences between the subsets once UL18 was removed). Because of this, we

356 feel that the data support the conclusion that removal of UL18 from AD169

357 allows better control by LIR1+ NK cells. When HCMV strain Merlin was used,

358 LIR1+ NK cells controlled virus spread better than LIR1- NK cells again

359 independently verifying our previous observations (Figure 2). Similar with AD169,

360 deletion of UL18 from this virus strain also resulted in the elimination of this

361 difference (Figure 3B). The experiments were repeated using NK cells derived from

362 four independent donors and in all but one donor the same pattern of results were

363 observed (Figure 3A-B): donor 302 showed a significant difference between subsets

364 when AD169-dUL18 was used (Figure 3A), while donor 405 showed a small but

365 significant difference between the subsets when Merlin-ΔUL18 was used (Figure 3B).

366

367 The results suggested that UL18 was responsible for the effects on LIR1+ and LIR1-

368 NK cell control of viral dissemination, and further suggested that UL18 from different

369 strains affected NK cell subsets in a different manner. The crystal structure of the

370 UL18 protein interacting with LIR1 receptor has been determined by Yang et al (57)

371 showing that the interactions occur between the α3 domain of UL18 and LIR1

372 receptor (57). There are three sites of interactions between UL18 and LIR1, compared

373 with two sites between human MHC Class I and LIR1, which has been suggested as
374 the reason for a 1000-fold higher binding affinity observed between UL18 and LIR1
375 compared to MHC Class I (57).
376
377 To investigate if strain-dependent sequence variability was responsible for the
378 differences observed in LIR1+ NK cell control of HCMV dissemination, UL18
379 sequences from the Genebank were identified and the amino acid sequences within
380 the α3 region were aligned (Figure 3C). In this region, HCMV strains TB40/e and
381 Merlin have a sequence of NKAPDD, while AD169 has DKVPED. These three 3
382 amino acid differences (N/D, A/V and D/E) are located adjacent to the additional site
383 of interaction with LIR1 proposed by Yang et al (57), which results in the increased
384 binding affinity compared to the MHC1-LIR1 interaction.
385
386 Although these amino acids have not been implicated in the direct interaction between
387 UL18 and LIR (57), we hypothesized that they maybe of significance because 12 out
388 of 13 unique UL18 sequences in the database have either amino acid sequences of
389 NKAPDD or DKVPED (Figure 3B). We further constructed a

390 neighboring-phylogenic tree to analyse the similarity in the α3 domains between the

391 HCMV viral strains (Figure 3D). The alignment shows that both the UL18 sequences

392 from TB40/e and Merlin were closely related to each other, while the AD169

393 sequence was located in a different cluster (Figure 3D). There were no differences

394 between the α1 and α2 regions of UL18 proteins from these three viruses (not shown).

395

396 **UL18 sequence variability affects NK cell control of HCMV dissemination**

397 If the 3 amino acids identified in figure 3 were responsible for the differences

398 observed in control of viral dissemination, mutating the UL18 from the sequence of

399 one virus strain to the sequence of the other, should reverse the pattern of control

400 observed with LIR1+ and LIR1- NK cells. Recombineering was used to mutate the

401 AD169 sequence from DKVPED to the Merlin sequence NKAPDD

402 (AD169-UL18Merlin) and a reciprocal mutation was made to Merlin sequence from

403 NKAPDD to the AD169 sequence DKVPED (Merlin-UL18AD169), and mutant

404 UL18 sequences verified by PCR amplification and sequencing (Figure 4A). These

405 mutant viruses were used to infected human fibroblasts and VDA performed using

406 LIR1+ and LIR1- NK cells derived from multiple independent donors.

407

408 As previously observed, LIR1+ NK cells from four different donors were less
409 effective at controlling AD169 dissemination (Figure 4B, left column). However,
410 when the three amino acids were mutated to the Merlin sequence the pattern of
411 recognition was changed to that previously seen with Merlin virus, whereby LIR1+
412 NK cells controlled virus spread more effectively than LIR1- NK cells (Figure 4B,
413 right column). Likewise, as before, LIR1+ NK cells from all donors were more
414 effective at controlling Merlin dissemination than LIR1- NK cells (Figure 4C, left
415 column), and the mutation of three amino acids within UL18 of Merlin to the AD169
416 sequence reversed this pattern whereby LIR1+ NK cells controlled virus spread less
417 effectively than LIR1- NK cells (Figure 4C, right column). Together these results
418 suggest that LIR1 expressing NK cell subsets can differentially control HCMV, but
419 that this ability can be altered by variation in the sequence of UL18, the viral ligand to
420 the LIR1 receptor.

421

422 **NKG2C expressing NK cells influence the functional responses of NK cells but**
423 **not the dissemination of HCMV**

424 It has been suggested that receptors other than LIR1 may also influence the activity of
425 NK cells against HCMV infected cells, in particular the activating receptor NKG2C.
426 Numerous reports have shown strong correlation between the acquisition of HCMV
427 infection and an increase in frequency of NKG2C-expressing NK cells in peripheral
428 blood (37-43), and one report has suggested that NKG2C binds with UL18 at very
429 low affinity (36). Although no reports to date have shown differential control between
430 NKG2C+ and NKG2C- NK cells of HCMV in standard cytotoxic assays, one recent
431 report does suggest that NKG2Cbright NK cells exhibits higher degranulation against
432 target cells in the presence of serum containing HCMV-specific antibodies (44).
433
434 We therefore investigated whether NKG2C+ NK cells mediated better control of
435 HCMV in our viral dissemination assay. Since we had already shown that LIR1
436 expression affected NK-mediated control of viral spread, cells were sorted into four
437 subsets based on both LIR1 and NKG2C expression (Figure 5A). We also analysed
438 activated NK cell lines with anti-NKG2A, NKG2C and LIR 1 in order to determine
439 the distribution of the inhibitory NK cell receptor NKG2A from four different
440 donors (2 HCMV seropositive 2 HCMV seronegative). The results show that there

441 was no or negligible expression of inhibitory receptor NKG2A (HCMV
442 seropositive 0.4% and 1.1%, HCMV seronegative 0% and 5%) cells and as such
443 was unlikely to have a significant impact on the functional assay.
444

445 To ensure these subsets maintained their effector functions post-sorting, their
446 cytotoxicity (CD107a degranulation) and cytokine secretion against K562 target cells
447 was determined. Both LIR1+ NKG2C+ and LIR1- NKG2C+ NK cells degranulated
448 significantly more strongly than the LIR1+ NKG2C- and LIR1- NKG2C- NK cells.
449 i.e. NK cell subsets expressing the NKG2C activating receptor had higher cytotoxicity
450 towards K562 target cells than subsets without NKG2C (Figure 5B, left), however,
451 expression of LIR1 made little difference in this effector assay. The cytokine release
452 assay showed no significant differences between any NK cell subsets (Figure 5B,
453 right). Interestingly, these results suggested that different NK cell effector
454 mechanisms could be independently activated.
455

456 The four NK cell subsets were also co-cultured with strain TB40/e infected target
457 cells and CD107a and cytokine responses measured. All of the subsets displayed low

458 cytotoxicity against TB40/e-infected fibroblasts (Figure 5C, left), similar to previous

459 studies using *in vitro* expanded NK cell lines (24, 53). In contrast, the cytokine release

460 assay showed that although all subsets maintained their CCL4 production in response

461 to TB40/e infected fibroblasts, however NKG2C+ NK cell subsets had significantly

462 higher CCL4 secretion than NKG2C- subsets ($p<0.05$), irrespective of LIR1

463 expression (Figure 5C, right). Together these results suggested that expression of

464 NKG2C enhances CCL4 release by NK cells when interacting with TB40/e infected

465 target cells, but the induction of cytokine release does not correlate with NK cell

466 degranulation.

467

468 The 4 NK subsets were also used simultaneously in a VDA (Figure 6) using NK cells

469 from four independent donors. In good agreement with our earlier results, NK cell

470 subsets with LIR1 expression, with or without NKG2C, resulted in a lower percentage

471 of viral spread than cells without LIR1 (Figure 6A). This was observed across all the

472 donors tested. However, when we compared the NKG2C+ NK cell subsets against

473 NKG2C- NK cell subsets, with or without LIR1 expression, three out of four donors

474 tested showed no difference in the degree of HCMV control (Figure 6B). In one donor,

475 the LIR1+NKG2C+ subset exerted worse control than LIR1+NKG2C- NK subset

476 (Figure 6B, left column); while with another donor, LIR1-NKG2C+ NK subset

477 exerted better control than LIR1-NKG2C- NK cells (Figure 6B, right column).

478 However, taken together, we concluded that there is no clear difference in the degree

479 of HCMV dissemination when NK cells express or lack NKG2C on their surface.

480

481 **Discussion**

482 To date, ten HCMV gene products and one HCMV microRNA have been shown to
483 interfere with NK cell immune responses, by disrupting both activating and inhibitory
484 signaling to NK cells during HCMV lytic infection (19, 20). Studies into the activity
485 of NK cells *in vitro* against HCMV infected cells has predominantly focused on
486 effector mechanisms such as cytokine production and cytotoxicity (28, 35). Moreover,
487 although the frequency of NK cells expressing several NK receptors is associated
488 with HCMV serostatus (37, 39, 58), enhancement in NK cell effector functions has
489 only been demonstrated in the presence of anti-HCMV antibodies (41). Currently
490 there is little *in vitro* evidence to suggest that these higher frequency NK cell subsets
491 confer better control of HCMV in longer term culture.
492
493 A viral dissemination assay that we had previously used to study CD8+ T cell
494 responses (51) was established to test NK cell responses against fibroblasts lytically
495 infected with HCMV. This was similar in concept to a recently published focal
496 expansion assay (45), which was used to investigate the control of viral spread by NK
497 cells during HCMV TB40/e infection, and the effect of deletion of known viral NK

498 immune evasion genes. This work concluded that NK cells can efficiently control
499 HCMV transmission in different cell types and the UL16 viral protein contributes to
500 the immune evasion of NK cells during HCMV transmission. The focal expansion
501 assay and the viral dissemination assay are new methodological approaches employed
502 for studying the longer term interaction between NK cells and HCMV infected cells
503 *in vitro*. Compared with conventional NK cell cytotoxicity and degranulation assays,
504 both focal expansion and viral dissemination assays aim to assess the longer-term
505 control of NK cells on HCMV infection by indirectly measuring changes in the spread
506 of virus in the presence of NK cells. While we did not investigate which NK cell
507 effector mechanisms (either cytokine secretion or direct cell cytotoxicity or both) are
508 effective at limiting growth and dissemination of HCMV in our VDA, it has been
509 demonstrated by Wu et al (45) that during long-term co-incubation of NK cells with
510 HCMV infected target cells, both direct cell contact and soluble factors like IFN γ are
511 contributing factors to the control of dissemination (45).
512
513 Our assay shows that NK cells can exert effector functions at an E:T ratio as low as
514 0.3125:1, which is considerably lower and more physiological than short-term NK

515 cell cytotoxicity and degranulation assays, which often required an E:T ratio of 10:1

516 and higher. Our data support the hypothesis that NK cells control low passage

517 strains such as Merlin less effectively than high passage laboratory mutants such as

518 AD169; this is expected as AD169 is lacking several immune evasion genes (59, 60).

519 TB40/e is known to contain a mixture of virus populations, including some that lack a

520 functional UL141 (an established NK immune evasion gene) (53, 61), and also

521 contains a non-functional UL40 gene (62). Thus it was interesting to note that the

522 dissemination assay was also able to distinguish between NK cell control of infections

523 with TB40/e and Merlin, underlining the advantages of working with defined strains

524 that express a full complement of HCMV genes when characterizing viral

525 pathogenesis.

526

527 Importantly, our assay demonstrated for the first time that the ability of *in vitro*

528 expanded, activated LIR1+ NK cells to inhibit viral dissemination differs from LIR1-

529 NK cells, and was dependent on natural sequence variation within the ligands

530 expressed by a viral strain. These strain differences are likely due to the differences in

531 the binding kinetics exhibited by UL18 proteins from different HCMV strains to

532 LIR-1 (63, 64). Vales-Gomez et al demonstrated that a particular isolate of the UL18
533 protein, Variant E, which has the identical amino acid sequences in the α3 region as
534 strain Merlin UL18, showed a binding affinity that was at least 50 fold lower than the
535 UL18 protein from AD169 (63). They also demonstrated that the UL18 protein of
536 Variant E exhibited weaker inhibition against a LIR1-expressing transformed NK cell
537 line as compared to UL18 derived from AD169 (63). A separate study carried out by
538 Cerboni et al also showed that purified AD169 UL18 protein was able to inhibit
539 cytotoxicity of the NKL cell line to a greater extent than UL18 protein from a clinical
540 isolate, which again had the same α3 sequence found in strain Merlin (64).
541 Interestingly the three amino acids that we identified as responsible for the differing
542 abilities of NK cells to control dissemination, have recently been shown to be under
543 positive selection in HCMV strains, towards the more inhibitory sequence (65),
544 suggesting that the need to avoid activating NK cells can directly drive virus
545 evolution.
546
547 Given the above considerations, it seems likely that UL18 from both the Merlin and
548 TB40/e strains have weaker affinity for LIR1, and as a result LIR1+ NK cells

549 received weaker inhibitory signals than from the AD169 strain, and as a result
550 therefore were better at controlling HCMV dissemination. It is also possible that
551 UL18 of TB40/e/Merlin also interacts with other NK receptors that leads to
552 enhancement in NK cell function, which have yet to be identified. Alternatively,
553 instead of inducing weaker inhibition signaling via LIR1, the UL18 protein of
554 Merlin/TB40/e might cause a change in clustering of LIR1 receptor and 'antagonize'
555 inhibitory signaling. This antagonistic ligand behavior had been suggested in certain
556 peptide:MHC-KIR interactions between HCV and NK cells (66), although the
557 mechanism has yet to be fully established.
558
559 Lastly, we examined the effect of expression of the activating receptor NKG2C (67).
560 An increase in the proportion of NKG2C+ NK cells is strongly associated with
561 HCMV serostatus (37, 38, 40, 43, 68). However, there is as yet no evidence to suggest
562 that the acquisition of NKG2C on NK cells without the presence of anti-HCMV
563 antibodies confers a stronger immune response against HCMV infection. More
564 recently, another study demonstrated that although HCMV seropositive patients
565 receiving allogeneic stem cell transplantations have higher proportions of NKG2C+

566 NK cells, there is no obvious change in NKG2C+ NK cells between patients with or
567 without HCMV DNAemia 60 days after transplantation (42). In accordance with this,
568 our results showed no differences between NKG2C+ and NKG2C- NK cell subsets in
569 their ability to directly control HCMV dissemination (despite NKG2C expression
570 being able to enhance CCL4 cytokine production), suggesting that there may not be a
571 specific functional role for NKG2C in the direct control of HCMV. However, in light
572 of recent published work suggesting the presence of HCMV seropositive-donor serum
573 can induce stronger responses of NKG2Cbright NK cells in the short-term assay (44),
574 it would be interesting to assess if the donor serum can influence the outcome of these
575 long-term dissemination assays.
576
577 In summary, we have presented here an improved, *in vitro* technique of assessing
578 long-term immune control of NK cells against HCMV dissemination. Conventional
579 NK cell cytotoxicity assays focus on the NK cell responses within a few hours of
580 co-cultured with target cells. The VDA uses a much lower, more physiological E:T
581 ratio than conventional NK cytotoxicity assays, and extends the analysis of NK cells
582 responses to 9 days. Through the viral dissemination assay, we have uncovered new

583 evidence that NK cell subsets respond differently to different variants of viral ligands,

584 but the expression of NKG2C made little difference to the outcome of the long-term

585 HCMV control by NK cells. The VDA could be adapted to analyze other NK cell

586 subsets that have been indicated as ‘memory-like’, and assess whether their *in vitro*

587 control over virus spreading may be similar to that described previously for the

588 murine Ly49H activating receptor and MCMV m157 protein (69). Taken together,

589 this data is the first description of a number of novel interactions between NK cells

590 and HCMV during long-term lytic lifecycles. These results may have implications for

591 susceptibility to HCMV infection, and to future approaches to vaccination strategies

592 that involve generation of immunological ‘memory-like’ responses of NK cells.

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607 TABLE AND FIGURE LEGENDS

608 **Table 1: Primers used for UL18 mutant virus generation**

609 Sequences used to generate AD169-UL18 Merlin mutant virus are designated as
610 Merlin-F (F: forward primer) and Merlin-R (R: reverse primer); Merlin-UL18AD
611 Mutant virus are designated as AD169-F and AD169-R. Primer-F and Primer-R were
612 used to amplify the a3 domain of HCMV UL18 protein in standard PCR.

613

614 **Figure 1– Establishment of viral dissemination assay.**

615 A) Human fibroblasts (HFFs) infected with HCMV strain TB40/e UL32-GFP at
616 MOI= 0.1 and *in vitro* expanded NK cells were co-cultured for 9 days at various
617 effector to target (E:T) ratios, starting from 1.25:1 to 0.3125:1, at 37°C and 5% CO_2.
618 NK cells were then washed off and the HFFs were observed by fluorescence
619 microscopy (top panel) or following trypsinisation analysed by flow cytometry
620 (bottom panel). Representative dot plots show the results from uninfected and
621 infected controls and the change percentage of fluorescent cells following
622 co-incubation with different ratios of NK cells: HFFs. B) The assay was also
623 performed using HFFs infected with untagged AD169 at MOI=0.1. The cells were

624 stained intracellularly with anti-IE-Alexafluor488 antibodies before being analysed by
625 flow cytometry. C) The summary results of dissemination assays on NK cells derived
626 from N=7 donors using AD169 infected HFFs; from N=4 donors using TB40/e
627 infected HFFs; and from N=9 donors using Merlin infected HFFs. MOI used was 0.1
628 and various effector to target (E:T) ratios, starting from 1.25:1 to 0.3125:1, were used.
629 At the end of the assay TB40/e-infected or Merlin-infected HFFs were analysed by
630 flowcytometry without additional staining, while AD169-infected HFFs were stained
631 with anti-IE-Alexafluor488 antibody before analysis by flowcytometry. Data was
632 analysed by one-way ANOVA, and significant results ($p<0.05$ *, $p<0.01$**) are
633 indicated. D) Three separate dissemination assays using HCMV strains AD169,
634 TB40/e or Merlin at a MOI of 0.1 were conducted using *in vitro* expanded NK cells
635 from either a seropositive (Donor 319) or a seronegative donor (Donor 401). The data
636 was normalized according to the uninfected and infected controls. The black triangles
637 are results from Merlin-infected HFF; grey triangles are TB40/e-infected HFF; and
638 grey squares are AD169-infected HFF. Each data point represents 3 independent
639 readouts, and error bars represent SEM. Data was analysed by two-way ANOVA, and
640 significant results ($p<0.05$ *, $p<0.01$**) are indicated.

641

642 **Figure 2– NK cells expressing LIR1 have similar effector cellular functions to**
643 **LIR1 non-expressing NK cells, but are more able to control viral dissemination**
644 **of HCMV strains TB40/e and Merlin.**

645 A) *In vitro* expanded NK cells were stained with anti-CD3, CD56 and LIR1
646 antibodies and sorted by flow cytometry. CD56+ CD3- NK cells were first collected
647 (NK) before further sorting into LIR1+ (LIR1+ NK) and LIR1- (LIR1- NK) subsets
648 based on LIR1 expression. Representative dot plots of the NK cells before and after
649 sorting are shown. B) NK, LIR1+ NK and LIR1- NK cells were co-cultured with
650 K562 target cells. The NK cell to target ratio is 1:1. K562 cells and NK cells only are
651 the controls. After culturing for 5 hours the percentage of CD107a+ cells were
652 measured by flow cytometry. CCL4 concentration is quantified using ELISA after
653 culturing overnight. Each data point represents the mean value of 3 repeats and error
654 bars represent SEM. The experiment was preformed using three different donors
655 (N=3) and the results showed the average value analysed by the Student T-test. Non-
656 significant results (p>0.05, NS) is indicated. C) LIR1+ and LIR1- NK cell subsets
657 were co-cultured with human HFFs infected with HCMV strain AD169, TB40/e or
658 with Merlin at a MOI of 0.1 in a viral dissemination assay. The NK cell to target

659 ratios range from 1.25:1, 0.625:1 and 0.312:1. The data was normalized according to

660 the uninfected and infected controls. The grey triangles are results from LIR- NK

661 cells; and black squares are the results from LIR+ NK cells. Each data point

662 represents 3 independent readouts, and error bars represent SEM. In total each assay

663 was repeated three times (N=3) using NK cells from three different donors. Data was

664 analysed by two-way ANOVA, and significant results ($p<0.05$*, $p<0.01$**) are

665 indicated.

666

667 **Figure 3–UL18 proteins from different HCMV strains influence the control of**

668 **virus dissemination by LIR1+ NK cells**

669 A) 67 sequences of HCMV UL18 proteins were identified from the NCBI protein

670 database (www.ncbi.nlm.nlh.gov). 6 sequences had a truncation and are not included

671 in the alignment. 13 unique amino acid sequences of the α3 region of UL18 were

672 identified. Each unique sequence is only shown once in the alignment and is

673 represented by a unique letter. The number following the letter in the sequence name

674 represents the number of times the sequence has appeared in the database. Grey boxes

675 indicate the sites interacting with LIR1 as suggested by crystal structure (57), while

676 the black line indicates the key differences between the UL18 of AD169 and Merlin

677 strains. B) The results of the neighbour-phylogenic tree analysis showing the

678 relationship between the sequences based on the protein α3 region. 20 virus strains

679 have NKAPED sequence, 37 have DKVPED, and 4 have DKAPDD. C) NK cells

680 from four different donors (N=4) were sorted based on LIR1 expression as previously

681 described before co-cultured in a viral dissemination assay with HFFs infected with

682 HCMV strain AD169 and AD169-ΔUL18 at an MOI of 0.1. Infected cells were

683 stained with IE-ALEXA FLUOR 488 antibodies before analysis using flow cytometry.

684 D) Viral dissemination assay was repeated with NK cells from four different donors

685 (N=4) sorted based on LIR1 expression and co-cultured with HFFs infected with

686 HCMV strain Merlin and Merlin-ΔUL18 at an MOI of 0.1. Infected HFFs were

687 analysed on the basis of UL32-GFP fluorescence. In C) and D), the grey triangles are

688 results from LIR- NK cells; black squares are the results from LIR+ NK cells. The

689 NK cell to target ratios range from 1.25:1, 0.625:1 and 0.312:1. Each data point

690 represents 3 independent readouts. The data was analysed using two-way ANOVA,

691 error bars represent SEM and significant results ($p<0.05$*, $p<0.01$**) are indicated.

692

693 **Figure 4–Mutation of HCMV UL18 proteins causes changes in the control of**

694 **viral dissemination by NK cells.**

695 A) A PCR and sequencing on the α3 region of UL18 was preformed to check the

696 mutant virus AD169-UL18Merlin and Merlin-UL18AD169 compared with parental

697 strains. The amino acid alignment is shown with the altered sites indicated. Viral

698 dissemination assays were then carried out using NK cell from four different donors

699 (N=4) co-cultured with HFFs infected at MOI 0.1 with virus strains B) AD169 and

700 AD169-UL18Merlin or C) Merlin and Merlin-UL18AD169 virus. AD169 and

701 AD169-UL18Merlin infected HFFs were stained with IE-ALEXA FLOR 488

702 antibodies before analysis using flow cytometry. Merlin and Merlin-UL18AD169

703 virus infected HFFs expressed GFP and did not require additional staining. The NK

704 cell to target ratios range from 1.25:1, 0.625:1 and 0.312:1. The grey triangles are

705 results from LIR- NK cells; black squares are the results from LIR+ NK cells. Each

706 data point represents 3 independent readouts. The data was analysed using two-way

707 ANOVA, error bars represent SEM and significant results ($p<0.05$*, $p<0.01$**) are

708 indicated.

709

710 **Figure 5– Expression of NKG2C on *in vitro* expanded NK cells has an effect on**

711 **NK cell effector functions.**

712 A) *In vitro* expanded NK cells were stained with anti-CD3, CD56 and LIR1 and

713 NKG2C antibodies and sorted by flow cytometry into four subsets based on LIR1 and

714 NKG2C expressions. CD56+ CD3- NK cells were first collected (NK) before further

715 sorting into LIR1- NKG2C+, LIR1+ NKG2C+, LIR1- NKG2C- and LIR1+ NKG2C-.

716 Representative dot plots of the NK cells before and after sorting are shown. Sorted

717 NK cells were then co-cultured with B) K562 targets or C) HFFs infected with

718 TB40/e at MOI of 5. The NK cell to target ratio is 1:1. The percentage CD107a+ cells

719 were measured by flow cytometry after 5 hours, and the CCL4 cytokine secretion by

720 NK cells were measured after overnight incubation. NK cell only and target cell only

721 are the control samples. Error bars represent SEM. The experiment was repeated

722 using 3 different donor NK cells (N=3) and the average results were analysed by the

723 Student T-test. Significant results ($p<0.05$ *) are indicated.

724

725 **Figure 6– Expression of NKG2C on *in vitro* expanded NK cells does not control**

726 **HCMV dissemination more effectively over long-term.**

727 Viral dissemination assays were carried out using the NK cells sorted based on LIR1

728 and NKG2C as previous described incubated with TB40/e-infected HFFs at an MOI

729 of 0.1. The experiment was repeated four times using NK cells from four different

730 donors. The NK cell to target ratios range from 1.25:1, 0.625:1 and 0.312:1. The

731 results were analysed using flow cytometry and then normalized according to

732 uninfected and infected controls. On each graph the circle represents LIR1 (+)

733 NKG2C (+) NK cells; the rectangle represents LIR (+) NKG2C (-) NK cells; the

734 upward triangle represents LIR1 (-) NKG2C (+) NK cells; and downward triangle

735 represents LIR1 (-) NKG2C (-) NK cells. The comparison of LIR1 expressing and

736 LIR1- non expressing subsets are shown in A); while the comparison of NKG2C

737 expressing and NKG2C non-expressing subsets are shown in B). Each data point

738 represents 3 independent readouts. The data was analysed using two-way ANOVA,

739 error bars represent SEM and significant results ($p<0.05$*, $p<0.01$**) are indicated.

740

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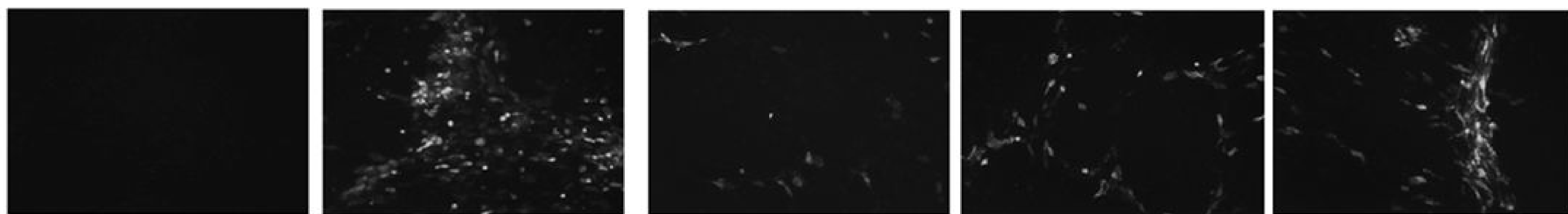
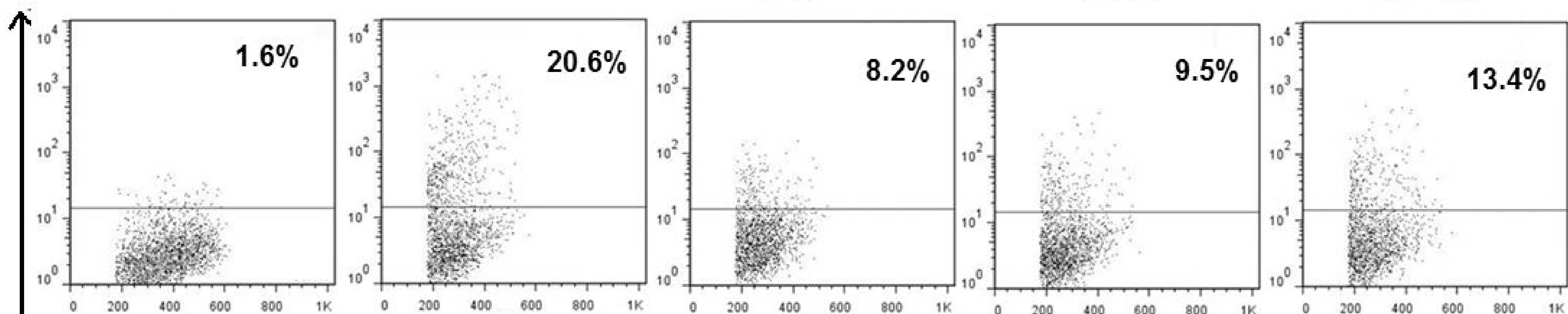
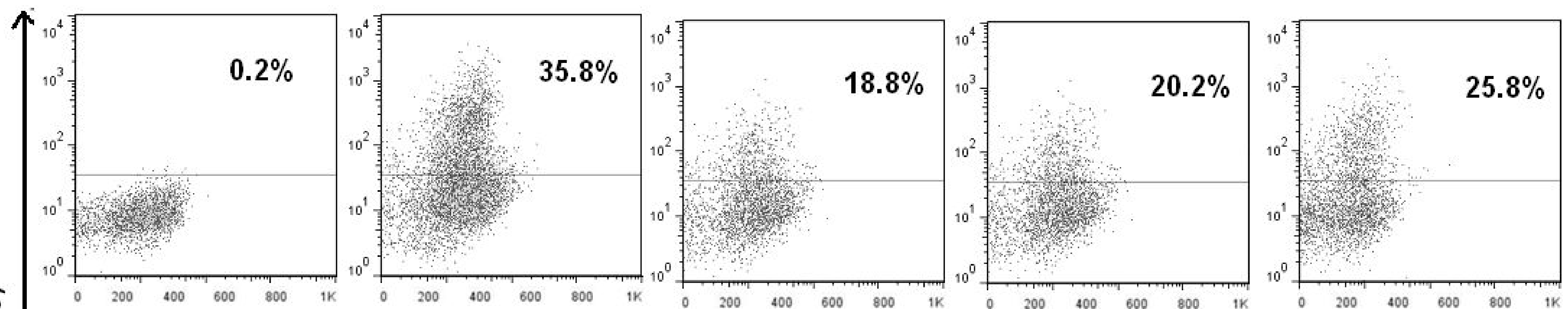
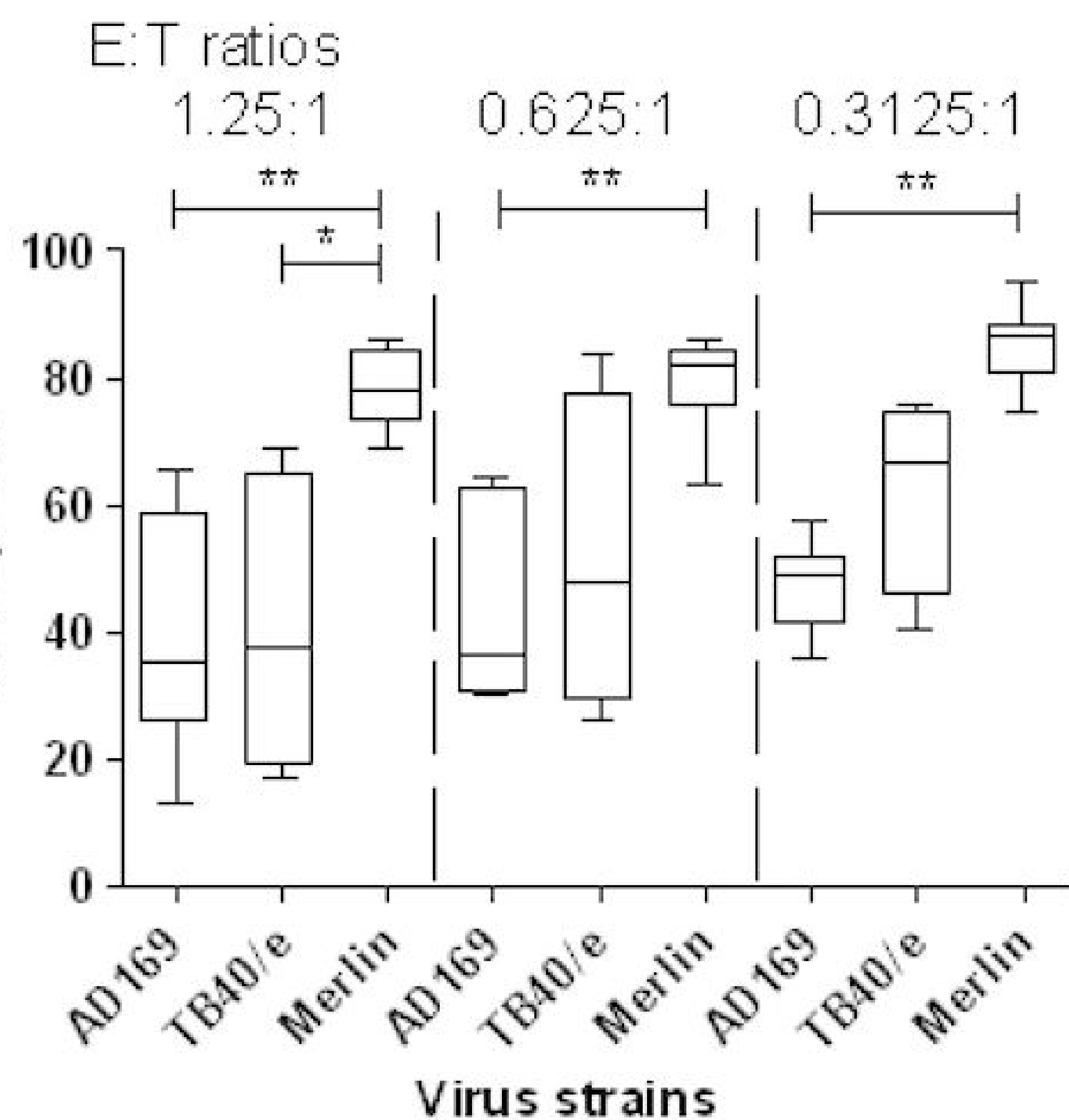
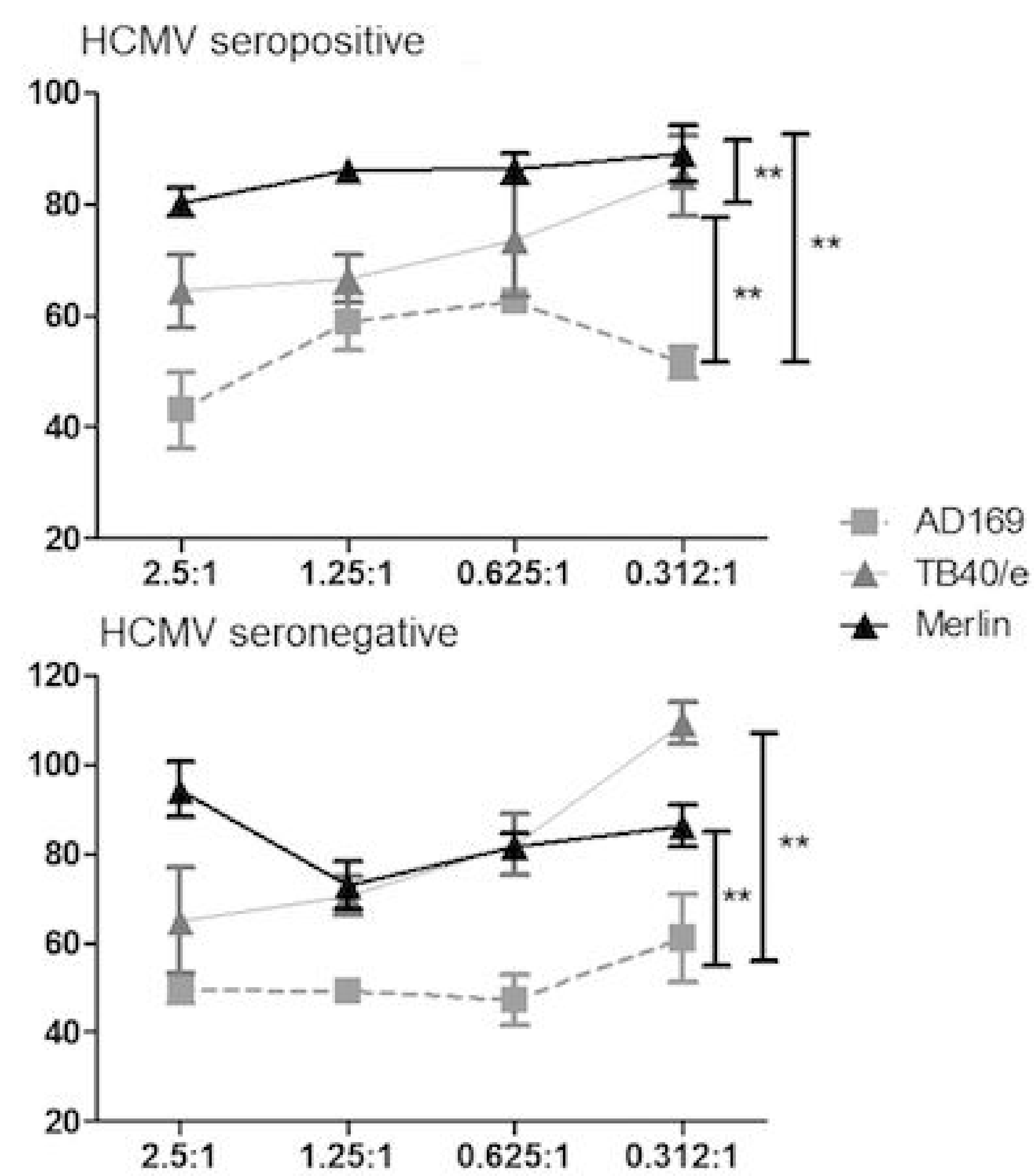
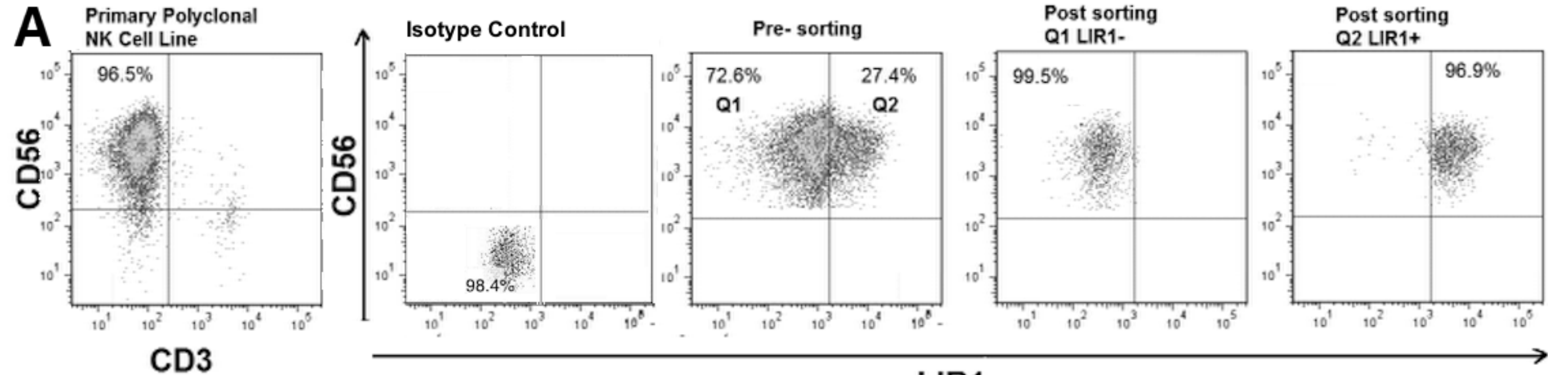

A
HFF only
Infected HFF
NK: HFF ratio
1.25:1
0.625:1
0.3125:1

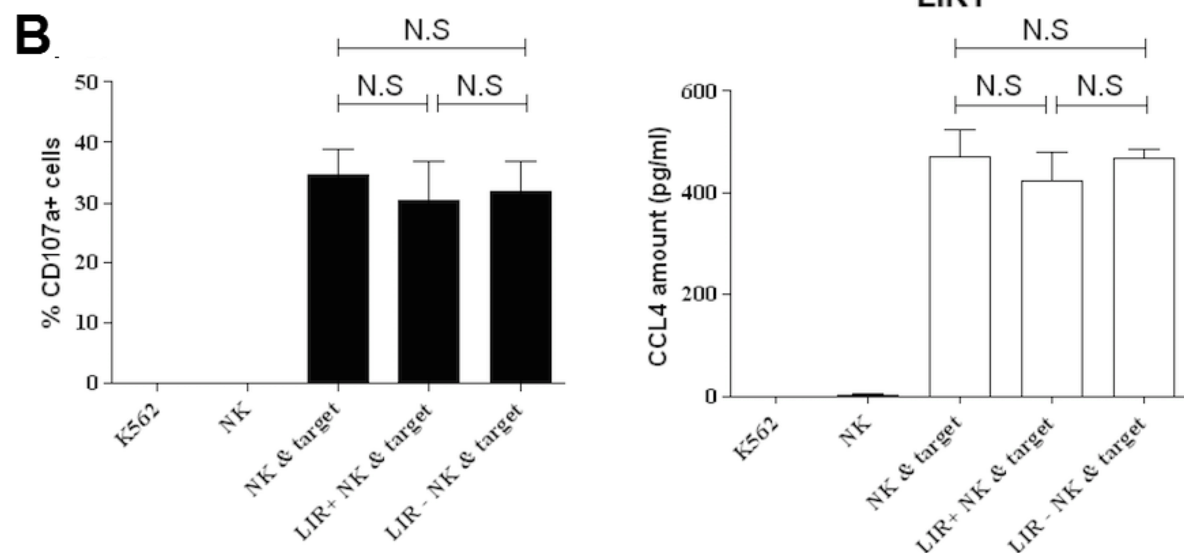

% of TB40/e-UL32GFP expression
1.6%
20.6%
8.2%
9.5%
13.4%
HFF only
Infected HFF
NK: HFF ratio
1.25:1
0.625:1
0.3125:1
FCS


B
% of IE-antibody stained cells (AD169)
0.2%
35.8%
18.8%
20.2%
25.8%
HFF only
Infected HFF
NK: HFF ratio
1.25:1
0.625:1
0.3125:1
FCS


C
E:T ratios
1.25:1
0.625:1
0.3125:1
Percentage Viral Spread
AD169
TB40/e
Merlin
Virus strains


D
HCMV seropositive
HCMV seronegative
AD169
TB40/e
Merlin
Normalised % viral spread
2.5:1
1.25:1
0.625:1
0.312:1
NK: HFF ratio

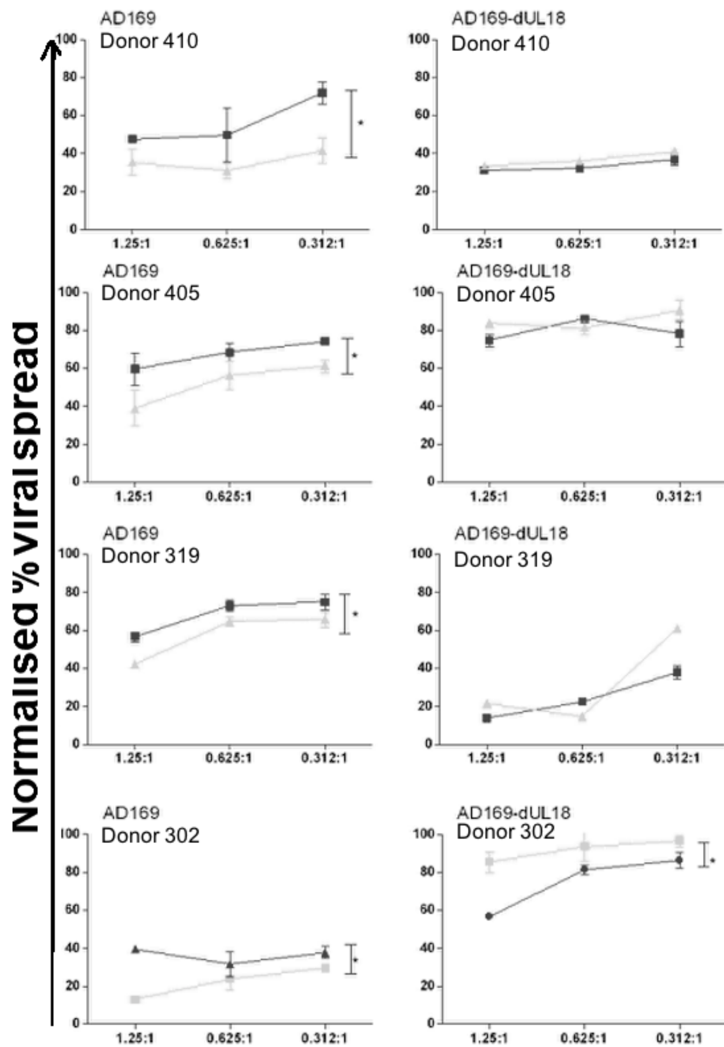


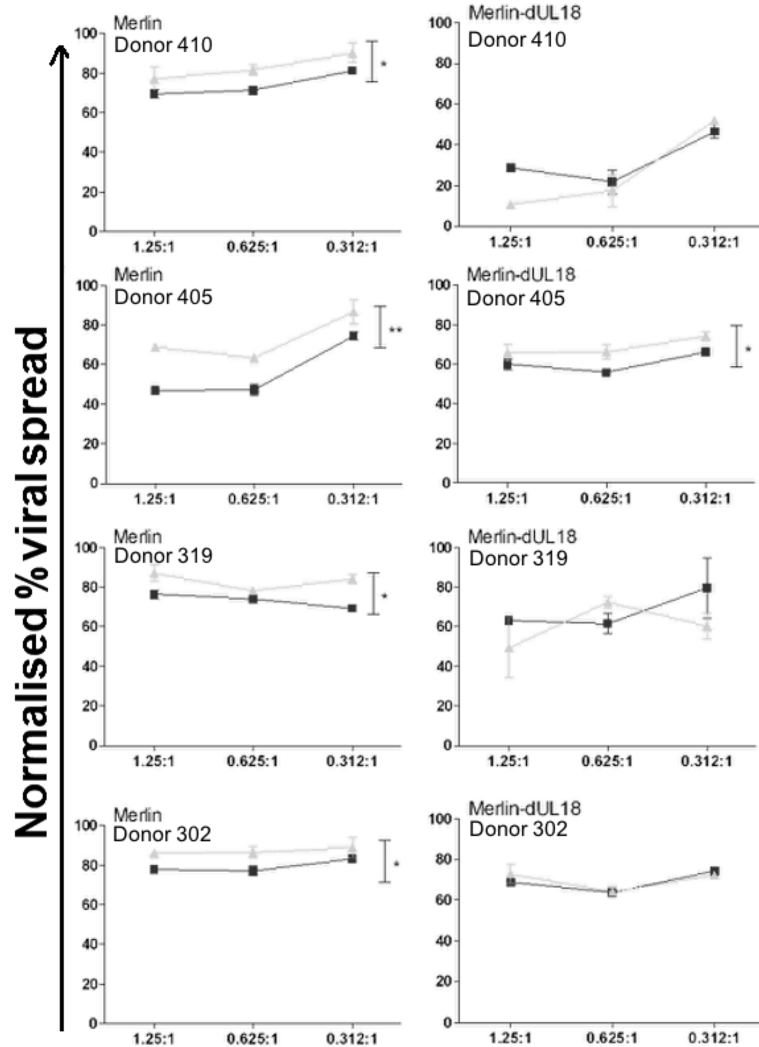


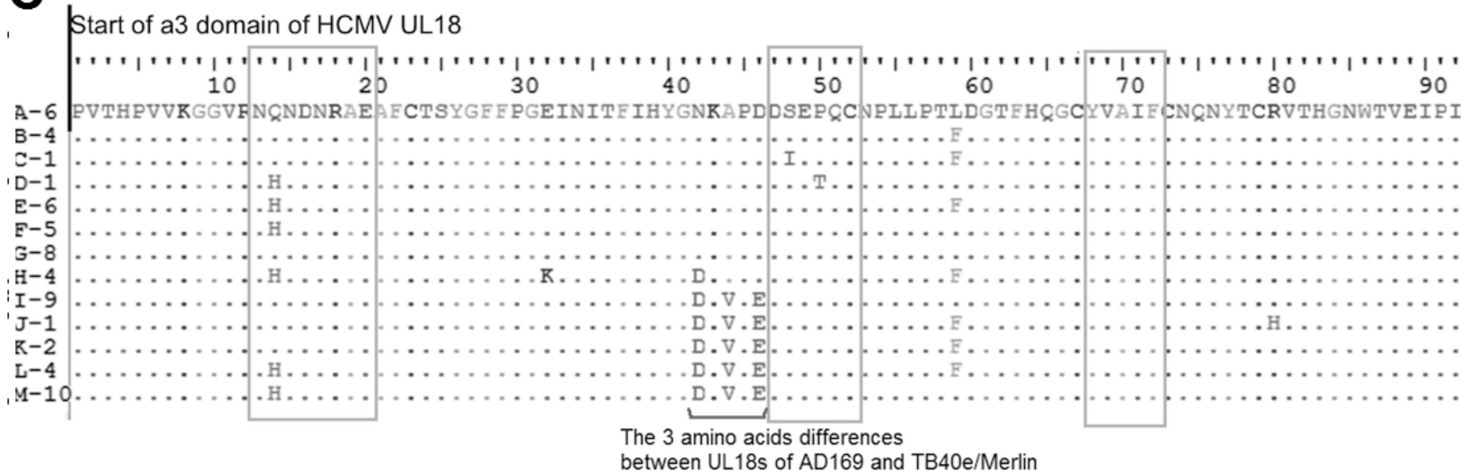
C
LIR1+ NK
LIR1- NK
AD169 Donor 407
AD169 Donor 409
AD169 Donor 323
Normalised % viral spread
1.25:1
0.625:1
0.312:1
NK: HFF ratio

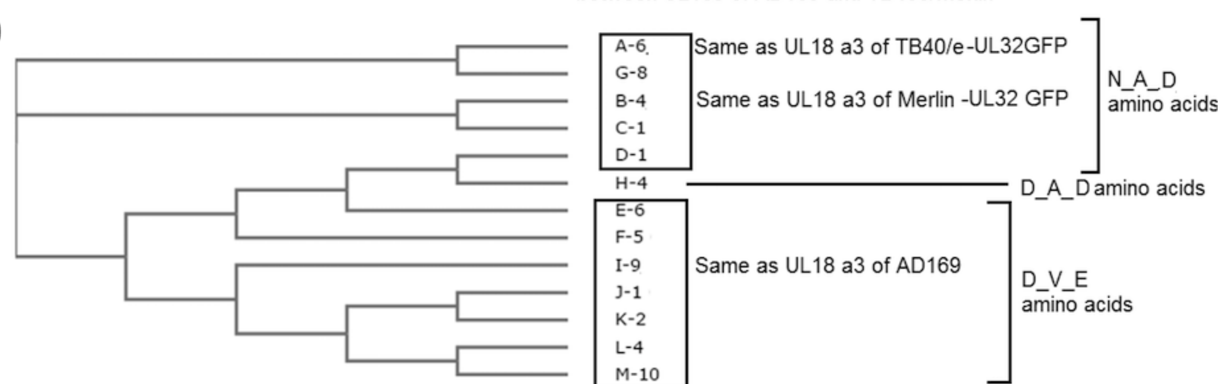
D
LIR1+ NK
LIR1- NK
TB40/e Donor 407
TB40/e Donor 409
TB40/e Donor 323
Normalised % viral spread
NK: HFF ratio

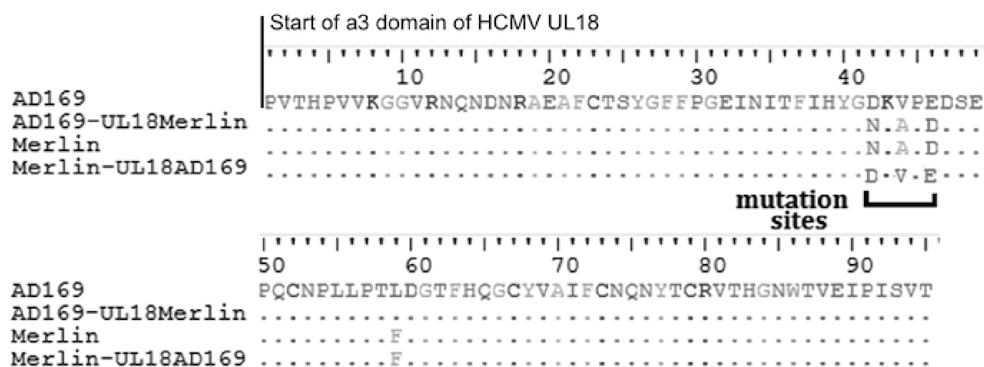
E
LIR1+ NK
LIR1- NK
Merlin Donor 407
Merlin Donor 409
Merlin Donor 323
Normalised % viral spread
NK: HFF ratio

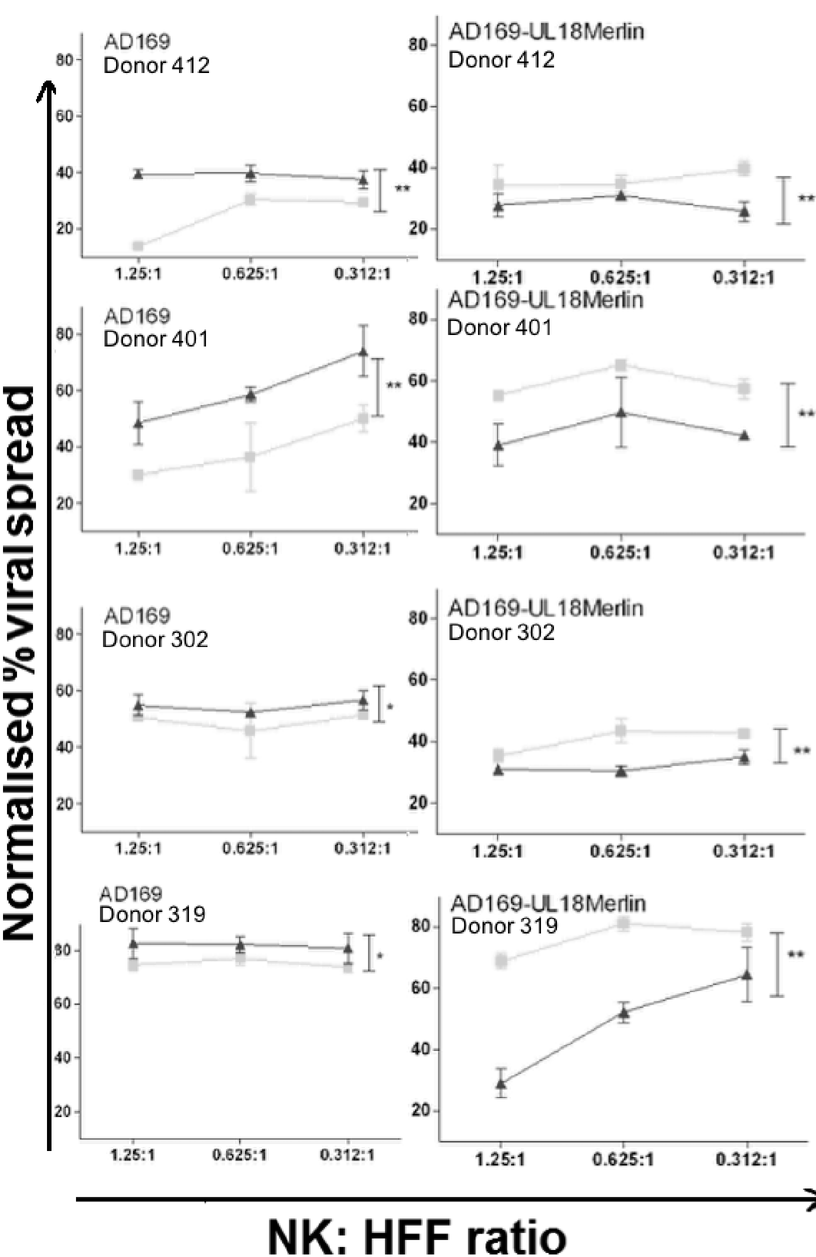

A
Virus strains
LIR1+ NK
LIR1- NK
Normalised % viral spread
AD169 Donor 410
AD169-dUL18 Donor 410
AD169 Donor 405
AD169-dUL18 Donor 405
AD169 Donor 319
AD169-dUL18 Donor 319
AD169 Donor 302
AD169-dUL18 Donor 302
1.25:1
0.625:1
0.312:1
NK: HFF ratio

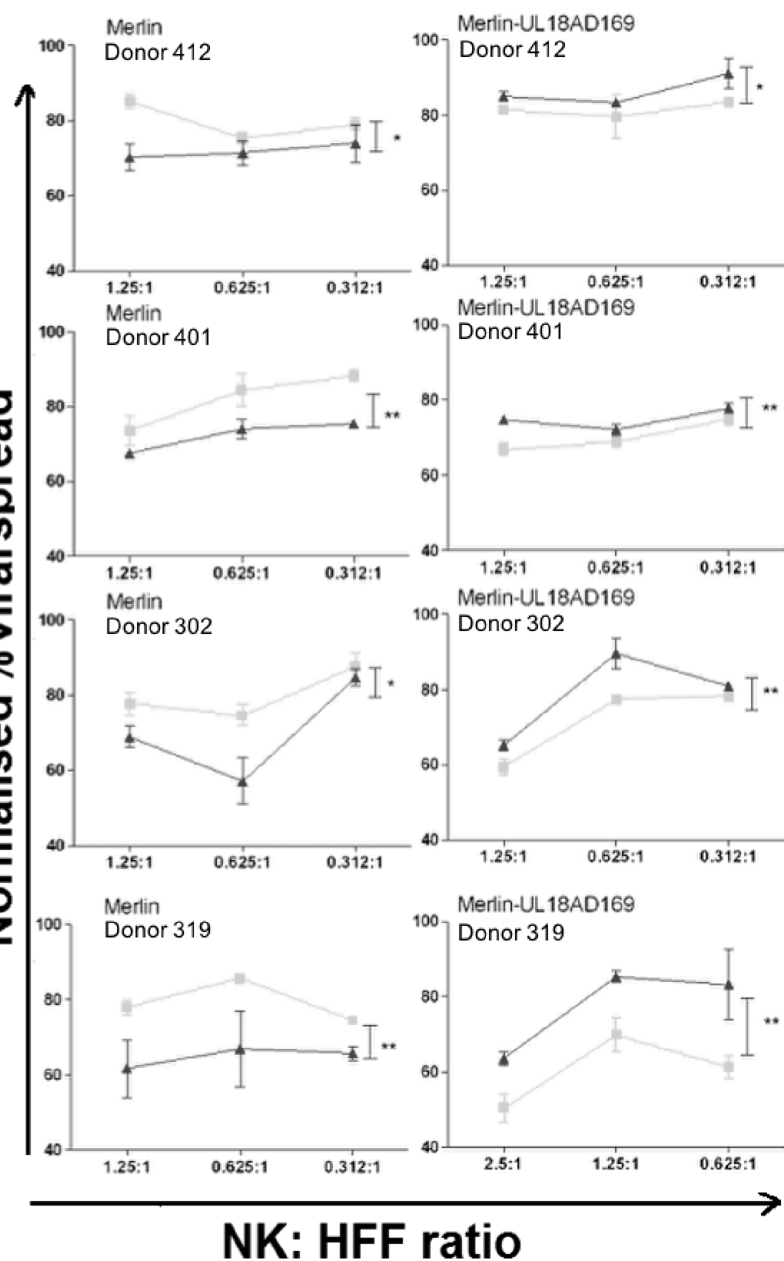

B
Virus strains
LIR1+ NK
LIR1- NK
Normalised % viral spread
Merlin Donor 410
Merlin-dUL18 Donor 410
Merlin Donor 405
Merlin-dUL18 Donor 405
Merlin Donor 319
Merlin-dUL18 Donor 319
Merlin Donor 302
Merlin-dUL18 Donor 302
1.25:1
0.625:1
0.312:1
NK: HFF ratio

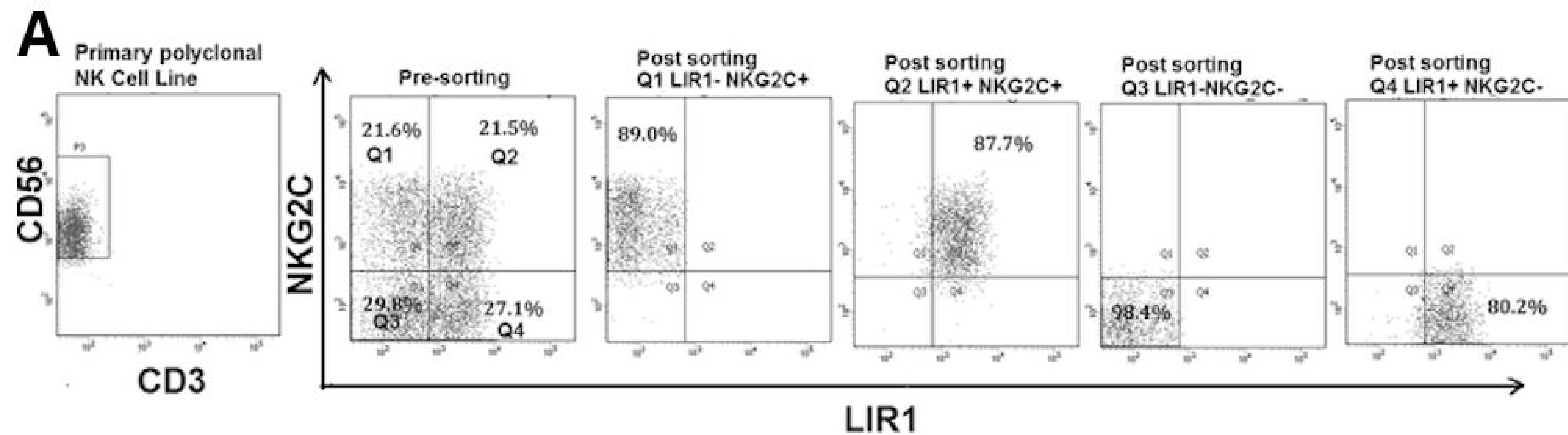

C
Start of a3 domain of HCMV UL18
A-6 PVTHPVVKGGVRNQNDNRAEAFCTSYGFFPGEINITFIHYGNKAPDDSEPQCNPLLPTLDGTFHQGCYVAIFCNQNYTCRVTHGNWTVEIPI
The 3 amino acids differences between UL18s of AD169 and TB40e/Merlin

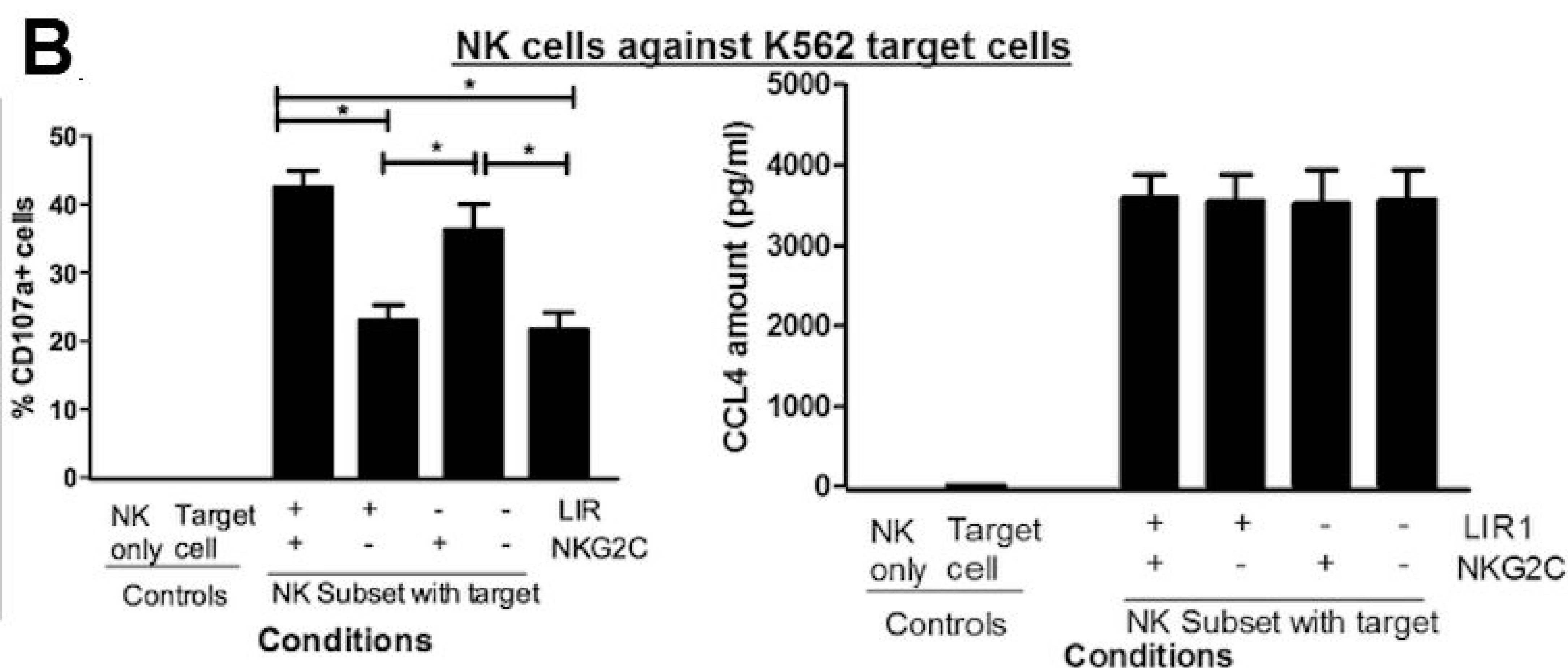

D
A-6
G-8
B-4
C-1
D-1
H-4
E-6
F-5
I-9
J-1
K-2
L-4
M-10
Same as UL18 a3 of TB40/e-UL32GFP
Same as UL18 a3 of Merlin -UL32 GFP
N_A_D amino acids
D_A_D amino acids
Same as UL18 a3 of AD169
D_V_E amino acids

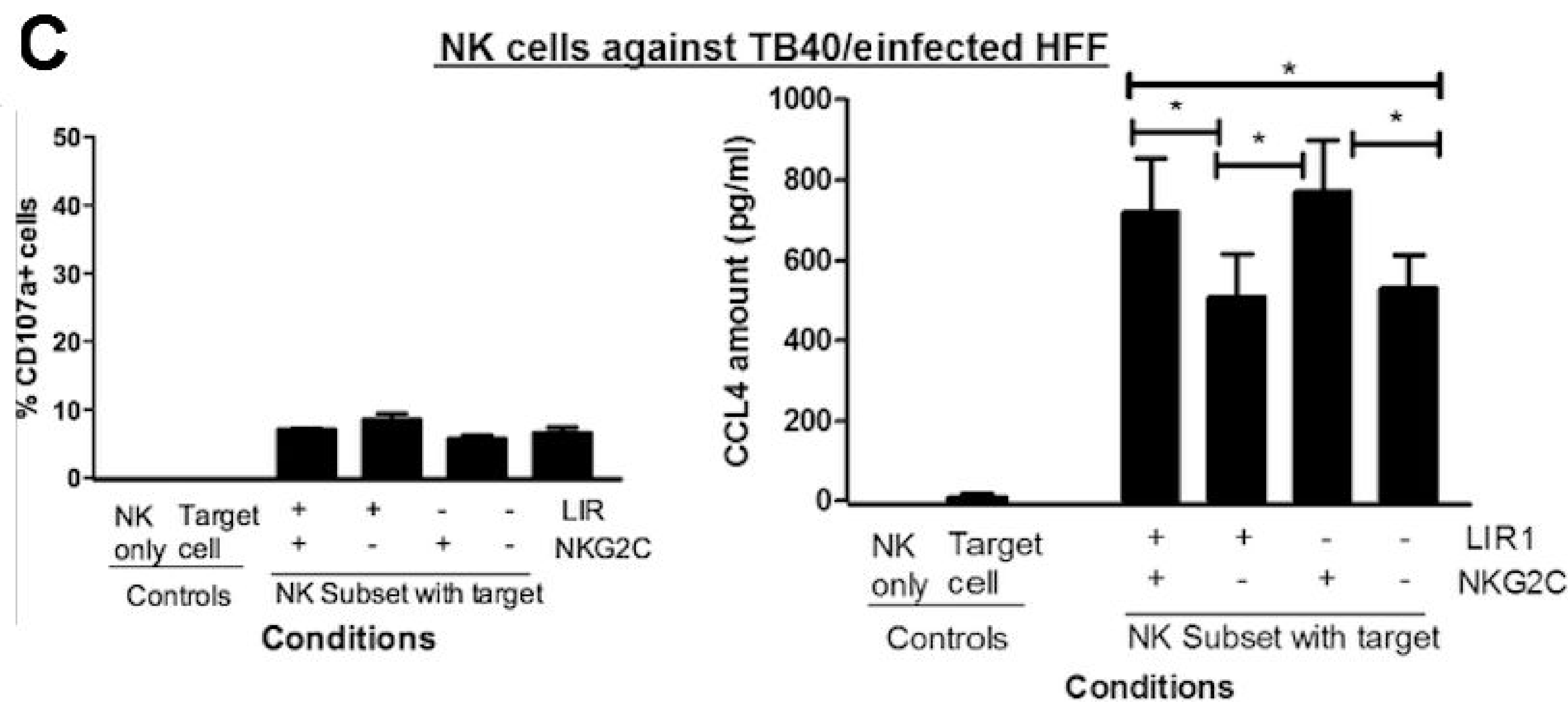

A
Start of a3 domain of HCMV UL18
AD169
AD169-UL18Merlin
Merlin
Merlin-UL18AD169
PVTHPVVKGGVRNQNDNRAEAFCTSYGFFPGEINITFIHYGDKVPEDSE
mutation sites
PQCNPLLPTLDGTFHQGCYVAIFCNQNYTCRVTHGNWTVEIPISVT


B
Virus strains
LIR1+ NK
LIR1- NK
AD169 Donor 412
AD169-UL18Merlin Donor 412
AD169 Donor 401
AD169-UL18Merlin Donor 401
AD169 Donor 302
AD169-UL18Merlin Donor 302
AD169 Donor 319
AD169-UL18Merlin Donor 319
Normalised % viral spread
NK: HFF ratio


C
Virus strains
LIR1+ NK
LIR1- NK
Merlin Donor 412
Merlin-UL18AD169 Donor 412
Merlin Donor 401
Merlin-UL18AD169 Donor 401
Merlin Donor 302
Merlin-UL18AD169 Donor 302
Merlin Donor 319
Merlin-UL18AD169 Donor 319
Normalised % viral spread
NK: HFF ratio


A
Primary polyclonal
NK Cell Line
CD56
CD3
NKG2C
Pre-sorting
21.6%
Q1
21.5%
Q2
29.8%
Q3
27.1%
Q4
Post sorting
Q1 LIR1- NKG2C+
89.0%
Post sorting
Q2 LIR1+ NKG2C+
87.7%
Post sorting
Q3 LIR1-NKG2C-
98.4%
Post sorting
Q4 LIR1+ NKG2C-
80.2%
LIR1


B
NK cells against K562 target cells
% CD107a+ cells
CCL4 amount (pg/ml)
NK only
Target cell
Controls
NK Subset with target
LIR
LIR1
NKG2C
Conditions


C
NK cells against TB40/einfected HFF
% CD107a+ cells
CCL4 amount (pg/ml)
NK only
Target cell
Controls
NK Subset with target
LIR
LIR1
NKG2C
Conditions

Viral Dissemination assay of LIR1/NKG2C expressing NK cell subsets against low-passage HCMV strain infected HFF

A

LIR1+ NKG2C+
LIR1- NKG2C+
LIR1+ NKG2C-
LIR1-NKG2C-

Donor 410 **
Donor 410 **
Donor 321 *
Donor 321 **
Donor 401 *
Donor 401 **
Donor 302 **
Donor 302 **

B

LIR1+ NKG2C+
LIR1+ NKG2C-
LIR1- NKG2C+
LIR1-NKG2C-

Donor 410 *
Donor 410
Donor 321
Donor 321
Donor 401
Donor 401
Donor 302
Donor 302 *

Normalised % viral spread

1.25:1 0.625:1 0.312:1

NK: HFF ratio

Table 1: Primers used for UL18 mutant virus generation

Primer Names	Sequence
Merlin-F	GACAACAGAGCTGAAGCATTCTGTACATCTTACGGGTTCTTTCCAGGGG AAATTAATATTACTTTTATCCATTACGGTCCTGTGACGGAAGATCACTTCG
Merlin-R	AAAAGATGGCTACGTAACATCCCTGATGGAAAGTCCCATCGAAGGTGG GAAGTAGCGGATTGCATTGAGGCTCGCTATCCTGAGGTTCTTATGGCTCTTG
AD169-F	GACAACAGAGCTGAAGCATTCTGTACATCTTACGGGTTCTTTCCAGGGG AAATTAATATTACTTTTATTCATTACGGTCCTGTGACGGAAGATCACTTCG
AD169-R	AAAAGATGGCTACGTAACATCCCTGATGGAAAGTCCCATCCAAGGTGG GAAGTAGCGGATTGCATTGAGGCTCGCTATCCTGAGGTTCTTATGGCTCTTG
Primer-F	AAACAAAACGTACATCGACGGTAA
Primer-R	AGCAAAGCGCATAAAAGCAGG