1   **The simultaneous insertion of two ligands in gD for the cultivation of oncolytic HSVs in non-**
2   **cancer cells and the retargeting to cancer receptors**
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43  **ABSTRACT**

44  Insertion of a single chain antibody (scFv) to HER2 (human epidermal growth factor receptor 2) in

45  gD, gH, or gB gives rise to herpes simplex viruses (HSVs) specifically retargeted to HER2-positive

46  cancer cells, hence in highly specific non-attenuated oncolytic agents. Clinical grade virus

47  production can not rely on cancer cells. Recently, we developed a double retargeting strategy

48  whereby gH carries the GCN4 peptide for retargeting to the non-cancer producer Vero-GCN4R cell

49  line, and gD carries the scFv to HER2 for cancer retargeting. Here, we engineered double retargeted

50  recombinants, which carry both the GCN4 peptide and the scFv to HER2 in gD. Novel, more

51  advantageous detargeting strategies were devised, so as to optimize the cultivation of the double-

52  retargeted recombinants. Nectin1 detargeting was achieved by deletion of aa 35-39, 214-223, or

53  219-223, and replacement of the deleted sequences with one of the two ligands. The latter two

54  deletions were not attempted before. All recombinants exhibited the double retargeting to HER2

55  and to the Vero-GCN4R cells, as well as detargeting from the natural receptors HVEM and nectin1.

56  Of note, some recombinants grew to higher yields than others. The best performing recombinants

57  carried a gD deletion as small as 5 amino acids, and grew to titers similar to those exhibited by the

58  singly retargeted R-LM113, and by the non-retargeted R-LM5. This study shows that double

59  retargeting through insertion of two ligands in gD is feasible and, when combined with appropriate

60  detargeting modifications, can result in recombinants highly effective *in vitro* and *in vivo.*

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63  **IMPORTANCE**

64  There is increasing interest in oncolytic viruses, following FDA and EMA approval of the oncolytic

65  HSV Oncovex$^{GM-CSF}$, and, mainly, because they greatly boost the immune response to the tumor

66  and can be combined with immunotherapeutic agents, particularly immune checkpoint inhibitors. A

67  strategy to gain high cancer specificity and avoid virus attenuation is to retarget the virus tropism to

68  cancer-specific receptors of choice. However, cultivation of retargeted oncolytics in cells

69  expressing the cancer receptor may not be approvable by regulatory agencies. We devised a strategy

70   for their cultivation in non-cancer cells. Here, we describe a double retargeting strategy, based on

71   the simultaneous insertion of two ligands in gD, one for retargeting to a producer, universal Vero

72   cell derivative, one for retargeting to the HER2 cancer receptor. These insertions were combined

73   with novel, minimally-disadvantageous detargeting modifications. The current and accompanying

74   studies teach how to best achieve the clinical-grade cultivation of retargeted oncolytics.

75

76 **INTRODUCTION**

77 Oncolytic viruses have come of age (1-5) since the approval by FDA and EMA of an oncolytic

78 herpes simplex virus (HSV), initially named Oncovex[GM-CSF] or T-Vec, for the treatment of

79 metastatic melanoma (6, 7). Several generations of oncolytic HSVs were designed and tested in

80 preclinical assays and in clinical trials. Many of them achieve cancer specificity by virtue of

81 attenuation, frequently obtained through the deletion of the $\gamma_1 34.5$ gene, whose product counteracts

82 the IFN and PKR response of the cell to the virus (7-10). In other examples, additional genes were

83 deleted (11, 12). The resulting recombinants exhibited varying degrees of attenuation. A drawback

84 of attenuation is that not all cancer cells sustain a robust replication of these viruses.

85 An alternative strategy to attenuation has been to obtain cancer specificity through the

86 modification of the HSV tropism and tropism retargeting to a cancer specific receptor of choice,

87 coupled with detargeting from natural receptors (13-21). In our laboratory the targeted cancer

88 receptor is HER2 (human epidermal growth factor receptor 2), expressed in breast, ovary, stomach,

89 lung and other cancers (22). While the HER2-positive cancers are usually treated with anti-HER2

90 monoclonal antibodies, exemplified by trastuzumab and pertuzumab, only a fraction of cancers is

91 sensitive to this treatment, and resistance develops frequently (23).

92 HSV enters cells through the concerted action of four envelope glycoproteins, named gD,

93 gH/gL and gB, which are activated in a cascade fashion by interaction with cognate receptors and

94 intermolecular signaling (24-29). Briefly, gD interacts alternatively with HVEM or nectin1 (30-32).

95 The receptor-bound gD activates gH/gL, which is additionally activated by $\alpha v\beta 6$ or $\alpha v\beta 8$-integrins

96 (33, 34). gH activation results in the displacement of gL (35), and is then transmitted to gB, which

97 executes the fusion between the virion envelope and the cell membrane (36). In the retargeted

98 viruses, a new ligand, exemplified by a single chain antibody to HER2, is engineered in gD, in gH,

99 or in gB, while appropriate deletions in gD ensure the detargeting from gD natural receptors (13,

100 15-17, 37, 38). The chimeric glycoproteins which carry the scFv to HER2 mediate HSV entry

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101 through HER2. Because of the detargeting-retargeting these oncolytic HSVs strictly depend on

102 HER2 for infection.

103      For clinical grade preparations of retargeted oncolytic HSVs, it is advisable to avoid the

104 virus cultivation in HER2-positive cancer cells. To meet these needs, we recently developed a

105 system for the cultivation in non-cancer cells of HSVs retargeted to HER2, and, potentially, to any

106 cancer-specific receptor of choice. The system is based on a double retargeting strategy. One

107 retargeting is to the HER2, or any cancer receptor of choice. The other retargeting is by way of the

108 20 aa long GCN4 peptide, which readdresses the tropism to Vero cells expressing the artificial

109 receptor named GCN4R (39). The latter is made by a single chain antibody to GCN4 (40) fused to

110 domains II, III, TM and C tail of nectin1. The choice of the Vero cells as recipients of GCN4R

111 rested on the notion that wt-Vero cells have been approved by FDA for the clinical grade

112 preparations of Oncovex$^{GM-CSF}$ (commercial name Imlygic), the derivative named Vero-His is

113 approved for clinical grade preparations of oncolytic Measles viruses (41), and, more generally, wt

114 Vero are approved for growth of a number human vaccines. The R-213 recombinant was

115 readdressed to GCN4R by engineering the GCN4 peptide in gH; simultaneously, it was readdressed

116 to HER2 by insertion of the scFv to HER2 in gD, in place of aa 6-38 (39). This deletion detargets

117 HSV tropism from HVEM and nectin1 (17).

118      The aims of this work were two-fold. First, to explore alternative ways to co-express the

119 scFv to HER2 for cancer retargeting and the GCN4 peptide for *in vitro* cultivation in the Vero-

120 GCN4R cells. Second, to define novel, less disadvantageous detargeting strategies, so as to

121 optimize the cultivation of retargeted oncolytic HSVs in the non-cancer cells.

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123

124 **RESULTS**

125 **Double gD retargeting and novel detargeting.** An aim of this work was to ascertain whether gD

126 can simultaneously accept two retargeting moieties, the GCN4 peptide and the scFv to HER2. To

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127    better accomplish this task, we reduced the size of the deletion in gD, so as to maintain the

128    detargeted phenotype, and preserve gD sequences, and possibly gD structure, as much as possible.

129    Our initial gD detargeted/retargeted viruses R-LM113 and R-LM249, which carry the deletion of aa

130    6-38, or 61-218 (17, 19), respectively, were designed at times when the regions of interaction

131    between gD and its receptors were known mainly through molecular biology approaches, and

132    through structural information on HVEM-bound gD (32). Indeed, the deletion of aa 38 in R-LM113

133    preceded the detailed knowledge of the nectin1 binding site in gD. Here, we took advantage of the

134    information on gD contact area with nectin1, inferred from the structure of gD bound to nectin1, as

135    determined by x-ray crystallography (42). According to the co-crystal structure, a tip in nectin1

136    protrudes into a groove in gD, whose critical residues include the previously known Y38 and the

137    adjacent residues, including H39, and residues 215 and 220-223. Those structural studies suggested

138    two alternative possibilities for nectin1 detargeting. One was the deletion of aa 35-39. The other

139    was the deletion of the region which includes aa 214-223 (42), not assayed before in detargeting

140    studies. Here we removed aa 214-223, or 219-223. The HVEM detargeting was achieved by the

141    simple insertion of the GCN4 peptide or of the scFv to HER2 between aa residues 24 and 25, which

142    are part of the HVEM binding site (32).

143        The list of double-insertion gD recombinants is reported in Table 1, which also summarizes

144    essential phenotypic features of the recombinants. The genome backbone is shown in Fig. 1 A. The

145    specific genotypes are shown in Fig. 1 B. The tropism was assayed in the HER2-positive cancer

146    cells SK-OV-3, in wt-Vero cells and in Vero-GCN4R, which express the artificial receptor to

147    GCN4 peptide (39) and in J cells derivatives. J cells express no receptor for HSV; derivatives

148    expressing a single receptor - HER2, nectin1, HVEM - were described (16, 43). R-LM113,

149    retargeted to HER2 but not to GCN4R, was included as control. The tropism of R-87, R-89, R-97,

150    R-99, R-99-2 is shown in Fig. 2, A-F. Cumulatively, the results show the following. (i) All

151    recombinants were detargeted from HVEM and from nectin1, since they failed to infect J-nectin1

152    and J-HVEM cells. (ii) All recombinants were retargeted to HER2, as inferred by the infection of J-

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153  HER2 and SK-OV-3 cells, and by inhibition of infection by trastuzumab, the MAb to HER2 from

154  which the scFv employed for retargeting was derived (44). This property is shared with R-LM113.

155  (iii) All recombinants, except R-89, infected wt-Vero cells. This infection was inhibited by

156  trastuzumab, hence most likely it occurred through the simian ortholog of hHER2. The genome

157  sequence of Vero cell is incomplete, and, so far, there is no documentation of a HER2 homologue.

158  However, Vero cells were isolated from an Africa Green Monkey (Chlorocebus sp.), and the

159  sequence of the Chlorocebus genome contains the HER2 homologue (Chlorocebus sabaeus;

160  REFSEQ: XM_008012845.1) with 98% identity with the human HER2 at the amino acid level. (iv)

161  All recombinants infected Vero-GCN4R cells. This infection was only in part decreased by

162  trastuzumab, indicating that it occurred in part through the GCN4 peptide present in the

163  recombinants, and its interaction with the GCN4R. (v) There was no difference in the recombinant

164  tropism whether the viruses were grown in Vero-GCN4R or in SK-OV-3 cells, as exemplified for

165  R-87 and R-99 (Fig. 2 G-H). Altogether, the results indicate that double retargeting through the

166  insertion of two different retargeting moieties in gD is feasible. All three nectin1-detargeting

167  strategies based on Δ35-39, Δ214-223, or Δ219-223 were effective. The detargeting through

168  deletion of the 214-223 or 219-223 regions were not attempted before.

169  **Comparative growth of double gD-retargeted recombinants.** We compared the yield of

170  the above recombinants to those of the wt HSV-1(F), the wt-gD recombinant named R-LM5, and

171  the singly HER2-retargeted R-LM113, in SK-OV-3 and in Vero-GCN4R cells. R-LM5 carries a wt-

172  gD, the BAC plus EGFP sequences, and is therefore the wt counterpart of the retargeted HSVs. A

173  representative experiment (Fig. 3, A, B) shows that at 48 h after infection the yield of the

174  recombinants R-87, R-97 and R-99-2 did not significantly differ one from the other, either in SK-

175  OV-3 or in Vero-GCN4R cells. We note that R-LM113 replicated for one passage in wt-Vero cells,

176  and its Vero-GCN4R derivative; however, numerous efforts to passage serially R-LM113 in these

177  cells were unsuccessful, and did not yield any progeny. The two recombinants with lower yields

178  were R-89 and R-99. R-87 and R-89, representative of the best performing and least well

179  performing recombinants, respectively, were further analyzed with respect to the extent of virus

180  release in the extracellular medium. Fig. 3 C, D shows that, for both viruses the extracellular virus

181  yield at 48 h was about 1 log lower than the intracellular virus yield, as was the case for R-LM113.

182      Next, we analyzed the ability of the recombinants to form plaques, with respect to plaque

183  size and plating efficiency. Fig. 4 A shows a typical plaque for each recombinant in Vero-GCN4R

184  and SK-OV-3 cells. The average plaque size of the recombinants is shown in Fig. 4 B. All

185  recombinants formed somewhat larger plaques in Vero-GCN4R than in SK-OV-3 cells. Also with

186  respect to the number of plaques, the efficiency was somewhat higher in the Vero-GCN4R than in

187  the SK-OV-3 (Fig 4 C). Altogether, these results show that the Vero-GCN4R cell line enables

188  efficient spread of the recombinants.

189      In the past, we observed that switching a virus from one cell line to a different cell line for

190  replication may sometime result in a lower replication rate at earliest passages after the switch.

191  Specifically, when a virus is grown in a certain cell line (e.g. Vero-GCN4R) and is then switched to

192  another cell line (e.g. SK-OV-3), there may be a decrease in the efficiency of virus growth at very

193  early passages. We analyzed whether the growth of R-87 and R-97 in Vero-GCN4R cells may

194  affect the extent of replication in the cancer SK-OV-3 cells. R-87 and R-97 were grown in Vero-

195  GCN4R (R-87$_{VG}$ and R-97$_{VG}$) or in SK-OV-3 cells (R-87$_{SK}$ and R-97$_{SK}$), and then employed to

196  infect SK-OV-3 cells. Fig. 5 shows that R-87$_{VG}$ grew as efficiently as R-87$_{SK}$ in SK-OV-3 cells.

197  Similarly, R-97$_{VG}$ grew as efficiently as R-97$_{SK}$ in SK-OV-3 cells. Thus, switching from Vero-

198  GCN4R to SK-OV-3 cells exerted no detrimental effect on the efficiency of viral growth.

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200      **Cell killing ability of double gD retargeted recombinants.** The above candidate oncolytic

201  recombinants were tested for ability to exert cytotoxic activity towards SK-OV-3 and Vero-GCN4R

202  cells. Fig. 6A shows all recombinants were cytotoxic for SK-OV-3 cells. The highest effect was

203  exhibited by the recombinants which replicated better. All the recombinants were cytotoxic also for

204  Vero-GCN4R cells (Fig. 6B). As expected, the exception was R-LM113 in Vero-GCN4R cells,

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205    since the virus infects these cells at low efficiency. As noted earlier, the HER2 retargeted viruses

206    infect Vero cells most likely through the simian HER2.

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208    **Oncolytic efficacy of a double gD retargeted recombinant in immunocompetent mice.**

209    We selected R-87, one of the best performing double gD retargeted recombinants, to evaluate the

210    oncolytic efficacy in immunocompetent mice. The animal model will be described elsewhere in

211    detail under different co-authorship (45). Essentially, it consists of the Lewis lung murine

212    carcinoma 1 (LLC-1) cells made transgenic for human HER2 (hHER2-LLC-1). The cancer cells

213    were implanted in a strain of the syngeneic C57BL6 mice, which are transgenic for, hence tolerant

214    to hHER2. Three days after implantation of the tumor cells, R-87 was administered intratumorally

215    (i.t.) at 3-4 days distance, with $1 \times 10^8$ PFU/injection, for a total of 4 treatments. As a comparison

216    we included in the experiment the prototypic R-LM113 and R-317 described in the accompanying

217    paper (46). Fig. 7 A-C shows that the antitumor efficacy of R-87 was very similar to those of R-

218    LM113 and of R-317, and the tumor size was significantly smaller than that in the untreated mice at

219    28 d (Fig. 7 D).

220

## DISCUSSION

221

222 Recently we developed a system for the cultivation in non-cancer cells of clinical grade oncolytic

223 HSVs retargeted to HER2, and, potentially, to any cancer-specific receptor of choice (39). The

224 potentially universal system is based on a double retargeting strategy. One retargeting is to the

225 cancer receptor, exemplified in our studies by HER2. The other retargeting is by way of the 20 aa

226 long GCN4 peptide, which readdresses the tropism to Vero cells expressing the artificial GCN4R.

227 Here, we asked whether a double retargeting *via* gD is feasible, and whether it can be optimized by

228 means of a less disadvantageous detargeting strategy, designed on the structural analysis of the gD-

229 nectin1 co-crystal (42). We report that gD simultaneously accepts two different heterologous

230 ligands for retargeting to two different receptors. The double retargeting can be combined with

231 novel nectin1-detargeting strategies, based on small deletions at two different loci in gD.

232 Analysis of the panel of gD recombinants shows that all of them were simultaneously

233 retargeted to the GCN4R and to the HER2, and detargeted from both HVEM and nectin1. A novel

234 finding to emerge from this investigation is that the modifications to the locus around aa 214-223 is

235 suitable for nectin1 detargeting, and retargeting. Each of the two heterologous receptors (HER2 and

236 GCN4R) can be used alternatively to the other, and independently of the other. The recombinants

237 switched readily from one cell system (GCN4R-positive cell) to the other (HER2-positive cell).

238 Not all the insertion sites were equivalent, and the combination of ligand to insertion site can

239 be optimized. This conclusion rests on the following examples.

240 Comparison of R-87 *versus* R-97. R-87 and R-97 share the following properties. They carry

241 the same deletion in gD (aa 35-39) for nectin1-detargeting. They carry one of the two inserts (the

242 260 aa long scFv to HER2 or the 20 aa long GCN4 peptide) between aa 24 and 25 for HVEM

243 detargeting. They differ in the relative position of the two inserts. Thus, in R-87 the gD deletion is

244 replaced by the scFv, whereas in R-97 the deletion is replaced by the GCN4 peptide. A comparison

245 of R-87 and R-97 shows that they grew to very similar yields. Hence, exchanging the short GCN4

246 peptide and the scFv at anyone of these two positions was irrelevant with respect to growth capacity.

247    Comparison of R-89 *versus* R-99. R-89 and R-99 share the following properties. They carry

248    the same deletion in gD (aa 214-223) for nectin1-detargeting. They carry one of the two inserts (the

249    scFv to HER2 or the GCN4 peptide) between aa 24 and 25 for HVEM detargeting. They differ in

250    the relative position of the two inserts. Thus, in R-89 the gD deletion is replaced by the scFv,

251    whereas in R-99 the deletion is replaced by the GCN4 peptide. The R-89 and R-99 recombinants

252    replicated in a similar manner, and there was no apparent effect of the relative position of the two

253    inserts. Of note, the yields of R-89 and R-99 were lower than those of R-87 and R-97. Hence, a 10

254    aa deletion at this locus does not enable a highly efficient replication.

255    A comparison of R-97 *versus* R-99-2 sheds light on the effects of performing the deletion in

256    the aa 35-39 locus *versus* the 219-223 locus. At 48 h these two recombinants replicated in a very

257    similar manner in both SK-OV-3 and Vero-GCN4R cells, although a difference was seen at 24 h.

258    Thus, a 5 aa deletion at anyone of these two loci results in very similar recombinants.

259    Comparison of R-99 *versus* R-99-2. These two recombinants differ in that R-99-2 carries a

260    smaller (5 aa) deletion than R-99 (10 aa deletion), and are otherwise identical. The important result

261    here is that R-99-2 grew one log more that R-99, suggesting that the size of the deletion may be

262    critical for a better preservation of gD functions. This may explain why R-87, R-97, and R-99-2,

263    which carry 5 aa long deletions replicated to similar yields. Of note, the differences in virus yield

264    were not fully recapitulated in the plaque size; the latter is influenced not only by virus replication

265    but also by ability to perform cell-to-cell spread.

266    The R-87 recombinant was selected to evaluate the antitumor efficacy *in vivo*, in an

267    immunocompetent mouse model. In general, the murine cancer cells are not as permissive to HSV

268    as the human cancer cells, hence this model underestimates the antitumor efficacy, a property

269    shared with the vast majority of murine cancer models for oncolytic HSVs (47, 48). Here, the

270    important result was that the anti-tumor efficacy of R-87 could not be differentiated from that of R-

271    LM113 and of the gB recombinant R-317, described in the accompanying paper (37). Thus, the

272    replication properties in cell cultures are recapitulated in the *in vivo* anti-tumor efficacy, and a

273  recombinant carrying two retargeting moieties in gD is not at disadvantage relative to R-LM113,

274  which carries a single retargeting moiety. Altogether, the double retargeting *via* gD was feasible.

275  By optimizing the detargeting strategies we generated double-retargeted gD recombinants which

276  replicated as efficiently as the singly retargeted R-LM113 or the non-detargeted R-LM5, and which

277  exerted anti-tumor activity *in vivo* as efficiently as R-LM113.

278       In an accompanying paper, we show that double retargeting is feasible also by insertion of

279  the GCN4 peptide in gB, and of the scFv in gD (46); even in that study (46), a novel gD detargeting

280  strategy was developed. In both studies, the comparison of a number of recombinants lead to

281  optimization of double retargeted recombinants. Together with the previous finding that the double

282  retargeting is achieved by insertion of the GCN4 peptide in gH, and of the scFv to HER2 in gD (39),

283  current data indicate that several alternative strategies have become possible, now that we have

284  enlarged the number of HSV glycoproteins that can serve as retargeting tools, and in the light of

285  accurate knowledge of gD-receptor structures (42). All in all the double gD recombinants and the

286  gB/gD simultaneous retargeting yielded recombinants which replicate at comparable yields and will

287  help move the field of retargeted oncolytic HSVs into the translational phase.

288

289     **MATERIALS AND METHODS**

290         **Viruses.** R-LM5 and R-LM113 were described (17) (see Table 1, for summary of genotypes

291     and tropism). R-LM5 carries wt-gD ORF, the bacterial artificial chromosome (BAC) sequences

292     cloned in the UL3-UL4 intergenic region, as in the parental pYeBac 102 (49) and the enhanced

293     green fluorescent protein (EGFP) ORF under the α27 promoter cloned within the BAC sequences

294     (17). It is not detargeted/retargeted, therefore is the wt-counterpart of R-LM113, R-87, R-89, R-97,

295     R-99, R-99-2. R-LM113 is identical to R-LM5, except that it carries a HER2-retargeted gD. In

296     particular the deletion of aa 6-38 of mature gD which removes critical residues for interaction with

297     HVEM and nectin1 and its replacement with the scFv to HER2 derived from trastuzumab (44),

298     detargets the virus tropism from the natural receptors. wt HSV-1 F was described (50).

299         **Engineering of R-87, R-89, R-97, R-99, R-99-2.**

300     To engineer the gD double retargeted recombinants we constructed two precursor BAC, BAC 81

301     and BAC 91, starting from LM55 BG BAC. BAC 81 carries GCN4 peptide between aa 24 and 25

302     of gD, whereas BAC 91 carries scFv HER2 in the same position. The HSV-1 recombinants R-87,

303     R-89 were derived from BAC 81 by insertion of the scFv HER2 in place of aa 35-39 (R-87), or in

304     place of aa 214-223 (R-89). The recombinants R-97, R-99, R-99-2 were derived from BAC 91 by

305     insertion of the GCN4 peptide in place of aa 35-39 (R-97), in place of aa 214-223 (R-99), or in

306     place of aa 219-223 (R-99-2). See Fig.1 B and Table 1. The aa sequence of the GCN4 peptide was

307     KNYHLENEVARLKKLG. The core YHLENEVARLKK residues represent the epitope

308     recognized by the single chain antibody C11L34-H6 (PDB # 1P4B) (40). In the recombinant

309     viruses, the GCN4 peptide was preceded and followed by GS linkers. The starting material for the

310     engineering of BAC 81 and BAC 91 was LM55 BG BAC which carries LOX-P-bracketed

311     pBeloBAC11 and eGFP sequences inserted between $U_L$3 and $U_L$4 of HSV-1 genome (17). The

312     engineering was performed in bacteria by means of galK recombineering, in two steps (38, 51). In

313     the first step, the galK cassette, with homology arms to gD, was inserted between aa 24 and 25 of

314     mature gD. In the second step, the galK insert was replaced with the GCN4 peptide cassette to

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315    generate the precursor BAC 81 or was replaced by the scFv HER2 cassette to generate the precursor

316    BAC 91.

317        To carry out the first step in the engineering of BAC 81, the galK cassette, with homology

318    arms to gD was amplified by means of primers gD24_galK_f and gD25_galK_r (Table 2), using p-

319    galK plasmid as template. The PCR-amplified galK cassette was then electroporated into SW102

320    bacteria, which carry the LM55 BG BAC, to generate BAC 80. To exclude galK false positive

321    colonies, the recombinant clones were plated on Mac Conkey agar base plates, supplemented with

322    1% galactose and 12 μg/ml chloramphenicol, and checked by colony PCR. Colony PCR was carried

323    out with primer galK_827_f and galK_1142_r (Table 2). To carry out the second step and generate

324    the precursor BAC 81, a cassette encoding the GCN4 peptide (GenBank accession number

325    AF416613.1) (40) bracketed by the downstream and upstream Gly-Ser linkers and by homology

326    arms to gD was generated, through annealing and extension of the partially overlapping

327    oligonucleotides gD24_GCN4_fB and gD25_GCN4_rB (Table 2). The oligonucleotides contained

328    a silent BamHI restriction site, for screening purposes. The amplimer encoding the GCN4 cassette,

329    with homology arms to gD, was electroporated into SW102 bacteria carrying BAC 80. The

330    recombinant BAC was named BAC 81. Positive bacterial clones were checked by means of BamHI

331    restriction analysis on colony PCR fragments, amplified with primers gD_ext_f and gD_ext_r

332    (Table 2).

333        The precursor BAC 91 carries the scFv to HER2 between aa 24 to 25 of gD. It was

334    generated from BAC 80. First, the scFv HER2 cassette bracketed by homology arms to gD was

335    amplified by means of primers gD24-scFvHer2-F and gD25-scFvHer2-R (Table 2). Bacterial

336    colonies were checked for the presence of sequence of choice by means of colony PCR with

337    primers gD_ext_f and scFv_456_r (Table 2).

338        To engineer R-87 and R-89, the scFv HER2 was inserted in gD Δ35-39 (R-87), or in gD

339    Δ214-223 (R-89), as detailed for BAC 81, by means of oligonucleotides reported in Table 2. To

14

340  engineer R-97, R-99, and R-99-2, the GCN4 peptide was inserted in gD Δ35-39 (R-97), gD Δ214-

341  223 (R-99), or gD Δ219-223 (R-99-2) of BAC 91, by means of oligonucleotides reported in Table 2.

342  To reconstitute the recombinant viruses, 500 ng of recombinant BAC DNA was transfected

343  into the Vero-GCN4R cell line and SK-OV-3 cell line by means of Lipofectamine 2000 (Life

344  Technologies), and then grown in these cells. Virus growth was monitored by green fluorescence.

345  The structure of the recombinant was verified by sequencing the entire gD. Virus stocks were

346  generated in Vero-GCN4R and SK-OV-3 and titrated in Vero-GCN4R and SK-OV-3 cells. All

347  other recombinants were engineered by the same procedure, by means of oligonucleotides described

348  in Table 2.

349  **Tropism of R-87, R-89, R-97, R-99, R-99-2.** The indicated cells were infected with the

350  indicated viruses at 1 PFU/cell, and monitored 24 h later with a Nikon Eclipse TS100 fluorescence

351  microscope. Where indicated, infection was carried out in the presence of MAb to HER2

352  (trastuzumab) at the concentration of 28 μg/ml.

353  **Determination of virus growth and extent of viral progeny release.** Vero-GCN4R and

354  SK-OV-3 cells were infected with wt HSV-1 F, R-LM5, R-LM113, R-87, R-89, R-97, R-99, R-99-

355  2 at 0.1 PFU/cell. Unabsorbed virus was inactivated by rinsing the cells with a pH 3 solution (40

356  mM citric acid, 10 mM KCl, 135 mM NaCl). Replicate cultures were frozen at 24 and 48 h after

357  infection. Progeny virus (intracellular plus extracellular) was titrated in SK-OV-3 cells. Results are

358  expressed as the mean of three independent experiments ± SD. In virus release experiments,

359  replicate cultures of Vero-GCN4R or SK-OV-3 infected with R-LM113, R-87 or R-89 at 0.1

360  PFU/cell were harvested 48 h after infection as cell lysates plus medium. Alternatively medium or

361  cellular fractions were harvested separately. Progeny virus was titrated in SK-OV-3 cells. Results

362  are expressed as the mean of three independent experiments ± SD.

363  **Plating efficiency and relative plaque size**. Replicate aliquots of R-LM5, R-LM113, R-87,

364  R-89, R-97, R-99, R-99-2 were plated on Vero-GCN4R and SK-OV-3 cells and the number of

15

365    plaques was counted 3 days later. Results represent the mean of three independent infections ± SD.

366    For plaque size determination, pictures of 6 individual plaques from each of the above samples

367    were taken 3 days after infection. Plaque areas were measured with Nis Elements-Imaging Software

368    (Nikon). Each result represents areas ± SD.

369    **Cytotoxicity assay.** SK-OV-3 and Vero-GCN4R cells were seeded in 96 well plates ($8 \times 10^3$

370    cell/well) and infected with wt HSV-1 F, R-LM5, R-LM113, R-87, R-89, R-97, R-99, R-99-2 (3

371    PFU/cell) or mock-infected. AlamarBlue (10 μl/well, Life Technologies) was added to the culture

372    media at indicated times after infection and incubated for 4 h at 37°C. Plates were read at 560 and

373    600 nm with GloMax Discover System (Promega Corporation). For each time point, cell viability

374    was expressed as the percentage of alamarBlue reduction in infected versus uninfected cells, after

375    subtraction of the background value (medium alone). Each point represents the average of at least

376    triplicate samples ± SD.

377    **In vivo anti-tumor efficacy.** C57BL6 mice transgenic for and tolerant to hHER2, received

378    from Jackson Laboratories, were implanted with the murine Lewis lung carcinoma 1 (LLC-1) cells

379    made transgenic for hHER2 (hHER2-LLC-1), $0.2 \times 10^6$ cells/mouse (45). Three days later mice

380    received R-87, R-LM113 and R-317 as control viruses, peri-intratumorally (i.t.), four

381    dosages/mouse at 3-4 days distance, $1 \times 10^8$ PFU/injection, 5 mice for each treatment group. Tumor

382    size was measured by means of a caliper at the indicated days as described (19). Animal

383    experiments were performed according to European directive 2010/63/UE, Italian laws 116/92 and

384    26/2014. Experimental protocols were reviewed and approved by the University of Bologna Animal

385    Care and Use Committee ("Comitato per il Benessere degli Animali, COBA"), and approved by the

386    Italian Ministry of Health, Authorization # 86/2017-PR to Prof. Anna Zaghini.

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**FIGURE LEGENDS**

**Fig. 1.** Genome arrangement of recombinants generated in this study. (A) Prototypic genome arrangement of recombinants. Each recombinant carries the BAC sequence and the α27-promoter driven EGFP (enhanced green fluorescence protein), bracketed by LoxP sites, cloned in the UL3 and UL4 intergenic region, the GCN4 peptide and the scFv to HER2 in appropriate sites of gD as detailed below. The Unique Long (UL) and Unique Short (US) portions of the genome, bracketed by terminal (TR) and internal repeats (IR), along with the location of gB and gH genes are shown. (B) Specific genotypic modifications in gD gene of each recombinant.

**Fig. 2.** Tropism of R-87, R-89, R-97, R-99, and R-99-2 recombinants, and, for comparison, of R-LM113 in the indicated cell lines. (A-F) The indicated cells were infected with R-87 (A), R-89 (B), R-97 (C), R-99 (D), R-99-2 (E) and for comparison, R-LM113 (F) at an MOI of 1 PFU/cell and monitored for EGFP expression by fluorescence microscopy 24 h post infection. J-cells express no receptor for wt HSV; J-HER2, J-nectin1, and J-HVEM express the indicated receptor. Infection was carried out in the absence of antibodies (no Ab), or in the presence of the humanized anti-HER2 monoclonal antibody trastuzumab at a concentration of 28 μg/ml. (G-H) Tropism of R-87 (G) and R-99 (H) recombinants grown in Vero-GCN4R cell. Cells were infected and monitored for EGFP expression as described above. The panels were adjusted by means of Adobe Photoshop software to match one to the other in the final gallery. The level, brightness and contrast of each panel were adjusted as follow R-87 (A) panels a,b,c,f +35 +75 +100, panels d,e,g,h,i,j,k +35 +25 +100; R-89 (B) panels a,b,e,f +35 +75 +100, panels c,g +35 +75 0, panels d,h,i,j,k +35 +25 +100; R-97 (C) panels a,b,e,f,g +35 +75 +100, panel c +35 +75 0, panels d,h +35 +25 0, panels i,j,k +35 +25 +100; R-99 (D) panels a,c,g +35 +75 0, panels b,e,f +35 +75 +100, panels d,h +35 +25 0, panels i,j,k +35 +25 +100; R-99-2 (E) panels a,b,e,f +35 +75 +100, panels c,g +35 +75 0, panels d,h,i,j,k +35 +25 +100; R-LM113 (F) panels a,b,e,f +35 +75 +100, panels c,g +35 +75 0, panels d,h,i,j,k +35 +25 +100. R-87$_{VG}$ (G) panels a,b +50 +75 +100, panel c +35 +100 +0, panels d,e,f,g +50 +25 +100,

22

584    panels h,i,j,k +50 0 +100; R-99$_{VG}$ (H) panels a,b,e,f +35 +75 +100, panel c +35 +75 0, panels

585    d,h,i,j,k +35 +25 +100, panel g +15 +75 0

586

587    **Fig. 3.** Yield of R-87, R-89, R-97, R-99, and R-99-2 recombinants, and of R-LM5, R-LM113 and

588    wt-HSV-1(F), for comparison. (A, B) SK-OV-3 (A) and Vero-GCN4R (B) cells were infected with

589    the indicated viruses at 0.1 PFU/cell. Progeny virus collected at 24 or 48 h after infection was

590    titrated in SK-OV-3 cells. Results represent the average of triplicates, ± SD. (C, D) Production of

591    intracellular and extracellular R-87, R-89, and R-LM113 in SK-OV-3 (C) and in Vero-GCN4R (D)

592    cells. Replicate cultures were infected as above. At 48 h after infection, media (extra) and cells

593    (intra) were harvested separately, or together (intra + extra). Progeny virus was titrated in SK-OV-3

594    cells. Results represent the average of triplicates, ± SD.

595

596    **Fig. 4.** Plating efficiency of the indicated recombinants in Vero-GCN4R and SK-OV-3 cells. (A) A

597    typical plaque is shown for each virus in the indicated cells. (B) Average plaque size of the

598    indicated recombinants in Vero-GCN4R and SK-OV-3 cells. Six pictures were taken for each

599    recombinants. Plaque areas were measured with Nis Elements-Imaging software (Nikon). (C)

600    Replicate aliquots of viruses were plated in SK-OV-3 and Vero-GCN4R cells. Plaques were scored

601    three days later. The relative number of plaques formed by each virus in the indicated cell line is

602    reported as percentage of the number of plaques formed in SK-OV-3 cells. Results represent the

603    average of triplicates, ± SD. The panels were adjusted by means of Adobe Photoshop software to

604    match one to the other in the final gallery. The level, brightness and contrast of each panel were

605    adjusted as follow. Panel a +30 +50 +100, panels b,h,n +20 +50 +100, panels c,d,k,l +50 +25 +50,

606    panels e,f +35 +20 +100, panel g +35 +50 +100, panels i,j +35 +25 +100, panel m +30 +75 +100.

607

608    **Fig. 5.** Comparative yield of R-87 and R-97 pre-cultivated in SK-OV-3 or Vero-GCN4R cells. R-87

609    and R-97 were cultivated in SK-OV-3 (R-87$_{SK}$, R-97$_{SK}$) or in Vero-GCN4R (R-87$_{VG}$, R-97$_{VG}$) cells

23

610    and employed to infect SK-OV-3 cells at 0.1 PFU/cell. Progeny virus harvested at 24 or 48 h after

611    infection was titrated in SK.OV-3 cells. Results represent the average of triplicates, ± SD.

612

613    **Fig. 6.** Cell killing ability of the indicated viruses for SK-OV-3 and Vero-GCN4R cells. (A, B) SK-

614    OV-3 (A) or Vero-GCN4R (B) cells were infected with the indicated recombinants, or with HSV-

615    1(F), R-LM5, or R-LM113 as controls, at 3 PFU/cell. Cell viability was quantified by alamarBlue

616    assay at the indicated days after infection. Results represent a typical experiment; each sample is the

617    average of triplicate assay ± SD.

618

619    **Fig. 7.** Antitumor activity of R-87. A-C. Groups of 5 mice from the hHER2-transgenic C57BL6

620    strain were implanted with hHER2-LLC-1 cells ($0.2 \times 10^6$ cell) in the left flank. Starting 3 d later,

621    mice received four intratumoral treatments with the indicated viruses, at 3-4 d distance, $1 \times 10^8$

622    PFU/treatment.. Tumor volumes for each treatment group are shown. D. Distribution of the tumor

623    size at 28 d after the initial treatment. This experiment is the same as that shown in Fig. 6 of the

624    accompanying paper (37).

625

626    Table 1. Summary of genotypes and major phenotypic properties of the listed recombinants

| Recombinant HSV-1 | GCN4 position in gD | scFv-HER2 position in gD | Retargeting to HER2 | Detargeting from nectin1/HVEM | Ref |
|---|---|---|---|---|---|
| R-87 | 24-25 | Δ35-39 | + | + | This paper |
| R-89 | 24-25 | Δ214-223 | + | + | This paper |
| R-97 | Δ35-39 | 24-25 | + | + | This paper |
| R-99 | Δ214-223 | 24-25 | + | + | This paper |
| R-99-2 | Δ219-223 | 24-25 | + | + | This paper |
| R-LM113 | none | Δ6-38 | + | + | (17) |
| R-LM5 | none | no scFv, no deletion | - | - | (17) |

627

628    Table 2. Oligonucleotides employed to engineer the indicated recombinants.

| BAC-81 | GCN4 peptide cassette inserted between aa 24 and 25 of mature gD of LM55 BAC |
|---|---|
| 1) galK insertion in gD 24-25 | Forward: gD24_galK_f CTC TCA AGA TGG CCG ACC CCA ATC GCT TTC GCG GCA AAG ACC TTC GGT CCC TG TTG ACA ATT AAT CAT CGG CA<br>Reverse: gD25_galK_r TGG ATG TGG TAC ACG CGC CGG ACC CCC GGA GGG TCG GTC AGC TGG TCC AGT CAG CAC TGT CCT GCT CCT T |
| 2) colony PCR for screening | Forward: galK_827_f GCG TGA TGT CAC CAT TGA AG<br>Reverse: galK_1142_r TAT TGT TCA GCG ACA GCT TG |
| 3) GCN4 cassette insertion in place of galK | Forward: gD24_GCN4_fB CTC TCA AGA TGG CCG ACC CCA ATC GCT TTC GCG GCA AAG ACC TTC GGT CG GAT CCA AGA ACT ACC ACC TGG AGA ACG AGG TGG CCA GAC TGA AGA AGC TGG TGG GCA GC<br>Reverse: gD25_GCN4_rB TGG ATG TGG TAC ACG CGC CGG ACC CCC GGA GGG TCG GTC AGC TGG TCC AGG CTG CCC ACC AGC TTC TTC AGT CTG GCC ACC TCG TTC TCC AGG TGG TAG TTC TTG GAT CC |
| 4) colony PCR for screening | Forward: gD_ext_f TCC ATA CCG ACC ACA CCG ACG AAT CCC<br>Reverse: gD_ext_r GAG TTT GAT ACC AGA CTG ACC GTG |
| **R-87** | **scFv HER2 inserted in gD Δ35-39 of BAC 81** |
| 1) galK insertion in gD Δ35-39 | Forward: galK_gD35_F TGA AGA AGC TGG TGG GCA GCC TGG ACC AGC TGA CCG ACC CTC CGG GGG TCC CTG TTG ACA ATT AAT CAT CGG CA<br>Reverse: galK_gD39_R GTG ATC GGG AGG CTG GGG GGC TGG AAC GGG TCT GGT AGG CCC GCC TGG ATT CAG CAC TGT CCT GCT CCT T |
| 2) scFv HER2 insertion in place of galK | Forward: gD-34-scFvHER2-F TGA AGA AGC TGG TGG GCA GCC TGG ACC AGC TGA CCG ACC CTC CGG GGG TCG AGA ATT CCG ATA TCC AGA T<br>Reverse: gD-40-scFvHER2-R GTG ATC GGG AGG CTG GGG GGC TGG AAC GGG TCT GGT AGG CCC GCC TGG ATG GAT CCA CCG AAC CAG AGC |
| 3) colony PCR for screening | Forward: gD_ext_f TCC ATA CCG ACC ACA CCG ACG AAT CCC<br>Reverse: scFv_456_r AGC TGC ACA GGA CAA ACG AGT GA GCC CCC |
| **R-89** | **scFv HER2 inserted in gD Δ214-223 of BAC 81** |

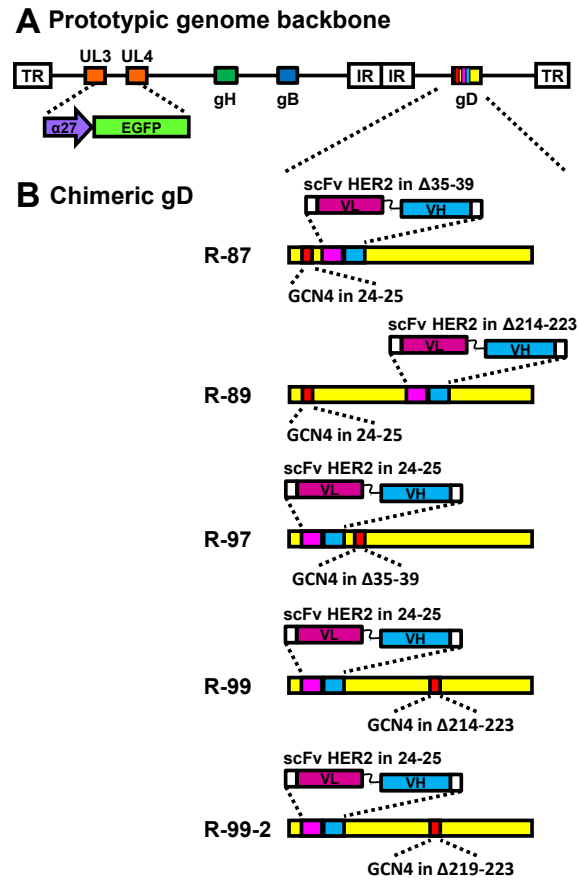| | |
|---|---|
| 1) galK insertion in gD Δ214-223 | Forward: galK_gD214_F CCT ACC AGC AGG GGG TGA CGG TGG ACA GCA TCG GGA TGC TGC CCC GCT TCC CTG TTG ACA ATT AAT CAT CGG CA<br>Reverse: galK_gD223_R CTC GTG TAT GGG GCC TTG GGC CCG TGC CAC CCG GCG ATC TTC AAG CTG TAT CAG CAC TGT CCT GCT CCT T |
| 2) scFv HER2 insertion in place of galK | Forward: gD213-scFvHER2f CCT ACC AGC AGG GGG TGA CGG TGG ACA GCA TCG GGA TGC TGC CCC GCT TCG AGA ATT CCG ATA TCC AGA T<br>Reverse: gD224-scFvHER2r CTC GTG TAT GGG GCC TTG GGC CCG TGC CAC CCG GCG ATC TTC AAG CTG TAG GAT CCA CCG GAA CCA GAG C |
| 3) colony PCR for screening | Forward: gDintforw CCC TAC AAC CTG ACC ATC GCT GG<br>Reverse: scFv_456_r AGC TGC ACA GGA CAA ACG GAG TGA GCC CCC |
| **BAC 91** | **scFv HER2 cassette inserted between aa 24 and 25 of mature gD of LM55 BAC** |
| 1) scFv HER2 insertion in place of galK | Forward: gD24-scFvHer2-F CTC TCA AGA TGG CCG ACC CCA ATC GCT TTC GCG GCA AAG ACC TTC GGT CGA GAT TCC GAT ATC CAG ATG<br>Reverse: gD25-scFvHer2-R TGG ATG TGG TAC ACG CGC CGG ACC CCG GAG GGT CGG TCA GCT GGT CCA GGA TCC ACC GGA ACC AGA G C |
| 2) Colony PCR for screening | Forward: gD_ext_f TCC ATA CCG ACC ACA CCG ACG AAT CCC<br>Reverse: scFv_456_r AGC TGC ACA GGA CAA ACG GAG TGA GCC CCC |
| **R-97** | **GCN4 inserted in gD Δ35-39 of BAC 91** |
| 1) galK insertion in gD Δ35-39 | Forward: gD35-galK-F GCT CTG GTT CCG GTg GaT CCC TGG ACC AGC TGA CCG ACC CTC CGG GGG TCC CTG TTG ACA ATT AAT CAT CGG CA<br>Reverse: gD39-galK-R GTG ATC GGG AGG CTG GGG GGC TGG AAC GGG TCT GGT AGG CCC GCC TGG ATT CAG CAC TGT CCT GCT CCT T |
| 2) GCN4 insertion in place of galK | Forward: gD35-GCN4-F GCT CTG GTT CCG GTg GaT CCC TGG ACC AGC TGA CCG ACC CTC CGG GGG TCG GAT CCA AGA ACT ACC ACC TGG AGA ACG AGG TGG CCA GAC TGA AGA AGC TGG TGG GCA GC<br>Reverse: gD39-GCN4-R GTG ATC GGG AGG CTG GGG GGC TGG AAC GGG TCT GGT AGG CCC GCC TGG ATG CTG CCC ACC AGC TTC TTC AGT CTG GCC ACC TCG TTC TCC AGG TGG TAG TTC TTG GAT CC |
| 3) colony PCR for screening | Forward: scFv4D5 651_f GGA CAC TGC CGT CTA TTA TTG TAG CCG CT<br>Reverse: gDintrev CCA GTC GTT TAT CTT CAC GAG CCG |
| **R-99** | **GCN4 inserted in gD Δ214-223 of BAC 91** |
| 1) galK insertion in gD Δ214-223 | Forward: galK_gD214_F CCT ACC AGC AGG GGG TGA CGG TGG ACA GCA TCG GGA TGC TGC CCC GCT TCC CTG TTG ACA ATT AAT CAT CGG CA<br>Reverse: galK_gD223_R CTC GTG TAT GGG GCC TTG GGC CCG TGC CAC CCG GCG ATC TTC AAG CTG TAT CAG CAC TGT CCT GCT CCT T |
| 2) GCN4 insertion in place of galK | Forward: gD213-GCN4-F CCT ACC AGC AGG GGG TGA CGG TGG ACA GCA TCG GGA TGC TGC CCC GCT TCG GAT CCA AGA ACT ACC ACC TGG AGA ACG AGG TGG CCA GAC TGA AGA AGC TGG TGG GCA GC<br>Reverse: gD224-GCN4-R CTC GTG TAT GGG GCC TTG GGC CCG TGC CAC CCG GCG ATC TTC AAG CTG TAG CTG CCC ACC AGC TTC TTC AGT CTG GCC ACC TCG TTC TCC AGG TGG TAG TTC TTG GAT CC |
| 3) Colony PCR for | Forward: gDintforw CCC TAC AAC CTG ACC ATC GCT GG<br>Reverse: HSV_139688_r CCG ACT TAT CGA CTG TCC ACC TTT CCC |

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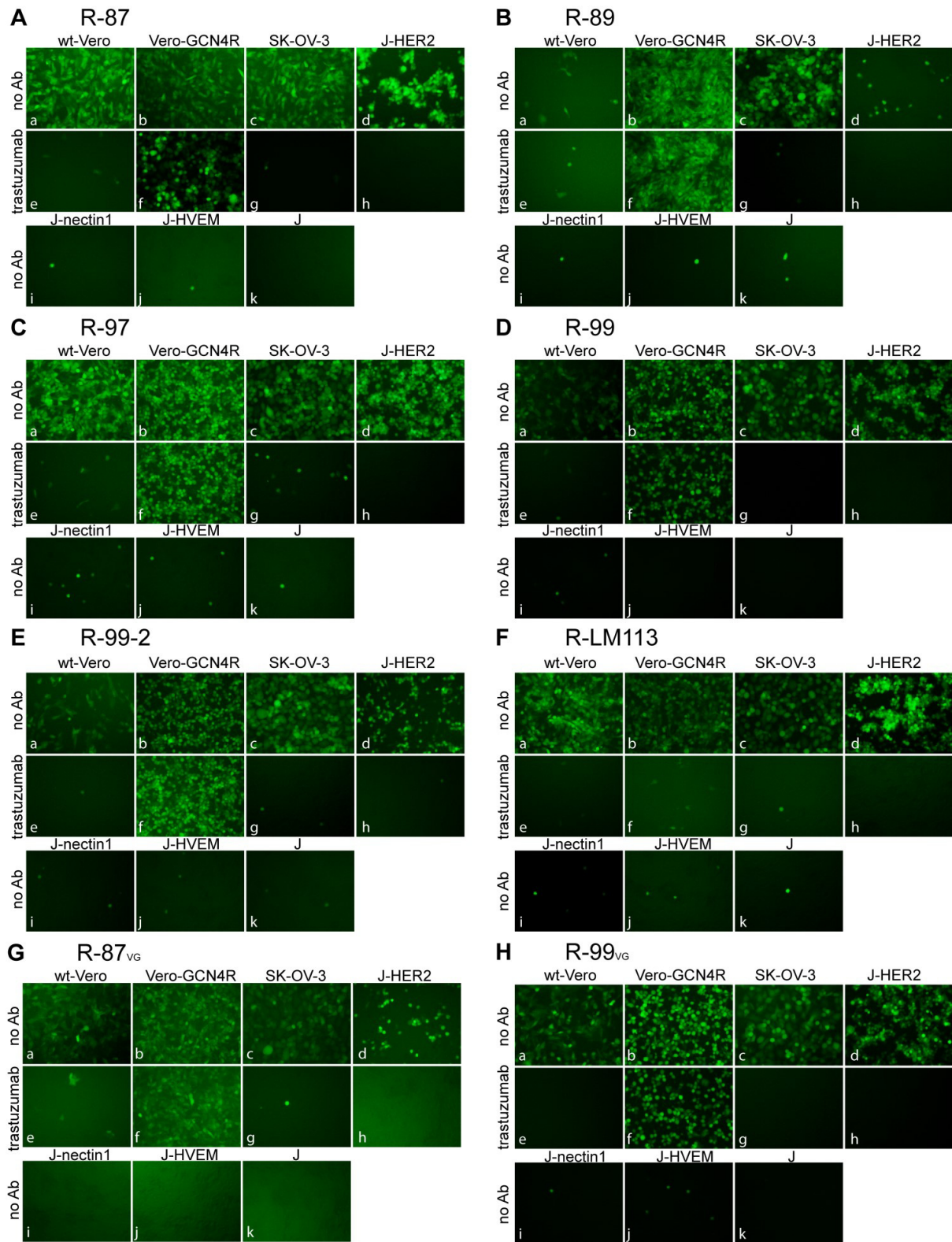| screening | |
|---|---|
| **R-99-2** | **GCN4 inserted in gD Δ219-223 of BAC 91** |
| 1) galK insertion in gD Δ214-223 | Forward: galK_gD214_F CCT ACC AGC AGG GGG TGA CGG TGG ACA GCA TCG GGA TGC TGC CCC GCT TCC CTG TTG ACA ATT AAT CAT CGG CA<br>Reverse: galK_gD223_R CTC GTG TAT GGG GCC TTG GGC CCG TGC CAC CCG GCG ATC TTC AAG CTG TAT CAG CAC TGT CCT GCT CCT T |
| 2) GCN4 insertion in place of galK | Forward: gD219-GCN4-F CCT ACC AGC AGG GGG TGA CGG TGG ACA GCA TCG GGA TGC TGC CCC GCT TCA TCC CCG AGA ACC AGC GCG GAT CCA AGA ACT ACC ACC TGG AGA ACG AGG TGG CCA GAC TGA AGA AGC TGG<br>Reverse: gD224-GCN4-R CTC GTG TAT GGG GCC TTG GGC CCG TGC CAC CCG GCG ATC TTC AAG CTG TAG CTG CCC ACC AGC TTC TTC AGT CTG GCC ACC TCG TTC TCC AGG TGG TAG TTC TTG GAT CC |
| 3) colony PCR for screening | Forward: gDintforw CCC TAC AAC CTG ACC ATC GCT TGG<br>Reverse: HSV_139688_r CCG ACT TAT CGA CTG TCC ACC TTT CCC |

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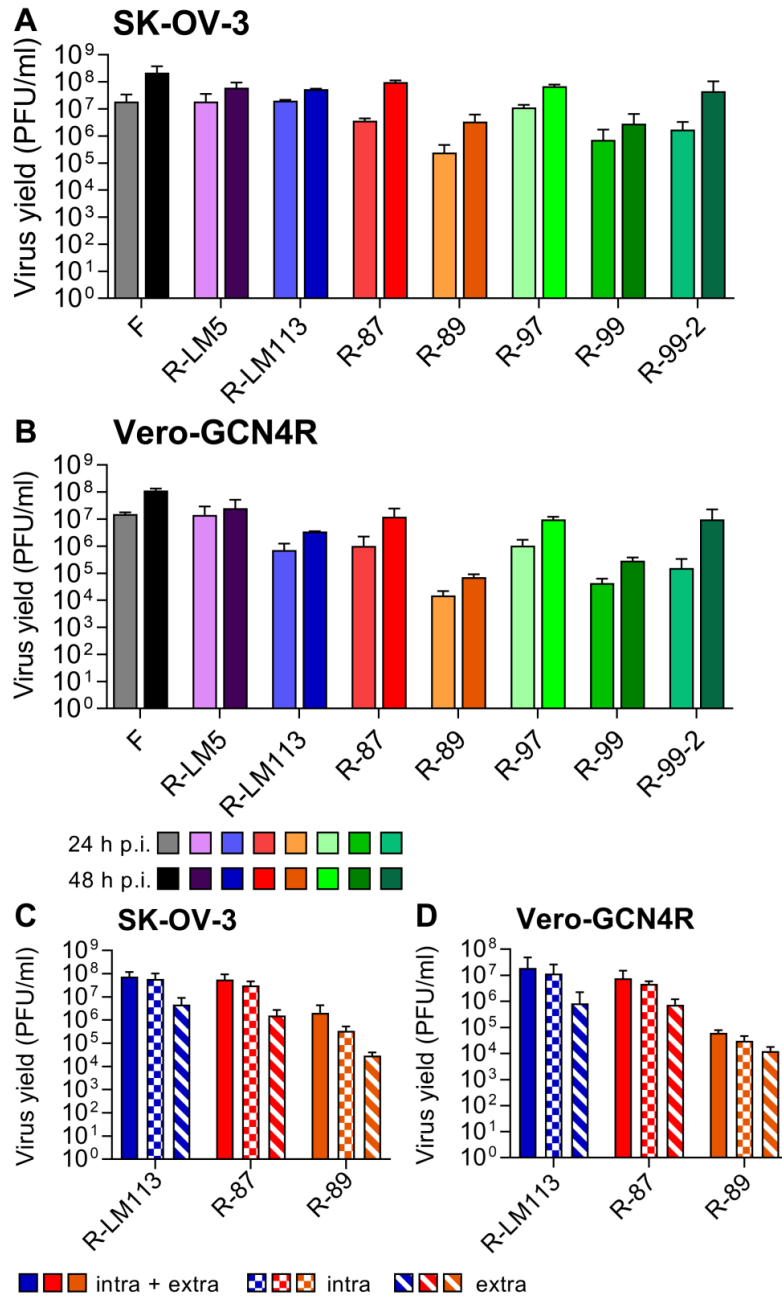**A** Prototypic genome backbone

**B** Chimeric gD

scFv HER2 in Δ35-39

R-87

GCN4 in 24-25

scFv HER2 in Δ214-223

R-89

GCN4 in 24-25

scFv HER2 in 24-25

R-97

GCN4 in Δ35-39

scFv HER2 in 24-25

R-99

GCN4 in Δ214-223

scFv HER2 in 24-25

R-99-2

GCN4 in Δ219-223

**Figure 1**

**Figure 2**

**Figure 3**
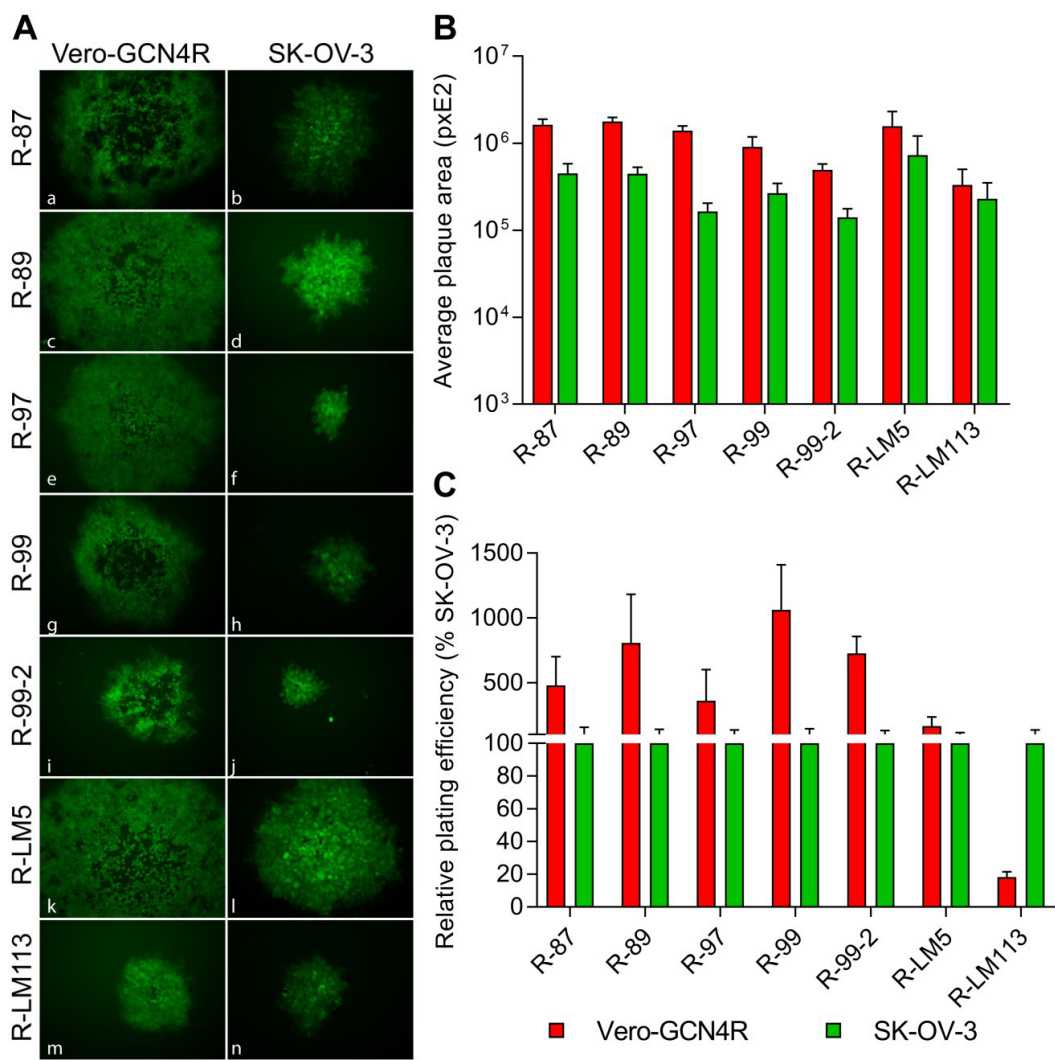
**Figure 4**
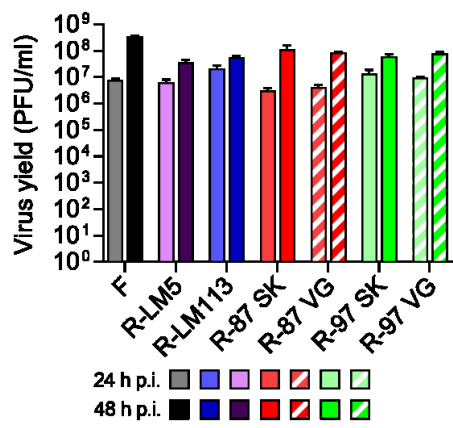
**Figure 5**

**Figure 6**

**Figure 7**