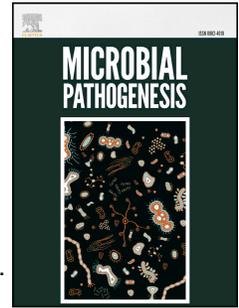


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Altered expression of cytokines in mice infected intranasally with two syncytial variants of Herpes simplex virus type 1

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1
2 **Altered expression of cytokines in mice infected intranasally with two syncytial**
3 **variants of Herpes simplex virus type 1**

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7 **Keywords:** herpes, virulence, cytokines, pathology, mouse

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30 **Abstract**

31

32 Immune evasion strategies are important for the onset and the maintenance of viral
33 infections. Many viruses have evolved mechanisms to counteract or suppress the host
34 immune response. We have previously characterized two syncytial (syn) variants of Herpes
35 simplex 1 (HSV-1) strain F, syn14-1 and syn17-2, obtained by selective pressure with a
36 natural carrageenan. These variants showed a differential pathology in vaginal and
37 respiratory mucosa infection in comparison with parental strain. In this paper we evaluated
38 the modulation of immune response in respiratory mucosa by these HSV-1 variants. We
39 observed altered levels of Tumor Necrosis Factor- α and Interleukin-6 in lungs of animals
40 infected with the syn14-1 and syn17-2 variants compared with the parental strain. Also, we
41 detected differences in the recruitment of immune cells to the lung in syn variants infected
42 mice. Both variants exhibit one point mutation in the sequence of the gene of glycoprotein
43 D detected in the ectodomain of syn14-1 and the cytoplasmic tail of syn17-2. Results
44 obtained in the present study contribute to the characterization of HSV-1 syn variants and
45 the participation of the cellular inflammatory response in viral pathogenesis.

46

47

48 **Main text**

49 Herpes simplex virus type 1 (HSV-1) is a human pathogen that infects and replicates in
50 epithelial cells of mucosal surfaces with the eventual establishment of latency in the ganglia
51 of sensory neurons. Important roles in limiting spread and early virus replication have been
52 ascribed to macrophages and natural killer cells (NK), which constitute the first line of
53 innate defense [1-3]. As expected, HSV-1 has evolved several strategies to counteract the
54 antiviral response by preventing the infected host to trigger a strong immune response and
55 thus facilitating viral replication. Furthermore, it has been previously reported that HSV is
56 able to suppress expression of proinflammatory cytokines by decreasing the stability of
57 mRNAs [4]. Secretion of proinflammatory cytokines is essential in the first line defense
58 against viruses [5, 6]. One of the macrophages-derived products that contribute to inhibit
59 HSV replication is the proinflammatory cytokine TNF- α [7] that plays an important role on
60 the early innate immune response. Furthermore, macrophages and other cell types rapidly
61 produce IL-6 at local tissue sites after HSV-1 infection [8]. IL-6 is a cytokine with
62 pleiotropic activities, including both proinflammatory and anti-inflammatory effects, that
63 has been characterized to contribute to immune response to HSV-1. At the same time, anti-
64 inflammatory cytokines are immunoregulatory molecules that control the proinflammatory
65 cytokine response to impair an excessive response [9, 10].

66 Natural carrageenans are known to be potent and selective inhibitors of HSV-1 and HSV-2,
67 affecting mainly the viral adsorption step [11]. As a result of a previous work we performed
68 we found that the selective pressure with the μ /v- carrageenan 1C3 allowed the isolation of
69 two syncytial (syn) variants of HSV-1 F strain, syn14-1 and syn17-2 which showed an
70 altered pathology in vaginal and respiratory mucosa infection [12]. In fact, intranasal
71 infection of BALB/c mice with syn variants induced 100% mortality at 7 days post-
72 infection (p.i.) and a differential infiltration of leukocytes was observed by
73 histopathological studies from lung mucosa.

74 Although HSV-1 is not a common respiratory virus in human, it can cause several
75 pathological conditions associated with the respiratory tract. In fact, herpetic respiratory
76 infections have been reported not only in neonates and immunosuppressed patients [13, 14],
77 but also in immunocompetent ones [15, 16]. Moreover, HSV-1 is able to penetrate the

78 basement membrane of human nasal respiratory mucosa and to replicate both in epithelium
79 and the underlying lamina propria of this tissue [17].

80 Based on these data, in the present work we wanted to deepen the study of the intranasal
81 infection of mice with variants syn14-1 and syn17-2 towards a better understanding of the
82 participation of the cellular inflammatory response in the pathogenicity of these viruses.
83 Taken into account the relevance of a rapid secretion of cytokines to counteract viral
84 infections, we focused on the analysis of cytokine modulation in lungs of infected mice. To
85 this end, BALB/c mice were infected with 10^6 PFU of syn14-1, syn17-2 or HSV-1 F strain,
86 as a control. At day 1 and 3 p.i., bronchioalveolar lavages (BAL) were performed using 1
87 ml of sterile saline solution. TNF- α , IL-6 and IL-10 levels were studied by ELISA
88 according to manufacturer's instructions (BD Biosciences) and virus yield was quantified
89 by plaque assay. Animals were maintained and handled in accordance with national and
90 international laws and policies from National Institutes of Health Guidelines and
91 regulations for care and use of test animals from Facultad de Ciencias Exactas y Naturales
92 (Buenos Aires, Argentina, CD 140/00).

93 Results shown in Fig. 1a (white bars) demonstrate that reduced levels of TNF- α could be
94 detected at day 1 p.i. for syn14-1 and syn 17-2. This low values were similar to those
95 observed in uninfected animals (1023,48 pg/ml). In contrast , at day 3 p.i. the level of
96 TNF- α for both syn variants was increased, while the parental strain showed a diminution
97 on TNF- α production (Figure 1a, grey bars). When IL-6 levels were analyzed, we found
98 that day 1 p.i. syn14-1 values showed an increase in IL-6 production while syn17-2 showed
99 a marked reduction in comparison with HSV-1 F, resembling the value of negative control
100 (28,31 pg/ml) (Figure 1b, white bars). However, at day 3 p.i. no significant differences
101 were observed for IL-6 levels in infected mice either with HSV-1 F or syn variants (Figure
102 1b, grey bars). The differential levels in cytokine production observed for syn variants
103 could be due to an over-expression of inhibitory cytokines. To test this possibility, our next
104 approach was to evaluate the production of IL-10, one of the major antiinflammatory
105 cytokines. As shown in Figure 1c, levels of IL-10 for syn14-1 and syn17-2 were lower at 1
106 day p.i. (white bars) similar of control without infected (1157,53 pg/ml), and increased at 3
107 days p.i. (grey bars) in comparison with parental virus. According to these results, the
108 pattern of IL-10 production observed for HSV-1 F and both syn variants was coincident

109 with the course of induction of TNF- α , suggesting that reduction of TNF- α observed 1 day
110 p.i was not due to an overproduction of IL-10. The proinflammatory cytokine IL-6 would
111 be also regulated by IL-10 [9], however the induction of this cytokine inhibitor would not
112 seem to explain completely the level of IL-6 observed at least for syn 14-1 variant.

113 With the aim to evaluate the correlation of cytokines pattern with virus replication, virus
114 titer was quantified in lung of infected mice. As can be seen in Figure 1d, at day 1 p.i., viral
115 titers for syn14-1 and syn17-2 were 10 and 113-fold higher, respectively, than those
116 registered for mice infected with HSV-1 F. At day 3 p.i., virus titers for syn variants as well
117 as for the parental strain were negligible.

118 These results suggest that intranasally inoculated syn variants would be able to modulate
119 differentially the immune response in association with a higher replication in lungs of
120 infected mice. At the same time, this modulation could explain the high mortality
121 previously described for mice intranasally infected with syn14-1 and syn17-2 [12].
122 Therefore, it may be speculated that the increased replication of syn variants might
123 contribute to their pathogenic phenotypes.

124 Considering that alveolar macrophages constitute the main cell population present in the
125 BAL fluid, we decided to analyze whether infection with HSV-1 F and syn variants
126 affected cytokine release in an *in vitro* model employing the murine macrophagic cell line
127 RAW 264.7. For this purpose, cells were infected with HSV-1 F, syn14-1 and syn17-2 and
128 at different times p.i., supernatants and cells monolayers were harvested and TNF- α and IL-
129 6 were determined by ELISA and RT-PCR, respectively. Cells stimulated with LPS
130 (1.5 μ g/ml) were used as positive control. For PCR analysis, cell monolayers were lysed in
131 Trizol (Invitrogen) and total RNA was isolated according to manufacturer's instructions.
132 cDNA was amplified with an initial incubation at 94°C during 10 min followed by 35
133 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C and a final incubation of 10 min
134 at 72°C. β -actin was used as an internal control. As can be seen in Fig. 2a, the levels of the
135 proinflammatory cytokines TNF- α and IL-6 were markedly reduced in cells infected with
136 syn14-1 and syn17-2, in contrast to those observed for HSV-1 F. In fact, the concentration
137 of both cytokines diminished by 84 to 100 % for the syn variants with respect to HSV-1 F
138 for every tested time. Furthermore, results obtained by RT-PCR correlated with those
139 obtained by ELISA since low levels of IL-6 and TNF- α mRNAs were also detected for the

140 syn variants (Fig. 2b) indicating that the transcription of these cytokines genes would not be
141 affected. Similar results were obtained using intraperitoneal macrophages harvested from
142 BALB/c mice (data not shown). The lower levels of cytokines observed by day 1 p.i. in
143 lung of mice infected intranasally with the syn 17-2 correlated with the results obtained
144 with macrophages *in vitro* whereas those observed for syn 14-1 did not. These results
145 suggest that the interaction between syn 14-1 and macrophages would not be the only factor
146 responsible for the up regulation of IL-6 as seen *in vivo*.

147 Taken into account that syn variants induced an altered profile of cytokines and that
148 glycoprotein D (gD) has been pointed out as an inducer of TNF- α [7, 18] and IL-6 [19]
149 during HSV-1 infection the sequence of this glycoprotein was analyzed. Results showed
150 that both syn variants presented a point mutation in gD in comparison to parental virus. For
151 syn14-1, the point mutation G889A induces a change D272N in the ectodomain of the
152 mature form gD. In the case of syn17-2, the point mutation G1119T induces a change
153 K348N in the cytoplasmic tail of the mature glycoprotein. Both mutations have not been
154 previously reported and would not be present within the four functional regions of gD
155 previously described [20].

156 As mentioned above, NK cells activity is also crucial for innate defenses. It has been
157 demonstrated that NK cells are recruited to the airways early after HSV-1 infection
158 restricting the early virus replication in the lung [21]. Moreover, Nandakumar *et al.*,
159 reported that NK cells activation by the virus contribute to the initial reduction in viral load
160 enhancing the stimulatory ability of the dendritic cells by enabling effective antigen
161 processing and presentation [22]. In order to study the proportion of NK cells and
162 monocytes in lung tissue of mice infected with syn variants, eight-week-old BALB/c mice
163 were infected intranasally with 10^6 PFU of HSV-1 F strain, syn14-1 or syn17-2 and lungs
164 were dissected for flow cytometric analysis. The marker NK 1.1 was used to detect NK
165 cells (APC Mouse Anti-Mouse NK-1.1, APC Mouse IgG2a κ , Isotype control); however,
166 the antigen is also a marker for specialized population of T lymphocytes (NK-T cells); and
167 CD11b was used to detect monocytes (FITC RAT Anti-Mouse CD11b, FITC Rat IgG2b, κ
168 Isotype Control) (BD Pharmingen). As can be seen in Fig. 3 (white bars) no differences
169 were observed in the percentage of monocytes present in lungs of mice infected with HSV-
170 1 F and syn variants, either at 3 or 5 days p.i. The number of NK cells at 3 days p.i. was

171 similar for the three viruses (Fig. 3, grey bars), however, at 5 days p.i lower percentages of
172 NK cells were observed for both HSV-1 F and syn17-2. Syn14-1 showed a significant
173 increase in NK cells in comparison with mock infected mice (Fig. 3, grey bars). In order to
174 address activation state of infiltrated cells, a combination of anti-NK 1.1 and anti-CD28
175 (PE Hamster Anti-mouse CD28, PE Hamster IgG2, λ 1 Isotype Control, BD Pharmingen)
176 antibodies were used to identify double positive cells as activated NK cells (NK+). As
177 shown in Table 1, lungs of mice infected with HSV-1 F showed a similar percentage of
178 NK+ cells at 3 and 5 days p.i. In contrast, more than 90% of NK were activated in lungs
179 infected with syn14-1 both at 3 days p.i. and 5 days p.i. (near 65%). Finally, in lungs of
180 mice infected with syn17-2 similar results to HSV-1 F were obtained at day 3 p.i. (71.9%),
181 while at 5 days p.i. the percentage decreased. These results are in accordance with previous
182 histopathological studies, in which infiltration of leukocytes was detected at day 5 p.i. in
183 lungs of mice infected with syn14-1 [12]. On the contrary, for syn17-2 the lower levels of
184 activated NK cells could be associated with the thickening of alveolar walls and the loss of
185 morphology as previously reported [12]. Reading *et al.* demonstrated that cytotoxicity of
186 lung NK cells is influenced by both NK number and their activation state [21]. Therefore,
187 the severe effect observed in infections carried out with syn17-2 might be associated to the
188 reduced levels of activated NK.

189 To summarize, the results presented in this paper suggest that syn variants have developed
190 a strategy to delay the activation of macrophages and hence the release of proinflammatory
191 cytokines. Thus, syn variants of HSV-1 could replicate and generate disease. In agreement
192 with this hypothesis, the lower levels of mRNAs cytokines observed in *in vitro*
193 experiments, would explain the early reduction of proinflammatory cytokines obtained for
194 syn variants. Mogensen *et al.* reported that HSV-1 down regulates the production of several
195 proinflammatory cytokines in a number of different cell types by mediating instability of
196 proinflammatory cytokine mRNAs [4]. In this way, although alterations of gD in syn
197 variants were detected in non-functional regions, it cannot be discarded that these
198 modifications could be responsible, at least in part, for the altered proinflammatory cytokines
199 pattern observed for syn variants. Nevertheless, it is important to note that the two variants
200 proved to be avirulent when inoculated by intrvaginal route whereas both killed all

201 intranasally inoculated animals [12]. Therefore, the immune response at genital mucosa
202 might be triggered in a different way comparing to the airway mucosa.

203 Clearance of HSV-1 infection requires a tightly coordinated interaction between innate and
204 adaptive response. NK cells are the major cell type recruited to the airways early after
205 HSV-1 infection [21], rapidly activated as demonstrated by the up-regulation of cytotoxic
206 capacity and production of IFN- γ . Our results showed that although similar numbers of NK
207 cells were detected at 3 days p.i. in lungs of mice inoculated with syn variants and parental
208 virus, the level of activation was higher for NK cells isolated from animals infected with
209 the syn variants. On the other hand, augmented levels of proinflammatory cytokines were
210 also detected at 3 days p.i. for syn variants. It is important to consider that infectious virus
211 could not be recorded from lungs of either HSV-1 or syn variants infected animals at this
212 time. In this regard, it is tempting to speculate that NK cells are able to control viral
213 replication but they are not required for an effective viral clearance. However, T cells in
214 lungs play an important role in lowering viral loads. In fact, Adler *et al.* reported that mice
215 deficient in NK and T cells were not able to survive after an intranasal infection, whereas
216 mice only lacking T cells could survive [23]. In this line, the differential NK infiltration
217 and/or activation observed for syn variants at 5 days p.i. would not be related to viral
218 clearance but instead may be responsible for the injury observed in lungs of mice infected
219 with the variants.

220 The ability of HSV to productively infect a wide range of hosts and cell types suggests that
221 HSV has evolved to gain usage of alternative receptors and pathways to facilitate entry into
222 multiple cell types [24]. Regardless of entry receptors or pathways utilized, HSV entry into
223 host cell has common features among various routes of virus entry, including HSV fusion
224 with the plasma membrane of the host cell. This indicates that HSV might recognize
225 structural features of receptors that are conserved among various cell types. On this regard,
226 it is tempting to speculate that the selective process exerted by carrageenans on HSV might
227 generate viruses that retain their ability to infect a wide variety of cells and compensate a
228 putative disadvantage at the stage of entrance with mutations at the level of TK and/or
229 DNA pol genes [12, 25]. The panorama *in vivo* should contemplate also compensating
230 mutations that would confer the virus the ability to evade the immune response.

231 In conclusion, our data provide evidence that the modulation of IL-6 and TNF- α seen *in*
232 *vitro* as well as *in vivo* suggests that HSV-1 targets the proinflammatory host response as a
233 mean of immune evasion. Results obtained in the present study are useful to understand the
234 factors defining HSV-1 virulence in the respiratory tract of mice. Moreover, HSV-1
235 variants constitute a valuable tool to understand the immune systems and to investigate the
236 contribution of specific components of mucosal immunity.

237

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245

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- 313

314 **Figure 1. Cytokines release and viral replication in mice intranasally infected.** Ten
315 (five for each time) female BALB/c mice were infected intranasally with 1×10^6 UFP of
316 HSV-1 F, syn14-1 and syn17-2. At 1 and 3 days p.i. three mice per group were sacrificed
317 and BALs were performed for quantification of (a) TNF- α , (b) IL-6 and (c) IL-10 by
318 ELISA. d) Viral titres were determined by plaque assay in homogenates of lungs from the
319 other two mice of the group. * denotes a p-value < 0.05; ** denotes a p-value < 0.05; #
320 denotes a p-value < 0.05.

321

322 **Figure 2. Cytokine analysis in an *in vitro* model.** RAW 264.7 murine macrophage cells
323 were infected with HSV-1 F, syn14-1 and syn17-2 at a MOI of 10 PFU/cell. At different
324 times p.i. supernatants and cell monolayers were harvested and IL-6 and TNF- α expression
325 was evaluated by ELISA (a) and mRNAs synthesis of this cytokines was evaluated by RT-
326 PCR assay (b). Band intensity was measured by using ImageJ program, and expressed as
327 fold changing of the ratio between the respective cytokine and β -actin. The data shown are
328 mean \pm SD of two independent experiments. cc: cell control.

329

330 **Figure 3. Cell influx in lungs of infected mice.** Five Eight-week-old BALB/c mice per
331 group were infected intranasally with 1×10^6 UFP of HSV-1 F, syn14-1 and syn17-2. At 3
332 and 5 days p.i. lungs were harvested. (a) Cell influx was analyzed by flow cytometry and
333 classified as monocytes (CD11b⁺) and NK cells (NK 1.1⁺). Histograms represent NK cells
334 Isotype (grey), Mock (dashed line) and syn14-1 infection (solid line). No significant
335 differences were detected with respect to control cells in the case of HSV-1 F and syn 17-2
336 histograms (data not shown) (b) Activated NK cells were classified as NK 1.1⁺/CD28⁺. The
337 data shown are mean \pm SD of two independent experiments. * denotes a p-value < 0.05; **
338 denotes a p-value < 0.05.

339

340 **Table 1. Activated NK cells in lungs of infected mice.** Five Eight-week-old BALB/c mice
341 per group were infected intranasally with 1×10^6 UFP of HSV-1 F, syn14-1 and syn17-2. At
342 3 and 5 days p.i. lungs were harvested. Activated NK cells were classified as NK
343 1.1⁺/CD28⁺.

344 **Glossary**

345 **Carrageenans:** are a family of linear sulfated polysaccharides that are extracted from
346 red seaweeds. They are widely used in the food industry, for their gelling, thickening and
347 stabilizing properties.

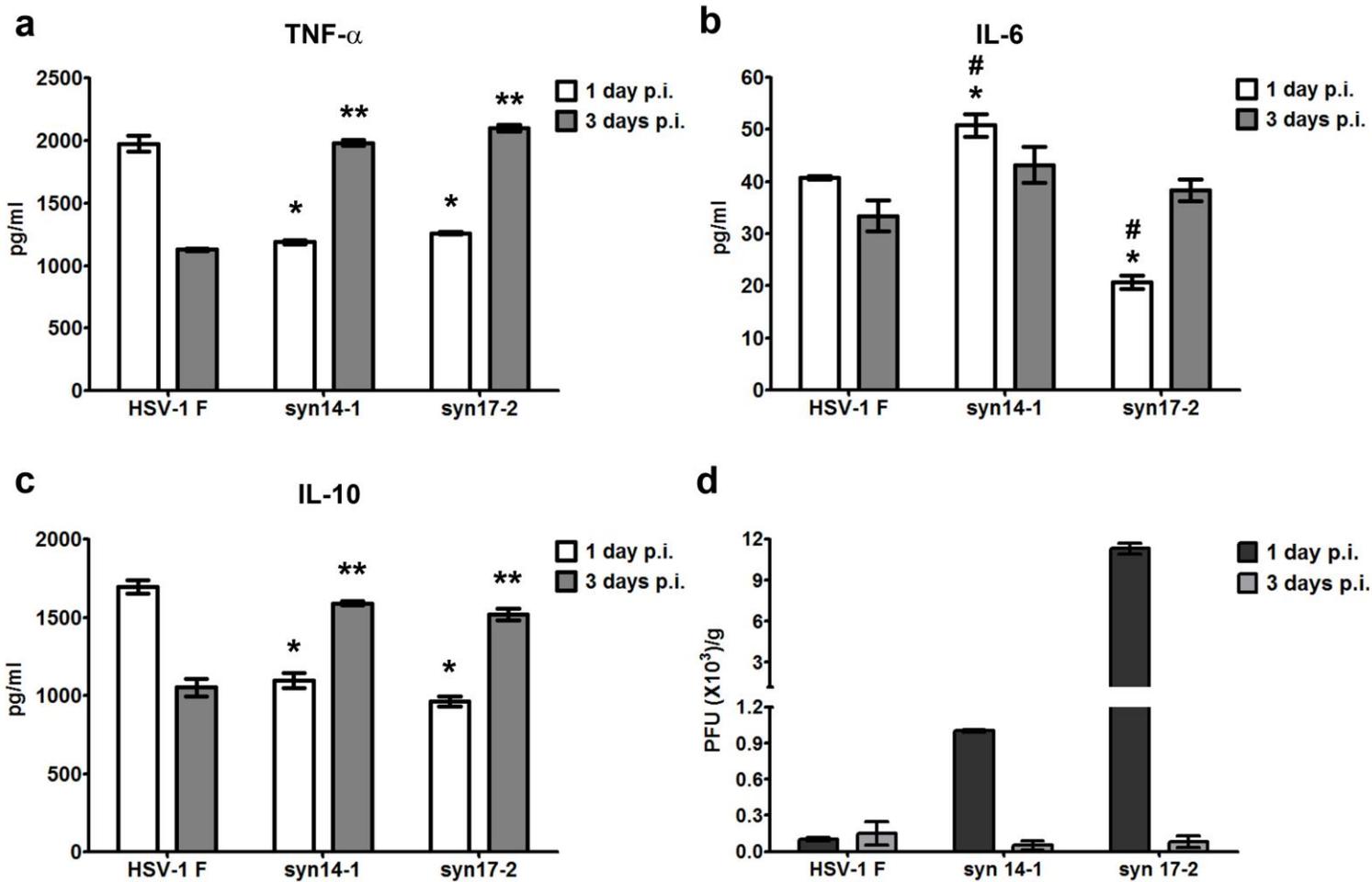
348 **Selective pressure:** It is a mutation-selective process used to obtain the variants. It consists
349 of serial passages of virus *in vitro* in the presence of carrageenans.

350 **Variants:** mutants of virus obtained under selective pressure.

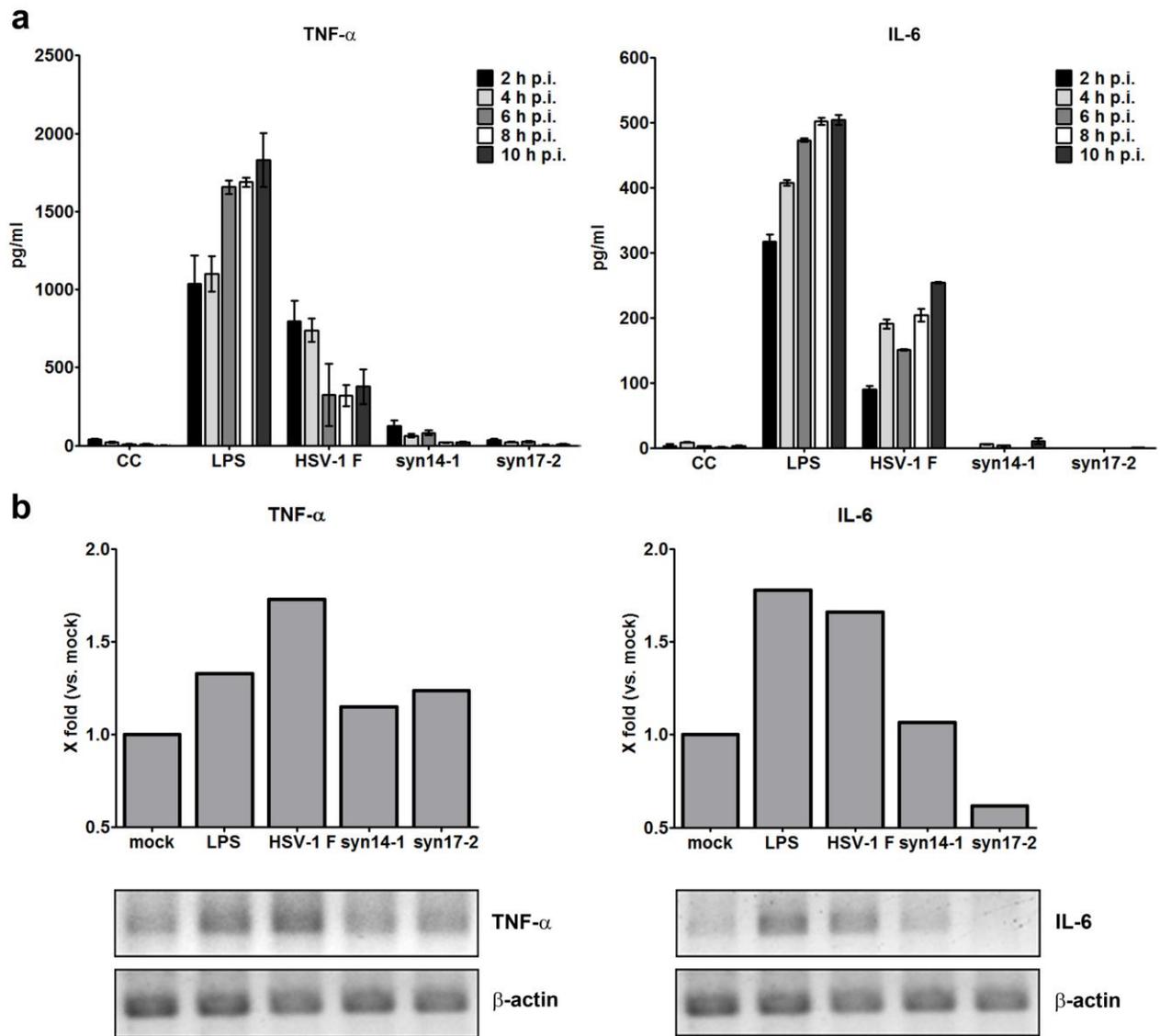
351 **Cytokine:** A small protein released by cells that has a specific effect on the interactions
352 between cells, on communications between cells or on the behavior of cells. The cytokines
353 includes the interleukins (as IL-6), lymphokines and cell signal molecules, such
354 as tumor necrosis factor (TNF) and the interferons, which trigger inflammation and respond
355 to infections. They are key molecules in modulating the immune response.

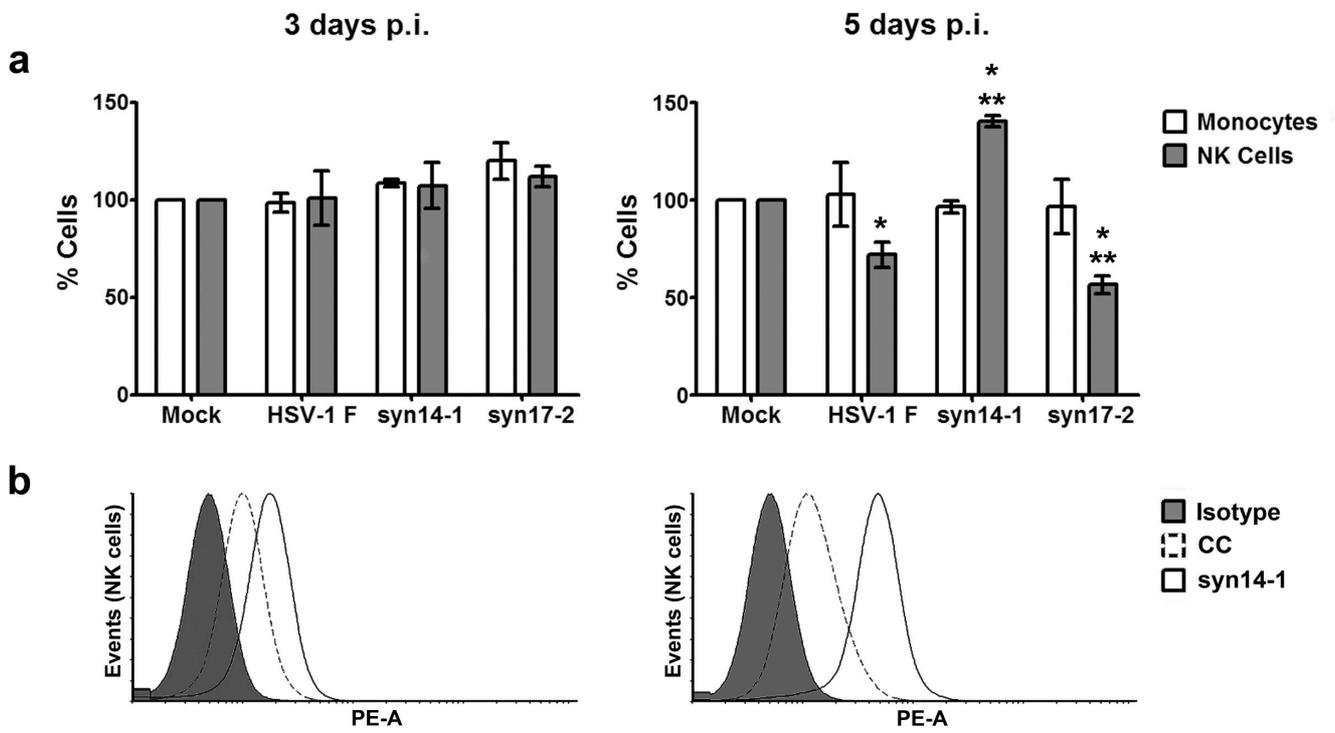
356 **Syncytial:** It is a multinucleate cell which can result from multiple cell fusions of
357 uninucleated cells.

| Virus | 3 days p.i. (% cells \pm SD) | 5 days p.i. (% cells \pm SD) |
|---------|-----------------------------------|-----------------------------------|
| HSV-1 F | 65.4 \pm 1.9 | 70.6 \pm 2.4 |
| syn14-1 | 91.6 \pm 3.6 | 95.0 \pm 3.7 |
| syn17-2 | 71.9 \pm 2.1 | 43.9 \pm 1.3 |



ACCEPTED





Highlights

- HSV-1 syn variants arise during in vitro serial passages with carrageenans.
- The pathology of HSV-1 syn variants depends on modulation of the innate immune response activation.
- Low level of TNF- α is not due to an overproduction of antiinflammatory cytokine .
- The virulence of HSV-1 syn variants by intranasal route correlates with an enhanced replication.