Systemic immunodominant CD8 responses with an effector-like phenotype are induced by intravaginal immunization with attenuated HSV vectors expressing HIV Tat and mediate protection against HSV infection

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Abstract

Mucosal HSV infection remains a public health issue in developing and developed world. However, an effective vaccine is still missing, partly because of the incomplete knowledge of correlates of protection. In this study we have investigated the kinetics and quality of immunity elicited by an attenuated HSV1 vector expressing the immunomodulatory Tat protein of HIV-1 (HSV1-Tat). Animals were immunized by intravaginal (IVag) or intradermal (ID) route with HSV1-Tat or with a control HSV1 vector expressing the LacZ gene (HSV1-LacZ) and immune responses were characterized in different anatomical districts.

IVag immunization with HSV1-Tat enhanced both expansion and memory phases of HSV-specific immunodominant CD8 responses at systemic, but not local, level and induced short- and long-term protection against mucosal challenge. Conversely, ID immunization with HSV1-Tat favored HSV-subdominant CD8 responses, which protected mice only at early time points after immunization.

IVag immunization, in particular with HSV1-Tat, compared to ID immunization, induced the differentiation of $CD8^+$ T lymphocytes into short-lived effector (SLEC) and effector memory (Tem) cells, generating more robust recall responses associated with increased control of virus replication. Notably, systemic SLEC and Tem contributed to generate protective local secondary responses, demonstrating their importance for mucosal control of HSV. Finally, IgG responses were observed mostly in IVag HSV1-Tat immunized animals, although seemed dispensable for protection, which occurred even in few IgG negative mice. Thus, HSV1 vectors expressing Tat induce protective anti-HSV1 immune responses.

Key words

HSV1; Tat; correlate of protection; mucosal infection; effector memory T cells; HSV vaccine.

1. Introduction

Type 1 and 2 herpes simplex viruses (HSV1 and HSV2) are pathogens that establish latency in the sensory ganglia giving rise to periodic reactivation whose consequences, when not asymptomatic, vary from cold sores to blindness, meningitis or encephalitis [1]. The prevalence of genital HSV1 infection is overcoming HSV2 in the western world [2-4], and more and more adolescents are lacking anti-HSV1 immunity at their sexual debut [5]. HSV reactivation cannot be controlled by currently available drugs, which act only against replicating HSV. For this reason, researchers have focused attention on immunization strategies capable of preventing the spread of the virus and/or blocking its reactivation. Despite numerous attempts to develop a vaccine [6], an effective immunization strategy is still missing. This is partly due to the fact that the correlates of protection are not clear, and some controversies exist on the role of humoral and cellular responses in controlling HSV infection [7, 8].

We have recently evaluated the adjuvant effects of HIV Tat on prophylactic immunization against HSV1, because the Tat protein displays peculiar immunomodulatory activities [9, 10]. Tat expressed by an attenuated HSV1 vector (HSV1-Tat) promoted full protection of immunized animals after a deadly challenge with wild-type (wt) HSV1 performed 28 days after immunization, as opposed to mice immunized with a control vector expressing the *lacZ* gene (HSV1-LacZ) instead of *tat* [11].

To identify correlates of protection which were not defined in the previous studies [11], here we have analyzed the duration, quality and tissue distribution of anti-HSV immune responses up to 5 months after immunization, as well as the long-term efficacy of vaccination with HSV1-Tat and HSV1-LacZ recombinants administered by different routes. Indeed, although the mouse model is not the most reliable to study HSV reactivation, it provides important knowledge to study HSV primary infection and the associated immune responses and, thus, to test prophylactic vaccine strategies [12-14].

The results show that the quality (i.e. the effector memory phenotype) rather than the quantity of systemic memory $CD8^+$ T cells is key for long-term protection and effectively induced by mucosal vaccination with HSV1-Tat.

2. Materials and Methods

2.1 Ethic statement

All animal experiments were conducted in conformity to European and Institutional guidelines for the housing and care of laboratory animals and performed under protocols approved by the Italian Ministry of Health.

2.2 Viruses and peptides

Attenuated, replication-competent HSV recombinants (HSV1-Tat and HSV1-LacZ) were generated and purified as previously described [11]. Wild-type HSV (HSV1 LV) was used for challenge experiments and purified as previously described [11]. The HSV1 Kb-restricted peptides SSIEFARL (SSI), derived from glycoprotein B, and QTFDFGRL (QTF), derived from ribonucleotide reductase 1, which correspond to immunodominant and subdominant CTL epitopes respectively [11], were synthetized by UFPeptides (Ferrara, Italy).

2.3 Mice inoculation and challenge

Seven days before IVag inoculation and challenge, female C57BL/6 mice (Charles-River, Bois des Oncins, Saint-Germain-Nuelles, France) were injected in the neck subcute with 2 mg/100 µl of Depo-Provera® (Depo-medroxy-progesterone acetate; Pharmacia & Upjohn, Pfizer S.r.l. Rome, Italy). For IVag immunization and challenge, mice were anaesthetized with 5% isofluorane (Merial Italia S.p.a., Padova, Italy) to allow scraping of the vagina with a pipe scraper (in order to remove the mucus that could trap the virus) and then inoculated with the purified virus using a pipette-tip. 10^4 PFU of HSV1-Tat and HSV1-LacZ were used for immunization and 10^8 PFU of wt HSV1 were used for challenge. For ID immunization, 10^4 PFU of HSV1-Tat and HSV1-LacZ were resuspended in 100 µl PBS (GIBCO, Life Technologies Italia, Monza, Italy) and injected in one site of the back. After immunization and challenge, mice were observed daily to determine weight and to monitor

94 the appearance of local and/or systemic clinical signs of infection including death. Disease signs
95 were classified as follows: 1 = ruffled hair, 2 = cold sores, 3 = limb paralysis, 4 = death.
96

97 *2.4 Mice sacrifice and tissue collection*

98 For mice sacrifice, animals were anesthetized intraperitoneally with 100 µl of isotonic solution
99 containing 1 mg of Zoletil (Virbac, Milano, Italy) and 200 µg Rompun (Bayer, Milano, Italy) to
100 collect spleens, inguinal lymph nodes (ILN) and lower genital tract (LGT). Splenocytes and cells
101 from ILN were purified from organs squeezed on filters as previously described [7]. LGT was
102 minced with sterile scissors and pieces of 1 mm were kept in RPMI supplemented with 1 mg/ml
103 Collagenase (Sigma, Milano, Italy) for 90 minutes at 37°C. Subsequently the medium was filtered
104 through a 70 µm cell strainer and with a 50 µm filcon (Becton Dickinson, Milano, Italy).
105 Blood samples for detection of HSV-specific T cells were collected from retro-orbital plexus in
106 heparinized tubes and, after red blood cells lysis, stained with dextramers and conjugated
107 antibodies.

108 *2.5 Flow cytometry*

109 Characterization of number and phenotype of HSV-specific $CD8^+$ T cells was done by flow
110 cytometry using dextramers (Immudex, Copenhagen, Denmark) to identify $CD8^+$ T cells specific
111 for the HSV1 Kb-restricted SSI and QTF epitopes as previously described [15]. The following
112 antibodies were used: anti-CD3 PerCP-Cy5.5, anti-KLRG1 APC and anti-CD127 PE-Cy7 (TONBO
113 Biosciences, Società Italiana Chimici Rome, Italy), anti-CD62L APC (Immunotools, Friesoythe,
114 Germany), anti-CD43 PE-Cy7 (activated isoform) and anti-CD44 BV510 (Biolegend, Campoverde
115 S.r.l. Milano, Italy), anti-CD103 BV510, anti-CD27 V450 and anti-CD8 APC-H7 (Becton
116 Dickinson Milano, Italy). Samples were acquired on FACS Aria flow cytometer (Becton
117 Dickinson). Flow cytometry data were analyzed using FlowJo (version 9.5.3; Tree Star Inc.,

Ashland, USA). Memory precursor effector cells (MPEC) were defined as KLRG1$^-$ CD127$^+$, short-lived effector cells (SLEC) as KLRG1$^+$ CD127$^-$, central memory T cells (Tcm) as CD44$^+$ CD62L$^+$ and effector memory T cells (Tem) as CD44$^+$CD62L$^-$.

2.6 Serology

Sera and vaginal lavages for antibodies determinations were collected and stored as previously described [11], and the presence and titers of anti-HSV IgG and IgA were assessed by Elisa as previously described [11, 16]. Briefly, samples collected from individual mice were assayed in 96-well immunoplates (Nunc Maxisorp, Milan Italy) previously coated overnight with 100 ng/well of HSV1 purified viral lysate (MacIntyre Strain, Tebu-bio, Milan, Italy) and blocked for 90 min at 37 °C with PBS containing 0.5% milk and 0.05% NaN3 (IgG) or PBS containing 1% BSA and 0.1% Tween 20 (IgA). After extensive washes, 100 µl/well of appropriate dilutions of each serum were dispensed in duplicate wells and then incubated for 90 (IgG) or 60 (IgA) minutes at 37 °C. Plates were washed again before the addition of 100 µl/well of HRP-conjugated goat anti-mouse IgG or IgA (Sigma-Aldrich). After incubation, plates were washed five times and subsequently a solution of 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Roche) was added. The absorbance values were measured at 405 nm with an automatic plate reader (Sunrise Tecan, Salzburg, Austria). The cut-off value was estimated as the mean optical density (OD) of 3 negative control sera plus 0.05. Each OD value was subtracted of the blank and cut-off values to obtain a net OD value. Antibody titers were calculated by intercept function using the Excel program.

Four days after the challenge, genital tract from infected mice were washed with PBS to determine HSV titers by plaque assay as previously described [11].

3. Results

3.1 Intravaginal immunization with HSV1-Tat promotes the expansion of HSV1-specific CD8$^+$ T cells at systemic level

To characterize the magnitude, the location and the kinetics of CD8$^+$ T cell responses elicited by immunization with HSV1 vectors, mice were immunized intravaginally (IVag) with HSV1-Tat or HSV1-LacZ recombinants. After immunization, mice were sacrificed during the expansion (day 7) or the memory (day 120) phases of the immune response for evaluation of the number of CD8$^+$ T cells specific for HSV1 immunodominant (SSI-specific) and subdominant (QTF-specific) epitopes in spleen, inguinal lymph nodes (ILN) and lower genital tract (LGT).

As shown in Fig. 1A (upper panels), at day 7 post-immunization (p.i.), mice immunized with HSV1-Tat showed an enhanced expansion of both SSI- and QTF-specific CD8$^+$ T cells in the spleen compared to mice immunized with HSV1-LacZ ($p < 0.01$). These responses decreased and reached similar levels during the memory phase (Fig. 1A, lower panels, day 120 p.i.). Instead, the numbers of SSI and QTF epitope-specific CD8$^+$ T cells in ILNs (Fig. 1B) and LGT (Fig. 1C) were comparable between the groups during all phases of immune response.

Analysis of CD8$^+$ responses in peripheral blood of HSV1-Tat mice during the expansion, contraction and memory phases (days 7, 14, 28 and 120) of the immune response revealed a significant higher and prolonged expansion of CD8$^+$ T lymphocytes specific for the immunodominant SSI epitope compared to mice immunized with HSV1-LacZ ($p < 0.05$, Fig. 1D). In particular, the SSI immunodominant CD8$^+$ response in the peripheral blood peaked at day 14 p.i in the HSV1-Tat group but at day 7 p.i. in HSV1-LacZ mice. In addition, Tat supported the persistence of a higher proportion of SSI-specific CD8$^+$ T cells in the blood. In fact, these responses remained significantly higher in mice immunized with HSV1-Tat, compared to HSV1-LacZ mice ($p<0.05$), both during the contraction (day 28) and the memory (day 120) phases (Fig. 1D). Apparently, the enhancing effect of Tat was limited to immunodominant SSI-specific cells since

165 circulating subdominant QTF-specific cells were persistently very low and no differences were
166 observed between the two groups (data not shown).
167 These results show that IVag immunization with HSV1-Tat promotes the expansion of
168 immunodominant and subdominant $CD8^+$ responses in the spleen and of immunodominant $CD8^+$
169 responses in the blood, resulting in higher percentages of circulating antigen-specific memory T
170 cells.
171
172 *3.2 Intravaginal immunization with HSV1-Tat promotes the differentiation of $CD8^+$ T lymphocytes*
173 *into short-lived effector and effector memory T cells*

174 Effector $CD8^+$ T cells may be subdivided in short-lived and memory precursor (SLEC and MPEC,
175 respectively) based on the expression of KLRG1 and CD127. To evaluate if the presence of Tat
176 within the HSV1 vector may affect the SLEC/MPEC balance, the phenotype of HSV1-specific
177 $CD8^+$ T cells was evaluated during the expansion phase. At day 7 p.i., HSV1-Tat and HSV1-LacZ
178 mice showed similar percentages of SSI- and QTF-specific SLEC and MPEC in spleen, lymph
179 nodes (Supplementary Figure S1A) and blood (data not shown). However, at day 14 p.i., HSV1-Tat
180 mice had significantly higher percentages of SSI- and QTF-specific SLEC and lower percentages of
181 MPEC in the blood compared to HSV1-LacZ mice (Fig. 2A), suggesting that Tat favors the
182 expansion of circulating short-lived effectors.
183 Next, we assessed the phenotype of the memory pool measuring the percentage of effector (Tem)
184 and central (Tcm) memory epitope-specific $CD8^+$ T cells at day 120 p.i.. While similar frequencies
185 of Tem and Tcm were observed between groups in lymphoid organs (Supplementary Figure S1B),
186 the percentage of SSI- and QTF-specific $CD8^+$ Tem cells were significantly higher in the blood of
187 mice immunized with HSV1-Tat than in HSV1-LacZ mice (Fig. 2B). Altogether, these results show
188 that Tat increases the number of circulating HSV1-specific short lived-precursors and effector
189 memory $CD8^+$ T cells.

190 *3.3 Intravaginal immunization with HSV1-Tat promotes the development of IgG responses*

191 To characterize the magnitude and kinetics of humoral responses, serum samples and vaginal
192 lavages were collected at 3 and 5 months p.i.. Anti-HSV1 serum IgG were undetectable in the
193 majority of animals. However, at 3 months p.i., HSV1-Tat mice showed higher frequencies and
194 titers of serum IgG (Fig. 3A) that persisted up to 5 months (Fig. 3B) after immunization. The
195 assessment of the IgG isotype revealed a pattern toward Th1-like responses as only IgG2a, and not
196 IgG1, were detected (not shown). No HSV1-specific IgG or IgA were present in vaginal lavages
197 (not shown).

198 *3.4 Intradermal immunization with HSV1-Tat elicits different immune responses compared to*
199 *immunization by the intravaginal route*

200 Attenuated HSV1 recombinants were administered also by the intradermal (ID) route, which is
201 more suitable for vaccination strategies.
202 As shown in Fig. 4A (upper panels), HSV1-Tat immunized mice showed a trend toward higher
203 numbers of epitope-specific $CD8^+$ T cells in spleen and ILN at day 7 p.i., while cellular responses
204 in blood were very low and no difference between groups were observed (Fig. 4B). The phenotype
205 of effector cells was comparable between HSV1-LacZ and HSV1-Tat ID immunized animals in all
206 tissues, with a strong predominance of memory precursors (MPEC) over SLEC (not shown).
207 During the memory phase (day 120 p.i.) mice of both groups had comparable levels of
208 immunodominant SSI-specific $CD8^+$ responses in all tissues. However, HSV1-Tat animals showed
209 significant higher numbers of QTF-specific $CD8^+$ T cells in the spleen ($p < 0.01$) but not in ILN
210 (Fig. 4A, lower panels) or in peripheral blood (Fig. 4B). Both groups showed similar percentages of
211 epitope-specific effector and central memory T cells (not shown).
212 Anti-HSV1 antibodies were undetectable in serum and vaginal lavages in both groups (data not
213 shown).

As compared to the IVag route of vaccination (Fig. 1), ID immunization was less immunogenic in respect to both the cellular and humoral response. However, ID administration of HSV1 vectors, and in particular of HSV1-Tat, favored the development of subdominant QTF-specific memory responses.

3.5 Intravaginal, but not intradermal, immunization with HSV1-Tat induces long-term protection against mucosal challenge

To assess the efficacy of immunization with HSV1-Tat and HSV1-LacZ, IVag or ID immunized mice were challenged at days 28 or 150 p.i. with a lethal dose of wt HSV1 administered by the IVag route.

After challenge at day 28, all mice immunized IVag with HSV1-Tat survived and only transiently showed very mild signs of disease, whereas mice immunized with HSV1-LacZ presented severe signs of disease, fatal in the majority of cases, in a fashion similar to PBS-treated mice (Supplementary Figure S2A), in agreement with previous studies [11]. Interestingly, HSV1-Tat induced protection at day 28 p.i. also when administered ID, as 67% of mice immunized ID with HSV1-Tat were protected from death, while mice immunized ID with HSV1-LacZ showed severe signs of disease and all but one died (Supplementary Figure S2B).

After challenge at day 150 p.i., all mice immunized IVag with HSV1-LacZ or HSV1-Tat showed mild signs of disease and survived, while all PBS mice died (Fig. 5A). In contrast, all mice immunized ID with HSV1-LacZ or HSV1-Tat developed a more severe disease (Fig. 5B) and 3 out of 7 animals died in each group (not shown). Thus, replication competent HSV1 recombinants *per se* confer long-term protection, especially if administered IVag. Daily assessment of mice weight, expected to increase in healthy animals, revealed that mice immunized IVag with HSV1-LacZ (Fig. 5C) as well as both groups of mice immunized ID (Fig. 5D) underwent loss of weight, in contrast to IVag HSV1-Tat immunized animals whose weight was not affected by virus challenge (Fig. 5C, $p < 0.05$). This suggests that only HSV1-Tat administered IVag induces full long-term protection.

239 Interestingly, four days after challenge HSV1 infectious particles were undetectable or barely
240 detectable in vaginal lavages of mice immunized IVag with HSV1-Tat as opposed to HSV1-LacZ
241 mice (Fig. 5E, $p < 0.05$) and both groups of mice immunized ID (Fig. 5F).
242 Overall, these data demonstrate that immunization with HSV1-Tat induces short-term protection
243 when administered either IVag or ID and long-term protection when administered IVag. Of note,
244 HSV1-LacZ mice either immunized IVag or ID showed a higher level of protection at day 150 than
245 at day 28 p.i. suggesting that memory T and B cells need time to develop and mature [17-20] to give
246 rise to protective responses which may be undetectable or ineffective before, especially during the
247 transition from effector to memory cells. Nonetheless, the presence of Tat, in particular for IVag
248 immunization, favors this process of maturation providing a higher level of protection.

249 *3.6 Tat fosters protective memory and recall responses*

250 While recall expansion is often used as a measure of protective memory responses, it has been
251 recently shown that splenic effector-like memory T cells characterized by low expression of CD43
252 and CD27, despite a poor recall proliferation, outperform other memory subsets in mediating
253 protection [21]. As it is unknown which of the two paradigms (recall proliferation vs. persistent
254 effector-like memory T cells) is important in mediating protection against HSV1 mucosal infection,
255 we assessed both CD43/CD27 expression on splenic memory HSV1-specific $CD8^+$ T cells as well
256 as cellular and humoral HSV1-specific recall responses.
257 CD43 expression was similar between IVag and ID immunized groups, except in the case of higher
258 values in ID HSV1-LacZ immunized animals (Supplementary Figure S3). In contrast, $CD8^+$ T cells
259 from ID-immunized mice displayed higher levels of expression of CD27 than IVag immunized
260 animals (Fig 6A). Moreover, SSI-specific $CD8^+$ T cells from IVag HSV1-LacZ immunized mice
261 expressed higher levels of CD27 than IVag HSV1-Tat immunized mice. These results suggest that
262 protection is associated with low CD27 expression on memory T cells specific for the
263 immunodominant SSI epitope.

Next, we evaluated secondary cellular responses in challenged mice. HSV1-specific $CD8^+$ responses were measured at the site of challenge, i.e. the lower genital tract (LGT), 4 days after infection, and a significant higher number of SSI-specific recall cells was observed in mice immunized IVag with HSV1-Tat compared to IVag HSV1-LacZ immunized animals (Fig. 6B, $p < 0.01$). Interestingly, the number of mucosal HSV1-specific $CD8^+$ T cells was dramatically higher after IVag immunization than after ID immunization (Supplementary Figure S4A).

Finally, serum recall humoral responses at different time points after challenge were measured. Anti-HSV1 IgG secondary responses developed more promptly in IVag HSV1-Tat mice, as demonstrated by both the increased frequency of responders as well as by higher IgG titers at days 2 and 7 post-challenge (Fig. 6C). Conversely, IgG recall responses were undetectable in the majority of ID immunized animals until day 14 post-challenge (Supplementary Figure S4B). Interestingly, even mice with undetectable pre-challenge IgG rapidly developed humoral secondary responses, suggesting an important role for memory B cells also in the absence of circulating antibodies, as already proposed for other vaccines [22].

These results suggest that the type of immune response (quality, quantity and localization) conferred by IVag immunization with HSV1-Tat results in more efficient recall responses. Taken together, these data show that effector-like memory cells give rise to robust local recall responses important to control mucosal HSV1 infection.

4. Discussion

HSV1 is a worldwide spread pathogen whose association with vaginal diseases is constantly increasing [23]. In this study we have characterized in mice the protective immune responses against HSV1 elicited by immunization with two different attenuated HSV1 recombinants, one of which expressing Tat, delivered by IVag and ID routes. Specifically, IVag immunization with HSV1-Tat, compared to immunization with HSV1-LacZ, enhanced the number of $CD8^+$ T cells specific for the immunodominant gB-derived SSI epitope (Fig. 1) and favored the development of short-lived effectors (SLEC, Fig. 2), resulting in a larger pool of memory T cells with an effector phenotype (Figs. 2 and 6). Moreover, IVag immunization with HSV1-Tat enhanced HSV1-specific IgG responses (Fig. 3) and both cellular and humoral recall responses (Fig. 6), which were associated to full protection up to 4 months (Supplementary Figure S2A and Fig. 5). Higher levels of protection were also observed at early time points in mice immunized ID with HSV1-Tat (Supplementary Figure S2B), but the effect was lost over time (Fig. 5), suggesting the need of a prime-boost strategy. In general, compared to mice immunized IVag, mice immunized ID with HSV1-Tat displayed lower cellular and humoral immune responses and were less protected from challenge. Interestingly, ID immunization, and in particular immunization with HSV1-Tat, favored the accumulation of memory $CD8^+$ T cells against the subdominant RR1-derived QTF epitope, suggesting that the route of administration impacts not only the priming of the immune system but also the hierarchy of antigen response. Our results indeed demonstrate that systemic and mucosal routes of administration may deeply affect the quantity and the quality of the immune response. Thus, more studies are necessary to explore how to improve systemic immunization or to test other mucosal routes (e.g. nasal) more suitable for mass immunization. In addition, further experiment to assess protection against challenges performed by different routes (e.g. ocular) must be done.

We show that IVag immunization with the HSV vector expressing Tat enhances SLEC expansion and promotes accumulation of HSV1-specific memory cells with an effector memory (Tem)

309 phenotype and low expression of CD27. Conversely, the ID route favored the expansion of memory
310 precursors (MPEC) and accumulation of memory $CD8^+$ T cells with high CD27 expression,
311 indicating that the route of immunization affects the phenotype of vaccine-induced T cells. Of note,
312 the CD27lo $CD8^+$ memory population has been recently identified as a subset of memory cells with
313 effector-like properties similar to SLEC and Tem, with low proliferative but high protective and
314 cytolytic capacity [21, 24]. This indicates that effector memory $CD8^+$ T cells are important for
315 protection as demonstrated in several infections [25-30], including ocular HSV, as asymptomatic
316 HSV1-infected individuals tend to develop HSV-specific $CD8^+$ T cells presenting mainly a SLEC
317 and Tem phenotype [12, 31, 32]. Consistently, our data show that $CD8^+$ T cells specific for the
318 immunodominant SSI epitope, which tend to display a more "effector" (SLEC/Tem) phenotype,
319 especially when generated in the presence of Tat, are more important for protection than those
320 directed to the subdominant QTF epitope, which predominantly develop into memory precursors
321 and central memory cells (MPEC and Tcm). In addition, it has been recently demonstrated that
322 HSV-specific $CD8^+$ T cells from asymptomatic subjects have an increased expression of T-bet,
323 Eomes, Blimp-1 and Bcl2 [32] transcription factors, that are upregulated by Tat in activated human
324 $CD8^+$ T cells [33] and by HSV1-Tat itself in murine $CD8^+$ T cells (not shown). Thus, our results
325 demonstrate for the first time the importance of effector memory $CD8^+$ T cells against mucosal
326 HSV1 infection and show that Tat is capable of driving their development.
327 The importance of effector memory cells in protection against HSV may reside in their capacity to
328 support the development of mucosal effective secondary responses, known to be crucial to control
329 HSV [34-36], as they are easily recruited into mucosal tissues [21, 36-38]. Consistently, mice
330 immunized IVag with HSV1-Tat displayed, compared to mice immunized IVag with HSV1-LacZ,
331 enhanced vaginal recall responses despite comparable numbers of local memory but higher number
332 of systemic effector memory HSV-specific $CD8^+$ T cells. However, it should be noted that, to
333 efficiently recruit effector memory cells at the site of infection, mucosal priming is needed, as
334 confirmed by the poor recall response observed in ID immunized mice.

In this study we found that humoral responses were not associated with protection, as also mice without IgG survived lethal challenge while few animals with detectable antibodies died. In accordance with other reports [34, 35, 39-44], this observation indicates that the humoral responses may contribute but are insufficient to mediate protection.

Despite live HSV viruses guarantee an antigenic breadth not provided by vaccination with single subunits [45], safety issues have been raised about their use. Exploratory analysis revealed the presence of the HSV recombinants in the spinal cord of some mice several weeks after immunization, especially in IVag treated mice (not shown). This suggests that attenuated HSV1 recombinants may establish latency in the central nervous system, although their capability to reactivate has not been assessed in this study and not demonstrated by others in similar mice models [45]. Anyhow, approaches based on defining and eliminating genes involved in latency or reactivation are currently being investigated. In addition, since HSV1 acquired by genital infection is less likely to recur and is less virulent than HSV2 [14, 46] and since the genomic sequences of HSV1 and HSV2 are closely related [47] and share several cross-reactive epitopes, HSV1-based vectors may represent promising and safe candidates against genital infection caused not only by HSV1 but also likely by HSV2. The potentiality of this immunization strategy against HSV2 is still under investigation.

In conclusion, in this report we describe an immunization strategy capable of inducing wide and long lasting humoral and cellular responses that controlled HSV1 infection. We observed that $CD8^+$ T cells of protected animals displayed a SLEC and Tem phenotype that contributed to generate high mucosal secondary responses, demonstrating for the first time the importance of these T cell subsets for the control of mucosal HSV infection. Of note, we also demonstrate that the presence of Tat within the recombinant HSV1 favored the development of such protective responses, constituting a proof of concept of its use as adjuvant for vaccines against HSV.

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Figure Legends

Fig. 1. Tat enhances the expansion of systemic HSV1-specific CD8+ T cells and the accumulation of antigen-specific circulating T memory cells. (A) Spleen, (B) ILN and (C) LGT from IVag immunized mice were harvested at days 7 or 120 p.i. and the number of SSI-specific and QTF-specific $CD8^+$ T cells was determined. Each dot represents single mouse data from 2 independent experiments (n = 3 each); horizontal lines represent the cumulative mean +/- SEM. (D) Blood from 5 immunized mice per group was collected at days 7, 14, 28 and 120 p.i. to determine the percentage of SSI-specific $CD8^+$ T cells. Mean +/-SEM from one representative experiment out of two is shown. For statistical analysis two-tailed Mann Whitney test was used. *P < 0.05, **P < 0.01.

Fig. 2. Tat promotes the development of HSV1-specific circulating $CD8^+$ T cells with a SLEC and Tem phenotype. (A) Expression of KLRG1 and CD127 was measured at day 14 p.i. on SSI- and QTF-specific $CD8^+$ T cells in the peripheral blood collected from IVag immunized mice. (B) Expression of CD44 and CD62L was measured at day 120 p.i. on SSI- and QTF-specific $CD8^+$ T cells in the peripheral blood collected from IVag immunized mice. Each dot represents data from a single mouse obtained in one representative experiment (n = 5) out of two; horizontal lines represent the cumulative mean +/- SEM. For statistical analysis two-tailed Mann Whitney test was used. *P < 0.05, **P < 0.01.

Fig. 3. Tat promotes the development of HSV1-specific IgG. Sera from mice IVag immunized mice were collected at days 100 (A) or 150 (B) p.i. to assess the presence and titers of HSV-specific IgG. Each dot represents single mouse data from one representative experiment (n = 10) out of two; horizontal lines represent the cumulative mean.

506 **Fig. 4.** ID immunization with HSV1 recombinants is less immunogenic than IVag immunization.
507 (A) Spleen and ILN from ID immunized mice were harvested at days 7 (upper panels) or 120
508 (lower panel) p.i. and the number of SSI-specific and QTF-specific $CD8^+$ T cells was determined.
509 Each dot represents single mouse data from 2 independent experiments (n = 3 each); horizontal
510 lines represent cumulative mean +/- SEM. For statistical analysis two-tailed Mann Whitney test was
511 used, **P<0.01. (B) Blood from immunized mice was collected at days 7, 14 or 120 p.i. to
512 determine the percentage of SSI-specific (upper panel) and QTF-specific (lower panel) $CD8^+$ T
513 cells. Mean +/- SEM from one representative experiment (n = 5) out of two is shown.
514
515 **Fig. 5.** Analysis of long-term protection induced by IVag and ID immunization with HSV1
516 recombinants. Mice immunized IVag (A, C and E) or ID (B, D and F) with HSV1-LacZ, HSV1-Tat
517 or PBS were challenged by IVag route at day 150 p.i.. Mice were monitored daily for appearance of
518 disease signs. (A) and (B) show mean disease signs +/- SEM. One representative experiment (n = 7)
519 out of two is shown. Mice weight was measured every two days after the challenge: (C) and (D)
520 show the percent variation of weight compared to the time of the challenge. One representative
521 experiment (n=7) out of two is shown. At day 4 post-challenge the presence of HSV1 particles was
522 assessed in vaginal lavages by plaque dilution assays: (E) and (F) show the number of PFU
523 measured in vaginal lavages from individual mice and lines represent cumulative mean +/- SEM.
524 Data from one representative experiment (n = 5) out of two are shown. For statistical analysis two-
525 tailed ANOVA test was used for (A, B, C, D) and two-tailed Mann Whitney test was used for (E,
526 F). *P < 0.05.
527
528 **Fig. 6.** Tat promotes the development of effector memory $CD8^+$ T cells and the induction of cellular
529 and humoral recall responses. (A) Expression of CD27 was measured on SSI- or QTF-specific
530 $CD8^+$ T cells from spleens collected at day 120 p.i. from IVag or ID immunized mice. Each dot
531 represents single mouse data from 2 independent experiments (n = 3 each). (B-C) IVag immunized

mice were challenged with wt HSV1 by IVag route at day 150 p.i. (B) LGT cells were harvested 4 days post-challenge and the number of SSI- or QTF-specific $CD8^+$ T cells was determined. (C) Sera were collected from IVag immunized mice to assess the presence and titers of HSV1-specific IgG at day 150 p.i. (day of challenge) and at days 2, 7 and 14 post-challenge. Each dot represents single mouse data from one representative experiment (n = 5-10) out of two. Horizontal lines represent cumulative mean +/- SEM. For statistical analysis two-tailed Mann Whitney test was used. *$P < 0.05$, **$P < 0.01$.

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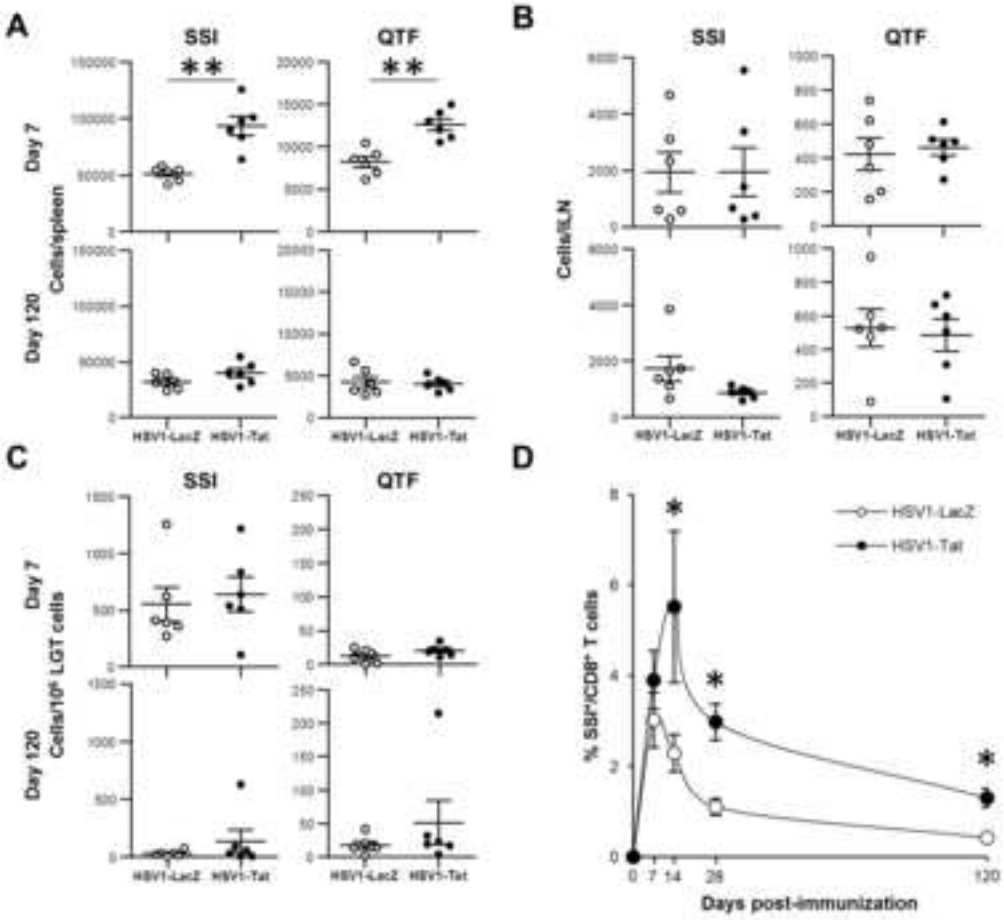

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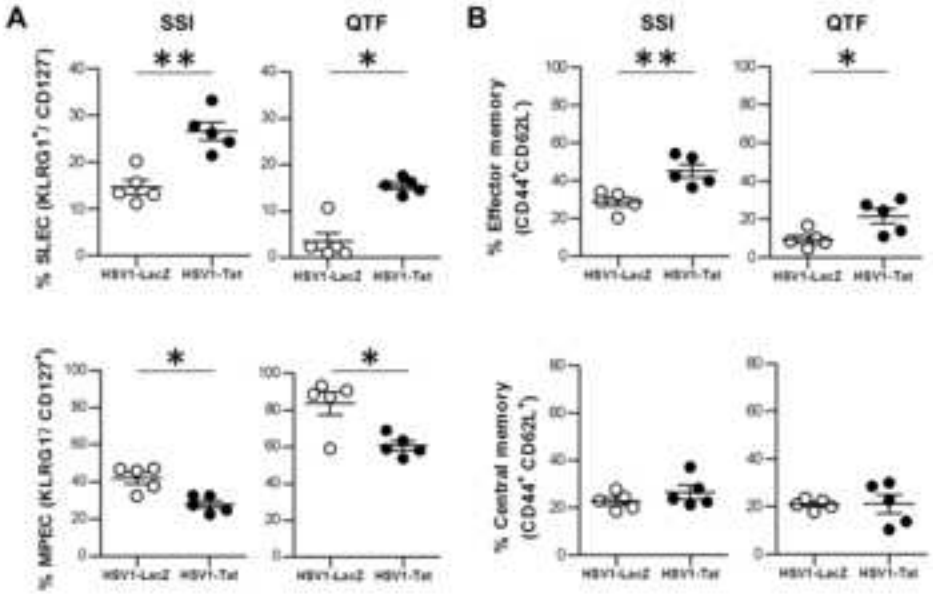

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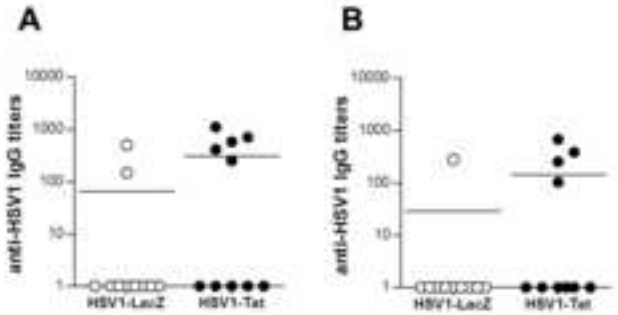

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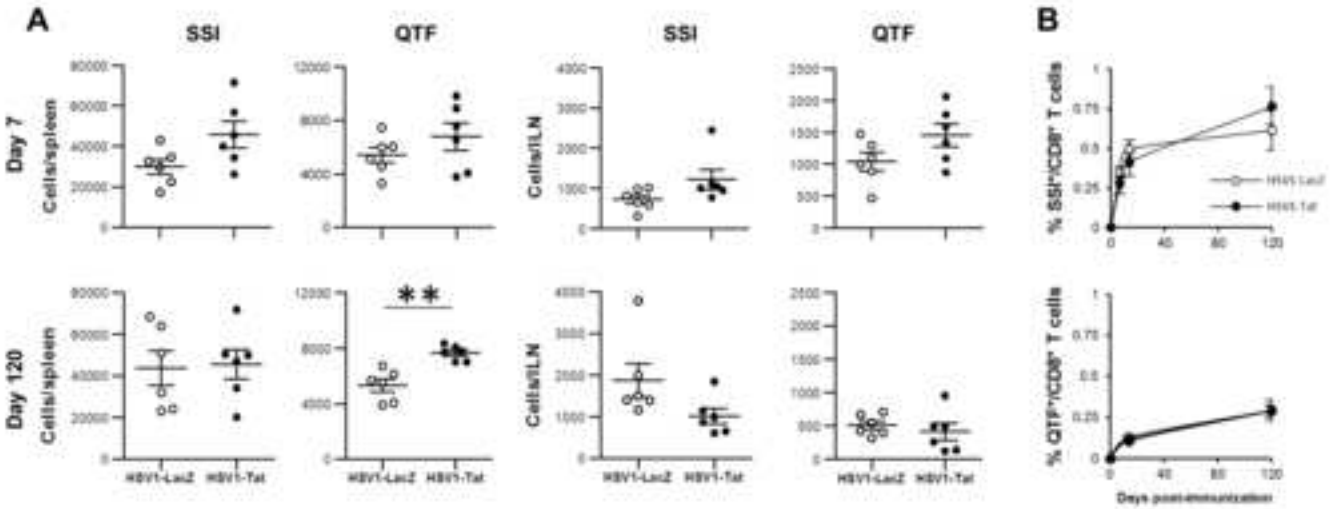

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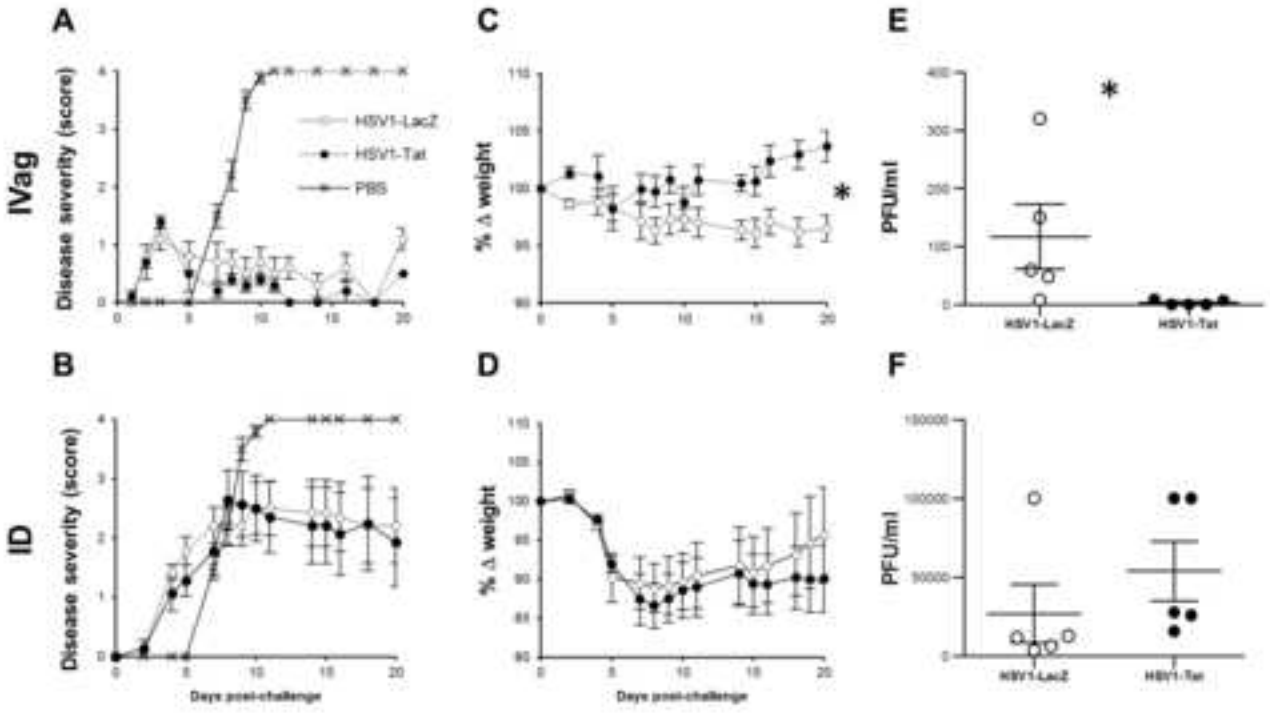

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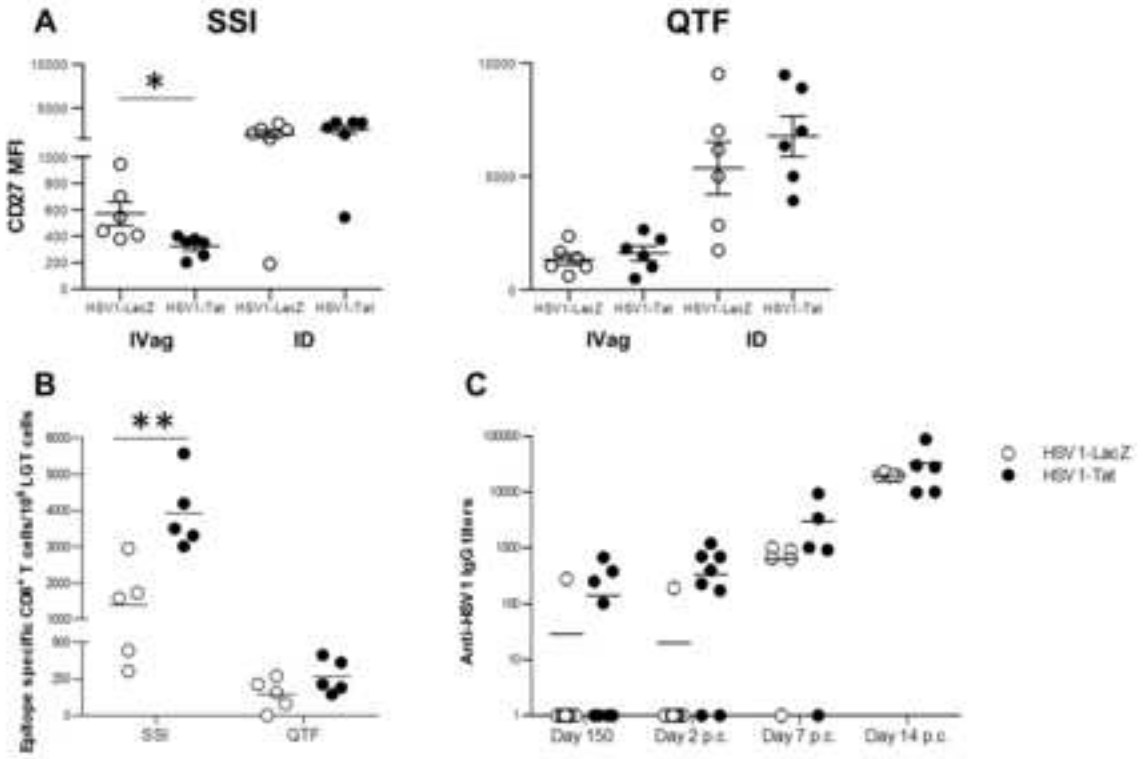

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