Research article

Differences in time of virus appearance in the blood and virus-specific immune responses in intravenous and intrarectal primary SIV_{mac251} infection of rhesus macaques; a pilot study

Liljana Stevceva[1], Elzbieta Tryniszewska[1], Zdenek Hel[1], Janos Nacsa[1], Brian Kelsall[2], Robyn Washington Parks[1] and Genoveffa Franchini*[1]

Address: [1]Basic Research Laboratory, National Cancer Institute, 41/D804, Bethesda, Maryland, 20892, USA and [2]Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, 10/11N238, Bethesda, MD, 20892-1890, USA

E-mail: Liljana Stevceva - stevcevl@mail.nih.gov; Elzbieta Tryniszewska - et74h@nih.gov; Zdenek Hel - zdenek@helix.nih.gov; Janos Nacsa - nacsa@nih.gov; Brian Kelsall - bk19v@nih.gov; Robyn Washington Parks - ParksR@intra.nci.nih.gov; Genoveffa Franchini* - veffa@helix.nih.gov

*Corresponding author

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Abstract

Background: HIV-I can be transmitted by intravenous inoculation of contaminated blood or blood product or sexually through mucosal surfaces. Here we performed a pilot study in the SIV_{mac251} macaque model to address whether the route of viral entry influences the kinetics of the appearance and the size of virus-specific immune in different tissue compartments.

Methods: For this purpose, of 2 genetically defined Mamu-A*01-positive macaques, 1 was exposed intravenously and the other intrarectally to the same SIV_{mac251} viral stock and virus-specific CD8+ T-cells were measured within the first 12 days of infection in the blood and at day 12 in several tissues following euthanasia.

Results: Virus-specific CD8+ T-cell responses to Gag, Env, and particularly Tat appeared earlier in the blood of the animal exposed by the mucosal route than in the animal exposed intravenously. The magnitude of these virus-specific responses was consistently higher in the systemic tissues and GALT of the macaque exposed by the intravenous route, suggesting a higher viral burden in the tissues as reflected by the faster appearance of virus in plasma. Differences in the ability of the virus-specific CD8+ T-cells to respond *in vitro* to specific peptide stimulation were also observed and the greatest proliferative ability was found in the GALT of the animal infected by the intrarectal route.

Conclusions: These data may suggest that the natural mucosal barrier may delay viral spreading. The consequences of this observation, if confirmed in studies with a larger number of animals, may have implications in vaccine development.

Background

Infection with human immunodeficiency vims (HIV) elicits an acute retroviral syndrome characterized by fever, pharyngitis, lymphadenopathy, myalgia, rash, and headache [1,2,3]. Sexual transmission of HIV infection occurs mostly via the intestinal or vaginal mucosa but

HIV-I is also effectively transmitted by the intravenous route [4] [5,6,7].

Recent studies have shown that the HIV-I or SIV virus rapidly penetrates vaginal, rectal, or oral mucosa attaching to and infecting primarily CD4+ T-cells where it replicates and consequently spreads to lymphoid tissue and systemic organs [8] [9,10,11,12].

Accumulating evidence has implicated virus-specific CTL in containing primary HIV/SIV infection and HIV-I/SIV-specific CD8+ CTL have been documented during the early weeks following infection, before a neutralizing Ab response is demonstrable [13] [14,15,16]. Despite the rapid dissemination of HIV-I by mucosal routes, productive mucosal transmission appears to be relatively inefficient and is estimated to occur once in 300 or more high-risk exposures [17]. Cell-mediated immunity and direct killing by cytotoxic lymphocytes from the vagina and colon lamina propria may be an important factor in containing viral infection at the site of primary infection [18] [19,20].

Mucosal T lymphocytes appear to be functionally distinct from those present in the peripheral circulation. While activated T-cells reenter lymphoid tissues and preferentially accumulate at the site of the initial activation, memory T-cells migrate continuously and randomly, similar to naive T-cells [21] [22]. The implication in terms of HIV infection is that, in the initial phase of an immune response, once primed, Ag-specific memory T-cells randomly enter and leave various lymphoid compartments but preferentially are retained in the lymphoid compartment where the antigen was presented at first [23] [24].

In humans, it is unfeasible to evaluate the immunological events that occur shortly after infection in the mucosal compartments. However, in the SIV_{mac251} macaque model, some of these issues can be addressed. SIV_{mac251} establishes persistent infection in rhesus macaques and causes an immunodeficiency syndrome closely resembling human AIDS [25] [26,27,28]. As in humans, the clinical course of SIV_{mac251} infection varies considerably among macaques. Recent evidence from our lab suggests that macaques that express the major histocompatibility class I Mamu-A*01 molecule restrict SIV_{mac251} replication following intrarectal exposure, as reported for HIV-I-infected individuals that express the HLA B*5701 [29], further validating this animal model of HIV-I infection. In this model, virus strain, dose, and especially route of infection can be defined and host-virus interactions under different conditions can be assessed. Here we used genetically defined Mamu-A*01 rhesus macaques to study the extent of virus-specific CD8+ T-cell response and the trafficking of lymphocytes to the gut during the first 12 days of intrarectal or intravenous transmission of the same stock of SIV_{mac251}.

Materials

Animals and procedure

Two female Mamu-A*01-positive macaques were involved in this study: animal 817 that was infected by undiluted $SIV_{mac251/561}$ stock virus (R. Pal *et al.,* unpublished data) by the intrarectal route and animal 819 by the intravenous route with a 1:3000 dilution of the same viral stock. Blood was drawn before infection and at days 4, 8, and 12 after inoculation with the virus. Animals were sacrificed at day 12 postinfection. The spleens and hilar, axillary, mesenteric, iliac, and inguinal lymph nodes as well as ileums, jejunums, and colons were collected in RPMI medium containing 10% FCS+ penicillin/streptomycin.

Isolation of tissue lymphocytes

Mononuclear cells were isolated from peripheral blood (PBMC), lymph nodes, spleens, and intestines. Mononuclear cells from spleens and lymph nodes were isolated by mechanical dissociation of the tissue and consecutive Ficoll gradient centrifugation. Tissues from ileums, jejunums, and colons were treated with 1 mM DTT (ICN Biomedicals, Aurora, OH) for 30 minutes followed by incubation in calcium/magnesium-free HBSS with EDTA (Life Technologies, Baltimore, MD) 4 times 1 hour with stirring at room temperature to remove the epithelial layer. At this stage, pieces of tissue were fixed in 10% neutral formalin and embedded in paraffin and sections were cut and stained with H&E. Microscopic examination was performed to ensure that all of the epithelium was removed and the lamina propria was intact.

Further, tissues were cut into smaller pieces and incubated at 37°C in Isocove's medium supplemented with 10% fetal calf serum and penicillin/streptomycin containing 400 U/ml Collagenase D (Boerrhinghem & Mannheim GmbH, Mannheim, Germany) and 25 U/ml DNAse (Worthington Biochemical, Lakewood, NJ) for 2–3 hours. The mononuclear cells were isolated from the supernatant containing dissociated cells by Percoll gradient centrifugation.

Flow cytometry

Fresh mononuclear cells were directly stained with PE-conjugated tetrameric complexes for Gag 181, Env 622, and Tat 28. PerCP-conjugated anti-CD3e (Pharmingen, San Diego, CA) and FITC-conjugated anti-CD8 (Becton Dickinson, San Jose, CA) were used in conjunction with the tetrameric complexes. In addition, cells were stained with a combination of anti-CD3 PerCP, anti-CD8 FITC

and anti CD4 PE (Becton Dickinson, San Jose, CA) . To assess their proliferative ability and to confirm the specificity of tetramer staining of *ex vivo* CD3+ CD8+ T-cells, lymphocytes were cultured at a concentration of 3 $\times 10^6$/ml in RPMI enriched with 10% human serum with addition of 10 μg/ml of the appropriate peptide and 20 U/ml of IL-2 for 7 days. Staining with tetrameric complexes was done afterward as described above.

Briefly, $5 \times lo^5$ lymphocytes isolated by Ficoll diatrizoate or Percoll gradient centrifugation were incubated with tetrameric complexes as previously described [30] and/or selected Ab for 30 minutes at room temperature. After washing the cells twice in Dulbecco's phosphate buffered saline supplemented with 2% FCS and fixation in 1% paraformaldehyde (Ph = 7.4), samples were analyzed by flow cytometry using FACScalibur (Becton Dickinson, San Jose, CA) instrument.

Viral load (NASBA)

SIV_{mac251} viral RNA copies in plasma was quantified by nucleic-acid-sequence-based amplification [31]. Briefly, RNA extracted from plasma was subjected to isothermal amplification with SIV_{mac251}-specific primers. A portion of the SIV wild type Gag gene was used to generate internal control. NASBA amplification products were detected by using chemiluminescence-based probe hybridization system. The assay can measure accurately

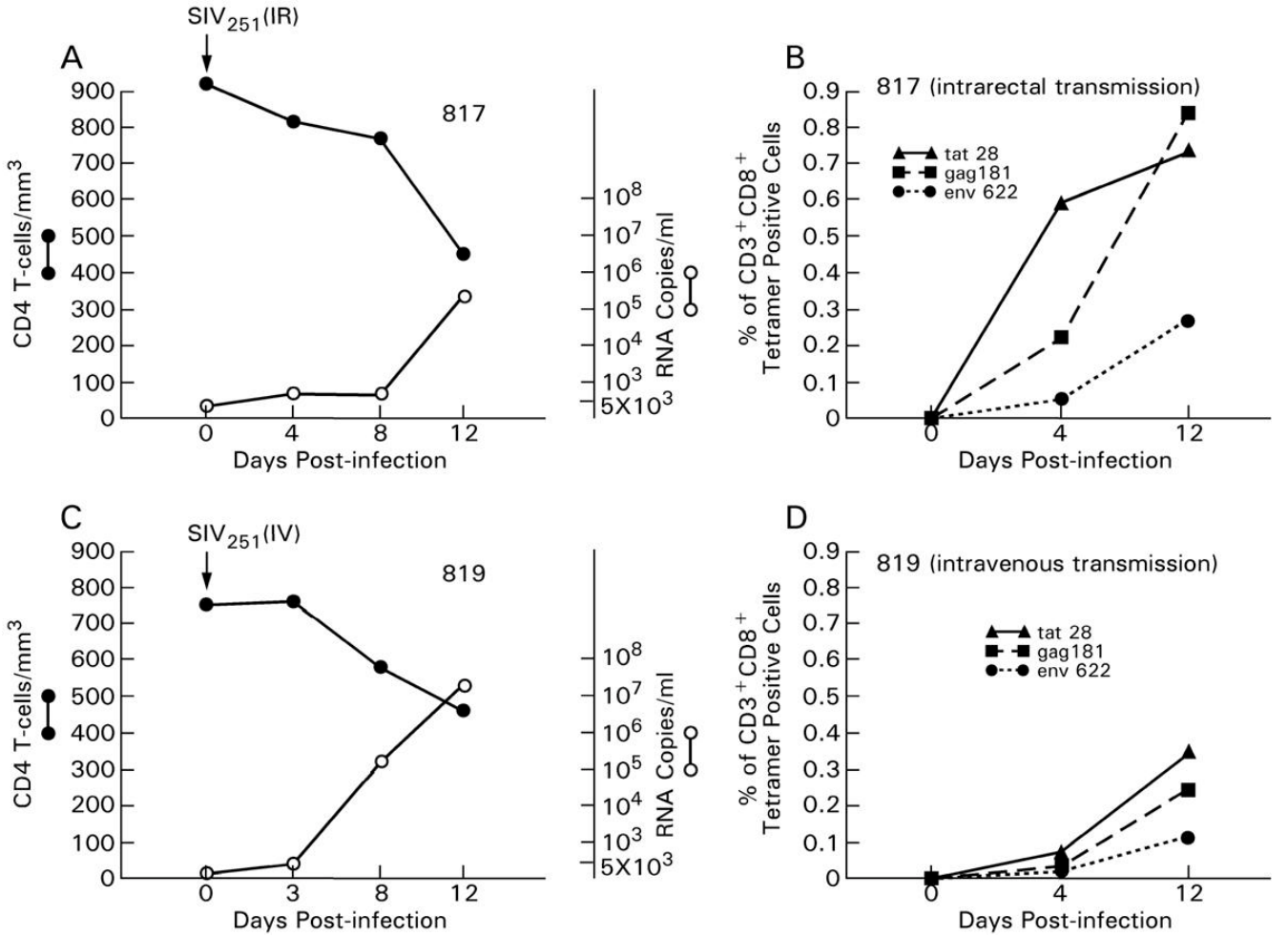

Figure 1

Plasma viral RNA appearance and the depletion of CD4+ T-cells were delayed (A) while virus-specific CD8+ T-cell response appeared earlier (B) in the animal infected intrarectally. The opposite pattern was observed in the animal infected intravenously (C, D). Plasma viral RNA was detected by NASBA. Total number of CD4+ T-cells was counted in the blood. Virus-specific CD8+ T-cell response was assessed by tricolor staining with CD3, CD8, and tetrameric complexes to Gag 181, Tat 28, and Env 622. CD8+ T-cells were defined by gating on CD3+ CD8+ cell population. An equivalent number of cells (1×10^4) was acquired through this gate in each analysis performed.

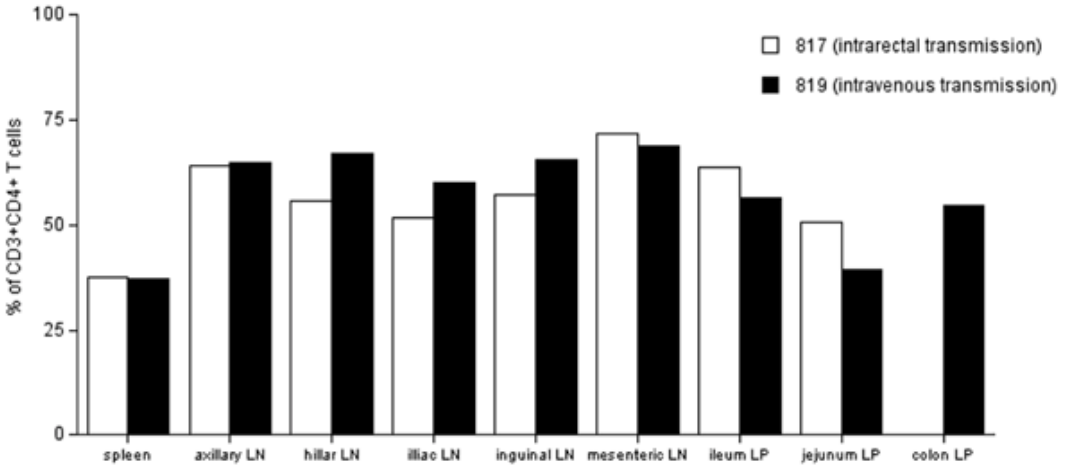

Figure 2
Depletion of CD4+ T-cells was not observed in tissues of either animal. The CD4+ T-cell population was defined by tricolor staining for CD3, CD4, and CD8. T-cells were defined by gating through CD3+ cells. An equivalent number of cells (1×10^4) was acquired through this gate for each analysis performed.

tenfold changes and is functional over a dynamic range spanning at least 10^4–10^7 copies.

Results

Kinetics of viral appearance and CD4+ T-cell decrease in the blood

Since the knowledge of the Mamu-A*01-restricted epitopes [32][33] allows for a quantitation of the CD8+ T-cell response using the tetramer technology [34], Mamu-A*01 macaques were chosen for this study. Following exposure to the same viral stock of SIV_{mac251} of macaque 817 by the intrarectal route and macaque 819 by the intravenous route, the kinetics of viral RNA appearance in the plasma was assessed by NASBA. Plasma viral RNA was detected as early as day 8 in animal 819, exposed by the intravenous route, and was highest at time of euthanasia (day 12), as demonstrated in Fig. 1C. In contrast, in animal 817, exposed by the intrarectal route, the appearance of plasma virus was delayed to day 12 postinfection (Fig. 1A) and at that time plasma viral RNA was 2 logs lower in animal 817 than in animal 819 (Figs. 1A and 1C). The absolute number of CD4+ T-cells/mm^3 blood decreased by 50% in both macaques by day 12 (Figs. 1A and 1C). However, the absolute number of CD8+ T-cells and CD3+ T-cells decreased as well, indicating that the apparent depletion of CD4+ T-cells may be due to redistribution rather than loss of CD4+ T-cells at this stage of infection.

Since animal 817 received three thousandfold more virus than animal 819, these data suggest that the kinetics of viral appearance in the blood in primary SIV_{mac251} infection may depend on the route of challenge rather than dose.

Virus-specific CD8+ T-cell response in the blood

Virus-specific CD8+ T-cells were quantitated in the blood of both animals at days 0, 4, and 12 postinfection using 3 tetrameric Mamu-A*01 molecules complexed with the SIV_{mac} Gag 181, Env 622, and Tat 28 peptides (T.M. Alien *et al.,* submitted).

In the blood, virus-specific tetramer-binding CD3+ CD8+ T-cells appeared earlier in macaque 817, exposed to SIV_{mac251} by the intrarectal route, than in 819 for all the 3 antigens studied (Figs. 1B and 1D). Overall, the size of the virus-specific CD8+ T-cell response in the blood was higher in animal 817 and, interestingly, a large population of Tat-specific CD8+ T-cells appeared in the blood as early as day 4, whereas in the same animal CD8+ T-cells specific to Gag 181 and Env 622 peaked later (Fig. 1). These data are consistent with previous observations on the kinetics of appearance of virus-specific CD8+ T-cells that recognize the Tat 28 epitope and underscore the importance of the immune response to this early SIV_{mac251} protein [35][36,37].

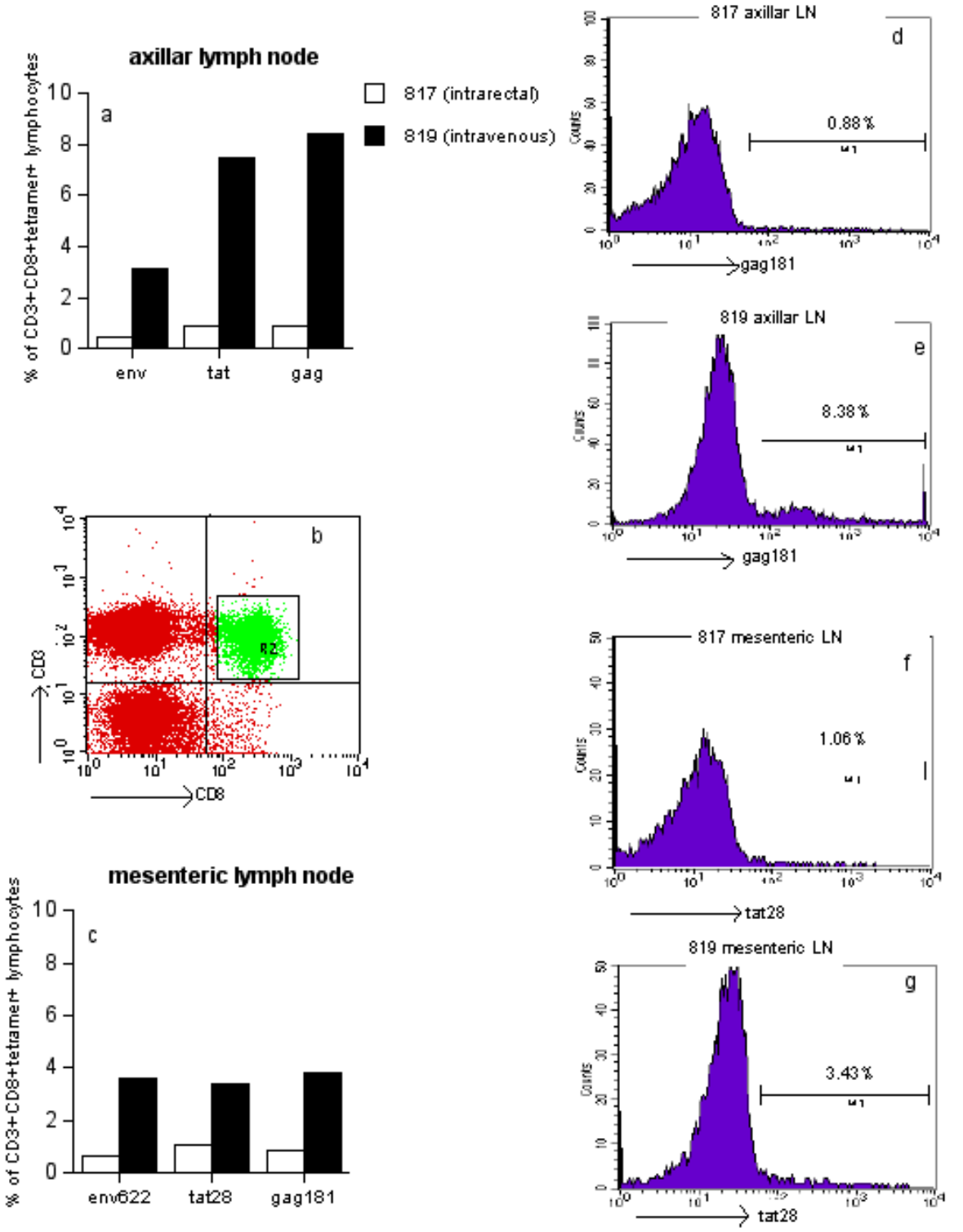

Figure 3
Tetramer-positive CD8+ T-cells were higher in axillary (A) and mesenteric lymph nodes (C) of the macaque infected intravenously (A, C, D, E, F, and G)12 days postinfection. The CD8+ T-cell population was defined by gating on CD3+ CD8+ lymphocyte population (B). The same number of cells (1×10^4), when possible, was acquired through this gate for every analysis performed.

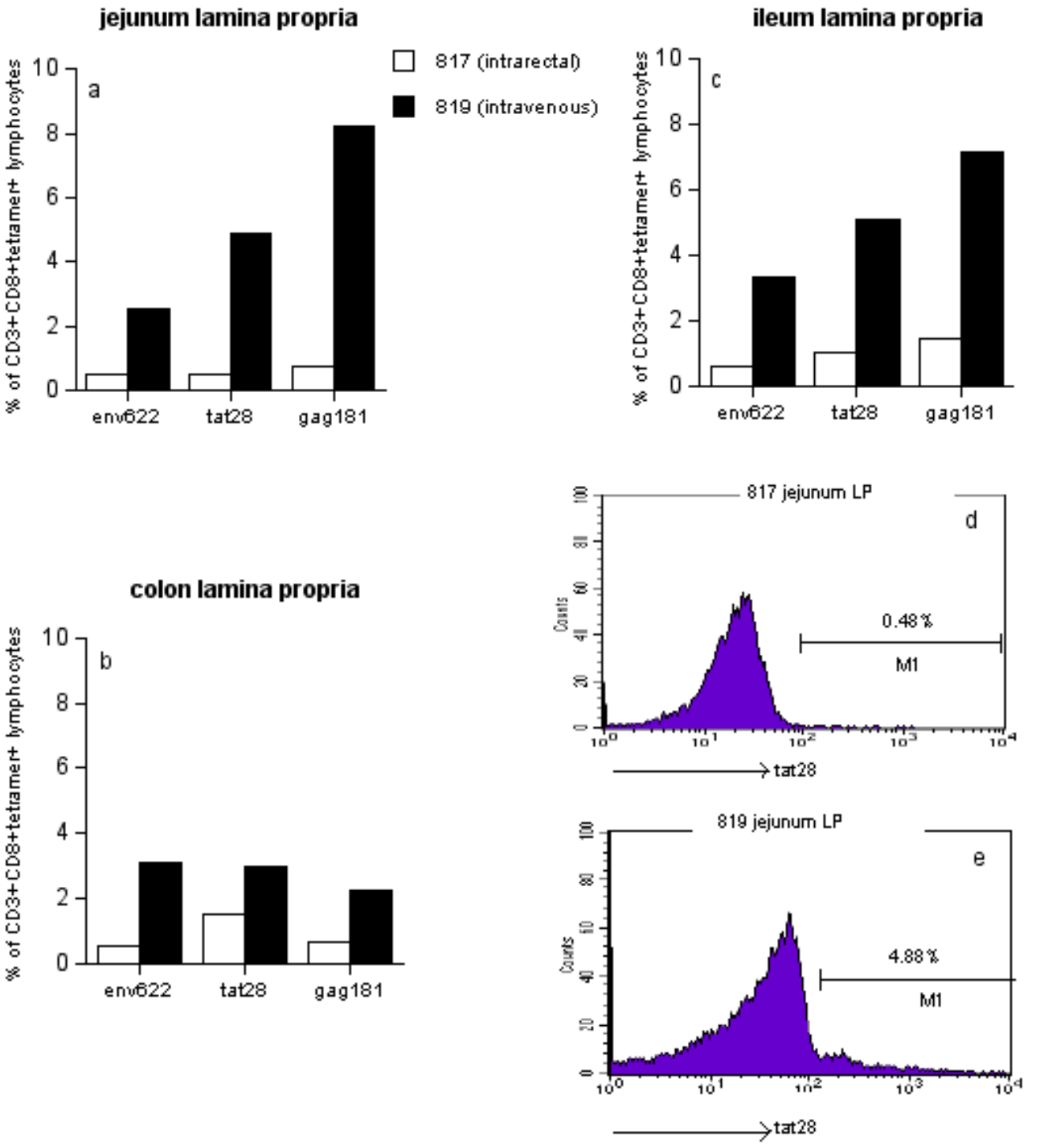

Figure 4
Tetramer-positive CD8+ T-cells from jejunum lamina propria (A), colon lamina propria (B), and ileum lamina propria (C) were higher in animal 819 that was infected intravenously. Figure D and E show the population of tat28+ cells in jejunum lamina propria of animal 817 (infected intrarectally, D) and animal 819 (infected intravenously, E).

Table 1:

a)

	817			819		
	env	tat	gag	env	tat	gag
Illiac sublumbar lymph node	0.35	0.91	0.37	0.64	0.91	0.42
Axillar lymph node	0.37	0.77	1.22	1.17	7.81	6.17
Hilar lymph node	0.92	0.74	0.86	1.04	1.16	0.95
Mesenteric lymph node	0.66	1.77	0.96	3.91	4.8	3.32
Inguinal lymph node	0.47	1.32	0.69	1.22	1.04	0.67

b)

	817			819		
	env	tat	gag	env	tat	gag
PBMC	0.27	0.74	0.84	0.12	0.35	0.24
spleen	0.61	0.60	0.56	0.55	0.23	1.4

c)

	817			819		
	env	tat	gag	env	tat	gag
Jejunum lamina propria	0.42	0.31	0.54	1.54	1.08	0.86
Ileum lamina propria	0.24	0.47	0.45	1.45	1.21	1.12
Colon lamina propria	0.28	1.17	0.42	2.89	0.73	2.21

*Absence of CD4+ T-cell depletion in the GALT of Mamu-A*01 macaques*

Both macaques were euthanized at day 12 and the relative percentages of CD4+ and CD8+ T-cells was assessed in various compartments. In contrast to previous reports [38] [39], depletion of CD4+ T-cells was not observed in any tissues, including the GALT of either animal (Fig. 2). It is possible that both animals could have controlled viral replication or that the tissues were examined too early and that depletion would have occurred at a latter stage of the infection. Preservation of CD4+ T-cell depletion in the GALT during primary infection may be a key event in delaying disease progression.

Quantitation of ex vivo CD8+ T-cell response in tissues

Virus-specific Gag 181 CD8+ T-cell response in the blood of macaques exposed intravenously to SIV_{mac251} appears to peak within the first weeks following intravenous exposure [40]. Therefore, both macaques were euthanized at day 12 and lymphocytes were harvested from several systemic tissues as well as the GALT. Quantitation of the ex vivo CD8+ T-cell population specific for Gag, Tat, and Env in the systemic tissues of both animals revealed that, in contrast to the blood, the highest virus-specific CD8+ T-cell response was found in most tissues of animal 819, challenged by the intravenous route (Figs. 3 and 4 and Table). In the same animal, the percentage of CD8+ T-cells specific for Tat 28 was as high as 8%, 6%, and 4% in the axillary and iliac lymph nodes and the spleen, respectively (Fig. 3A and Table). The number of CD8+ T-cells with different specificities was also higher in animal 819 than 817 in the GALT and particularly in the jejunum and ileum lamina propria (Figs. 4A,4B,4C) while the size of these virus-specific responses in the remaining tissues of both animals did not differ significantly (Table). These data indicate that both the Gag 181 and Tat 28 responses

are codominant and suggest that the overall higher CD8+ T-cell response in animal 819 may be related to an ongoing higher viral replication in the tissues of this animal, as reflected by the higher level of plasma viremia present at the time of euthanasia.

Differential proliferative ability of tetramer-binding CD8+ cells in tissues of animals 817 and 819

Blood, lymph nodes, and lamina propria lymphocytes were cultured *in vitro* in the presence of IL-2 and the virus-specific Gag 181, Tat 28, and Env 622 peptides to assess the ability of the tetramer-positive CD8+ T-cell population to expand upon antigen stimulation. In our experimental conditions, expansion of CD8+ T-cells specific for Tat, Env, and Gag occurred to a comparable extent in most tissues and was highest in the blood of animal 819 (Fig. 5B). In animal 817, the ability of virus-specific CD8+ T-cells to proliferate was highest in the jejunum and was higher than that observed in the same compartment in animal 819 (Fig. 5A). The finding that the highest level of CD8+ T-cells with proliferative ability was in the lamina propria of the jejunum of animal 817 challenged intrarectally is suggestive of a qualitative difference of CD8+ T-cells present at the site of induction and of heterogeneity in the virus-specific CD8+ response.

Discussion

The impact that the route of viral entry of HIV/SIV may have on the virus-specific immune response and control of viral replication to it is not yet fully understood. Here we conducted a small pilot study using genetically defined Mamu-A*01 macaques to study the vims-specific immune response following parenteral or rectal exposure to SIV_{mac251} This study was designed to begin to investigate the specificity of the immune response and its kinetics as well as the kinetics of viral replication according to the site of primary exposure and viral entry.

Within the limitation of the small number of animals used here, the mucosal barrier delayed the appearance of virus in the plasma whereas the decrease in the absolute CD4+ T-cell counts was equivalent. In addition, in contrast to other reports [41] [38], depletion of CD4+ T-cells in the GALT at day 12 postinfection was not observed in either animal regardless of the route of challenge. It is unclear what is the reason for this finding. It is possible that both animals could have controlled the infection or that the tissues were examined too early and that depletion would have occurred at a latter stage of the infection.

The finding of a higher percentage of tetramer-positive CD8+ T-cells in tissues of macaque 819, infected intravenously, than in animal 817, infected intrarectally, may reflect a higher rate of viral replication in tissues of this

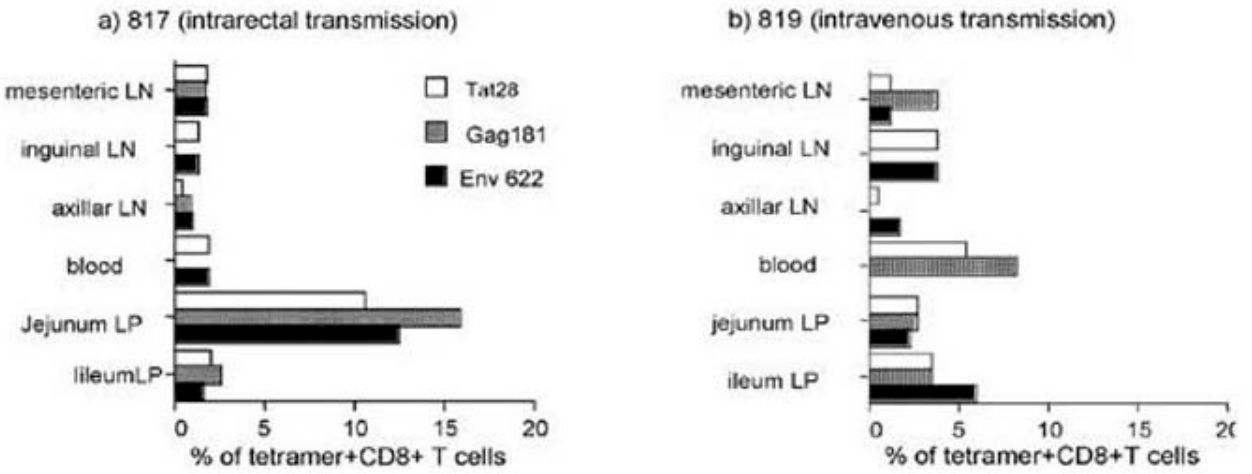

Figure 5
Proliferative ability of tetramer-binding CD8+ cells is higher at the site of primary virus encounter. Cells were cultured for 7 days in the presence of IL-2 and Gag 181, Tat 28, or Env 622 peptide. The percentage of tetrameric Mamu-A*01/Gag 181; Tat 28; and Env 622 complex binding CD8+ T-cells was established by tricolor staining, as described previously.

animal at the time of sacrifice. Of interest, the intrarectal route of infection resulted in faster kinetics of appearance of virus-specific CD8+ T-cells, particularly in the Tat epitope in the blood, indicating the importance of this immune response early in infection. In the same animal, a higher number of tetramer-specific CD8+ T-cells able to proliferate in response to all epitopes tested (Gag 181, Tat 28, and Env 622) was found in jejunum lamina propria than in other tissues. Unfortunately, at present, it is not possible to assess whether the differences in the proliferative ability of these cells reflect functional status and/or differentiation differences (effector versus memory) or others, in part because markers to define accurately these cells in rhesus macaques are not available.

Conclusions

In conclusion, this study, within the limitation of the small number of animals used, appears to suggest that exposure by the mucosal site, a natural barrier to pathogens, delays viral appearance in the blood. The differences in the relative percentage of homing markers may not necessarily reflect a true qualitative difference in the overall lymphocyte trafficking related to the mode of viral encounter and may depend on the time of analysis, since the kinetic of viral replication was delayed in the macaque exposed intrarectally. Nevertheless, these data demonstrate that a short window of opportunity to contain viral infection following mucosal exposure exists and that potentiating the effectiveness of the mucosal natural barrier by local immunization may further limit or halt viral replication. In fact, in a previous study, we have observed that it appears to be easier to protect vaccinated macaques by intrarectal exposure than intravenous exposure to SIV_{251} [42]. In particular, since a CD8+ T-cell response to Tat appears to be the earliest, this protein may be a key component of a preventive vaccine, as suggested by other [35][36,37,43].

List of abbreviations

HIV (human immunodeficiency virus)

HEV (high endothelial venules)

PBMC (peripheral blood mononuclear cells)

Declaration of Competing Interests

Competing interests: none declared.

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