Monitoring of Vaccine-Specific Gamma Interferon Induction in Genital Mucosa of Mice by Real-Time Reverse Transcription-PCR

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Monitoring of T-cell responses in genital mucosa has remained a major challenge because of the absence of lymphoid aggregates and the low abundance of T cells. Here we have adapted to genital tissue a sensitive real-time reverse transcription-PCR (TaqMan) method to measure induction of gamma interferon (IFN-γ) mRNA transcription after 3 h of antigen-specific activation of CD8 T cells. For this purpose, we vaccinated C57BL/6 mice subcutaneously with human papillomavirus type 16 L1 virus-like particles and monitored the induction of CD8 T cells specific to the L1_{165-173} H-2D^b-restricted epitope. Comparison of the responses induced in peripheral blood mononuclear cells and lymph nodes (LN) by L1-specific IFN-γ enzyme-linked immunospot assay and TaqMan determination of the relative increase in L1-specific IFN-γ mRNA induction normalized to the content of CD8b mRNA showed a significant correlation, despite the difference in the readouts. Most of the cervicovaginal tissues could be analyzed by the TaqMan method if normalization to glyceraldehyde-3-phosphate dehydrogenase mRNA was used and a significant L1-specific IFN-γ induction was found in one-third of the immunized mice. This local response did not correlate with the immune responses measured in the periphery, with the exception of the sacral LN, an LN draining the genital mucosa, where a significant correlation was found. Our data show that the TaqMan method is sensitive enough to detect antigen-specific CD8 T-cell responses in the genital mucosa of individual mice, and this may contribute to elaborate effective vaccines against genital pathogens.

The mucosal surfaces, such as those of the genital tract, are common entry sites for many sexually transmitted infectious agents like human immunodeficiency virus, herpes simplex virus, human papillomaviruses (HPV), or chlamydia. The generation of mucosa-specific cytotoxic T-cell responses thus plays a crucial role in the defense against infection or tissue dissemination of these pathogens. However, the monitoring of specific T-cell responses in the genital mucosa has been difficult because of the absence of lymphoid aggregates and the low abundance of T cells in such tissues. Antigen-specific gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays or chromium release cytotoxicity assays using genital tissues have been reported (4, 6, 7, 14), but they required animal pools or large surgical pieces together with in vitro antigen-specific restimulation/expansion steps, which may bias the magnitude and quality of the initial immune response. Alternatively, knowledge of antigen-specific T-cell receptor sequences has allowed direct ex vivo detection of CD8 T cells in mucosal tissues by reverse transcription (RT)-PCR (15), but this type of assay may not reflect the functional status of the cells. Recently, studies on the priming/trafficking of specific CD8 T cells in the mouse genital tract using flow cytometry analysis were reported (12, 20), but this required the in vivo transfer of T cells expressing a particular antigen-specific T-cell receptor into recipient mice, which cannot be easily translated to other antigen/pathogen systems. IFN-γ expression/secretion is an early and functional response of antigen-activated CD8 T cells, and here we have exploited the high sensitivity of real-time RT-PCR analysis using TaqMan chemistry to measure a 3-h antigen-driven IFN-γ induction in CD8 T cells isolated from the genital mucosa of individual mice. C57BL/6 mice were vaccinated subcutaneously (s.c.) with HPV type 16 (HPV16) virus-like particles (VLPs) as a model antigen, and induction of IFN-γ mRNA expression in response to stimulation with the H-2D^b-restricted L1_{165-173} epitope (17) was measured after normalization to a noninducible molecule. We first analyzed samples (lymph node [LN] cells and peripheral blood mononuclear cells [PBMC]) in which the conventional IFN-γ ELISPOT assay technique could be run in parallel to ensure that the two methods yield similar measurements of the L1-specific CD8 T-cell response. The results obtained with cervicovaginal (CV) tissues by TaqMan were further compared to results obtained by IFN-γ ELISPOT assay of PBMC and LN from individual animals. Our findings illustrate the importance of determining the immune response in genital tissue, and not only in the periphery, in the design of efficient vaccine strategies to fight against genital pathogens.

MATERIALS AND METHODS

Immunization of mice. Six- to 8-week-old female C57BL/6 mice (Iffa Credo; France) were used in all experiments, and the ethical directives of the Swiss veterinary authorities were followed. Baculovirus-derived purified HPV16 VLPs were administered s.c. into the back at the base of the tail (2, 16). A dose of 75

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or 100 µg, depending on the VLP stock, was delivered in a 100-µl inoculum volume. For each experiment, the number of mice per group is mentioned in Results.

Preparation of LN and CV tissue suspensions. Mice were sacrificed by inhalation of CO₂, and LN and genital tracts were harvested. Single-cell suspensions were obtained by pressing the LN onto a 70-µm filter (Falcon) with a syringe piston and subsequently passing the cells through a 40-µm filter (Falcon). Dissociated cells were resuspended in complete high-glucose Dulbecco’s modified Eagle medium containing glutamax-1 and sodium pyruvate supplemented with 10 mM HEPES, 1/10 nonsolvent amino acids, 100 U of penicillin-streptomycin/ml, 10% fetal calf serum (FCS) (all from Invitrogen), and 20 µM 2-mercaptoethanol (Sigma).

For preparation of CV cells, the uterus horns were removed. The remaining cervix and vagina were minced and washed twice in extraction buffer (Hanks-balanced salt solution and 10 mM dithiothreitol). Minced tissues were then digested with 0.5 mg/ml thermolysin (Roche) in extraction buffer for 45 min at 4°C under agitation and then filtered through 150-µm-pore-diameter nylon filters. Isolated cells were kept at 4°C, and the remaining tissues were digested with 1 mg/ml collagenase/dispase (Roche) in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and 10 mM HEPES, 10 mM HEPES, 1/10 nonsolvent amino acids, 100 U of penicillin-streptomycin/ml, and 20 µM 2-mercaptoethanol (Sigma).

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Quantification of the cDNAs was performed with the TaqMan RT reagents (P/N N808-0234; Applied Biosystems) in 23-µl final volumes. Aliquots of 5 µl of cDNAs were directly used in duplicate for the quantification of IFN-γ and CD8b, while cDNA was diluted 20-fold for GAPDH quantification. Results were considered only when the difference between the Ct obtained for the duplicates was less than 1. In case the difference exceeded 1, the RT-PCR was performed again with a new cDNA aliquot of the same sample. Normalization of samples was performed by dividing the amount of IFN-γ cDNA by the amount of CD8b or GAPDH cDNA, depending on the experiments. Relative increases in IFN-γ were calculated by dividing the normalized number of IFN-γ cDNA copies in L1-stimulated wells by those measured in medium-stimulated wells. If samples were stimulated with an unrelated HPV peptide instead of medium alone, the baseline normalized IFN-γ cDNA levels were similar (data not shown). Therefore, medium alone was used as a control in the experiments described here.

Statistical analysis. Spearman correlation analysis with two-tailed P values was performed with Prism 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Method overview. The amplitude of the antigen-specific CD8 T-cell response observed after vaccination was determined by the induction of IFN-γ mRNA expression that could be achieved after 3 h of ex vivo antigen-specific stimulation. A real-time RT-PCR was set up to determine the quantities of IFN-γ, CD8b, and GAPDH mRNAs by a two-step method that consisted of RT with random hexamers, followed by a real-time PCR with the specific primer, reverse primer, and reporter dye FAM (6-carboxyfluorescin), and the CD8b and GAPDH probes were labeled with TET (tetrahydro-6-carboxyfluorescin). Reaction mixtures had a total volume of 25 µl, and the thermal cycler parameters included 2 min at 50°C, 10 min at 95°C, and 45 cycles involving denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Real-time monitoring of fluorescence emission from cleavage of sequence-specific probes by the nuclease activity of Taq polymerase allowed definition of the cycle threshold (Ct) during the exponential phase of amplification. The Ct was defined as the cycle at which fluorescence exceeds 10 times the SD above the mean of the background of the early cycles. All real-time PCR assays were performed in duplicate, and the mean cDNA quantities were calculated. Standard curves were generated for each gene quantified with 10-fold dilutions of cloned cDNAs (see below). The reagents were found to have good PCR amplification efficiency as determined by the slope of the standard curves (between −3.3 and −3.7), and linear regression analysis showed that all standard curves had an R² value of >0.99. To determine the intra-assay variability of the real-time PCR, 10-fold serial dilutions of CD8b, IFN-γ, and GAPDH cDNAs (ranging from 10³ to 10⁶ copies) were amplified in quadruplicate in the same run. For interassay variability, the same cDNA dilutions were analyzed in six different experiments performed over a 1-month period.

Determination of antigen-specific relative increase in IFN-γ by real-time RT-PCR. Isolated cells (50,000 to 400,000/well) were incubated in round-bottom 96-well plates (Nuncatod 16332) in the presence of the L1-165,173 peptide at 1 µg/ml for 3 h at 37°C in 5% CO₂. This peptide pulse duration appeared to be optimal, as additional 2 h of incubation, the IFN-γ mRNA amount was decreased more than threefold (data not shown). Cells were harvested, and RNA was extracted with an RNeasy mini-kit (Qiagen), eluted in 30 µl of H₂O₂ and kept at −20°C. Aliquots of 0.5 µg of purified RNA were reverse transcribed with random hexamers with the TaqMan RT reagents (P/N N808-0234; Applied Biosystems) in 23-µl final volumes. Aliquots of 5 µl of cDNAs were directly used in duplicate for the quantification of IFN-γ and CD8b, while cDNA was diluted 20-fold for GAPDH quantification. Results were considered only when the difference between the Ct obtained for the duplicates was less than 1. In case the difference exceeded 1, the RT-PCR was performed again with a new cDNA aliquot of the same sample. Normalization of samples was performed by dividing the amount of IFN-γ cDNA by the amount of CD8b or GAPDH cDNA, depending on the experiments. Relative increases in IFN-γ were calculated by dividing the normalized number of IFN-γ cDNA copies in L1-stimulated wells by those measured in medium-stimulated wells. If samples were stimulated with an unrelated HPV peptide instead of medium alone, the baseline normalized IFN-γ cDNA levels were similar (data not shown). Therefore, medium alone was used as a control in the experiments described here.

Statistical analysis. Spearman correlation analysis with two-tailed P values was performed with Prism 4.00 for Windows (GraphPad Software, San Diego, CA).
CD8b as CD8 T cells are those that express L1165-173-inducible-as the mean relative increase in L1165-173-specific IFN-

CV tissues from five naive mice to assess the background levels of

ELISPOT assay results of a compilation of four experiments in

3.1% for GAPDH and were therefore comparable for each

GAPDH (Table 1). The interassay coefficients of variation ranged from 1.5 to 2.9% for IFN-γ, 1.3 to 3.6% for CD8b, and 1.5 to

Time course analysis of L1-specific CD8 T-cell response in

PBMC after s.c. vaccination with HPV16 VLPs. To set up a method that allows determination of the antigen-specific CD8 T-cell response in the genital mucosa of individual mice, we evaluated first the amplitude and kinetics of this response in PBMC by conventional IFN-γ ELISPOT assay. The aim was twofold: first to determine the peak of the CD8 T-cell response in PBMC in order to analyze the immune response in the genital mucosa at a time when a maximal number of CD8 T cells are migrating to effector sites and second to generate a great number of samples/data for comparing the conventional method to the new TaqMan method. Figure 1 shows the L1165-173-specific IFN-γ ELISPOT assay results of a compilation of four experiments in which a total of 41 mice were vaccinated once with 75 or 100 µg of HPV16 VLPs s.c. at day 1, sampled on 2 consecutive days between day 4 and day 8, and finally sacrificed between day 7 and day 9. The L1-specific response peaks at day 7 and then tends to decrease over the next days, meaning that a maximal number of L1-reactive CD8 T cells circulates in the periphery at this time point and is therefore susceptible to reach peripheral tissues such as the genital tract.

Correlation between the data obtained by the TaqMan method and the conventional IFN-γ ELISPOT assay in the periphery. As a further step in the validation of the TaqMan method, we compared the immune responses induced in PBMC and LN by conventional ex vivo L1165-173-specific IFN-γ ELISPOT assays and TaqMan determination of the relative increase in L1165-173-specific IFN-γ mRNA normalized to the content of CD8b mRNA. PBMC samples of 21 mice s.c. immunized with 100 µg of HPV16 VLPs, for which the IFN-γ ELISPOT assay results are shown in Fig. 1, were also analyzed by the TaqMan method. Figure 2A shows the data for samples taken at different time points between days 7 and 9. Cells isolated from pools of specific LN (cervical, ili-o-sacral, inguinal, mesenteric, and brachial) of the same mice were also analyzed by both methods (Fig. 2B). Despite the difference between the readouts (the number of IFN-γ secreting cells versus the increase in IFN-γ mRNA expression), the two methods showed a significant correlation (Spearman ρ = 0.68 and 0.77 for PBMC and LN, respectively; P < 0.0001), suggesting that the two methods yield similar measurements of antigen-specific CD8 T-cell responses. The TaqMan method may thus advantageously replace the classical IFN-γ ELISPOT assay for the investigation of vaccine-induced CD8 responses in individual CV tissue samples, where limited numbers of cells, which harbor a low immune cell content (<1% of T cells; data not shown), are available.

Analysis of the L1165-173-specific CD8 response in CV cells by the TaqMan method after s.c. vaccination of mice with HPV16 VLPs. Analysis of L1-specific IFN-γ induction in CV tissue samples was performed by the TaqMan method with 20 mice that were immunized s.c. with 75 µg of HPV16 VLPs at day 1 and sacrificed at day 7, for which the IFN-γ ELISPOT

### Table 1. Intra- and interassay variations

| cDNA and no. of copies | Intra-assay variation | Interassay variation |
|------------------------|-----------------------|----------------------|
|                        | Mean Ct (SD)          | Mean Ct (SD)         |
|                        | CV (%)                | CV (%)               |
| IFN-γ                  | 24.20 (0.11)          | 24.38 (0.72)         |
| 10^5                   | 0.47                  | 2.90                 |
| 10^4                   | 27.74 (0.47)          | 27.78 (0.81)         |
| 10^3                   | 31.13 (0.27)          | 31.19 (0.82)         |
| 10^2                   | 34.80 (0.57)          | 34.89 (0.53)         |
| 10^1                   | 37.82 (0.83)          | 37.98 (0.69)         |
| CD8                    | 20.91 (0.31)          | 21.22 (0.31)         |
| 10^5                   | 1.50                  | 1.46                 |
| 10^4                   | 24.65 (0.34)          | 24.78 (0.45)         |
| 10^3                   | 28.02 (0.43)          | 28.64 (0.37)         |
| 10^2                   | 31.55 (0.38)          | 31.93 (0.53)         |
| 10^1                   | 36.15 (0.31)          | 35.76 (1.30)         |
| GAPDH                  | 21.12 (0.14)          | 21.44 (0.64)         |
| 10^5                   | 0.66                  | 2.90                 |
| 10^4                   | 24.76 (0.26)          | 25.17 (0.45)         |
| 10^3                   | 28.32 (0.25)          | 28.70 (0.44)         |
| 10^2                   | 31.49 (0.22)          | 32.18 (0.62)         |
| 10^1                   | 35.14 (1.23)          | 36.64 (1.14)         |

* Input number of standard cDNA copies.

* CV, coefficient of variation.
The assay results of PBMC samples are shown in Fig. 1. The amount of RNA recovered from CV cells allowed us to perform three independent RT-PCR runs for each sample and each gene of interest (IFN-γ, CD8b, and GAPDH). When relative increases in IFN-γ induction were calculated after normalization to CD8b, only 15 out of 20 samples could be analyzed because in the remaining 5 samples, the CD8b copy number was too low to be reproducibly detectable after three attempts (Fig. 3A). When normalization to GAPDH was performed, 18 samples could be analyzed because in the other 2 samples, the number of IFN-γ cDNA copies measured was also very low and thus not reliable. Five mice were found to have a relative increase in IFN-γ in the CV tissues above the significance threshold after normalization to both CD8b and GAPDH. An additional mouse, for which the CD8b copy number was too low to be reproducibly detectable, was also positive after normalization to GAPDH. In one mouse, the relative increase in IFN-γ was shown to be positive after normalization to CD8b (4.8-fold) and not after normalization to GAPDH (2.7-fold), and in another mouse, it was the opposite (1.6-fold when normalized to CD8b and 4.3-fold when normalized to GAPDH), but the values were close to the respective detection limits. The mean relative increases calculated according to both normalization methods were very similar. Moreover, there was a strong correlation between the relative increases calculated by the two methods (Fig. 3B). In conclusion, out of 20 mice, 18 samples of CV tissues incubated with medium alone. Dashed lines indicate respective thresholds of significance. (B) Relative increases in L1-specific IFN-γ obtained for individual animals after normalization to GAPDH (horizontal axis) were plotted against the relative increases obtained after normalization to CD8b (vertical axis).
In a subset of 10 mice, the L1-specific IFN-γ ELISPOT assay response was, in addition, examined in the cervical and sacral LN (Fig. 4B). As reported by Soderberg et al., we found a sacral LN in 7/10 mice; interestingly, there was a strong correlation (Spearman \( r = 0.93, P < 0.01 \)) between the relative increases in L1-specific IFN-γ mRNA measured in CV tissue and the number of L1-specific IFN-γ spots detected in the sacral LN, but not with the number of spots enumerated in the cervical LN (Fig. 5B). In conclusion, the L1-specific response measured by the TaqMan method in the CV tissue correlates only with the response detected in the sacral LN and not with the response measured in the PBMC or lumbar cervical LN.

**DISCUSSION**

Monitoring of vaccine-induced T cells in the genital tract has rarely been done. This is mainly due to the paucity of immune cells that can be recovered from the genital mucosa of individual mice and the relatively high number of cells necessary to perform the conventional assays generally used to detect activated vaccine-specific CD8 T cells. Here we were interested in exploiting a sensitive method to quantify the expression of IFN-γ mRNA as a measurement of CD8 T-cell activation in CV cell samples from individual mice. We chose the HPV16 VLP as a model antigen and first determined the kinetics of the CD8 T-cell response specific for a known CTL epitope of HPV16 L1, L1165-173, in the PBMC of mice by the IFN-γ ELISPOT assay. We found that the L1165-173-specific response peaked at day 7 after s.c. vaccination with VLPs, which is in accordance with the kinetics observed after infection with viruses such as lymphocytic choriomeningitis virus (10) and influenza virus (5, 9) or after vaccination with synthetic peptides (13). We also observed that the highest number of specific CD8 T cells was present at the same time point in the spleen; however, the response declined less rapidly in that organ (data not shown). Analysis of PBMC and LN samples by IFN-γ ELISPOT assay and real-time RT-PCR in parallel showed that despite the difference between the readouts (the number of IFN-γ-secreting cells after overnight stimulation versus the increase in IFN-γ mRNA expression after 3 h of stimulation), the data obtained by the two methods present a significant correlation. Interestingly, out of 32 PBMC samples analyzed, 23 were scored positive and 7 were negative by both methods and only 2 were scored negative with the ELISPOT assay but showed positive, albeit small (3- and 7.1-fold), relative increases in IFN-γ by real-time RT-PCR. Out of the 22 LN samples, 17 were scored positive and 3 were negative by both methods and only 2 were scored negative by the TaqMan method but harbored a significant, although low (67 and 63 spots/10⁶ cells), number of L1-specific IFN-γ-secreting cells in the ELISPOT assay. Therefore, 92.6% of the samples showed concordant results by both methods; the four remaining samples were near the threshold of significance of both techniques and appeared to be positive by either the TaqMan or the ELISPOT assay. Thus, as previously reported (25), the two assays appear to be comparable in sensitivity. In our experience, around 50 L1-specific spots/10⁶ PBMC or LN cells can be routinely measured with the IFN-γ ELISPOT assay when as few as 100,000 cells/well are used, which corresponds to a

![Graph A](image1.png)

**FIG. 4.** L1-specific IFN-γ ELISPOT assay for PBMC and LN of mice immunized with HPV16 VLPs. PBMC and lumbar LN cells of 20 VLP-immunized mice (A) and cervical and sacral LN of 10 VLP-immunized mice (B) were evaluated for L1165-173-specific IFN-γ secretion by ELISPOT assay. Results are shown as the number of L1-specific IFN-γ spots/10⁶ cells. Dashed lines indicate respective thresholds of significance.

Correlation of the L1-specific TaqMan response of CV cells with the IFN-γ ELISPOT assay response in the sacral LN. In the same 20 mice in which we investigated the L1-specific IFN-γ response in CV cells by the TaqMan method, we analyzed the L1-specific IFN-γ response by ELISPOT assay in the cervical LN (distant from the site of immunization) and the LN of the ilio-sacral region, in addition to PBMC. According to Soderberg et al., these latter LN comprise the lumbar LN (two or three LN) and the sacral LN (23). This sacral LN is located just underneath the lumbar LN and has been described as a “mucosal” LN because, in contrast to the lumbar LN, for example, it is maintained in lymphoexin-β knockout mice, which are known to harbor only mucosal LN (23). In addition, the high endothelial venules of the sacral LN express the mucosal addressin MadCAM-1. All of the mice harbored L1165-173-specific IFN-γ spots in the PBMC and lumbar LN (Fig. 4A). Interestingly, we found no correlation between the magnitude of the responses detected in these immune compartments by IFN-γ ELISPOT assay and the induction of IFN-γ mRNA measured in CV tissue by the TaqMan method (Fig. 5A). Indeed, the mice in which we could observe L1-specific IFN-γ mRNA induction in the genital cells were not the ones that presented the highest ELISPOT assay response in the periphery.

![Graph B](image2.png)
frequency of 1 specific CD8 T cell out of a total of 20,000 cells. This
is indeed close to what we observed by TaqMan analysis; i.e., 2 cells
from a CD8 T-cell clone specific for a defined peptide were
detected among 100,000 unrelated cells not producing IFN-γ
(corresponding to a frequency of 1/50,000; unpublished results).
We have to keep in mind, however, that individual effector cells
likely produce less IFN-γ upon specific stimulation than do T-cell clones; therefore, their
detection frequency may be lower. PBMC and LN cell populations
harbor >10% CD8 T cells, which is at least 1 log higher than the
level in CV cells. Tenfold more CV cells/well may thus be
needed in an IFN-γ ELISPOT assay to detect immune
responses close to the threshold of significance. Such a number
of cells is clearly not achievable from a single mouse and
cannot be handled in 96-well plates without leading to high
background levels (unpublished observations). In conclusion,
the main advantage of the real-time RT-PCR assay resides in
the capacity to analyze smaller samples than those necessary
for the ELISPOT assay or tetramer or flow cytometry analysis.
Handling of cells for TaqMan analysis takes longer and re-
quires multiple steps (such as extraction of total RNA, RT, and
real-time PCR), compared to the ELISPOT assay. The latter
will therefore remain the method of choice for the analysis of
larger cell samples such as those isolated from the blood,
spleen, and LN, although a real-time RT-PCR technique has
already been set up to measure vaccine-induced IFN-γ mRNA
in PBMC from melanoma patients previously vaccinated with
a synthetically modified peptide epitope from the melanoma
self-antigen gp100 (11). Here, we measured the induction of
IFN-γ mRNA in response to a particular peptide, L1_{165-173},
after vaccination with HPV16 VLPs as a model antigen.

Rather than targeting the response to a particular CTL

![Correlation TaqMan/ELISPOT](image-url)
epitope, a more general pattern of activated CD8 T cells could be analyzed by using antigen-presenting cells that have been engineered to express entire protein antigens or overlapping peptides spanning these antigens during the in vitro 3-h stimulation step. This would bypass the need to identify immunodominant CTL epitopes and therefore to be limited to particulation step. This would bypass the need to identify immunopeptides spanning these antigens during the in vitro 3-h stimulation. Application of the real-time RT-PCR method described here to human samples is therefore feasible, especially when a small amount of material is available, as in the case of tissue biopsy. In such a situation, it could be necessary to perform an in vitro expansion of the biopsy-isolated cells before the 3-h antigen exposure to reach a detectable number of vaccine-activated cells.

The ability to investigate the L1-specific IFN-γ mRNA induction in CV cells of individual mice by TaqMan analysis allowed us to compare the responses elicited in different immune compartments after vaccination with VLPs. Our data clearly show that the L1-specific immune response measured in genital tract cells only correlates with the response measured in the sacral LN and not with that detected in the population of circulating lymphocytes, as well as in the distant cervical LN and the local lumbar LN. It has to be noted that there is an ambiguity concerning the murine LN nomenclature in the scientific literature (24). In particular, several publications refer to the iliac LN but without clearly describing which LN were indeed taken. Our results underline the existence of a compartmentalization of the immune response in the anatomically juxtaposed LN of the ilio-sacral region, and they suggest that the sacral LN may represent a surrogate of the genital tract when analyzing the T-cell response. Alternatively, there may be a correlation between the L1-specific immune responses measured in the genital tract and the sacral LN because this particular LN, in contrast to the other LN of the iliac region, does not drain the s.c. immunization site we used in our experiments. Thus, the L1-specific CD8 T cells detected in the lumbar LN comprise, in addition to genital lymphocytes, lymphocytes preferentially recirculating through these LN because they have been primed there. The immune response observed in the lumbar LN would therefore not uniquely reflect the response detected in the genital tract. Knowing that four or five LN are localized in the ilio-sacral region and that they differ in their mechanisms of development, as well as in the expression of addressins on their high endothelial venules (19), further studies are needed to address the difference in the behavior of the lymphocytes present in these LN and their role in providing immunity to the genital mucosa. Development of sensitive assays to monitor the induction of immune responses in the genital tract is of particular relevance in order to fight against infection with many sexually transmitted infectious agents, such as human immunodeficiency virus, herpes simplex virus, HPV, or chlamydia. Measurements of immunological parameters have almost always focused on the systemic immune system, while vaccines capable of protecting against those pathogens most likely need to induce long-term mucosal immune responses, as the mucosal surfaces are the most natural routes for their transmission. The real-time RT-PCR approach described here provides a practical method to analyze the CD8 immune responses elicited in the genital tract of mice after vaccination. It may contribute to the optimization of immunization regimens in preclinical studies to better understand the quantitative and qualitative aspects of the immune responses that are needed to achieve an effective clinical response. In addition, our results suggest that determining the immune response in the genital tissue (or the sacral LN as a potential surrogate of the genital mucosa) and not only in the periphery may help in designing efficient vaccine strategies to fight against genital pathogens.

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