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# Non-replicating mucosal and systemic vaccines: quantitative and qualitative differences in the Ag-specific CD8$^+$ T cell population in different tissues

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**Abstract**

Directed dissemination of Ag-specific CD8$^+$ T cells to infected organs or cancerous tissues is a prerequisite for optimal immunotherapy. Ag-specific CD8$^+$ T cells were quantitated in systemic and mucosal tissues after nasal, rectal, or cutaneous immunization with CTL epitope peptide and the adjuvant cholera toxin (CT). Mucosal and cutaneous immunization induced Ag-specific CD8$^+$ lymphocytes that were detectable in both mucosal and systemic compartments, suggesting a less strict distribution pattern than that known for B cells. However, optimal localization, activation and phenotype of these cells correlated with the route of immunization. In accordance with this observation, protection against a mucosal challenge with a virus expressing the CTL epitope was superior in mucosally-immunized animals.


*Keywords:* Mucosal/systemic vaccines; Non-replicating; CT; CTL; Virus; Cancer

Immunization strategies that induce homing and localization of Ag-specific lymphocytes to mucosal tissues may be required for protection against pathogens that infect at mucosal tissues or cancers that develop at or metastasize to mucosal tissues. The route of immunization might influence the type, magnitude and localization of Ag-specific immunity within mucosal and systemic compartments. We have reported that nasal immunization, but not systemic immunization, induced Ag-specific IgA in mucosal secretions [1,2]. These studies and others have suggested that systemic route of immunization, with a non-replicative vaccine, generates Ag-specific Ab-secreting cells that may not travel to mucosal organs [1–3]. We and others have also reported that mucosal immunization with a non-replicative vaccine, composed from peptide immunogen and CT adjuvant, induced Ag-specific CTL in mucosal and systemic compartments [4–6]. Belyakov et al. further showed that systemic immunization with IFA + peptide induced CTL responses that were detected in systemic tissues only [5]. Similarly, we compared CT + peptide nasal vaccine to CFA + peptide cutaneous vaccine. We observed that in the peritoneal cavity, which is considered a systemic compartment, both vaccines induced similar numbers of Ag-specific CD8$^+$ T cells. Yet, in the lung, a mucosal organ, CT + peptide nasal vaccine potently induced specific CTL and Ag-specific CD8$^+$ T cells, while no CTL activity or Ag-specific CD8$^+$ T cells were detected following the CFA-based cutaneous vaccine (data not shown). Thus, it might be concluded that systemic immunization with a non-replicative vaccine is not capable of inducing a CTL that travel to mucosal sites. However, because cutaneous immunization utilized Freund's adjuvants while nasal immunization utilized CT as the adjuvant, differences in the accumulation of Ag-specific CTL in different tissues may have been a result of the adjuvant utilized and not the route of immunization. Therefore, to accurately measure the effect of route of delivery, we revisited the issue of CD8$^+$ T cell distribution induced by a non-replicating vaccine, composed of a CTL epitope peptide and the same CT adjuvant.

We analyzed the effect of different routes of immunization on the number and phenotype of CD8$^+$ T cells in non-lymphoid mucosal organs as compared to the spleen. Vaccine composed from the H-2K$^b$-presented ovalbumin

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257–264 aa peptide, SIINFEKL, and the CT adjuvant [4]. C57BL/6 mice were injected i.v. with OT-1 transgenic T cells [7], which are specific for H-2K$^b$–SIINFEKL complex, and immunized twice via the nasal, rectal or footpad route with CT + SIINFEKL. The number of Ag-specific CD8$^+$ T cells in the lung, spleen and salivary glands (SG) was monitored 9 days after the last immunization (Fig. 1A). To monitor the specific CD8$^+$ T cells responding to immunization with CT + SIINFEKL, we used Cy5-conjugated K$^b$/β2m/SIINFEKL tetramers that would

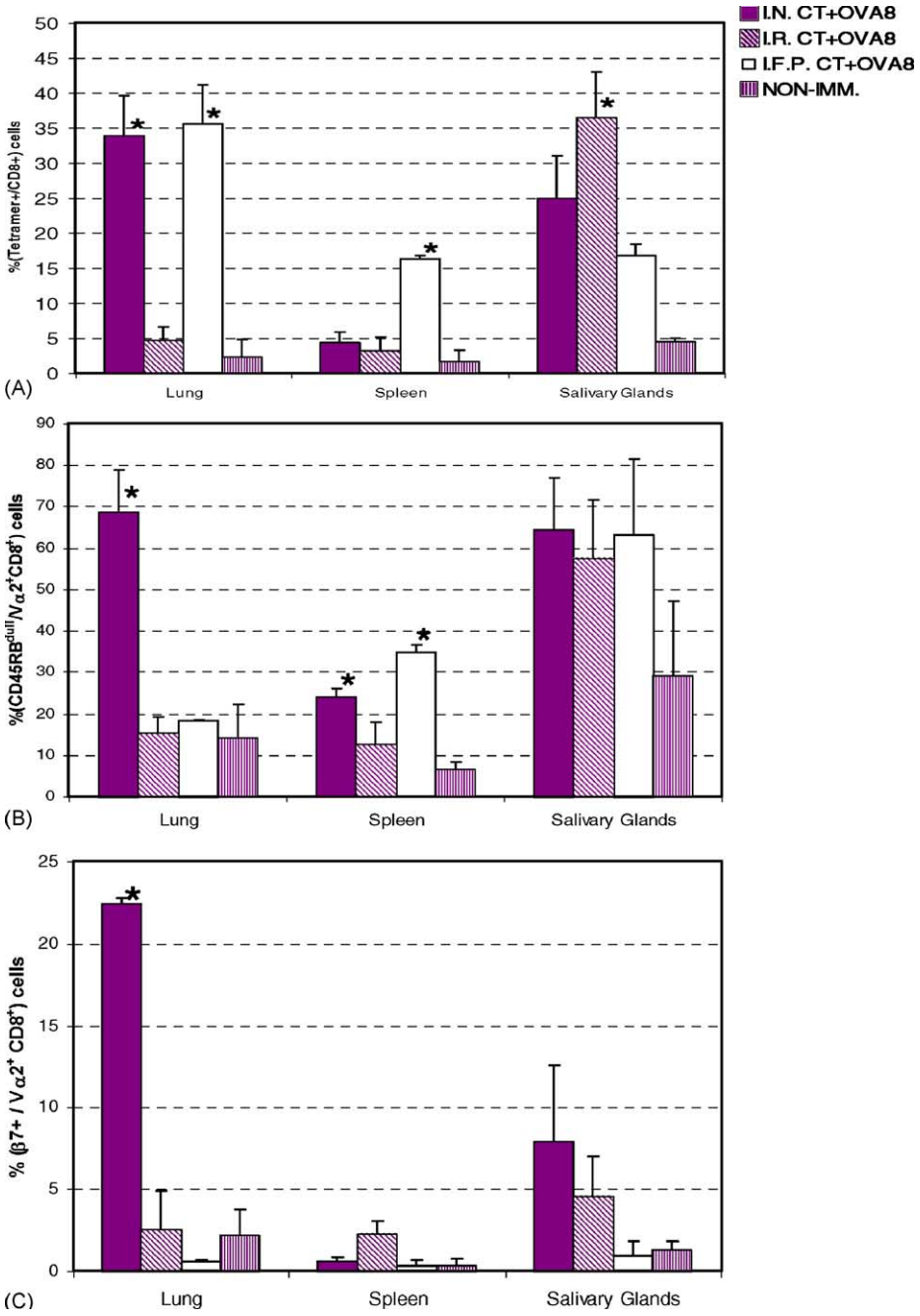


Fig. 1. The route of immunization affects the tissue distribution of tetramer$^+$, CD45RB$^{dull}$, and β7$^+$ Ag-specific CD8$^+$ T cells. OT-1 splenocytes ($2 \times 10^7$) were transferred to C57BL/6 mice by i.v. injection. Mice were immunized intrafootpad, intranasal or intrarectal with CT + SIINFEKL on Days 2 and 16. Nine days after second immunization spleen, lung and SG were removed and mononuclear immune cells were purified as described [4–6]. Cells were: (A) stained with tetramer and anti-CD8, results are shown as the percentage of tetramer$^+$CD8$^+$ T cells of all CD8$^+$ T cells; (B) stained for CD8, CD45RB and Vα2.1 expression, results are shown as the percentage of CD45RB$^{dull}$ (activated cells) of CD8$^+$Vα2.1$^+$ T cells; and (C) stained for CD8, β7 integrin and Vα2.1 expression, results are shown as the percentage of β7$^+$ cells of CD8$^+$Vα2.1$^+$ T cells. All results are the average of three independent experiments. Bars, ± S.D.; (*) immunization route that induces significantly greater: (A) % tetramer$^+$CD8$^+$, (B) % CD45RB$^{dull}$/CD8$^+$Vα2.1$^+$, (C) % β7$^+$/CD8$^+$Vα2.1$^+$ cells in indicated tissue vs. non-immunized mice, $P = 0.05$ ANOVA.

stain both endogenous and transferred T cells that recognize the $K^b$/β2m/SIINFEKL complex. Intranasal and intrafootpad immunization induced a significantly increased percentage of tetramer$^+$CD8$^+$ cells in the lung as compared to non-immunized mice (34.0 ± 7%, 35.7 ± 6.8%, respectively, versus 2.3 ± 2.6%; $P = 0.05$). The level of Ag-specific CD8$^+$ T cells in the lung was comparable with both routes of immunizations, whereas in the spleen, intrafootpad immunization was the only route of immunization to induce significantly increased numbers of tetramer$^+$CD8$^+$ cells (16.3 ± 0.6%; $P = 0.05$); nearly four-fold greater than the percentage of tetramer$^+$CD8$^+$ cells induced by nasal or rectal immunization. Comparing this result to the observations with the IFA or CFA adjuvants, indicates that not only the route of immunization, but also the adjuvant used might affect the magnitude of the response. It also indicates that the distribution of Ag-specific CD8$^+$ T cells, induced by a non-replicative vaccine, might have a different pattern than that observed for B cells and that a "mucosal barrier" in CTL is not as strict as thought. Intrarectal immunization was the only route of immunization to induce a significant increase in the percentage of tetramer$^+$CD8$^+$ cells in the SG (Fig. 1A). Comparison of intranasal and intrarectal routes (Fig. 1), indicates that even within the mucosal compartment, distinct routes of vaccination influence differently the outcome of Ag-specific CD8$^+$ T cells. Similarly in the systemic compartment, subcutaneous immunization with CT + peptide results in poor induction of CD8$^+$ T cells activity as compared to cutaneous (intrafootpad) immunization with CT + peptide (Fig. 1 and [4]).

Most of the expanded and activated Ag-specific CD8$^+$ T cells in the immunized mice, both in mucosal and systemic compartments, were originated from the transferred OT-1 cells as assessed by their expression of Vα2.1, which is the Vα of the OT-1 transgenic TCR (data not shown). OT-1 CD8$^+$ T cells to be transferred were harvested from the OT-1 spleen and manifested naïve phenotype (CD45RB$^{high}$) [8] just before transfer. Naïve T cells are preprogrammed to migrate and recirculate through spleen and secondary mucosal or systemic LN [9]. Thus, harvesting naïve OT-1 CD8$^+$ T cells from the OT-1 spleen for the transfer, should not restrict their distribution to mucosal inductive sites in the immunized mice. Indeed, our results indicate that this assumption is correct (Fig. 1A).

The "mucosal barrier" reappears in our results when analyzing the activation phenotype (CD45RB$^{dull}$) [8] and integrins pattern of the Ag-specific CD8$^+$ T cells in the immunized mice. Nasal immunization resulted in activation of 68.5 ± 12.5% of the Ag-specific CD8$^+$ T cells detected in the lung as compared to <20% for all other groups tested ($P = 0.05$; Fig. 1B). In the spleen, both the intranasal and intrafootpad route of immunization were associated with a significant increase in the percentage of Ag-specific CD8$^+$ T cells expressing an activated phenotype (24.0 ± 2.6% and 34.8 ± 2.4%, respectively; $P = 0.05$). We did not detect differences in α4β7 expression on the Ag-specific CD8$^+$ T cells isolated from the lung, spleen or salivary gland (data not shown). However, when tested for β7 expression, nasal immunization resulted in a significantly higher percentage of Ag-specific CD8$^+$ T cells expressing β7 in the lung (22.4 ± 0.5%; $P = 0.05$, Fig. 1C). Staining for αE integrin revealed similar results (data not shown) suggesting that nasal immunization was associated with induced expression of αEβ7 integrin on Ag-specific CD8$^+$ T cells in the lung. Others have reported that the β7 integrin was required for activated OT-1 T cells to enter the small and large intestinal mucosal and that the αEβ7 integrin appeared to play no role in OT-1 migration during a primary immune response [10]. However, the association between αEβ7 or α4β7 expression and the migration of activated CD8$^+$ T cells to the lung was not studied in this previously published paper. Although integrin αE-deficient mice had no decrease in the number of CD3$^+$ T cells in the lung as compared to wild-type, the affect of αE-deficiency on Ag-specific CD8$^+$ T cells was not studied [11].

We next compared vaccine-induced CD8$^+$ T induction and spreading in the Peyer's patches (PP) and spleen as examples of mucosal and systemic inductive lymphoid tissues, respectively. Again, we immunized OT-1-transffered mice with the same vaccine formulation, CT + SIINFEKL, by the intranasal route or intrafootpad route. Immunization via the footpad induced a significantly greater percentage of tetramer-positive, CD8$^+$ T cells in the spleen (11.0 ± 3%; $P = 0.05$) as compared to intranasal immunization (2.8 ± 1.9%). However, nasal immunization induced a significantly greater percentage of tetramer$^+$CD8$^+$ cells in the PP as compared to footpad immunization, with 31.3 ± 1.2% ($P = 0.05$) and 11.3 ± 8.5% of the CD8$^+$ cells that are tetramer positive after nasal and footpad immunization, respectively (Fig. 2A). Our results indicate that mucosal immunization is superior to systemic immunization for the induction of Ag-specific CD8$^+$ T cells that home to mucosal inductive lymphoid tissues such as PP, while systemic immunization is superior to mucosal immunization for the induction of Ag-specific CD8$^+$ T cells that are detectable in systemic lymphoid tissues. Analysis of blood peripheral lymphocytes revealed phenotype similar to the spleen (data not shown).

We further determined if Ag-specific CD8$^+$ T responses induced by mucosal or systemic immunization were able to protect against an infectious challenge at a mucosal surface. Mice (without pre-transfer of OT-1 cells) were immunized twice with CT + SIINFEKL by the nasal or intrafootpad route and then infected rectally with a recombinant vaccinia expressing ovalbumin and β-galactosidase (rVV-OVA–β-gal) 9 days after the last immunization. This vaccinia strain has been found to home and replicate best in the ovaries; however, its pathway to the ovaries after intrarectal application involves gut inductive sites such as the PP. β-Galactosidase activity was monitored in the ovary 3 days after challenge and used as a surrogate marker for viral load. Both routes of immunization showed protection against intrarectally administered rVV-OVA–β-gal

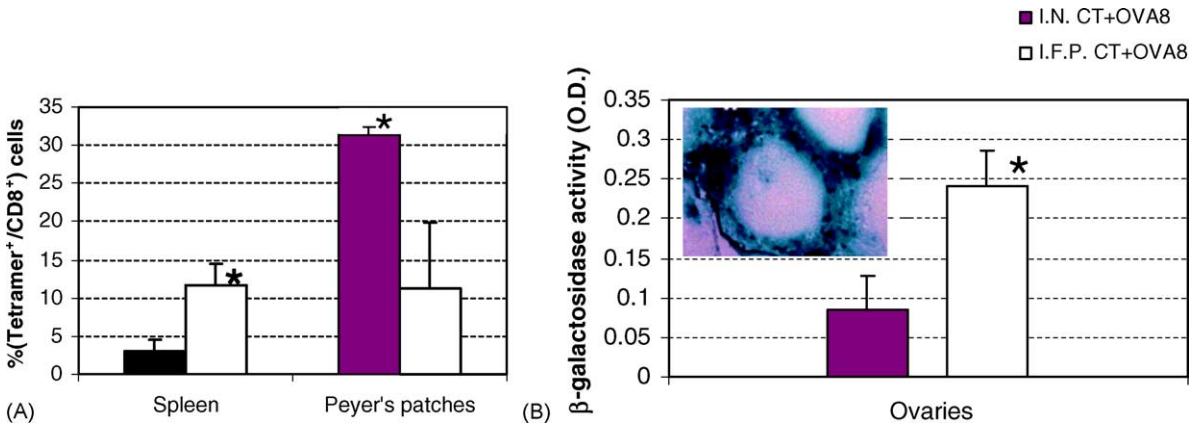


Fig. 2. The route of immunization influences the lymphoid tissue distribution of Ag-specific $CD8^+$ T cells and protection against a mucosal viral challenge. (A) Flow cytometry-based quantitation of parenteral and nasal routes of CT + SIINFEKL vaccine in PP and spleen. OT-1 splenocytes ($2 \times 10^7$) were transferred to C57BL/6 mice by i.v. injection. Mice were immunized intrafootpad or intranasal with CT + SIINFEKL on Days 2 and 16. Nine days after second immunization, Spleen and PP were removed and mononuclear immune cells were purified as described [4–6]. Cells were stained with tetramer and anti-CD8. Results are shown as the percentage of $tetramer^+CD8^+$ T cells of all CD8 positive T cells. SIINFEKL is termed as OVA8 in graph. Results are the average of three independent experiments. Bars, ±S.D. (*) immunization route that induces significantly greater % $tetramer^+CD8^+$ cells in indicated tissue, $P = 0.05$ ANOVA. (B) Load of rVV-OVA–β-gal in the ovary in mice pre-immunized parenterally or nasally with CT + SIINFEKL. Mice were immunized intrafootpad or intranasal with CT + SIINFEKL on Days 2 and 16. Nine days after second immunization rVV-OVA–β-gal ($2 \times 10^7$ pfu per mouse, three mice per group) was applied intrarectally. Three days after, ovaries were harvested, pooled and homogenated, and β-galactosidase activity in the homogenate was tested. Reaction of β-galactosidase with CPRG substrate was measured in triplicates at wavelength of 570 nm. Results are the average of three independent experiments. O.D., optical density; Bars, ± SD. (*) immunization route that induces significantly greater protection against vaccinia dissemination to the ovary after rectal infection, $P = 0.05$ ANOVA. Inset: immunohistochemical staining of β-gal activity in an rVV-OVA–β-gal-infected ovary.

(Fig. 2B), but did not affect protection against a control rVV-β-gal (data not shown). However, nasal immunization provided protection against rVV-OVA–β-gal dissemination to the ovary that was superior to protection provided by intrafootpad immunization, suggesting that mucosal immunization is superior to systemic immunization for protection against a pathogenic challenge that initiates at a mucosal surface ($P = 0.05$; Fig. 2B). Intrarectal immunization with CT + SIINFEKL abolished the growth and dissemination of any intrarectal challenge of vaccinia, regardless of OVA antigen expression. This is possibly due to the induction of innate immunity by the rectal application of CT [12]. Others have shown that intrarectal immunization, but not cutaneous immunization with peptide and adjuvant, protected against an intrarectal challenge with vaccinia expressing the CTL antigen [13]. Our results confirm that mucosal (nasal) immunization is superior to systemic immunization for protection against a rectal viral challenge.

Our results suggest that not only the route of immunization but also the adjuvant formulation affects the induction and distribution of Ag-specific $CD8^+$ T cells induced by non-replicating vaccines. The observations also implicate that systemic immunization with an appropriate adjuvant can evoke immune response in mucosal organs. However, optimal localization, activation and phenotype of the cells correlated with the route of immunization. Ag-specific $CD8^+$ T cells at mucosal tissues may play an important role in protection against pathogens that infect via mucosal tissues (this study and [5,14]) and cancers that develop at or metastasize to mucosal tissues and, therefore, more studies regarding tissue distribution and phenotype of vaccine induced T cells are needed.

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