Expression of Mucosal Homing Receptor α4β7 by Circulating CD4+ Cells with Memory for Intestinal Rotavirus

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Abstract

The integrin α4β7 mediates lymphocyte binding to mucosal addressin cell adhesion molecule-1, and its expression defines lymphocytes capable of trafficking through the intestines and the intestinal lymphoid tissues. We examined the ability of discrete $\alpha4\beta7^{hi}$ and $\alpha4\beta7^{-}$ subsets of circulating memory phenotype ($CD45RA^{-}$) $CD4^{+}$ T cells to proliferate in response to rotavirus, a ubiquitous intestinal pathogen. $\alpha4\beta7^{hi}$ memory ($CD45RA^{-}$) $CD4^{+}$ T cells displayed much greater reactivity to rotavirus than $\alpha4\beta7^{-}$ memory or naive ($CD45RA^{+}$) $CD4^{+}$ T cells. In contrast, $\alpha4\beta7^{-}$ memory cells were the predominant population responsive to mumps antigen after intramuscular vaccination. Our results are consistent with the conclusion that natural rotavirus infection, an enteric pathogen, results in a specific circulating memory $CD4^{+}$ response that is largely limited to the gut-homing $\alpha4\beta7^{+}$ subpopulation. This phenotype is not shared with memory cells elicited by intramuscular immunization (shown here) or by skin contact allergens. The results support the hypothesis that gut trafficking memory $CD4^{+}$ T cells comprise cellular memory for intestinal antigens and suggest that regulated expression of α4β7 helps target and segregate intestinal versus systemic immune response. (*J. Clin. Invest.* 1997. 100:1204–1208.) Key words: human • T lymphocytes • mucosal immunity • infectious immunity • adhesion molecules

Introduction

The integrin α4β7 mediates binding of lymphocytes to the mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1) (1, 2), an interaction required for efficient trafficking of lymphocytes into the large and small intestines and the Peyer's patches. Recent studies have shown that α4β7 expression defines a specific subset of memory T cells characterized by the capacity to bind to the mucosal vascular addressin in vitro (3), to home to mucosal lymphoid tissues (4), and to recirculate through the gastrointestinal tract and presumably the lamina propria (5, 6). In contrast, T cells that lack α4β7 are largely excluded from homing to mucosal Peyer's patches (4), and recirculate preferentially through nonmucosal tissues (6, 7). These latter memory cells characteristically display higher levels of α4β1, and include the skin-associated cutaneous lymphocyte antigen (CLA^{1})$^{+}$ T cell memory subset (3). It is widely held that the selective trafficking of $\alpha4\beta7^{hi}$ versus $\alpha4\beta7^{-}$ memory T cells exists to target and segregate mucosal (intestinal) versus nonmucosal immunity, but the association of α4β7 expression with immunologic memory for intestinal antigens and pathogens has not been tested.

Rotavirus is the primary cause of acute gastroenteritis in children and is a leading cause of infant mortality in developing countries. Rotavirus infects and replicates in the mature villous tip enterocytes of the small intestine (8, 9). Although extra-intestinal spread of rotavirus has been documented in immunodeficient people and animals, in most circumstances, infection is restricted to the small bowel. The selective localization of rotavirus to the gut suggests that the cell mediated immune response must occur through the actions of mucosal effector cells (10). Earlier studies have shown that transfer of $CD8^{+}$ T cells from rotavirus-immunized mice into chronically rotavirus infected SCID mice could promote clearance of the virus (11). In concurrent in vivo mouse studies, we have demonstrated further that $CD8^{+}$ T cells capable of clearing rotavirus infection are found among the $\alpha4\beta7^{hi}$ memory subset (Rosé, J.R., M.B. Williams, L.S. Rott, E.C. Butcher, and H.B. Greenberg, manuscript submitted for publication). Rotavirus reactive $CD4^{+}$ T cells are also elicited during rotavirus infection, and their presence has been correlated with immunity. For example, the emergence of rotavirus-specific $CD4^{+}$ T cells is observed during the convalescence of young children after documented rotavirus infection (12).

This study was designed to assess intestinal homing receptor expression by circulating rotavirus-specific $CD4^{+}$ T cells resulting from natural rotavirus exposure. We report that CD4 memory for this mucosal pathogen is found selectively in the $\alpha4\beta7^{hi}$ subset. In contrast, memory $CD4^{+}$ T cells responsive to mumps antigen after intramuscular vaccination are almost exclusively $\alpha4\beta7^{-}$. The findings support the hypothesized segregation of mucosal versus systemic immunity through specialized expression of homing receptors on responsible memory T cell populations.

Methods

Cell preparation. Peripheral blood for evaluation of rotavirus reactivity was collected from young children convalescing after acute rotavirus infection (diagnosed by antigen shedding in stool) and from

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1. *Abbreviations used in this paper:* CLA, cutaneous lymphocyte adhesion molecule; PBMC, peripheral blood mononuclear cell.

adult volunteers. Although virtually all adults have serologic evidence for previous exposure to rotavirus, the level of demonstrable CD4 reactivity is variable, probably due in part to the time interval from the last exposure. Therefore, in order to enhance the probability of obtaining good proliferative responses in peripheral memory $CD4^+$ T cells, volunteers were recruited who had an infant or toddler family member with diarrhea and who subsequently became symptomatic themselves. We reasoned that parents of young children had a higher likelihood of recent rotavirus exposure and that this likelihood would be further increased if the infant or toddler had had recent diarrheal infection. Children's blood samples (4 ml) were acquired 2–4 wk after onset of illness and adult samples (60–70 ml) were obtained from volunteers 4–6 wk after onset of illness. Adult volunteers for mumps assessment were given a single dose of the live attenuated mumps vaccine, MUMPSVAX® (Merck, Sharp & Dohme, Westpoint, PA) intramuscularly and gave 60–70 ml blood 3–5 wk later. Almost all adults have immunity to mumps and the vaccine was used to boost memory cell frequency. Two volunteers had worked previously in pediatrics wards and were assessed for reactivity to both rotavirus and mumps. Blood was applied to a Ficoll-Hypaque density gradient (Histopaque 1077; Sigma Chemical Co., St. Louis, MO) to separate the peripheral blood mononuclear cells (PBMC). Cells were washed twice in Ca^{2+}/Mg^{2+}–free HBSS and resuspended in RPMI 1640 (Bio-Whittaker, Walkersville, MD) supplemented with 10% normal human serum. $CD4^+$ cells were subsequently purified with Dynal magnetic beads according to the manufacturer's protocol and resuspended in DME (Bio-Whittaker) supplemented with 10% FCS, glutamine (0.29 mg/ml), penicillin (100 U/ml), and streptomycin (0.1 mg/ml) in preparation for cell surface staining and sorting.

Monoclonal antibodies. Cells, suspended at 10^7/ml, were stained with mouse-derived anti-α4β7 (Act-1) (13) or rat-derived anti-β7 (FIB504) (3) antibodies versus FITC-conjugated anti-CD45RA (Immunotech, Inc., Westbrook, ME). Act-1 was a gift from Charles Mackay (LeukoSite, Boston, MA) and A. Lazarovits (Robarts Research Institute, London, Ontario) and was biotin conjugated in our laboratory. The second stage for Act-1 Biotin was Streptavidin PE (PharMingen, La Jolla, CA) at 0.25 μg per 10^6 cells and for FIB 504, mouse anti–rat Ig PE (Chromoprobe, Mountain View, CA) at 0.5 μg per 10^6 cells. Anti-CD4 FITC (PharMingen) or biotin (Immunotech) followed by streptavidin PE was used to assess purity of the $CD4^+$ isolated cells.

Staining and cell sorting. Cell suspensions were incubated interchangeably with Act-1 biotin (anti-α4β7 heterodimer) and FIB504 (anti-β7 chain) (since high expression of both identifies virtually the same $CD4^+$ T cell population in peripheral blood [3]) and anti-CD45RA FITC for 30 min and washed once. Either streptavidin PE or mouse anti–rat PE second stage was then added as appropriate and incubated for 30 min followed by washing. Cells were incubated on ice and covered with aluminum foil. DME (as described in cell preparation above) was used for washes. Sorted cells were collected in DME supplemented with 20% FCS, glutamine, penicillin, and streptomycin. Cell sorting was performed on a FACStar (Becton Dickinson, San Jose, CA) equipped with an argon 488-nm laser and Consort 30 software.

Viral antigens. The rotavirus used in proliferation assays was rhesus rotavirus (RRV). Virus was grown in MA104 cells as described previously (14). Virus was partially purified by ultracentrifugation of clarified infected cell lysates at 38,000 rpm in a Ti45 rotor. The viral pellet was resuspended in TNC buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 5 mM $CaCl_2$) and extracted with GenesolvD (1,1,2-trichloro-1,2,2-trifluorothane; AlliedSignal Inc., Morristown, NJ). The virus was concentrated by centrifugation through 40% sucrose at 28,000 rpm in an SW-28 rotor. The pellet from this step was resuspended in a small volume of TNC and titered as described previously (14). Inactivated virus was prepared as described (15) by UV-irradiation of sucrose-purified RRV for 20 min in the presence of 40 μg/ml psoralen. The concentration of rotavirus determined by plaque titration before inactivation was 2×10^8 pfu/ml. Mumps virus antigen was obtained commercially from Bio-Whittaker.

Proliferation assay. For proliferation assays, PBMCs, or sorted cells, were washed and resuspended at a concentration of 1×10^6 per ml in RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (292 μg/ml), 10% heat inactivated AB^- serum (Sigma Chemical Co), and 50 μg/ml gentamicin (Sigma Chemical Co.). Antigen specific proliferation was examined using commercial mumps virus antigen (50 μl/ml of a 1:100 dilution of stock virus antigen (Bio-Whittaker) or inactivated rotavirus (50 μl/ml). The concentration of rotavirus before inactivation was 2×10^8 pfu/ml. Concanavalin A at 2 μg/ml was used as a positive control for lymphocyte viability. Cells were incubated for 3 d in 96-well U-bottom tissue culture plates (Costar Corp., Cambridge, MA) in a final volume of 200 μl. Cells were labeled by addition of 1 μCi of $[^3H]$thymidine (Amersham Life Science Inc., Arlington Heights, IL) for 18 h, followed by harvesting of cells with a Skatron semi-automated cell harvester (Skatron Inc., Sterling, VA). Quantitation of incorporated $[^3H]$thymidine was performed on a Beckman LS 1701 scintillation counter.

Autologous plastic-adherent monocytes were used as antigen presenting cells when sorted cell populations were to be tested for proliferation in response to viral antigens. U-bottom plates were incubated with crude PBMCs at 1×10^6 cells per well for 4 h at 37°C. Nonadherent cells were removed by washing plates three times with RPMI 1640. 1×10^5 sorted lymphocytes were added to precoated plates with or without antigens in a final volume of 200 μl, as described above.

Results

In initial experiments, we examined $β7^{hi}$ and $β7^-$ subsets in a pediatric blood sample acquired 18 d after onset of illness (child C in Table I) that had sufficient cells for sorting. $β7^{hi}$ blood lymphocytes showed a 2.6-fold greater proliferative response to rotavirus than $β7^-$ cells. Memory cell number (16) and volume of blood available from children was very limited, however, and restricted our ability to characterize proliferative activity in well-defined lymphocyte subsets.

Because of this limitation, subsequent studies were carried out in adults. Essentially all adults have circulating antibody to rotavirus, consistent with the universal exposure to this pathogen (17). However, many adults do not display a detectable CD4 proliferative response to rotavirus antigen, presumably because of the absence of recent re-exposure. In preliminary studies, 10 individuals without exposure to very young children (in whom we confirmed universal exposure to rotavirus by presence of serum antibody) did not have a detectable PBMC proliferative response to rotavirus (data not shown). Therefore volunteers were preselected for recruitment based on a history of recent contact with young children (family members or patients < 2-yr-old) with gastroenteritis during the rotavirus season (autumn–winter). Of the six individuals preselected, all but one displayed a significant in vitro CD4 response to rotavirus. The greater number of cells accessible from adult blood samples enabled an examination of each of the three T cell populations of interest (naive $CD4^+$ cells, $α4β7^{hi}$ and $α4β7^-$ memory $CD4^+$ cells).

Fig. 1 illustrates β7 versus CD45RA expression on $CD4^+$ cells from a representative adult volunteer. Within the $CD45RA^-$ (memory) cells there are discrete $α4β7^{hi}$ and $α4β7^-$ subpopulations while $CD45RA^+$ (naive) cells are unimodal for α4β7 at an intermediate level. The average percentage of cells sorted was 8.6% (range 4.0–11.8%) for the memory $α4β7^{hi}$ population, 27.8% (range 8.0–55.2%) for the memory $α4β7^-$ population, and 43.3% (range 15.9–62.6%) for the naive population ($n = 10$). Gates used for sorting the $α4β7^{hi}$ and $α4β7^-$ memory phenotype and naive phenotype cells are shown.

*Table I. Stimulation Index**

	Rotavirus nonresponsive			Rotavirus reactive			Mumps not immunized			Mumps reactive		
	$(\alpha 4)\beta 7^+$	$(\alpha 4)\beta 7^-$	Naive	$(\alpha 4)\beta 7^+$	$(\alpha 4)\beta 7^-$	Naive	$(\alpha 4)\beta 7^+$	$(\alpha 4)\beta 7^-$	Naive	$(\alpha 4)\beta 7^+$	$(\alpha 4)\beta 7^-$	Naive
Child C		ND		4.07	1.54	ND		ND			ND	
No. 1	0.48	0.33	0.74		ND		0.57	0.27	1.05		ND	
No. 2	1.79	0.82	1.43		ND		1.62	0.31	0.88		ND	
No. 3	2.82	3.00	ND	6.27	2.31	2.30		ND			ND	
No. 4		ND		3.81	2.05	0.58	1.02	1.39	ND		ND	
No. 5		ND		3.87	1.15	0.97		ND			ND	
No. 6[‡]		ND		5.18	3.91	2.04		ND		1.97	6.25	2.57
No. 7[‡]		ND		7.71	3.81	3.16	2.8	3.0	1.6		ND	
No. 8[mv]		ND			ND			1.07 (crude)[§]		3.00	6.28	1.21
No. 9[mv]		ND			ND			8.52 (crude)[§]		1.34	6.16	1.45
No. 10[mv]	2.80	3.03	0.97		ND			7.79 (crude)[§]		5.27	18.1	5.91

All are adult subjects except Child C. *Stimulation index was calculated as proliferative response with antigen divided by proliferative response with no antigen. [‡]Denotes individuals who had worked recently in pediatric wards. Neither subject received mumps vaccine as part of the study, nor did they have specific memory of experiencing acute gastroenteritis. [§]Represents unsorted PBMC response. [mv], individuals received mumps vaccine; ND, not done.

Five adults exhibited significant $CD4^+$ T cell proliferation to rotavirus antigen. Three of these individuals (No.s 3, 4, and 5) were family members of sick children and two (No.s 6 and 7) had worked recently in pediatrics wards. These two individuals had no specific memory of having gastroenteritis themselves nor had they any known recent exposure to mumps or mumps vaccine. There was a dramatic difference in the proliferative response of the specific $CD4^+$ subsets to rotavirus antigen. $\alpha 4\beta 7^{hi}$ memory cells were more reactive to rotavirus than the other subsets, demonstrating an average 3.7-fold (SE = 1.6; range = 2.4–6.6) increased stimulation index over the naive subset, and 2.3-fold (SE = 1.0; range 1.3–3.4) over $\alpha 4\beta 7^-$ memory cells. Paired student's t test comparisons confirmed the statistical significance of enhanced $\alpha 4\beta 7^{hi}$ cell responsiveness ($P < 0.004$ versus $\alpha 4\beta 7^-$ memory; $P < 0.001$ versus naive). For the one individual (No. 3) with both pre- and postdiarrheal infection stimulation indices, there was a specific increase in responsiveness only in the $\alpha 4\beta 7^{hi}$ subset. In three out of the five rotavirus reactive individuals (No.s 3, 5, and 7), the $\alpha 4\beta 7^-$ and naive subset indices were equivalent. Assuming the naive cell stimulation index indicates a true negative or background response, reactivity in these individuals is exclusively in the $\alpha 4\beta 7^{hi}$ population. However, for the other two individuals, we observed an $\alpha 4\beta 7^-$ response that was higher than the naive subset, possibly indicating a low level of reactivity to rotavirus among $\alpha 4\beta 7^-$ cells in some individuals. However, in all cases of natural exposure, $\alpha 4\beta 7^+$ memory cells responded to rotavirus antigen substantially better than either naive or $\alpha 4\beta 7^-$ memory cells.

These results contrast with those of four patients studied with no history of recent personal or occupational exposure to gastroenteritis (No.s 1, 2, 3, and 10). None of these patients displayed a significant difference in response indices of $\alpha 4\beta 7^{hi}$ versus $\alpha 4\beta 7^-$ memory phenotype cells and only one of three assayed showed a significant difference between memory and naive phenotype responses.

Four adults were evaluated for mumps-specific proliferative responses 3–5 wk after intramuscular mumps vaccination (No.s 6, 8, 9, and 10—note that No. 10 was assessed for rotavirus reactivity 7 mo before mumps vaccination). Table I contains $CD4^+$ subset stimulation index values for individuals who had no reactivity to mumps (No.s 1, 2, 4, and 7), as well as comparisons of crude (unsorted) prevaccine versus postvaccine values on three individuals (No.s 8, 9, and 10). Two of these three individuals had an increased response to mumps after the vaccine (No.s 8 and 10) while one (No. 9) appeared to have no increase in the stimulation index. Irrespective of the relative pre- and postmumps levels of reactivity, however, all individuals with $CD4^+$ T cell memory to mumps displayed the same pattern of response. In contrast to the natural rotavirus response, CD4 proliferative memory to mumps antigen after intramuscular vaccination was enriched among $\alpha 4\beta 7^-$ memory cells, which displayed an average 3.7-fold (SEM = 1.9, range = 2.4–5.2) increased proliferative index over that of naive cells and 3.3-fold (SEM = 1.7, range 2.1–4.6) over $\alpha 4\beta 7^{hi}$ memory cells. Student's t test confirmed statistically significant differences between $\alpha 4\beta 7^-$ versus naive or $\alpha 4\beta 7^{hi}$ responses ($P < 0.04$).

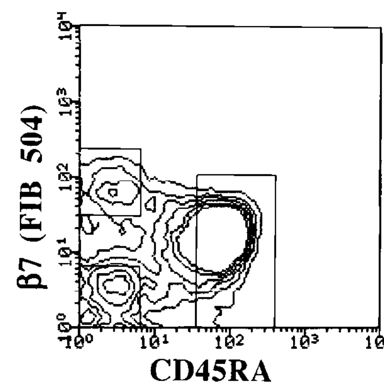

Figure 1. $CD4^+$ cells from a representative volunteer consist of memory ($CD45RA^-$) $\alpha 4\beta 7^{hi}$, memory ($CD45RA^-$) $\alpha 4\beta 7^-$, and naive ($CD45RA^+$) subpopulations. The contour plot illustrates α4β7 versus CD45RA staining on purified $CD4^+$ cells (purity averaged 98%). The windows show the sort gates used for separation of the populations studied.

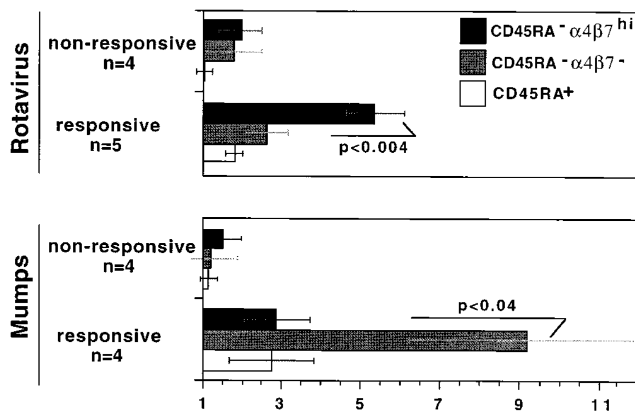

Figure 2. Summary of proliferative responses of sorted subsets to rotavirus and mumps antigen. The bar graphs represent the mean memory $\alpha4\beta7^{hi}$, memory $\alpha4\beta7^{-}$, and naive $CD4^{+}$ proliferative responses to rotavirus and mumps antigen. The stimulation index was calculated by dividing the cpm [^{3}H]thymidine incorporated in the presence of antigen by the cpm [^{3}H]thymidine incorporated in the presence of medium alone. Standard error is shown.

Fig. 2 summarizes the results from adult subjects, emphasizing the clear compartmentalization of the cellular response to the two antigens. There was a significant enrichment of rotavirus reactivity among the $\alpha4\beta7^{hi}$ versus $\alpha4\beta7^{-}$ and naive cell fractions. In contrast, enhanced responsiveness to mumps was found solely in the $\alpha4\beta7^{-}$ subpopulation.

Discussion

Recently, we have shown that memory phenotype T cells capable of homing to mucosal Peyer's patches, and of recirculating from blood to intestinal lymph, are largely if not exclusively $\alpha4\beta7^{hi}$, and that memory cells lacking α4β7 are largely excluded from these intestinal trafficking patterns (4, 6). It is widely held, but has not been previously demonstrated, that these $\alpha4\beta7^{hi}$ gut-homing memory phenotype T cells comprise immunologic memory to intestinal pathogens, and that the subdivision of $\alpha4\beta7^{+}$ versus $\alpha4\beta7^{-}$ memory populations exists to help target and segregate these immune responses within the body. Here we have tested this hypothesis by asking whether $CD4^{+}$ T cell memory induced by an intestinal pathogen, rotavirus, is found preferentially within the α4β7 expressing memory subset. Rotavirus proliferates exclusively within the epithelial cells of the small intestine and rotavirus exposure, occurring generally in the first 3 yr of life, is universal in the population. We found that circulating $CD4^{+}$ T cells that proliferate in response to rotavirus are predominantly $\alpha4\beta7^{hi}$ and of memory ($CD45RA^{-}$) phenotype. $\alpha4\beta7^{-}$ memory $CD4^{+}$ cells most often displayed rotavirus reactivity at the background level characterized by naive $CD4^{+}$ cell responses and always had significantly less reactivity than $\alpha4\beta7^{hi}$ memory $CD4^{+}$ cells.

In contrast, reactivity to mumps antigen after intramuscular vaccination was found exclusively in the memory $\alpha4\beta7^{-}$ subset. Although background (naive) proliferation levels varied, there was a consistently heightened responsiveness in the $\alpha4\beta7^{-}$ subset for each individual, while the naive and memory $\alpha4\beta7^{hi}$ subsets responded similarly.

Some of our subjects responded to neither viral antigen, some responded only to rotavirus or mumps, which generally correlated with recent known exposure to gastroenteritis or mumps vaccine, and one responded to both antigens. Of fundamental importance, there was absolute consistency in the phenotype of the responding memory subpopulation. When an individual had a proliferative response to rotavirus, we found the reactive $CD4^{+}$ cells in the memory $\alpha4\beta7^{hi}$ subset and conversely mumps reactive cells were in the memory $\alpha4\beta7^{-}$ subset.

Our findings are consistent with models in which the site of antigen presentation determines the homing phenotype of memory and effector cells (18–21). However, as this is the first model in which α4β7 expression by $CD4^{+}$ T cells has been correlated with functional T cell memory for an intestinal antigen, it is formally possible that α4β7 upregulation is a feature of the response to rotavirus rather than of the intestinal site of the immune response in natural infection. This seems unlikely for the following reasons. First, in studies of B cell responses to unrelated experimental antigens, circulating B cells capable of secreting antigen-specific antibodies are enriched by anti-α4β7 magnetic bead separation after oral but not systemic inoculation (22). Moreover, previous studies of $CD4^{+}$ T cell responses to cutaneous antigens (23) have shown that epidermal allergens yield a response by memory T cells which display the cutaneous lymphocyte antigen CLA, a skin lymphocyte homing receptor. As CLA^{+} T cells are $\alpha4\beta7^{-}$ (3), these results are consistent with our findings of mumps reactive cells in the $\alpha4\beta7^{-}$ population after systemic immunization. Thus results in these unrelated models, as shown here for the immune response to intestinal rotavirus and systemic mumps antigen, suggest that gastrointestinal but not systemic immune responses generate $\alpha4\beta7^{+}$ lymphocyte memory.

In conclusion, our results indicate that natural rotavirus infection of the intestine is associated with an $\alpha4\beta7^{hi}$ memory $CD4^{+}$ T cell proliferative response, whereas the $CD4^{+}$ T cell response to systemically presented mumps antigen is limited to the $\alpha4\beta7^{-}$ subpopulation. Taken together with recent studies in other models, the findings provide the first direct experimental support for models of targeted trafficking of memory T cells for regionally restricted antigens, and suggest that the expression of tissue-selective homing receptors may help segregate intestinal from systemic immune responses.

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