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A DNA prime-oral *Listeria* boost vaccine in rhesus macaques induces a SIV-specific CD8 T cell mucosal response characterized by high levels of $\alpha_4\beta_7$ integrin and an effector memory phenotype

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Abstract

In this study in Rhesus macaques, we tested whether IL-12 or IL-15 in a DNA prime-oral *Listeria* boost amplifies the SIV-Gag-specific CD8 mucosal response. SIV-specific CD8 T cells were demonstrated in the peripheral blood (PB) in all test vaccine groups, but not the control group. SIV-Gag-specific CD8 T cells in the PB expressed $\alpha_4\beta_7$ integrin, the gut-homing receptor; a minor subset co-express $\alpha_E\beta_7$ integrin. SIV-Gag-specific CD8 T cells were also detected in the gut tissue, intraepithelial (IEL) and lamina propria lymphocytes (LPL) of the duodenum and ileum. These cells were characterized by high levels of β_7 integrin expression and a predominance of the effector memory phenotype. Neither Il-12 nor IL-15 amplified the frequency of SIV-specific CD8 T cells in the gut. Thus, the DNA prime-oral *Listeria* boost strategy induced a mucosal SIV-Gag-specific CD8 T cell response characterized by expression of the $\alpha_4\beta_7$ integrin gut-homing receptor.

Keywords: Vaccination; Mucosal immunity; Adhesion molecule

Introduction

HIV is a major health problem in the western world and has reached pandemic proportions in third world countries. The most common mode of infection is sexual transmission through mucosal tissue. Furthermore, active replication of HIV (Fackler et al., 1998; Kotler et al., 1991; Markowitz et al., 1999; Smith et al., 1994) and eradication of activated/memory CD4 T cells (Schneider et al., 1994, 1995; Veazey et al., 1998) occurs in the GALT early in HIV infection. Therefore, a successful HIV vaccine needs to induce a strong mucosal immune response to prevent mucosal infection and the rapid reduction of CD4 T cells in the GALT. SIV infection in rhesus macaque monkeys has been used extensively to model HIV infection in humans and to test vaccines. Prior studies demonstrated that the target population for SIV infection in the GALT (Li et al., 2005), reduction in memory CD4 T cells (Reynolds et al., 2005) and SIV-specific CD8 T cell responses are similar to HIV in humans (Mattapallil et al., 2004; Veazey et al., 2000, 2001; Vingert et al., 2003). Containment of SIVmac251 infection in vaccinated rhesus macaques correlates with the magnitude of SIV-specific CD4 and CD8 T cell responses (Hel et al., 2002). In addition, SIV disease progression is thought to be associated with impairment of SIV-specific T cell function (Hel et al., 2001). SIV vaccines have been tested in rhesus macaques with a focus on inducing mucosal (Evans et al., 2003; Zhao et al., 2003) or systemic (Radaelli et al., 2003) immunity. Mucosal immunity

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can be induced by oral immunization; effector T cells migrate locally from inductive to effector sites in the gut as well as to other mucosal effector sites of the body (Cromwell et al., 2000; Evans et al., 2003).

We have been using *Listeria* as a vaccine vector to deliver target antigens as a vaccine for HIV, reviewed in Paterson and Johnson (2004) or tumors, reviewed in Paterson and Maciag (2005). The *Listeria*-based vaccines are capable of inducing a powerful cell-mediated immune response to target antigens. This is due to a number of factors including the innate immune response to *Listeria* and its unique life cycle. The bacterium has an intracellular lifecycle that intersects with both MHC class I and II processing pathways; proteins secreted by a *Listeria* vector can be processed through both the cytosolic or endosomal compartments onto MHC class I or II, contributing to both a CD4 and CD8 response to the target antigen (Bishop and Hinrichs, 1987; De Libero and Kaufmann, 1986) with a Th1 phenotype (Mata and Paterson, 1999). In a recent study we demonstrated that a SIV DNA prime-*Listeria* boost immunization strategy induced a cellular response capable of partial suppression of SIV239 viral replication (Boyer et al., 2005b). Also, in prior studies, IL-12 (Boyer et al., 2005a; Egan et al., 2005) or IL-15 (Kutzler et al., 2005) plasmids enhanced the SIV-Gag-specific T cell response to the SIV-Gag DNA vaccine (Calarota et al., 2003). Therefore, we designed a study to see whether a DNA prime-oral *Listeria* boost combined with either IL-15 or IL-12 plasmid vaccines would amplify mucosal CD8 T cell responses to SIV. In order to explore this hypothesis, we used multi-parameter flow cytometry and ELISPOT to measure the CD8 T cell responses to the DNA prime-oral *Listeria* strategy in the peripheral blood, lymph nodes and the gut. We used flow cytometry to assess the binding of SIV-Gag tetramers to CD8 T cells in conjunction with the expression of beta 7 integrin. Beta 7 integrin is an adhesion molecule that facilitates normal intestinal immune cell trafficking to the gut (Butcher et al., 1999; Kuklin et al., 2000; Lefrancois et al., 1999; von Andrian and Mackay, 2000) and serves as a 'gut-homing' marker on T cells. In addition, we investigated the functional state of the CD8 T cells in each sample by examining expression of CD28 and CD95. This enabled us to assess the relative frequency of CD8 T cells in a naive or effector memory state (Pitcher et al., 2002). We found that the DNA prime-oral *Listeria* boost strategy induced a SIV-Gag-specific CD8 T cell response in the gut, lymph nodes and peripheral blood. This response was accompanied by a change in PB CD8 T cell subsets, including a decrease in the frequency of naive and an increase in effector memory cells. Although there was an increase in the number of monkeys responding to SIV-Gag in the PB in the vaccine group which received IL-15, there was no difference in the magnitude (the % of CD8 T cells with elevated tetramer binding or the number of spot forming cells by IFN-γ ELISPOT) of SIV-Gag responses by CD8 T cells between vaccine groups. Although the inclusion of IL-12 clearly enhanced the DNA priming of SIV-specific CD8 T cells in the PB and IL-15 enhanced the response to the first *Listeria* boost, the inclusion of these cytokines in the DNA vaccine prime did not enhance the final vaccine response.

Results

The aim of this study was twofold; firstly, to assess whether the DNA prime-oral *Listeria* boost immunization strategy induces a SIV-specific mucosal response; and secondly, to assess whether this mucosal response could be amplified by IL-12 or IL-15. Therefore, initially monkeys were sorted into four immunization groups of five individuals, based on their MHC class I haplotype. Test groups were primed with three rounds of SIV *gag* and *env* DNA vaccine *i.m.* followed by oral *Listeria* boost four times (see Table 1 for details). Each vaccine group contained individuals that express MHC class I haplotype MamuA*01 and/or MamuA*02, as well as controls that were non-A01/A02. Following the immunization, samples were harvested from the peripheral blood, lymph nodes and gut tissue to evaluate the immune response.

Initially, we found that all test vaccines induced SIV-Gag- and Env-specific CD8 T cells (detected by IFN-gamma ELISPOT) in the PB, 14 days after the 1st three DNA vaccines and the 1st *Listeria* boost. However after the 2nd *Listeria* boost the SIV Env-specific CD8 T cells had decreased markedly (Fig. 1Aii), we ceased testing for SIV Env CD8 response in the PB from this point. Statistical

Table 1
Immunization schedule and monkey test/control groups

Group	Weeks 0, 4, 8	Weeks 16 and 20	Weeks 24 and 38	Monkey no.	Haplotype
1	DNA 2 mg SIVgag 2 mg SIVenv	Lm Gag and Env oral 3×10^{10}/dose	Lm Gag and Env oral 3×10^{11}	4113	A08, B01
				4107	A01, A02, B17
				4110	A01
				4111	A02
				4116	A02, A08, B01
2	DNA 2 mg SIVgag 2 mg SIVenv +IL-15 plasmid	*Lm* Gag and Env vectors oral 3×10^{10}/dose	*Lm* Gag and Env vectors oral 3×10^{11}	4104	A08, B01
				4105	A01, A02
				4108	Unknown
				4109	A08
				4114	A02
3	DNA 2 mg SIVgag 2 mg SIVenv +IL-12 plasmid	*Lm* Gag and Env vectors oral 3×10^{10}/dose	*Lm* Gag and Env vectors oral 3×10^{11}	4112	Unknown
				4117	A01, A08
				4118	A02
				4120	A02, B01
				4123	A02
Control	DNA Variola	*Lm*-E7 Oral 3×10^{10}/dose	*Lm*-E7 Oral 3×10^{11}	4106	A02, A08
				4115	B01
				4119	A01,A02, B01
				4121	Unknown
				4122	B01

Rhesus monkeys were sorted into 5 individuals per group to include an even spread of MamuA*01 and MamuA*02 haplotypes as well as non-A01/A02 haplotypes. As a result each test vaccine and control group had haplotype-negative and -positive members for the SIV-Gag tetramers. Details of the DNA vaccine for each test and control group are given; the same dose and construct was used for all test vaccine groups for the oral *Listeria*.

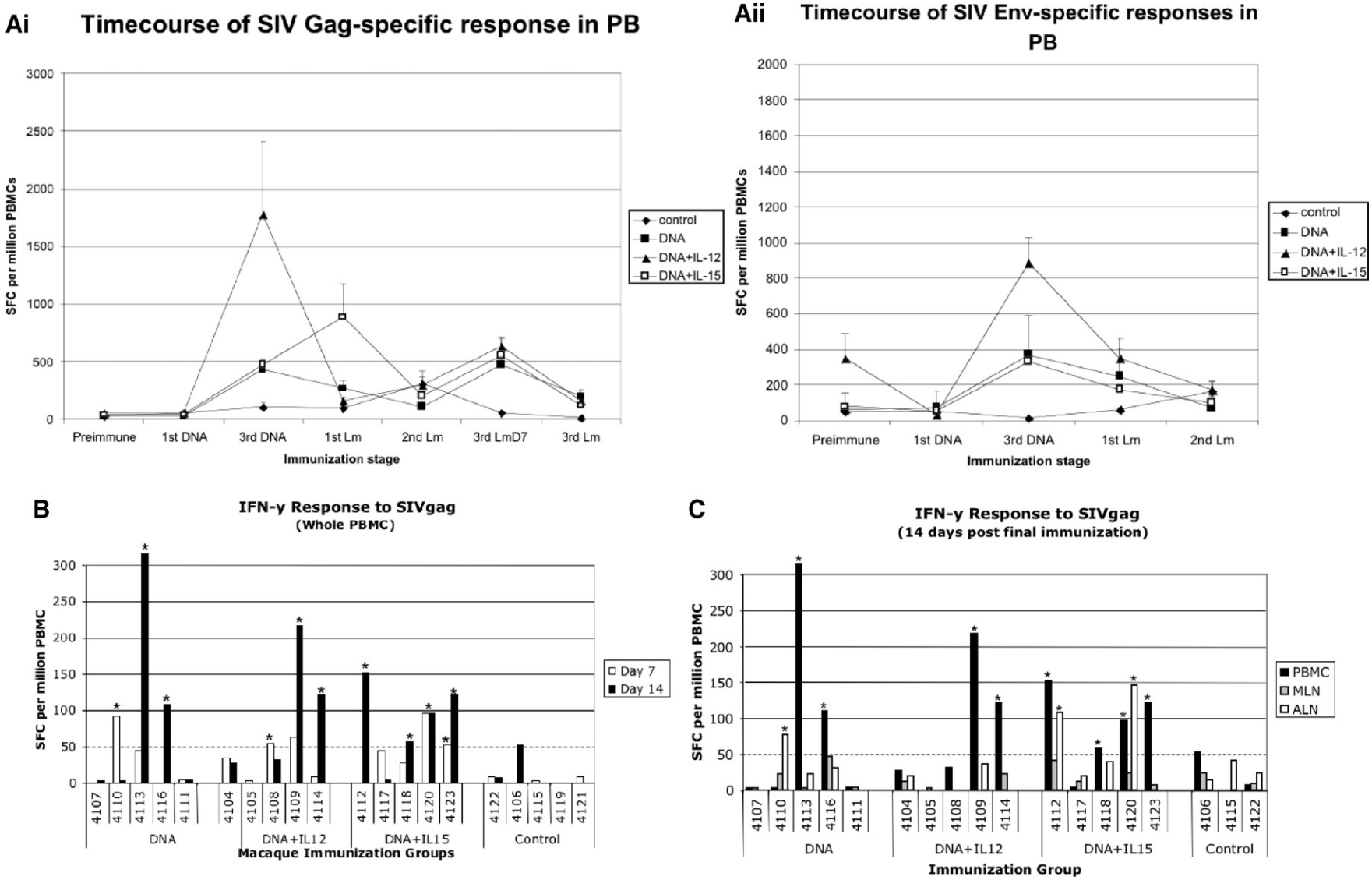

Fig. 1. DNA prime-*Listeria* boost immunization-induced SIV-Gag-specific CD8 T cells in the peripheral blood and axillary lymph node but not the mesenteric lymph node. SIV-Gag-specific CD8 T cells were detected by ELISPOT for IFN-gamma secretion in response to SIV-Gag and SIV Env overlapping peptides (as per methods). The ELISPOT assay was performed on (A) PBMC's harvested during DNA and *Listeria* immunizations, samples were collected 14 days after each immunization except 3rd LmD7, which was collected 7 days after the 3rd *Listeria* immunization (B) PBMCs harvested days 7 and 14 post-final *Listeria* immunization, and (C) PBMCs, axillary and mesenteric lymph nodes harvested day 14 post-final *Listeria* immunization. Samples are positive when they have >50 SFC per million PBMCs. Samples marked by an asterisk (*) have >50% reduction in SFC/million PBMCs after CD8 depletion of the PBMCs. In (A) the ELISPOT data has been plotted as the mean±standard error for Gag (Ai) and Env (Aii) responses for each immunization group over the time course of the DNA prime-*Listeria* boost; whereas in panels B, C the individual data for each monkey has been plotted on samples collected day 14 after the last *Listeria* boost.

analysis of the SIV Env-specific CD8 T cell response by the non-parametric test (Kruskal-Wallis) demonstrated a significant difference between vaccine groups at two time points only. This occurred following the 3rd DNA vaccine ($p=0.012$) and the first *Listeria* boost ($p=0.046$). Subsequently, a Mann–Whitney test was used to demonstrate a significant difference between the control and test vaccine groups at two immunization points (post-3rd DNA and -1st *Listeria* immunization). The DNA only ($p=0.008$), DNA+IL-12 ($p=0.008$) and DNA+IL15 ($p=0.05$) vaccine groups were significantly different to the control group post-3rd DNA immunization. In addition, after the 1st *Listeria* immunization the DNA+IL-12 ($p=0.021$) and DNA+IL-15 were significantly different to the control.

The SIV-Gag responses persisted through to the 3rd *Listeria* boost (Fig. 1Ai), at this time point the SIV-Gag response was detected at day 7 and this decreased to day 14 (Fig. 1Ai). Statistical analysis of the SIV-Gag-specific CD8 T cell response by the Kruskal-Wallis test showed a significant difference between vaccine groups following the 3rd DNA vaccine and following the 3rd *Listeria* dose. Subsequent analysis by Mann–Whitney of the data at these time points showed that all three test vaccines were significantly different to the control vaccine. Following the 3rd DNA immunization compared to the control group the vaccine groups DNA only ($p=0.047$), DNA+IL-12 ($p=0.047$) and DNA+IL-15 ($p=0.028$) were all significantly different. Also, 7 days following the 3rd *Listeria* dose, the vaccine groups DNA only ($p=0.016$), DNA+IL-12 ($p=0.009$) and DNA+IL-15 ($p=0.047$) were also significantly different to the control group.

DNA prime-Listeria boost induce SIV-Gag-specific T cells in the peripheral blood

Peripheral blood samples were collected on days seven and fourteen after the last *Listeria* boost and the presence of SIV-Gag-specific CD8 T cells was assessed by ELISPOT (Fig. 1B) and tetramer analysis (Fig. 2 and Supplementary Table 1). The ELISPOT data in Fig. 1B shows that the PBMCs secrete IFN-gamma in all three test groups. Three responders out of five monkeys were found in DNA and DNA plus IL-12 vaccine groups, whereas four responders out of five monkeys were detected in the DNA plus IL-15 group. Furthermore, depletion of CD8 T cells reduces the number of cells secreting IFN-gamma by >50% in all monkeys that were positive (marked by a * in Fig. 1B), indicating that the CD8 T cells are significant contributors to the IFN-gamma secretion by PBMCs as detected by ELISPOT. When the ELISPOT data for SIV-Gag-specific CD8 T cells in the PB at day 14 was collated with the same data set from the lymph node samples (Fig. 1C) we found two responders (monkeys 4120 and 4112) in the DNA+IL-15 group who had SIV-Gag-specific CD8 T cells in the PB and the axillary lymph node. Conversely, there were a number of monkeys with circulating SIV-Gag-specific CD8 T cells (by ELISPOT) in the PB (4113, 4116, 4109, 4114, 4118 and 4123) that did not have evidence of a response by this assay in either lymph nodes (Fig. 1C).

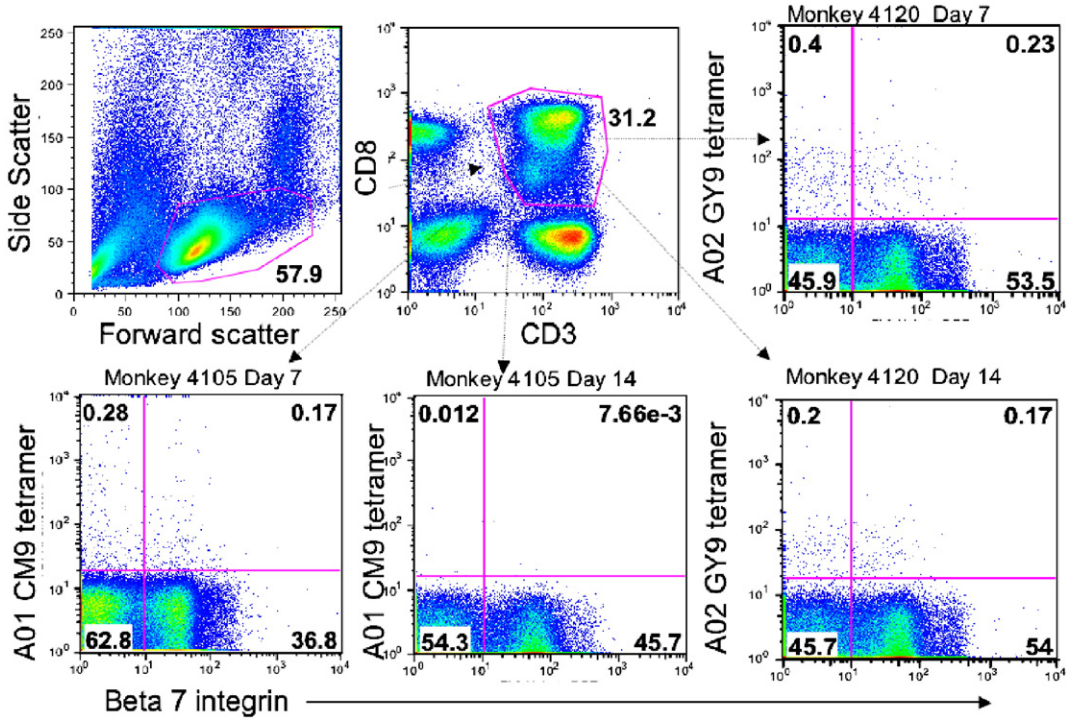

Fig. 2. DNA prime-*Listeria* boost vaccine induces SIV-gag-specific CD8 T cells that are present in the periphery. Dot plots from representative samples demonstrating the binding of SIV-Gag tetramer and surface expression of beta 7 integrin on CD8 T cells from PB. CD8 T cells were first selected based on a lymphocyte scatter gate followed by dual expression of CD3 and CD8.

Interestingly, the ELISPOT assay did not detect any SIV-Gag-specific CD8 T cells in the mesenteric lymph node of immunized monkeys (Fig. 1C). Representative dot plots in Fig. 2 demonstrate the gating strategy to define CD8 T cells, followed by A01 or A02 tetramer binding to the CD8 T cells in the peripheral blood taken on days seven and fourteen post-last oral *Listeria* boost. Monkey 4120 had positive tetramer binding with the A02 SIV-Gag tetramer on both days seven and fourteen; in contrast monkey 4105 has positive binding only on day seven. The frequency of SIV-Gag-specific CD8 T cells in the PB is shown in Supplementary Table 1, a summary of the A01 and A02 SIV-Gag tetramer binding in all peripheral blood samples is shown in Table 2. These data show that each test vaccine-induced SIV-Gag-specific CD8 T cells, whereas CD8 T cells induced by the control vaccine were negative for A01/A02 SIV-Gag tetramer. When tetramer binding to only A01 or A02 monkeys were assessed, three out of four monkeys were positive in group one, both monkeys were positive in group two and three of four were positive in group three. The A02 monkeys positive for tetramer analysis were positive on both days seven and fourteen after the last *Listeria* boost in five of six monkeys. In contrast, positive binding of the A01 SIV-Gag tetramer was detected only on day seven samples.

When the SIV-Gag ELISPOT and tetramer data from PB samples were collated (see Table 2), we demonstrated SIV-Gag-specific CD8 T cells in 5/5 monkeys in group one, 4/5 monkeys in group two and 4/5 monkeys in group three. Furthermore, individual monkeys within these vaccine groups were demonstrated to be either ELISPOT$^+$/Tetramer$^+$, ELISPOT$^-$/Tetramer$^+$ or ELISPOT$^+$/Tetramer$^-$. The ELISPOT$^-$/Tetramer$^+$ group are likely to represent SIV-Gag-specific CD8 T cells that are secreting cytokines other than IFN-gamma, which has been observed previously (Loffredo et al., 2004). Whereas, the ELISPOT$^+$/Tetramer$^-$ group represents monkeys that are responding to epitopes other than those bound by the A01 or A02 tetramer.

DNA prime-Listeria boost immunization induces SIV-Gag-specific CD8 T cells in the gut, draining and distal lymph nodes

In order to assess the mucosal response to DNA prime-oral *Listeria* boost, samples were collected from the gut (jejunum and ileum), mesenteric (draining lymph node) and axillary (distant lymph node) lymph nodes. The gut tissue was separated into two fractions, intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL). SIV-Gag tetramer binding was assessed on all samples; whereas IFN-gamma ELISPOT was only performed on lymph node samples. Representative dot plots depicting positive binding of the A02 SIV-Gag tetramer to CD8 T cells for the lymph nodes and gut tissue are shown in Fig. 3, the frequency of SIV-Gag-specific CD8 T cells in each sample is shown in Supplementary Table 2. In general, higher levels of SIV-Gag tetramer binding were seen in the gut tissue of monkeys than in either of the two lymph nodes. Monkey 4123 was the only individual to respond to the DNA prime-oral *Listeria* boost immunization in all samples tested except the MLN (see Table 2). Furthermore, pooling the gut tissue results showed

Table 2
Summary of SIV-gag-specific T cells in the PB, the gut, draining and distant lymph nodes detected by tetramer staining (T) and ELISPOT (E)

Group	Immunization	Monkey	Haplotype	PBMC				ALN		MLN		Jejunum		Ileum	
				D7 T	D7 E	D14 T	D14 E	T	E	T	E	IEL	LPL	IEL	LPL
1	DNA prime	4113	A08, B01	−	−	−	+	−	−	−	−	−	−	−	−
	Lm boost	4107	A01, A02, B17	+	−	−	−	+	−	+	−	+	−	−	−
	No cytokine	4110	A01	+	+	−	−	+	+	+	−	−	−	−	+
		4111	A02	+	−	−	−	−	−	+	−	−	+	−	+
		4116	A02, A08, B01	−	−	−	+	−	−	−	−	−	−	−	−
2	DNA prime	4104	A08, B01	−	−	−	−	−	−	−	−	−	−	−	−
	Lm boost	4108	none	−	+	−	−	−	−	−	−			−	
	IL-12	4109	A08	−	+	−	+	−	−	−	−	−	−	−	−
		4105	A01, A02	+	−	−	−	+	−	+	−	+	−	+	+
		4114	A02	−	−	+	+	−	−	−	−	+	+	−	+
3	DNA prime	4112	none	−	−	−	+	−		−	−	−	−	−	−
	Lm boost	4117	A01, A08	−	−	−	−	+	+	+	−	−	−	−	+
	IL-15	4118	A02	+	−	−	+	−	−		−	+	−	−	+
		4120	A02, B01	+	+	+	+	−	−	−	−	−	−	−	+
		4123	A02	+	−	+	+	−	+	−	−	+	+	+	+
4	Control DNA	4121	none	−	−	−	−								
	LmE7 boost	4122	B01	−	−	−	−		−	−	−	−	−	−	−
		4115	B01	−	−	−	−	−	−	−	−	−	−	−	−
		4106	A02, A08	−	−	−	−	−	−	−	−	−	−	−	−
		4119	A01, A02, B01	−	−	−	−								

PBMC samples were collected either day 7 (D7) or day 14 (D14) post-*Listeria* boost, whereas axillary LN (ALN), mesenteric lymph node (MLN), jejunum and ileum were all collected on day 14. Sections of jejunum and ileum were processed to recover intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL), tetramer analysis only was performed on these samples. Tetramer binding was calculated as the % CD8 T cells staining positive for the A01-restricted tetramer CM9 or the A02-restricted tetramer GY9. This was calculated by the addition of the upper left and upper right quadrants for the beta 7 versus tetramer binding dot plots, as per Fig. 2 for PB and Fig. 3 for tissue samples. Positive tetramer binding or ELISPOT results were determined as per the methods and is represented by "+", a negative result "−".

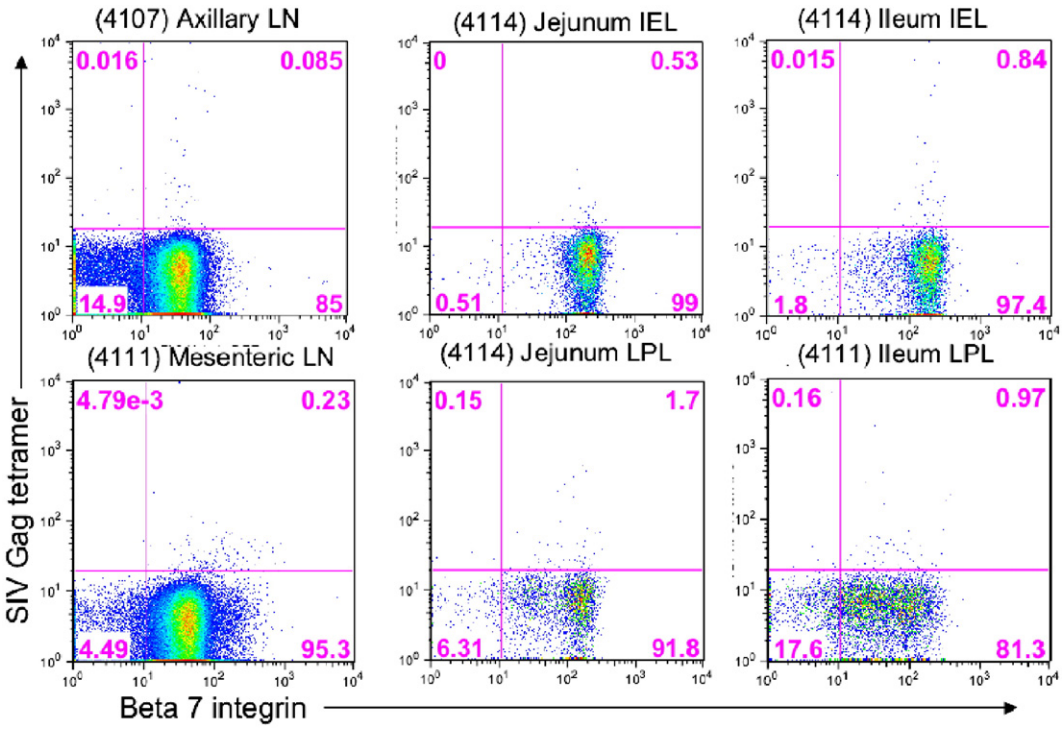

Fig. 3. DNA prime-*Listeria* boost vaccine induces SIV-gag-specific CD8 T cells that are present in the gut and lymph nodes. Dot plots from representative samples (monkey no. in brackets) demonstrating the binding of SIV-Gag tetramer and surface expression of beta 7 integrin on CD8 T cells from gut tissue (jejunum and ileum), mesenteric and axillary lymph nodes. CD8 T cells were selected based on a lymphocyte scatter gate and dual expression of CD3 and CD8.

elevated SIV-Gag tetramer binding to CD8 T cells in 4/5 possible responders in group one, 2/2 in group two and 3/4 in group three. No monkeys from vaccine control group four had detectable SIV-Gag-specific CD8 T cells by IFN-gamma ELISPOT or tetramer staining.

Beta 7 integrin expression on SIV-Gag-specific CD8 T cells in the peripheral blood does not differ between different vaccine groups

Beta 7 integrin was detected on a significant proportion of the SIV-Gag-specific CD8 T cells in the PB (see representative dot plots in Fig. 2). Alpha 4 beta 7 integrin must be expressed on antigen experienced CD8 T cells in order for them to adhere and migrate through gut blood vessels (Butcher et al., 1999; Lefrancois et al., 1999). Therefore, beta 7 integrin expression can serve as a marker of mucosal-homing CD8 T cells on peripheral blood CD8 T cells. Beta 7 integrin expression was assessed on CD8 T cells concomitantly with SIV-Gag tetramer binding, this enabled assessment of beta 7 integrin on antigen-specific CD8 T cells as well as the total CD8 T cell population in the PB. Firstly, the CD8 T cells from monkeys with elevated binding of A01/A02 SIV-Gag tetramer were examined (see Fig. 4). We detected a range of (35–83%) of SIV-Gag-specific CD8 T cells that co-express beta 7 integrin. Furthermore, the beta 7 integrin expression on these cells was predominantly at moderate levels. There was no difference between vaccine groups regarding the expression of beta 7 integrin on SIV-Gag-specific CD8 T cells.

CD8 T cells from immunized monkeys have an increased frequency and expression of beta 7 integrin in the peripheral blood

Next, the expression of beta 7 integrin was assessed on all CD8 T cells in the peripheral blood. A sequential gating technique was used to define T cells and then display beta 7 integrin expression on CD8 T cells (see Fig. 5). Beta 7 expression on CD8 T cells was separated into low, moderate and high levels and the relative percentage in each group recorded for all samples. Analysis of the frequency of CD8 T cells with low, moderate or high beta 7 levels in the peripheral blood demonstrated a change in a subset of the immunized monkeys in CD8 T cells that express moderate or high levels of beta 7 integrin (see Fig. 6), there was no apparent change in the beta 7 low subgroup (data not shown). When CD8 T cells with moderate beta 7 expression were assessed, a number of immunized monkeys showed increasing frequency of this phenotype from day 7 to day 14 post final *Listeria* boost. Furthermore, many of these monkeys (with the increasing frequency of CD8 T cells expressing beta 7 moderate) were previously shown to have SIV-Gag-specific CD8 T cells (arrowed in Fig. 6A). When the frequency of CD8 T cell expressing high beta 7 integrin were assessed a similar picture emerged. However, in this case, there was a preponderance of immunized monkeys from group three that showed increasing levels of beta 7 high CD8 T cells and were SIV-Gag specific (arrowed in Fig. 6B). Interestingly, monkey 4123 (from vaccine group three) had increased levels of CD8 T cells with beta 7 integrin^{hi} by day seven which subsequently dropped by

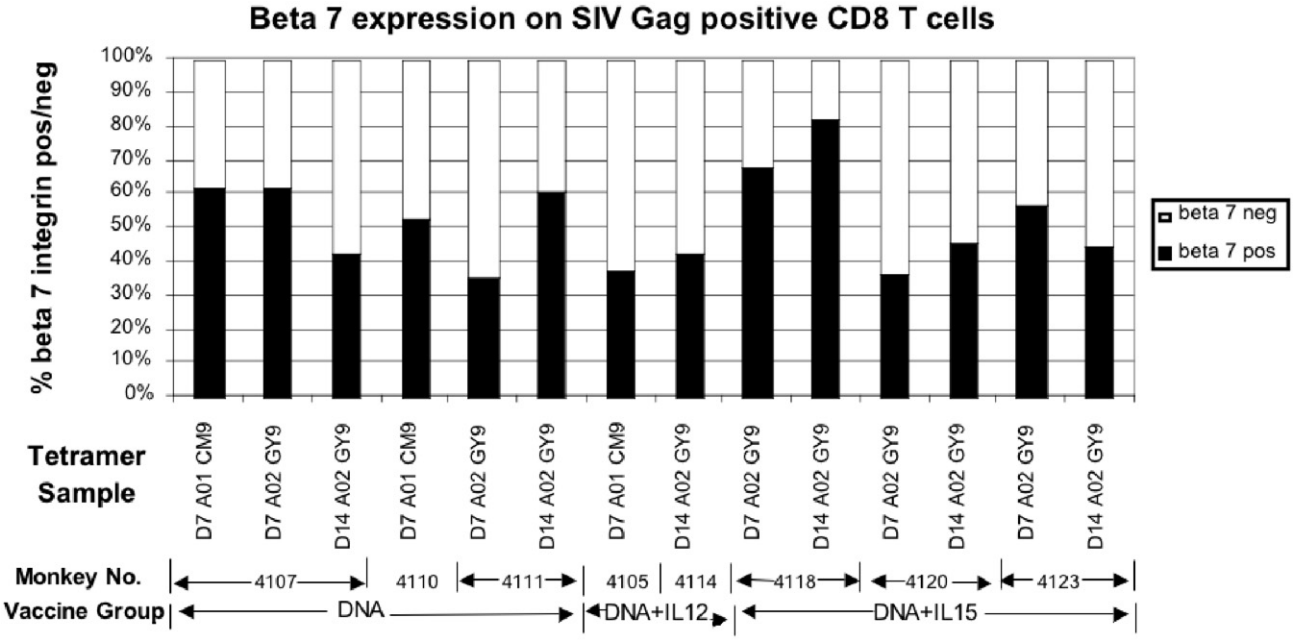

Fig. 4. Beta 7 integrin expression on SIV-Gag-specific CD8 T cells in the peripheral blood does not differ between different DNA prime-*Listeria* boost vaccine groups. Results show the frequency of SIV-Gag tetramer-positive CD8 T cells that co-express beta 7 integrin (black) or lack beta 7 integrin (white). Data are presented on SIV-Gag tetramer (A01 or A02)-positive cells from day 7 (D7) or day 14 (D14) post-last *Listeria* boost. Also indicated are the monkey number and the vaccine group to which individual monkeys belong.

day fourteen. This same monkey had SIV-Gag-specific CD8 T cells in the peripheral blood, gut tissue and axillary lymph nodes.

When the beta 7 expression on CD8 T cells was examined in the PB of pre-immunized monkeys there was no difference in the frequency of beta 7 moderate or high CD8 T cells compared to day 7 (data not shown). Further analysis of the alpha chain expression coupled with beta 7 integrin on PB CD8 T cells showed that the majority of beta 7 integrin-positive CD8 T cells co-express the alpha 4 subunit (CD49d). Furthermore, when the CD8 T cells were separated into beta 7 integrin moderate or high clusters we demonstrated that both subgroups co-expressed alpha 4 as well as alpha-E (CD103) subunits (see Fig. 6C). The population of CD8 T cells that were beta 7/alpha-E positive is increased in frequency in the beta 7 high cluster, although it is only a minor subgroup compared to the gut-homing beta 7/alpha 4-positive CD8 T cells.

Beta 7 integrin expression on CD8 T cells in the lymph nodes

When just SIV-Gag-specific CD8 T cells were assessed, beta 7 integrin was co-expressed in both the axillary and mesenteric lymph nodes. Furthermore, these cells expressed beta 7 integrin at moderate levels only (see examples in Fig. 3).

When the total CD8 T cell population in the mesenteric lymph node was assessed for beta 7 integrin expression there were (81–97%) CD8 T cells that were beta 7 integrin positive (see Supplementary Fig. 1). The vast majority of these CD8 T cells express beta 7 at moderate levels (67–90%) and a smaller subset were beta 7^{hi} (1–12%). When the beta 7^{hi} CD8 T cells were examined, six monkeys that received the test vaccines had more than 5% CD8 T cells with this phenotype. However, there was no difference in the frequency of this phenotype between vaccine groups or between monkeys that had SIV-Gag-specific CD8 T cells in the mesenteric lymph node. In contrast, when the axillary lymph nodes were examined, only two of the monkeys that had received a test vaccine had beta 7^{hi} CD8 T cells greater than 5%. Nonetheless, the CD8 T cells harvested from axillary lymph nodes do express beta 7 integrin at a high frequency (78–98%) (see Supplementary Fig. 1).

Beta 7 integrin expression on gut CD8 T cells

Beta 7 integrin was investigated on both the IEL and LPL fraction of jejunum and ileum tissue. Example dot plots of CD8 T cell beta 7 expression can be seen in Fig. 5 for each of these gut tissues. These dot plots show some interesting features of CD8 T cells from the gut. Firstly, all CD8 T cells from the IEL and LPL fractions express beta 7 integrin. Secondly, in general, CD8 T cells from the IEL fraction express beta 7 integrin at higher levels than the LPL's. Furthermore, beta 7 integrin was expressed at high levels on SIV-Gag tetramer-positive CD8 T cells. More detailed analysis of the level of beta 7 expression on ileal CD8 T cells can be seen in Fig. 7, the data for the jejunal CD8 T cell fractions are similar (data not shown). The ileal IEL fraction contains CD8 T cells which are largely beta 7^{hi}, only three monkeys immunized with the test vaccine had less than 50% CD8 T cells with the beta 7^{hi} phenotype. In contrast, a more heterogeneous expression of beta 7 integrin was observed on CD8 T cells from the ileal LPL fraction (see Fig. 7). While all

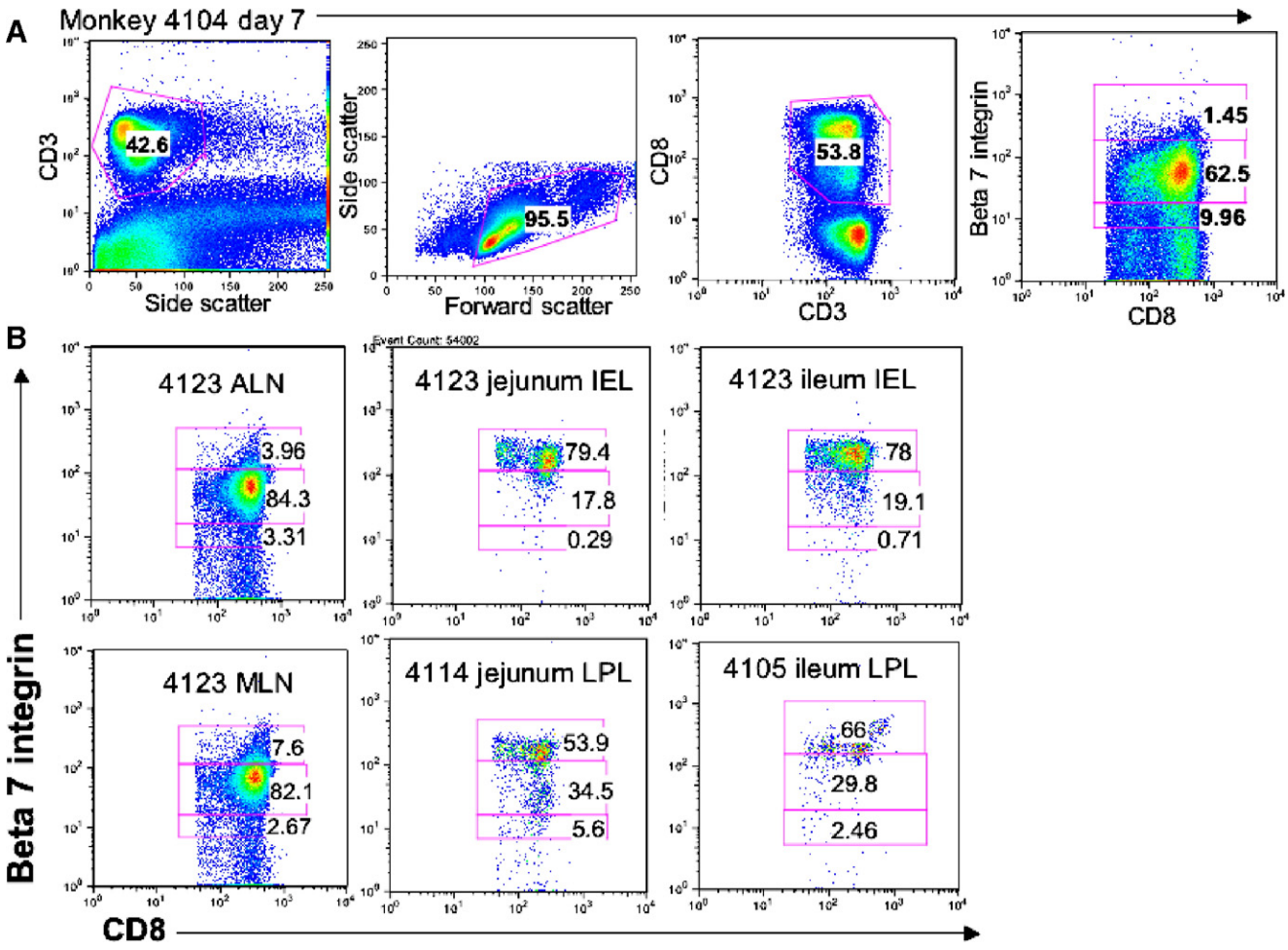

Fig. 5. Beta 7 integrin expression on CD8 T cells differs between the peripheral blood, lymph nodes and the gut tissue. Dot plots from representative samples depict the relative expression of beta 7 integrin on CD8 T cells isolated from peripheral blood(A), mesenteric or axillary lymph nodes and gut tissue (B). T cells were selected based on expression of CD3 and a lymphocyte scatter gate.

the CD8 T cells express beta 7 integrin, more than half of the immunized monkeys' express predominantly beta 7 integrin at moderate levels in the ileal LPL fraction.

Central and effector memory CD8 T cells are induced by a DNA prime-Listeria boost strategy

The relative frequency of naive, central and effector CD8 T cells was evaluated in all peripheral blood, lymph node and gut tissue samples using expression of CD28 and CD95 as a phenotypic indicator of functional type. The phenotype for these 3 functional subgroups in the lymphocytes of rhesus macaques has been previously established by Pitcher et al. (2002). In this study, they identified that CD8 T cells in the PB can be divided into naive ($CD28^+/CD95^-$), effector memory ($CD28^-/CD95^+$) and central memory ($CD28^+/CD95^+$) cells. They identified these CD8 T cell subgroups using intra-cellular cytokine staining for cytokines and co-expression of CD markers following stimulation by the superantigen SEB plus anti-CD49d or rhesus macaque CMV IE-1 peptide mixes.

In our study, the pattern of CD28 and CD95 expression on CD8 T cells was identical to that reported by Pitcher et al. in the peripheral blood, lymph nodes and the gut IELs (see Fig. 8 for example dot plots). On day 7, the peripheral blood contains largely naive CD8 T cells; however, the naive CD8 T cells decrease in frequency from day 7 through to day 14 in immunized monkeys, $p=0.01$ (see Fig. 9). Simultaneously, the effector memory CD8 T cell population increased in the peripheral blood from day 7 through to day 14 in most immunized monkeys, $p=0.06$ (see Fig. 9). The frequency of naive and effector CD8 T cells in the PB samples of pre-immune monkeys is similar to that seen on day 7 post last *Listeria* boost (data not shown).

The frequency of naive, effector and central memory CD8 T cell subsets in the lymph nodes of immunized monkeys differed from peripheral blood. Naive CD8 T cells are the major subgroup in the axillary and mesenteric lymph nodes and range from (55–85%) of CD8 T cells (see Supplementary Fig. 2). The central memory CD8 T cells are the next most common subgroup in both sets of lymph nodes and constitute (10–50%) of CD8 T cells. In contrast,

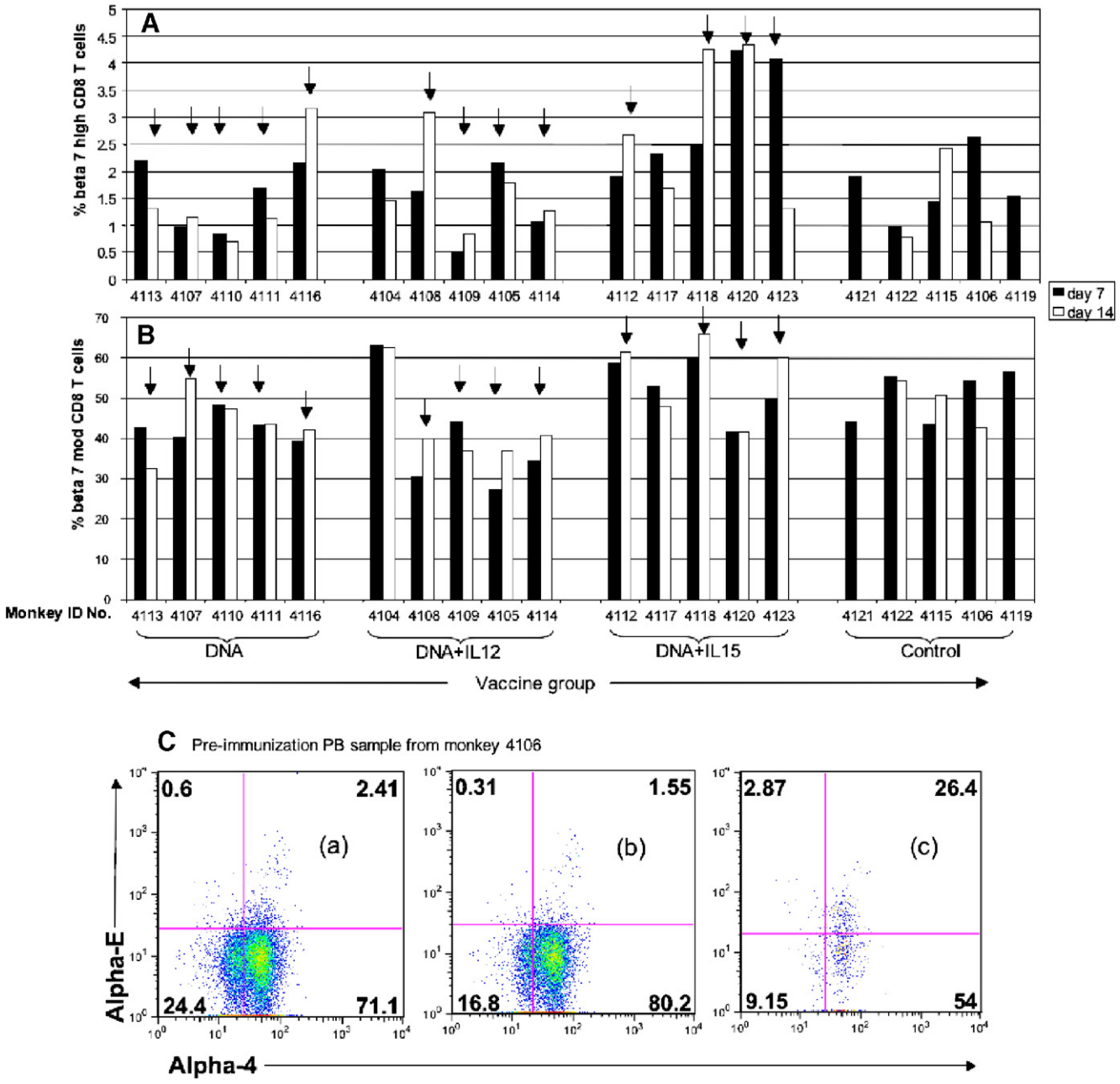

Fig. 6. CD8 T cells expressing $\alpha_4\beta_7$ integrin increase in the peripheral blood of some immunized monkeys from day 7 to day 14 post-*Listeria* boost. The percentage of CD8 T cells expressing beta 7 integrin was monitored in the peripheral blood on days 7 and 14 after the final *Listeria* boost immunization. This graph represents the frequency of CD8 T cells expressing beta 7 at high (A) or moderate (B) levels. CD8 T cells were selected based on co-expression of CD3 and CD8 and a lymphocyte scatter gate. Arrows indicate monkeys with CD8 T cells positive for SIV-Gag tetramer binding and/or IFN-gamma ELISPOT. The integrin alpha chain co-expressed with the beta 7 subunit was demonstrated in panel C. Representative dot plots in panel C depicting the alpha chain expression on CD8 T cells that are beta 7 integrin positive (a), beta 7 moderate (b) and beta 7 high (c).

the gut tissue IELs consisted almost exclusively of CD8 T cells with an effector memory phenotype (see Fig. 8 for representative dot plots, and Supplementary Fig. 3 for individual data) and a few (<5%) CD8 T cells with a central memory phenotype. Statistical analysis (Mann–Whitney) of the naive, effector and central memory CD8 T cell frequency in the gut demonstrated a significant difference ($p=0.048$) between the DNA+IL-15 versus DNA groups in the ileum effector memory CD8 subset only. Otherwise, there was no difference in the frequency of naive, effector or central memory CD8 T cells between vaccine groups in the lymph node or gut tissue.

Discussion

Inducing a mucosal cell-mediated immune response has become a clear aim for successful HIV vaccines. This is based on knowledge that HIV is commonly transmitted across

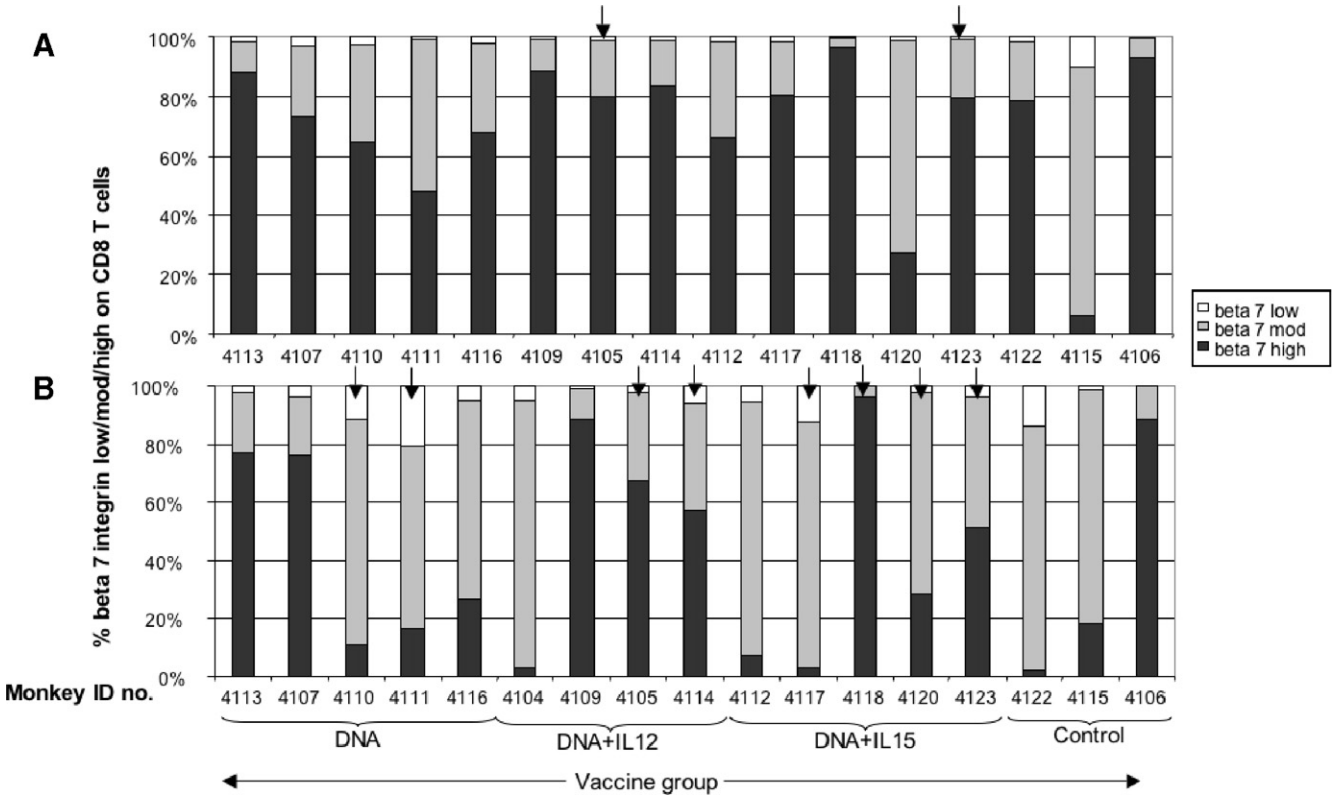

Fig. 7. High beta 7 integrin expressing CD8 T cells are prevalent at gut effector sites. The percentage of CD8 T cells expressing beta 7 integrin at high, moderate or low levels was monitored in the ileum, intraepithelial cells (A) and lamina propria lymphocytes (B) day 14 after the final *Listeria* boost immunization. CD8 T cells were selected based on co-expression of CD3 and CD8 and a lymphocyte scatter gate, beta 7 expression on CD8 T cells was divided into either low, moderate or high levels (as per Fig. 5). Arrows indicate monkeys with elevated numbers of CD8 T cells that express beta 7 at high levels and are positive for SIV-Gag tetramer and/or IFN-gamma ELISPOT.

mucosal surfaces following sexual contact, HIV establishes an active infection in memory CD4 T cells in the gut and depletes them. This massive assault on the immune system in the gut happens early in the infection before the virus is evident in the periphery. Therefore, a successful vaccine should induce HIV-specific CD8 T cells that would prevent the active replication of the virus in the gut and avoid the subsequent elimination of CD4 T cells.

SIV infection in rhesus macaque monkeys has been commonly used to model HIV infections in humans. In fact, a series of studies have measured the mucosal cell-mediated immune response to SIV vaccines (Baig et al., 2002; Cranage et al., 1997; Cromwell et al., 2000; Evans et al., 2003; Imaoka et al., 1998; Klavinskis et al., 1996; Lehner et al., 1999; Shacklett et al., 2002; Sharpe et al., 2003; Wang et al., 2000) in rhesus macaques. Two of these studies are noteworthy because the vaccines initiate an immune response in different ways. The first study uses a modified Salmonella vector with a type III secretion system which targets antigens to the cytoplasm and induces a CD8 T cell response (Evans et al., 2003). The second study uses attenuated strains of SIV (administered *i.v.*), which are thought to induce a mucosal immune response because the virus replicates in the gut CD4 T cell population (Cromwell et al., 2000). In this study, we used a novel *Listeria* vaccine vector to deliver target antigens as a vaccine for SIV. Our current study tested if we could amplify the mucosal CD8 T cell response to SIV by combining the DNA prime-oral *Listeria* with either IL-15 or IL-12 plasmid vaccines. We designed the study to have 4 vaccine groups, three received the test SIV DNA vaccines with or without IL-12 or IL-15. The control group received a DNA prime-oral *Listeria* boost vaccine with irrelevant antigens.

Initially, SIV-Gag and Env-specific CD8 T cells were demonstrated in the PB; however the Env responses decreased sharply after the 1st *Listeria* boost. After completion of the immunization schedule, we monitored the SIV-Gag CD8 T cell response in the peripheral blood, lymph nodes and the gut. SIV-Gag-specific CD8 T cells were demonstrated in the PB from monkeys within all test vaccine groups on both days 7 and 14 post-last *Listeria* boost. The combination of ELISPOT and tetramer data was more compelling for the DNA + IL-15 group compared to the other test groups, in that there was a larger number of responding monkeys in this group in the PB and the axillary lymph node. Further examination of these SIV-Gag-specific CD8 T cells in the PB demonstrated a variable frequency of expression of the gut-homing molecule, beta 7 integrin. There was no difference in beta 7 integrin expression on SIV-Gag CD8 T cells in the PB between vaccine groups. Further characterization of the CD8 T cells in the PB revealed that the beta 7 integrin-positive cells expressed the alpha 4 subunit, a minor subset also co-express alpha-E subunit. SIV-Gag-specific CD8T cells were also demonstrated in gut tissue, gut draining lymph nodes (mesenteric) and distant lymph node

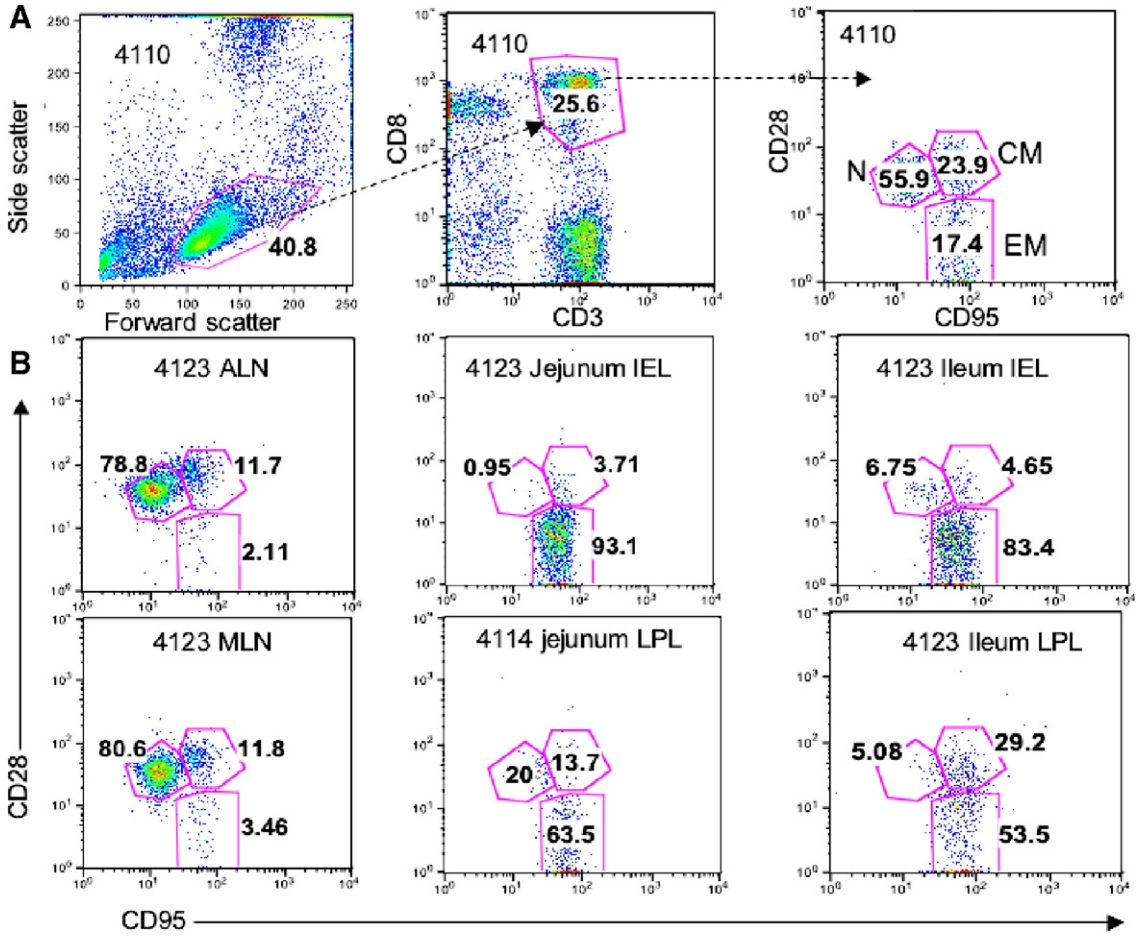

Fig. 8. Gut tissue, lymph nodes and peripheral blood contain different proportions of naive and memory CD8 T cells. Dot plots from representative samples showing (A) a peripheral blood sample with the gating procedure to define CD8 T cells and then CD95 versus CD28 expression to define the naive (N), central (C) and effector (EM) memory cell. Dot plots in (B) show representative distribution of naive, central and effector memory cells in lymph nodes and gut tissue.

(axillary). The SIV-Gag-specific CD8 T cells in these tissues all expressed beta 7 integrin, but at varying density on the cell surface. In the gut IEL fraction, and to a lesser extent the LPL fraction, the SIV-Gag-specific CD8 T cells express beta 7 integrin at high levels. This most likely represents the co-expression of two separate heterodimeric integrin molecules, alpha-E as well as alpha 4 with beta 7 (Farstad et al., 1996). Alpha 4 beta 7 is required for adhesion of the CD8 T cell to the gut endothelium, this occurs via its ligand MadCAM-1 and from this point the CD8 T cell can migrate into the gut lamina propria (Andrew et al., 1996; Rott et al., 1996). Subsequently, the CD8 T cell may also express alpha-E beta 7, a feature of IELs, this enables the cell to bind E-Cadherin expressed by the epithelial cells of the gut mucosa.

Interestingly, we also observed beta 7 integrin expression on SIV-Gag-specific T cells in both the axillary and mesenteric lymph nodes. Beta 7 integrin expression on CD8 T cells in lymph nodes has been described in previous studies (Andrew et al., 1996; Farstad et al., 1996; Pitcher et al., 2002), loss of beta 7 integrin partially decreases migration to mesenteric lymph nodes but does not affect migration to peripheral lymph nodes (e.g., axillary lymph node) (Wagner et al., 1996). This effect is due to the differential expression of ligands on the high endothelial venules at the respective sites, mesenteric lymph nodes express peripheral node addressin (L-selectin ligand) and/or MadCAM-1 (alpha 4 beta 7 ligand) whereas peripheral lymph node HEV only express peripheral node addressin.

Our data describing the induction of SIV-specific CD8 T cells in the PB, gut and lymph nodes is similar to that described previously by other studies, which used a smaller number of monkeys to test induction of mucosal immunity by a SIV vaccine (Baig et al., 2002; Cromwell et al., 2000; Evans et al., 2003; Sharpe et al., 2003). Cromwell et al. (2000) observed SIV-Gag-specific CD8 T cells in response to an attenuated SIV vaccine in PB and the gut. We have a similar frequency of SIV-Gag-specific CD8 T cells as well as induction of both beta 7-positive and -negative CD8 T cells to SIV-Gag in the PB. This suggests that we have induced SIV-Gag-specific CD8 T cells that have the capacity to home to mucosal and other effector sites. In another study, a *Salmonella* vector was used to deliver SIV-Gag (Evans et al., 2003) and was focused on mucosal priming. Similar to the *Listeria* vector, this vector delivers antigen to both MHC class I and II processing pathways (Russmann et al., 1998). The authors demonstrated a SIV-Gag-specific CD8 T cell response in 2 rhesus macaques that received the *Salmonella* Gag plus MVA Gag boost, both these monkeys had beta 7 integrin-positive and -negative SIV-Gag CD8 T cells

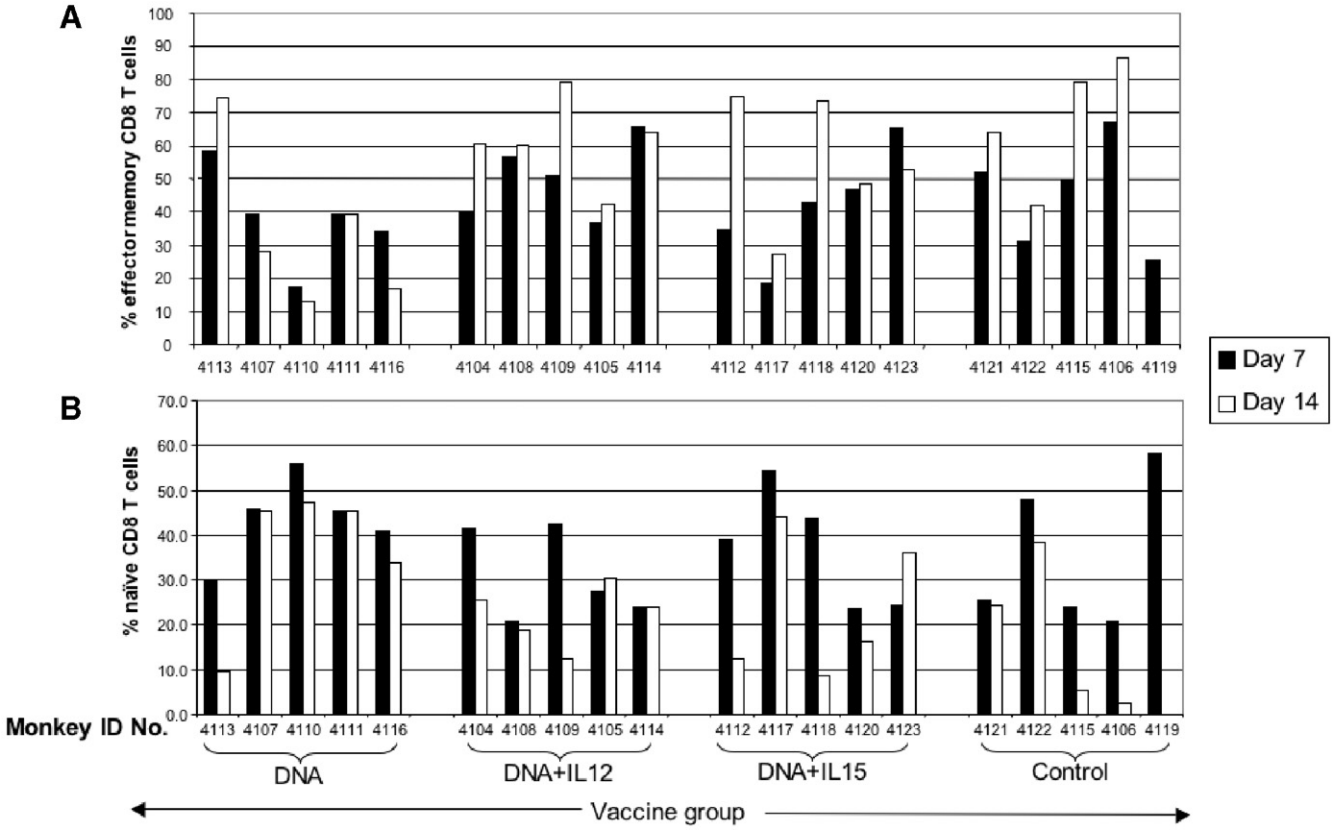

Fig. 9. Effector memory and naive CD8 T cells alter in the peripheral blood after immunization. Effector memory and naive CD8 T cells were assessed in the peripheral blood on days 7 and 14 post-*Listeria* boost. Relative expression of CD95 and CD28 on CD8 T cells was used to define the effector memory (A) and naive (B) cells. CD8 T cells were defined by scatter gates and co-expression of CD3 and CD8.

in the PB. Baig et al. (2002) showed SIV-Gag-specific T cells in the PB and colonic mucosa following *i.m.* immunization with plasmid DNA containing the SIVmac239 gag, pol, nef genes and HIV 89.6 *env* epitope genes boosted by MVA expressing SIC gag-pol and HIV 89.6 (17 monkeys). In addition, Sharpe et al. (2003) demonstrated SIV-Gag-specific CTL in mesenteric, iliac and inguinal lymph nodes following intra-rectal immunization with HIV and SIV CTL epitope DNA in microparticles (3 monkeys). We have demonstrated a similar magnitude of SIV-Gag-specific CD8 T cells in the periphery and the gut mucosa in response to our vaccine in a larger group of monkeys and found this response to be consistent across all test vaccine groups.

We have evidence of a CD8 T cell response to the SIV vaccine in most monkeys in all test vaccine groups. Although there was no marked difference in the final gut mucosal response between vaccine groups there was a difference detected in the PB during the priming phase of the study, specifically after the 3rd DNA and 1st *Listeria* boost. This suggests that the IL-15 or IL-12 plasmid may not be giving additional benefit to the mucosal immune response to SIV DNA-oral *Listeria* boost. However, it is possible that these two cytokines could be inducing functionally different CD8 T cells. Therefore, further studies could be done to examine the SIV-Gag CD8 T cell functional phenotype (Betts et al., 2005) after completion of the last oral *Listeria* boost as well as looking for a memory response.

In addition, we describe a change in the frequency of effector memory and naive CD8 T cells in the PB in response to immunization. Naive CD8 T cells decrease and effector memory CD8 T cells increase in response to immunization. The frequency of naive and effector memory CD8 T cells in the gut was similar to that described previously (Pitcher et al., 2002). Although there was some individual variation (especially in the gut lamina propria lymphocyte fraction), the only significant change in the frequency of these functional CD8 T cell subsets occurred in the ileum IEL fraction in response to the DNA + IL-15 immunization.

In our previous study (Boyer et al., 2005b), a DNA prime-*Listeria* boost vaccine-induced SIV-Gag and Env-specific T cells. When the immunized monkeys were challenged with SIVMac239, it was found the monkeys that received the DNA prime-*Listeria* boost vaccine had significantly better control of viral loads for a longer period than the control monkeys. In this study, we characterized the mucosal CD8 T cell response to the DNA prime-*Listeria* boost vaccine; because this required sacrifice of the animals, it was not logistically possible to simultaneously perform a SIV challenge in the same group of immunized monkeys. Nonetheless, based on the data from our prior study (Boyer et al., 2005b), we would expect that following DNA prime-*Listeria* boost the monkeys would be more resistant to SIVMac239 challenge than control monkeys.

In summary, this study has demonstrated that a DNA prime-oral *Listeria* boost strategy induces a mucosal SIV-Gag-specific CD8 T cell response. We provided evidence of the SIV-Gag-specific CD8 T cells circulating in the PB, many of which had gut-homing capacity. Importantly, these antigen-specific CD8 T cells were present in the gut, both the lamina propria and between the epithelial cells of the gut mucosa. This SIV-specific mucosal immunity has the potential to prevent SIV infection at mucosal surfaces and limit the rapid expansion of SIV in the gut.

Methods

Animals

Male and female Indian rhesus macaques (*Macaca mulatta*) 3–8 years old were evaluated at the start of the study. The rhesus macaques were housed at Bioqual, Rockville, MD, under USDA approved conditions. These facilities are accredited by the American Association for the Accreditation of Laboratory Animal Care International and meet National Institute of Health standards as set forth in the Guidelines for Care and Use of Laboratory Animals. Animals were tested for MHC class I SLA expression by HLA/Molecular Diagnostics Laboratory at the University of Wisconsin. They were assigned to different vaccine groups as shown in Table 1 to evenly spread the distribution of haplotypes.

Vaccines and immunization

DNA vaccines

The pCSIVenv and the pCSIVgag plasmids expressing the SIV envelope and core protein, Gag, are Rev-independent expression vectors that have been codon optimized for high level expression as previously described (Nappi et al., 2001). Codon optimization mutates the DNA sequence without disrupting the amino acid sequence. Ultimately, there is mutation or elimination of the RRE inhibitory sequence which leads to the more abundant translation of tRNA. The final construct was characterized by restriction digest and DNA sequence.

The dual promoter IL-12 expression vector encodes the macaque IL-12 p35 and p40 genes (Boyer et al., 2005a). The rhesus IL-12 p35 subunit is expressed under control of the HCMV immediate early promoter and SV40 polyadenylation signal, whereas the rhesus IL-12 p40 subunit is expressed under control of the simian CMV promoter (SCMV) and BGH polyadenylation signal.

The cloning of the macaque IL-15 construct (Sequence from GenBank, accession number U19843) into pVAX1 cloning vector (Invitrogen, Carlsbad, CA) was carried out by PCR amplification with the following 5′ to 3′ primers. Sense: GCCCCCGTCGACGCCGCCACCATGAGAATTTCGAAA-CCACATTTGAG, Antisense: ATCGGGCTCGAGTCAA-GAAGT GTTGATGAACATTTGG. The features of pVAX1 cloning vector that make it suitable for use as a vector designed for DNA vaccines include a CMV promoter for high-level expression in a wide range of mammalian cells, BGH polyadenylation signal for efficient transcription termination and polyadenylation of mRNA and kanamycin resistance gene for selection in *Escherichia coli*. PCR conditions for all reactions were 1 cycle at 97 °C for 3 min, 30 cycles at 94 °C for 1 min, 55 °C for 75 s and 72 °C for 75 s, followed by final extension at 72 °C for 10 min. The 488 base pair PCR product was ligated into pVAX1 cloning vector following a restriction enzyme digestion using *Eco*RI and *Xho*I that were designed into the PCR primers and are in the multiple cloning region of pVAX1. All positive clones were verified by sequence analysis.

Plasmids were purified by Puresyn (Malvern, PA). DNA was formulated in 0.15 M citrate buffer and 0.25% bupivicaine at a pH of 6.5. Expression of the IL-12 (Boyer et al., 2005a) and IL-15 (Kutzler M and Boyer J, unpublished observations) plasmid constructs were demonstrated following transient transfection of RD cells. RD cell supernatants were harvested after 72 h and analyzed to verify the presence of IL-12 or IL-15 protein by commercial ELISA assay.

Listeria vaccines

The recombinant *Listeria monocytogenes* vaccines used in this study have been described in detail in a previous study (Boyer et al., 2005b). Three *Listeria* constructs were used for the oral boost segment of the immunization protocol, Lm-Gag secretes SIV-Gag protein. Due to difficulties in *Listeria* secreting SIV Env, two separate *Listeria* constructs were made that together overlap the entire gp120 sequence. Lm-SIV-Env-5′ expresses fragment residues 30–298 (no signal sequence) and Lm-SIV-3′ expresses residues 198–525 (Boyer et al., 2005b). Prior to their use all strains of bacteria were mouse-passaged in order to stabilize their behavior *in vivo* (Peters and Paterson, 2003). Expression of the SIV proteins by recombinant *Listeria* was confirmed by Western blot. The bacteria were propagated on BHI (Difco, Detroit, MI), bacterial aliquots were stored at −70 °C. An inoculum of bacteria was prepared for oral immunization by thawing an aliquot and appropriately diluting it in sterile PBS.

Immunization schedule

Four groups of Indian rhesus macaques were immunized as outlined in Table 1. In three of the immunization groups, five rhesus macaques were immunized *i.m.* at weeks 0, 4, 8 and 12 with 2 mg each of the pCSIVgag and pCSIVenv DNA plasmids. The DNA immunizations were followed by four boosts with recombinant *L. monocytogenes* at weeks 16, 20, 24 and 38. The *L. monocytogenes* was delivered orally as an equal mixture of 10^{10} Lm-SIV-Gag and Lm-SIV-Env-5′ and -3′ providing a total dose of 3×10^{10} bacteria for the first two immunizations and 3×10^{11} bacteria/dose for the final two immunizations. A control group received 2 mg Variola DNA (genes A27L and A4L) followed by three boosts with recombinant *L. monocytogenes* expressing HPV-16 E7 (Gunn et al., 2001) at the same time intervals as the test groups. Thirty minutes prior to the oral immunization, monkeys were given Zantac (ranitidine hydrochloride) 2.5 mg/kg *i.m.* The animal was then anesthetized

with ketamine hydrochloride 10 mg/kg *i.m.* Then, 40 ml CeraVacx Buffer was administered *p.o.* via a nasogastric tube. Each dose of vaccine was thawed in 1 ml lots and administered orally via the gastric tube.

Collection of samples and preparation of cells

Peripheral blood was collected during the immunization schedule and on days 7 and 14 after the last oral *Listeria* immunization. PBMCs were prepared by standard density gradient centrifugation and a viable cell count performed.

On day 14 after the last oral *Listeria* immunization, a necropsy was performed and tissue samples collected, including axillary and mesenteric lymph nodes and 10 cm segments of jejunum and ileum. Excision of the jejunum was performed 30–40 cm distal to the pylorus to obtain proximal jejunum tissue. Lymphocytes were isolated from the lymph nodes following physical disaggregation and passing the cells through a 70-μm sieve followed by a Tris-buffered ammonium chloride lysis step. In contrast, lymphocytes were isolated from the gut tissue to obtain intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) as described previously (Veazey et al., 1997). Briefly, each gut tissue segment was incubated in 30 ml Hank's Balanced Salt Solution (HBSS) containing 1.0 mM EDTA for 30 min at 37 °C in an orbital shaker. Supernatant was collected, the cells were isolated by centrifugation and resuspended in RPMI and stored on ice. The process was repeated with the tissue samples and the harvested cells were pooled, washed in calcium/magnesium-free PBS and resuspended in RPMI1640 medium containing penicillin, 25 mM HEPES buffer, 2 mM L-glutamine and 5% FBS, these cells form the IEL fraction. LPLs were collected by cutting the residual intestinal segments into 1–2 mm pieces and transferring the tissue into a flask of RPMI 1640 medium containing 0.75 mg/ml collagenase (90 U/ml), penicillin, 25 mM HEPES buffer, 2 mM L-glutamine and 5% FBS. Flasks were incubated for two 30 min periods at 37 °C; at the end of each interval, intestinal pieces were further separated by repeated pipetting with a 10-ml pipette. The resulting medium was passed through a sieve (size 40 μm) to separate the tissue from the LPLs. The IEL and LPL cell suspensions were further enriched using a Percoll bilayer density gradient centrifugation. The Percoll gradient included 2 ml of 60% Percoll under 2 ml of 35% Percoll. The cell suspensions were layered over the Percoll bilayers and centrifuged for 20 min at 1000×*g*. Cells were harvested and washed and resuspended in RPMI for immunological assessment.

Antibodies and tetramers

The following antibodies from BD Pharmingen were used in this study CD8 (RPTA-8), beta 7 integrin (FIB504), CD3 (SP34-2), CD95 (DX2) and CD28 (CD28.2). In addition, the antibodies CD8 beta (2ST8.5H7), CD103 or alpha-E (2G5) and CD49d or alpha 4 (HP2/1) were provided by Beckman Coulter.

Two SIV-Gag tetramers, which were generously provided by David Watkins (University of Wisconsin), were used in this study, the CM9 tetramer (CTPYDINQM, $Gag_{181-189}$) (Allen et al., 1998; Kuroda et al., 1998) a MamuA*01 epitope and GY9 tetramer (GSENLKSLY, Gag_{71-79}) (Vogel et al., 2002) a MamuA*02 epitope.

Flow cytometry

Staining

Two sets of stains were performed on each sample. Firstly, SIV-Gag tetramer binding to CD8 T cells was combined with beta 7 integrin expression. Therefore, 10^6 cells were stained with either APC-conjugated tetramer, CM9 (A01 haplotype monkeys) or GY9 (A02 haplotype monkeys), for 1 h at 37 °C followed by direct labeling with beta 7 integrin-PE, CD3-PerCPCy5.5 and CD8-FITC. The second set of direct stains were performed to define the CD8 T cells as either naive, effector or central memory. In this case, 5×10^5 cells were directly stained with CD95-FITC, CD28-PE, CD8-PECy7 and CD3-APC.

In addition, a subset of peripheral blood samples were prepared to characterize the alpha chain coupled with beta 7 integrin on CD8 T cells. These samples were stained by CD8beta-PE, CD103-biotin, CD49d-FITC, integrin beta 7-PECy5 followed by streptavidin-APC. Isotype controls for each CD marker were included for all samples.

Data acquisition and analysis

Data were acquired on the BD FACSCalibur flow cytometer using the CellQuest PRO software. List mode files were analyzed using FlowJo (Tree Star Inc.), a sequential gating strategy was used to define CD8 T cells before assessment of tetramers or additional CD markers.

Tetramer analysis

Two forms of controls were used for analysis of the tetramer binding to CD8 T cells. Firstly, each vaccination group contained non-A01/A02 haplotype monkeys. In addition, group 4 monkeys received a non-SIV control vaccine. The quadstat regions were set for each test sample based on negative controls for the sample (isotype and 'fluorescence minus one' controls) as well as non-A01/A02 monkeys stained with the same batch and run on the same day. In order to establish, a reference value for positive tetramer binding, the mean value for tetramer binding to non-A01/A02 monkeys was calculated. A test sample was deemed 'positive' if the A01 or A02 tetramer binding was greater than twice the mean of non-A01/A02 monkeys. This value was established for both the A01 CM9 and A02 GY9 tetramers on samples collected days 7 and 14 post final *Listeria* boost. For example, in PB samples day 7, the positive cut-off for A01 tetramer CM9 binding to CD8 T cells was 0.128% and the A02 tetramer GY9 binding was 0.156%, respectively. The same process was used to determine the cut-off for A01 or A02 tetramer binding to gut and lymph node samples.

ELISPOT

Peptides

One hundred and twenty-five peptides corresponding to the entire coding region of SIVmac239 Gag and Env protein were obtained from the AIDS Reagent Reference Repository (NIH). These 15-mers overlapping by 11 amino acids were resuspended in DMSO at a final concentration of 100 μg/ml for ELISPOT analysis.

ELISPOT

ELISPOT was performed using IFN-gamma reagents purchased from MabTech (Sweden) and nitrocellulose plates from Cellular Technologies (Cleveland Ohio). Plates were coated with IFN-gamma capture antibody and incubated overnight at 4 °C. After washing with sterile PBS, the peptide pools were diluted 1:200 in culture medium (RPMI 1640 containing 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin and 10% fetal bovine serum) and 100 μl was transferred into 3 wells. Cells in culture media were added to the wells in triplicate at a concentration of 200,000 cells per well. The plate was incubated for 24 h at 37 °C. Biotinylated antibody at a concentration of 1 μg/ml was added and incubated overnight at 4 °C. After washing, streptavidin-AP was added at a concentration of 1 ng/ml and incubated for 2 h at room temperature. Plates were washed with PBS. BCIP/NBT was added and incubated until spots appeared. The reaction was stopped. A positive response is defined as greater than 50 spot forming cells (SFC) per 1 million PBMCs.

Depletion studies

A second set of PBMCs was depleted of CD8 lymphocytes with anti-CD8 depletion beads according to manufacturer's protocol (Dynal) before plating cells in triplicate with peptides as described in the Methods section for the ELISPOT assay. The ELISPOT protocol as described above was completed on the PBMCs depleted of CD8 lymphocytes.

Acknowledgments

We thank David Watkins for the generous donation of the CM9 and GY9 tetramers for this study, and Matt Reynolds for advice on the optimum staining protocol with these tetramers. Wendeline Wagner and the laboratory animal support team at BIOQUAL for the care and treatment of the animals described in this study and Xiaohui Peng for production of the *Listeria* vaccines. Yvonne Paterson acknowledges that she has a financial interest in Advaxis Inc., a vaccine and immunotherapeutic company that is developing *L. monocytogenes* as a cancer vaccine vector.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2006.06.036.

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