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Protective mucosal immunity elicited by targeted iliac lymph node immunization with a subunit SIV envelope and core vaccine in macaques

THOMAS LEHNER[1], YUFEI WANG[1], MARTIN CRANAGE[2], LESLEY A. BERGMEIER[1], ELAINE MITCHELL[1], LOUISA TAO[1], GRAHAM HALL[2], MIKE DENNIS[2], NICOLA COOK[2], ROGER BROOKES[1], LINDA KLAVINSKIS[1], IAN JONES[3], CARL DOYLE[3] & ROBERT WARD[1]

[1]*Department of Immunology, United Medical & Dental Schools of Guy's & St. Thomas' Hospital, London SE1 9RT, UK*
[2]*Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG, UK*
[3]*Institute of Virology and Environmental Microbiology, Mansfield Road, Oxford OX1 3SR, UK*
Correspondence should be addressed to T.L.

Prevention of sexually transmitted HIV infection was investigated in macaques by immunization with a recombinant SIV (simian immunodeficiency virus) envelope gp120 and core p27 vaccine. In two independent series of experiments, we used the novel targeted iliac lymph node (TILN) route of immunization, aiming close to the iliac lymph nodes draining the genitorectal mucosa. Rectal challenge with the SIVmac 32H J5 molecular clone in two series induced total protection in four out of seven macaques immunized by TILN, compared with infection in 13 of 14 unimmunized macaques or immunized by other routes ($P = 0.025$). The remaining three macaques showed either a decrease in viral load (>90%) or transient viremia, indicating that all seven TILN-immunized macaques showed total or partial protection ($P = 0.001$). Protection was associated with significant increase in the iliac lymph nodes of IgA antibody-secreting cells to p27 ($P < 0.02$), CD8-suppressor factor ($P < 0.01$), and the chemokines RANTES and MIP-1β ($P < 0.01$).

HIV infection occurs most commonly during homosexual intercourse in developed countries and heterosexual intercourse in developing countries[1-3]. Rectal transmission of HIV-1 is likely to be facilitated by the thin columnar epithelium (24.6 ± 9.7 µm thick), which is about 10 times thinner than the vaginal stratified squamous epithelium[4]. Hence, rectal mucosa is vulnerable to be breached during anal intercourse, providing direct access of infected seminal fluid to the submucosal tissues. Rectal epithelial cells, however, can be infected by semen containing HIV (ref. 5), and HIV nucleic acid has been detected by *in situ* hybridization in rectal epithelium from patients with AIDS and diarrhea[6]. The target for HIV might be the columnar epithelial cells, as cultured human colorectal or ileal epithelial cells[7] and fetal enterocytes have been infected with HIV (ref. 8). CD4 glycoprotein, which is the principal receptor for HIV-1, and Langerhans cells have not been found in rectal epithelium[4,9]. M cells are abundant over Peyer's patches in rectal epithelium and are capable of transmitting HIV-1 (ref. 10). Galactosyl cerebroside is an alternative receptor for HIV-1 in the rectal epithelial cells and can mediate CD4-independent infection by binding defined gp120 epitopes (ref. 11, 12). Fc receptors for IgG (ref. 9) and complement receptors[13] were identified in rectal epithelial cells and may enable HIV/SIV to cross the epithelial barrier by means of virus–IgG antibody–C3 complexes binding to these receptors.

Transmission of SIV *in vivo* through intact rectal epithelium has been demonstrated in macaques[14-16]. Prevention of rectal infection with SIV has been reported by intramuscular immunization with inactivated SIV cells grown in human T cells[14]. Intravenous exposure to low subinfectious doses of live SIV in *Macaca fascicularis* and *Macaca nemestrina* protected the animals against an infectious rectal challenge with SIV, in the absence of detectable antibodies but demonstrable T-cell proliferative responses to SIV (ref. 15). In both above experiments SIV was grown in human T cells, so that antibodies to HLA antigens are likely to have been involved in the protection[17,18]. However, prevention of rectal infection by stimulating the regional mucosal-lymphoid immunity has so far not been tested. Direct rectal mucosal immunization augmented by oral and intramuscular (i.m.) immunization with recombinant SIV core p27, expressed as hybrid Ty virus-like particles (p27:Ty-VLP) and linked to cholera toxin B subunit, elicited secretory IgA (and IgG) antibodies in rectal washings, serum IgA and IgG antibodies, and $CD4^+$ T-cell proliferative responses in the draining iliac and inferior mesenteric lymph nodes, blood and spleen[19].

There is a great deal of evidence to suggest that $CD8^+$ T cells may function as MHC class I-restricted cytotoxic cells[20,21] and as MHC-unrestricted, nonlytic suppressor cells in HIV replication[22,23]. However, cytokines have failed to account for the CD8 suppressor activity[23], although recently very significant homology was reported between the CD8 suppressor factor and the three chemokines: RANTES, MIP-1α and MIP-1β (ref. 24), and IL-16 (ref. 25). Indeed, some seronegative subjects with a high risk of sexual exposure show increased concentrations of T-cell-generated chemokines[26].

The lymph nodes have gained special significance, as they function as HIV reservoirs[27] and major sites for viral replication.

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Table 1 Virus isolation and virus loads in PBMCs in two series of experiments

Group	Macaque	Vaccine	Route	Challenge	Virus isolation after challenge (infected cells/10^6 PBMCs) at weeks 2	4	6	8–9	10	12
Series I										
A	R11	gp120 + p27	TILN	1	<1	<1	<1	<1	NT	NT
	931	gp120 + p27	TILN	1	<1	<1	<1	<1	NT	NT
	932	gp120 + p27	TILN	1	312	312	28	62	NT	NT
	200	gp120 + p27	TILN	1	62	140	62	12	NT	NT
B	187	nil	nil	1	>7692	140	312	140	NT	NT
	196	nil	nil	1	7692	1562	312	28	NT	NT
	199	nil	nil	1	2	>7692	140	62	NT	NT
	581	nil	nil	1	>7692	140	312	699	NT	NT
Series II										
A	89	gp120 + p27	TILN-i.m.	1	<1	<1	<1	<1	<1	<1
			TILN-i.m.	2	<1	<1	<1	<1	<1	<1
	91	gp120 + p27	TILN-i.m.	1	<1	<1	<1	<1	<1	<1
			TILN-i.m.	2	<1	<1	<1	<1	<1	<1
	92	gp120 + p27	TILN-i.m.	1	3509	<1	5.5	<1	<1	<1
B	84	gp120 + p27	Na-Re-i.m.	1	≥1	≥1	≥1	≥1	≥1	≥1
	85	gp120 + p27	Na-Re-i.m.	1	≥1	≥1	≥1	≥1	≥1	≥1
	93	gp120 + p27	Na-Re-i.m.	1	<1	<1	<1	<1	<1	<1
			Na-Re-i.m.	2	≥1	≥1	≥1	≥1	≥1	≥1
C	95	gp120 + p27	i.d.	1	≥1	≥1	≥1	≥1	≥1	≥1
	97	gp120 + p27	i.d.	1	≥1	≥1	≥1	≥1	≥1	≥1
	94	gp120 + p27	i.d.	1	<1	<1	<1	<1	<1	<1
			i.d.	2	≥1	≥1	≥1	≥1	≥1	≥1
D	877	nil	nil	1	1562	5.5	62.5	28	62.5	28
	399	nil	nil	1	<1	<1	<1	<1	<1	<1
			nil	2	3509	312	28	5.5	5.5	NT
	895	nil	nil	1	<1	<1	<1	<1	<1	<1
		nil		2	699	NT	5.5	28	5.5	NT
	923	nil	nil	1	<1	<1	<1	<1	<1	<1
		nil		2	<1	NT	<1	<1	<1	<1

Peripheral blood mononuclear cell (PBMC)-associated virus loads were determined by limiting dilution. The results are expressed as the number of infected cells per 10^6 PBMCs (ref. 6). Nested PCR for *nef* (ref. 32) was used in all macaques, and the results were in agreement with those of cocultures. In series II, all the immunized and unimmunized macaques that were not infected after the first rectal challenge were rechallenged with the same dose of SIVmac J5 virus pool (25 MID_{50}) as used in the first application.
Abbreviations: TILN, targeted iliac lymph node; TILN-i.m., TILN intramuscular; Na-RE-i.m., nasal, rectal, then intramuscular; i.d., intradermal.

The turnover of HIV-1 in the circulation is rapid, with a half-life of about 2 days[28,29], and the replicating CD4 population probably resides in the lymphoid tissue. An alternative route of immunization has therefore been developed by subcutaneously (s.c.) targeting close to the internal and external iliac lymph nodes[30]. This elicited a broad local immune response, with specific IgA and IgG antibodies being detected not only in the rectal secretions but also in urine, urethral washings and seminal fluid. Specific T-cell proliferative responses to SIV p27 were elicited in the draining iliac lymph nodes, blood and spleen.

The objective of this investigation was to find out whether administration of SIV gp120 and p27 by the TILN route, compared with other routes of immunization, prevents infection when a macaque is subsequently challenged with live SIV applied to the rectal mucosa. In addition to the specific secretory and serum antibodies, and T-cell proliferative responses, we investigated antibody-secreting B cells, CD8-suppressor factor (CD8-SF) and the three β chemokines in the targeted iliac as compared with the unrelated axillary lymph nodes.

Virus isolation after rectal challenge in macaques

A recombinant SIV envelope gp120 and core p27 vaccine was administered s.c., with aluminum hydroxide as the adjuvant, to four rhesus macaques by targeting the iliac lymph nodes[30]. In the first series, four male rhesus macaques were immunized three times with this vaccine (200 μg each of SIV gp120 and SIV p27) and four control macaques were not immunized. The eight macaques were challenged about 2 weeks after the last immu-

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nization with 9500 median tissue culture infectious dose ($TCID_{50}$) [equivalent to at least 34 median infectious doses (MID_{50})] of the SIVmac 32H J5 molecular clone[31]. Total protection was found by coculture and nested polymerase chain reaction (PCR)[32] in two of the four immunized macaques or decrease in viral load of 94% and 97.4% in the two other immunized macaques (Table 1). In contrast all four unimmunized control macaques were infected. Analysis of variance (ANOVA) of viral load between the four immunized and four control macaques over the experimental period was very significant ($F = 5.86$, $P < 0.001$).

In the second series, three male macaques were immunized with the same vaccine and by the same TILN route (×3), but in addition, they received an i.m. boost into the right and left gluteal muscles. They were challenged with 25 MID_{50} of a cell-free virus pool derived from the spleen of an SIVmac J5-infected macaque. Two macaques remained free of any detectable virus in peripheral blood mononuclear cells (PBMCs) by coculture and nested PCR, despite two rectal challenges with live SIV. The third macaque showed a transient infection, with virus being isolated at week 2 and 6 (3509 and 5.5 cells per 10^6 PBMCs, respectively), but virus was not detectable at weeks 4, 8, 10 and 12 (Table 1). In contrast three of the four unimmunized controls became infected after rectal challenges with SIV. Surprisingly, all six macaques that were immunized either by intradermal (i.d.) scarification of the inguinal skin (×5) or by nasal (×2) followed by rectal (×3) and i.m. gluteal (×1) route became infected (Table 1).

Antibody responses

All immunized macaques, irrespective of the route of immunization, developed secretory IgA and IgG antibodies to gp120 and p27 in rectal washings after their last dose of immunization (Table 2), unlike the preimmunized or unimmunized macaques in which antibodies were not detected. There was no obvious difference in the rectal antibody titers, although the intradermally immunized macaques showed very low antibody titers (1:1) up to the fourth immunization dose. In contrast, urinary SIgA and IgG antibodies were detected only in the TILN-immunized macaques (Table 2), suggesting a regional immune response. TILN immunization, especially with i.m. boost, elicited the highest serum IgA and IgG antibody titers to both gp120 and p27 (up to 1:6400 with one at 1:25,600), whereas i.d. immunization yielded significantly lower titers (up to 1:200). However, the antibody levels failed to differentiate between total, partial or no protection in the immunized macaques. Serum neutralizing antibody levels were measured (by T. Corcoran, National Institute for Biological Standards and Control, Potters Bar, UK), and the antibodies showed low titers and were not correlated with protection (results not presented).

Antibody-secreting B cells

Antibody-secreting B cells were then assayed for IgA and IgG antibodies to gp120 and p27, with particular reference to the iliac lymph nodes, as compared with the unrelated control axillary lymph node cells (Table 3). Only IgA p27 antibody-secreting cells in the iliac lymph nodes showed significant variation between the four group means of macaques (ANOVA: $F = 3.86$, $P = 0.05$). IgA antibody-secreting cells to p27 in the iliac lymph nodes of the TILN-immunized macaques were significantly increased, as compared with the other three groups of animals (unpaired t-test = 2.987, d.f. 11, $P < 0.02$) or compared with the axillary lymph nodes (paired t-test = 11.283, d.f. 2, $P < 0.01$). However, antibody-secreting B cells were not detected in the axillary lymph nodes or PBMCs of the TILN-immunized macaques and were found only in some of the infected macaques (Table 3). IgG antibody-secreting cells to p27 and gp120 were increased in

Table 2 IgA and IgG antibody titers to SIV p27 and gp120 and T-cell proliferative responses

Route of immun.	No.	Rectal washings IgA p27	Rectal washings IgA gp120	Rectal washings IgG p27	Rectal washings IgG gp120	Urine (×10^{11}) IgA p27	Urine IgA gp120	Urine IgG p27	Urine IgG gp120	Serum IgA p27	Serum IgA gp120	Serum IgG p27	Serum IgG gp120	Stimulation index gp120	Stimulation index p27
TILN	R11	4	4	4	4	10	10	8	10	1,600	1,600	1,600	1,600	28.8	6.4
TILN	931	16	16	4	4	4	4	2	2	3,200	3,200	3,200	3,200	24.2	24.2
TILN	932	2	2	2	2	4	4	4	4	3,200	1,600	3,200	800	26.9	14.2
TILN	200	16	16	4	4	4	8	4	4	800	800	3,200	6,400	19.1	3.6
TILN-i.m.	89	4	4	4	4	4	8	4	8	1,600	6,400	6,400	3,200	21.3	19.0
TILN-i.m.	91	4	4	4	4	8	4	2	2	6,400	6,400	6,400	6,400	4.9	3.7
TILN-i.m.	92	4	4	1	4	8	8	8	8	1,600	1,600	25,600	12,800	47.0	19.1
Na-Re-i.m.	84	8	8	4	4	0	0	0	0	1,600	800	3,200	3,200	3.1	7.0
Na-Re-i.m.	85	4	4	4	4	0	0	0	0	1,600	800	6,400	6,400	63.5	12.0
Na-Re-i.m.	93	8	8	4	8	0	0	0	0	100	100	200	200	6.5	12.1
i.d.	95	4	4	2	2	0	0	0	0	200	200	200	200	1.1	13.5
i.d.	97	4	4	2	2	0	0	0	0	200	100	200	100	1.0	10.9
i.d.	94	4	4	2	4	0	0	0	0	100	200	100	200	2.9	3.5

IgA and IgG antibody titers in rectal washings, urine and serum to SIV p27 and gp120 were determined by ELISA. The T-cell proliferative responses were assayed in 4-day cultures, with and without gp120 or p27, and pulsed with [^{3}H]thymidine. Antigen-stimulated T-cell proliferation was expressed in stimulation indices, that is the ratio of counts with and without antigen.

Abbreviations: immun., immunization; TILN, targeted iliac lymph node; TILN-i.m., TILN intramuscular; Na-RE-i.m., nasal, rectal, then intramuscular; i.d., intradermal.

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Table 3 The number of IgA and IgG antibody-secreting cells to gp120 and p27 per 10^6 cells

			Antibody secreting cells											
			Iliac lymph nodes				Axillary lymph nodes				PBMCs			
Route of immun.	No.	Challenge	IgA gp120	IgA p27	IgG gp120	IgG p27	IgA gp120	IgA p27	IgG gp120	IgG p27	IgA gp120	IgA p27	IgG gp120	IgG p27
TILN	89	Protected	3.3	63.3	42.5	538	0	0	0	0	0	0	0	0
	91	Protected	0	60.0	6.7	202	0	0	0	0	0	0	0	0
	92	Tr. viremia	50.0	46.7	128.0	106.7	0	0	41.7	0	0	0	0	0
N-R-i.m.	84	Infected	0	0	0	197.0	0	20.0	0	43.0	0	0	0	0
	85	Infected	0	70.0	0	3.3	0	13.3	10.0	16.6	0	10	0	26.6
	93	Infected	0	0	6.7	38.3	0	33.3	16.7	23.3	0	0	0	3.7
i.d.	95	Infected	0	6.7	93.3	51.7	0	0	40.0	20.0	0	12.6	0	3
	97	Infected	0	0	20.0	40.0	0	0	0	0	0	6.7	0	4
	94	Infected	10.0	35.0	57.0	55.0	0	0	46.7	60.0	0	6.7	18.8	11.2
Unimmun.	877	Infected	0	0	82.5	46.7	0	0	36.7	26.7	0	27.3	13.3	0.3
	399	Infected	0	3.3	53.3	63.3	0	10.0	13.3	10.0	0	0	0	1.3
	995	Infected	0	6.7	43.3	110.0	0	0	35.0	35.0	0	3	0	1.3
	923	Not infected	0	0	0	0	0	0	0	0	0	0	0	0

The numbers of IgA and IgG antibody-secreting cells to gp120 and p27 were determined in cells eluted from the internal iliac and axillary lymph nodes and in PBMCs at autopsy. The results are presented as antibody-secreting cells per 10^6 cells in the protected TILN-immunized macaques, and compared with the two infected immunized and one unimmunized group of macaques.
Abbreviations: PBMCs, peripheral blood mononuclear cells; immun., immunized; TILN, targeted iliac lymph node; Tr. viremia, transient viremia; N-R-i.m., nasal, rectal, then intramuscular; i.d., intradermal; unimmun., unimmunized.

the iliac lymph nodes but were not detected (with one exception) in the axillary lymph nodes of the TILN-immunized macaques. IgA gp120 antibody-secreting cells failed to show a significant increase in the TILN-immunized macaques. IgG antibody-secreting cells were found in the iliac and axillary lymph nodes in all four groups, except in the axillary lymph nodes of the TILN-immunized macaques, suggesting that IgG antibody-secreting cells are induced both as a result of anamnestic response to infection and to TILN immunization.

T-cell proliferative responses

T-cell stimulation with p27 showed significant increase in proliferation (stimulation index (SI) > 3) in all 13 immunized macaques (Table 2) and response to gp120 in all but the i.d. immunized macaques. Hence, the lack of protection in the latter group might have been related to a lack of T-cell responses to SIV gp120 (and very low serum antibodies to gp120 and p27). This, however, would not account for the lack of protection in the naso-rectal-i.m.-immunized macaques in which significant T-cell responses to gp120 and p27 were present in all three macaques.

Fluorescent tracking of cells with the PKH-26 dye

The protective immune efficacy of the TILN route raised the question whether the iliac lymph nodes function as an inductive site of immunization to the rectal mucosa. This was investigated by means of the fluorescent tracking dye (PKH-26)[33,34], after TILN as compared with i.m. immunization into the deltoid (×3) with 200 μg of the particulate SIV p27:Ty-virus-like particles. At the same time and a week later, the immunizing sites were injected with 10 μM PKH26, and SIV p27:Ty-VLP (200 μg) was applied to the rectal mucosa, so as to encourage homing of labeled mononuclear cells from the iliac lymph nodes or deltoid muscle. A week later the macaques were killed, a number of tissues were removed, and mononuclear cells were eluted from the lymphoid and mucosal tissues. Flow cytometry showed that the proportion of PKH26-labeled mononuclear cells found in the internal iliac lymph nodes after TILN immunization (mean ± s.e.m.; 5.5% ± 1.1) was higher than that after i.m. immunization (2.3% ± 1.1), as was also observed in the external iliac lymph nodes (10.6% ± 3.9 and 3.5% ± 2.6, respectively). Preferential homing to the rectum (8.2% ± 5.1 and 2.0% ± 1.1) and ascending colon (11.4% ± 2.2 and 3.1% ± 1.6), respectively, but not to the small intestine (2.0% and 1.4%), was evident after TILN, as compared with i.m. immunization. This was also clearly seen following TILN immunization when labeled cells were compared with those after i.m. immunization in the inferior mesenteric lymph nodes (6.7% ± 1.2 and 3.6% ± 0.3). However, the proportion of labeled cells in the unrelated submandibular lymph nodes showed no difference between the two routes of immunization (5.3% ± 0.3 and 4.2% ± 0.9). Double-labeled flow cytometry confirmed that the PKH26-labeled cells in the internal iliac lymph nodes and the rectum were $CD4^+$ and $CD8^+$ T cells, as well as B cells and macrophages (data not presented). These results are consistent with the concept that the iliac lymph nodes may function as an inductive site for T and B cells to home to the rectum and ascending colon.

CD8-suppressor factor

We then investigated the CD8-SF, in view of its potential significance in preventing SIV replication in $CD4^+$ cells[22,23]. Soluble

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antigens usually do not induce CD8⁺ class I-restricted cytolytic cells[35], and indeed, we were unable to demonstrate CD8⁺ cytolytic cells, although, using particulate SIV p27 antigen, we generated cytolytic CD8 cells in the iliac lymph nodes and PBMCs (ref. 36). Significant variation in inhibition of replication of SIV in CD4⁺ T cells was found with iliac lymph node CD8-SF between the four group means, as well as PBMCs (Table 4, ANOVA test $F = 4.437$ and 5.394, $P < 0.05$ at 1:2 and 1:5 dilution, respectively). CD8-SF generated from the unrelated axillary lymph nodes as well as PBMCs failed to show a significant variation in SIV inhibition (Table 4). Furthermore, the TILN-immunized macaques showed a significant increase in CD8-SF activity when compared with the immunized control or unprotected macaques (unpaired t-test = 4.286 and 3.509, d.f. 9, $P < 0.01$ at 1:2 and 1:5 dilutions, respectively). Comparison of CD8 suppression between the iliac and axillary lymph nodes of the TILN-immunized macaques also showed significantly higher suppression of SIV replication in the iliac lymph node cells (paired t-test = 6.318, d.f. 2, $P < 0.05$). Notably, the only unimmunized macaque that was not infected after rectal challenge yielded the highest CD8-SF (greater than 98%) not only in the iliac but also the axillary lymph nodes (Table 4, No. 923). This result suggests that macaques may develop resistance to SIV infection by acquiring CD8-SF through some natural, possibly cross-reacting, immunity.

Table 4 The effect of CD8 suppressor factor on SIV replication in simian CD4⁺ T cells

Route of immun.	No.	Challenge	CD8 suppression of RT (%) Iliac LN 1:2	Iliac LN 1:5	Axillary LN 1:2	Axillary LN 1:5	PBMCs 1:2	PBMCs 1:5
TILN	89	Protected	64.3	49.1	42.1	19.7	69.1	55.1
TILN	91	Protected	62.6	51.0	43.4	7.2	73.1	43.6
TILN	92	Tr. viremia	62.7	52.0	17.0	0	53.0	42.5
N-R-i.m.	84	Infected	24.1	0	5.6	29.4	74.6	54.6
N-R-i.m.	85	Infected	35.5	37.7	32.0	13.0	53.7	38.3
N-R-i.m.	93	Infected	22.0	4.0	19.0	11.0	7.0	16.0
i.d.	95	Infected	0	0	6.7	0	31.5	25.7
i.d.	97	Infected	33.3	27.3	40.9	18.9	18.4	2.7
i.d.	94	Infected	19.9	15.5	19.1	19.2	12.9	2.5
Unimmun.	877	Infected	NT	NT	14.0	19.0	2.0	18.0
Unimmun.	399	Infected	37.8	27.5	40.1	34.4	32.1	14.1
Unimmun.	895	Infected	45.4	34.1	18.4	15.3	56.4	42.1
Unimmun.	923	Not infected	99.1	47.1	98.2	48.1	96.3	33.1

The CD8 suppressor factor was prepared from CD8-enriched cells that were eluted from the internal iliac and axillary lymph nodes (LN) and from peripheral blood mononuclear cells (PBMCs) at autopsy. The results are expressed as percent inhibition of reverse transcriptase (RT) of the control culture, and is given for the culture supernatant at dilutions of 1:2 and 1:5.
Abbreviations: TILN, targeted iliac lymph node; Tr. viremia, transient viremia; N-R-i.m., nasal, rectal, then intramuscular; i.d., intradermal; unimmun. unimmunized.

The chemokines RANTES, MIP-1α and MIP-1β

The chemokines RANTES, MIP-1α and MIP-1β, which have recently been reported to be responsible for some of the CD8-SF activity of HIV replication[24], were also assayed (Table 5). One-way ANOVA between TILN-immunized macaques and the other immunized or control macaques failed to show a significant variation between the group means in the iliac lymph nodes, unless only the infected macaques are included (that is, all but macaque 923, Table 5): RANTES ($F = 6.46$, $P < 0.025$), MIP-1α ($F = 6.05$, $P < 0.025$), MIP-1β ($F = 4.57$, $P < 0.05$) and IL-8 ($F = 6.507$, $P < 0.025$). Significant increase in RANTES ($P < 0.01$) and MIP-1β ($P < 0.01$) were found using the unpaired t-test in the TILN-immunized as compared with the infected macaques in the three other groups. Although MIP-1α and the unrelated chemokine IL-8 also showed significant variation between the group means ($P < 0.025$), comparison of the TILN-immunized with the infected macaques in the other three groups failed to show a significant difference. Similar analysis of the axillary lymph node cells showed significant variations between the group means for MIP-1α ($P < 0.005$), MIP-1β ($P < 0.001$) and IL-8 ($P < 0.01$) but not for RANTES. However, all chemokines except MIP-1β ($P < 0.001$) failed to show a significant increase in the TILN-immunized as compared with the infected macaques from the other three groups. Furthermore, significantly increased concentrations of RANTES ($P < 0.01$), MIP-1α ($P < 0.025$) and MIP-1β ($P < 0.05$), but not IL-8, were found in the iliac as compared with the axillary lymph nodes of the TILN-immunized macaques.

MIP-1α and MIP-1β generated from CD8-enriched PBMCs showed significant variation between the group means ($P < 0.05$) but the 5% level of significance was not reached for RANTES ($F = 3.567$) and was not found with IL-8 ($F = 1.984$). The corresponding analysis of TILN-immunized as compared with the infected macaques also showed significantly higher concentrations of the two chemokines ($P < 0.01$).

Discussion

Mucosal challenge experiments with SIVmac suggest in two independent series that rectal infection by SIV can be totally prevented in 4 out of 7 macaques immunized by the TILN route as compared with 13 out of 14 unimmunized or immunized by other routes (Fisher's exact test, one-tailed $P = 0.025$; each series separately, $P = 0.214$ and $P = 0.108$, giving a joint one-tailed probability of 0.023). The remaining three macaques immunized by the TILN route showed either decreases in viral load (>90%) or transient viremia, indicating that all seven TILN-immunized macaques showed total or partial protection of rectal transmission by SIV, compared with seven out of eight unimmunized macaques that became infected (Fisher's exact test, one-tailed $P = 0.0012$; each series separately $P = 0.071$ and 0.029, giving a joint one-tailed probability of 0.002). However, the number of macaques used was limited, and a larger series will be required to confirm the significance of this protective strategy against mucosal infection. Although the same vaccine was used in the i.d. and naso-rectal-i.m. immunizations, these failed to induce protection. It appears that the route of immunization is another determining factor in the successful outcome of vaccination.

It should be noted that the SIVmac J5 molecular clone has been derived from the SIVmac 32H virus stock, which originates from

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Table 5 The concentrations of RANTES, MIP-1α and MIP-1β and the unrelated IL-8 chemokine

			Chemokines (pg/ml)											
			Iliac lymph nodes				Axillary lymph nodes				PBMCs			
Route of immun.	No.	Result of Challenge	RANTES	MIP-1α	MIP-1β	IL-8	RANTES	MIP-1α	MIP-1β	IL-8	RANTES	MIP-1α	MIP-1β	IL-8
TILN	89	Protected	133	170	125	652	54	114	89.5	697	526	818	809	651
TILN	91	Protected	86	214	139	525	13	134	110	695	832	650	1024	590
TILN	92	Tr. viremia	177	280	42	644	82	185	146	600	52	151	53	836
N-R-i.m.	84	Infected	22.5	0	43	602	40	42	30	614	116	164	69	586
N-R-i.m.	85	Infected	125	238	35	572	0	0	0	600	108	291	110	556
N-R-i.m.	93	Infected	10	102	19	303	16.5	103	3.4	326	61	77	32	498
i.d.	95	Infected	17.5	176	32	986	11.5	134	16	710	26	139	34	1158
i.d.	97	Infected	7	191	31	601	7	206	35	607	12	179	25	575
i.d.	94	Infected	11	174	20	679	13	141	39	616	8	131	30	636
Unimm.	877	Infected	15	6	8	304	15	6	18	377	34	8	24	579
Unimm.	399	Infected	8	7	11	309	7	9	10	337	21	12	71	245
Unimm.	895	Infected	3.5	7	54	187	7	7	6	323	24	10	13	498
Unimm.	923	Not infected	230	299	36	599	126	147	116	534	222	61	41	512

The concentrations of RANTES, MIP-1α and MIP-1β and the unrelated IL-8 chemokine were assayed in the culture supernatants generated for the CD8-SF from the iliac and axillary lymph nodes and in PBMCs. The results for the four groups of macaques are given in picograms per milliliter at 1:8 dilution of the supernatants, which was optimal for the specific enzyme immunoassay.
Abbreviations: immun., immunized; TILN, targeted iliac lymph node; Tr. viremia, transient viremia; N-R-i.m., nasal, rectal, then intramuscular; i.d., intradermal; unimm., unimmunized.

SIVmac 251 (ref. 31). The SIVmac J5 molecular clone encodes a full-length nef protein, unlike the *nef* genes in SIVmac 251 (pBK28), SIVmac 239 or the SIVmac C8 molecular clone. The viral clone infects Indian and Chinese rhesus macaques, as well as cynomolgus macaques and appears more virulent than the SIVmac C8 molecular clone; the viremia persists longer and the macaques develop AIDS, as with the uncloned SIVmac 251 from which it is derived[31]. The speed of disease expression, however, is variable[37].

Significant secretory IgA and IgG antibodies in rectal fluid, serum IgA and IgG antibodies, and circulating T-cell proliferative responses to SIV gp120 and p27 were found in all TILN and naso-rectal-i.m. and to a lesser extent in the i.d., but not in the unimmunized control macaques. T-cell proliferative responses to both gp120 and p27 were significantly increased in all 13 immunized macaques except for gp120 in the intradermally immunized animals. The results suggest that SIgA, or IgG to gp120 or p27 did not clearly differentiate protected from infected, immunized macaques and this may also apply to the corresponding serum antibodies. Urinary SIgA and IgG antibodies to both antigens were found only in TILN-immunized macaques, and although they may prevent the spread of the infection among sexual partners, their role in preventing rectal mucosal transmission of SIV is not clear. The speculative possibility that urinary antibodies to SIV antigens might be an index of protection needs to be explored. T-cell proliferative responses to gp120 and p27 also failed to show a clear relationship between protection and infection among the immunized macaques.

We have also studied the proportion of B cells in the lymph nodes and PBMCs capable of generating IgA and IgG antibodies. IgA antibody-secreting cells to p27 were significantly increased in the iliac lymph nodes of the TILN-immunized and protected macaques, compared with the infected macaques ($P < 0.02$) or with the axillary lymph nodes in the TILN-immunized macaques ($P < 0.01$). IgG antibody-secreting cells to p27 were also increased in the iliac lymph nodes of the protected macaques, but this was also observed in the infected animals. Indeed, similar results were found with IgG antibody-secreting cells to gp120, suggesting that, whereas IgG antibody-secreting cells in the iliac lymph nodes of the protected macaques were elicited by the TILN immunization, those in the infected macaques may have been generated by anamnestic response, as IgG antibody-secreting cells were found in both iliac and axillary lymph nodes. The paucity of antibody-secreting cells in PBMCs reflects the narrow window of about 7 days postimmunization when antibody-secreting cells can be detected. (The macaques were killed 3–4 months after the last immunization.)

The rationale for selecting the internal and external iliac lymph nodes was that these were enlarged after rectal or genital mucosal immunization[19,38], and they are known to drain the rectal and genital tract. Furthermore, the internal iliac lymph nodes were identified after submucosal vaginal injection of colloidal iron in macaques[39], by targeting these lymph nodes by injecting colloidal carbon before autopsies[30] and by identifying these lymph nodes after pelvic immunization in mice[40]. We have now demonstrated with the PKH26 fluorescent tracking dye that these lymph nodes may function as one of the inductive sites for T- and B-cell immunity, from which $CD4^{+}$,$CD8^{+}$ T cells and B cells home to the rectal mucosa. Indeed, the proportion of labeled cells in these lymph nodes was significantly higher after TILN than i.m. (deltoid) immunization, whereas no difference was found in the unrelated

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submandibular lymph nodes. Furthermore, preferential homing of labeled cells was found in the rectum and ascending colon, as compared with that to the small intestine.

The CD8 suppressor factor inhibiting replication of SIV in $CD4^+$ T cells >50% was found in the immunized protected as compared with immunized unprotected or control macaques ($P < 0.001$). Furthermore, significantly higher suppression of SIV replication was found in the targeted iliac as compared with axillary lymph node cells ($P < 0.05$). These results suggest that the CD8-SF can be elicited by immunization, and it may play a part in preventing mucosal SIV/HIV infection. This finding is consistent with the correlation of CD8 suppression with the clinical state of the infected subjects[41], but extends the CD8 activity to prevention of infection by immunization.

In view of the recent report that recombinant RANTES, MIP-1α and MIP-1β induced dose-dependent inhibition of HIV or SIV replication[24], we have assayed the three β chemokines, as well as a control (IL-8) chemokine generated by phytohemagglutinin (PHA)-stimulated CD8 cells from PBMCs and the iliac and axillary lymph nodes. The results suggest that generation of RANTES and MIP-1β are significantly increased in CD8-enriched cells derived from the iliac lymph nodes, whereas only MIP-1β is increased in axillary lymph nodes. All three chemokines are increased significantly in PBMCs of TILN-immunized and protected macaques as compared with the infected macaques ($P < 0.02$ to $P < 0.01$). The control IL-8 did not yield significantly increased levels in the TILN-immunized macaques. Whether CD8 suppressor activity and the three β chemokines account for the protection from rectal infection is not clear and needs to be further investigated by *in vivo* experiments with these chemokines. However, this is the first evidence that CD8-SF and the concentrations of RANTES, MIP-1β and possibly MIP-1α are increased after protective immunization with a recombinant SIV vaccine. It is noteworthy that the only unimmunized macaque that was not infected after two rectal challenges yielded the highest CD8-SF (greater than 98%) not only in the iliac but also the axillary lymph nodes (Table 4, No. 923). This macaque also showed raised concentrations of RANTES, MIP-1α and MIP-1β, similar to the natural resistance to HIV-1 infection very recently reported in a cohort of seronegative subjects with a high risk of sexual exposure to HIV-1 (ref. 26). The results suggest that macaques may develop resistance to SIV infection by acquiring CD8-SF and β chemokines through some natural, possibly cross-reacting immunity.

We suggest that prevention of sexual transmission of SIV/HIV by immunization might not be necessarily governed by any one immune mechanism but by several factors functioning at three successive immune barriers. Rectal epithelium (barrier 1, or that of the genital tract), has specific surface IgA and IgG (ref. 19, 30, 38) and intraepithelial SIgA (ref. 42), antibody-secreting B cells[43], CD8-SF (Y.W. *et al.*, manuscript in preparation) and $CD8^+$ cytotoxic T cells[36], which may prevent viral transmission or decrease the viral load. However, if the virus were to breach the epithelial barrier, it is carried by dendritic cells, Langerhans cells or macrophages to the iliac lymph nodes (barrier 2), as was found for vaginal infection with SIV (ref. 39, 44). We have demonstrated SIV p27-specific IgA antibody-secreting B cells and CD8-SF in the iliac lymph nodes here, and $CD4^+$ T helper cells[30] and $CD8^+$ cytotoxic cells[36] are also upregulated at this inductive site for genitorectal immunity and may prevent the formation of a viral reservoir of infection[27]. Dissemination of any residual viral load from the iliac lymph nodes into the circulation may be controlled by the same T-cell functions and serum antibodies (barrier 3).

The evidence suggests that targeting the iliac lymph nodes for immunization elicits significant antibody-secreting B cells, CD8 suppressor factor, chemokines, CD4 proliferative and helper T cells, and is associated with total or partial protection from rectal mucosal infection with SIV. The essential role of immunity in the regional lymph nodes in protection against local mucosal infection is consistent with the function of the regional lymph nodes in HIV infection[27,45].

Methods

Immunization schedule. In series I, a group of four male rhesus monkeys (*Macaca mulatta*) were immunized by the s.c. TILN route[30] (×3), at about monthly intervals with gp120 and p27 (200 μg each) in alum (AluGel, Uniscience, London). Another group of four control macaques did not receive any vaccine. Recombinant SIVmac 251 gp120 was expressed in recombinant Baculovirus-infected cells[46], and rSIV p27 was expressed in pGEX-3X as a glutathione *S*-transferase fusion protein. TILN immunization was performed by s.c. injection, first by inserting the needle medially to the femoral pulse just above the inguinal ligament and to a depth of 1–2 cm, dispensing about 0.15 ml, and then directing the needle upwards, and laterally toward the pelvic floor, to a depth of about 4 cm and injecting the rest of about 0.35 ml of the vaccine. This procedure was repeated on the other side of the inguinal region. In series II, about 14 weeks later, 13 male rhesus monkeys were used. Three macaques were immunized by the TILN route (×3) as in series I, but a fourth dose of 100 μg of the vaccine was injected i.m. into each buttock. Another group of three macaques were immunized with the same vaccine but without alum by scarification at four sites just above and below the inguinal ligament on each side (×5) at about monthly intervals. The rationale was to enable epidermal Langerhans cells to carry the vaccine to the draining iliac lymph nodes. Three macaques were given the same vaccine except that for the mucosal routes, SIV gp120 and p27 were covalently linked to the mucosal adjuvant cholera toxin B subunit (Sigma, Poole, Dorset, UK) as described elsewhere[47]. The vaccine was applied monthly by means of lubricated pediatric nasogastric tubes by nasal (×2), followed by rectal (×3) and then i.m. (into the buttock, ×1) routes. The control group consisted of four unimmunized macaques.

Virus challenge by the rectal route. In series 1, viral challenges were carried out by topical application to the rectal mucosa of 9500 $TCID_{50}$ of cell-free, rhesus macaque PBMC-grown SIVmac J5 molecular clone virus pool[31]. Series 2 macaques were inoculated with 25 MID_{50} (median monkey infectious dose derived by intrarectal titration) of a cell-free virus pool derived from the spleen of an SIVmac J5-infected macaque, 167 days after infection. In view of the poor rectal infectivity in the unimmunized controls in series 2 (1 out of 4), all the uninfected macaques were rechallenged rectally after an interval of about 14 weeks. However, 1 week before the challenge, all the immunized uninfected macaques were boosted according to the original immunization schedule; two macaques by TILN, one by nasal-rectal-i.m. and one by the i.d. route. Macaque R11 (series 1) was challenged and protected on two occasions.

Virus isolation and virus load. The isolation and characterization of the SIVmac J5 molecular clone used in this study[31] and the nested PCR for *nef* (ref. 32) have been described before. PBMC-associated virus loads were determined by limiting dilution. Briefly, simian PBMCs were separated from whole blood by centrifugation on Ficoll Paque (Pharmacia). Cells were diluted from 10^6 to 4×10^5 and subsequently in fivefold steps to 130 cells, and duplicate cultures were

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cocultivated with the human T-cell line C8166 in 25-cm^2 flasks. Medium and C8166 cells were replenished every 3 to 4 days, and the total culture volume was maintained at approximately 15 ml. All cultures were kept for 30 days or until cytopathic effect was apparent. Virus isolation was confirmed by indirect immunofluorescence using polyclonal anti-SIV serum. Fifty percent end points were calculated using the Kärber formula, and the results were expressed as the number of infected cells per 10^6 PBMCs.

Antibody assays by ELISA. IgA and IgG antibodies to p27 and gp120 in rectal washings, urine and serum were determined by ELISA as described previously[19,30]. Samples of fluid were collected before and at monthly intervals after each immunization. Blood collected from the femoral vessels was defibrinated and the serum separated. A constant volume of about 6.0 ml of rectal washings was collected without trauma, with the aid of flexible, lubricated pediatric nasogastric tubes, as described previously[30]. Urine was collected by direct aspiration of the bladder. All experimental procedures were performed under sedation with ketamine hydrochloride (10 mg/kg) and Domitor (10 mg/kg).

IgA and IgG antibodies to gp120 and p27 were determined by ELISA as described previously[19]. Briefly plates were coated with a predetermined optimal concentration of gp120 or p27 (at 1 µg/ml) and with a random 20-base peptide (R20) as a control antigen; they were incubated with doubling dilutions of test samples. Bound antibody was detected by incubation with rabbit IgG anti-monkey IgA (8 µg/ml; Nordic Immunological Laboratories, Tilburg, The Netherlands) or IgG (2 µg; Sigma), followed by affinity-purified goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma). For rectal washings (concentrated ×4), urine (concentrated ×10), the starting samples were used neat and then diluted 1:2, 1:4 and so on, whereas serum samples were diluted 1:100, 1:200 and so on. The fluids were concentrated by lyophilization and the concentration step for the washings was taken into account when the results were calculated.

T-cell proliferative assay. T-cell cultures were set up by separating mononuclear cells from defibrinated blood by Lymphoprep (NYCOMED, Oslo) density gradient centrifugation before and after immunization from all macaques[30]. The cells were cultured without antigen and with 1 and 10 µg/ml of gp120, p27, a control peptide (R20) or concanavalin A in 96-well round-bottomed plates (Costar, Cambridge, Massachusetts), containing RPM1 1640 (Gibco), as described before[30]. The results were expressed as stimulation indices (SI, ratio of counts with and without antigen), for cultures stimulated with the optimum (10 µg/ml) gp120 or p27. All cultures yielded high stimulation indices and counts with concanavalin A, and no significant increase in counts was seen with the control peptide (R20; data not presented).

Antibody-secreting cells. Iliac and axillary lymph nodes were removed at autopsy about 4 months after the last immunization. They were treated conventionally[30], and they were suspended at 1×10^6 cells/ml. Antibody-secreting cells were assayed in Microtitre Multiscreen HA plates (Millipore UK Ltd., Watford, Herts, UK) with nitrocellulose bases and coated with SIVgag p27, gp120 or KLH control, each at 10 µg/ml (ref. 48). After washing with Iscoves medium, 100 µl of the cell suspension was transferred into the plates (in triplicates) at concentrations of 10^4, 5×10^4 and 10^5 cells of the lymph node tissues or 10^5, 2×10^5 and 5×10^5 PBMCs, for 16 h, at 37 °C in 5% CO_2. The plates were developed for antibody-secreting cells by removing the cells and washing the plates (×3) and then treating them with glutaraldehyde for 10 min. The plates were then incubated with biotinylated Fcγ-specific goat anti-human IgG (100 µl of 1:1500 dilution; Tago Immunobiologicals, Camarillo, California) or biotinylated Fcγ-specific goat anti-monkey IgA (10 µg/ml; Nordic Immunological Laboratories) for 16 h at 4 °C, washed (×3) and then treated with avidin-conjugated peroxidase (4 µg/ml Extravidin horseradish peroxidase; Sigma) for 60 min at room temperature. After three washes biotinylated rabbit anti-peroxidase (2 µg/ml) was added for 2 h at 4 °C, and the reaction was amplified by repeating the incubation step with the avidin-conjugated peroxidase. The plates were washed (×6), allowed to dry and developed with AEC/hydrogen peroxide (Sigma). As soon as the spots developed (within 3–8 min), the reaction was stopped by washing the plates with cold water, and the spots were enumerated under low magnification (×20) with a binocular microscope and expressed per 10^6 cells.

Assay of SIV suppression in CD4 cells by CD8-suppressor factor. CD8-SF was assayed[49] from PBMCs, iliac and axillary lymph nodes in 13 macaques. CD8$^+$ cells were enriched by negative selection, after removal of CD4 and B cells and monocytes from PBMCs. The cells were suspended in RPMI medium supplemented with 10% FCS, 2 mM of glutamine and 100 µg/ml penicillin and streptomycin (3×10^6 cells/ml) and stimulated for 3 days with 10 µg/ml PHA. The culture supernatant was discarded, and the lymphoblasts were then cultured in 10% IL-2 medium, and after 2 days, the cultures were centrifuged and the supernatant collected. The cells were passaged the same way three times, resulting in up to 10 ml of supernatant. The latter was filtered through a 0.45-µm filter, and 100 µl of CD8 supernatant was added to 2×10^5 SIV-infected, PHA-stimulated CD4$^+$ T cells. SIV replication was assayed by determining reverse transcriptase (RT) activity on day 9 using the RT detection and quantification method (Quan-T-RT assay kit, Amersham). Suppression of RT was assayed at a dilution of the culture supernatant of 1:2 and 1:5, and the result was expressed as a percent inhibition of the control culture. Inhibition greater than 50% is considered to be significant suppression of SIV replication.

Assay of concentrations of four chemokines. RANTES, MIP-1α, MIP-1β and IL-8 chemokines were assayed in the culture supernatants generated for the CD8-SF from PBMCs, iliac and axillary lymph nodes. Specific enzyme immunoassays were used (R and D Systems Europe Ltd., Abingdon, UK). The optimum conditions were established with the CD8 cell culture supernatant diluted at 1:8, and all the results are presented in picograms per milliliter at that dilution.

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