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Maternal immune status influences the HIV-specific immune responses in pups after DNA prime protein boost using mucosal adjuvant

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Abstract

This study was designed to determine the impact of maternal HIV-1 specific immunity on HIV-DNA immunization of 2 weeks old pups during the breast-feeding period.

Adult female mice received intranasal or intradermal HIV DNA (gp160Env, p37Gag, Nef, Tat and Rev) prime and recombinant protein booster immunizations that induced mucosal and systemic HIV-1 specific B and T cell responses. Intranasal administration of the immunogens induced higher serum IgG titers to HIV antigens than the intradermal immunization. Furthermore, predominantly higher HIV-1 specific fecal IgA titers were obtained in nasally immunized mice. The capacity to respond to one single prime with DNA and one boost with recombinant protein was then compared in pups born to mothers displaying HIV-1-specific immune responses and in pups born to non-vaccinated mothers. Immune responses to the greatest number of antigens were detected in pups born to mothers having the highest HIV-1 specific immune responses. These data suggest that HIV-1 DNA-plasmid immunization during breast-feeding and recombinant protein boosting shortly thereafter enhances the breadth of the humoral HIV-1 specific immune responses.

1. Introduction

In early life, during and a few weeks after birth neonates are normally deficient in their ability to respond potently to invading pathogens. Newborn individuals thus rely heavily upon passively transferred antibodies from the mother. In mammalians, the transfer of antibodies may occur both during fetal life and through breast-feeding after birth. However, with the risk of early life exposure to certain microorganisms it would be highly desirable to induce adaptive immunity in neonates, immunity that can combat infectious agents when the passively transferred maternal immune protection recedes. Several infections, such as diarrhoeal infections or infections that can be transmitted from mother to child, such as Cytomegalovirus (CMV), Hepatitis B virus (HBV) or Human immunodeficiency virus (HIV-1) can be fatal in the neonatal period, particularly in developing countries. Hence, immunization during early life would be an attractive mean to provide solid and long-lasting immunity. As HIV-1 remains one of the major global killers it is of importance to develop vaccines that can be used early after birth and have the capacity to provide long-lasting both cell-mediated and humoral neutralizing immunity the virus.

It is important to understand the plasticity and functionality during development of the early fetal or neonatal immune system when aiming at inducing long-lasting immunity by early age vaccination. The classical view is that antigen presented during the fetal life may result in immunological tolerance against the antigen. However, exceptions to this dogma exist as shown by the importance of cell-mediated immunity against intracellular pathogens during early life.

Transfer of antibodies from mother to child via mothers' milk or via the intestinal epithelium and placenta protects the neonate or infant from invading pathogens. The transferred antibodies may act passively to directly neutralize invading pathogens or in the form of idiotypic antibodies as

immunogens. Both mechanisms can have important impact on childhood/infant vaccinations [1-3].

Immunized female mice will deliver a potent mucosal immunity in form of secretory IgA to the mucosal surface of their children during the breast-feeding. It is important to study how this transfer of antibodies will influence the immune responses in the pups after mucosal immunization and also to understand, which vaccine strategy that will provide potent immunity in the pups. Therefore, this study attempted to analyze the efficacy of early life immunization utilizing HIV-1 DNA plasmid vaccination of 18-20 days old pups. The mothers were either untreated or had previously been immunized with HIV-1 DNA and recombinant protein. DNA plasmids, live viral/bacterial vectors or even protein immunization may stimulate a cell-mediated adult-like immunity in neonates, pups and newborns [1-7]. For this reason, this work focuses on the humoral immune responses against both structural and regulatory proteins of HIV-1.

2. Materials and Methods

2.1. Animals, vaccine candidates, adjuvants and immunization

The groups of females, immunization protocol and the grouping of the pups are summarized in Table 1. Female C57Bl/6 mice of the H-$2^{b/d}$ haplotype were immunized three times with 4 week intervals 10 μg plasmids encoding HIV-1subtype B gp160Env, p37Gag, Nef, Tat and Rev [8,9] and subsequently boosted once with 10 ug recombinant gp160Env, p24Gag and Rev. The female mice were divided into four groups and were immunized either intranasally (group 1 and 2) or intradermally (group 3 and 4). Group 1 did not receive any adjuvant whereas group 2, received 25 μg of the adjuvant poly (di(carboxlatophenoxy)phosphazene) (PCPP, kindly provided by

Table 1. Immunization groups and schedules. In both mothers and pups the same doses of antigen were used 10 ug DNA (2 ug/plasmid expressing Gagp37, Envgp160, Rev, Nef and Tat) and 5 ug recombinant HIV antigen (1 ug/antigen Gagp24, gp160, Rev, Nef, Tat).

	Group	**Immunization of offspring**					
		Group name	**Route**	**Prime at age**		**Booster**	
Mothers immunized intranasally with HIV-1 DNA and recombinant HIV-1 antigens without adjuvant							
Prime and boost days							
Day 0, 26 and 52 DNA	1 and 2	P1	I.m.	Days 15–17	HIV-DNA with saline	Day 36	rHIV-Ag with saline
Day 72, rHIV-Ag		P2	I.n.a.	Days 15–17	HIV-DNA with saline	Day 36	rHIV-Ag with saline
		P3	I.m.	Days 15–17	HIV-DNA/PCPP	Day 36	rHIV-Ag/PCPP
		P4	I.n.a.	Days 15–17	HIV-DNA/PCPP	Day 36	rHIV-Ag/PCPP
Mothers immunized intradermally with HIV-1 DNA and recombinant HIV-1 antigens with rGMCSF adjuvant							
Prime day							
Day 0, 26 and 52 DNA	3 and 4	P1	I.m.	Days 15–17	HIV-DNA with saline	Day 36	rHIV-Ag with saline
Day 72, rHIV-Ag		P2	I.n.a.	Days 15–17	HIV-DNA with saline	Day 36	rHIV-Ag/PCPP
		P3	I.m.	Days 15–17	HIV-DNA/PCPP	Day 36	rHIV-Ag with saline
		P4	I.n.a.	Days 15–17	HIV-DNA/PCPP	Day 36	rHIV-Ag/PCPP
Mothers not HIV-immunized							
Prime day							
Day 0, 26 and 52 DNA	5	P5	I.m.	Days 15–17	HIV-DNA with saline	Day 36	rHIV-Ag with saline
Day 72, rHIV-Ag		P6	I.n.a.	Days 15–17	HIV-DNA with saline	Day 36	rHIV-Ag/PCPP
		P7	I.m.	Days 15–17	HIV-DNA/PCPP	Day 36	rHIV-Ag with saline
		P8	I.n.a.	Days 15–17	HIV-DNA/PCPP	Day 36	rHIV-Ag/PCPP
		P9	I.n.a.	Days 15–17	Saline	Day 36	Saline

Parallell Solution, Cambridge, USA) in 50 µl PBS [10]. Group 3 was immunized intradermally without adjuvant wheras group 4 received recombinant murine GM-CSF (1 µg/mouse, ProSpec-Tany TechnoGene Ltd, Rehovot, Israel). All HIV-genes were carried by the plasmid pKCMV [8]. One control -group of eight saline treated mice was included (Group 5) as well as one group of 6 mice given PCPP only (Group 6). One booster immunization with 1 µg of each recombinant HIV-1 antigens gp160Env, p24Gag, Nef, Rev and Tat antigen dissolved in PBS with or without PCPP adjuvant was given.

After the final booster immunization the female mice were selected for mating and the off-spring immunized within 15-18 days after birth, still during the breast feeding-period. All immunizations were performed under anesthesia with 4% isofluran (Abbott Scandinavia, Solna, Sweden). The pups (enumerated P1-P9) were at day 15-17 after birth given one single dose of 10 µg HIV-1 DNA, either intramuscularly or intranasally, with or without PCPP adjuvant. The pups received the DNA in a total volume of 20µl and the immunization was divided into two time points with 7 hours interval in order to reduce the risk that the immunogens would reach the lungs. Three weeks later (day 36 after birth) the pups immunized with 5 µg recombinant HIV-proteins. Mice were sacrificed at three or six weeks after the final immunization.

Each group of HIV-1 immunized pups was divided into pups receiving the HIV-DNA intramuscularly into the hind right and left quadriceps muscle without adjuvant (Group P1 or P5) or with PCPP as adjuvant (Group P3 or P7). Furthermore, HIV-1 immunized pups were divided into pups receiving the HIV-DNA intranasally without adjuvant (Group P2, P6) or with PCPP as adjuvant (Group P4 and P8). Finally, one group of pups born to non-vaccinated mothers and given only saline or PCPP intranasally was used as controls (Group P9).

2.2. Samples collection

Blood was obtained from each mouse by tail bleedings at two-week intervals after each immunization, and at the same time feces were collected. Serum was stored at –20ºC until used. Mucosal samples were collected as previously described [11-14]. Briefly, the fecal pellets were collected in PBS supplemented with protease inhibitors (Sigma-Aldrich, S:t Louis) and stored at –70ºC until used. HIV-1 antigen-specific serum IgG and IgA as well as fecal IgA reactivity was analyzed by ELISA and in HIV-1 neutralization assays. T-cell immunity was analyzed by T-cell proliferation and ELIspot IFN-γ and IL-2 cytokine release assays.

2.3. Enzyme-linked immunosorbent assay (ELISA) for detection of IgG and IgA antibodies

Ninety-six well plates (NUNC-Maxisorp, Odense, Denmark) were coated with recombinant HIV-1 proteins p24Gag (0.5 μg/ml), recombinant gp160Env (0,5 μg/ml, BioSciences Int, CT), Nef (0,5 μg/ml, kindly provided by Dr. V.Erfle, GSF, Munich, Germany) and Tat (1 μg/ml, kindly provided by C, Svanholm, KI, Stockholm). Sera were diluted in phosphate buffer saline (pH 7.4) with 0.5% bovine serum albumine (BSA, Boehring Mannheim, Mannheim, Germany) and 0.05% Tween 20 (Sigma, Aldrich, Sweden, AB) and 100μl of dilutions 1/50, 1/100, 1/1000, 1/10 000 and 1/100 000 were added to each well and incubated at +37ºC for 1,5 h. Mucosal samples were analyzed as previously described [13-15]. Horseradish peroxidase conjugated anti-mouse IgG (Biorad, Richmond), diluted 1/3000 or anti-mouse IgA (Southern Biotechnologies, Birmingham, AL) diluted 1/1000, was added and samples were incubated at +37 ºC for 2 h were after OPD (2 mg/ml orthophenylendiamine in 0.05M sodium citric acid pH 5,5 with 0.003% H_2O_2) was added as substrate at 100 μl/well. After 30 minutes of incubation, the reaction was stopped by adding 100 μl of 2.5M H_2SO_4 to each well and the optical density was measured at 490nm. Sera, vaginal

secretions and feces from samples obtained prior to the immunizations were used as negative controls.

2.4. ELISA for detection of B-cell secreted IgG in vitro

Ninety-six well plates (NUNC-Maxisorp, Odense, Denmark) were coated with recombinant HIV-1 p24Gag or gp160Env. Splenocytes were diluted at a concentration of 10^6 cells/ml in complete RPMI 1640 medium containing 5% bovine serum (Gibco, Life Technologies, Paisley, Scotland) and 200µl of the cell suspensions were added to each well and incubated at +37°C for 72 hours. At day 3, plates were washed three times with PBS containing 0.05% Tween 20 and tracing of bound murine IgG and IgA was performed as described above.

2.5. HIV-1 neutralization assay

Sera collected from immunized and control mice were tested for their HIV-1 neutralizing capacity in vitro as previously described [8,16]. In brief, serially diluted heat-inactivated serum (75 µl/well) was mixed with HIV-1 IIIB, HIV-1 SF2 or patient isolate 6920 (75 µl/well) at a virus concentration of 50 TCID50. The serum/virus mixture was incubated for 1 hour at +37°C before 100 000 PHA-IL-2 activated human PBMCs (75 µl/well) were added. The mixtures were kept at +37 °C in 5% CO_2/air over night. The cells were thereafter washed 3 times with 200 µl RPMI1640/well before 250 µl/well complete RPMI-10% FCS was added and incubated for 5 days at +37 °C. Subsequently the medium supernatants were collected and assayed for HIV-1 p24Gag content by capture ELISA [15].

2.6. ELISpot

Ficoll–Paque Plus (Amersham Biosciences Europe GmbH, Uppsala, Sweden) was used to purify lymphocytes derived from the spleen (splenocytes). Splenocytes from individual animals were suspended in RPMI 1640 (Sigma) supplemented with penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA, USA) and 10% fetal calf serum (Sigma) and were distributed in anti-interferon-γ or anti-IL2 (Mabtech, Nacka, Sweden) antibody-coated 96-well polyvinylidene fluoride bottomed plates (MAIPN 4510; Millipore Corp., Bedford, MA, USA). Splenocytes, $2x10^5$/well in triplicate wells, were stimulated with 1 μg/peptide/well of 15-mers with 10-amino-acid overlaps (Thermo Hybaid, Germany) covering the HIV-1 proteins p55Gag and gp160Env. A control peptide library consisting of 18 peptides derived from HIV-1 protein reverse transcriptase was used in addition to the medium alone control. Concanavalin A (1 μg/well) was used as a positive control to test cell viability. The plates were then developed as described by the manufacturer. Results are given as cytokine-producing spot-forming cells (SFC) per million splenocytes and responding animals were defined as having above 50 SFC per million plated cells and twice the number of SFC for nonstimulated cells from the same animal.

2.7. T-cell proliferation

The antigen-specific proliferation of lymphocytes was analyzed as described previously [11,12]. Antigens used were recombinant proteins gp160Env, p24Gag, Nef and Tat and synthetic 15-mer peptides representing HIV-1 gp120 V3.

2.8. Peptide synthesis

The peptides corresponding to the gp120 V3-loop region (aa 298-322/IHIGPGRAFV) and gp41 neutralizing epitope synthesized were: aa661-675/ELDKWAS (Hybaid T-peptides, Ulm, Germany). The p55Gag and gp160Env-peptide pools were obtained from the US National Institutes of Health HIV-reagent repository (NIH, Washington, MA) and used in the ELISpot analyses [17].

2.9. Statistical analyses

Statistical comparisons between the groups were performed using the nonparametric Mann-Whitney U test. A significant difference was considered when a p-value of <0.05 was obtained. One-way ANOVA with Dunnetts post-test was performed using GraphPad Prism version 4.0a for MacIntosh, OS 9, Apple GraphicPad Software, San Diego, CA was used for comparisons of medians between groups at $p<0.001$ and $p<0.05$ levels.

3. Results

3.1. Humoral immune responses in adult mice

Humoral immune responses studied during 6 weeks after the protein booster immunization (for immunization protocol see Table 1), by testing the presence of HIV-1 specific p24Gag, gp160Env, Nef and Tat IgG and IgA in serum of adult mice. The HIV-1 specific serum antibody titers and the presence of HIV-1 neutralizing activity in the sera are shown in Table 2A and 2C, respectively. After protein boost, all HIV-immunized adult mice developed HIV-1 specific immunity against the four studied HIV-1 antigens, independently of route of immunization and use of adjuvant (Table 2B). Serum antibody responses against HIV-1 antigens were induced to higher titers after intranasal immunization than after intradermal immunization, both with and

Table 2A. Immunization protocol and study groups of immunized adult female mice. Immunization was performed three times with 10 ug HIV-1 DNA (days 0, 26, 52) and once with 5 ug recombinant proteins (day 78) representing the same HIV-1 proteins (gag, gp160, Nef and Tat).

Group	**Immunization**	**Adjuvant**	**Route**	**Median ELISA IgG titer serum**				**Median ELISA IgA titer serum**				**Median ELISA IgA titer fecal**			
				Gagp24	**Env gp160**	**Nef**	**Tat**	**Gagp24**	**Env**	**Nef**	**Tat**	**Gagp24**	**Env**	**Nef**	**Tat**
1	3× DNA + 1× rProt	No	I.n.a.	1200 (600–3400)	8,200 (200–23,000)	1200 (200–2800)	480 (380–100)	240 (200–1560)	3200 (100–5200)	440 (400–540)	240 (220–260)	4 (4)′	4 (4–6)	8 (6–8)	6 (2–6)
2	3× DNA + 1× rProt	PCPP	I.n.a.	4800 (400–7800)	18,000 (400–22,600)	1200 (200–1900)	440 (200–800)	400 (360–2600)	240 (100–1400)	120 (100–200)	100 (100–160)	14 (8–24)	16 (8–24)	24 (12–64)	8 (6–12)
3	3× DNA + 1× rProt	No	I.d.	400 (100–2200)	1,200 (100–2000)	200 (100–300)	200 (100–300)	300 (100–1400)	100 (100–300)	100 (100–280)	<100 NA	2 (2–4)	2 (2–4)	<2 NA	<2 NA
4	3× DNA + 1× rProt	GMCSF	I.d.	1400 (400–6200)	21,000 (800–24,000)	1600 (300–2400)	120 (100–180)	340 (200–1000)	400 (200–1000)	200 (140–360)	<100 NA	2 (2–4)	2 (2–4)	4 (4–8)	6 (2–6)
5	4× Saline	No	I.n.a.	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<2 NA	<2 NA	<2 NA	<2 NA
6	4× Saline	PCPP	I.n.a.	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<100 NA	<2 NA	<2 NA	<2 NA	<2 NA
Positive controls															
Mab anti-p24				74,000	<100	<100	<100								
Mab anti-gp120				<100	960,000	<100	<100								
Mab anti-Nef				<100	<100	5500	<100								
Mab anti-Tat				<100	<100	<100	1100								

Abbreviations: I.m = Intramuscular, I.n.a = Intranasal, I.d = Intradermal. GM-CSF = Granulocyte-macrophage colony stimulating factor. Values within parenthesis represents the 25th and 75th quartiles

Table 2B. Study groups of immunized adult female mice and percent (%) of antibody responding animals per group 21 days after each immunization.

Group	Immunization	Adjuvant	Route	Time point for sampling	Frequency (%) of immune responders to			
					Gagp24	Env gp160	Nef	Tat
1	3× DNA + 1× rProt	No	I.n.a.	After 1× DNA	33	0	0	0
				After 2× DNA	100	66	66	50
				After 3× DNA	100	100	100	100
				After 3× DNA-rProt	100	100	100	100
2	3× DNA + 1× rProt	PCPP	I.n.a.	After 1× DNA	17	0	33	33
				After 2× DNA	83	66	66	50
				After 3× DNA	100	100	100	100
				After 3× DNA-rProt	100	100	100	100
3	3× DNA + 1× rProt	No	I.d.	After 1× DNA	0	0	33	0
				After 2× DNA	66	17	83	50
				After 3× DNA	100	100	100	100
				After 3× DNA-rProt	100	100	100	100
4	3× DNA + 1× rProt	GMCSF	I.d.	After 1× DNA	33	17	50	33
				After 2× DNA	83	100	66	83
				After 3× DNA	100	100	100	100
				After 3× DNA-rProt	100	100	100	100
5	Saline	No	I.n.a.	After 1× DNA	0	0	0	0
				After 2× DNA	0	0	0	0
				After 3× DNA	0	0	0	0
				After 3× DNA-rProt	0	0	0	0
6	Saline	PCPP	I.n.a.	After 1× saline	0	0	0	0
				After 2× saline	0	0	0	0
				After 3× saline	0	0	0	0
				After 4× saline	0	0	0	0

Abbreviations: I.m = Intramuscular, I.n.a = Intranasal, I.d = Intradermal. GM-CSF = Granulocyte-macrophage colony stimulating factor.

Table 2C. HIV-1 neutralization in vitro against three HIV-isolates with serum collected from HIV-immunized mothers (group 1-5) and in their pups after immunization (groups P1-P7). Two laboratory strains and one clade B patient isolate.

Group	**Immunogen**	**Adjuvant**	**Route**	**HIV-1 isolates**		
				HIV-1 IIIB	**HIV-1 SF2**	**HIV-1 6920**
1	3× DNA + 1× rProt	No	I.n.a.	480 (200–880)	800 (600–1200)	40 (20–80)
2	3× DNA + 1× rProt	PCPP	I.n.a.	600 (400–1000)	1800 (1200–3400)	160 (40–440)
3	3× DNA + 1× rProt	No	I.d.	80 (20–100)	200 (60–300)	20 (20–40)
4	3× DNA + 1× rProt	GMCSF	I.d.	1200 (600–1800)	1800 (1000–2600)	100 (40–240)
5	Saline	No	I.n.a.	<20	<20	<20
P1	1× DNA + 1× rProt	No	I.m.	<20 NA	ND	ND
P2	1× DNA + 1× rProt	No	I.n.a.	<20 NA	ND	ND
P3	1× DNA + 1× rProt	PCPP	I.m.	40 (20–40)	ND	<20 NA
P4	1× DNA + 1× rProt	PCPP	I.n.a.	20 (20)	ND	<20 NA
P5-7	1× DNA + 1× rProt	No	I.n.a.	<20 NA	ND	<20 NA
Positive controls						
Mab anti-p24			<20	<20	<20	
Mab anti-gp120			2200	2400	140	

Abbreviations: P1-P7 (Pups of female adult mice in Group 1-5). I.m = intramuscular, I.d = intradermal, I.n.a = Intranasal, NA = Not applicable, ND= Not determined

without PCPP. Only against gp160Env were the binding serum IgG titers significantly lower in group 3 (i.d. no adjuvant) than observed in group 2 (i.na with PCPP, $p<0.022$) and 4 (i.d and rGM-CSF, $p<0.022$). Furthermore, the titers of serum IgA against Tat were significantly lower in groups 3 and 4 than in group 1 (i.na, no adjuvant $p=0.043$) and 2 (i.na with PCPP, $p=0.043$). After completing the immunization schedule of four doses (3x DNA plus 1x recombinant

proteins) no significant differences in the breath of the HIV-1 specific serum IgG titers were observed when comparing responses induced by the different routes of immunization or the use of adjuvants (Tables 2A and B). Differences in the frequency of serum IgG/IgA responders were seen mainly after the first and the second immunizations, where animals given DNA-plasmids together with PCPP responded earlier, more broadly and more strongly than animals immunized without adjuvant (Table 2B). The difference in levels of serum IgG titers, but not serum IgA titers, induced by the two different routes of immunization showed a trend towards higher titers after intranasal immunization, especially against p24Gag, gp160Env and Nef, when compared to the non-adjuvant immunized intradermal animals. Control animals in Group 5 and 6 remained ELISA negative against all four HIV-1_antigens. The offspring of the immunized mice were grouped together (groups P1-P4) and subsequently compared to the offspring (Group P5-P9) of the untreated mice.

3.2. Systemic and fecal humoral immune responses in pups born to HIV-immune or non-immune mothers

The addition of PCPP to intramuscular or intranasal DNA-immunizations augmented the vaccine-specific immune responses in the pups. A majority of the pups, both born to HIV-immune and naïve mothers displayed detectable immune responses against at least two HIV-1 antigens (p24Gag and Nef, Table 3). However, a significantly broader immune responses (i.e. reactivity directed against more of the included HIV-proteins) was detected in mice born to HIV-immune mothers (Table 4 $p<0.001$, Figures 1 and 2 A-D).

To more sensitively investigate if the pups had developed systemic humoral immune responses, B cells were isolated from spleen 6 weeks after protein immunization and cultured for three days

Table 3A. Immunization protocol and study groups of immunized pups of HIV-immunized and control female mice. Immunization was performed once with 10 ug HIV-1 DNA p37gag, gp160, Nef, Tat and Rev (age day 15-17) and once with 5ug recombinant HIV-1 proteins (day 37) with or without adjuvant. HIV-1 specific IgG and fecal IgA reactivity at day 72 post-birth.

Group	**Immunogen**	**Adjuvant**	**Route**	**Median ELISA IgG titer**				**Median ELISA fecal IgA titer**			
				Gagp24, day 72	**Env gp160, day 72**	**Nef, day 72**	**Tat, day 72**	**Gagp24**	**Env gp160**	**Nef**	**Tat**
P1	1× DNA + 1× rProt	No	I.m.	220 (100–1200)	<100 NA	140 (100–260)′	<100 NA	<2 NA	<2 NA	<2 NA	<2 NA
P2	1× DNA + 1× rProt	No	I.n.a.	<100 NA	<100 NA	120 (100–140)	<100 NA	2 (2–8)	3 (4–8)	4 (2–10)	4 (<2–4)
P3	1× DNA + 1× rProt	PCPP	I.m.	400 (300–1260)	110 (100–200)	380 (240–480)	120 (100–260)	<2 NA	<2 NA	<2 NA	<2 NA
P4	1× DNA + 1× rProt	PCPP	I.n.a.	600 (400–800)	150 (100–180)	300 (300–400)	100 (100–140)	32 (4–72)	4 (2–8)	16 (4–36)	8 (4–8)
P5	1× DNA + 1× rProt	No	I.m.	100 (100–360)	<100 NA	120 (200–440)	100 (100)′	<2 NA	<2 NA	<2 NA	<2 NA
P6	1× DNA + 1× rProt	PCPP	I.m.	120 (100–600)	<100 (100–140)	120 (100–500)	<100 NA	<2 NA	<2 NA	<2 NA	<2 NA
P7	1× DNA + 1× rProt	No	I.n.a.	<100 NA	<100 NA	<100 NA	<100 NA	2 (2–12)	<2 NA	2 (2–4)	<2 NA
P8	1× DNA + 1× rProt	PCPP	I.n.a.	240 (120–480)	<100 NA	<100 NA	<100 NA	8 (2–8)	<2 NA	12 (4–36)	<2 NA
P9	Saline	PCPP	I.n.a.	<100	<100	<100	<100	<2	<2	<2	<2
Positive controls											
Mab anti-p24				68,000	<100	<100	<100				
Mab anti-gp120				<100	880,000	<100	<100				
Mab abti-Nef				<100	<100	4600	<100				
Mab anti-Tat				<100	<100	<100	1200				

Abbreviations: I.m = Intramuscular, I.n.a = Intranasal, I.d = Intradermal. GM-CSF = Granulocyte-macrophage colony stimulating factor.NA=Not applicable

Table 3B. HIV-1 specific serum antibodies in blood of 28-44 days young pups born of HIV-1 seropositive and HIV-1 seronegative mothers. These pups remained unimmunized and were tested for passively transferred HIV-specific serum antibodies from the mother.

Group	**Pups (*n*)**	**Day**	**Median ELISA IgG titer**			
			Gagp24	**Env gp160**	**Nef**	**Tat**
Mother						
1 + 2	4	28	1200 (400–2200)	440 (300–4400	120 (100–340)	110 (<100–200)
	4	44	380 (<100–600)	<100 (<100–100)	<100 (<100)	<100 (<100)
3 + 4	3	28	880 (800–1000)	700 (300–700)	200 (100–200)	100 (100–200)
	3	44	260 (100–300)	100 (100–400)	<100 (<100)	<100 (<100)
	2	58	<100	<100	<100	<100
5	5	28	<100	<100	<100	<100

in plates coated with HIV-1 antigens. The production of HIV-specific antibodies was subsequently measured by ELISA. Only in cultures with cells derived from HIV-immunized pups could HIV-1 specific IgG secreting cells be found. Furthermore, B cells from HIV-DNA immunized mice born to HIV-1 seropositive mothers responded with the broadest HIV-1 antigen recognition (Figure 3A-B). These data suggest that the reactivity observed in the mice was not only due to passively transferred antibodies from the HIV+ mothers. Nonimmunized pups, either born to HIV-immune or non-immune mothers, did not demonstrate any HIV-1 Env-, Nef- or Tat-specific antibodies 6 weeks after the separation from the mother (Table 3B). No anti-gag antibodies were detected in the pups 8 weeks after separation from the mother (data not shown).

3.3. Presence of HIV-1 neutralizing antibodies

Serum antibodies capable of neutralizing HIV-1 in vitro were observed in the adult mice (Table 2C). The immunogens used were based upon the two viral strains IIIB and SF2 and high HIV-1 neutralizing serum titers were obtained against these two tissue culture adapted HIV-1 isolates

Table 4. Comparison of HIV-1 specific immune responders among pups to HIV-1 immune and HIV-1 naive mothers after immunization with or without the use of adjuvant. Antigens tested against: p24gag, gp160Env, Nef and Tat.

Group	**Adjuvant**	***n***	**Percentage (%) of immune responders after a primary immunization to**			
			At least one HIV-1 antigen	**Three HIV-1 antigens**	**Four HIV antigens**	**Significance**
Pups born to HIV-1-immune mothers						
	No	24	62%	50%	12.5%	
	Yes	29	100%	66%	38%	
Total		53	81%	31%	14%	$p < 0.001$
Pups born to HIV-1-naive mothers						
	No	16	50%	0%	0%	
	Yes	12	75%	0%	0%	
Total		28	57%	0%	0%	$p < 0.001$

(TCLA). Approximately, ten-fold lower serum neutralizing titers were seen against primary clade B isolate (6920) suggesting that this immunization schedule have capacity to induce neutralizing antibodies against circulating HIV-1 isolates, but the antibodies against the primary HIV-1 isolates are more difficult to obtain at high titer. There was a trend toward higher serum neutralizing titers in sera collected from group 1 (i.na. no adjuvant), 2 (i.na. plus PCPP) and 4 (i.d plus rGM-CSF) then in sera from group 3 (i.d., no adjuvant) against the TCLA HIV-1 strains, but not against the 6920 primary isolates. Since, the sera were pooled to allow repeated neutralization

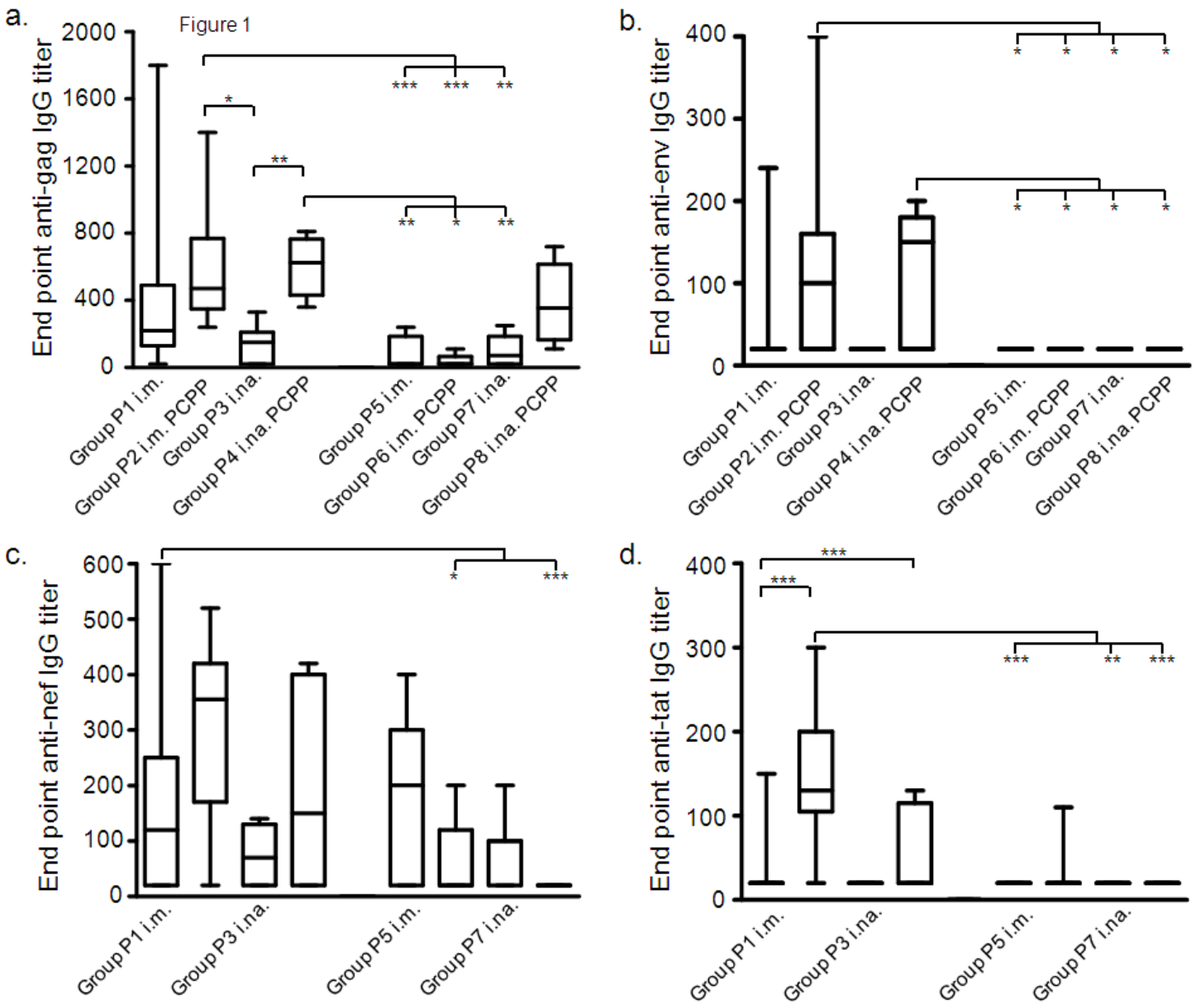

*Figure 1. Median serum IgG titers in pups born to HIV-1 immunized (Groups P1 to P4) or HIV-1 naïve (Group P5-P8) four weeks after HIV-1 gp160Env, p37Gag, nef and tat DNA and one recombinant HIV-1 protein immunization. The box-plots show the median titers and the 25% and 75% quartiles for each study group of pups and in (A) against p24Gag, with indicated significant differences in the IgG titers between the study groups (B) against gp160Env, with significant differences in the IgG titers between indicated groups. Significant differences in serum IgG titers indicated in (C) against Nef and significant titer differences shown (D) against Tat. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

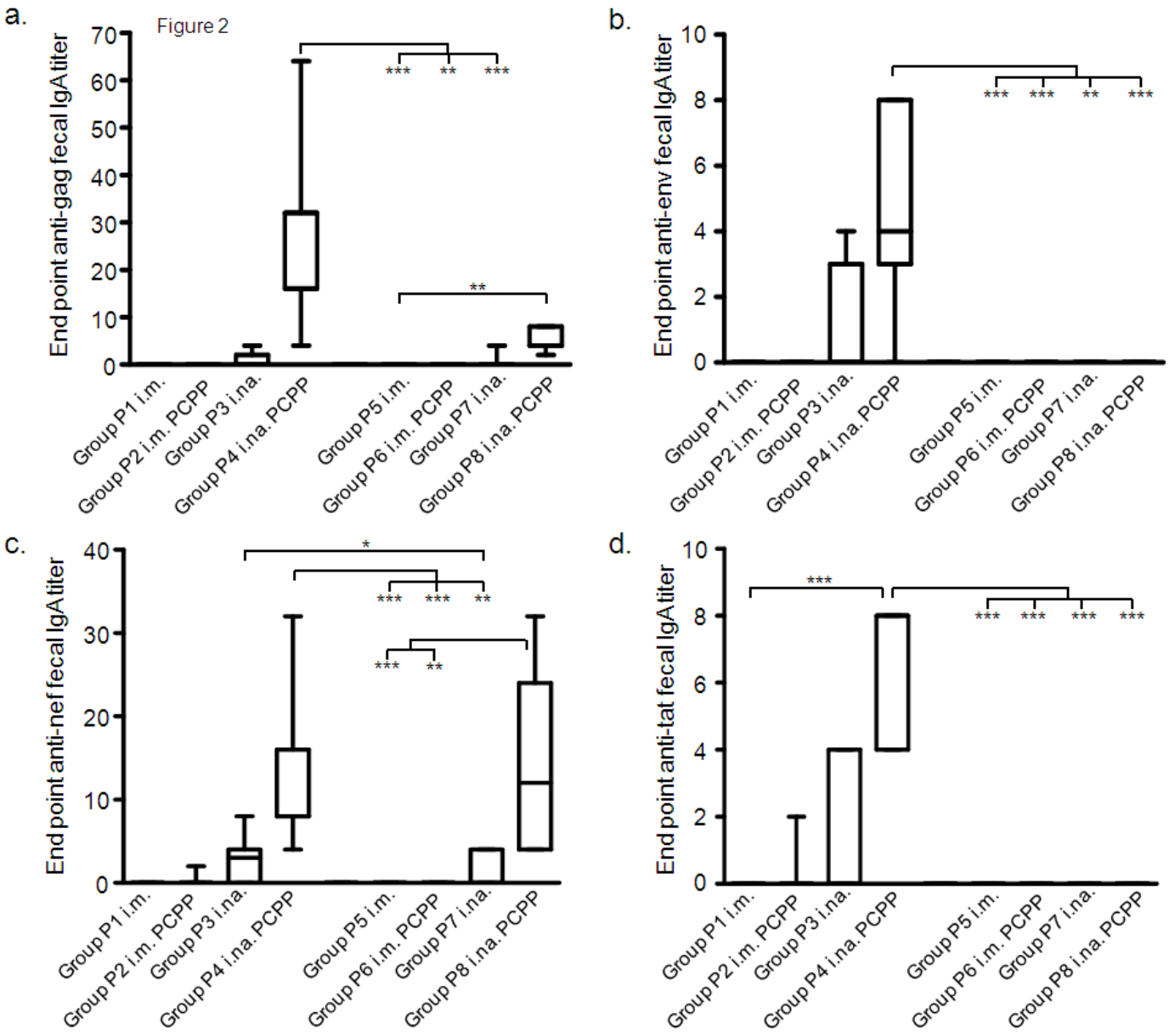

*Figure 2. Fecal IgA titers in pups born to HIV-1 immunized (Groups P1-P4) or born of HIV-1 naïve mothers (Group P5-P8) six weeks after one HIV-1 gp160Env, p37Gag, nef and tat DNA and recombinant HIV-1 protein immunization. The box-plots show the median titers and the 25% and 75% quartiles for each study group of pups and in (A) against p24Gag, with indicated significant differences in the IgG titers between the study groups (B) against gp160Env, with significant differences in the IgG titers between indicated groups. Significant differences in serum IgG titers indicated in (C) against Nef and significant titer differences shown (D) against Tat. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

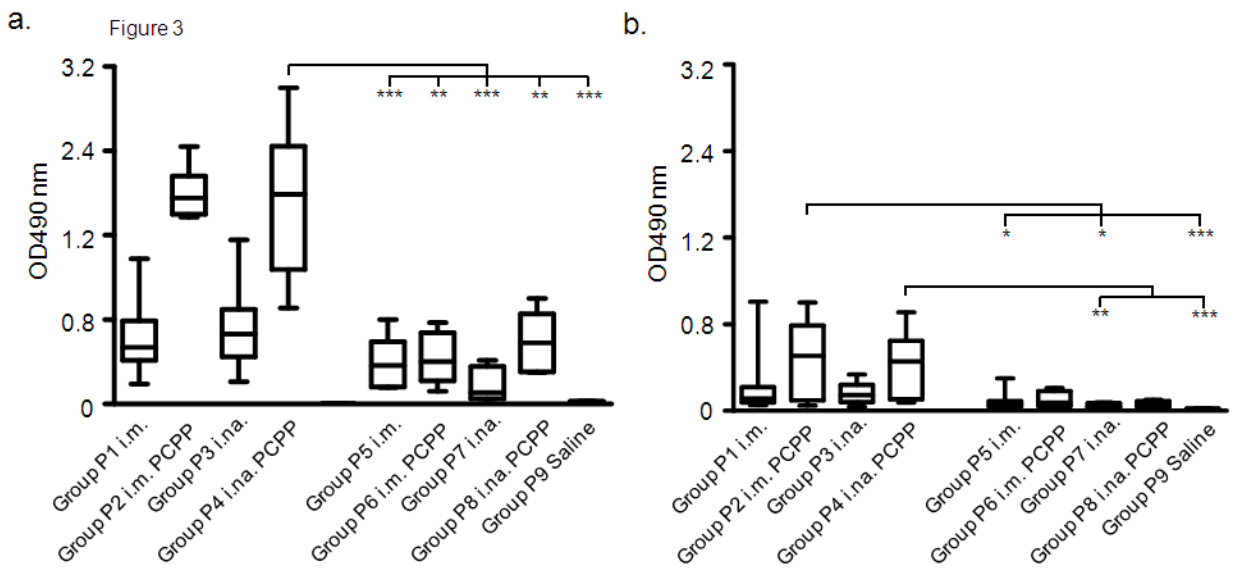

*Figure 3. In vitro B-cell IgG synthesis against HIV antigens and control antigen of 200 000 spleen cells collected from HIV-1 DNA immunized pups at 6 weeks post-immunization. Groups P1 to P4 represents spleen B-cells from HIV-immunized pups born from HIV+ mothers and groups P5-P8 represents spleen B-cells from HIV-immunized pups born from HIV- mothers and group P9 represents B cells from pups born to non-vaccinated mothers but given saline or PCPP only. The box-plots show the median OD values and the 25% and 75% quartiles for each study group of pups and in (A) against p24Gag where the OD values of B-cells are shown and significant differences indicated and (B) against gp160Env where significant differences are indicated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

assays no reliable statistical analyses could be performed to compare the differences in titers between the groups. In the pooled serum collected from the pups at 6 weeks post-immunization no or only weak HIV-1 neutralizing activity was seen, suggesting that further booster immunizations would be needed in order to obtain higher HIV-1 neutralizing titers.

3.4. HIV-1 specific IgA in fecal washes after immunizations

A mucosal (intestinal, IgA) response was observed in addition to systemic immune response (Table 2A and 3A, Figure 2A-D). In pups in groups P2, P4 and P8 significant HIV-1 specific fecal IgA antibody titers were observed contrasting the results observed in intramuscularly immunized pups or pups immunized without the addition of adjuvant.

3.5. Breadth of humoral immune responses induced by HIV-1 DNA immunization.

Immunization of pups born to HIV-1 seropositive mothers resulted in significantly broader HIV-1 antigen recognition than immunization of pups born to HIV-1 seronegative mothers ($p<0.001$, Table 4). Pups in groups P3 and P4 receiving HIV-1 immunogens combined with the PCPP adjuvant showed the strongest serum responses to all four tested HIV-antigens. Furthermore, pups in group P4 (i.na. plus PCPP) developed the strongest fecal HIV-1 specific IgA response. Pups in group P2 (i.na., no adjuvant) recognize most of the HIV-antigens but responded with lower titers than pups in group P4. Pups born to HIV seronegative mothers and immunized intranasally with addition of PCPP (P8) developed fecal IgA recognizing Gag and Nef antigens.

3.6. Cell-mediated immunity

Splenocytes of the DNA immunized pups at 6 weeks post immunization were analyzed for vaccine-specific cellular immune responses. DNA-immunization with 10 μg plasmid in PCPP adjuvant emulsions could induce IFN-γ and IL-2 responses against p24Gag. The median IFN–γ spots/10^6 cells ranged from 38-40 in intranasally immunized animals and 50 in intramuscularly immunized animals and 0-8 in controls receiving only adjuvant without HIV-1 DNA plasmids. IFN–γ responses against gp160Env was also observed. The intranasally DNA/PCPP immunized animals responded with 30-400 spots/10^6 cells while unadjuvanted intramuscular immunization resulted in 10-15 spots/10^6. No vaccine-specific IL-2 responses could be observed from any animal. HIV-1 antigen-specific T-cell proliferation against all four HIV-1 recombinant antigens were detected in equal frequency in all HIV-1 immunized study groups, no significant differences

in magnitude between the groups were detected. No significant differences in cell-mediated responses between the different groups of DNA immunized animals were seen (data not shown).

4. Discussion

The HIV-1 specific serum and fecal antibody titers of the mothers clearly did not negatively influence the responses in the pups. On the contrary, the data obtained in this study suggest that broader HIV-1 specific humoral immunity was obtained when breast-feeding pups were immunized with HIV-1 DNA prime and protein boost. Pups born of HIV-1 sero-negative mothers showed significantly lower as well as less broad immune responses. There are several possible explanations for the observed differences.

Firstly, the immunogens used, which includes DNA-plasmids. Plasmids are considered to be clean antigen-expressing agents and plasmids are not hampered by pre-existing immunity to the vector, as is the case for viral or bacterial vaccine vectors. Secondly, in the intranasal tissue, the concentrations of potentially blocking or inhibiting antibodies are lower then in, for instance serum. Thus allowing for more efficient antigen uptake and presentation by antigen-presenting cells. Thirdly, when it comes to the DNA prime and recombinant antigen boost, the maternal antibodies may bind to the antigen and form immune complexes that more efficiently are taken up by dendritic cells or B cells via FcR-mediated uptake mechanisms [18]. Lastly, since the pups had been fed with mother's milk containing HIV-specific immunoglobulins perhaps they have induced anti-idiotypic antibodies (complementary determining regions) which may have triggered and enhanced the B-cell immune responses in the pups [19]. The remarkable result was that the pups of the HIV+ mothers developed serum and fecal IgA antibodies against all four studied HIV-antigens. While the truly HIV-1 naïve pups responded immunologically very similar to untreated adult mice, the HIV-1 DNA-primed once and boosted with a low dose recombinant

antigens once, resulting in humoral antibodies against one or two antigens. One possible explanation is that some of the immune reactivities can be due to remaining HIV-1 specific antibodies that are passively transferred from the mother to the offspring, even though we were able to detect them in the 20 control mice that we studied without immunization (Table 3). Furthermore, perhaps soluble factors in the mothers milk such as cytokines or chemokines, may influence the immune responsiveness of the breast-fed pup. This in combination with the nutrient-rich mothers milk may create a favorable immunological milieu in the pup making it more capable of responding to vaccination.

However, even though (at the time of immunization) the maternal antibody titers were relatively high (Table 1) and clearly detectable in pups at 4 weeks after terminated breast-feeding, the titers were not dramatically high. The neutralizing antibody titers were low which may at, to some degree, explain why the maternal antibodies did not have negative influence. In previous studies, using live vectors showed that when maternal antibody titers reach above a certain level, they were able to suppress vaccine-specific immune responses [20,21], probably by binding to and facilitating the clearance of vaccine antigen in the pups. Previous studies have been performed on much younger mice (1-7 days post-partum, or even in utero [1-3,22-24]) than the pups used in our study, in which the pups had reached their final days of breast-feeding (day 15-17) where after they were separated from the mother two to three days later.

It may also be of interest to discuss the immunity to HIV evoked in the adult female mothers. In, these studies we evaluated the primary immune responses against four HIV-antigens in both adults and pups that both were immunized with a heterologous DNA-prime and later with a recombinant protein boost. In, both populations the administrations were performed intranasally or parentally where the parental dose in the mothers was given intradermally and in the pups

intramuscularly. Finally, the numbers of immunizations differed; the adult females were given three DNA-immunizations before they were finally boosted with protein while the pups were DNA-primed and protein-boosted once. The reasons for these differences were the following, it was important to obtain a full and clearly detectable humoral immunity in the mothers to be able to study if this complete HIV-1 antigen-specific repertoire could have effect when transferred to the pups. In the pups it was instead more important to investigate how broad and fast a HIV-1 specific humoral immunity could be obtained with as few immunizations as possible. The idea behind this decision was that in certain high risk HIV-1 exposure situations i.e HIV-infected mothers providing breast-milk to their children it may be critical to obtain the HIV-1 immunity rapidly and early in life to prevent infection and disease, as with many gastrointestinal or diarrheal orally transmitted diseases [23]. We did not immunize mothers intramuscularly since this route seldom gives efficient mucosal secretory IgA immunity. The aim was to investigate the impact of maternal antibodies transferred both via placenta (IgG) and via breast milk, both IgA and IgG.

Regarding the role of adjuvants in these experiments; in the mothers we tested two types of adjuvants, in the intranasally immunized mothers we delivered DNA and proteins with the PCPP, an adjuvant described as a antigen-deposit induced delivery. Thus, the DNA or the protein may for a longer time become released a situation where the antigen-exposure time would be prolonged allowing a larger amount of immune cells to meet that immunogen. The other adjuvant, the recombinant GM-CSF, used in the intradermally immunized mice is a known inflammatory substance that causes antigen-presenting cells to become activated and to mature, thus more efficiently presenting engulfed of expressed antigen to T-cells. In this study only the adult mice obtained GMCSF adjuvant intradermally. GMCSF had a positive effect on the

antibody response and on the HIV-1 neutralizing capacity (Group 4, Tables 2B and 2C) in adult mouse serum, but did not significantly influence the immune reactivity of the HIV-immunized pups when compared with pups of PCPP immunized mothers..

The pups receive the maternal antibodies both through transplacental transfer and through milk by breast-feeding. In this study, the pups were older then what have been reported in previous studies performed with 1-7 days old pups [1-3,22-24]. Our aim was however to analyze the influence of nasal administration preferably avoiding lung tissue involvement during antigen delivery. The young mice were given such small volumes of 5 µl/nostril of immunogen that most of the volume should be taken up in the nasal cavity (NALT). This would have been difficult in smaller mice, where perhaps oral mucosal administration would perform better [23].

This route would probably better reflect the natural situation as most pathogens enter across mucosal surfaces and that mothers thus often are both mucosally and systemically immune when delivering their offspring.

It is possible that with this immunization strategy for the adult mice was capable of providing immune-enhancing effects in the pups; this has been reported with other protein-based vaccines [25]. In other studies, with other vaccine candidates, problems with vaccine-take in the offspring after DNA immunization have been described if a very strong maternal humoral immunity is present. In these reports it seems as if the maternal antibody levels may have masked the vaccine antigen, even when they were given parentally as killed vaccines [1,2,22]. In this study, these difficulties were not seen, likely because the maternal antibody-titers against HIV antigens were not high enough to interfere with immunization. Furthermore, especially in mucosally immunized pups the presence of neutralizing antibodies may not have reach sufficient levels to interfere with vaccine administration and uptake. It seems clear from several previous reports that DNA-

immunization of neonates, newborns or even in utero can induce vaccine-specific cellular immunity, as shown for Influenza virus, respiratory syncytial virus and Herpes virus [3,26-32]. Occasionally also antibody responses have been reported after DNA-prime and MVA-boost immunization against malaria, in this case antibodies were mainly found in neonates born of sero-negative mothers [33]. It is today clear that most HIV-1 transmissions occur via mucosal transmission, (reviewed in [34]) and an immune response elicited by vaccination should act at mucosal surfaces to be efficient. To achieve this, a vaccine would probably be most efficacious if delivered via the mucosa, preferably intranasal or orally using needle-free devices. In addition, the systemic humoral and cellular responses are also believed to be important [35,36]. It may thus be a good idea to search for immunization strategies that provide both strong systemic and mucosal CTL and neutralizing antibody responses against HIV-1.

In this study, we show that with the use of HIV-1 DNA and PCPP adjuvants it was possible to induce systemic and mucosal HIV-1 specific immunity, both cell-mediated and humoral. Moreover, the responses were significantly broader in immunized pups born to mothers displaying HIV-1 specific immune responses. Although the duration of the immune responses in the pups is currently unknown, our data indicates a place for immune intervention in early life.

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