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Genetic immunization with GPI-anchored anthrax protective antigen raises combined CD1d- and MHC II-restricted antibody responses by natural killer T cell-mediated help

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ABSTRACT

Studies have demonstrated that lipid rafts ultimately regulate the endocytosis of anthrax toxin via clathrin dependent pathway. Interestingly, GPI-anchored protein rich rafts have also been shown to be transported down to the endocytic pathway to reducing late endosomes. Taking advantage of this parallelism, we tried translating the anthrax toxin natural intoxication mechanism by administering a DNA chimera that encoded protective antigen attached to a mammalian GPI-anchor sequence at its C-terminus (pGPI-PA63). We also designed a chimera that had an additional N-terminal TPA leader sequence (pTPA.GPI-PA63) with an aim to target GPI-PA63 to ER where new CD1 molecules are synthesized. Analysis of antibody titers demonstrated successful priming and potential IgG titers after the first boost. In vitro cell proliferation studies in the presence of GPI-attached PA63 peptides revealed that there was a clonal expansion of $CD4^+$ $NK1.1^+$ helper T cell population which rapidly produced IL-4 in response to T cell receptor ligation. These cells provided direct B cell help that aided IgG formation. Effector responses generated by NKT cells were found to be MHC II-independent and CD1d-restricted. In addition, the group pTPA.GPI-PA63 also displayed low magnitude MHC-II restricted (CD1d-independent) NKT cell and $CD4^+$ T cell helper responses in response to non-GPI attached PA63 peptides which overall resulted in the heightened responses seen for this group. Importantly, DNA vaccination mediated the generation of high avidity neutralizing antibodies that mediated protection against lethal toxin challenge.

1. Introduction

Bacillus anthracis, the causative agent of anthrax, has developed an ingenious strategy to target cells by secreting a soluble proteinaceous exotoxin which belongs to a large family of binary (AB type) pore forming toxins. Formation of anthrax toxins is initiated once the 'B' subunit (protective antigen; PA83) binds to a cell surface anthrax toxin receptor (ATR), TEM8 and CMG2 [1,2]. PA83 subsequently undergoes cleavage by furin resulting in PA63, a membrane bound protein which forms a heptameric pre-pore of individual monomers with a β-barrel structure [3,4]. Pre-pore to pore conversion occurs upon endocytosis triggered by the low pH in the endosome to form a membrane spanning pore [5,6]. Thus, the ATR which initially resides in the glycerolipidic, i.e. non-raft regions of the plasma membrane, upon binding and heptamerization of PA, associates with lipid-raft like domains and undergoes rapid endocytosis via clathrin-dependent pathway [7]. All this is undertaken to ensure the translocation of the catalytically active 'A' subunit, edema factor (EF) and lethal factor (LF), into the cytosol where it can hijack the cellular machinery [8]. Binding of EF or LF to PA63, which forms edema toxin (EdTx) and lethal toxin (LeTx), respectively, occurs at the cell surface after the heptamerization has occurred [9]. Once in the cytosol, EF, an adenylate cyclase, induces substantial increase in conversion of intracellular ATP to cAMP in the presence of calmodulin [10]. Subsequently, water homeostasis and cellular signaling of host are disrupted resulting in edema [10,11]. LF is a zinc-dependent metalloprotease that cleaves short N-terminal fragments from mitogen or extracellular signal-regulated protein kinase kinase1 (MEK1), MEK2 and MEK3, the upstream activators of ERK1, ERK2 and p38, respectively [12–14] that disrupts antigen receptor signaling resulting in macrophage function inhibition and cell death [14,15].

The depredation thus, resulting from anthrax toxin action in affected individuals has riveted attention. Early treatment of anthrax is essential, as antibiotics may not be effective late in the disease owing to the accumulation of high levels of LeTx and EdTx even with the elimination of the organism. The licensed

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ccine, anthrax vaccine absorbed (AVA), suffers from the criticisms incomplete characterization of the composition of the vaccine, casional reactogenicity, and the need for frequent administration boosters [16]. Therefore, the current scenario necessitates the arch for newer anthrax vaccines. In this context the potential of novel approach that uses DNA for vaccination has been exploited. ost of the DNA vaccination strategies, tested so far against anthrax, ve relied mainly on DNA-adjuvant combinations for generating otective immunity [17–25].

The observation that ATR couples raft translocation and medi- es endocytosis of EF/LF along with the oligomerization of PA made hypothesize that a DNA chimera encoding protease-cleaved frag- ent of PA (PA63) attached to a C-terminal glycosylphosphatidyl ositol (GPI) anchor sequence might ensure lateral association of e cell-surface GPI-anchored PA with liquid-ordered, cholesterol- d sphingolipid-rich domains or rafts. Biochemical, morpho- gical and functional approaches have been able to trace that I-anchored protein rich rafts are transported down to the endo- tic pathway to reducing late endosomes in mammalian cells [26]. erefore, adoption of such an approach opens up a possibility that I anchored PA63 will undergo its natural cycle upon endocytosis hich might improve DNA vaccine potency.

Based on this rationale we constructed a DNA chimera encoding I-anchored form of PA63 utilizing the mammalian PLAP (Pla- ntal Alkaline Phosphatase) GPI anchor sequence. The GPI signal pears to have been conserved, however, a mammalian GPI signal s utilized with the understanding that GPI signals from para- ic protozoa are not recognized by mammalian cells [27]. We also signed a chimera in which PA was attached to an N-terminal TPA ader along with a C-terminal GPI anchor. TPA is expressed by vas- lar smooth muscle and binds to a specific cellular receptor p63, hich occurs in fibroblasts as an intracellular protein associated th the ER [28,29]. The TPA leader peptide can therefore, target e expressed antigen directly to the ER thus, obviating the need r the antigen to be processed and translocated to this structure 0]. Furthermore, ER is also known to be the site where new MHC d CD1 molecules are synthesized. Both human and murine CD1d olecules (Group 2 CD1 molecules) have been shown to bind to I-anchored proteins and present them to a variety of $CD4^+NK1.1^+$ lper T cells that have been shown to control humoral immunity ainst parasites [31] and elicit cell-mediated immune responses ainst tumors [32].

Therefore, with these observations in mind we designed the esent study and we successfully illustrated the generation of not ly classical MHC II-restricted immunoglobulin (Ig) responses but o non-classical CD1d-restricted Ig responses. These responses nerated by GPI-anchored PA63 were found to be superior as com- red to those generated by native form of PA63 and attributed the generation of high frequency of PA-specific NKT cells aris- g as a result of DNA vaccination. Also, NKT cells were restricted B cells producing antibodies against the GPI-anchored protec- e antigen that was captured, internalized and presented in the 1d context. Importantly, DNA vaccination mediated the genera- n of high avidity toxin neutralizing antibodies which efficiently otected immunized animals against anthrax LeTx challenge.

Materials and methods

. DNA vaccine plasmids and recombinant proteins

DNA manipulations were performed according to standard olecular biology procedures [33] using the *E. coli* DH5α strain. e structural gene for PA63 was cloned in eukaryotic expression ctors bearing the targeting signals to generate the DNA vaccine asmids (for primer sequences refer Table 1, Supplementary information, SI). Recombinant PA83 (rPA83) and rPA63 proteins were obtained from chimeras pMW*pag* and pSM*pag*, respectively, cloned in our lab previously [34]. Peptides (mapped epitopes of PA) used for in vitro stimulation for cells were synthesized commercially (Clover Scientific Pvt. Ltd., India) at >95% purity. In addition peptides representing the mapped epitopes of PA attached to a mammalian GPI-anchor sequence were also synthesized commercially at >95% purity. (For Peptide sequences refer Table 2, SI).

2.2. Transfection and immunoblotting

J774A.1 murine macrophage cells were seeded at a concentration of $2–3 \times 10^7$ cells into a 75-cm^2 flask (Corning costar) until the cells reached approximately 50–70% confluence. Plasmid DNA transfection was performed with LipofectAMINE 2000 (Invitrogen) reagent, as specified by the manufacturer. For Western blot analysis, the transfected cells were washed twice with PBS (pH 7.4) and lysed in lysis buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) 48 h post-transfection to prepare total cell lysate. Cell membrane protein fraction was prepared by Qproteome membrane protein kit (Qiagen) according to the manufacturer's protocol. The presence of cell membrane in the fractions was determined by the associated NADH oxidase activity [35]. Culture supernatant proteins were precipitated by ice-cold acetone. Proteins from the total cell-lysate, cell membrane fractions and culture supernatants were subjected to SDS–polyacrylamide gel electrophoresis under denaturing conditions with 50 mM dithiothreitol (DTT). Proteins from the gel were transferred on to a nitrocellulose membrane and probed with rabbit anti-PA polyclonal serum followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG (Amersham biosciences). Blot was developed using BCIP/NBT (Sigma–Aldrich) as substrate.

2.3. Vaccination and lethal toxin challenge

Six- to eight-week-old female Swiss albino outbred mice (National Institute of Nutrition, Hyderabad, India) were immunized intramuscularly (i.m.) with 100 μg of DNA (endotoxin-free) on days 0, 28 and 56. Mice immunized with rPA83 received recombinant protective antigen protein formulated with alhydroxy gel on days 0 and 28. Pre-immune sera were collected before immunization and thereafter, sera were collected 2 weeks after each immunization. At different time intervals post the last vaccination (at week 12, 14, 16, 18, 20), immunized mice were challenged with anthrax LeTx (50 μg of PA83 and 22 μg of LF; approximately 4–5 LD_{50}) injected intravenously via tail vein, and the challenged mice were closely monitored for 21 days. All experiments were performed in accordance with 'Indian Animal Ethics Committee' regulations.

2.4. ELISA detection of anti-PA reactivity in mouse serum

The anti-PA reactivity of sera from immunized animals was determined by direct ELISA. Briefly, ELISA plates were coated with rPA63 (10 μg/ml) diluted in PBS. Following blocking and washes in PBS-Tween 20 (0.2%), serum dilutions were added and incubated for 2 h at room temperature. Further, upon washes with PBS-Tween 20 (0.2%), plates were incubated either with goat anti-mouse IgG or IgM HRP conjugate (Santacruz Biotechnology) for 1 h at room temperature. Thereafter, plates were washed with PBS-Tween 20 (0.2%), and developed with TMB substrate (Amersham biosciences). The reaction was stopped with 1N sulfuric acid, and the plates were analyzed at 450 nm in an ELISA reader (Benchmark Plus Microplate spectrophotometer, BioRAD). The negative controls included sera from mice immunized with PBS and vector. Endpoint antibody

titers were expressed as the reciprocal of sample dilution at which the absorption at 450 nm was greater than two times the background signal.

2.5. Detection of toxin neutralizing antibody (TNA) titers

The protective effect of anti-toxin antibodies was determined using a previously described assay that measures their capacity to protect the J774A.1 cells from LeTx [36]. Briefly, J774A.1 cells (5×10^4 cells/well) were seeded in 96-well, flat bottom plates. After the cells reached 50–70% confluency, serum dilutions were added together with LeTx (1.0 μg/ml each of PA and LF) and incubated for 4 h. After an additional incubation of 30 min after the addition of MTT (0.5 mg/ml) (Sigma–Aldrich), the cells were lysed with acidic isopropanol (0.04–0.1N HCl in 90% isopropanol). The A_{450} of 100% viable cells was calculated from the average of four wells receiving no LeTx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an A_{450} ≥90% of the value of wells receiving no LeTx.

2.6. In vitro cell proliferation assay

After isolation, 4×10^7 spleen lymphocytes from naive or DNA vaccinated mice were cultured in the presence of pooled peptides [either GPI-attached PA peptides or non-GPI PA peptides (10 μg/ml of each peptide)] with IL-2 (10 U/ml) for a period of 4 days. After that NK1.1$^+$TCR-α/β$^+$ cells and CD4$^+$ T cells were analyzed by flow cytometry using fluorescence-activated cell sorting (FACS VantageTSE Flow Cytometry System, Becton Dickinson India Pvt. Ltd., Gurgaon 122001, Haryana, India). Monoclonal antibodies against NK1.1, CD4, and TCR-α/β) were purchased from BD PharMingen India Pvt. Ltd. The details of the staining and sorting have been described previously [37]. Clonal expansion in the presence of GPI-anchor peptide only (Table 2, SI) was subtracted to negate any proliferation response generated specifically against the glycerolipidic region.

2.7. Isolation of lymphoid cell subsets by FACS

Spleen cells were incubated on nylon wool column for 45 min, and the nonadherent cells were used for the isolation of NKT cells and CD4$^+$ T cells by cell sorting by FACS. NK1.1, CD4$^+$, and TCR-α/β cells were identified using monoclonal antibodies (BD PharMingen India Pvt. Ltd.). Unless otherwise noted, CD4$^+$NK1.1$^+$TCR-α/β$^+$ cells were used as purified NKT cells. The stained cells were isolated using the FACS Vantage^{TM}. The purity of the sorted cells was >98%.

2.8. In vitro IL-4 production

NKT cells and CD4$^+$ T cells (10^5 cells/ml) were incubated with pooled peptides [either GPI-attached peptides or non-GPI peptides (10 μg/ml of each peptide)] in the presence of splenocyte APCs (syngeneic splenocytes that were pulsed with peptides and then given mitomycin-C treatment). Antigen induced IL-4 production was determined in the presence and absence of anti-MHC II, anti-MHC I and anti-CD1 monoclonal antibody (BD PharMingen India Pvt. Ltd.). Cell-free culture supernatants were harvested 24 h post-stimulation and cytokine-specific sandwich ELISA was performed using OptEIA kit for the specific cytokine (BD PharMingen India Pvt. Ltd.) according to manufacturer's protocols. IL-4 production in the presence of GPI-anchor peptide only (Table 2, SI) was subtracted to negate non-specific stimulation due to the glycerolipidic region only.

2.9. B-lymphocyte purification

After isolation, splenocytes were incubated with anti-CD43 and anti-Mac-1 antibody-conjugated microbeads (Miltenyi Biotec India Pvt. Ltd.). The bead-bound cells (positive fraction) were separated from unbound cells (negative fraction) using an AutoMacs magnetic cell sorter. The enriched cell population was subjected to multiparameter cell sorting by FACS to separate cells expressing B220, a marker present on cells committed to the B lineage [38]. The preparation was 96% B220$^+$, the few contaminants were CD43$^+$/Mac-1$^+$.

2.10. Helper assays

B lymphocytes (2.5×10^5/ml) were co-cultured in the presence of NKT cells, CD4$^+$ helper T cells or alone in the presence of either, GPI or non-GPI-PA peptides (pooled peptides, 10 μg/ml) and IL-2 (10 U/ml). Antigen specific IgG production was quantified upon addition of anti-class II and anti-CD1 mAb by ELISPOT. IgG production in the presence of GPI-anchor peptide only (Table 2, SI) was subtracted to negate non-specific stimulation due to the glycerolipidic region only. Spots were developed using Alkaline Phosphatase-linked secondary antibody and BCIP/NBT (Sigma–Aldrich) as substrate. Results were expressed as number of SFU (Spot Forming Units) per 10^6 cells.

2.11. Determination of avidity of PA-specific IgG antibodies

Antibody avidity was measured by using a previously described ELISA that utilizes urea as chaotropic agent to dissociate low-affinity antigen–antibody complexes [39].

2.12. Statistical analysis

The experimental data were analyzed by software programs Sigma Plot 8.1 or Excel (Microsoft). The statistical significance of differences was analyzed by a two-tailed Student's *t*-test for independent groups (followed by Bonferroni's correction to adjust for multiple comparisons). A p value of <0.05 determined was considered statistically significant. Correlation coefficients were determined by linear regression analysis.

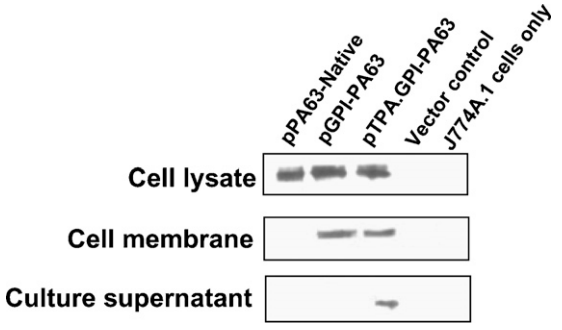

Fig. 1. The address tags efficiently target PA63 to various sub-cellular locations. Shows expression of different forms of protease-cleaved fragment of protective antigen in J774.A1 mouse MΦ-like cells. Cells were transfected with DNA vaccine constructs. Cell lysates and cell membrane fractions were prepared 48 h post-transfection. Along with that cell culture supernatant proteins were harvested by acetone precipitation. Subsequently, the protein samples were subjected to 12% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane. Blot was probed with anti-PA polyclonal serum followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG and developed using BCIP/NBT as substrate.

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ti-PA antibody titers in the serum of DNA immunized animals.

nstruct	Antibody endpoint titers[a], $10^3 \pm$ SD								
	IgM			IgG			TNA		
	Priming	1st Boost	2nd Boost	Priming	1st Boost	2nd Boost	Priming	1st Boost	2nd Boost
PI-PA63	0.48 ± 0.1	<0.05	<0.05	25.0 ± 4.0	47.0 ± 6.5[b,c]	79.0 ± 4.1[b,c]	−BD	0.35 ± 0.11[b]	0.51 ± 0.2[b]
PA.GPI-PA63	0.55 ± 0.2	<0.05	<0.05	13.5 ± 1.5	88.0 ± 5.5[b,c]	100 ± 4.0[b,c]	−BD	0.75 ± 0.23[b]	1.0 ± 0.15[b]
A63-Native	0.37 ± 0.2	<0.05	<0.05	3.5 ± 0.5	10.0 ± 2.0	20 ± 2.1	−BD	0.10 ± 0.01	0.15 ± 0.1
83	0.40 ± 0.4	<0.05	<0.05	18.5 ± 0.5	58.0 ± 5.0[b,c]	70.0 ± 2.1[b,c]	−BD	0.27 ± 0.05	0.39 ± 0.1[b]
ctor control	−BD[d]	−BD	−BD	−BD	−BD	−BD	−BD	−BD	−BD

Mice were immunized on days 0, 28 and 56; and anti-PA titers were measured in the serum of the immunized mice 14 days after each immunization. Results represent rs obtained for 8–10 mice tested in a group in three independent experiments and results are expressed as mean ±SE.
Significantly higher ($p < 0.001$) titers as compared to the pPA63-Native group as calculated by Student's *t*-test followed by Bonferroni's correction to adjust for multiple nparisons.
Significantly higher ($p < 0.01$) titers as compared to those developed after priming.
Below detection.

Results

. Transfection of DNA vaccine chimeras in J774A.1Φ-like cells llowed by detection with Western blotting

The structural gene for PA63 was cloned in DNA vaccine plas-ds bearing the address tags (GPI, TPA-GPI). Authenticity of e DNA constructs was evaluated by transient transfection in 74A.1 cells with DNA chimeras followed by subcellular frac-nation and immunoblot analysis (Fig. 1). Results indicated at the PA63 protein along with the GPI and TPA signals was ing properly recognized by the mammalian cells. The DNA imeras, pGPI-PA63 and pTPA.GPI-PA63, expressed membrane-chored form of PA63 (Fig. 1). In addition, the chimera pTPA.GPI-PA63 also mediated secretion of PA63 in the culture supernatants.

3.2. Significant high avidity antibody responses were generated in the vaccinated mice

Following i.m. injection of the DNA constructs, 100% of the mice had strong serum IgM titers (Table 1) which peaked after priming and then declined after administering the first and second booster dose. Pronounced PA-specific IgG titers were demonstrated by groups that were immunized with chimeras encoding the GPI-anchored form of PA63. Highest titers were elicited by the chimera pTPA.GPI-PA63 (≈100,000), followed closely by the groups pGPI-PA63 (≈79,000) and rPA83 (≈70,000) which also generated

ble 2
tibody response and protection against *B. anthracis* LeTx challenge at different time points post-vaccination.

oup	Time of challenge[a] (week)	Antibody titers post-challenge, $10^3 \pm$ SD			
		Total anti-PA antibody	LeTx neutralizing antibody	Survival% (No. of survivors/No. challenged)	MTD[b] ± SE (days)
PI-PA63	12	85.5 ± 5.5	0.88 ± 0.20	100 (9/9)	
	14	91.4 ± 8.8[c]	0.90 ± 0.10[c]	100 (8/8)	
	16	90.5 ± 7.0[c]	0.94 + 0.20[c]	100 (7/7)	
	18	80.1 ± 6.5	0.70 ± 0.15	75 (6/8)	9.5 ± 0.50[c]
	20	80.5 ± 7.5	0.69 ± 0.12	75 (5/7)	8.9 ± 0.40
PA.GPI-PA63	12	120 ± 5.5	1.30 ± 0.50	100 (8/8)	
	14	199 ± 6.0[c]	1.95 ± 0.40[d]	100 (8/8)	
	16	175 ± 8.0[c]	1.70 ± 0.20[d]	100 (5/5)	
	18	155 ± 3.0	1.55 ± 0.15	100 (7/7)	
	20	140 ± 2.0	1.51 ± 0.11	100 (8/8)	
A63-Native	12	26.0 ± 4.0	0.19 ± 0.05	43 (3/7)	3.6 ± 0.61
	14	41.0 ± 6.5	0.25 ± 0.02	56 (5/9)	5.9 ± 0.25
	16	34.0 ± 3.5	0.24 ± 0.03	56 (5/9)	5.7 ± 0.52
	18	20.0 ± 2.5	0.19 ± 0.03	44 (4/9)	3.6 ± 0.55
	20	19.0 ± 2.5	0.18 ± 0.05	44 (4/9)	3.3 ± 0.60
83	12	53.5 ± 9.0	0.58 ± 0.10	63 (5/8)	5.6 ± 0.60
	14	88.0 ± 15.5[c]	0.72 ± 0.15[d]	78 (7/8)	9.5 ± 0.20[c]
	16	87.9 ± 12.5[c]	0.68 ± 0.12[d]	78 (7/8)	9.7 ± 0.15[c]
	18	78.0 ± 15.5	0.60 ± 0.21	70 (7/9)	8.9 ± 0.30
	20	79.5 ± 14.5	0.59 ± 0.20	69 (7/9)	8.8 ± 0.20
ctor control	12	−BD[e]	−BD	0 (0/5)	0.70 ± 0.12
	14	−BD	−BD	0 (0/4)	0.65 ± 0.15
	16	−BD	−BD	0 (0/4)	0.75 ± 0.20
	18	−BD	−BD	0 (0/5)	0.70 ± 0.16
	20	−BD	−BD	0 (0/6)	0.70 ± 0.20

The immunized mice were challenged at 12, 14, 16, 18, 20 weeks post-immunization with LeTx injected i.v. through tail vein and monitored for 21 days. Results represent rs obtained for $n = 9$ mice tested in a group in three independent experiments and results are expressed as mean ±SE.
MTD, mean time to death was determined by determining the average time to death for each group, excluding the survivors. Results are represented as MTD ± SE.
Significantly higher ($p < 0.01$) titers as compared to the pPA63-Native group as calculated by Student's *t*-test followed by Bonferroni's correction to adjust for multiple nparisons.
Significantly higher ($p < 0.001$) titers as compared to the pPA63-Native group.
BD, below detection.

Table 3
Avidity indices of anti-PA antibodies in the serum of immunized mice.

Construct	Antibody avidity index[a]		
	Day 70	Day 100 challenge	Day 28 post-challenge
pGPI-PA63	33	62[b,c]	78[b,c]
pTPA.GPI-PA63	39	79[b,c]	87[b,c]
pPA63-Native	23	34	53[c]
rPA83	30	75[b,c]	88[b,c]
Vector control	−BD[d]	−BD	−BD

[a] Avidity index = (endpoint titer in the presence of urea)/(endpoint titer in the absence of urea)) × 100.
[b] Significantly higher ($p < 0.001$) avidity index as compared to the pPA63-Native group as calculated by Student's *t*-test followed by Bonferroni's correction to adjust for multiple comparisons.
[c] Significantly higher ($p < 0.01$) as compared to the AI at day 70.
[d] No PA-specific antibody was detected.

significant titers ($p < 0.001$ vs. pPA63-Native group) after the second boost. Importantly, these titers were maintained until week 20 post-priming (Table 2). Evaluation of TNA titers revealed that Letx neutralization activity was measurable only after the first booster dose (Table 1). TNA titers were approximately 2–3 times higher in the serum of animals immunized with pTPA.GPI-PA63 and pGPI-PA63 than the animals vaccinated with the native construct lacking the GPI anchor. Sera from control mice did not neutralize anthrax LeTx at all. The group immunized with rPA83 formulated with alhydroxy gel also generated significant TNA titers measuring ≈390 which was twice as compared to the pPA63-Native group but only after the second booster dose was administered ($p < 0.001$ vs. pPA63-Native group, Table 1). Evaluation of the antibody avidities revealed that high avidity antibodies were generated only at the time of challenge on day 100 (Table 3). Groups immunized with pTPA.GPI-PA63, pGPI-PA63 and rPA83 showed highest avidity indices (AI) of 79, 62 and 75, respectively, whereas the group pPA63-Native showed a significantly lower AI value of 34. Four weeks post-challenge, a further increase in AI was observed for all the groups (Table 3) with groups pTPA.GPI-PA63, pGPI-PA63 and rPA83, showing avidities as high as 87, 78 and 88, respectively, as opposed to an AI = 53 for the pPA63-Native group.

3.3. $CD4^+$ $NK1.1^+$ helper-T cells were generated upon vaccination with GPI anchored form of PA63

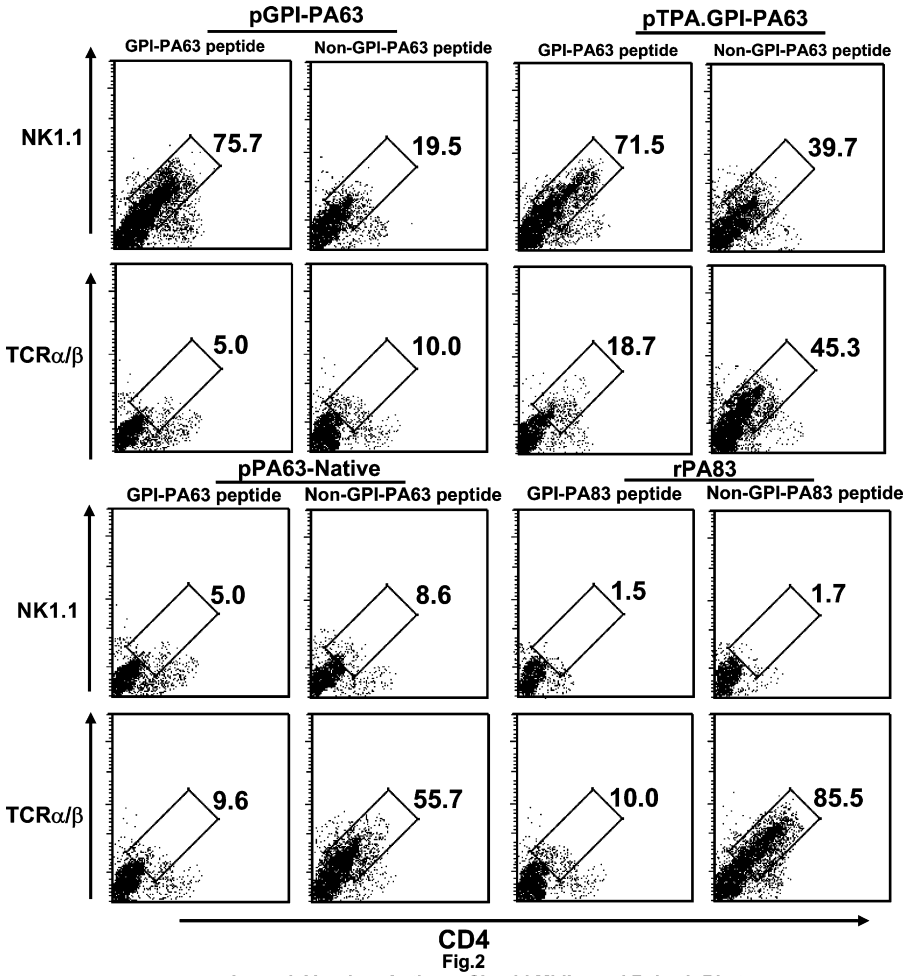
Flow cytometric analysis revealed that there was a marked increase in both the absolute and relative numbers of $CD4^+$ $NK1.1^+$ helper T cell (NKT) population after 5-day culturing in the presence of GPI-attached PA63 peptides (Fig. 2). The relative numbers of NKT cells after in vitro proliferation was astonishingly higher for the groups immunized with chimera pGPI-PA63 (75.7%) and pTPA.GPI-PA63 (71.5%), as compared to those immunized with pPA63-Native (5%) and rPA83 (1.5%). Importantly, this proliferation response also resulted in 18.7% $CD3^+CD4^+$ ($CD4^+\alpha/\beta$ TCR^+) cells from the pTPA.GPI-PA63 group which otherwise displayed a higher number of $CD3^+CD4^+$ (45.3%) when the splenocytes were cultured in the presence of non-GPI-PA63 peptides. The NKT cell population from this group also proliferated rapidly in the presence of non-GPI PA63 peptides but the relative number of NKT cells proliferating in response to GPI-attached PA63 peptides (71.5%) was quite higher as compared to those proliferating in response to non-GPI PA63 peptides (39.7%). The groups immunized with the chimera pPA63-Native and rPA83, on the other hand, solely gave rise to $CD4^+$ T cell population (55.7% and 85.5%, respectively) in the presence of non-GPI PA63 peptides only. Overall, chimera pTPA.GPI-PA63 resulted in proliferation of both $CD4^+$ T and $CD4^+$ NKT-cell population whereas pGPI-PA63 chimera resulted in clonal expansion of NKT-cell population in majority, in response to GPI-attached PA63 peptides. Thus, the NKT cell population from the group pTPA.GPI-PA63 responded well against both GPI-attached and free PA63 peptides whereas pGPI-PA63 responded only against the GPI-attached PA63 peptides.

3.4. Combined CD1d- and MHC II-restricted IL-4 responses were generated by chimera pTPA.GPI-PA63

Fig. 3 shows IL-4 production by NKT cells isolated from immunized mice when co-cultured with splenocyte APCs in the presence of GPI-attached and free PA63 peptides. NKT cell-mediated IL-4 production in the presence of GPI-attached PA63 peptides was completely abrogated by anti-CD1 mAb in both the groups that received pTPA.GPI-PA63 and pGPI-PA63 chimeras (Panels A and E). These responses on the other hand were completely unaffected by anti-MHC II mAb pointing that GPI-anchored PA63 was efficiently processed and presented in context with CD1 molecule to NKT cells in these groups. The group immunized with pPA63-Native did not show any NKT cell mediated IL-4 production in the presence of GPI-attached or non-GPI PA63 peptides (Panels I and K). On the contrary, NKT cells isolated from the group pTPA.GPI-PA63 displayed NKT cell mediated IL-4 production in the presence of non-GPI PA63 peptides (Panel F). Interestingly, these responses were totally abrogated by anti-MHC II mAb but were completely unaffected by anti-CD1 mAb. Also, evaluation of $CD4^+$ α/β TCR^+ T cell-mediated IL-4 responses indicated that the $CD4^+$ T cells isolated from the group pTPA.GPI-PA63 and pPA63-Native produced IL-4 only when they were presented with non-GPI PA63 peptides (Panels H and L). This lysis was completely abrogated by anti-MHC II mAb and was totally unaffected by anti-CD1 mAb indicating that the antigen was presented in context with MHC-II molecules. On the other hand IL-4 production by $CD4^+$ helper T cells isolated from the group pGPI-PA63 was insignificant in the presence of both the forms of PA63 peptides (Panels C and D). Furthermore, the IL-4 secretion pattern of the group that received rPA83 formulated with alhydroxy gel was found to be similar to that obtained for the pPA63-Native group and the responses were not significantly different. Overall, the results provided evidence that NKT cells from the group pTPA.GPI-PA63 mediated CD1d- and MHC-II restricted IL-4 responses against GPI- as well as non-GPI forms of PA63, respectively. $CD4^+$ T cells generated IL-4 responses exclusively against the non-GPI form of PA63. Also, MHC I-restricted responses had no role to play.

3.5. CD1d- and MHC II-restricted help to B cells aided IgG formation

We co-cultured autologous B lymphocytes with NKT cells in the presence of GPI-attached PA63 peptides to assess NKT cell aided IgG formation (Fig. 4). As expected, NKT cells isolated from the group pTPA.GPI-PA63 and pGPI-PA63 extended efficient help to B lymphocytes for IgG formation (Panels A and E, Fig. 3). This response was also dependent upon CD1d-restriction of the GPI-attached PA63 peptides as high SFCs (Spot Forming Cells) were obtained only in the absence of anti-CD1d antibody. However, such a response was slightly higher for the group pGPI-PA63 which developed SFU ≥600 as compared to the group pTPA.GPI-PA63 which also displayed a high SFU of ≈600 (Panels A and E). Importantly, the group pTPA.GPI-PA63 also displayed MHC II-restricted NKT cell mediated IgG responses against non-GPI PA63 peptides (Panel F) as opposed to only CD1d-restricted responses seen for the group pGPI-PA63 (Panels A and B). In addition to this, $CD4^+$ T cell-mediated B cell helper responses to non-GPI PA63 peptides were also evaluated. These results were in corroboration with those obtained for IL-4 production and also detailed the generation of MHC II-restricted IgG responses to non-GPI PA63 peptides, by the groups pTPA.GPI-PA63, pPA63-Native and rPA83 (Panels H, L and P; respectively).



. 2. $CD4^+$ $NK1.1^+$ helper-T cells were generated upon vaccination with GPI anchored form of PA63. Mice were immunized i.m. with different DNA vaccine constructs. At day mice were euthanized to take out the spleens and spleen cells were cultured in the presence of pooled peptides [either GPI-attached PA peptides or non-GPI PA peptides μg/ml of each peptide)] with IL-2 (10 U/ml) for a period of 4 days. Thereafter, $NK1.1^+TCR\text{-}\alpha/\beta^+$ cells and $CD4^+$ T cells were analyzed by flow cytometry.

. Correlation between TNA/anti-PA titers and protection of ce against Letx toxin challenge

Immunized mice were challenged with a LeTx mixture (50 μg and 22 μg LF, ≈4–5 LD_{50}) in a total volume of 100 μl via tail vein ection at different time intervals (week 12, 14, 16, 18 and 20) post-munization. Toxin challenge results as obtained are summarized Table 2. Control mice (vector immunized) died after receiving an thrax LeTx injection with a MTD (Mean Time to Death) around ± 0.2 days. All the mice immunized with pTPA.GPI-PA63 resisted xin challenge and elicited a survival percentage of 100% at all the ne intervals post-immunization (Table 2). The average TNA and ti-PA titers for this group corresponded to $\geq 10^3$ and $\geq 10^5$, respec-ely. On the other hand, the group pGPI-PA63 showed a 100% rvival till week 16 with average TNA titers for protection ranging tween 880 and 940. TNA titers below this range (680–700) con-rred protection to 75% of the mice and increased MTD to 8.9–9.5 ys. Results also depicted that both anti-PA and TNA titers lev-d off at titers about $\geq 10^5$ and $\geq 10^3$ respectively, for the group PA.GPI-PA63; and ≥80,000 and 700, respectively, for the group PI-PA63 post-week 14. For the group pPA63-Native, TNA titers nging from 180 to 250 conferred protection to 43–56% of the ce with an average MTD ranging from 3.3 to 5.9 days. On the her hand, TNA and anti-PA titers of ≈700 and ≈70,000 provided otection to 78% of the animals immunized with rPA83, with an erage MTD of 9.0–9.5 days.

Statistical differences between the delays in time-to-death as measured by log rank statistic for the groups immunized with GPI-bearing chimeras compared to the vector control group showed that the differences were quite significant ($p < 0.01$) and correlated well with survival ($r = 0.85$). Analysis of post-challenge TNA titers showed that survival at week 14 and 16 correlated well with each other ($r = 0.89$). The results therefore, suggested that mice immunized with DNA vaccine chimeras bearing GPI-anchor sequence developed significant TNA titers which protected the mice against anthrax lethal toxin challenge. Importantly, DNA immunization alone with the chimera pTPA.GPI-PA63 provided 100% protection to animals till week 20 post-priming.

4. Discussion

Although the currently licensed anthrax vaccine generates potential anti-anthrax immunity, it suffers from drawbacks like occasional reactogenicity, an extended dosage schedule, and frequent administration of boosters. Clearly, there is reason to explore alternatives. Therefore, based on the hypothesis that GPI-anchored form of PA will improve DNA vaccination against anthrax, we constructed a DNA chimera encoding the protease-cleaved fragment of protective antigen (PA63) attached to a C-terminal mammalian PLAP (Placental Alkaline Phosphatase) GPI anchor sequence. Along with that we constructed another chimera bearing PA63 gene with an N-terminal TPA leader along with a C-terminal PLAP-GPI anchor.

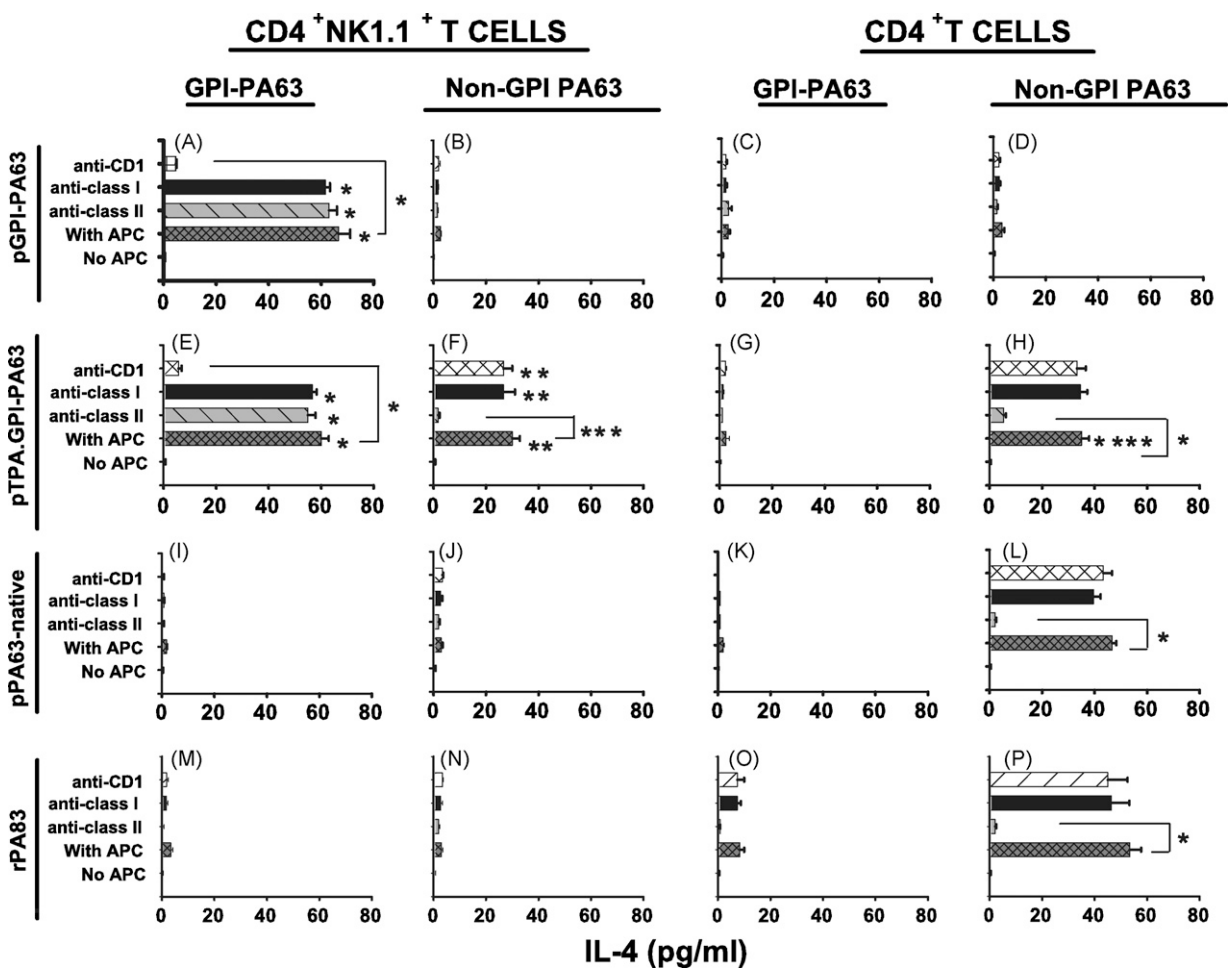


Fig. 3. Combined CD1d- and MHC II-restricted IL-4 responses were generated pTPA.GPI-PA63 chimera. Mice were immunized i.m. with different DNA vaccine constructs. At day 70 mice were euthanized to take out the spleens and spleen cells were used for the isolation of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ T cells by cell sorting using FACS Vantage^{TM} instrument. NKT cells and $CD4^+$ helper T cells were cultured in the presence 10 μg/ml GPI-PA63 and non-GPI-PA63 peptides to determine the cytokine secretion in vitro. Cell-free supernatants were collected 24 h. Each bar represents the concentration of IL-4 secreted by NKT cells/$CD4^+$ T cells from 9 mice in a group tested in three different experiments and error bars represent the ±SEM. (The error bars represent 95% confidence intervals calculated from the group wise mean concentration. *, **, ***, and ****, represent significant difference of the response relative to pPA63-Native immunization defined as $p < 0.0001$, $p < 0.001$, $p < 0.01$and $p < 0.05$, respectively).

In vitro expression of the chimeras in the J774A.1 cells was evaluated to test whether the gene for PA63 and the signals were being properly recognized in the mammalian cells. Subcellular fractionation and Western blotting indicated that the PA63 protein along with the GPI and TPA signal was being properly recognized by the mammalian cells. The chimeras, pGPI-PA63 and pTPA.GPI-PA63, expressed membrane-anchored form of PA63. In addition, the chimera pTPA.GPI-PA63 also mediated the secretion of PA63 in the cell culture supernatants.

Earlier studies have underscored the importance of anti-PA antibodies in anti-spore activity and suggested their role in impeding the early stages of infection with *B. anthracis* spores [40]. Also, passive transfer of anti-PA antibodies has been shown to provide protection against anthrax infection in guinea pigs [41]. Therefore, to test the ability of GPI anchored PA63-expressing chimeras to generate protective humoral responses in vivo, we injected mice with the DNA chimeras and determined the anti-PA reactivity in the serum of the immunized animals. Evaluation of IgM titers post-inoculation suggested successful priming. Remarkable serum IgG antibody titers were observed after the second booster for both the groups immunized with the constructs encoding GPI-anchored form of PA. These responses were approximately two to three times higher than those generated by pPA63-Native chimera indicating that GPI-anchored forms evoked better PA-specific humoral responses than the non-GPI anchored form of PA. Importantly, electron microscopic studies have shown that spore-associated proteins can be recognized by anti-PA antibodies, and PA-immune serum from several species enhanced the phagocytosis of spores of the virulent Ames and Sterne strains by murine peritoneal macrophages [40,42,44]. In addition, it has been shown that IgG antibody displays highest affinity towards PA [43]. Therefore, development of high endpoint titers of anti-PA IgG antibody upon DNA vaccination gains high merit from anti-spore activity point of view.

TNA titers were also analyzed and were again found to be higher for the groups that received chimeras encoding GPI-anchored form of PA. TNA titers after attaining a peak at week 14, first declined and then plateaued. This decline in neutralization titer paralleled the declining survival percentage supporting the notion that antibodies involved in neutralization are the ones that afford protection against LeTx challenge. This was also shown by the increased delay in death in partially protected mice as compared to the control mice.

Statistical differences between the delays in time-to-death as measured by log rank statistic for the groups immunized with GPI-bearing chimeras compared to the vector control group showed that the differences were quite significant ($p < 0.01$) and correlated well with survival ($r = 0.85$). Further, survival at week 14 and 16 correlated well with the TNA titers developed at these two time points ($r = 0.89$). Anti-PA titers of $\geq 10^5$ and TNA titers $\geq 10^3$ mediated resis-

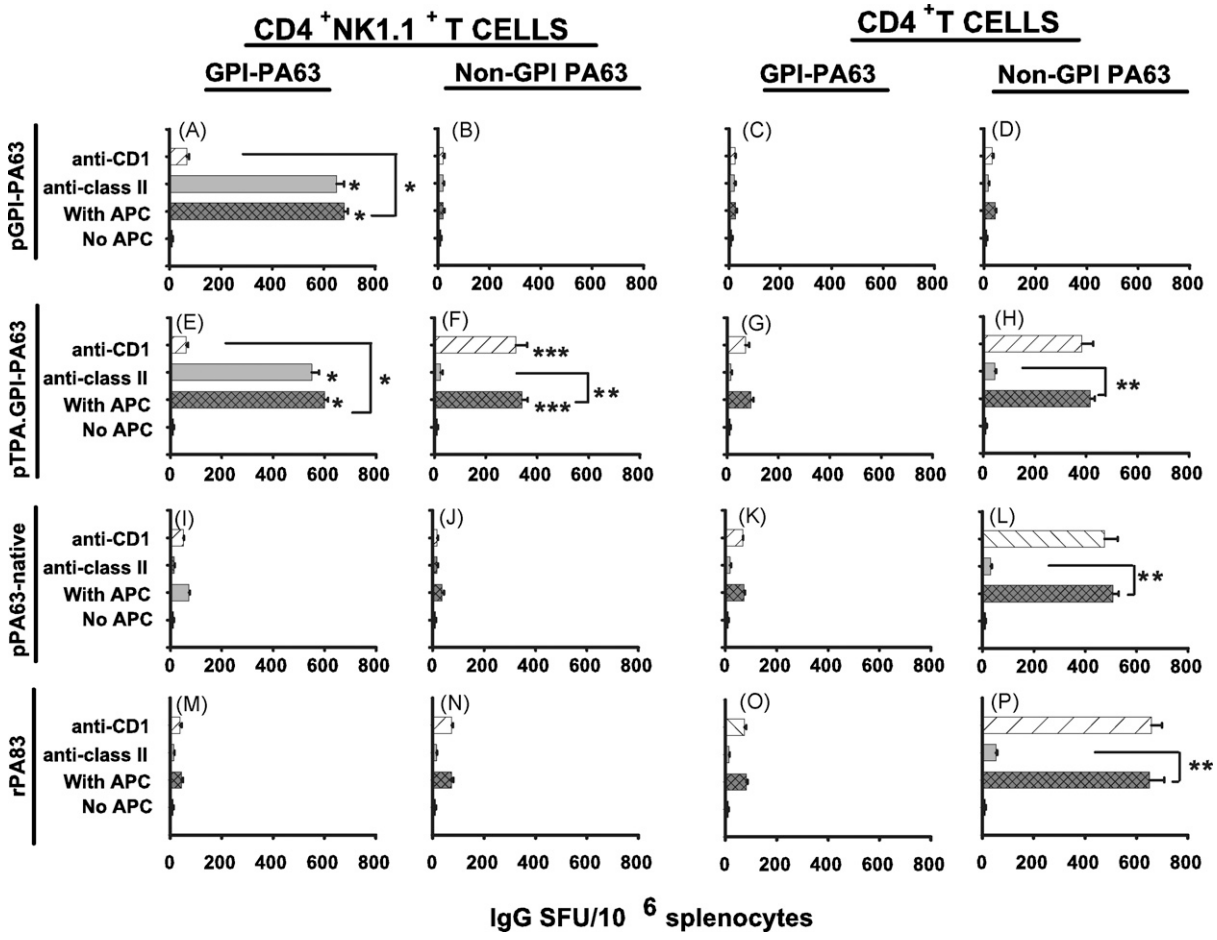


Fig. 4. CD1d- and MHC II-restricted help to B cells aided IgG formation. Mice were immunized i.m. with different DNA vaccine constructs. At day 70 mice were euthanized take out the spleens and spleen cells were used for the isolation of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ T cells by cell sorting using FACS Vantage^{TM} instrument. NKT cells d $CD4^+$ helper T cells were cultured in the presence 10 μg/ml GPI-PA63 and non-GPI-PA63 peptides to determine IgG formation in vitro as determined by ELISPOT assay. SPOT data is expressed as mean Spot Forming Cells (SFC's) ± SE of the Ag-stimulated cells minus un-stimulated cells. Each bar represents number of SFC's obtained for 9 ce in a group tested in three different experiments. (The error bars represent 95% confidence intervals calculated from the group wise mean SFC. *, **, ***, and ****, represent nificant difference of the response relative to pPA63-Native immunization defined as $p < 0.0001$, $p < 0.001$, $p < 0.01$and $p < 0.05$, respectively.).

nce against toxin challenge and conferred 100% protection to the imals in the group pTPA.GPI-PA63. Recombinant PA83 immu-zation, on the other hand, conferred only 78% protection response th average TNA and anti-PA titers of ≈700 and ≈70,000. Such a rrelation between protection and neutralization titers has also en observed in other animal models like guinea pigs [45,46] d rabbits [47] suggesting that this phenomenon is not species-ecific. Additionally, evaluation of antibody avidities depicted a ogressive increase in the avidity of the antibodies on subse-ent immunizations. Further investigation of memory responses terms of affinity maturation of antibodies (antibody avidity) on challenge indicated that a challenge engendered anamnes-immune response was mounted. Taken together, these TNA and ti-PA antibodies generated upon immunization with the GPI-chored forms of PA can efficiently confer protection against both xemia and bacteremia, thus, providing armour against infection.

To determine the fine specificity of the T cell subsets generated on DNA vaccination we examined the in vitro proliferation of e splenocytes in the presence of GPI-attached- as well as non-I-PA63 peptides. Our results revealed that NKT cells were the in target for activation by GPI-anchored PA63 peptides. These sults were consistent with the seminal study which illustrated at cellular GPI and glycolipids are natural ligands of mouse CD1d, member of CD1 family of evolutionarily conserved MHC-like olecules [48]. These CD1d molecules in turn have been shown to directly control the function of $NK1^+$ natural killer T (NKT) cells, a heterogenous subset of T cells displaying a $CD4^+$ or $CD4^-CD8^-$ double negative phenotype, and co-expressing the natural killer cell receptor NK1.1/NKR P1A and a semi-invariant TCR encoded in mice [49]. Interestingly, an appreciable number of NKT cells also proliferated in the presence of non-GPI PA63 peptides, from the group pTPA.GPI-PA63 indicating that NKT cells isolated from this group responded well against both GPI as well non-GPI attached forms of PA63. However, NKT cells clonally expanded to relatively higher numbers in the presence of GPI-attached PA peptides as compared to non-GPI-associated PA peptides. Results therefore, point that somehow the post-translational modifications of the expressed antigen in the mammalian systems in vivo affected their ability to activate innate immunity.

Generation of Ig responses against an antigen is as a result of classical MHC-II-dependent "cognate-help" which requires BcR-mediated antigen internalization and presentation of specific epitopes to antigen-specific T cells [50]. Studies however, suggest a direct role for invariant $CD4^+$ NKT cells in regulating CD1d-restricted B cell help which regulates B lymphocyte proliferation and effector functions [51]. Therefore, to establish the restriction requirements of this NKT cell lineage, we evaluated the IL-4 responses and NKT cell mediated IgG formation. We show here that the recognition of GPI-anchored PA63 by the NKT cells (from groups pGPI-PA63 and pTPA.GPI-PA63) is MHC-independent and

CD1-restricted. However, analysis of similar responses by $CD4^+$ helper T cells isolated from the groups, pTPA.GPI-PA63, pPA63-Native and rPA83 indicated that these responses were on account of MHC II-mediated restriction of non-GPI-attached PA63. Interestingly, NKT cells from the group pTPA.GPI-PA63 displayed MHC II-restriction and CD1-independence when presented with the non-GPI PA63 peptides.

Why the groups pTPA.GPI-PA63 and pGPI-PA63 mainly displayed CD1d restricted responses: was the next question we addressed. There are reports that have shown that GPI-anchored proteins occur as microdomains in cell membrane in living cells [52]. In this manner the alkyl acyl chain of the GPI-anchored proteins can make contacts with the CD1 pocket on the cell surface on account of the unique capacity of CD1 molecules to bind and exchange lipids also on the cell surface [53]. Therefore, the generation of predominantly CD1d-restricted NKT cell responses by the group pGPI-PA63 and pTPA.GPI-PA63 could probably be as a result of the cell surface recognition of membrane-anchored PA63 by CD1d molecules. However, the NKT cells and $CD4^+$ helper T cells isolated from the group pTPA.GPI-PA63 also displayed Ig responses to non-GPI anchored PA63 that were MHC-II restricted and CD1-independent which suggested that the antigen expressed by this chimera was being channeled to MHC II pathway as well. This could possibly be as a result of deacylation of the expressed GPI-anchored antigen either in ER or upon secretion by endogenous host phospholipases.

TPA is a protein that is expressed by vascular smooth muscle and binds to a specific cellular receptor p63, occurs in fibroblasts as an intracellular protein associated with the ER [28,29]. The TPA leader peptide possibly mediated targeting of the expressed antigen directly to the ER. As matter of fact, TPA leader peptide has also been shown to mediate the secretion of the proteins attached to them [54,55]. This was also seen in our case following in vitro transfection and immunoblot analysis (Fig. 1). This secreted (possibly deacylated) protein was taken up as an extracellular protein by endocytosis and channeled to MHC II pathway as indicated by abrogation of effector responses generated by NKT cells and $CD4^+$T helper T cells upon addition anti-MHC II mAb (Figs. 3 and 4). Overall, there was successful antigen trafficking to both pathways of antigen presentation that is CD1 as well as MHC II in response to GPI-anchored and non-GPI anchored forms of PA, respectively.

To our knowledge there is no published work demonstrating the development of NKT cell mediated helper responses generated by combined CD1d- and MHC II-restriction of the antigen delivered via i.m DNA vaccination. These NKT cells were CD1d reactive and upon antigen recognition responded *en masse*, leading in turn to a variety of effects on the immune system. Generation of such rapid responses in vivo by NKT cells arising as a result of DNA vaccination can contribute to the development of intermediate immunity between pattern recognition and adaptive immune system [31]. Also, NKT cells can restrict B cells in vivo resulting in antibodies against the GPI-anchored antigen that would be captured, internalized and presented in the CD1d context [51].

Importantly, both mouse and human CD1d molecules are widely broadly distributed on most cells of hematopoietic origin, with the highest levels observed on leukocytes such as dendritic cells, B cells or monocytes that will assure constant immune surveillance. Additional help provided by the classical MHC II restricted NKT- and $CD4^+$ T-helper cell responses further resulted in the augmentation of useful responses. Overall, we successfully illustrate here the generation of efficient humoral responses against anthrax protective antigen that generated protective immunity against anthrax lethal toxin challenge. Further work can gain a complete understanding regarding the immune pathways involved which can ultimately lead to the design and development of better human vaccines against anthrax to counter emergency.

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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.vaccine.2009.01.042.

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