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(54) Title: TH1-POLARIZING ADJUVANTS FOR ENHANCING IMMUNOGENICITY OF HIV ANTIGENS

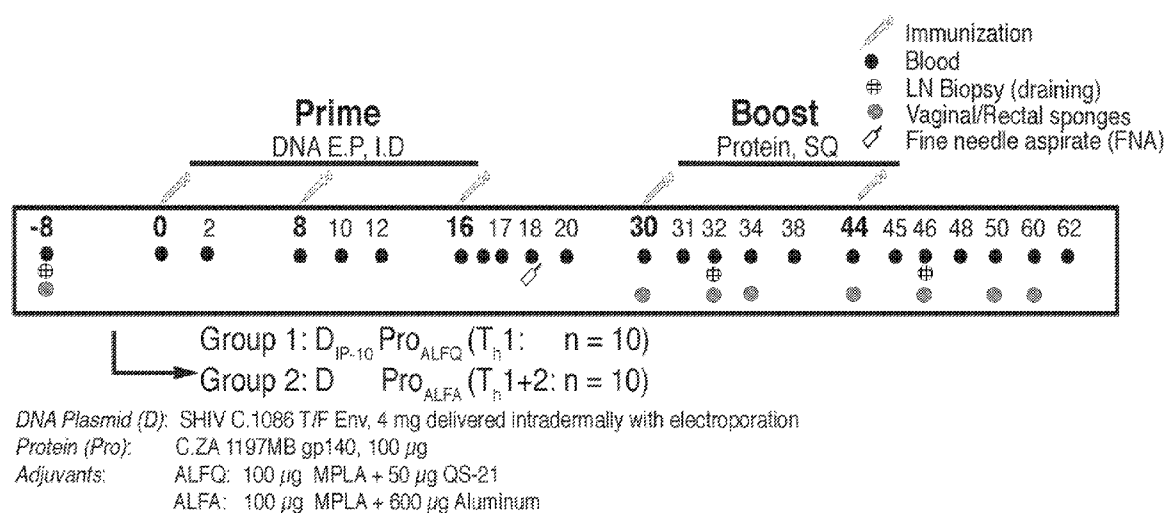


FIG. 1D

(57) Abstract: The present disclosure provides methods and compositions for enhancing an immune response in a subject undergoing HIV vaccination. In particular, the present disclosure provides adjuvants and methods for increased Th1 polarization of T cells in a subject in order to enhance the immune reaction against an HIV antigen.

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TH1-POLARIZING ADJUVANTS FOR ENHANCING IMMUNOGENICITY OF HIV ANTIGENS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Application No. 62/853,543, filed May 28, 2019, and U.S. Provisional Application No. 62/927,607, filed October 29, 2019, the disclosures of which are hereby incorporated by reference in their entirety for all purposes.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] This invention was made with Government support under Grant No. OD023034, awarded by the National Institutes of Health (NIH). The Government has certain rights in the invention.

BACKGROUND

[0003] Antiretroviral therapy (ART) has a remarkable impact on the morbidity and mortality associated with HIV but significant barriers hamper its global implementation, limiting its full potential as a preventative tool. Moreover, the intersection of aging-related conditions and the consequences of long-term ART is predicted to adversely impact the healthcare system as well as the HIV patient. These challenges underscore the need to pursue strategies that will provide both sustainable HIV prevention and a functional HIV cure.

[0004] The results of the RV144 trial demonstrated that vaccination may prevent HIV transmission in humans, and longevity of anti-Env antibodies may be the key to this protection. Efforts to improve upon the prime-boost vaccine regimen used in RV144 have indicated that booster immunizations increase serum anti-Env antibody titers, but only transiently. Poor antibody durability hampers efforts to develop an effective HIV-1 vaccine.

[0005] CD4 T follicular helper cells (Tfh) are a specialized subset of CD4 T cells that migrate to germinal centers (GC) within secondary lymphoid organs and provide growth and

differentiation signals to GC B cells within a few days of immunization. GCs are populated by antigen-activated, rapidly proliferating B cell clones, which rely on cytokines and co-stimulatory signals from Tfh cells to undergo immunoglobulin affinity maturation, class-switch recombination, and differentiation to memory B cells and plasma cells. The maturation of GC B cells to plasma cells and the resulting long-lived humoral immunity hinges on effective Tfh help. Tfh cells are heterogeneous and, depending on inflammatory signals during T cell priming, differentiate into Th1, Th2, Th17-type Tfh cells. The polarization of a Tfh cell influences cytokine profile and co-stimulatory molecule expression.

[0006] There is a critical need to identify strategies that will augment vaccine-mediated humoral immunity for a successful HIV vaccine. The present disclosure satisfies this need and provides other advantages as well.

BRIEF SUMMARY

[0007] In one aspect of the present disclosure, a method is provided of enhancing the anti-Env antibody response during HIV vaccination in a subject, the method comprising (i) administering a first Th1-polarizing adjuvant to the subject together with a DNA vaccine comprising a polynucleotide encoding a first HIV Env polypeptide, wherein the first adjuvant comprises a polynucleotide encoding interferon-induced protein (IP)-10; and (ii) administering a second Th1-polarizing adjuvant to the subject together with a booster comprising a second HIV Env polypeptide, wherein the second adjuvant comprises QS-21.

[0008] In some embodiments of the method, the second HIV Env polypeptide is a gp140 polypeptide. In some embodiments, the DNA vaccine further comprises one or more additional polynucleotides encoding one or more additional HIV polypeptides selected from the group consisting of Gag, protease, reverse transcriptase, Tat, Rev, and combinations thereof. In some embodiments, the first adjuvant and the DNA vaccine are administered transdermally with electroporation. In some embodiments, the HIV Env polypeptide, HIV gp140 polypeptide, and/or one or more additional HIV polypeptides are from HIV-1. In some embodiments, the HIV-1 is of clade C origin. In some embodiments, the booster further comprises a lipid and/or liposomal adjuvant. In some embodiments, the liposomal adjuvant comprises Army Liposome Formulation (ALF) liposomes. In some embodiments, the lipid adjuvant comprises monophosphoryl lipid A (MPLA). In some embodiments, the first Th1-polarizing adjuvant and DNA vaccine are administered to the subject 1, 2 or 3 times prior to

the administration of the booster. In some embodiments, the first Th1-polarizing adjuvant and DNA vaccine are administered to the subject at 0, 8, and 16 weeks. In some embodiments, the booster is administered to the subject 1 or 2 times.

[0009] In some embodiments of the method, peripheral and germinal center (GC) Tfh cells isolated from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster display higher proportions of anti-Env Th1 cells than do peripheral and germinal center (GC) Tfh cells taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant. In some embodiments, blood taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster shows higher anti-gp140 extrafollicular and/or plasma cell-derived titers than does blood from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant. In some embodiments, serum IgG antibodies taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster show a broader cross-clade anti-Env response and/or increased specificity for gp120 V1V2 loops than do serum IgG antibodies taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant. In some embodiments, serum IgG antibodies taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster display elevated anti-Env titers that persist longer than do serum IgG antibodies taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

[0010] In some embodiments of the method, serum IgG antibodies taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster show higher avidity against gp140 than do serum IgG antibodies taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant. In some embodiments, serum taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster have greater neutralization activity against HIV-1 than does serum taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant. In some embodiments, the neutralization of HIV-1 is assessed using the TZM-bl assay. In some embodiments, serum taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster have greater antibody-dependent cellular toxicity (ADCC) and/or antibody-dependent phagocytosis (ADP) activity

against HIV-infected cells than does serum taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant. In some embodiments, the HIV-infected cells are infected with HIV-1. In some embodiments, the HIV-1 is of clade C origin. In some embodiments, IgG and/or IgA antibodies isolated from the rectal and/or vaginal mucosa of the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster show higher anti-gp140 titers than do IgG and/or IgA antibodies isolated from the rectal and/or vaginal mucosa of a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

[0011] In another aspect, the present disclosure provides a pharmaceutical composition for vaccinating a subject against HIV, the composition comprising a DNA vaccine comprising a polynucleotide encoding an HIV Env polypeptide, a polynucleotide encoding interferon-induced protein (IP)-10, and a pharmaceutically acceptable carrier. In some embodiments of the composition, the DNA vaccine further comprises one or more additional polynucleotides encoding one or more additional HIV polypeptides selected from the group consisting of Gag, protease, reverse transcriptase, Tat, Rev, and combinations thereof. In some embodiments, the HIV Env polypeptide and/or one or more additional HIV polypeptides are from HIV-1. In some embodiments, the HIV-1 is of clade C origin. In some embodiments, the polynucleotide encoding the HIV Env polypeptide and the polynucleotide encoding IP-10 are present within a single DNA vector. In some embodiments, the composition is formulated for transdermal delivery with electroporation.

[0012] In another aspect, the present disclosure provides a pharmaceutical composition comprising an HIV Env polypeptide, QS-21, and a pharmaceutically acceptable carrier. In some embodiments, the HIV Env polypeptide is a gp140 polypeptide. In some embodiments, the composition further comprises a lipid and/or liposomal adjuvant. In some embodiments, the liposomal adjuvant comprises Army Liposome Formulation (ALF) liposomes. In some embodiments, the lipid adjuvant comprises monophosphoryl lipid A (MPLA). In some embodiments, the HIV Env polypeptide is from HIV-1. In some embodiments, the HIV-1 is of clade C origin.

[0013] Other objects, features, and advantages of the present invention will be apparent to one of skill in the art from the following detailed description and figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] FIGS. 1A-1D. Immunization schedule for subtype C HIV-1 Envelope DNA prime and protein boost vaccine regimen. **FIG. 1A:** Flow cytometric plots illustrate expression of HIV Env, SIV Gag, and IP-10 by 293-T cells transfected with DNA and DNAIP-10 plasmids. Grey overlay shows expression in non-transfected cells. **FIG. 1B:** Bar graph shows surface expression of HIV Env based on detection with a panel of monoclonal antibodies as indicated. **FIG. 1C:** IP-10 titers in supernatants of transfected 293T cells show accumulation of IP-10 following transfection with DNAIP-10. **FIG. 1D** shows immunization schedule and sampling. Two groups of 10 rhesus macaques each were immunized three times with DNA followed by two immunizations with protein. DNA was delivered intradermally and three seconds later electrical pulses were delivered around the injection site using the ICHOR TriGrid Array. Group 1 animals (n=10) received DNA plasmid expressing IP-10 and an ALFQ-adjuvanted C.gp140 boost (DIP-10ProALFQ). Group 2 (n=10) animals were immunized with DNA and boosted with ALFA-adjuvanted C.gp140 protein (DProALFA).

[0015] FIGS. 2A-2G. DIP-10ProteinALFQ vaccine induces robust anti-Env antibody titers with cross-clade breadth. **FIG. 2A:** Antibody kinetics against C. 1086 Env gp140 in serum at weeks 0, 2, and 8 following each protein boost assessed by binding antibody multiplex assay (BAMA); right panel shows scatter plot values for each animal at weeks 0, 2, and 8 post 2nd protein boost. **FIG. 2B:** Kinetics of C. 1086 specific anti-Env titers after 2nd protein boost measured by ELISA; right panel shows titers for each individual animal. BAMA assay was used to measure responses against CH505 (**FIG. 2C**), Con C (**FIG. 2D**), Con S (**FIG. 2E**), and gp70 V1 V2 (**FIG. 2F**). **FIG. 2G** shows fold change in antibody titers at indicated time points after 2nd protein boost relative to the first. Animals receiving the DProALFA vaccine are represented by blue circles and animals receiving the DIP-10ProALFQ vaccine by red circles. Kinetic data show geometric means. Vertical dotted lines show immunization time points. In dot plots, geometric means are indicated as horizontal lines. Statistical significance across vaccine regimens was tested using unpaired, two-tailed Mann-Whitney U test; *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001.

[0016] FIGS. 3A-3M. DIP-10 ProteinALFQ vaccine elicits high avidity anti-Env antibody with ADCC and ADP activities. **FIG. 3A:** Surface Plasmon Resonance (SPR) was used to determine the avidity index (AI) in serum at 2 weeks after final DNA and each protein boost using C. 1086 gp140 protein immobilized onto sensor chips. **FIG. 3B** shows SPR-based AI

values in the two vaccine regimens over time. **FIG. 3C** shows significantly higher avidity in DIP-10 ProteinALFQ. at 2 weeks post 2nd protein boost. AI measured against C.1086 gp140 using 2 M sodium thiocyanate (**FIG. 3D**) and 0.1 M sodium citrate (**FIG. 3E**) at week 8 post 2nd protein. AI against Con C (**FIG. 3F**) and Con S (**FIG. 3G**) measured using 0.1 M sodium citrate at week 8 post 2nd protein boost. **FIG. 3H**: Serum-neutralizing antibody responses were assessed against tier 1A (MW965.26) pseudovirus and the 50% infective dose (ID₅₀) was determined. **FIG. 3I** shows ADCC activity against SHIV CH505 infected target cells. **FIG. 3J**: Antibody-dependent phagocytosis using gp120-coated beads was measured using sera from week 8 post 2nd protein boost at serum dilutions ranging from 1:100 to 1:2500. **FIG. 3K** shows individual ADP scores at 1:500 serum dilution. **FIG. 3L**: C.1086 gp120 subclass analysis was performed on week 8 post 2nd protein boost to measure IgG1, IgG2, IgG3, and IgG4. **FIG. 3M** shows IgG1/IgG4 ratio across vaccine groups at week 8 post 2nd protein boost. Statistical significance across vaccine regimens was tested using unpaired, two-tailed Mann-Whitney U test and within group differences over time were tested using Wilcoxon matched-pairs signed rank test; *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001.

[0017] FIGS. 4A-4E. DNAIP-10 ProteinALFQ vaccine elicits robust anti-Env antibody in vaginal and rectal mucosal secretions. Vaginal and colorectal secretions were evaluated for C.1086 concentrations. **FIGS. 4A-4B** show kinetics of C.1086 IgA responses in vaginal and rectal secretions. **FIGS. 4C-4D** show IgA and IgG titers in rectal secretions. **FIG. 4E** shows gp140 IgA kinetics in sera. Horizontal broken lines represent the assay limit of detection. Kinetic data show geometric means. Vertical dotted lines show immunization time points. In dot plots, geometric means are indicated as horizontal lines. Statistical significance was tested using unpaired, two-tailed Mann-Whitney U test; *p < 0.05, **p < 0.01, *** p < 0.001, **** p < 0.0001.

[0018] FIGS. 5A-5D. DIP-10 ProteinALFQ vaccine induces Env-specific Tfh cells in peripheral blood. **FIG. 5A**: Flow cytometric gating of CXCR5⁺ cells in PBMCs to identify OX40⁺ CD25⁺ activated CD4 T subsets after stimulation with C.1086 protein and Con C peptide pools. **FIG. 5B** shows frequency of Env-specific CD4 T cells at week 1 post 1st protein boost which strongly correlate with responses against the C.ZA.1197MB boosting immunogen (**FIG. 5C**). **FIG. 5D** shows robust induction of IP-10 in sera after 1st protein immunization. Statistical significance was tested using unpaired, two-tailed Mann-Whitney U

test. Spearman coefficient of correlation values were computed to determine associations; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

[0019] FIGS. 6A-6L. DIP-10 ProteinALFQ vaccine induces GC Tfh cells with distinctive Th1 signatures. **FIG. 6A:** Gating strategy to identify GC Tfh cells and GC B cells in lymph node at 2 weeks post 1st protein boost. Histograms show higher relative expression of Bcl-6 and ICOS in GC Tfh cells. **FIG. 6B:** *Ex vivo* co-culture experiments with sorted GC Tfh cells demonstrates B helper capacity. **FIG. 6C** shows kinetics of GC Tfh responses in lymph node at specified time points. **FIG. 6D:** Dot plot shows higher relative frequency of GC B cells in Th1 vaccine group, and correlation of GC B cells with GC Tfh cells. Frequencies of Env-specific CD4 T cells in lymph node correlate with GC Tfh cells. **FIG. 6E:** Histogram shows relative CXCR3 expression in GC Tfh cells and GC B cells and dot plot shows significantly higher CXCR3 expression on GC Tfh cells and GC B cells in Th1 vaccine regimen. **FIG. 6F:** Flow plot illustrates higher expression of CXCR3 on T-bet⁺ memory B cells. **FIG. 6G:** Antibody titers at week 18 post 2nd protein predicted by frequency GC Tfh cells and proportion of CXCR3-expressing GC Tfh cells at 2 weeks post 1st protein. Antibody avidity at week 8 post 2nd protein predicted by GC Tfh cells at 1st protein. **FIG. 6H** shows animals selected (triangles) for RNA-seq and Principal component analysis of RNA-Seq data (**FIG. 6I**); colors represent populations as indicated in **FIG. 6A**. **FIG. 6J:** Heatmap shows expression of genes differentially expressed in Tfh relative to naïve across four sorted CD4 subsets indicated in **FIG. 6I**. **FIG. 6K:** Volcano plot of differentially expressed transcripts (adjusted $p < 0.05$ in red) for Tfh cells versus naïve cells. **FIG. 6L:** Log fold change values of key Tfh and Th1 genes in Tfh (red) and memory Tfh (blue) cells in lymph node Th1 vaccinated animals. Statistical significance was tested using unpaired, two-tailed Mann-Whitney U test. Spearman coefficient of correlation values were computed to determine associations; * $p < 0.05$, **** $p < 0.0001$.

[0020] FIGS. 7A-7G. DNAIP-10 immunization induces systemic expansion of pro-inflammatory monocytes and enhances GC Tfh responses. **FIG. 7A** shows gating strategy to identify activated CXCR5⁺ cells in blood at day 0 and day 14 following DNA3 immunization. Kinetic data show transient accumulation of ICOS⁺ PD-1⁺ CXCR5⁺ cells in blood at day 14 following DNA3 when expressed as relative frequencies (left) and absolute counts (right). **FIG. 7B:** Flow cytometric gating to identify inflammatory CD14⁺CD16⁺ monocytes which increase at day 3 following DNA immunization (**FIG. 7C**). **FIG. 7D:** Higher relative increase in pro-inflammatory monocytes in DNA-IP-10 primed animals. **FIG.**

7E shows induction of IL-1b in sera 7 days following DNA immunization. **FIG. 7F**: Fine needle aspirates of draining lymph nodes show increased frequencies of GC Tfh cells in DNA-IP-10 primed animals at day 14 post DNA. **FIG. 7G** shows that the frequency of pro-inflammatory monocytes predicts C.1086C gp140 antibody titers at week 8 post 2nd protein boost. Statistical significance was tested using unpaired, two-tailed Mann-Whitney U test. Spearman coefficient of correlation values were computed to determine associations; * $p < 0.05$, ***, $p < 0.001$, **** $p < 0.0001$.

[0021] FIGS. 8A-8D. Th1 DNA prime enhances frequency of pro-inflammatory monocytes and GC Tfh cells. **FIG. 8A**: Increase in net frequency of pro-inflammatory monocytes in Th1 vaccine group. **FIG. 8B**: Increase in IP-10, IL-1 β , IL-6 and IL-21 after DNA prime. **FIG. 8C**: Increase in GC Tfh cells with Th1 vaccine. **FIG. 8D**: Frequency of pro-inflammatory monocytes after DNA prime predicts antibody response at memory.

[0022] FIGS. 9A-9D. Th1 DNA prime enhances frequency of Env-specific Tfh cells. **FIG. 9A** shows time points of blood collection for T cell kinetic analysis. **FIG. 9B**: Flow plot illustrates gating strategy to identify activated CD4 Tfh cells based on coexpression of ICOS and PD-1. Kinetics of relative frequencies and absolute counts of ICOS+PD-1+ Tfh cells. **FIG. 9C** shows gating strategy to identify Env-specific Tfh cells based on co-expression of OX40 and CD25. **FIG. 9D** shows higher Env-specific Tfh cells in Th1 regimen.

[0023] FIGS. 10A-10B. Schematic shows study design with three DNA primes (SHIV 1086C, 4 mg given I.D with E.P) followed by two protein boosts with Clade C gp140 Envelope, HIV-1 isolate C.ZA.1197MB, 100 μ g delivered subcutaneously (SQ). In the Th1 group, DNA was adjuvanted with IP-10 and protein with ALFQ and the Th1+2 group got DNA alone with ALFA-adjuvanted protein boost. **FIG. 10A**: Kinetics of antibody responses following 2nd protein boost. **FIG. 10B**: Scatter plot showing antibody titers in all animals within the experimental groups at weeks 0, 2, 8, and 18 following 2nd protein boost with antibody fold-change in Th1 relative to Th1+2 group.

[0024] FIGS. 11A-11D. After Th1 vaccine regimen, antibodies demonstrate increased breadth, avidity, and broader specificity. Antibody breadth (**FIG. 11A**), Avidity index (**FIG. 11B**), and specificity (**FIG. 11C**) and neutralization (**FIG. 11D**) titers at indicated time points after 2nd protein boost.

[0025] FIG. 12. Polyfunctional Th1/Th17 CD4 T cells predict durability of anti-Env titers in sera and mucosa.

[0026] FIG. 13. Experimental design of vaccine trial 1 (S1). All groups are immunized intradermally using a Bioject electroporation device with plasmid DNA-SHIV construct expressing 1086 Clade C Env, tat, and rev; and SIV239 Gag (3 mg/dose, week 0 and 4). Groups 2 and 3 receive a similar dose of IP-10 adjuvanted DNA-SHIV construct. The MVA-SHIV boost is intramuscular (10^8 PFU/dose, week 16,24). Animals are co-immunized with gp140 protein (Clade C Env, 100 μ g), in the thigh opposite to that of the MVA boost, formulated either in Alum (500 μ g, Groups 1, 2) or AS01B (50 μ g MPL+50 μ g Q21) Group 3). Animals in Groups 1 and 3 are compared for vaccine efficacy and all groups are compared for immunogenicity.

[0027] FIGS. 14A-14F. DNA/MVA vaccine induces robust Env antibody. Monkeys were immunized with DNA (wks 0, 8) and MVA (wks 16, 32). In **FIG. 14B**, some animals received gp140 (Pro) +Alum with 2nd MVA ;**, $p < 0.01$ using a two-tailed t test; # $p = 0.05$.

[0028] FIGS. 15A-15E show a transient accumulation of CXCR5+ Tfh cells expressing the cell-cycle marker Ki-67 at the peak CD4 effector response following the MVA boost (**FIGS. 15A-15B**, * $p < 0.05$). Analogous to functionality of Gag and Env-specific CD4 T cells co-expressing the Tfh cytokine interleukin (IL) 21 and the Th1 cytokine IFN γ (**FIG. 15C**); Ki-67+ pTFH expressed CXCR3, a marker of Th1 polarity (**FIG. 15D**). Notably, the proportion of CXCR3+Ki-67+ pTFH cells markedly increased following MVA immunization relative to baseline (**FIG. 15E**, *** $p < 0.001$).

[0029] FIGS. 16A-16F show aspects of the GC response following the 2nd MVA boost and how Pro+Alum modulated this response. GC Tfh were examined by high expression of programmed death (PD)1 and CXCR5, and Tfh cells based on the CXCR5+PD-1+ phenotype (**FIG. 16A**). M+Pro vaccine increased Th1-polarized Tfh cells (**FIG. 16B**) even in the presence of Alum. The proportion of CXCR3+ GC Tfh correlated with frequency of GC B cells (**FIG. 16C**), antibody avidity (**FIG. 16D**), durability (**FIG. 16E**), and neutralization (**FIG. 16F**).

[0030] FIGS. 17A-17C. IP-10 induces CXCR3+ Tfh cells. Flow plot (**FIG. 17A**) and histogram (**FIG. 17B**) show CXCR3 expression on Tfh cells at increasing IP-10 doses. **FIG. 17C:** Increase in CXCR3+ Tfh cells with IP-10.

[0031] FIGS. 18A-18C. Construction of IP-10-DNASHIV vaccine. **FIG. 18A:** The DNA vaccine includes IP-10 as a fusion to IRES or as a fusion to the 2A peptide downstream of Env. **FIG. 18B:** Characterization of CD40L-DNA vaccine. **FIG. 18C:** Significantly higher magnitude and proportion (% responders indicated in grey area) vaccine-elicited CD4s after 1st prime in CD40L-DNA (blue) relative to DNA (clear); ****, $p < 0.0001$.

[0032] FIGS. 19A-19C. Identifying vaccine-elicited Th1 and Th2 Tfh cells. The cell-cycle marker Ki-67 was utilized to interrogate CXCR5⁺ CD4 T cells at peak effector time points following each immunization for expression of CXCR3 and CCR4, as outlined in **FIG. 19A**. This is complemented by intracellular cytokine staining (ICS)-based assays designed to examine vaccine-specific responses (at peak and memory) after stimulation with relevant Gag and Env peptide pools. After stimulation, Ag-specific Tfh cells are identified based on cytokine positivity and CXCR5 expression, as shown in **FIGS. 19B-19C**.

[0033] FIG. 20. CD40L⁺ Tfh are Env specific. Vaccine-specific TFH cells are identified by translocation of CD40L after 5 hour stimulation with Env peptides in the presence of co-stimulatory molecules.

[0034] FIGS. 21A-21B. The frequency of Env-specific IFNG+IL-21⁺ cells at 1 week post 2nd MVA, and the frequency of CXCR3⁺ GC Tfh cells at 2 weeks post 2nd MVA, correlate with antibody durability measured at 20 weeks post 2nd MVA.

[0035] FIGS. 22A-22B. Assays to measure humoral immune responses. **FIG. 22A:** ELISPOT assay captures increase in gp140 ASCs with Pro+MVA relative to MVA alone. In **FIG. 22B**, ADCC increases following a 3rd protein boost, while no increase was observed in ADP.

[0036] FIGS. 23A-23B. CXCR3⁺ Tfh cells express CCR5. **FIG. 23A:** CXCR3⁺ Tfh cells comprise higher frequency of CCR5 cells. **FIG. 23B:** Higher levels of pro-viral DNA in X3⁺ Tfh cells in lymph node; ***, $p < 0.001$; * $p < 0.05$.

[0037] FIGS. 24A-24B. Th1 vaccine regimen induces robust and durable serum HIV anti-Env antibody titers. Schematic shows study design with 3 DNA primes (SHIV 1086C, 4 mg given I.D with E.P) followed by two protein boosts with Clade C gp140 Envelope, HIV-1 isolate C.ZA.1197MB, 100 µg delivered subcutaneously (SQ). In the Th1 group, DNA was adjuvanted with IP-10 and protein with ALFQ and the Th1+2 group got DNA alone with ALFA-adjuvanted protein boost (**FIG. 24A**). Kinetics of antibody responses following 2nd

protein boost (**FIG. 24B**). Scatter plot showing antibody titers in all animals within the experimental groups at week 0, 2, 8, and 18 following 2nd protein boost with antibody fold-change in Th1 relative to Th1+2 group.

[0038] FIGS. 25A-25D. After Th1 vaccine regimen antibodies demonstrate increased breadth, avidity, and broader specificity. Antibody breadth (**FIG. 25A**), Avidity index (**FIG. 25B**), and specificity (**FIG. 25C**) and neutralization (**FIG. 25D**) titers at indicated time points after 2nd protein boost.

[0039] FIGS. 26A-26B. Robust induction of anti-Env-specific IgA (**FIG. 26A**) and anti-Env-specific IgG (**FIG. 26B**) in vaginal and rectal mucosa with Th1 vaccine regimen. Data are post 2nd protein boost.

[0040] FIGS. 27A-27D. Th1 DNA prime induces higher magnitude Env-specific Tfh cells. **FIG. 27A** shows time points of blood collection for T cell kinetic analysis. **FIG. 27B:** Flow plot illustrates gating strategy to identify activated CD4 Tfh cells based on co-expression of ICOS and PD-1. Kinetics of relative frequencies and absolute counts of ICOS+PD-1+ Tfh cells. **FIG. 27C** shows gating strategy to identify Env-specific Tfh cells based on co-expression of OX40 and CD25. **FIG. 27D** shows higher Env-specific Tfh cells in Th1 regimen.

[0041] FIGS. 28A-28G. Induction of polyfunctional Th1/Th17 CD4 Tfh cells in Th1 vaccine regimen. **FIG. 28A** shows transient induction of pro-inflammatory Th1 cytokine IP-10, Th17 cytokine IL-17A and IL-6 and IL-21. **FIG. 28B** shows higher frequencies of Env-specific Tfh cells in PBMCs and **FIG. 28C** illustrates a robust GC response in Th1 vaccine regimen. **FIG. 28D** illustrates gating for GC Tfh cells with higher relative expression of Bcl-6, Th1 marker CXCR3 and Th17 marker CCR6. **FIG. 28E** shows higher CD40L expression if IFNG+IL-17+ cells. **FIG. 28F:** RNA-sequencing analysis of GC Tfh cells in Th1 vaccine treatment animals (n=3) reveals upregulation of Th1/ Th17 genes BATF, PRKCQ and MYD88. **FIG. 28G:** Phenotypic examination of GC subsets confirmed increase in proportion of CXCR3+CCR6+ GC Tfh cells in Th1 vaccinated group.

[0042] FIG. 29. Polyfunctional Th1/Th17 CD4 T cells predict durability of anti-Env titers in sera and mucosa. The frequency of polyfunctional Th1/Th17 GC TFH cells is directly predictive of binding titers as measured by ELISA and BAMA.

[0043] **FIGS. 30A-30D.** A single CAF01 adjuvanted protein immunization induces robust antibody responses. **FIG. 30A:** Structure of the cationic liposome CAF01. Single subcutaneous immunization with CAF01-adjuvanted Chlamydia antigen (CTH 522, 5 µg; **FIG. 30B**) or 4-Hydroxy-3-nitrophenylacetyl hapten conjugated to ovalbumin (NP-OVA, 5 µg; **FIG. 30C**) in mice shows robust induction of antibody responses at levels comparable or superior to that induced by the MF59 analog adjuvant, Addavax. **FIG. 30D:** Notably, the frequency of OVA-bound cells at the site of injection was significantly higher at 24- and 48-hours following immunization, indicating formation of antigen depot at the site of immunization with CAF01.

[0044] **FIG. 31.** Robust protein expression of Env and Gag in DNA 1086C plasmid co-expressing IP-10 and IL-6.

[0045] **FIGS. 32A-32D.** Th1 vaccine enhances frequency of pro-inflammatory monocytes and GC Tfh cells. **FIG. 32A:** Increase in net frequency of pro-inflammatory monocytes in Th1 vaccine group. **FIG. 32B:** Increase in IP-10, IL-1β, IL-6 and IL-21 after DNA prime. **FIG. 32C:** Increase in GC Tfh cells with Th1 vaccine. **FIG. 32D:** Frequency of pro-inflammatory monocytes after DNA prime predicts antibody response at memory.

[0046] **FIGS. 33A-33B.** Increased Env gp140 1086.C Antibody avidity (kd off-rate) in Th1 vaccine group. Data represent quadruplicate samples for each animal. **FIG. 33A** shows higher avidity after sequential immunization in both vaccine groups. **FIG. 33B** shows increased avidity in Th1-Tfh vaccine regimen.

[0047] **FIGS. 34A-34B.** Higher levels of Env IgG1 (**FIG. 34A**) and IgG3 (**FIG. 34B**) with Th1 vaccine regimen. Titers were measured at week 8 post 2nd protein.

DETAILED DESCRIPTION

1. Introduction

[0048] The present disclosure relates to methods and compositions for enhancing the immune response in subjects undergoing HIV vaccination. In particular, the present methods and compositions can be used in conjunction with prime-boost vaccine regimens, *e.g.*, using DNA vaccines against HIV, in order to enhance the Th1 polarization of Tfh cells in a subject. The present disclosure is based in part on the discovery that Th1 polarization in conjunction with a DNA prime substantially increases antigen-specific, *e.g.*, Env-specific, Tfh cells, and

that Th1 polarization in conjunction with a protein boost, *e.g.*, using gp140, results in greater production of the IgG1 subclass with enhanced longevity, breadth, avidity, ADCC, and ADP activities of the antigen-specific antibody. In particular, the present disclosure provides DNA constructs expressing both HIV (and/or SIV) genes and interferon protein 10 (IP-10), as well as compositions comprising an HIV protein boost, *e.g.*, gp140, with a Th1-polarizing adjuvant such as QS-21. The present methods and compositions are effective at inducing, *e.g.*, pro-inflammatory monocytes, Env-specific CD4 T follicular helper cells, and higher germinal center responses relative to DNA alone (*i.e.*, in the absence of Th1 polarization). In addition, the localized expression of IP-10 using the present constructs enhances immunogenicity by concerted effects on antigen-presenting cells and T cells, and limits the potential for non-specific, systemic effects, thereby reducing side effects.

2. Definitions

[0049] As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

[0050] The terms “a,” “an,” or “the” as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to “a cell” includes a plurality of such cells and reference to “the agent” includes reference to one or more agents known to those skilled in the art, and so forth.

[0051] The terms “about” and “approximately” as used herein shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Typically, exemplary degrees of error are within 20 percent (%), preferably within 10%, and more preferably within 5% of a given value or range of values. Any reference to “about X” specifically indicates at least the values X, 0.8X, 0.81X, 0.82X, 0.83X, 0.84X, 0.85X, 0.86X, 0.87X, 0.88X, 0.89X, 0.9X, 0.91X, 0.92X, 0.93X, 0.94X, 0.95X, 0.96X, 0.97X, 0.98X, 0.99X, 1.01X, 1.02X, 1.03X, 1.04X, 1.05X, 1.06X, 1.07X, 1.08X, 1.09X, 1.1X, 1.11X, 1.12X, 1.13X, 1.14X, 1.15X, 1.16X, 1.17X, 1.18X, 1.19X, and 1.2X. Thus, “about X” is intended to teach and provide written description support for a claim limitation of, *e.g.*, “0.98X.”

[0052] The term “antigen” refers to a molecule, or a portion thereof, that is capable of inducing an immune response (*e.g.*, in a subject). While in many instances an immune response involves the production of an antibody that targets or specifically binds to the antigen, as used herein the term “antigen” also refers to molecules that induce immune responses other than those that specifically involve the production of an antibody that targets the antigen, *e.g.*, a cell-mediated immune response involving expansion of T cells that target antigen-derived peptides presented on the surface of target cells. The term also refers to molecules that do not necessarily induce immune responses by themselves but can be made to induce an immune response when presented in the right context, and/or in combination with other molecules that facilitate immune responses. In particular embodiments of the present disclosure, an antigen refers to an HIV protein, or immunogenic fragment thereof, *e.g.*, an HIV Env protein (*e.g.*, gp120, gp140, gp160).

[0053] The term “nucleic acid sequence encoding a peptide” refers to a segment of DNA, which in some embodiments may be a gene or a portion thereof, that is involved in producing a peptide chain (*e.g.*, an HIV protein or IP-10). A gene will generally include regions preceding and following the coding region (leader and trailer) involved in the transcription/translation of the gene product and the regulation of the transcription/translation. A gene can also include intervening sequences (introns) between individual coding segments (exons). Leaders, trailers, and introns can include regulatory elements that are necessary during the transcription and the translation of a gene (*e.g.*, promoters, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions, etc.). A “gene product” can refer to either the mRNA or protein expressed from a particular gene.

[0054] The terms “expression” and “expressed” refer to the production of a transcriptional and/or translational product, *e.g.*, of a nucleic acid sequence encoding a protein (*e.g.*, an HIV protein or IP-10). In some embodiments, the term refers to the production of a transcriptional and/or translational product encoded by a gene (*e.g.*, a gene encoding an antigen) or a portion thereof. The level of expression of a DNA molecule in a cell may be assessed on the basis of either the amount of corresponding mRNA that is present within the cell or the amount of protein encoded by that DNA produced by the cell.

[0055] The term “recombinant” when used with reference, *e.g.*, to a polynucleotide, protein, vector, or cell, indicates that the polynucleotide, protein, vector, or cell has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. For example, recombinant polynucleotides contain nucleic acid sequences that are not found within the native (non-recombinant) form of the polynucleotide.

[0056] The term “immune response” refers to any response that is induced (*e.g.*, in a subject) by an antigen, including the induction of immunity against pathogens (*e.g.*, viruses such as HIV). Immune responses induced by systems, recombinant polynucleotides, compositions, and methods described herein are typically desired, intended, and/or protective immune responses. The term includes the production of antibodies against an antigen, as well as the development, maturation, differentiation, and activation of immune cells (*e.g.*, B cells and T cells). In some instances, an immune response comprises inducing pro-inflammatory monocytes, Env-specific CD4 T follicular helper cells, and germinal center responses. The term also includes increasing or decreasing the expression or activity of cytokines that are involved in regulating immune function (*e.g.*, in a subject). Other markers of immune responses include, but are not limited to, Th1-Tfh responses, dendritic cell (DC)-T cell interactions, the magnitude of vaccine-specific T cells, Tfh differentiation, Th1 polarization, IL-6 production in B cells, plasma cell differentiation, induction of CD80 and CD40 on DCs, the strength of cross-priming and magnitude of cytolytic responses, effects on APCs and T cells, and others.

[0057] “HIV” refers to the human immunodeficiency virus, and can include any type, group, sub-group, clade, strain, variant, or isolate, *e.g.*, HIV-1, HIV-2, HIV-1 Group M (including sub-groups or clades A, B, C, D, F, G, H, J), Group N, Group O, C.1086, Con C, Con S CH505, *etc.* HIV is also used to describe genes within the HIV genome (*e.g.*, gag, pol, env, tat, rev, nef, vpr, vif, and vpu), or to proteins encoded by the genes, including Env (or gp120, gp140, gp160, gp41), Gag (or MA, CA, SP1, NC, SP2, P6), Pol (or RT, RNase H, IN, PR), Tat, Rev, Nef, Vpr, Vif, and Vpu.

[0058] “IP-10”, or interferon-gamma-induced protein 10 (also known as CXCL10, or C-X-C motif chemokine 10, INP10, small-inducible cytokine B10, and other names) is an 8.7 kDa protein belonging to the CXC chemokine family. In humans, the gene encoding IP-10 corresponds, *e.g.*, to NCBI Gene ID No. 3627. IP-10 also encompasses polynucleotides

comprising the human IP-10/CXCR3 mRNA of NCBI Reference Sequence NM_001565, or polynucleotides having a sequence comprising at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more identity to the nucleotide sequence of NM_001565. IP-10 also encompasses polypeptides comprising (or polynucleotides encoding) the human IP-10/CXCR3 protein of NCBI Reference Sequence NP_001556.2, or comprising (or polynucleotides encoding) a polypeptide sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more identity to NP_001556.2, as well as to derivatives, variants, and fragments thereof.

[0059] “Th1-polarization” refers to the differentiation of CD4⁺ T cells into specific subsets in response to antigens presented by antigen presenting cells (APCs) and depending on, *e.g.*, the particular cytokines present in the microenvironment. Polarization involves in part the modulation of particular transcription factors that results in the production of autocrine cytokines, producing a positive feedback loop that contributes to the polarization of the cells. CD4⁺ subsets include Th1, Th2, Th3, Th9, Th17, Treg, Tr1, and Tfh (*see, e.g.,* Martinez-Sanchez *et al.*, *Front. Physiol.* (2018); doi.org/10.3389/fphys.2018.00877). Th1 polarization involves, *e.g.*, the transcription factor T-bet and the cytokine IFN γ ; other markers of Th1 polarization include, *e.g.*, bcl-6, CXCR3, BCL-6, TBX21, SemA4A, and CCR5.

[0060] “Gp140” is a soluble version of the Env protein that contains the entire ectodomain (gp120) and a portion of the transmembrane glycoprotein (gp41). As used herein, Gp140 encompasses forms, variants, and derivatives of Gp140, including monomeric, dimeric, trimeric, *etc.*, forms, forms in which the gp120 and gp41 components are linked, *e.g.*, by a disulfide bond, mutated forms, *e.g.*, with an I559P change within the gp41 component, and others. “Gp140” also comprises immunogenic fragments of full-length gp140, *e.g.*, comprising one or more portions of the gp120 and/or gp41 components that are useful for eliciting immune responses in a subject.

[0061] “QS-21” is a purified plant extract containing water soluble triterpene glycoside saponins that is derived from the soap bark tree, *Quillaja saponaria*. In particular embodiments, QS-21 comprises a quillaic acid triterpene substituted with a branched trisaccharide and a linear tetrasaccharide, which is connected to an acyl chain via a hydrolytically labile ester. In particular embodiments, QS-21 is a 65:35 mixture of the apiose- and xylose-substituted variants of the above-described molecule. As used herein, QS-21 can be natural or semi-synthetic, *e.g.*, prepared by purifying the prosapogenin part of the

molecule and synthetically adding the rest of the molecule. Fully synthetic forms of the molecule are encompassed as well. QS-21 can be obtained from commercial sources, *e.g.*, from Desert King International (San Diego, CA).

[0062] As used herein, an “adjuvant” refers to a compound administered to a subject in conjunction with an antigen for enhancing an immune response to the antigen. Adjuvants can increase the immunogenicity of vaccines in any of a number of ways, and can include inorganic compounds such as salts, *e.g.*, aluminum salts, as well as organic compounds and mixtures of compounds, including extracts and preparations, *e.g.*, Freund’s incomplete adjuvant, squalene, MF59, monophosphoryl lipid A, QS-21. In some embodiments, an adjuvant is a polypeptide such as IP-10. In particular embodiments, an “adjuvant” as used herein refers to adjuvants that increase the Th1 polarization of Tfh cells, *e.g.*, IP-10 and/or QS-21.

[0063] A “prime-boost” or “prime-booster” vaccine strategy refers to an immunization approach that is administered in two or more stages and in which the same antigen is presented to a subject in identical (in a homologous protocol) or different (in a heterologous protocol) forms. For example, in one stage a polynucleotide, *e.g.*, DNA molecule encoding an immunogenic protein, is administered one or more times, wherein the protein encoded by the polynucleotide is expressed by cells in the body, and in a second stage the protein itself is administered. The form of the protein administered in the second stage need not be identical to that of the encoded protein; for example, in numerous embodiments of the present disclosure, a prime DNA vaccine is administered encoding the Env protein, and the gp140 form of the Env protein is administered during the boost stage. Generally the first, *i.e.*, DNA prime, stage precedes the protein boost stage, but other sequences can be performed as well, including approaches in which the two stages overlap to some extent.

[0064] “Tfh” cells, or T follicular helper cells, are a subset of CD4⁺ T cells that help B cells produce antibodies against foreign pathogens (*e.g.*, against HIV). Tfh cells are located both in circulation and in secondary lymphoid organs, *e.g.*, tonsils, spleen, and lymph nodes, in particular in the B cell zones, where they interact with and stimulate B cells (*e.g.*, through CD40 and by producing IL-21). Tfh cells are involved in the formation of the germinal centers (GCs), structures that form within the B cell zones during an immune response. Tfh cells are defined by, *e.g.*, the expression of the transcription factor Bcl6 and cell surface markers such as CXCR5, PD1, and ICOS.

[0065] The term “antigen-presenting cell” or “APC” refers to a cell that displays or presents an antigen, or a portion thereof, on the surface of the cell. Typically, antigens are displayed or presented with a major histocompatibility complex (MHC) molecule. Almost all cell types can serve as APCs, and APCs are found in a large number of different tissue types. Professional APCs, such as dendritic cells, macrophages, and B cells, present antigens to T cells in a context that most efficiently leads to their activation and subsequent proliferation. Many cell types present antigens to cytotoxic T cells.

[0066] As used herein, the terms “polynucleotide,” “nucleic acid,” and “nucleotide,” refer to deoxyribonucleic acids (DNA) or ribonucleic acids (RNA) and polymers thereof. The term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, and DNA-RNA hybrids, as well as other polymers comprising purine and/or pyrimidine bases or other natural, chemically modified, biochemically modified, non-natural, synthetic, or derivatized nucleotide bases. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid. Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions), homologs, and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); and Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)).

[0067] The terms “vector” and “expression vector” refer to a nucleic acid construct, *e.g.*, plasmid or viral vector, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular nucleic acid sequence (*e.g.*, encoding an HIV antigen and/or a protein having IP-10-like activity) in a host cell or engineered cell. In some embodiments, a vector includes a polynucleotide to be transcribed, operably linked to a promoter. Other elements that may be present in a vector include those that enhance transcription (*e.g.*, enhancers), those that terminate transcription (*e.g.*, terminators), those that confer certain binding affinity or antigenicity to a protein (*e.g.*, recombinant protein) produced from the vector, and those that enable replication of the vector and its packaging (*e.g.*, into a viral particle). In some embodiments, the vector is a viral

vector (*i.e.*, a viral genome or a portion thereof). A vector may contain nucleic acid sequences or mutations, for example, that increase tropism and/or modulate immune function.

[0068] The terms “polypeptide,” “peptide,” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. All three terms apply to amino acid polymers in which one or more amino acid residues are an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. As used herein, the terms encompass amino acid chains of any length, including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

[0069] The terms “subject,” “individual,” and “patient” are used interchangeably herein to refer to a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, mice, rats, simians, humans, farm animals, sport animals, and pets. Tissues, cells and their progeny of a biological entity obtained *in vivo* or cultured *in vitro* are also encompassed.

[0070] As used herein, the term “administering” includes oral administration, topical contact, administration as a suppository, intravenous, intraperitoneal, intramuscular, intralesional, intratumoral, intrathecal, intranasal, intraosseous, or subcutaneous administration to a subject. Administration is by any route, including parenteral and transmucosal (*e.g.*, buccal, sublingual, palatal, gingival, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, *e.g.*, intravenous, intramuscular, intra-arterial, intradermal, subcutaneous, intraperitoneal, intraventricular, intraosseous, and intracranial. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, *etc.*

[0071] The term “treating” refers to an approach for obtaining beneficial or desired results including, but not limited to, a therapeutic benefit and/or a prophylactic benefit. “Therapeutic benefit” means any therapeutically relevant improvement in or effect on one or more diseases, conditions, or symptoms under treatment. Therapeutic benefit can also mean to effect a cure of one or more diseases, conditions, or symptoms under treatment. Furthermore, therapeutic benefit can also mean to increase survival. For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, condition, or symptom, or to a subject reporting one or more of the physiological symptoms of a disease, even though the disease, condition, or symptom may not yet be present.

[0072] The term “therapeutically effective amount” or “sufficient amount” refers to the amount of a system, recombinant polynucleotide, or composition described herein that is sufficient to effect beneficial or desired results. The therapeutically effective amount may vary depending upon one or more of: the subject and disease condition being treated or prevented, the weight and age of the subject, the severity of the disease condition, the immune status of the subject, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The specific amount may vary depending on one or more of: the particular agent chosen, the target cell type, the location of the target cell in the subject, the dosing regimen to be followed, whether it is administered in combination with other compounds, timing of administration, and the physical delivery system in which it is carried.

[0073] For the purposes herein, an effective amount is determined by such considerations as may be known in the art. The amount must be effective to achieve the desired therapeutic effect in a subject suffering from, *e.g.*, HIV infection, or to prevent an infection, *e.g.*, by HIV. The desired therapeutic effect may include, for example, amelioration of undesired symptoms associated with the disease, prevention of the manifestation of such symptoms before they occur, slowing down the progression of symptoms associated with the disease, slowing down or limiting any irreversible damage caused by the disease, lessening the severity of or curing the disease, or improving the survival rate or providing more rapid recovery from the disease. Further, in the context of prophylactic treatment the amount may also be effective to prevent the development of the disease or to prevent an infection (*e.g.*, HIV infection).

[0074] The term “pharmaceutically acceptable carrier” refers to a substance that aids the administration of an active agent to a cell, an organism, or a subject. “Pharmaceutically acceptable carrier” also refers to a carrier or excipient that can be included in the compositions described herein and that causes no significant adverse toxicological effect on the patient. Non-limiting examples of pharmaceutically acceptable carriers include water, sodium chloride (NaCl), normal saline solutions, lactated Ringer’s, normal sucrose, normal glucose, binders, fillers, disintegrants, lubricants, coatings, sweeteners, flavors and colors, liposomes, dispersion media, microcapsules, cationic lipid carriers, isotonic and absorption delaying agents, and the like. The carrier may also comprise or consist of substances for providing the formulation with stability, sterility and isotonicity (*e.g.*, antimicrobial preservatives, antioxidants, chelating agents and buffers), for preventing the action of microorganisms (*e.g.*, antimicrobial and antifungal agents, such as parabens, chlorobutanol,

phenol, sorbic acid and the like) or for providing the formulation with an edible flavor, etc. In some instances, the carrier is an agent that facilitates the delivery of a polypeptide, fusion protein, or polynucleotide to a target cell or tissue. One of skill in the art will recognize that other pharmaceutical carriers are useful in the present disclosure.

[0075] The term “vaccine” refers to a biological composition that, when administered to a subject, has the ability to produce an acquired immunity to a particular pathogen or disease in the subject. In particular embodiments of the present disclosure, a vaccine is used to produce an acquired immunity against HIV infection. Typically, one or more antigens, fragments of antigens, or polynucleotides encoding antigens or fragments of antigens that are associated with the pathogen or disease of interest are administered to the subject. In some instances, cells are engineered to express proteins such that, when administered as a vaccine, they enhance the ability of a subject to acquire immunity to an organism that causes an infectious disease, *e.g.*, HIV. As used herein, the term “vaccine” includes, but is not limited to, systems and recombinant polynucleotides described herein, as well as viral particles, host cells, and pharmaceutical compositions that comprise systems or recombinant polynucleotides described herein. In particular embodiments, a vaccine refers to an HIV (or SIV) vaccine, *e.g.*, a DNA vaccine encoding one or more HIV (and/or SIV) proteins, *e.g.*, Env, Gag, protease, reverse transcriptase, Rev, and combinations thereof, or a composition comprising an HIV protein, *e.g.*, gp140.

3. Detailed Description of the Embodiments

[0076] The present disclosure provides methods and compositions for enhancing the anti-Env antibody response during HIV vaccination in a subject, *e.g.*, during vaccination using a prime DNA vaccine encoding one or more HIV (or SIV) proteins, and/or during a boost step in which an HIV protein is administered. In particular, the methods and compositions involve the administration of one or more adjuvants, *e.g.*, during the prime and/or boost step, in order to enhance the Th1-Tfh responses in the subject.

[0077] Any of a number of methods can be used to enhance Th1 polarization in a subject. For example, one or more Th1-polarization promoting compounds can be administered in coordination with a prime vaccination step, *e.g.*, by administering to the subject a DNA vaccine encoding one or more HIV polypeptides and a Th1-polarizing protein. In particular embodiments, the adjuvant is a protein such as IP-10, and a polynucleotide encoding IP-10 is

administered to the subject. In particular embodiments, a single DNA vector is administered one or more times to the subject that comprises polynucleotides encoding both the one or more HIV (or SIV) proteins and the adjuvant such as IP-10.

[0078] In particular embodiments, the Th1-polarizing protein administered in coordination with the DNA prime is interferon-induced protein 10 (IP-10). IP-10 is also known as, *e.g.*, CXCL10 or INP10, and corresponds, *e.g.*, to NCBI Gene ID No. 3627. In some embodiments, a polynucleotide comprising the human IP-10/CXCL10 mRNA of NCBI Reference Sequence NM_001565, or of a polynucleotide sequence comprising at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more identity to the nucleotide sequence of NM_001565, or encoding the polypeptide of NCBI Reference Sequence NP_001556.2, or encoding a polypeptide having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more identity to NCBI Reference Sequence NP_001556.2, is used. In some embodiments, a polypeptide comprising the human IP-10/CXCL10 protein of NCBI Reference Sequence NP_001556.2, or comprising a polypeptide sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more identity to NP_001556.2, is used. In particular embodiments, a polynucleotide encoding IP-10 is used, and is administered to the patient on a DNA plasmid, *e.g.*, a single DNA plasmid comprising polynucleotides encoding one or more HIV polypeptides and encoding IP-10.

[0079] Th1 polarization can also be promoted by administering to the subject a Th1-polarizing compound, *e.g.*, adjuvant, in conjunction with one or more protein boosts, *e.g.*, administration of an HIV protein such as gp140. In particular embodiments, the boost protein, *e.g.*, gp140, is administered together with the adjuvant QS-21. Any form of QS-21 can be used in the present methods, including natural, semi-synthetic, and synthetic forms, and that QS-21 can be used at a variety of amounts, *e.g.*, at 50 µg/500 µl of vaccine dose, or 40-60 µg/500 µl, 30-70 µg/500 µl, 20-80 µg/500 µl, 10-90 µg/500 µl, or 1-100 µg/500 µl of vaccine dose.

[0080] When a Th1-polarizing agent (*e.g.*, a polynucleotide encoding a Th1-polarizing protein such as IP-10, or an adjuvant such as QS-21) is administered in coordination with an HIV antigen, *e.g.*, a DNA molecule encoding an HIV protein or a protein boost, the agent and the antigen can be administered in any of a variety of ways with respect to one another. For example, the agent can be co-administered with the antigen, *e.g.*, co-formulated and administered together to a subject. In some embodiments, as described elsewhere herein, a

single DNA plasmid is administered that encodes one or more HIV (or SIV) proteins, *e.g.*, Env, and also encodes IP-10. In other embodiments, multiple plasmids or vectors, *e.g.*, one or more plasmids encoding one or more HIV (or SIV) proteins, and a separate plasmid encoding IP-10, are formulated together. In some embodiments, a polypeptide boost such as gp140 is co-formulated with a Th1-promoting adjuvant, *e.g.*, QS-21.

[0081] In other embodiments, the Th1-polarizing agent is formulated independently from the HIV antigen, but they are administered at about the same time (*e.g.*, within 10 minutes, 30 minutes, or 1 hour of each other, or during a single clinical visit, or on the same day). In other embodiments, the Th1-polarizing agent is formulated independently from the HIV antigen, and they are administered at different times (*e.g.*, not during a single clinical visit, or not on the same day, or at least 1, 2, 3 or more days apart). In such embodiments, the Th1-polarizing agent can be administered before or after the administration of the HIV antigen.

Immunization

[0082] The HIV vaccines described herein can take on any of a number of forms, including through the administration of proteins, peptides, and nucleic acids including RNA or DNA encoding one or more HIV (or SIV) proteins.

Nucleic acid vaccines

[0083] In a particular embodiment, the vaccine is a DNA vaccine, *e.g.*, a DNA vector such as a DNA plasmid comprising one or more polynucleotides encoding one or more HIV proteins, operably linked to one or more promoters. In particular embodiments, the DNA vaccine comprises a polynucleotide encoding the Env protein, or an immunogenic fragment or derivative thereof. In some embodiments, the DNA vaccine encodes multiple HIV proteins, *e.g.*, any combination of Env, Gag, protease, reverse transcriptase, tat, and rev, or immunogenic fragments or derivatives thereof. The HIV proteins can be from HIV-1 or HIV-2, from any group, *e.g.*, HIV-1 group M, group N, group O, or group P, or HIV-2 group A, B, C, D, E, F, G, or H, and from any sub-group or clade, *e.g.*, HIV-1 group M clade (sub-type) A, B, C, D, E, F, G, H, I, J, or K, or any strain, variant, or isolate from within any of such groups, sub-groups or clades. In particular embodiments, one or more of the proteins used for vaccination are from HIV-1 clade C. It will be appreciated that the precise HIV origin of each encoded protein is considered independently for the purposes of the present disclosure, *e.g.*, one or more proteins can be from one clade, strain or variant/isolate, one or more proteins can be from a second clade, strain, or variant/isolate, *etc.* Any of these proteins can be encoded as

a full-length protein, or can be encoded as a fragment of the full-length protein, *e.g.*, as a minimal peptide sequence capable of eliciting an immune response in a subject. Guidance to the selection of HIV proteins, protein fragments, peptides, variants or derivatives of HIV proteins, protein fragments, or peptides can be found, *e.g.*, in Rappuioli and Bagnoli (eds.) (2011) “Vaccine Design: Innovative Approaches and Novel Strategies,” (Caister Academic Press, UK) or Agudelo and Patarroyo (2010), “Quantum chemical analysis of MHC-peptide interactions for vaccine design” *Mini Rev Med Chem* 10(8): 746-758, the entire disclosures of which are herein incorporated by reference. Any protein encoded by the HIV genome, *e.g.*, gp120 env, gp140 env, gp160 env, p18, gag, pol, vif, vpr, vpu, tat, rev, and nef, or any fragment, derivative, or combination of any of these proteins, can be used during the prime immunization phase (*e.g.*, using polynucleotides encoding the protein, peptide, fragment, or derivative) and/or during the protein boost phase.

[0084] When polynucleotides, *e.g.*, DNA plasmids, encoding one or more HIV proteins and/or IP-10 are used, the polynucleotides are typically present within one or more expression cassettes, *i.e.*, the polynucleotides are operably linked to one or more promoters. Any promoter capable of driving expression of the polynucleotides in one or more cells of a subject can be used, including inducible and constitutive promoters. In some embodiments, the CMV promoter is used. In some embodiments, all of the one or more HIV proteins and IP-10 are operably linked to a single promoter, *e.g.*, a CMV promoter, that drives constitutive expression in cells, leading to the expression of a single mRNA from which the different proteins are expressed by subgenomic splicing and frameshifting.

[0085] In particular embodiments, the DNA vaccine comprises a pGA2/JS2 plasmid, *i.e.*, a DNA vector that encodes one or more of the HIV-1 Env, Tat, Rev, Gag, Protease, and reverse transcriptase proteins under the control of the CMV vector. In particular embodiments, a polynucleotide encoding IP-10 is integrated into the vector, *e.g.*, downstream of the env gene.

[0086] Plasmid constructs can be produced, maintained, and cultured using standard molecular biology techniques, *e.g.*, as taught in Sambrook *et al.*, (1989), “Molecular Cloning: A Laboratory Manual”, (2nd ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.; and Ausubel *et al.* (Eds), (2000-2010), “Current Protocols in Molecular Biology”, John Wiley and Sons, Inc. Before being used for vaccination, the function of the constructs can be assessed, *e.g.*, by transfection into human cells, *e.g.*, 293 T cells, and then grown, harvested, and assessed with respect to the expression of HIV proteins using, *e.g.*, flow cytometry using

monoclonal antibodies. In addition, cellular and secreted expression of IP-10 or other Th1-polarizing proteins can be assessed, *e.g.*, by intracellular cytokine signaling and/or ELISA.

[0087] Recombinant polynucleotides, (*e.g.*, DNA plasmids, expression cassettes, polynucleotides encoding HIV antigens or IP-10) can be prepared using standard molecular biology methods. Polynucleotides encoding protein antigens can be identified, *e.g.*, by searching a human or other model organism DNA sequence database for any gene segment that has a certain percentage of sequence homology to a known nucleotide sequence, such as one encoding the antigen. Any DNA sequence so identified can be subsequently obtained by chemical synthesis and/or a polymerase chain reaction (PCR) technique such as the overlap extension method. For a short sequence, completely *de novo* synthesis may be sufficient; whereas further isolation of full length coding sequence from a human or other model organism cDNA or genomic library using a synthetic probe may be necessary to obtain a larger gene.

[0088] Alternatively, a nucleic acid sequence can be isolated from a cDNA or genomic DNA library (*e.g.*, human or rodent cDNA or human, rodent, bacterial, or viral genomic DNA library) using standard cloning techniques such as polymerase chain reaction (PCR), where homology-based primers can often be derived from a known nucleic acid sequence. Commonly used techniques for this purpose are described in standard texts, *e.g.*, Sambrook and Russell, *supra*.

[0089] Based on sequence homology, degenerate oligonucleotides can be designed as primer sets and PCR can be performed under suitable conditions (*see, e.g.*, White *et al.*, PCR Protocols: Current Methods and Applications, 1993; Griffin and Griffin, PCR Technology, CRC Press Inc. 1994) to amplify a segment of nucleotide sequence from a cDNA or genomic library. Using the amplified segment as a probe, the full-length nucleic acid encoding a protein of interest is obtained. Oligonucleotides used in the construction of the recombinant polynucleotides that are not commercially available can be chemically synthesized, *e.g.*, according to the solid phase phosphoramidite triester method first described by Beaucage & Caruthers, *Tetrahedron Lett.* 22: 1859-1862 (1981), using an automated synthesizer, as described in Van Devanter *et. al.*, *Nucleic Acids Res.* 12: 6159-6168 (1984). Purification of oligonucleotides is performed using any art-recognized strategy, *e.g.*, native acrylamide gel electrophoresis or anion-exchange HPLC as described in Pearson & Reanier, *J. Chrom.* 255: 137-149 (1983).

[0090] Upon acquiring a nucleic acid sequence encoding a protein of interest, the coding sequence can be further modified by a number of well-known techniques such as restriction endonuclease digestion, PCR, and PCR-related methods to generate coding sequences, including mutants and variants derived from the wild-type protein. The polynucleotide sequence encoding the desired polypeptide can then be subcloned into a vector, for instance, an expression vector, so that a recombinant polypeptide can be produced from the resulting construct. Further modifications to the coding sequence, *e.g.*, nucleotide substitutions, may be subsequently made to alter the characteristics of the polypeptide.

[0091] A variety of mutation-generating protocols are established and described in the art, and can be readily used to modify a polynucleotide sequence encoding a protein of interest. *See, e.g.*, Zhang *et al.*, *Proc. Natl. Acad. Sci. USA*, 94: 4504-4509 (1997); and Stemmer, *Nature*, 370: 389-391 (1994). The procedures can be used separately or in combination to produce variants of a set of nucleic acids, and hence variants of encoded polypeptides. Kits for mutagenesis, library construction, and other diversity-generating methods are commercially available.

[0092] In some embodiments, a recombinant polynucleotide or vector described herein contains a nucleic acid sequence that encodes a selectable marker. A selectable marker is useful, for example, when a polynucleotide described herein is being recombinantly modified. Taking antibiotic resistance genes as an example of a selectable marker, treating the cells that contain the recombinant polynucleotides with the antibiotic will identify which cells contain recombinant polynucleotides that have incorporated the antibiotic resistance gene (*i.e.*, the cells that survive after antibiotic treatment must have incorporated the antibiotic resistance gene). If desired, the recombinant polynucleotides can be further screened (*e.g.*, purified from the cells, amplified, and sequenced), in order to verify that the desired modification has been recombinantly introduced into the polynucleotide at the correct position.

Protein Booster

[0093] Following immunization using the prime vaccine, the subject will receive one or more boosts with HIV polypeptide antigens, *e.g.*, gp140. The boost can be administered any of a number of times following the immunization, *e.g.*, 1, 2, 3, 4, 5 or more times, and at different intervals, *e.g.*, every 1, 2, 3, 4, 5, 6, 7, 8, weeks or more. In one embodiment, the booster is administered two times, *e.g.*, at weeks 30 and 44 (with week 0 corresponding to the

date of the first prime immunization). The boost can be administered at any of a variety of amounts, *e.g.*, 100 µg protein/boost, or 10-50, 50-100, 100-150, or 150-200 µg protein/boost, or 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 200, 250, 300 or more µg protein, *e.g.*, gp140, per boost.

[0094] In addition to a Th1-promoting adjuvant such as QS-21, the booster polypeptide can be administered with one or more other adjuvants. For example, the booster can be administered with a liposomal and/or lipid adjuvant, *e.g.*, Army Liposome Formulation (ALF) liposomes. ALF liposomes comprise dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG) saturated phospholipids, cholesterol (chol), and monophosphoryl lipid A (MPLA), *e.g.*, synthetic MPLA (*e.g.*, 3D-PHAD), each of which can be obtained commercial suppliers such as Avanti Polar Lipids (Alabaster, AL). Other liposomes that can be used comprise CAF01, an adjuvant that promotes Th1 polarization and which can therefore be used in the place of, or in addition to, QS-21. For formulations adjuvanted with ALFQ (*i.e.*, ALF + QS-21), lipids can be mixed in a molar ratio of, *e.g.*, 9:1:12.2:0.36 (DMPC:DMPG:Chol:MPLA), dried, rehydrated with, *e.g.*, Sorenson PBS (pH 6.2), followed by microfluidization and filtration. The HIV protein, *e.g.*, gp140, can then be mixed with ALFQ in a 1:1 volume ratio, and vaccine doses can comprise, *e.g.*, 100 µg MPLA, 100 µg protein, and 50 µg QS-21 in a total volume of 500 µl.

Subjects

[0095] The present methods and compositions can be used for the immunization of any subject, *e.g.*, a human, that could benefit from an enhanced immune response against HIV infection, *e.g.*, an enhanced anti-Env antibody response during HIV vaccination. In some embodiments, the subject is male. In some embodiments, the subject is female. In some embodiments, the subject is an adult (*e.g.*, an adult male). In some embodiments, the subject is an adolescent. In some embodiments, the subject is a child.

[0096] In some embodiments, the subject has not been infected with HIV, *e.g.*, and the methods and compositions are used to enhance the subject's immune defenses against HIV in order to prevent future infection. In other embodiments, the subject is infected with HIV, and the methods are used to enhance the subject's immune response against HIV in order to slow or potentially reverse the original infection.

Immune Response and Th1 Polarization

[0097] The present methods and compositions provide for enhanced immune responses with vaccination against HIV (and/or SIV). In particular, the present methods and compositions provide for enhanced Th1 polarization of Tfh cells. Without being bound to the following theory, it is believed that increased Th1 polarization enhances the immune response by increasing the Env-specific Tfh cells when delivered with the DNA prime, and that Th1 polarization with a protein boost results in greater production of the IgG1 subclass with enhanced longevity, breadth, avidity, ADCC, and ADP activities of the anti-Env antibody.

[0098] The enhanced immune response and increased Th1 polarization provided by the present methods and compositions can be detected, characterized, or quantified in any of a number of ways. For example, any of the assays described in any of the Examples to assess Th1 polarization or the nature or strength of an immune response in a subject can be used. In some embodiments, Tfh cells, *e.g.*, germinal center (GC) Tfh cells, are isolated from a subject, and the expression of one or more marker of Th1 polarization assessed. Suitable markers include, but are not limited to, CXCR3, T-bet, IFN γ , CXCR3, BCL-6, TBX21, SEMA4A, and CCR5. In some embodiments, the administration of a Th1-polarizing adjuvant, *e.g.*, with a DNA prime and/or with a protein boost, leads to an increase in or more of the herein-described Th1 polarization markers, *e.g.*, to an increase relative to the level in the absence of the Th1-polarizing adjuvant.

[0099] In some embodiments, the efficacy of an Th1-polarizing adjuvant is assessed by detecting one or more aspects of the immune response in a subject to an HIV antigen, *e.g.*, Env. For example, the extrafollicular and/or plasma cell-derived titers, *e.g.*, vs. gp140, can be evaluated using a binding antibody multiplex assay (BAMA) or an enzyme-linked immunosorbent assay (ELISA). In particular embodiments, the administration of a Th1-polarizing agent in coordination with the administration of an HIV antigen leads to an increase in an extrafollicular and/or plasma cell-derived antibody response against an HIV protein, *e.g.*, gp140. In some embodiments, the increase in antibody response can be, *e.g.*, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10 or more fold higher in subjects receiving a Th1-polarizing adjuvant than in subjects not receiving a Th1-polarizing adjuvant.

[0100] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in the breadth, *e.g.*, cross-clade breadth, of serum IgG antibodies relative to the breadth in a subject not

receiving a Th1-polarizing adjuvant. In some embodiments, the breadth of the antibodies is assessed by determining the kinetics of the antibody response against different HIV antigens, *e.g.*, titers against Env from different HIV clades. Antibody binding against different HIV proteins, *e.g.*, against HIV Env proteins from different Groups, Clades, or isolates, *e.g.*, against Con S (group M consensus), Con C, or isolate Case A2 or CH505 proteins, can be assessed, *e.g.*, using a binding antibody multiplex assay (BAMA) or ELISA. In some embodiments, binding to gp120 V1V2 loops is assessed, *e.g.*, scaffolded on murine leukemia virus (MLV) gp170.

[0101] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in the longevity of the serum IgG antibody response that is greater than any increase in longevity that may be observed in a subject not receiving a Th1-polarizing adjuvant. For example, the elevation of anti-Env titers observed following one or more HIV protein boosts can be detected over time and the duration of the elevated antibody response assessed. In one embodiment, titers are assessed at, *e.g.*, week 8 and week 18 following the second protein boost, and the elevation observed in subjects receiving the Th1-polarizing adjuvant at the second time point (*e.g.*, at 18 weeks following the second protein boost) is at least as high as any elevation observed in subjects not receiving the Th1-polarizing adjuvant.

[0102] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in the avidity (*e.g.*, expressed as dissociation constants, K_d) of serum IgG antibodies relative to the avidity in a subject not receiving a Th1-polarizing adjuvant. Increases in avidity can be assessed, *e.g.*, by Surface Plasmon Resonance (SPR) or by 2M sodium thiocyanate or 0.1 M sodium citrate displacement ELISA with, *e.g.*, gp140 protein or with Con C, Con S, or other HIV proteins from other groups, clades, variants or isolates.

[0103] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in the capacity of immune sera to neutralize HIV, *e.g.*, HIV-1, *e.g.*, as assessed using a TZM-bl assay, in which neutralization titers are measured in TZM-bl cells infected with HIV vs. cells infected with a control virus such as MLV. In such embodiments, for example, titers can be assessed against, *e.g.*, subtype C tier 1A variants, tier 1B variants, and tier 2 isolates. In particular embodiments, the induction of tier 1A neutralizing antibodies in a subject receiving a Th1-

polarizing adjuvant is at least as high as any induction observed in a subject not receiving a Th1-polarizing adjuvant, at one or more time points following one or more protein boosts.

[0104] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in antibody-dependent cellular toxicity (ADCC) and/or antibody-dependent phagocytosis (ADP) triggered by engagement of Fc receptors on antibody-bound target cells by innate cells. ADCC can be assessed, *e.g.*, by measuring killing of HIV-infected target cells, *e.g.*, by NK cells (*e.g.*, CD16+/FcγR3 NK cells) in the presence of immune serum. ADP can be assessed, *e.g.*, by measuring the phagocytosis of gp120-coated beads by CD32+ (FcγR2) and/or CD64+ (FcγR1) THP-1 monocytic cells. In particular embodiments, the increase in ADCC and/or ADP is at least as high in a subject receiving a Th1-polarizing adjuvant as it is in a subject not receiving a Th1-polarizing adjuvant.

[0105] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in anti-gp120 IgG1 antibodies relative to IgG2, IgG3, or IgG4 antibodies, *e.g.*, as measured by ELISA. In particular embodiments, Th1-polarizing adjuvants lead to a specific increase in anti-gp120 IgG1 antibodies in subjects, and in particular to an increase that is as high or higher than any increase observed in subjects not receiving a Th1-polarizing adjuvant. In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to a specific increase in the IgG1/IgG4 ratio among anti-gp120 antibodies, and in particular to an increase that is as high or higher than any increase in the IgG1/IgG4 ratio in subjects not receiving a Th1-polarizing adjuvant.

[0106] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in mucosal anti-gp120 IgG and IgA antibodies relative to IgG2, IgG3, or IgG4, and in particular to an increase that is as high or higher than any increase observed in subjects not receiving a Th1-polarizing adjuvant. For example, in some embodiments, Th1-polarizing adjuvants lead to a specific increase in anti-gp140 IgG and/or IgA antibodies in vaginal and/or rectal secretions, wherein the increase is stronger and/or has greater longevity than any increase seen in subjects not receiving a Th1-polarizing adjuvant.

[0107] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in Env-specific

Tfh cells and GC Tfh cells with distinctive Th1 signatures. For example, peripheral blood mononuclear cells (PBMCs) can be isolated and stimulated with overlapping peptide pools comprising one or more gp140 proteins, and the induction of activation markers such as CD25 and OX40 assessed by flow cytometry after stimulation. In some embodiments, the frequency of Env-specific CD4 T cells is higher in subjects receiving a Th1-polarizing adjuvant than in subjects not receiving a Th1-polarizing adjuvant, as is the percentage of Env-specific CD95+ CD4 T cells.

[0108] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to an increase in germinal center (GC) Tfh cells (as assessed, *e.g.*, by virtue of CXCR5+, PD-1+++ expression) and GC B cells (as assessed, *e.g.*, by virtue of Ki-67+, Bcl-6+, CD20 expression), and in particular to an increase that is as high as or higher than any increase seen in subjects not receiving a Th1-polarizing adjuvant. For example, subjects receiving Th1-polarizing adjuvants can show higher Env-specific Tfh cell frequencies in the lymph nodes (LN) and can show a correlation between Tfh cell frequencies in the LN and GC Tfh cells, but not with memory Tfh cells, indicating that GC Tfh cells are enriched for vaccine-induced follicular cells.

[0109] In some embodiments, the administration of a Th1-polarizing adjuvant in coordination with the administration of an HIV antigen leads to a systemic expansion of pro-inflammatory monocytes and an increase in GC Tfh responses, *e.g.*, during the priming immunization phase. For example, Th1-polarizing antibodies increase the relative frequencies and absolute counts of ICOS+ PD-1 + CXCR5+ CD4 T cells in blood, *e.g.*, at day 14 following a priming immunization step, as well as an increase in CD14+ CD16+ HLA-DR+ cells in blood. In some embodiments, Th1-polarizing adjuvants lead to an increase in IL-1 β levels, *e.g.*, as determined using a flow-based Legendplex assay, and to an increase in GC Tfh cell frequencies within fine-needle aspirates of the draining LN, that is as high or higher than any increase observed in subjects not receiving a Th1-polarizing adjuvant.

[0110] Any of these parameters or effects, or any of the parameters or effects described in the Examples or elsewhere herein, can be used to assess the efficacy of a Th1-polarizing adjuvant. In some embodiments, a Th1-polarizing adjuvant leads to an increase of at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, or more in any of the herein-described parameters or effects relative to a control value (*e.g.*, to the value observed in a subject not receiving a Th1-polarizing adjuvant). As such, a Th1-

polarizing adjuvant can refer to any agent that, when administered with either a DNA prime against HIV, or with a protein boost against HIV, can lead to an increase of at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 150%, 200%, 250%, 300%, or more in any of the herein-described parameters or effects relative to a control value (*e.g.*, the value observed in a subject not receiving a Th1-polarizing adjuvant), at any point of the immunization process (*e.g.*, at any one or more points of the priming phase and/or booster phase).

Pharmaceutical Compositions and Administration

[0111] The present disclosure provides compositions comprising an immunogenic component (*e.g.*, a DNA vaccine or an immunogenic polypeptide) capable of inducing immunity against a targeted agent (*e.g.*, HIV Env protein), comprising a Th1-polarizing adjuvant (*e.g.*, a polynucleotide encoding IP-10 or an agent such as QS-21), and potentially comprising one or more additional adjuvants or compounds such as Army liposome formulations. In some embodiments, the compositions further comprise a pharmaceutically acceptable carrier. As such, the present disclosure provides pharmaceutical compositions for inducing an anti-Env (or other HIV protein or antigen) in a subject. In some embodiments, the composition comprises one or more polynucleotides encoding one or more HIV proteins, *e.g.*, Env, and one or more polynucleotides encoding a Th1-polarizing polypeptide, *e.g.*, IP-10, and a pharmaceutically acceptable carrier. In some embodiments, the composition comprises one or more HIV polypeptides, *e.g.*, gp140, one or more Th1-promoting adjuvants, *e.g.*, QS-21, and a pharmaceutically acceptable carrier.

[0112] The compositions may be formulated for, *e.g.*, injection, inhalation, or topical administration, *e.g.*, facilitating direct exposure of host cells and tissues to the immunogenic component and Th1-polarizing adjuvant. In particular embodiments, the compositions, *e.g.*, prime DNA vaccines, are formulated for transdermal injection, *e.g.*, by electroporation. In particular embodiments, the compositions, *e.g.*, protein boost, are formulated for subcutaneous or intramuscular administration, *e.g.*, delivery into the thigh. In particular embodiments, the compositions, *e.g.*, protein boost, are formulated in liposomes, *e.g.*, Army liposome formulations (ALFA), *e.g.*, by the lipid deposition method. In other embodiments, the compositions are formulated in nanoparticles or in dry powder form, *e.g.*, suitable for delivery by particle bombardment. In some embodiments, the compositions, *e.g.*, DNA

vaccine, are formulated as naked DNA (*see, e.g.*, US Patent Nos. 6,265,387, 6,972,013, and 7,922,709).

[0113] In particular embodiments, the DNA vaccines are prepared as DNA vectors or plasmids, *e.g.*, a pGA2 plasmid, or pGA2/JS2 plasmid, or derivative thereof. In some embodiments, the compositions, *e.g.*, DNA vaccine, are prepared as recombinant viruses, *e.g.*, by modifying a parent virus to incorporate exogenous genetic material, *e.g.*, one or more polynucleotides encoding one or more HIV proteins or antigens and/or a polynucleotide encoding IP-10. A non-limiting list of suitable viruses that can be used for the purposes of the present disclosure include lentiviruses (*e.g.*, HIV, HIV-1, HIV-2, FIV, BIV, EIAV, MW, CAEV, SIV), adenoviruses and adeno-associated viruses, alphaviruses, flaviviruses, and poxviruses. For methods and examples concerning the use of suitable viral vectors, *see, e.g.*, US Patent Nos. 5,219,740, 7,250,299, 7,608,273, 6,465,634, 7,811,812, 5,744,140, 8,124,398, 5,173,414, 7,022,519, 7,125,705, 6,905,862, 7,989,425, 6,468,711, 7,015,024, 7,338,662, 5,871,742, and 6,340,462. In such embodiments, the viruses are typically recombination-competent (*i.e.*, capable of reproducing in an infected host cell). Modification of such viruses and vectors or plasmids for the preparation of the present DNA vaccines can be achieved using standard molecular biology techniques, *e.g.*, as taught in Sambrook *et al.* (1989) "Molecular Cloning: A Laboratory Manual" (2nd ed. Cold Spring Harbor Press) and Ausubel *et al.* (Eds.) (2000-2010) "Current Protocols in Molecular Biology" (John Wiley and Sons).

[0114] The pharmaceutical compositions described herein may comprise a pharmaceutically acceptable carrier. In certain aspects, pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of the pharmaceutical compositions described herein (*see, e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, 18TH ED., Mack Publishing Co., Easton, PA (1990)).

[0115] The pharmaceutical compositions described herein will often further comprise one or more buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants (*e.g.*, ascorbic acid, sodium metabisulfite, butylated hydroxytoluene, butylated hydroxyanisole, *etc.*), bacteriostats, chelating agents such as

EDTA or glutathione, solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, suspending agents, thickening agents, preservatives, flavoring agents, sweetening agents, and coloring compounds as appropriate.

[0116] The pharmaceutical compositions described herein are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically or prophylactically effective. The quantity to be administered depends on a variety of factors including, *e.g.*, the age, body weight, physical activity, hereditary characteristics, general health, sex, and diet of the individual, the condition or disease to be treated or prevented, and the stage or severity of the condition or disease. In certain embodiments, the size of the dose may also be determined by the existence, nature, and extent of any adverse side effects that accompany the administration of a therapeutic or prophylactic agent(s) in a particular individual. Other factors that can influence the specific dose level and frequency of dosage for any particular patient include the activity of the specific compound employed, the metabolic stability and length of action of that compound, the mode and time of administration, and the rate of excretion.

[0117] Generally, for administering the compound (*e.g.*, vaccine or adjuvant) for therapeutic or prophylactic (*e.g.*, vaccination) purposes, the compound is given at a therapeutically or prophylactically effective dose. In particular, an effective amount of a pharmaceutical composition described herein is an amount that is sufficient to obtain an enhanced immune response against an HIV vaccine, *e.g.*, in view of any of the parameters or indices described herein, and/or a sufficient amount to enhance the immunity of a subject to HIV infection or to the propagation of an already-existing HIV infection in the subject.

[0118] In certain embodiments, the dose may take the form of solid, semi-solid, lyophilized powder, or liquid dosage forms, such as, for example, tablets, pills, pellets, capsules, powders, solutions, suspensions, emulsions, suppositories, retention enemas, creams, ointments, lotions, gels, aerosols, foams, or the like, preferably in unit dosage forms suitable for simple administration of precise dosages.

[0119] As used herein, the term “unit dosage form” refers to physically discrete units suitable as unitary dosages for humans and other mammals, each unit containing a predetermined quantity of a therapeutic or prophylactic agent calculated to produce the desired onset, tolerability, and/or therapeutic or prophylactic effects, in association with a suitable pharmaceutical excipient (*e.g.*, an ampoule). In addition, more concentrated dosage

forms may be prepared, from which the more dilute unit dosage forms may then be produced. The more concentrated dosage forms thus will contain substantially more than, *e.g.*, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more times the amount of the therapeutic or prophylactic compound.

[0120] Methods for preparing such dosage forms are known to those skilled in the art (*see, e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, *supra*). The dosage forms typically include a conventional pharmaceutical carrier or excipient and may additionally include other medicinal agents, carriers, adjuvants, diluents, tissue permeation enhancers, solubilizers, and the like. Appropriate excipients can be tailored to the particular dosage form and route of administration by methods well known in the art (*see, e.g.*, REMINGTON'S PHARMACEUTICAL SCIENCES, *supra*).

[0121] The pharmaceutical compositions described herein can be administered locally or systemically to the subject, *e.g.*, intraperitoneally, intramuscularly, intra-arterially, orally, intravenously, intracranially, intrathecally, intraspinally, intralesionally, intranasally, subcutaneously, intracerebroventricularly, topically, and/or by inhalation. In particular embodiments, the compositions are administered by intradermal injection with electroporation (*e.g.*, for the priming DNA vaccine) or subcutaneously (*e.g.*, for the protein boost).

[0122] In some embodiments, the DNA vaccines and/or polynucleotides encoding IP-10 or other Th1-polarizing protein are administered, *e.g.*, by intradermal injection with electroporation (*e.g.*, using the ICHOR TriGrid Array; Ichor Medical Systems), and can be administered any of a number of times, *e.g.*, 1, 2, 3, 4, 5 or more times, and following any of a number of vaccination regimens, *e.g.*, every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more weeks. In a particular embodiment, the immunization is performed 3 times, *e.g.*, at weeks 0, 8, and 16. DNA vaccines can be administered at any of a number of levels, *e.g.*, 4 mg of plasmid DNA vector per subject per vaccination, or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more mg of plasmid DNA vector per subject per vaccination, or 1-10, 1-20, 1-8, 1-7, 2-6, 3-5 mg or plasmid DNA vector per subject per vaccination.

[0123] In some embodiments, the protein boost and/or QS-21 or other Th1-polarizing adjuvant is administered by intradermal injection, *e.g.*, into the thigh, and can be administered any of a number of times following the immunization, *e.g.*, 1, 2, 3, 4, 5 or more times, and following any of a number of regimens, *e.g.*, every 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,

15, 16, 17, 18, 19, 20 weeks or more. The protein boost can be administered at any of a number of levels, *e.g.*, 100 µg protein/boost, or 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 200, 250, 300 or more µg protein, *e.g.*, gp140, per boost. In some embodiments, the protein boost, *e.g.*, gp140, is mixed with ALFQ in a 1:1 volume ratio, and vaccine doses comprise, *e.g.*, 100 µg MPLA, 100 µg protein, and 50 µg QS-21 in a total volume of 500 µl.

Kits

[0124] In another aspect, kits are provided herein. In some embodiments, the kit comprises a DNA prime composition (*e.g.*, a DNA vaccine encoding one or more HIV proteins, *e.g.*, Env, and also encoding a Th1-polarizing protein, *e.g.*, IP-10, and optionally a pharmaceutically acceptable carrier). In some embodiments, the kit comprises a protein boost composition (*e.g.*, an HIV protein, *e.g.*, gp140, a Th1-polarizing adjuvant, *e.g.*, QS-21, optionally one or more additional adjuvants, *e.g.*, ALF, and optionally a pharmaceutically acceptable carrier). In some embodiments, the kit comprises one or more DNA prime compositions and one or more protein boost compositions. In some embodiments, the kit is for inducing an enhanced (*e.g.*, stronger, broader, longer) immune response against an antigen, *e.g.*, HIV Env protein (*e.g.*, in a subject). In other embodiments, the kit is for preventing or treating a disease, *e.g.*, HIV.

[0125] The kits described herein can be packaged in a way that allows for safe or convenient storage or use (*e.g.*, in a box or other container having a lid). Typically, the kits described herein include one or more containers, each container storing a particular kit component such as a reagent, a control sample, and so on. The choice of container will depend on the particular form of its contents, *e.g.*, a kit component that is in liquid form, powder form, etc. Furthermore, containers can be made of materials that are designed to maximize the shelf-life of the kit components. As a non-limiting example, kit components that are light-sensitive can be stored in containers that are opaque.

[0126] In some embodiments, the kit contains one or more elements, *e.g.*, syringe, useful for administering compositions (*i.e.*, a pharmaceutical composition described herein) to a subject. In yet other embodiments, the kit further comprises instructions for use, *e.g.*, containing directions (*i.e.*, protocols) for the practice of the methods described herein (*e.g.*, instructions for using the kit for enhancing an immune response in a subject to an HIV protein). While the instructional materials typically comprise written or printed materials,

they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this disclosure. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

4. Examples

[0127] The following examples are offered to illustrate, but not to limit, the claimed invention.

Example 1. Adjuvant-Dependent Modulation of CD4 T Follicular Helper Cells Impacts Longevity and Functional Quality of Antibody Responses to a Subtype C HIV-1 Envelope Vaccine in Rhesus Macaques

Abstract

[0128] Enduring humoral immunity through vaccination is dependent upon effective interaction of follicular helper CD4 T cells (Tfh) with germinal center (GC) B cells. Th1 polarization of Tfh cells is an important process shaping the success of Tfh-GC B cell interactions by influencing co-stimulatory and cytokine-dependent Tfh help to B cells. However, the question remains whether adjuvant-dependent modulation of Th1-Tfh cells enhances HIV-1 vaccine-mediated antiviral envelope (Env) antibody responses. In this study, we investigated whether an HIV-1 vaccine platform designed to increase the number of Th1-polarized Tfh cells enhances the functional quality and longevity of anti-Env antibodies. Utilizing a novel interferon-induced protein (IP)-10-adjuvanted HIV subtype C DNA prime, followed by an MPLA+QS-21-adjuvanted subtype C Env protein boost in rhesus macaques (Th1 group), we observed higher anti-Env serum IgG antibody titers with significantly increased cross-clade reactivity and heightened specificity to V1V2 loops when compared to macaques primed with DNA lacking IP-10 and boosted with MPLA+alum-adjuvanted Env protein (Th1+2 group). The Th1 vaccine regimen increased serum antibody effector functions, induced higher IgG1 and lower IgG4 antibodies, increased circulating Env-specific Tfh cells and generated greater anti-Env mucosal IgA responses. Within lymph nodes, we observed augmented GC B cell responses and promotion of Th1 gene expression profiles in GC Tfh cells. The frequency of GC Tfh cells predicted both the magnitude and avidity of anti-Env serum IgG response at memory time points. Together, these data demonstrate that

adjuvant-induced stimulation of Th1-Tfh cell production during the prime and the boost is an effective strategy to enhance the longevity and functional quality of the anti-Env antibody response.

Introduction

[0129] In this study, we investigated whether an HIV-1 vaccine platform designed to increase the number of Th1-polarized Tfh cells would enhance the functional quality and longevity of HIV-1 anti-Env antibodies. Our data show that a Th1 prime followed by a Th1 boost increases HIV-1 anti-Env specific binding antibody in serum and mucosal compartments. The Th1 vaccine regimen augmented GC B cell responses and promoted Th1 gene expression profiles in GC Tfh cells. The number of GC Tfh cells positively correlated with both longevity and avidity of anti-Env specific antibody responses. Together, these data show that by stimulating production of Th1-Tfh cells during the prime and boost regimen using an adjuvanted vaccine, we can enhance the longevity and function of the anti-HIV-1 - Env antibody response.

Results

[0130] *Vaccination regimen.* Twenty female rhesus macaques were assigned to one of two experimental groups: For Group 1 (n = 10), the Th1 vaccine group, the Th1 chemokine, interferon-induced protein (IP)-10, a ligand for and an inducer of CXCR3, was used as a molecular adjuvant to a DNA vaccine, (DIP-10) to prime Th1-type Tfh cells. Group 2 (n=10) animals received the same DNA vaccine without adjuvant (**FIG. 1D**). The DNA plasmid expressed SIVmac239 Gag, protease, reverse transcriptase, Tat, Rev, HIV C. 1086 Env, and the DIP-10 plasmid additionally expressed rhesus IP-10. The DNA was delivered intradermally (ID) with electroporation (EP) in both experimental groups.

[0131] Prior to immunizing animals, we evaluated plasmid constructs using 293 T cells. At 48 h following transfection, cells were harvested and expression of HIV proteins was assessed by flow cytometry using monoclonal antibodies (PG9, PG16, PGT121 for surface Env, 2F12 for intracellular SIV Gag, and J034D6 for intracellular IP-10). As illustrated in **FIG. 1A** flow plots, both constructs expressed comparable levels of Env and Gag proteins as determined by staining with PGT121 and 2F12, respectively. Cellular and secreted IP-10 as determined by intracellular cytokine staining (**FIG. 1B**) and ELISA (**FIG. 1C**), respectively, was specific to the DNA IP-10 construct. Following DNA immunization, we used clade C C.ZA 1197MB gp140 protein adjuvanted with Army Liposome Formulation (ALF)

liposomes, monophosphoryl lipid A (MPLA) and a detoxified saponin derivative, QS-21 (ALFQ) (20) to boost Th1 primed responses (DIP-10 PALFQ; Th1 group) (**FIG. 1D**). Group 2 animals received an unadjuvanted ID, EP delivered DNA prime and protein adjuvanted with MPLA + aluminum-adsorbed ALF formulation (ALFA) (21), wherein the protein was adsorbed to aluminum hydroxide and then added to ALF (DPALFA; Th1+2 group). Blood was collected at weeks -8 and 0 of vaccination, and at weeks 1, 2, 4, 8, 18, and 20 following each vaccination, as indicated. Fine needle aspirates of lymph nodes (LN) or LN biopsies (draining) were collected to examine GC responses, and rectal and vaginal secretions were sampled to assess mucosal antibodies (**FIG. 1D**).

[0132] *DIP-10 ProteinALFQ vaccine induces robust and durable anti-Env antibody titers with cross-clade breadth.* To ascertain whether induction of Th1 versus mixed Th1+2 inflammatory responses elicited anti-Env antibody responses of different magnitudes, we first evaluated responses against C.1086 gp140 Env using a binding antibody multiplex assay (BAMA)(22). We have previously shown that the transient extrafollicular plasmablast response contributes to peak serum IgG antibody titers following the boost, while titers at week 8 and beyond are mainly plasma cell derived (12). Therefore, we assessed antibody levels at weeks 0, 2, and 8 following each of the protein boosts to capture both extrafollicular (week 2) and plasma cell-derived (week 8 and beyond) titers. The data showed robust induction of anti-C.1086 Env responses following the 1st protein immunization in all 20 animals and potent recall of memory B cells following the 2nd protein immunization as evidenced by a robust boost in antibody responses (**FIG. 2A**). Strikingly, Env ALFQ boosted animals developed significantly higher responses against C.1086 gp140; median AUC values in ALFA (Th1+2) and ALFQ (Th1) vaccine groups were: wk 0, 7496 and 20301, $p < 0.01$; wk 2, 46481 and 63469, $p < 0.001$; wk 8, 20714 and 36709, $p < 0.0001$ 2nd protein boost.

[0133] We confirmed these findings by using an independent enzyme linked immunosorbent assay (ELISA) assay to explore C.1086 gp140 anti-Env antibody kinetics after the 2nd protein boost (**FIG. 2B**). The assay revealed that anti-Env titers exhibited a median 5-fold increase at week 2 post-2nd protein immunization relative to week 0 indicating a successful booster response. In affirmation of the BAMA data, antibody titers were significantly higher in the Th1 group in comparison to the Th1+2 group at week 0 (median ng/mL of anti-gp140 IgG; Th1+2 = 257 and Th1 = 1314; $p < 0.01$), at week 2 (Th1+2 = 9942 and Th1 = 29104; $p < 0.0001$), week 8 (Th1+2 = 1830 and Th1 = 7076; $p < 0.001$), and week 18 (Th1+2 = 450 and Th1 = 2431; $p < 0.001$).

[0134] We next assessed the breadth of the serum IgG antibody response and found that AUC values against CH505 subtype C Env were also significantly higher in the Th1 group relative to the Th1+2 group ($p < 0.01$, **FIG. 2C**). Similarly, increased responses against the Con S (group M consensus) and Con C proteins at week 2 following the 2nd protein boost in the Th1 group were sustained at week 8 demonstrating greater induction of antibodies with cross-clade breadth using the Th1 vaccine regimen ($p < 0.05$, **FIGS. 2D** and **2E**). We also assessed binding to gp120 V1V2 loops from isolate Case A2, scaffolded on murine leukemia virus (MLV) gp70, at weeks 2 and 8 and found that significantly higher specificity to these important regions was induced in the Th1 vaccine regimen following the second protein boost ($p < 0.05$, **FIG. 2F**).

[0135] Based on significantly elevated anti-Env antibody responses in the Th1 vaccine regimen we sought to quantify antibody longevity. To this end, we calculated fold change in titers at week 8 and 18 following 2nd protein boost relative to titers at week 8 post 1st protein boost. Significantly higher titers at week 8 (mean 1.7-fold in Th1+2 versus 4.5-fold in Th1 group; $p < 0.05$) and week 18 (mean 0.3-fold in Th1+2 versus 1.3-fold in Th1 group; $p < 0.01$) post 2nd protein boost in Th1 vaccinated animals demonstrated that the Th1 vaccine regimen was effective at enhancing durability of anti-Env serum IgG titers (**FIG. 2G**). Together, the data show that the Th1 group had higher induction of cross-clade breadth, elicited stronger binding to a gp70-V1V2 protein, and enhanced antibody longevity relative to the Th1+2 group.

[0136] *DIP-10 ProteinALFQ vaccine elicits high avidity anti-Env antibody with ADCC and ADP activities.* Next, we quantified avidity of IgG binding antibodies (as disassociation constants, kd) in sera collected at 2 weeks post final DNA prime and after each of the protein boosts using Surface Plasmon Resonance (SPR) to C.1086 gp140 protein (23). The data showed that gp140-specific antibodies reached higher avidity with each sequential immunization in both vaccine groups ($p < 0.0001$, **FIGS. 3A** and **3B**). Increased avidities in the Th1 vaccine group relative to the Th1+2 group was suggestive of more productive GC reactions in the Th1 vaccine group ($p < 0.05$, **FIG. 3C**). To confirm that higher avidity antibodies in the Th1 vaccine group were sustained, we determined avidity at 8 weeks following the 2nd protein boost using a 2M sodium thiocyanate displacement ELISA with C.1086C gp140 antigen (12). The data showed sustained induction of higher avidity antibodies in the Th1 group ($p < 0.05$, **FIG. 3D**), which was further corroborated with a 0.1 M sodium citrate ELISA ($p < 0.01$, **FIG. 3E**). Notably, higher avidity antibodies against Con C

and Con S gp140 proteins were also induced in the Th1 vaccine regimen ($p < 0.01$, **FIGS. 3F** and **3G**).

[0137] After establishing induction of higher avidity antibodies in the Th1 vaccine group, we next evaluated capacity of immune sera to neutralize HIV-1 using the classic TZM-bl assay (12). We detected robust activity against MW965.26, a subtype C tier 1A variant (**FIG. 3H**) whereas neutralization of tier 1B and tier 2 isolates was sporadic (24). The data showed higher induction of tier 1A neutralizing antibodies in the Th1 vaccine group (ID50 range at week 2 post 2nd protein boost Th1+2: 37 -1126; Th1: 195-4977, $p < 0.01$). These titers dropped to an ID50 value of 20 in the Th1+2 group but were maintained between 24-1057 in the Th1 group ($p < 0.001$). To assess generation of Fc-mediated antibody effector responses, we measured antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP) triggered by engagement of the Fc receptors on antibody-bound target cells by innate cells (25) (26, 27). ADCC was assessed by measuring killing of Clade C CH505 SHIV-infected CEM.NKR target cells by a rhesus CD16+ (FcγR3) NK cell line in the presence of immune serum. As shown in **FIG. 3I**, serum from Th1 vaccinated animals demonstrated significantly greater ADCC activity at week 2 and week 8 after the 2nd protein boost when compared to Th1+2 immunized animals ($p < 0.01$). Serum collected from Th1 vaccinated animals at week 8 post protein boost 2 also mediated significantly greater phagocytosis of C.1086 gp120-coated beads by the CD32+ (FcγR2) and CD64+ (FcγR1) THP-1 monocytic cell line (**FIGS. 3J** and **3K**). To determine if the adjuvants given to animals in the Th1 and Th1+2 groups generated different IgG subclass antibody repertoires, we quantified C.1086 gp120-specific IgG1, IgG2, IgG3, and IgG4 by ELISA. We found that IgG2 and IgG3 antibodies were extremely low and did not differ between groups (**FIG. 3L**). However, Th1 vaccinated animals had higher gp120-specific IgG1 ($p < 0.0001$, **FIG. 3L**) while Th1+2 vaccinated animals had higher gp120-specific IgG4 resulting in markedly elevated IgG1/IgG4 ratio in the Th1 vaccine group ($p < 0.001$, **FIG. 3M**). These results are consistent with the report that antibodies of the IgG1 subclass are the most functional in rhesus macaques (28, 29).

[0138] *DNAIP-10ProteinALFQ vaccine elicits robust anti-Env antibody in vaginal and rectal mucosal compartments.* Having established induction of higher serum IgG antibody titers in Th1 vaccinated animals, we next sought to determine whether mucosal anti-Env antibodies were also correspondingly increased. To this end, we assayed rectal and vaginal

secretions for C.1086 gp140-specific IgG and IgA antibodies at baseline and longitudinally after each of the protein boosts.

[0139] The appearance of gp140-specific IgG in secretions closely mimicked the kinetics of the serum IgG antibody response, with each protein boost increasing levels of Env-specific IgG antibodies in vaginal and rectal secretions (**FIGS. 4A** and **4B**). As in serum, the Th1 vaccine regimen generated higher levels of specific IgG in secretions when compared to the Th1+2 vaccine. The gp140-specific IgA in vaginal and rectal secretions was also increased to a greater extent by the Th1 vaccine regimen (**FIGS. 4C** and **4D**). Notably, at week 16 post 2nd protein boost, vaginal IgA antibodies were still above the limit of detection in most Th1 vaccinated animals but in only 2 of 10 Th1+2 vaccinated animals. Analysis of gp140-specific IgA in serum revealed higher induction in the Th1 group (**FIG. 4E**). However, the kinetics of the serum IgA response in Th1 as well as Th1+2 animals differed strikingly from the mucosal IgA responses, especially in the reproductive tract (**FIGS. 4C** and **D**), suggesting a true mucosal (locally-derived) IgA response was generated in these animals. This was most evident after the 2nd protein boost, when vaginal IgA antibodies to gp140 were found to be dramatically increased but serum IgA antibodies were reduced (**FIGS. 4A** and **4C**). Together, these data demonstrate that the Th1 vaccine regimen was more effective than the Th1+2 regimen for generating long-lived anti-Env binding antibodies in serum and secretions, as well as serum IgG antibodies with greater breadth, avidity, and function.

[0140] *DIP-10 ProteinALFQ vaccine induces Env-specific Tfh cells and GC Tfh cells with distinctive Th1 signatures.* The DIP-10 PALFQ Th1 vaccine promoted anti-Env antibody longevity and functionality following the 1st protein boost. Based upon this finding, we wanted to determine whether this vaccine regimen also correspondingly enhanced Tfh responses in the periphery and LNs. To this end, we first assessed whether higher magnitude of Env-specific Tfh cells were induced 7 days after 1st boost, corresponding to the peak of the effector response. PBMCs were stimulated with overlapping peptide pools representing Con C gp140 together with C.1086 gp140 protein. The induction of the activation markers CD25 and OX40 was assessed by flow cytometry after stimulation (**FIG. 5A**, flow plot) (30). The analysis revealed a higher frequency of Env-specific CD4 T cells in DIP-10 PALFQ animals. When expressed as a percentage of CD95+ CD4 T cells, median frequencies of Env specific-CD4 T cells were on average 10-fold higher in Th1 group, indicative of a higher magnitude Env-specific Tfh response ($p < 0.001$, **FIG. 5B**). The frequency of Env-specific CD4 T cells responding to the C.1086 gp140 priming immunogen correlated positively with

CD4 T cells responding to the C.ZA gp140 boosting immunogen ($p < 0.0001$, **FIG. 5C**). Evaluation of IP-10 levels in sera using a flow-based Legend plex assay at day 0 and 3 of 1st protein boost in the ALFQ-adjuvanted animals confirmed induction of IP-10 following the Th1 protein boost ($p < 0.05$, **FIG. 5D**).

[0141] Next, we assessed LN responses using biopsies collected at day 14 post 1st protein boost and identified GC Tfh cells as CXCR5⁺, PD-1⁺⁺⁺ cells (red population, **FIG. 6A**) and GC B cells as Ki-67⁺, Bcl-6⁺ CD20 cells. As expected, Bcl-6 expression was highest on GC Tfh cells relative to PD1⁺⁺ Tfh and PD1⁻ memory Tfh populations. GC Tfh cells also expressed higher relative levels of ICOS compared to other CD4 subsets, and consistent with the functional ability of Tfh cells (12), our *ex vivo* analysis of sorted GC Tfh cells revealed their capacity to support IgG production from autologous B cells (**FIG. 6B**). Evaluation of GC TFH frequencies over the course of immunization revealed a significant induction of GC Tfh cells 2 weeks after protein boost 1, and significantly higher frequencies 2 weeks after protein boost 2 (**FIG. 6C**).

[0142] Following the 1st protein boost, we found that GC B cell frequencies were significantly higher in the Th1 vaccine regimen ($n=10$ animals in each group; median Th1+2: 14.2% (of CD20⁺ cells) versus Th1: 25%, $p < 0.05$), and the frequency of GC Tfh cells strongly correlated with GC B cell responses. (**FIG. 6D**). Importantly, Env-specific Tfh cell frequencies in the LN directly correlated with GC Tfh cell frequencies but not memory Tfh cells, indicating that GC Tfh cells were enriched for vaccine-induced follicular cells ($p < 0.0001$, **FIG. 6D**). Next we assessed expression of CXCR3, which is heterogeneously expressed by GC Tfh cells (histogram, **FIG. 6E**), and found higher relative expression of CXCR3 both within GC Tfh and GC B cells in the Th1 vaccine group ($p < 0.05$, **FIG. 6E**). Notably, T-bet expression on B cells, a marker of memory B cells (31), corresponded with CXCR3 expression, suggesting a mechanistic basis for enhanced antibody responses in the Th1 vaccine group. Consistent with this hypothesis, the magnitude of Tfh cells and the relative frequency of CXCR3⁺ Tfh cells within the GC was directly predictive of serum antibody titers at week 18 post 2nd protein boost. GC responses predicted antibody avidity at week 8 after the 2nd protein boost, supporting our hypothesis that induction of Th1-Tfh cells enhanced the quality of anti-Env antibody responses ($p < 0.05$, **FIG. 6G**).

[0143] To gain insights into the molecular mechanisms underlying successful antibody responses, we next determined transcriptional signature in GC Tfh cells. To achieve this goal,

we sorted four different CD4 subsets from the LNs of 3 Th1 group animals with highest antibody titers at week 8 post 1st protein boost. These subsets were naive cells (CD4+CD95-), Tfh cells (CD95+CXCR5+PD-1+/+++), memory Tfh cells (CD95+CXCR5+ PD-1-), and memory non-Tfh cells (CD95+ CXCR5-PD-1-). Similar subsets were sorted from 2 high responder animals in the Th1+2 vaccine group. (animal sorted identified by triangles, **FIG. 6H**).

[0144] RNA samples meeting quality control checks were sequenced using a 3'-Tag-RNA-Seq library prep protocol on the Illumini HiSeq 4000 platform. Prior to analysis of sequenced reads, genes with fewer than 40 counts per million reads were filtered, leaving 7,086 genes. Differential expression analyses were conducted using the limma-voom Bioconductor pipeline. We performed significance analysis of microarrays using the Dseq package from R/Bioconductor to compare the transcriptome profiles. Principal component analysis of the top 500 differentially expressed genes showed that the majority of variation in the data was driven by CD4 subsets. Memory CD4 T cells (CXCR5+ and CXCR5-) clustered closely together relative to naive and Tfh subsets (**FIG. 6I**). Segregation of Tfh cells between the Th1 and Th1+2 groups indicated adjuvant dependent modulation of transcriptional activity within the germinal center, suggestive of qualitative differences in Tfh cells between vaccine groups. To extract information on biologically relevant gene-sets, we performed gene set enrichment analysis with the goal of determining biological pathways that were enriched in Tfh cells in the Th1 vaccine regimen. Genes regulating interleukin (IL)-12, tumor necrosis factor (TNFa), interferon gamma (IFNG), and IL-6 production were strongly enriched in Tfh cells. Consistent with metabolic activity of effector cells and functional capacity of Tfh cells, pathways regulating cellular metabolism, glucose homeostasis, and B cell proliferation were also enriched (**FIG. 6J**).

[0145] To determine transcriptional activity of Tfh cells in the Th1 vaccine group, we focused on differentially induced genes in Tfh cells relative to naive cells (n=89, adj. p < 0.05, **FIG. 6K**), of which induction of key Tfh transcripts including CXCR5, ICOS, and Bcl-6 was common to both Tfh cells and memory Tfh cells (**FIG. 6L**). Consistent with representation of Th1 genes in GSEA, Tfh cells showed higher expression of TBX21 and IFNG (**FIG. 6L**). The class IV semaphorin protein (SEMA4A), a co-stimulatory molecule expressed by Th1 cells (32), was significantly induced, as was high-mobility group box 1 (HMGB1), an inflammatory mediator regulating TNF and IL-6 production (33). Induction of IL-18R suggested the capacity of IL-18 to drive IFN γ production within the GC (34).

Likewise, we noted higher expression of receptor interacting serine/threonine kinase 2 (RIPK2), which drives IFN γ in Th1 cells and contributes to Th1 differentiation (35). The corresponding downregulation of IL-4R in Tfh cells indicated enrichment of the Th1 program within Tfh cells in DIP-10 ProALFQ vaccinated animals. This, together with increased protein expression of CXCR3 within the GC, supports the conclusion that CD4 T cell help for humoral immunity was driven by Th1 Tfh cells in the Th1 vaccine regimen (**FIG. 6I**).

[0146] *DNAIP-10 immunization induces systemic expansion of pro-inflammatory monocytes and enhances GC Tfh responses.* Based on increased frequencies of Env-specific Tfh cells and evidence for induction of a Th1 transcriptome program in Th1 vaccinated animals following the 1st protein boost, we sought to assess Tfh responses during the priming immunization phase. First, we evaluated blood to quantify activated CXCR5⁺ CD4 T cells (**FIG. 7A**). Based on co-expression of ICOS and PD-1, activation markers induced upon TCR stimulation, the data showed that DNA immunization significantly increased the relative frequencies and absolute counts of ICOS⁺ PD-1⁺ CXCR5⁺ CD4 T cells in blood at day 14 (n=20; median frequencies, day 0: 3.38%; day 14: 6.7%, p < 0.0001; n =20; absolute counts, day 0: 3.04; day 14, 8.7 day 14, p < 0.01, **FIG. 7A**). Based on the robust induction of activated CXCR5⁺ CD4 T cells, we next assessed whether a concomitant acute induction of pro-inflammatory monocytes (innate cells that drive Tfh responses) preceded the activated CXCR5⁺ response (36, 37). We quantified frequencies of CD14⁺ CD16⁺ HLA-DR⁺ (lineage-) cells in blood (**FIG. 7B**) and discovered rapid and robust expansion of pro-inflammatory monocytes with significantly higher induction in the Th1 vaccine group (**FIGS. 7C and 7D**). Consistent with increased pro-inflammatory monocyte frequencies at day 3, IL-1 β levels, determined using a flow-based Legendplex assay, were elevated at day 7 following the 3rd DNA boost. Based on evidence showing that monocyte-derived IL-1 β drives effective Tfh and CD4 T cell differentiation, we examined LN responses (**FIG. 7E**). Strikingly, the GC Tfh cell frequencies within the fine-needle aspirates of the draining LN were higher in the Th1 vaccine group following the 3rd DNA prime (**FIG. 7F**). Notably, the greater inflammatory response was associated with increased levels of antibodies, linking the innate immune response to priming of effective CD4 Tfh help (**FIG. 7G**).

Discussion

[0147] The present study gives rise to three main conclusions; first, that an HIV-1 vaccine platform designed to promote Th1-polarized Tfh cells increases the number of circulating

Env-specific Tfh cells, enhances GC responses, increases anti-Env binding antibody titers in sera, stimulates serum antibody effector functions. Second, that a Th1 vaccine regimen can elicit anti-Env vaginal and rectal IgA responses; and third that induction of high avidity antibodies, reflective of productive GC responses, are engendered by a Th1 vaccine regimen. Collectively, the data demonstrate that adjuvant-induced stimulation of Th1-Tfh cell production during the vaccine prime and boost is an effective strategy to enhance the longevity and functionality of the anti-Env antibody response.

[0148] Productive T cell responses critically depend on cytokine signals during priming, and recent studies demonstrate that monocyte-derived IL-1 β drives effective CD4 T cell differentiation and Tfh responses (37-39). Here, investigation of the kinetics of pro-inflammatory monocytes – cellular innate biomarkers of adjuvanticity – revealed a transient increase in CD14⁺CD16⁺ monocytes in blood with a higher relative increase in the Th1 vaccine group and a corresponding transient increase in IL-1 β . Strikingly, fine-needle aspirates of the draining LNs showed higher GC frequencies in the Th1 vaccine group, indicating active/productive GC responses. Notably, the improved inflammatory response was associated with increased antibody longevity linking the innate immune response to effective induction of CD4 Tfh cells. Indeed, several recent studies show that potent priming of the immune response sets the stage for stronger boosting of cellular and humoral immunity in the setting of DNA prime, NYVAC boost and Ad5 prime, NYVAC boost vaccine regimens (25, 40). The effectiveness of priming is not limited to CD4 T cells and B cells; a DNA vaccine targeting conserved elements of SIV Gag robustly primes cytotoxic T cells which are effectively boosted following a long rest period (41, 42). These data open the possibility to a critical window of opportunity during the priming phase. This window can be exploited to prime for long-lasting, durable CD4, CD8 T cell, and antibody responses to HIV-1 vaccination.

[0149] The HVTN studies 070 and 080 employed the IL-12 DNA adjuvanted plasmid with the subtype B PENNVAX-B (PV) DNA plasmid and showed 80% response rates after the third DNA vaccination in PV+IL-12 recipients compared to a 44% response rate with the PV alone vaccine. A subsequent follow up study demonstrated robust recall of binding anti-Env antibody titers with ADCC activity following an MVA boost in PV+IL-12 recipients (43, 44). Because IL-12 is a classic innate mediator of Th1 responses, the data suggest that an increase in Th1 GC Tfh cells may underlie the observed effects. Correspondingly, studies in rhesus macaques with an ALVAC prime, ALVAC + gp120 protein boost using SIV immunogens

showed higher SIV Env titers with MF59 compared to alum adjuvanted protein boosts 2 weeks following the final immunization (45). While Tfh responses and memory antibody titers were not examined, a recent study in humans showed enhanced binding antibody titers 26 weeks after booster immunization with a Th1 GLA-SE-adjuvanted malaria antigen relative to one formulated in aluminum (46). These studies in conjunction with the present study provide support to the immune potential of Th1-Tfh cells in fostering long-lived antibody titers. In contrast, a study using a homologous subtype C protein immunization reported induction of higher anti-Env antibody titers with aluminum-hydroxide Ahydrogel[®] relative to Addavax[™], an MF59 analog, in rabbits (47). Collectively, these data indicate the importance of detailed studies to understand the context in which Th1 responses are superior to Th1+2 responses and how viral versus DNA vectors and subunit proteins influence this paradigm.

[0150] Our findings raise the question of the mechanisms underlying the DIP-10ProALFQ vaccine-mediated enhancement in Tfh responses. A few possibilities can be explored; IP-10 increases dendritic cell-T cell interactions, which could have favored Tfh differentiation (48). IP-10 also increases IL-6 production in B cells which is known to support Tfh differentiation and enhance plasma cell differentiation (49). This, together with the potent immune stimulatory potential of MPL+QS-21 boost, may have synergized to enhance Tfh responses numerically and favored Th1 differentiation program within Tfh cells (50). Indeed, GC Tfh cells induced following viral infections where Th1 inflammatory responses predominate express Bcl-6, Tbx21, IFNG, and IL-21 consistent with the induction of Th1-type Tfh cells (51). Our transcriptomic analysis of Tfh cells following the 1st protein boost in the Th1 vaccine regimen show coordinate expression of Th1 regulated genes as evidenced by enrichment of pathways related to IFNG signaling. The higher relative expression of the Th1 chemokine receptor CXCR3 in GC Tfh cells and GC B cells validate the gene expression data. Together, our transcriptomic and phenotypic data on Tfh cells indicate a role for adjuvant induced quantitative (increased Tfh numbers) and qualitative (increased proportion of Th1-Tfh cells) effects on antibody longevity. Mechanistic studies are needed to discern the respective contribution of increased Tfh numbers versus Th1 skewing of Tfh cells on antibody responses as both these characteristics are inextricably linked in the current study.

[0151] In addition to adjuvant-dependent modulation of Tfh responses, we made the striking observation that the Th1 vaccine regimen induced significantly higher anti-Env IgG1 titers while the Th1+2 vaccine regimen induced relatively higher anti-Env IgG4 titers. These

data provide the strongest evidence for adjuvant impacting antibody subclass profile in rhesus macaques to an Clade C HIV vaccine regimen. Thus, the predominance of IgG1 over IgG4 at week 8 following the last boost, a time point when majority of the circulating antibody is plasma cell derived, supports the conclusion that the Th1 vaccine regimen induced qualitatively different GC responses relative to a Th1+2 vaccine regimen. Enhancement of antibody effector functions – both ADCC and ADP in the Th1 vaccine group are consistent with report antibodies of the IgG1 subclass are the most functional in rhesus macaques (28, 29) and corroborate our conclusions.

[0152] Another notable observation was induction of robust mucosal anti-Env IgA responses in the reproductive tract and in rectal secretions. Interestingly, unlike mucosal anti-Env IgA responses, which were boosted after the 2nd protein immunization, serum IgA responses were not correspondingly enhanced. This incongruity between mucosal and serum IgA responses suggests that a true mucosal (locally-derived) response was generated in vaccinated animals and that the Th1 vaccine was more effective in inducing mucosal immunity. These findings raise the possibility that induction of Th17-type Tfh cells within the mucosal draining lymph nodes could underlie mucosal IgA responses. Although our studies were not designed to evaluate this possibility, the effectiveness of the Th1/Th17 adjuvant cationic liposomal formulation (CAF01) suggests that induction of Th1/Th17-type Tfh cells may be extremely important to drive robust mucosal IgA responses while correspondingly enhancing serum anti-Env IgG responses and functionality (52).

[0153] In summary, our findings demonstrate that Th1-DNA prime substantially increases the Env-specific Tfh cells relative to a Th1+Th2 vaccine regimen and that Th1-protein boost results in greater production of the IgG1 subclass with enhanced longevity, breadth, avidity, ADCC, and ADP activities of anti-Env antibody.

Materials & Methods

[0154] *Rhesus Macaques.* Twenty adult female colony-bred rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center and maintained in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. All studies were approved by the University of California Davis Institutional Animal Care and Use Committee (IACUC). At study initiation, animals were 3.5 - 4.5 years of age with a median weight of 5.3 kg, were SIV- STLV- SRV-, had no history of dietary or pharmacological manipulation, and had intact ovaries.

[0155] *Immunizations.* DNA immunizations were administered via intradermal injection with electroporation utilizing the ICHOR TriGrid Array (Ichor Medical Systems, San Diego) at weeks 0, 8, and 16. For each DNA immunization, two groups of 10 animals received 4 mg of the pGA2/JS2 plasmid DNA vector (53) encoding either SHIV C.1086 T/F Env + interferon-induced protein (IP)-10 (Group 1) or SHIV C.1086 T/F Env alone (Group 2). Details of the SHIV DNA construct have been described (54). At weeks 30 and 44, Group 1 animals received boosts with 100 µg C.ZA 1197MB gp140 protein (Immune Technology, New York, NY) adjuvanted with 100 µg MPLA + 50 µg QS-21 (ALFQ) and Group 2 animals received 100 µg C.ZA 1197MB gp140 adjuvanted with 100 µg MPLA + 600 µg Aluminum (ALFA). The protein formulation was delivered in a 250 µl volume (50 µg protein) subcutaneously in each thigh during both boosts.

[0156] *Adjuvants.* Dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) saturated phospholipids, cholesterol (Chol), and synthetic monophosphoryl lipid A (MPLA, 3D-PHAD) were purchased from Avanti Polar Lipids (Alabaster, AL). DMPC and Chol were dissolved in chloroform, and DMPG and MPLA were dissolved in chloroform:methanol (9:1). Alhydrogel[®], aluminum hydroxide (AH) in a gel suspension was purchased from Brenntag (Frederikssund, Denmark). Saponin, QS-21 were purchased from Desert King International (San Diego, CA) and was dissolved in Sorensen PBS, pH 5.6.

[0157] Army liposome formulations (ALF) containing DMPC, DMPG, Chol, and MPLA were prepared by the lipid deposition method. For vaccine preparations adjuvanted with ALFA, dissolved lipids were mixed in a molar ratio of 9:1:7.5:0.36 (DMPC:DMPG:Chol:MPLA) and dried by rotary evaporation followed by overnight desiccation. Liposomes were formed by molecular biology grade water (Quality Biological, Gaithersburg, MD), microfluidized, and sterile filtered, followed by lyophilization. 100 µg of gp140 protein was adsorbed to 600 µg of Alhydrogel in PBS, pH 7.4, and incubated on a tilted roller at room temperature (RT) for 1 h prior to adding to lyophilized ALF. For vaccine preparations adjuvanted with ALFQ (ALF containing QS-21), lipids were mixed in a molar ratio of 9:1:12.2:0.36 (DMPC:DMPG:Chol:MPLA), dried, rehydrated by adding Sorensen PBS, pH 6.2, followed by microfluidization and filtration. gp140 was mixed with ALFQ in a 1:1 volume ratio. Each vaccine dose in 500 µl volume contained 100 µg MPLA (and 100 µg protein) and either 600 µg aluminum or 50 µg QS-21.

[0158] *Specimen collection and processing.* Lymph node (LN) biopsies were obtained 2 weeks following each of the protein boosts and were manually processed by disassociation through 100µM cell strainers and washing in complete media, as described previously (12). Two weeks after the 3rd DNA immunization, fine needle aspirates of LN were obtained using a 22 gauge needle, as previously described (55). PBMCs were isolated from whole blood collected in CPT vacutainer tubes by density gradient centrifugation as previously described (12). For serum, coagulated blood was centrifuged at 800g for 10 min to pellet clotted cells, followed by extraction of fluid and storage at -80° C. Rectal and vaginal secretions were collected using premoistened Weck-Cel sponges and eluted as described (56).

[0159] *C.1086 gp140-specific serum IgG ELISA.* Serum IgG titers against HIV-1 C.1086 Env gp140 were determined by ELISA. In brief, 96-well microtiter plates with high binding capacity (Thermo Fisher, MA) were coated overnight at 4° C with 1 µg/mL C.1086 Env gp140C from the NIH AIDS Reagent Program (ARP) diluted in 0.1M carbonate-bicarbonate buffer, pH 9.2. Plates were washed with PBS containing 0.1% Tween-20 (PBST) and blocked with 5% w/v nonfat dry milk in PBS for 2h at RT followed by four washes with PBST. Standard (PG9 monoclonal antibody from the ARP) and serum samples were run at 3 dilutions/sample (1:50-1:450) in sample dilution buffer and incubated at RT for 2 h on a microplate shaker. After washing, the plate was incubated for 1 h with 1:10,000 HRP conjugated goat anti-monkey IgG (Nordic MUBio, Netherlands). The plates were washed and then developed with TMB substrate (Thermo Fisher) and the reaction was quenched with 2N H₂SO₄ (Sigma, St. Louis, MO). Absorbance was recorded at 450nm with a reference filter at 570 nm using a Spectramax 5 plate reader (Molecular Devices, Sunnyvale, CA). Baseline sera from each animal served as negative control and OD values 2-fold above baseline were considered positive and extrapolated to determine anti-Env antibody concentrations.

[0160] *Sodium thiocyanate avidity assay.* C.1086 Env gp140C-specific IgG antibody avidity was determined using a chaotropic displacement ELISA with NaSCN. Serum samples were incubated in duplicate at 6000 pg per well for 2h at RT. The plate was washed five times. For the dissociation step, one well of each sample was manually treated with 100 µL of 2 M NaSCN (Sigma-Aldrich) to dissociate antigen-antibody complexes and a second well of the same sample was treated with PBS as a control. The plate was incubated for 15 min at RT, followed by washing three times. The plate was then developed as described above for the C.1086 gp140C ELISA. For each sample, antibody avidity was reported as an avidity

index value (a percentage), which was calculated as the ratio of absorbance in the well treated with NaSCN to that in the well treated with PBS.

[0161] *Biacore binding and avidity analysis.* Binding and avidity determination were conducted using Surface Plasmon Resonance (SPR) Biacore 4000 system. The immobilizations were performed in 10 mM HEPES and 150 mM NaCl pH 7.4 using a standard amine coupling kit, as previously described (23, 57). The CM5-S series chip surface was activated with a 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 0.1 M N-hydroxysuccinimide (NHS) for 600s (GE Healthcare). For the cyclic biotinylated V2 C.1086 peptide, 1 μ M Streptavidin (Life Technologies) in 10 mM sodium acetate pH 4.5 (5,800 - 7,400 RU) was coupled for 720s. The immobilized surface was then deactivated with 1.0 M ethanolamine-HCl pH 8.5 for 600s. Spot 3 in each flow cell was left unmodified to serve as a reference. Following surface deactivation, 0.06 - 1.5 μ M cyclic biotinylated V2 C.1086 peptide was captured, resulting in two range of densities; high density (1,900 – 2,300 RU) and low/medium density (340 – 580 RU). For C.1086 gp140C, 0.56 – 15 μ g/mL protein was immobilized directly on the sensor CM5 chip, resulting in four ranges of densities; very high density (9,800 – 10,100 RU); high density (3,400 – 4,100 RU); medium density (960 – 1,700 RU) and low density (240 - 670 RU). Following surface preparation, heat inactivated serum samples were diluted 1:50 in the running buffer (10 mM Hepes, 300 mM NaCl and 0.005% Tween 20, pH 7.4). The diluted samples were injected onto the V2 peptide or gp140 protein surface for 320 s followed by 1,800 s dissociation period. The bound surface was then enhanced with a 240 s injection of 30 μ g/mL secondary antibody goat anti-monkey IgG. To regenerate the bound surface, 175 mM HCl was injected for 70s. For each serum sample or controls, 4 - 8 replicates were collected at a rate of 10 Hz, with an analysis temperature of 25° C. All sample injections were conducted at a flow rate of 10 μ L/min. Data analysis was performed using Biacore 4000 Evaluation Software 4.1 with double subtractions for unmodified surface and buffer for blank. The fitting was conducted using the dissociation mode integrated with the Evaluation software 4.1.

[0162] *Binding Antibody Multiplex Assay (BAMA) and sodium citrate avidity assay.* SIV-specific serum IgG BAMA was performed with a panel of Env and V1V2 antigens: C.1086 gp140, CH505 TF gp140, Con S (group M consensus) gp140, and Con C (clade C consensus) gp140, gp70-V1V2 Clade B/Case A2 scaffolded protein and C.1086 V1V2 avitagged protein. Samples were titrated in 5-fold serial dilutions starting at 1:80 and binding magnitude is reported as AUC. Positivity criteria (determined at dilution 1:80) was as follows: (1) MFI

>100; (2) MFI > Ag-specific cutoff (95th percentile of all baseline binding per antigen); (3) MFI 3-fold > than that of the matched baseline before and after blank/MuLV subtraction. All BAMA and avidity assays were performed in a blinded fashion using magnetic beads. For avidity assays, samples were tested with and without sodium citrate (0.1 M, pH 3.0) at 2 dilutions for each antigen based on BAMA titration for maximum coverage of samples in the linear range of the assay. The dilutions were 1:80 and 1:400 for gp70-V1V2, 1:400 for C.1086 V1V2, 1:2000 for CH505TF gp140, 1:2000 for ConC gp140, and 1:10000 for C.1086 gp140 and ConS gp140. Antibody avidity is reported as avidity index, which was calculated as $100 \times (\text{MFI in the citrate-treated well} / \text{MFI in the untreated well})$. Avidity index is reported for sample-antigen combinations that were (1) identified as positive responders in the IgG BAMA assay and (2) had an MFI within the linear range for the untreated sample. The pre-set assay criteria for sample reporting were coefficient of variation per duplicate values of <15% for each sample and >100 beads counted per sample.

[0163] *Neutralization.* Neutralization assays were performed as previously described (58) using TZM-bl cells. We measured neutralization activity against the tier 1 clade C pseudovirus MW965.26 using MLV-pseudotyped virus as an indicator of non-HIV-specific activity in the assay. Neutralization titers were measured at week 2 and week 8 post 2nd protein boost and were considered to be positive for neutralizing antibody activity based on the criterion of signal >3x detected against the MLV negative control virus. The majority of positive titers detected were against the tier 1 virus MW965.26 with occasional very weak neutralization titers against the tier 2 viruses.

[0164] *Antibody-dependent cellular cytotoxicity.* Engineered human cell lines CD16+ KHYG-1 (effector cells) and CEM.NKR-CCR5-sLTR-Luc (target cells) provided by D. Evans were maintained in R10 cell culture medium consisting of RPMI medium supplemented with 10% fetal bovine serum, 25 mM HEPES, 2 mM l-glutamine, and 0.1 mg/ml Primocin (59, 60). The growth medium of CD16+ KHYG-1 cells was additionally supplemented with cyclosporine (CsA) and interleukin-2 (IL-2) at a concentration of 1 µg/ml and 5U/mL, respectively.

[0165] Luciferase based ADCC assays were carried out as previously described with some modifications (59). Two million CEM.NKR-CCR5-sLTR-Luc target cells were spinoculated with SHIV.C.CH505.375H.dCT (38 ng p27) for 2 hours at 2,600 rpm at 30° C in the presence of 1 µg/mL polybrene. Subsequently, the target cell/virus mixture was incubated overnight at

37° C 5% CO₂. The next day, virus was removed and cells were incubated for another 72 hours prior to the ADCC assay. For the ADCC assay, serum: effector cells: target cells were plated in a 1:1:1 volumetric ratio. Sera was heat inactivated and diluted (1:50 dilution in R10 containing 10 U IL-2 per mL, with no CsA), mixed with PBS-washed, infected target cells (1×10^4 cells per well), and effector cells (5×10^4 cells per well). Assay plates were incubated overnight at 37°C and 5% CO₂. Plates were then centrifuged at 1,800 rpm for 5 min at room temperature and 100 µL of the supernatant was removed. The cell pellets were resuspended and mixed with 50 µl of the luciferase substrate reagent BriteLite Plus (Perkin Elmer, MA). Luciferase activity was read in black 96-well plates according to the manufacturer's instructions using a Synergy 2 micro plate luminometer (BioTek). Percent ADCC activity of each tested animal immune serum (week 2 and week 8 post 2nd protein) measured as reduction in RLUs, was calculated based on respective week 0 pre-immune serum (100% RLU). All samples were tested in triplicates and experiments were performed twice.

[0166] *Antibody Dependent Phagocytosis.* Serum antibodies were tested for ability to enhance phagocytosis of gp120 expressing beads by THP-1 cells using methods similar to those previously described (58). Briefly, 5 µL of 1 µm avidin-coated Fluorospheres (Invitrogen) were labeled with 2 µg biotinylated anti-His tag antibody (Pierce), then 3.5 µg His-tagged Clade C gp120 Du151 protein (Immune Technologies) per plate. The gp120 beads and triplicate 5-fold dilutions of heat-inactivated serum in a 50 µL volume were then pre-incubated at 37° C in V-bottom plates. After 1 h, 2×10^4 THP-1 cells in 50 µL were added to each well. After 5 h at 37°C in 5% CO₂, the cells were washed in Ca⁺² and Mg⁺² - free DPBS and resuspended in 180 µL of warm 0.12% Trypsin/EDTA. After 5 min at 37° C, the trypsin was removed and the cells were resuspended in 1% paraformaldehyde. Fluorescence was evaluated using a FACS Canto (BD Biosciences) and Flo-jo software. Phagocytosis was measured by multiplying the % fluorescent cells by their median fluorescence intensity. The phagocytic score was then calculated by dividing phagocytosis of test samples by the average phagocytosis measured with preimmune serum.

[0167] *IgG subclass antibodies.* Ten rows of a 96-well Immulon 4 microtiter plate (VWR) were coated overnight at 4° C with 50 ng per well of C.1086 gp120 Δ7 K160N protein (61) in PBS. The remaining 2 rows were coated with duplicate 2-fold serial dilutions of rhesus IgG1, IgG2, IgG3 or IgG4 (Nonhuman Primate Reagent Program) starting at 25 ng/mL in PBS to generate a standard curve. Plates were washed with PBS containing 0.05% Tween 20 and blocked for 30 min at RT with reagent buffer (0.1% bovine serum albumin in wash buffer).

Two- or three-fold dilutions of serum in reagent buffer were then added to the wells coated with gp120. Reagent buffer was added to wells coated with standard. Following overnight storage at 4° C, the plate was washed and reacted for 1 h at 37° C with 1 µg/mL of the relevant monoclonal antibody from the Nonhuman Primate Reagent Program: anti-rhesus IgG1 (mouse IgG2a clone 3C10), anti-rhesus IgG2 (mouse IgG1 clone 3C10), anti-rhesus IgG3 (mouse IgG1 clone 2G11) or anti-rhesus IgG4 (mouse IgG1 clone 7A8). The plate was then consecutively washed and treated with 100 ng/mL of biotinylated goat anti-mouse IgG1 or IgG2a for 1 h at 37° C, neutralite-avidin peroxidase for 30 min at RT, and TMB (all from SouthernBiotech). Absorbance was recorded at 370 nm. SoftMax Pro software (Molecular Devices) was used to construct a standard curve and determine concentrations of antibody. Preimmune serum samples had < 10 ng/mL of antibody in these assays.

[0168] *Mucosal antibodies and serum IgA.* BAMA with C.1086 gp140 K160N-labeled magnetic beads (MagPlex, BioRad) was used as previously described (61) to measure concentrations of antigen-specific IgG in secretions and IgA in both secretions and serum depleted of IgG. Briefly, beads reacted with dilutions of standard (62) and specimens at 1100 rpm and 4° C overnight were washed and developed with biotinylated anti-monkey IgG or -monkey IgA (Rockland) followed by Phycoerythrin-labeled Neutralite avidin (SouthernBiotech). Construction of standard curves and interpolation of antibody concentrations was done using Bioplex Manager software after measurement of fluorescence in a Bioplex 200 (BioRad). Concentrations of gp120-specific IgG or IgA in secretions were divided by the total IgG or IgA measured in the sample by ELISA (63) to obtain the specific activity (ng IgG or IgA antibody per µg total IgG or IgA).

[0169] *Activation induced Marker (AIM) assay.* Cells were stimulated with overlapping peptide pools of HIV consensus C and HIV-1 C.1086 Env gp140C protein in AIM media as previously described (30). All antigens were used at a final concentration of 2 µg/mL in a stimulation cocktail made with using 0.2 µg of CD28 and 0.2 µg CD49d costimulatory antibodies per test. Unstimulated controls were treated with volume-controlled DMSO (Sigma-Aldrich). Tubes were placed in 5% CO₂ incubator at 37° C and incubated overnight. Following an 18 hour stimulation, the cells were stained, fixed, and acquired the same day. Phenotype panel on lymph nodes and PBMCs was performed using standard flow cytometry assays.

[0170] *Legend Plex assay.* A Legendplex assay was performed to evaluate cytokines in rhesus macaque sera (Biolegend). The assay was performed according to the manufacturer's protocol. Samples were acquired on a BD LSR Fortessa cell analyzer.

Table 1.

S. No.	Reagent	Fluorochrome	Clone	Catalog number	Source
1.	CD28		CD28.2	555725	BD Biosciences
2.	CD49d		9F10	555501	BD Biosciences
3.	CD3	AF700	SP34-2	557917	BD Biosciences
4.	CD4	BV650	L200	563737	BD Biosciences
5.	CD8	BV510	SK1	563919	BD Biosciences
6.	CD20	Pacific blue	2H7	302328	BioLegend
7.	CXCR5	PE	MU5UBEE	12-9185	Thermo Fischer
8.	CD25	APC	BC96	20-0259	Tonbo Bio
11.	CD134	BV786	L106	744746	BD Biosciences
12.	CD95	Pe-Cy5	DX2	564710	BD Biosciences
13.	Live/dead	Aqua/BV510	NA	L-34966	Invitrogen
14.	ICOS	BV786	C398.4A	313534	BioLegend
15.	CXCR3	APC	IC6	550967	BD Biosciences
16.	PD-1	APC-Cy7	EH12.2H9	329922	BioLegend
17.	IP-10	PE	J034D6	519503	BioLegend
18.	Bcl-6	A488	K112-91	561524	BD Biosciences
19.	Ki-67	BUV 395	B56	564071	BD Biosciences

[0171] *Flow cytometry and cell sorting.* Cells were acquired on a BD FACSymphony using FACS Diva version 8.0.1. Compensation, gating and analysis were performed using FlowJo (Versions 9 and 10). Cell sorting was performed using BD FACSAria III. Reagents used for flow cytometry are listed in Table 1.

[0172] *Statistical analysis.* Statistical analysis was performed using GraphPad Prism 7. Results for groups were compared using the two-tailed nonparametric Mann-Whitney rank sum test. For correlation analysis, the two-tailed Spearman rank correlation test was used.

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Example 2. Targeting CD4 Tfh Cells to Enhance HIV Vaccine-Induced Humoral Immunity

Summary

[0173] With over 2.5 million new HIV infections per year, the majority in resource-poor countries with limited access to antiretroviral therapy, an effective HIV vaccine continues to remain among the most promising and safe strategies for preventing infection and reducing the burden of AIDS. While designing an effective HIV vaccine is a daunting goal, the modest efficacy of the RV144 Thai trial indicates that it is possible to prevent HIV infection in humans by vaccination, and that long-lasting antibody contributes significantly for this protection. Because vaccine-elicited CD4 T follicular helper cells (Tfh) are essential for establishing long-lived antibody, harnessing Tfh help is vital for an effective HIV vaccine. This Example focuses on achieving this goal using a recombinant DNA-prime, modified vaccinia Ankara (MVA)-boost vaccine platform in path for clinical development. Our data show that generation of Tfh cells expressing the chemokine receptor CXCR3 correlates strongly with multiple attributes of protective humoral immunity. Furthermore, CXCR3⁺ Tfh cells express higher levels of B cell helper factors relative to CXCR3⁻ Tfh cells. Without being bound by the following theory, it is believed that HIV vaccines designed to induce high magnitude CXCR3⁺ Tfh cells increases antibody persistence, enhancing protection against a mucosal HIV challenge.

Approach

[0174] Long-lived plasma cells are primarily responsible for antibody durability, and the survival and differentiation of plasma cells within germinal centers (GC) depends on optimal

help from CD4 T follicular helper cells (Tfh). Therefore, it is imperative to design vaccines that can harness optimal Tfh cell help to establish durable anti-HIV antibodies.

[0175] Akin to CD4 T helper (h) cells, Tfh cells also encompass distinct Th1 or Th2 subsets, and several recent studies have demonstrated that Th polarity of Tfh cells critically determines outcome of antibody responses to various vaccines/infections. Yet, it remains unclear whether Th1 or Th2-type Tfh cells regulate HIV vaccine induced antibody. Bridging this gap will greatly advance vector-based vaccine design strategies and rational selection of adjuvants for protein boosts to extend the functional quality and durability of the vaccine-elicited antibody against HIV. Our recent studies using a DNA(D)-prime/modified vaccinia Ankara(MVA)+Alum-adjuvanted protein (Pro)- boost regimen showed that generation of CXCR3⁺ Th1-polarized Tfh cells within GCs associates with Env antibody durability, neutralization potential and avidity (Iyer SS, Amara R et al., JI, 2015). Furthermore, CXCR3⁺ Tfh cells express higher levels of B cell helper co-stimulatory molecules and cytokines relative to CXCR3⁻ Tfh cells.

[0176] We use the well-established DNA/MVA vaccine platform, which is in clinical development, for two integrated approaches; first, we employ the Th1 chemokine, interferon induced protein (IP)-10, a ligand for and an inducer of CXCR3, as a molecular adjuvant to a DNA vaccine (DIP-10) to prime Th1-type Tfh cells. Second, we employ AS01B as a strong Th1 adjuvant to the gp140 protein (ProAS01B) to boost Th1-type Tfh cells. This vaccine regimen is evaluated in a monkey study with three vaccine groups to determine whether increase in Th1-polarized Tfh cells during the prime with a DIP-10MProAlum vaccine and during both the prime and the boost with a DIP-10MProAS01B vaccine enhances durable and protective HIV antibody relative to a reference DMProAlum platform.

[0177] The first approach is to delineate immune determinants and characterize gene expression profiles of vaccine-specific Th1 versus Th2-polarized Tfh cells. Specifically, a novel IP-10-DNA-SHIV vaccine is constructed and characterized *ex vivo*. In addition, cytokine/chemokine determinants are delineated, and single-cell transcriptional profiling is performed of Env-specific Th1 and Th2 Tfh cells in all three outlined vaccine groups.

[0178] The second approach is to determine whether Th1-polarized Tfh cells enhance magnitude, quality and persistence of Env antibody titers. Specifically, it is determined whether the DIP-10MProAlum and DIP-10MProAS01B vaccines increase Env binding titers in sera and mucosa and enhance neutralizing and non-neutralizing antibody titers.

[0179] The third approach is to determine whether a Th1-polarized vaccine regimen enhances mucosal protection against a SHIV challenge and augments viral control. Specifically, it is determined whether the DIP-10MProAS01B vaccine enhances protection against a vaginal heterologous SHIV challenge and potentiates viral control in infected animals. In addition, the susceptibility of CXCR3⁺ CD4 Tfh cells to SHIV infection is determined *ex vivo*.

Introduction

[0180] The advent of highly effective antiretroviral therapy (ART) and its use in pre-exposure prophylaxis (PrEP) has resulted in major strides in HIV/AIDS prevention (1). However, this has not diminished the need for a protective vaccine regimen, which, unlike PrEP, does not require conscious effort, continued adherence or frequent follow-up to be effective (2, 3). Therefore, development of an effective HIV/AIDS vaccine continues to be the most sustainable and cost-effective approach and a top priority for HIV/AIDS prevention. To date, there have been six HIV vaccine efficacy trials that have tested four different vaccine modalities. The first indication of efficacy was observed in the RV144 Thai trial, which tested a poxvirus vector prime (ALVAC, months 0,1) and ALVAC+gp120 protein boost (months 3, 6) and demonstrated an estimated vaccine efficacy of 31% (4). Protection was linked with durability of anti-HIV Envelope (Env) antibody titers (5) emphasizing the need to enhance antibody persistence by induction of long-lived plasma cells (6, 7). Plasma cells are the product of germinal center (GC) reactions within lymphoid tissues, and the survival and differentiation of GC B cells to plasma cells critically depends on help from CD4 T follicular helper cells (Tfh) (8, 9). Therefore, it is imperative to design vaccines to harness optimal Tfh cell help to establish durable humoral immunity (10).

[0181] Similar to canonical CD4 T cells (11), Tfh cells also encompass characteristics of Th1, Th2, and Th17-type cell subsets which can be identified by expression of specific chemokine receptors (12). Several recent studies have demonstrated that Th1 and Th2 Tfh cells possess differential capacity for B cell help, and the induction of a specific Tfh subset critically determines outcome of the antibody response to vaccination/infection (13-15).

[0182] With respect to vaccination, Th1-polarized CXCR3-expressing Tfh cells have been associated with durable antibody titers following influenza immunization (13, 16). In our HIV vaccine preclinical study, we recently showed that the magnitude of Th1-polarized CXCR3⁺ Tfh responses correlated with Env antibody durability, neutralization potential and

avidity following a modified vaccinia Ankara (MVA) boost (17). However, in a recent study in HIV+ individuals, CXCR3- Tfh cells were found to be associated with neutralizing titers (14) implying divergent functions of Th1-Tfh cells in vaccination versus chronic infection. These reports underscore a pressing need for unequivocally determining whether Th1 or Th2 type Tfh cells regulate durable and protective antibody to viral vector and/ subunit protein HIV immunizations. Furthermore, the surge of interest in employing Th1 adjuvants such as MF59 and AS01B over the Th2 adjuvant Alum in Phase I/II HIV vaccine trials makes this a highly timely and clinically relevant question in HIV/AIDS vaccine research (18-20).

Study Design

[0183] As shown in **FIG. 13**, all animals receive two DNA (D) primes and two MVA+gp140 protein boosts (MPro). Vaccine groups 1, 2, and 3 are used to investigate the outcome of graded increase in Th1 immune response on antibody durability in systemic and mucosal compartments. Vaccine groups 1 and 3, at the opposite ends of the Th1 response spectrum, are directly compared for efficacy against an intravaginal heterologous Clade C SHIV.

Data

[0184] *DNA/MVA vaccine induces robust anti-Env antibody.* We have demonstrated that intramuscular immunization with DNA/MVA vaccines elicits immunologically coordinated humoral immune responses in periphery and mucosa (27, 28). Determination of Env-specific binding titers by ELISA over the course of immunization shows a temporal increase in Env titers, which achieve robust peak after the 2nd MVA boost (**FIG. 14A**).

[0185] Our data combining Pro+Alum with the 2nd MVA (**FIG. 14B**) demonstrate a modest but significant increase in peak titers in sera (**FIG. 14C**) and rectal mucosa (**FIG. 14D**). We also noted increase in heterologous tier 1-neutralization titers in sera (**FIG. 14E**), and a trend for increase in antibody against cyclic V1V2 peptides in mucosa (**FIG. 14F**). These data demonstrate the feasibility of combining MVA with subunit protein immunogens to enhance humoral responses. Preliminary data from the HIV Vaccine Trials Network (HVTN) demonstrating high magnitude, durable anti-V1V2 antibodies in humans with AS01B relative to Alum as an adjuvant provide a strong rationale for utilizing AS01B to further enhance the magnitude and persistence of DNA/MVA+protein elicited humoral immunity.

[0186] *DNA/MVA vaccine elicits Tfh responses, which are discernable in peripheral blood.* Our data demonstrate a transient accumulation of CXCR5⁺ Tfh cells expressing the cell-cycle marker Ki-67 at the peak CD4 effector response following the MVA boost (**FIGS. 15A and B**, * $p < 0.05$). Analogous to functionality of Gag and Env-specific CD4 T cells co-expressing the Tfh cytokine interleukin (IL) 21 and the Th1 cytokine IFNG (**FIG. 15C**); Ki-67⁺ pTfh expressed CXCR3, a marker of Th1 polarity (**FIG. 15D**). Notably, the proportion of CXCR3⁺Ki-67⁺ pTfh cells markedly increased following MVA immunization relative to baseline (**FIG. 15E**, *** $p < 0.001$). These data demonstrate that DNA/MVA vaccination elicits Tfh cells of distinct Th polarity and establishes our ability to sensitively capture this response in peripheral blood (17).

[0187] *Lymph node Tfh responses following DNA/MVA+Protein immunization.* To capture salient aspects of the GC response following the 2nd MVA boost and how Pro+Alum modulated this response, we examined GC Tfh cells by high expression of programmed death (PD)1 and CXCR5, and Tfh cells based on the CXCR5⁺PD-1⁺ phenotype (**FIG. 16A**). Our data showed that M+Pro vaccine increased Th1-polarized Tfh cells (**FIG. 16B**) even in the presence of Alum.

[0188] Strikingly, the proportion of CXCR3⁺ GC Tfh correlated with frequency of GC B cells (**FIG. 16C**), antibody avidity (**FIG. 16D**), durability (**FIG. 16E**), and neutralization (**FIG. 16F**). These data provide a compelling incentive for determining whether a strong Th1 DNA prime and a strong Th1 MVA boost will enhance durable and protective antibody.

[0189] *IP-10 increases induction of CXCR3⁺ Tfh cells.* To confirm that utilizing IP-10 as a molecular adjuvant to a DNA vaccine induces Th1-type Tfh cells, we examined whether exogenous IP-10 induces CXCR3 expression on Tfh cells *ex vivo*. Rhesus peripheral blood mononuclear cells (PBMC) were stimulated using CD3/CD28 beads in the absence or presence of increasing doses of IP-10 for 48h. We utilized a dose of IP-10 based on the range of serum concentrations reported following MVA immunization in macaques (29). Our data show that IP-10 induces a distinct increase in proportion of Tfh cells expressing CXCR3 (**FIGS. 17A and B**) and importantly increases frequency of CXCR3⁺ Tfh cells (**FIG. 17C**).

Approaches

First approach

[0190] In order to effectively harness Tfh help for HIV vaccine design, understanding heterogeneity of the vaccine-elicited Tfh response and identifying factors contributing to this heterogeneity are critical. The goal of the first approach is to extensively define the cytokine/chemokine response to vaccination and how this influences Tfh cell magnitude and polarity. This approach is further designed to acquire comprehensive insights into division of labor within vaccine-elicited Tfh cells by interrogating the transcriptional signature of Env-specific CXCR3⁺ Th1 and CCR4⁺ Th2 Tfh cells.

[0191] We utilize a DNA-SHIV construct expressing 1086 Clade C Env, tat and rev; and SIV239 Gag, protease, and reverse transcriptase using a CMV promoter. As previously described, the DNA vaccines express multiple HIV/SIV proteins from a single RNA by subgenomic splicing and frameshifting (28). We construct IP-10-DNA-SHIV vaccine by inserting the rhesus macaque IP-10 sequence (297 base pairs) as a fusion to the (a) internal ribosome entry site (IRES) or (b) the peptide from foot and mouth disease (F2A) downstream of Env. Our rationale for testing two constructs *ex vivo* is to be able to select an optimal construct for *in vivo* vaccine experiments.

[0192] Based on ongoing studies using a CD40L-DNA-SHIV construct, we have observed robust expression of CD40L on 293-T transfected cells with the 2A DNA vaccine. Higher vaccine specific responses with the DNA-SHIV CD40L vaccine provide evidence for enhanced immunogenicity *in vivo*. These data provide an important proof-of-principle that the IP-10 DNA vaccine can be designed and that enhanced immunogenicity can be quantified *in vivo*. We compare the IP-10-DNA-SHIV IRES and F2A constructs *ex vivo* using a variety of different approaches which include determining antigenicity of constructs by evaluating expression of HIV-1 Env (surface, clone PGT121), SIV Gag (intracellular, clone 2F12) and IP-10 (intracellular, clone J034D6) by flow cytometry on transfected 293-T cells. Cell supernatants from 293-T transfected cells are also be collected for measurement of secreted IP-10 by ELISA and for functional activity of IP-10 using cell supernatants to stimulate PBMCs. We compare supernatants for ability to activate DCs and induce CXCR3 expression on CD4 T cells. We select either the IRES or the 2A construct based on efficiency of Env and IP-10 expression for *in vivo* experiments.

[0193] Another objective is to understand how the *in vivo* chemokine/cytokine response immediately following immunization influences CD4 Tfh development and differentiation, which is directed by cues early in the immune response (36). Studies in a mouse model of

acute viral infection show that IP-10 is rapidly induced in a type I IFN dependent manner (37), and studies in macaques show transient induction of pro-inflammatory factors including IP-10 early after MVA immunization (29). Therefore, to optimally capture the early and transient induction of immune factors, we collect blood at 0, 12, 24, 48, and 76 hours post immunization to establish a comprehensive cytokine /chemokine profile temporally, and across vaccine platforms. In addition, we collect CVL at 0 and 24 hours after each immunization to evaluate the immune milieu in the genital mucosa. We determine (i) cytokine, chemokine profile using a multiplex assay to measure soluble factors driving Th1 polarization: interleukins (IL)-12, IL-1 β , IL-8, IL-2; macrophage inflammatory protein (MIP)1 α , MIP1 β , MIP3 α , MIP3 β ; macrophage chemotactic protein (MCP)-1; CXCR3 ligands; monokine induced by gamma (MIG/CXCL9), IP-10/CXCL10, interferon (IFN)-inducible T-cell alpha chemoattractant (I-TAC/CXCL11); tumor necrosis factor (TNF) α ; IFNs IFN α , β , γ . We also examine a panel of Th2/regulatory factors including IL-4, IL-10, IL-R α , transforming growth factor (TGF) α , TGF β and additional CCR4 ligands including CCL5, CCL17, and CCL22.

[0194] To determine how the innate response influences (ii) vaccine-specific Tfh responses, we quantify vaccine-elicited Tfh cells in systemic, lymphoid, and mucosal compartments. We have demonstrated that the DNA/MVA vaccine induces robust frequencies of vaccine-specific CD4 T cells that are broadly distributed systemically (17, 27), within the spleen and lymph nodes, and in the rectal and genital mucosa. Due to evidence for CXCR3 in directing homing of T cells to the vaginal mucosa (38), we evaluate T cell responses in the genital tract. We examine blood and lymph node Tfh cells for $\alpha\beta\gamma$ expression to understand how potentiating Th1 responses impacts trafficking to the gut associated lymphoid tissue (39).

[0195] We study vaccine-specific Tfh cells utilizing two complementary approaches we have optimized and established (17). We utilize the cell-cycle marker Ki-67 to interrogate CXCR5⁺ CD4 T cells at peak effector time points following each immunization for expression of CXCR3 and CCR4 as outlined in **FIG. 19A**. This is complemented by intracellular cytokine staining (ICS)-based assays designed to examine vaccine-specific responses (at peak and memory) after stimulation with relevant Gag and Env peptide pools. After stimulation, Ag-specific Tfh cells are identified based on cytokine positivity and CXCR5 expression utilizing the panel as shown in **FIGS. 19B** and **19C**. We evaluate GC

responses using flow cytometry and examine GC Tfh, Tfh and GC B cells for CXCR3 and CCR4 expression (similar to analysis shown above).

[0196] To gain a comprehensive understanding of molecular heterogeneity within vaccine elicited Tfh cells, we determine transcription profile of CXCR3⁺ vs. CCR4⁺ Tfh cells within the draining lymph node. Because robust antibody titers are established after the 2nd MVA boost, and active germinal centers are observed at 14 days post 2nd MVA (27), we collect biopsies from the draining lymph node at this time. Vaccine-specific Tfh cells are identified by translocation of CD40L after 5 hour stimulation with Env peptides in the presence of co-stimulatory molecules (**FIG. 20**) (40). Samples from 5 animals within each of three vaccine groups are selected to understand heterogeneity within Tfh cell subsets and how it compares across vaccine regimens. Because changes in CXCR5, CXCR3, and PD-1 likely occur upon stimulation, CD3⁺CD4⁺Fas⁺CXCR5⁺PD-1^{+/++} (comprising Tfh and GC Tfh) cells are sorted using fluorescence-assisted cell sorting (FACS) into CXCR3⁺ and CCR4⁺ single positive subsets prior to stimulation. We also sort CXCR3⁻ CCR4⁻ double negative Tfh cells. Naive CD4 T cells (CD4⁺Fas⁻ CXCR5⁻PD-1⁻CXCR3⁻) serve as a reference population. After stimulation with Env peptides in the presence of co-stimulatory molecules using established protocols (17), cells are stained with surface CD40L and twenty-four CD40L⁺ cells from each of the two subsets are singly sorted into a 96-well plate. The single-cell RNA sequencing methodology allows us to capture the heterogeneity of the Tfh cell response to stimulation and give us the ability to resolve important associations between cell phenotype, stimulation status (by quantifying genes encoding Tbet (Th1) and IL-4 (Th2) that are rapidly up regulated upon TCR stimulation (41)), and cellular transcriptional program.

Second approach

[0197] Several recent studies have demonstrated functional heterogeneity within Tfh cells based on Th polarization, which is intertwined with chemokine receptor expression (12-15). Our data provide two strong pieces of evidence supporting a role of Th1-polarized Tfh cells in enhancing antibody durability. First, the frequency of Env-specific IFNG⁺IL-21⁺ cells at 1 week post 2nd MVA, and second, the frequency of CXCR3⁺ GC Tfh cells at 2 weeks post 2nd MVA correlate with antibody durability measured at 20 weeks post 2ndMVA (**FIG. 20**). In the second approach, we conduct a systematic and comprehensive assessment of antibody responses across three vaccine regimens designed to elicit graded increase in Th1-polarized Tfh cells.

[0198] We perform a broad repertoire of quantitative and functional assays to comprehensively assess the humoral response in the systemic and mucosal compartments. We perform Enzyme-linked immunosorbent spot (ELISPOT) assays using standard protocols (43, 44) to measure Env-specific antibody secreting cells (ASCs) using fresh PBMCs, at day 5 following 1st and 2nd MVA boost. We have established kinetics of gp140-ASCs in our studies (17) (**FIG. 22A**). We quantitate plasma cells using bone marrow aspirates at memory post 2nd MVA. We measure Env-specific IgG and IgA binding titers and antibody IgG subclass (45) against gp140 and ConA captured virus like particle trimeric gp160 antigen by ELISA using established protocols (17, 27). We utilize the highly sensitive binding antibody multiplex assay (BAMA) to quantify Env responses against gp120, gp41, V1V2, and V3 antigens in the mucosa and sera. We measure neutralization responses against tier 1 and tier 2 autologous and heterologous viruses using the TZM-bl assay after the 2nd MVA boost (46). Initial assays are done with the challenge SHIV, the immunogen SHIV, and clade-matched tier 1A, tier 1B and tier 2 viruses, which include SHIV-1157ipEL-p and Ce1086_B2. Further assays are performed against a multi-clade global virus panel described previously (47). To assess non-neutralizing antibody effector functions, we measure antibody-dependent phagocytosis (ADP) using the Ackerman flow cytometric assay (48) and antibody dependent cellular cytotoxicity (ADCC) using the Evans assay (49). Our data (**FIG. 22B**) show induction of antibodies that direct ADCC and ADP following a DNA/MVA immunization. We also measure antibody glycosylation (23).

Third approach

[0199] A significant impediment to enhancing vaccine efficacy is the waning of Env antibody titers following vaccination (5, 50). While booster protein immunizations are a means to augment antibody titers, they present the concern of inefficient CD8 T cell recall and potential generation of target cells, which together can create an environment that favors initial viral replication upon exposure (51). Consequently, vaccine strategies to establish durable humoral immunity while inducing robust cytolytic responses are highly desirable.

[0200] To determine vaccine efficacy, we determine protection against weekly intravaginal repeat low-dose challenge with heterologous clade C SHIV-1157ipd3N4 (35) for a maximum of 12 weeks (12 challenges). We utilize the stock titrated to infect about 30% of the unvaccinated animals at the first challenge. We determine whether DIP-10MProAS01B

vaccine delayed rate of acquisition and decreased peak viremia in infected animals relative to DMPProAlum.

[0201] *Statistical Analyses:* To arrive at sample sizes, we conducted power analyses using per-challenge infection rates computed from our prior trial using the SIV251 challenge virus. Our power analyses determined that we should achieve a minimum per-challenge infection rate of about 0.02 in group 3 to see a statistically significant (80% power at the alpha level of 0.05) difference between groups 1 and 3. We evaluate two main parameters to determine protection: 1) Status of Infection and 2) number of challenges to infection. Comparison of proportions of infected and uninfected animals between vaccine groups and controls is performed using Chi-square test. Comparisons of number of challenges until infection between groups is conducted using the two-sided log-rank test and visualized using Kaplan-Meier curves. Models of the protective effect of vaccination proposed by Hudgens and Gillbert are fitted, and the Akaike Information Criteria (AIC) is used to select the most parsimonious model (54). In addition, we determine peak viral load at 2 and 3 weeks post-infection to determine whether DIP-10MProAS01B vaccine enhanced viral control relative to DMPProAlum vaccine.

[0202] The goal of this approach is to understand whether vaccine approaches that induce Th1 polarized CD4 responses generate HIV target cells. Our data demonstrate enrichment of CCR5+ cells within the CXCR3+ Tfh cell subset (**FIG. 23A**). Furthermore, our data show significantly higher levels of pro-viral DNA in X3+ Tfh cells (**FIG. 23B**) suggestive of enhanced permissivity to infection. Therefore, we determine the frequency of CXCR3+ CD4 T cells in the context of CCR5 and CXCR5 in the vaginal mucosa, lymph node and blood prior to challenge and determine if this correlates with either acquisition or peak viremia. As an additional functional assay to quantitate vaccine-induced increase in susceptibility to infection (55), archived cells from vaginal biopsies at baseline, weeks 1 and 8 post 2nd MVA booster immunization are exposed to the SHIV challenge virus *ex vivo* for 18 hours; washed and cultured for 48 hours. We quantitate virus in the supernatant and infected cells are assessed by intracellular Gag staining. This assay yields information on whether vaccine-induced changes in the mucosal immune environment favor viral replication.

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Example 3. Targeting CD4 Follicular Helper T Cells for Enhancing HIV Vaccine-Induced Humoral Immunity

Summary

[0203] Anti-retroviral therapy (ART) has dramatically altered the HIV pandemic landscape by rendering the disease manageable - but still incurable. Significant barriers are associated with the global implementation of ART that limit its utility for sustainable prevention of HIV. Moreover, the intersection of aging-related conditions and the consequences of long-term ART will have a substantial impact on the healthcare system as well as on HIV patients. These challenges underscore the need to pursue strategies that will provide both sustainable HIV prevention and a functional HIV cure. The results of the RV144 trial indicate that vaccination may prevent HIV transmission in humans and that durability of anti-envelope (Env) HIV antibodies may be the key to this protection. Efforts to improve upon the RV144 trial have demonstrated that booster immunizations increase serum anti-Env antibody titers only transiently. This “anti-Env antibody persistence problem” impedes our efforts to develop an effective HIV vaccine.

[0204] Here, we use a DNA-prime/Protein-boost vaccine regimen in rhesus macaques. In the first approach we focus on establishing how Th1 versus Th1/Th17 priming impacts innate inflammatory response, Env Tfh frequencies, phenotype, transcriptional profile, and B cell helper capacity, as well as systemic and mucosal anti-Env antibody titers. In the second approach we determine whether combining the state-of-the-art soluble Env mimic, an Env SOSIP trimer protein immunogen with a highly potent and robust adjuvant (to induce Th1/Th17 response, results in increased production of a high-quality, longlasting anti-Env antibodies relative to Th1 vaccine regimen. We further investigate whether vaccine-mediated

induction of the polyfunctional Tfh response improves protection when faced with a mucosal simian HIV challenge.

Overview

[0205] Antiretroviral therapy (ART) has a remarkable impact on the morbidity and mortality associated with HIV but significant barriers hamper its global implementation, limiting its full potential as a preventative tool. Moreover, the intersection of aging-related conditions and the consequences of long-term ART is predicted to adversely impact the healthcare system as well as the HIV patient. These challenges underscore the need to pursue strategies that will provide both sustainable HIV prevention and a functional HIV cure. The results of the RV144 trial indicate that vaccination may prevent HIV transmission in humans and that durability of anti-envelope (Env) HIV antibodies may be the key to this protection. Efforts to improve upon the RV144 trial have demonstrated that booster immunizations increase serum anti-Env titers but do so transiently. This short-lived impact demonstrates an ineffectiveness in the induction of long-lived Env-specific plasma cells, the source of the durable antibody. This “anti-Env antibody persistence problem” is impeding our progress in developing an effective HIV vaccine.

[0206] The answer lies in the CD4 T follicular helper cells (Tfh), a subset of CD4 helper (Th) cells, vital for differentiation of short-lived germinal center B cells to long-lived antibody-producing plasma cells. Tfh cells are heterogeneous and encompass Th1, Th2, Th17-type helper attributes. The proportion of each helper attribute determines the durability and functional quality of the antibody response. These helper attributes are programmed during T-cell priming and are dictated by the initial inflammatory response following vaccination. This provides researchers a window of opportunity to manipulate the CD4 Tfh response in order to maximize the generation of durable anti-Env humoral immunity. Because the characteristics that make up an optimal Tfh helper cell for induction of durable anti-Env antibody have not yet been clearly defined, our long-term goal is to determine how manipulating the Tfh helper profile impacts the longevity of anti-Env antibodies within the setting of priming and boosting immunizations.

[0207] Here, we establish whether improved durability and neutralization potential of anti-Env antibodies can be influenced by manipulating vaccine-induced generation of Th1/ Th17 Tfh cells. These investigations are supported by rigorous data demonstrating that: 1) a Th1-DNA prime substantially increases the Env-specific Tfh cells relative to a Th1+Th2 vaccine

regimen; 2) Th1-protein boost results in greater production of the IgG1 and IgG3 subclasses with enhanced durability, breadth, specificity and avidity of anti-Env antibody; and 3) Th1 vaccine generation of polyfunctional Th1/Th17 GC Tfh cells predicts the durability of anti-Env IgG titers in sera and anti-Env IgA titers in mucosa.

[0208] Using a DNA/Protein vaccine platform, we describe two integrated approaches; 1) we employ interferon protein-10 and interleukin 6 as molecular adjuvants to a DNA (DIP-10-IL-6) vaccine to prime Th1/Th17-type Tfh cells; 2) we use a cationic liposomal adjuvant, CAF01, a strong Th1/Th17 adjuvant to a state-of-the-art soluble Env mimic, an Env SOSIP trimer protein, to boost Th1/Th17-type Tfh cells. Using monkeys, we evaluate this regimen with three vaccine groups to determine whether an increase in Th1/Th17-type Tfh enhances the longevity of anti-Env, the protective HIV antibody, relative to a Th1 platform only during the prime or during both the prime and the boost.

[0209] The first approach is to demonstrate that a Th1/Th17 DNA-prime is more effective at increasing the magnitude of Env-specific Tfh cells relative to a Th1 vaccine regimen. Here, we a) delineate innate determinants of Env-specific Tfh cell differentiation during the DNA prime; and b) delineate Env-specific Tfh cells induced in response to a Th1 versus Th1/Th17 DNA prime.

[0210] The second approach is to demonstrate that synergistic boosting of Th1/Th17 polarized Tfh cells elicits robust and durable anti-Env antibody titers in systemic and mucosal compartments. Here, we a) determine anti-Env antibody quantity, quality, neutralization potential, and effector functions in systemic and mucosal compartments following vaccination; and b) determine whether specific immunity established by Th1/Th17 regimen is more effective at preventing mucosal tier 2 SHIV acquisition.

Experimental approach and data.

[0211] Th1 vaccine regimen induces robust and durable serum HIV Env antibody titers. Detailed kinetic analysis revealed that HIV anti-Env titers peaked after the final boost with a median 5-fold boost in antibody titers at week 2 post-2nd protein relative to week 0. Notably, antibody titers were significantly higher in the Th1 regimen at week 0 (median gp140 titers $\mu\text{g/ml}$; Th1+2 = 257.2 and Th1 = 1314; $p < 0.01$), at week 2 (Th1+2 = 9942 and Th1=29104; $p < 0.0001$), week 8 (Th1+2=2067 and Th1 = 6904; $p < 0.001$), and week 18 (Th1+2= 450 and Th1= 2431; $p < 0.001$) (**FIG. 24**). We used binding antibody multiplex assay (BAMA) to assess the breadth and specificity of the serum antibody response. Responses to both 1086.C

and CH505 Clade C Env immunogens were significantly higher in the Th1 group. Similarly, the increased titers against Con C and Con S were sustained at week 8 demonstrating induction of cross-clade breadth (**FIG. 25A**). Avidity index measured using 2M sodium thiocyanate revealed the presence of higher affinity antibodies in the Th1 group (**FIG. 25B**). We also assessed binding to linear V1V2 and conformational epitopes at week 2 and 8 and found that significantly higher specificity to these important regions in the Th1 vaccine regimen (**FIG. 25C**). Evaluation of HIV neutralization activity demonstrated higher induction of Tier 1A clade C isolate (ID50 range at week 2 post 2nd protein boost Th1+2: 37 -1126; Th1: 195-4977, $p < 0.01$) These titers dropped to an ID50 value of 20 in Th1+2 group and were maintained between 24-1057 in the Th1 group (**FIG. 25D**).

[0212] We also measured secretions from vaginal and rectal sponges to assess anti-Env IgA responses. Similar to observed serum responses, the Th1 vaccine initiated a significant increase in mucosal 1086.C gp140-specific IgA and IgG titers in the vaginal and rectal mucosa relative to Th1+2 regimen (**FIGS. 26A and B**). Notably, at week 8 post 2nd protein boost, rectal IgA titers were below the limit of detection in all Th1+2 animals relative to detectable levels in 8/10 of Th1 vaccinated animals. Together these data demonstrate that Th1 vaccine regimen is more effective in generating durable binding titers in sera and mucosa, as well as HIV anti-Env antibodies with greater breadth, avidity, broader specificity, and neutralization potential relative to a mixed Th1+2 vaccine regimen. Th1 vaccine induces higher frequencies of Env-specific CD4 T cells. To study Tfh responses in detail following the prime we examined whether Tfh frequencies were increased following the third DNA prime. A significant increase in frequencies and absolute counts of ICOS+PD-1+activated Tfh cells was observed (**FIG. 27B**). Consistent with this observation, Env-specific Tfh cells were induced at significantly higher frequencies in the Th1 group (**FIGS. 27C and D**). These data establish the importance of a strong Th1 DNA prime in eliciting strong Tfh cell responses.

[0213] *Induction of polyfunctional Th1/Th17 CD4 Tfh due to Th1 vaccine regimen.* Following the first protein boost, we characterized the innate inflammatory response and observed induction of cytokines important for Th1, Th17, and Tfh programming (**FIG. 28A**). Following the boost, Env-specific Tfh cells were higher in the Th1 vaccine regimen and were associated with increase in GC B cell frequencies (**FIGS. 28B and C**). GC Tfh cells expressed higher levels of Bcl-6, CXCR3, CCR6 and IFNG, IL-17+ cells expressed relatively higher levels of CD40L (**FIG. 28E**). Gene expression profiles revealed enrichment of Th1 /

Th17 and classical Tfh genes within GC Tfh cells in the Th1 vaccine regimen (**FIG. 28F**). Correspondingly, we found that the frequency of Th1/Th17 Tfh cells was higher in the Th1 vaccine regimen suggesting the induction of polyfunctional CD4 Tfh cells (**FIG. 28G**).

[0214] *Induction of polyfunctional Th1/Th17 CD4 Tfh cells predicts durability of anti-Env antibody titers in systemic and mucosal compartments.* We performed correlational analysis to determine GC correlates of antibody titers, which showed the frequency of polyfunctional Th1/Th17 GC Tfh cells to be directly predictive of binding titers measured by ELISA and BAMA (**FIG. 29**). Consistent with the role of Th17 cells in inducing IgA responses, an association with mucosal Env IgA titers was also observed.

[0215] *A single immunization with CAF01-adjuvanted protein induces robust antibody responses.* Our data strongly indicate that synergistic induction of polyfunctional Th1/Th17 CD4 Tfh cells enhances HIV anti-Env antibody titers. Therefore, we sought to procure the CAF01 adjuvant – the strongest known adjuvant shown in multiple studies to induce Th1/Th17 cells – for the studies described. Data in mice using CAF01 (44) (**FIG. 30**) adjuvanted to either a chlamydia (**FIG. 30B**) or ovalbumin protein (**FIG. 30C**) show gradual increase in Ag-specific titers in serum at levels comparable or superior to the squalene-based oil-in-water nano-emulsion adjuvant Addvax™, an MF59 analog. Quantification of antigen-bound cells at the site of immunization show higher antigen uptake with CAF01 suggestive that antigen depot induction at the immunization site (**FIG. 30D**) results in robust germinal center responses at the draining lymph node and subsequent enhancement in antibody titers. Together with CAF01 mediated skewing to a Th1 and Th17 inflammatory response (39) and robust induction of IgA plasma cells in genital mucosa (41), these data provide a strong scientific rationale for determining whether an HIV vaccine platform designed to prime and reproducibly boost Th1/Th17 CD4 Tfh cells employing a CAF01 adjuvant will engender high titer serum and mucosal HIV anti-Env responses.

Methods

[0216] *Study design:* The S1 and S2 studies are designed to investigate the outcome of synchronous priming and boosting of polyfunctional Th1/Th17 CD4 Tfh cells on antibody durability, breadth, specificity, and neutralization potential in both the systemic and mucosal compartments. All groups are directly compared for immunogenicity and for efficacy against intrarectal simian (S) HIV BG505 S375Y virus (weekly challenge with 1.4×10^7

virions/challenge). The DNA SHIV BG505-SOSIP.664 plasmid is used, into which we clone in IP-10 and IL-6.

[0217] *Animals:* SIV- STL- SRV-negative Indian origin rhesus macaques from the CNPRC breeding colony, between the ages of 3-6 years are selected for the study. Each group has equal males and females.

[0218] *Sample Size:* There are 8 macaques in Group 1, and 12 macaques in Groups 2 and 3. We have worked with a statistician to ensure our studies are sufficiently powered to detect differences in immunogenicity. We evaluate individual innate, T cell, and antibody trajectories over time and compare mean trajectories across experimental groups. We then correlate individual trajectories of the innate inflammatory response with those of Tfh responses and humoral immunity. Our study design (sample sizes, repeated measures) provides confidence limits of about $\pm 0.5s$ for the difference in rates of change of markers, compared to the variation s in individual trajectories (R package longpower, Edland 2009). We have 80% power to detect differences comparable to those found in our previous studies.

[0219] *Sampling and assays:* The sampling schedule is designed to optimally and rigorously capture immunological and biological dynamics to link innate immune parameters to Tfh responses and ultimately to antibody responses following vaccination. To this end, all animals are sampled at various time points at baseline and after each of the immunizations. We evaluate clinical chemistries immediately following and weeks after immunization to monitor safety (26).

First approach

[0220] Part 1a) Delineate innate responses to a Th1 versus Th1/Th17 DNA-prime to define predictive correlates of Env-specific Tfh cells during the prime.

[0221] *Experimental Approach:* Productive T cell responses critically depend on cytokine responses during priming, and recent studies demonstrate that monocyte-derived IL-1 β drives effective Tfh and CD4 T cell differentiation (28, 60, 61). Detailed investigation of the kinetics of pro-inflammatory monocytes – cellular innate biomarkers of adjuvanticity – revealed a transient increase in CD14⁺CD16⁺ monocytes in blood with a higher relative increase in the Th1 vaccine group (**FIG. 32**). Strikingly, the quantity of GC Tfh cells within the fine-needle aspirates of the draining lymph node showed higher GC frequencies in Th1 vaccine group (**FIG. 32C**). Notably, the improved inflammatory response was associated

with increased levels of antibody durability linking the innate immune response to priming of effective CD4 Tfh help (**FIG. 32D**). Based on these data, we compare induction of innate immune responses between Th1 and Th1/Th17 priming regimens. To optimally capture the early and transient induction of immune factors, we collect blood at 0, 48, and 72 hours post immunization to establish a comprehensive cytokine /chemokine profile temporally, and across vaccine platforms. We determine (i) Cytokine, chemokine profile using a multiplex assay to measure soluble factors driving Th1 polarization: interleukins (IL)-12, IL-1 β , IL-8, IL-2; macrophage inflammatory protein (MIP)1 α , MIP1 β , MIP3 α , MIP3 β ; macrophage chemotactic protein (MCP)-1; CXCR3 ligands; monokine induced by gamma (MIG/CXCL9), IP-10/CXCL10, interferon (IFN)-inducible T-cell alpha chemoattractant (I-TAC/CXCL11); tumor necrosis factor (TNF) α ; IFNs IFN α , β , γ . We examine a panel of Th17 factors including IL-17, IL-6, TGF- β , IL-21, IL-23 and the CCR6 ligand CCL20. In addition, we capture dynamics of pro-inflammatory monocyte frequencies following immunization as shown in **FIG. 32**. Together, this approach provides a comprehensive in-depth overview of the inflammatory profile during priming.

[0222] Part 1b): Delineate Env-specific Tfh responses to a Th1 versus Th1/Th17 DNA-prime.

[0223] *Experimental Approach:* We quantify vaccine-specific Tfh cells in systemic and lymphoid compartments. We have demonstrated that the DNA EP vaccine induces robust frequencies of vaccine-specific CD4 T cells in the blood and lymph node. We study vaccine-specific Tfh cells utilizing two complementary approaches we have optimized and established (17). We utilize the cell-cycle marker Ki-67 to interrogate CXCR5⁺ CD4 T cells at peak effector time points following each immunization for expression of CXCR3 and CCR6. This is complemented by activation induced marker (AIM) and standard intracellular cytokine staining (ICS)-based assays designed to examine vaccine-specific responses (at peak and memory) after stimulation with relevant Gag and Env peptide pools (62, 63). After stimulation, Ag-specific Tfh cells are identified based on co-expression of AIM marker positivity and CXCR5 expression. We evaluate GC responses using flow cytometry and examine GC Tfh, Tfh and GC B cells for CXCR3 and CCR6 expression.

[0224] To gain a comprehensive understanding of molecular heterogeneity within vaccine elicited Tfh cells, we determine transcription profiles of single-cell sorted CXCR3⁺ vs. CCR6⁺ Tfh cells within the draining lymph node. Since antibody titers are established after

the 3rd DNA boost in the majority of animals, and active germinal centers are observed at 14 days post 3rd DNA, we collect biopsies from the draining lymph node at this time. Vaccine-specific Tfh cells are identified by induction of OX40 and CD25 after 5 hour stimulation with Env peptides in the presence of co-stimulatory molecules (40). Samples from 5 animals within each vaccine group are selected to understand heterogeneity within Tfh cell subsets and how it compares across vaccine regimens. Because changes in CXCR5, CXCR3, CCR6, and PD-1 likely occur upon stimulation, CD3+CD4+Fas+CXCR5+PD-1+/++ (comprising Tfh and GC Tfh) are sorted using fluorescence-assisted cell sorting (FACS) into CXCR3+ and CCR6+ single positive subsets prior to stimulation. We also sort CXCR3-CCR6- double negative Tfh cells. Naive CD4 T cells (CD4+Fas-CXCR5-PD-1-CXCR3-) serve as a reference population. After stimulation with Env peptide pools in the presence of co-stimulatory molecules using established protocols (17), cells are stained with surface OX40 and CD25 and twenty-four double positive cells from each of the two subsets are singly sorted into a 96-well plate. The single-cell RNA sequencing methodology allows us to capture the heterogeneity of the Tfh cell response to stimulation and gives us the ability to resolve important associations between cell phenotype, stimulation status, and cellular transcriptional program.

[0225] Part 1c): Delineate anti-Env binding antibody titers elicited following the prime to Th1 versus Th1/Th17 DNA immunization.

[0226] *Experimental Approach:* We assess the humoral response in the systemic and mucosal compartments over the course of DNA immunization. We perform ELISA assays using standard protocols to measure Env-specific antibody in sera and mucosa.

Approach 2

[0227] Our data show marked potency of a Th1-Tfh vaccine regimen in eliciting humoral immunity and demonstrates that increasing the frequency of Th1/Th17 GC Tfh cells within the draining lymph node may result in enhanced durability of antibody titers at the memory time point. These data together with published evidence in the literature provide a strong scientific rationale to utilize CAF01 adjuvanted trimeric Env protein immunogen BG505 SOSIP.664 boost to test the hypothesis that induction of polyfunctional Th1 and Th17 Tfh cells will enhance antibody titers in systemic and mucosal compartments.

[0228] Part 2a): Delineate innate responses and Env-specific Tfh responses to a Th1 versus Th1/Th17 boost. As outlined for Approach 1, we perform an in-depth and comprehensive interrogation of innate and Tfh responses following the boost.

[0229] Part 2b): Perform an in-depth characterization of systemic and mucosal titers following vaccination.

[0230] *Experimental Approach:* We perform a broad repertoire of quantitative and functional assays to comprehensively assess the humoral response in both the systemic and mucosal compartments. We measure anti-Env-specific IgG and IgA binding titers and antibody IgG subclass (45) against the BG505 SOSIP gp140 by ELISA using established protocols. We utilize the highly sensitive binding antibody multiplex assay (BAMA) to quantify anti-Env responses against gp120, gp41, V1V2, and V3 antigens in the mucosa and sera.

[0231] We measure neutralization responses against tier 1 and tier 2 autologous and heterologous viruses using the TZM-bl assay after the 2nd protein boost (64, 65). Initial assays are done with the challenge SHIV, the immunogen SHIV, and clade-matched tier 1A, tier 1B and tier 2 viruses. Encouraging tier 2 nAb responses stimulates further assays against a multi-clade global virus panel and bnAb mapping mutants for breadth and specificity of the Tier2 nAb response. In the event of absent or weak tier2 nAb, we assay viruses that can detect precursors to bnAbs that have been developed for V2 glycan and CD4bs lineages.

[0232] To assess non-neutralizing antibody effector functions, we measure antibody-dependent phagocytosis (ADP) using the Ackerman flow cytometric assay and antibody dependent cellular cytotoxicity (ADCC) using the Evans assay. We measure antibody avidity using surface plasmon resonance as described previously (66). Our data show generation of higher avidity gp140 anti-Env antibodies with sequential immunization and significantly higher antibody avidity reached with Th1-Tfh group indicative of productive germinal center reaction (**FIG. 33**). Furthermore, Th1 vaccine regimen results in improved IgG1 and IgG3 subtype antibody responses (**FIG. 34**) prompting the question of whether this led to a corresponding increase in antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated virus inhibition (ADCVI) activities.

[0233] To follow up on these data, we measure ADCC and ADCVI functionalities using gp120 coated and virus infected target cells (67). Given recent evidence of IgG1 subclass

antibodies playing a role in neutralization, we also delineate the relationship between IgG subclasses and neutralization potential of sera (68).

[0234] Part 2c): Determine whether specific immunity established by Th1/Th17 regimen is more effective at preventing mucosal tier 2 acquisition.

[0235] *Experimental Approach:* To determine vaccine efficacy, we determine protection against weekly Intrarectal repeat low-dose challenge with clade B BG505 N332 S375Y for a maximum of 12 weeks (12 challenges) (69). Age and sex-matched unvaccinated controls are derived from the CNRPC colony and we determine whether DIP-10/IL-6 SOSIPCAF01 vaccine delayed rate of acquisition and decreased peak viremia in infected animals relative to DIP-10/IL-6 SOSIPALFQ vaccine.

Statistical Analyses

[0236] To arrive at sample sizes, we conducted power analyses using per-challenge infection rates computed from our prior trials using the SIV251 challenge virus. Our power analyses determined that we should achieve a minimum per-challenge infection rate of about 0.02 in group 3 to see a statistically significant (80% power at the alpha level of 0.05) difference between groups 1 and 3.

[0237] We evaluate two main parameters to determine protection: 1) Status of Infection and 2) number of challenges to infection. Comparison of proportions of infected and uninfected animals between vaccine groups and controls are performed using Chi-square test. Comparisons of number of challenges until infection between groups are conducted using the two-sided log-rank test and visualized using Kaplan-Meier curves. Models of the protective effect of vaccination proposed by Hudgens and Gillbert are fitted, and the Akaike. Information Criteria (AIC) are used to select the most parsimonious model. In addition, we determine peak viral load at 2 and 3 weeks post-infection to determine whether DIP-10/IL-6 SOSIPCAF01 vaccine enhanced viral control relative to DIP-10/IL-6 SOSIPALFQ vaccine.

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5. Exemplary Embodiments

[0238] Exemplary embodiments provided in accordance with the presently disclosed subject matter include, but are not limited to, the claims and the following embodiments:

1. A method of enhancing the anti-Env antibody response during HIV vaccination in a subject, the method comprising:

(i) administering a first Th1-polarizing adjuvant to the subject together with a DNA vaccine comprising a polynucleotide encoding a first HIV Env polypeptide, wherein the first adjuvant comprises a polynucleotide encoding interferon-induced protein (IP)-10; and

(ii) administering a second Th1-polarizing adjuvant to the subject together with a booster comprising a second HIV Env polypeptide, wherein the second adjuvant comprises QS-21.

2. The method of embodiment 1, wherein the second HIV Env polypeptide is a gp140 polypeptide.

3. The method of embodiment 1 or 2, wherein the DNA vaccine further comprises one or more additional polynucleotides encoding one or more additional HIV polypeptides selected from the group consisting of Gag, protease, reverse transcriptase, Tat, Rev, and combinations thereof.

4. The method of any one of embodiments 1 to 3, wherein the first adjuvant and the DNA vaccine are administered transdermally with electroporation.

5. The method of any one of embodiments 1 to 4, wherein the first HIV Env polypeptide, second HIV Env polypeptide, and/or one or more additional HIV polypeptides are from HIV-1.

6. The method of embodiment 5, wherein the HIV-1 is of clade C origin.

7. The method of any one of embodiments 1 to 6, wherein the booster further comprises a lipid and/or liposomal adjuvant.

8. The method of embodiment 7, wherein the liposomal adjuvant comprises Army Liposome Formulation (ALF) liposomes.

9. The method of embodiment 7 or 8, wherein the lipid adjuvant comprises monophosphoryl lipid A (MPLA).

10. The method of any one of embodiments 1 to 9, wherein the first Th1-polarizing adjuvant and DNA vaccine are administered to the subject 1, 2 or 3 times prior to the administration of the booster.

11. The method of embodiment 10, wherein the first Th1-polarizing adjuvant and DNA vaccine are administered to the subject at 0, 8, and 16 weeks.

12. The method of any one of embodiments 1 to 11, wherein the booster is administered to the subject 1 or 2 times.

13. The method of any one of embodiments 1 to 12, wherein peripheral and germinal center (GC) Tfh cells isolated from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster display higher proportions of anti-Env Th1 cells than do peripheral and germinal center (GC) Tfh cells taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

14. The method of any one of embodiments 1 to 13, wherein blood taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster shows higher anti-gp140 extrafollicular and/or plasma cell-derived titers than does blood from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

15. The method of any one of embodiments 1 to 14, wherein serum IgG antibodies taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster show a broader cross-clade anti-Env response and/or increased specificity for gp120 V1V2 loops than do serum IgG antibodies taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

16. The method of any one of embodiments 1 to 15, wherein serum IgG antibodies taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster display elevated anti-Env titers that persist longer than do serum IgG antibodies taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

17. The method of any one of embodiments 1 to 16, wherein serum IgG antibodies taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster show higher avidity against gp140 than do serum IgG antibodies taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

18. The method of any one of embodiments 1 to 17, wherein serum taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster have greater neutralization activity against HIV-1 than does serum taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

19. The method of embodiment 18, wherein the neutralization of HIV-1 is assessed using a TZM-bl assay.

20. The method of any one of embodiments 1 to 19, wherein serum taken from the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster have greater antibody-dependent cellular toxicity (ADCC) and/or antibody-dependent phagocytosis (ADP) activity against HIV-infected cells than does serum taken from a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

21. The method of embodiment 20, wherein the HIV-infected cells are infected with HIV-1.

22. The method of embodiment 21, wherein the HIV-1 is of clade C origin.

23. The method of any one of embodiments 1 to 22, wherein IgG and/or IgA antibodies isolated from the rectal and/or vaginal mucosa of the subject subsequent to the administration of the second Th1-polarizing adjuvant and booster show higher anti-gp140 titers than do IgG and/or IgA antibodies isolated from the rectal and/or vaginal mucosa of a second subject that has received the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

24. A pharmaceutical composition for vaccinating a subject against HIV, the composition comprising a DNA vaccine comprising a polynucleotide encoding an HIV Env polypeptide, a polynucleotide encoding interferon-induced protein (IP)-10, and a pharmaceutically acceptable carrier.

25. The pharmaceutical composition of embodiment 24, wherein the DNA vaccine further comprises one or more additional polynucleotides encoding one or more additional HIV polypeptides selected from the group consisting of Gag, protease, reverse transcriptase, Tat, Rev, and combinations thereof.

26. The pharmaceutical composition of embodiment 24 or 25, wherein the HIV Env polypeptide and/or one or more additional HIV polypeptides are from HIV-1.

27. The pharmaceutical composition of embodiment 26, wherein the HIV-1 is of clade C origin.

28. The pharmaceutical composition of any one of embodiments 24 to 27, wherein the polynucleotide encoding the HIV Env polypeptide and the polynucleotide encoding IP-10 are present within a single DNA vector.

29. The pharmaceutical composition of any one of embodiments 24 to 28, wherein the composition is formulated for transdermal delivery with electroporation.

30. A pharmaceutical composition for boosting vaccination against HIV, the composition comprising an HIV Env polypeptide, QS-21, and a pharmaceutically acceptable carrier.

31. The pharmaceutical composition of embodiment 30, wherein the HIV Env polypeptide is a gp140 polypeptide.

32. The pharmaceutical composition of embodiment 30 or 31, wherein the composition further comprises a lipid and/or liposomal adjuvant.

33. The pharmaceutical composition of embodiment 32, wherein the liposomal adjuvant comprises Army Liposome Formulation (ALF) liposomes.

34. The pharmaceutical composition of embodiment 32 or 33, wherein the lipid adjuvant comprises monophosphoryl lipid A (MPLA).

35. The pharmaceutical composition of any one of embodiments 30 to 34, wherein the HIV Env polypeptide is from HIV-1.

36. The pharmaceutical composition of embodiment 35, wherein the HIV-1 is of clade C origin.

[0239] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, one of skill in the art will appreciate that certain changes and modifications may be practiced within the scope of the appended claims. In addition, each reference provided herein is incorporated by reference in its entirety to the same extent as if each reference was individually incorporated by reference.

WHAT IS CLAIMED IS:

1 1. A method of enhancing the anti-Env antibody response during HIV
2 vaccination in a subject, the method comprising:

3 (i) administering a first Th1-polarizing adjuvant to the subject together with a
4 DNA vaccine comprising a polynucleotide encoding a first HIV Env polypeptide, wherein
5 the first adjuvant comprises a polynucleotide encoding interferon-induced protein (IP)-10;
6 and

7 (ii) administering a second Th1-polarizing adjuvant to the subject together
8 with a booster comprising a second HIV Env polypeptide, wherein the second adjuvant
9 comprises QS-21.

1 2. The method of claim 1, wherein the second HIV Env polypeptide is a
2 gp140 polypeptide.

1 3. The method of claim 1, wherein the DNA vaccine further comprises
2 one or more additional polynucleotides encoding one or more additional HIV polypeptides
3 selected from the group consisting of Gag, protease, reverse transcriptase, Tat, Rev, and
4 combinations thereof.

1 4. The method of claim 1, wherein the first adjuvant and the DNA
2 vaccine are administered transdermally with electroporation.

1 5. The method of claim 1, wherein the first HIV Env polypeptide, second
2 HIV Env polypeptide, and/or one or more additional HIV polypeptides are from HIV-1.

1 6. The method of claim 5, wherein the HIV-1 is of clade C origin.

1 7. The method of claim 1, wherein the booster further comprises a lipid
2 and/or liposomal adjuvant.

1 8. The method of claim 7, wherein the liposomal adjuvant comprises
2 Army Liposome Formulation (ALF) liposomes.

1 9. The method of claim 7, wherein the lipid adjuvant comprises
2 monophosphoryl lipid A (MPLA).

1 10. The method of claim 1, wherein the first Th1-polarizing adjuvant and
2 DNA vaccine are administered to the subject 1, 2 or 3 times prior to the administration of the
3 booster.

1 11. The method of claim 10, wherein the first Th1-polarizing adjuvant and
2 DNA vaccine are administered to the subject at 0, 8, and 16 weeks.

1 12. The method of claim 1, wherein the booster is administered to the
2 subject 1 or 2 times.

1 13. The method of claim 1, wherein peripheral and germinal center (GC)
2 Tfh cells isolated from the subject subsequent to the administration of the second Th1-
3 polarizing adjuvant and booster display higher proportions of anti-Env Th1 cells than do
4 peripheral and germinal center (GC) Tfh cells taken from a second subject that has received
5 the DNA vaccine and booster but that has not received the first and/or second Th1-polarizing
6 adjuvant.

1 14. The method of claim 1, wherein blood taken from the subject
2 subsequent to the administration of the second Th1-polarizing adjuvant and booster shows
3 higher anti-gp140 extrafollicular and/or plasma cell-derived titers than does blood from a
4 second subject that has received the DNA vaccine and booster but that has not received the
5 first and/or second Th1-polarizing adjuvant.

1 15. The method of claim 1, wherein serum IgG antibodies taken from the
2 subject subsequent to the administration of the second Th1-polarizing adjuvant and booster
3 show a broader cross-clade anti-Env response and/or increased specificity for gp120 V1V2
4 loops than do serum IgG antibodies taken from a second subject that has received the DNA
5 vaccine and booster but that has not received the first and/or second Th1-polarizing adjuvant.

1 16. The method of claim 1, wherein serum IgG antibodies taken from the
2 subject subsequent to the administration of the second Th1-polarizing adjuvant and booster
3 display elevated anti-Env titers that persist longer than do serum IgG antibodies taken from a
4 second subject that has received the DNA vaccine and booster but that has not received the
5 first and/or second Th1-polarizing adjuvant.

1 17. The method of claim 1, wherein serum IgG antibodies taken from the
2 subject subsequent to the administration of the second Th1-polarizing adjuvant and booster
3 show higher avidity against gp140 than do serum IgG antibodies taken from a second subject
4 that has received the DNA vaccine and booster but that has not received the first and/or
5 second Th1-polarizing adjuvant.

1 18. The method of claim 1, wherein serum taken from the subject
2 subsequent to the administration of the second Th1-polarizing adjuvant and booster have
3 greater neutralization activity against HIV-1 than does serum taken from a second subject
4 that has received the DNA vaccine and booster but that has not received the first and/or
5 second Th1-polarizing adjuvant.

1 19. The method of claim 18, wherein the neutralization of HIV-1 is
2 assessed using a TZM-bl assay.

1 20. The method of claim 1, wherein serum taken from the subject
2 subsequent to the administration of the second Th1-polarizing adjuvant and booster have
3 greater antibody-dependent cellular toxicity (ADCC) and/or antibody-dependent
4 phagocytosis (ADP) activity against HIV-infected cells than does serum taken from a second
5 subject that has received the DNA vaccine and booster but that has not received the first
6 and/or second Th1-polarizing adjuvant.

1 21. The method of claim 20, wherein the HIV-infected cells are infected
2 with HIV-1.

1 22. The method of claim 21, wherein the HIV-1 is of clade C origin.

1 23. The method of claim 1, wherein IgG and/or IgA antibodies isolated
2 from the rectal and/or vaginal mucosa of the subject subsequent to the administration of the
3 second Th1-polarizing adjuvant and booster show higher anti-gp140 titers than do IgG and/or
4 IgA antibodies isolated from the rectal and/or vaginal mucosa of a second subject that has
5 received the DNA vaccine and booster but that has not received the first and/or second Th1-
6 polarizing adjuvant.

1 24. A pharmaceutical composition for vaccinating a subject against HIV,
2 the composition comprising a DNA vaccine comprising a polynucleotide encoding an HIV

3 Env polypeptide, a polynucleotide encoding interferon-induced protein (IP)-10, and a
4 pharmaceutically acceptable carrier.

1 25. The pharmaceutical composition of claim 24, wherein the DNA
2 vaccine further comprises one or more additional polynucleotides encoding one or more
3 additional HIV polypeptides selected from the group consisting of Gag, protease, reverse
4 transcriptase, Tat, Rev, and combinations thereof.

1 26. The pharmaceutical composition of claim 24, wherein the HIV Env
2 polypeptide and/or one or more additional HIV polypeptides are from HIV-1.

1 27. The pharmaceutical composition of claim 26, wherein the HIV-1 is of
2 clade C origin.

1 28. The pharmaceutical composition of claim 24, wherein the
2 polynucleotide encoding the HIV Env polypeptide and the polynucleotide encoding IP-10 are
3 present within a single DNA vector.

1 29. The pharmaceutical composition of claim 24, wherein the composition
2 is formulated for transdermal delivery with electroporation.

1 30. A pharmaceutical composition for boosting vaccination against HIV,
2 the composition comprising an HIV Env polypeptide, QS-21, and a pharmaceutically
3 acceptable carrier.

1 31. The pharmaceutical composition of claim 30, wherein the HIV Env
2 polypeptide is a gp140 polypeptide.

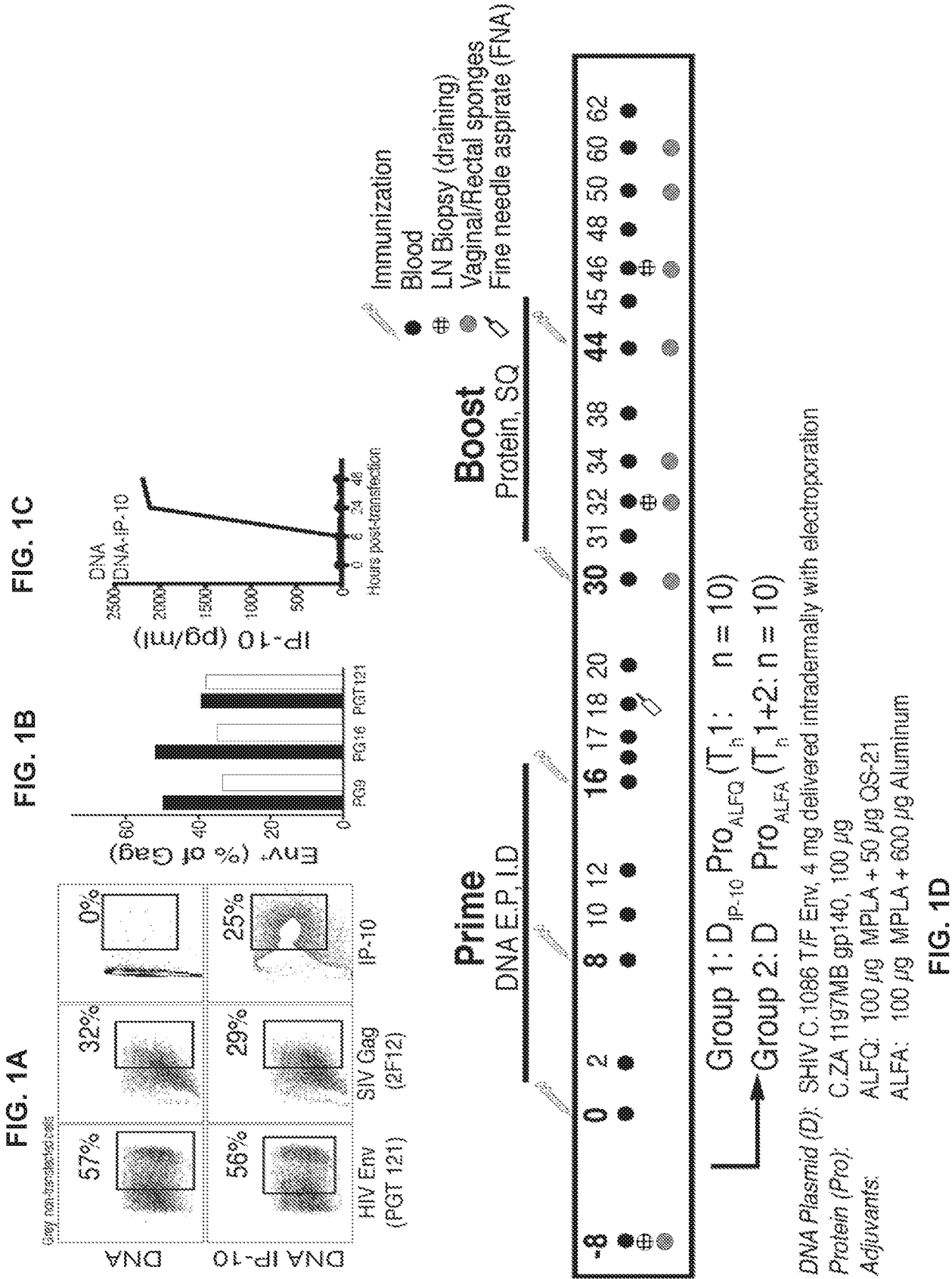
1 32. The pharmaceutical composition of claim 30, wherein the composition
2 further comprises a lipid and/or liposomal adjuvant.

1 33. The pharmaceutical composition of claim 32, wherein the liposomal
2 adjuvant comprises Army Liposome Formulation (ALF) liposomes.

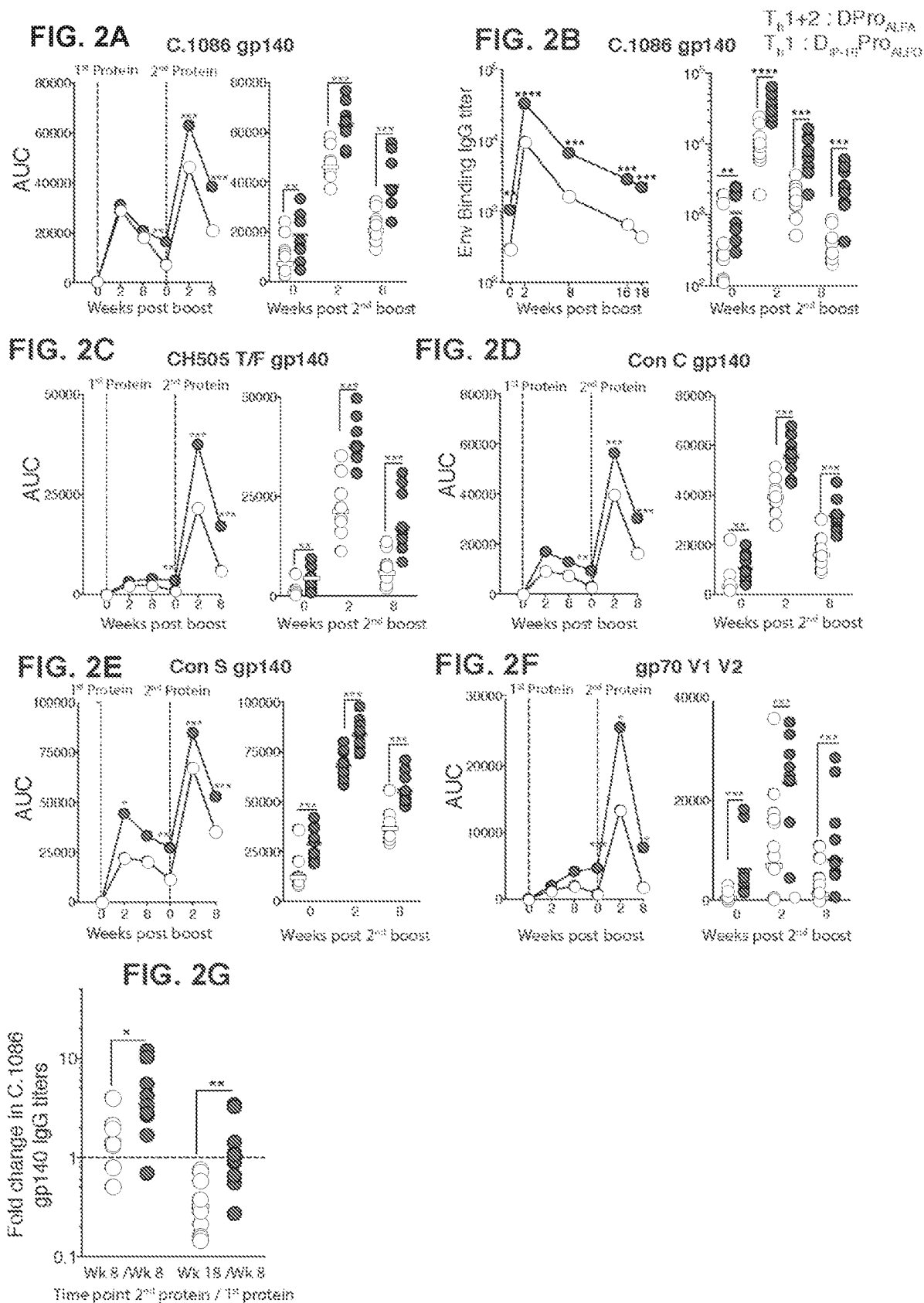
1 34. The pharmaceutical composition of claim 32, wherein the lipid
2 adjuvant comprises monophosphoryl lipid A (MPLA).

1 35. The pharmaceutical composition of claim 30, wherein the HIV Env
2 polypeptide is from HIV-1.

1 36. The pharmaceutical composition of claim 35, wherein the HIV-1 is of
2 clade C origin.



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FIG. 3A

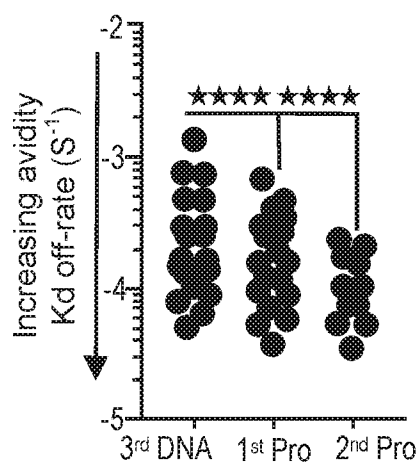


FIG. 3B

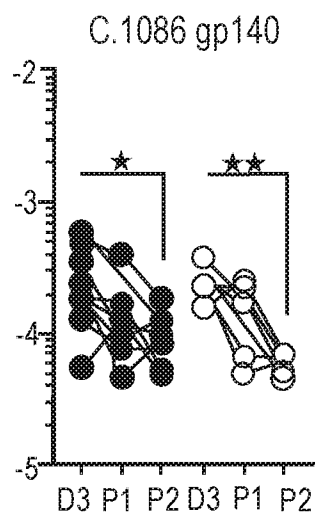


FIG. 3C

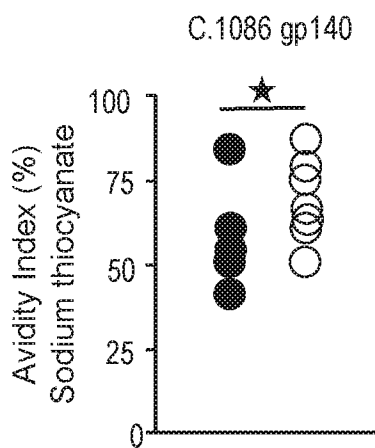
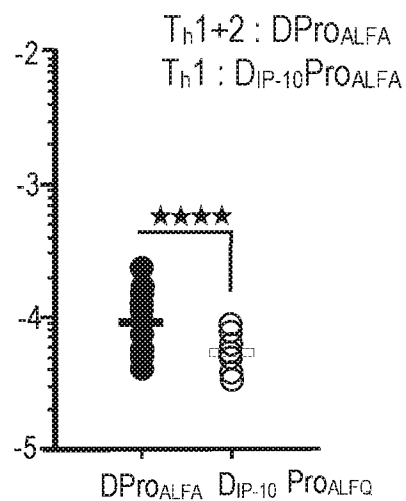


FIG. 3D

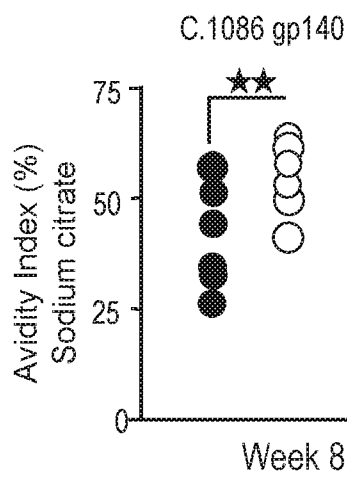


FIG. 3E

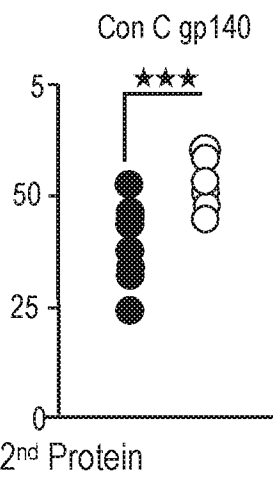


FIG. 3F

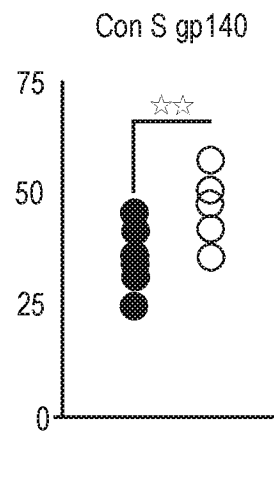


FIG. 3G

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FIG. 3H

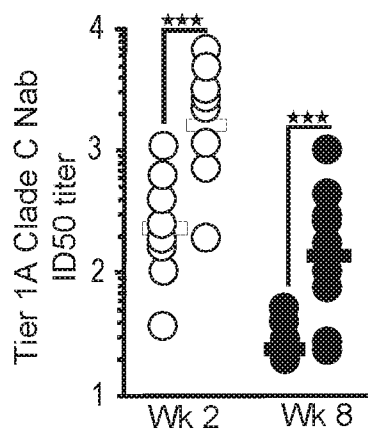


FIG. 3I

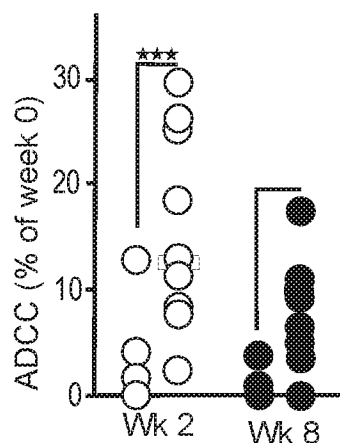


FIG. 3J

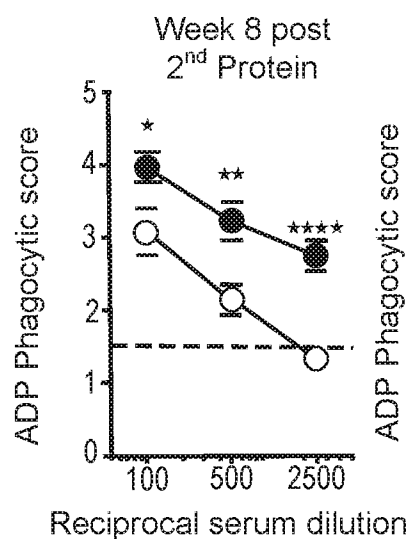


FIG. 3K

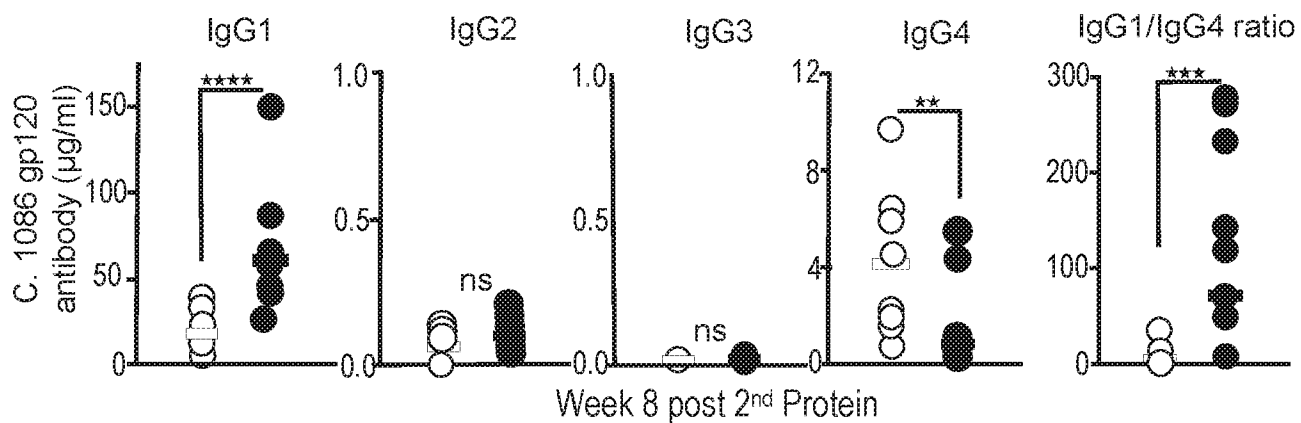
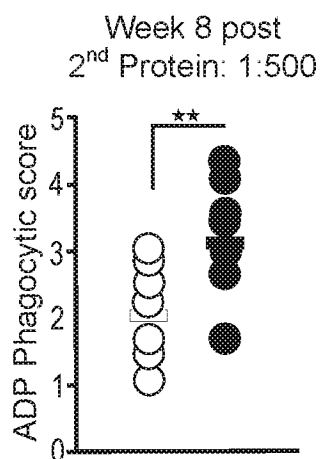
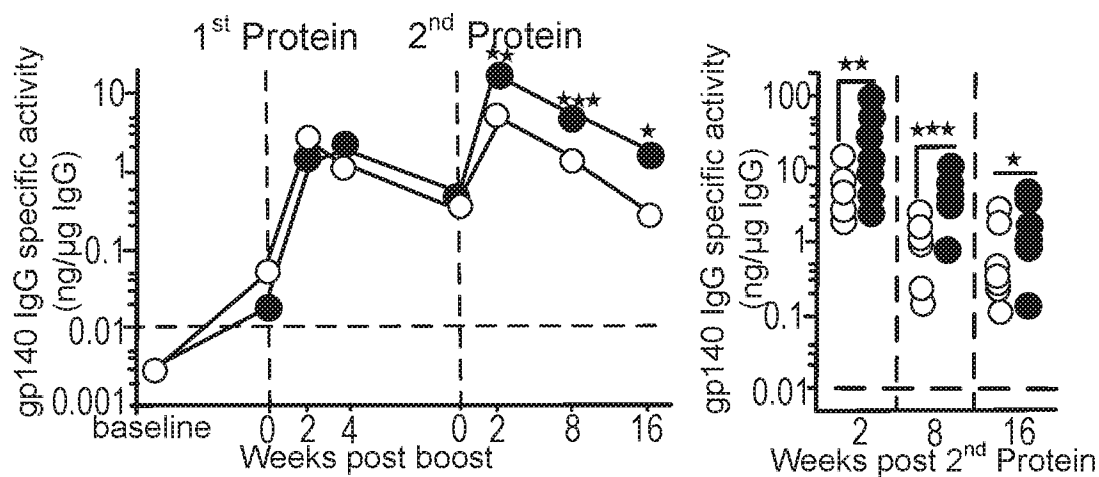
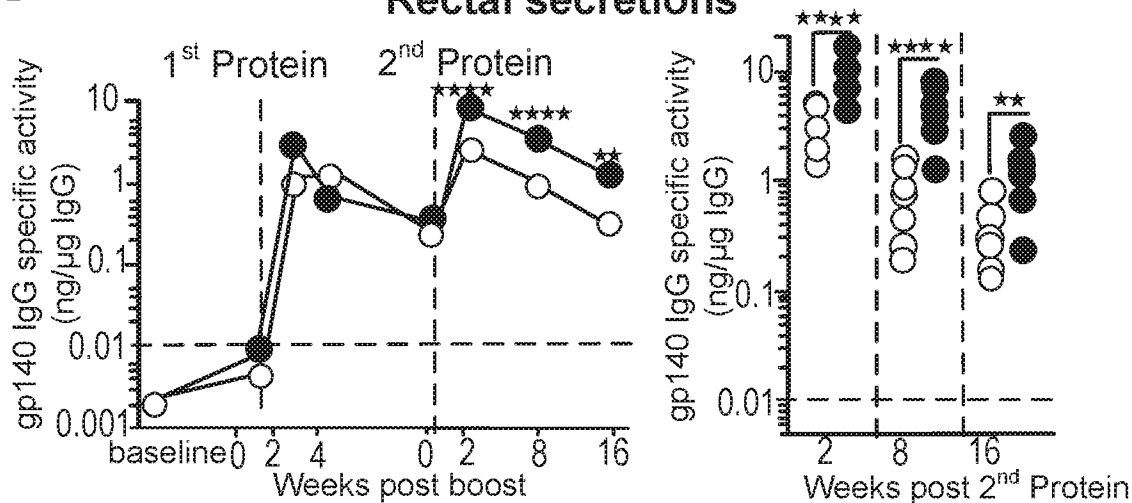
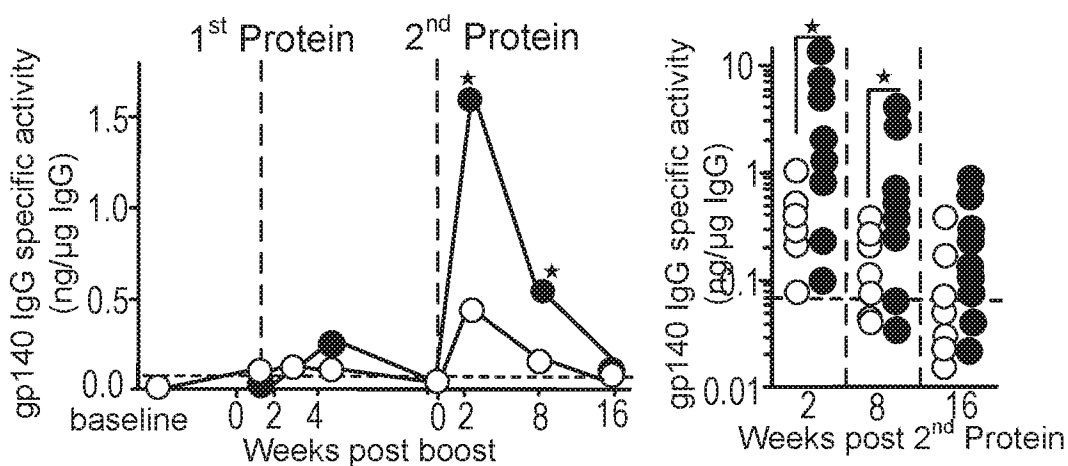


FIG. 3L

FIG. 3M

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 T_{h1+2} : DPrO_{ALFA} T_{h1} : DIP-10PrO_{ALFQ}**FIG. 4A****Vaginal secretions****FIG. 4B****Rectal secretions****FIG. 4C****Vaginal secretions**

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FIG. 4D

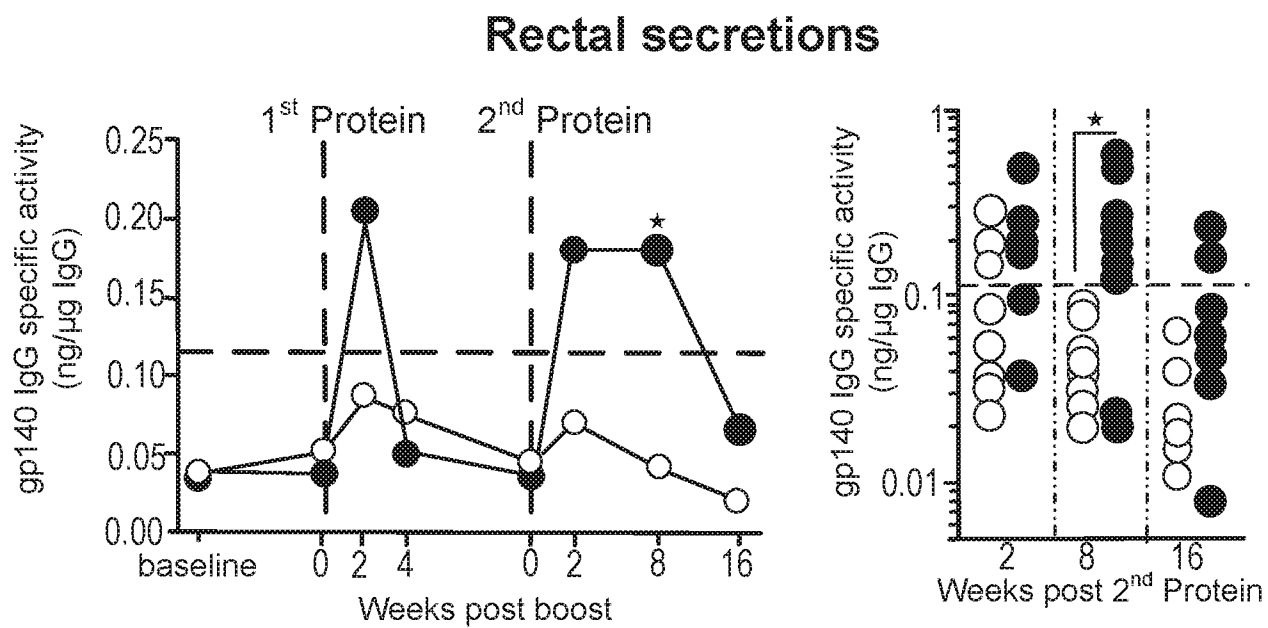


FIG. 4E

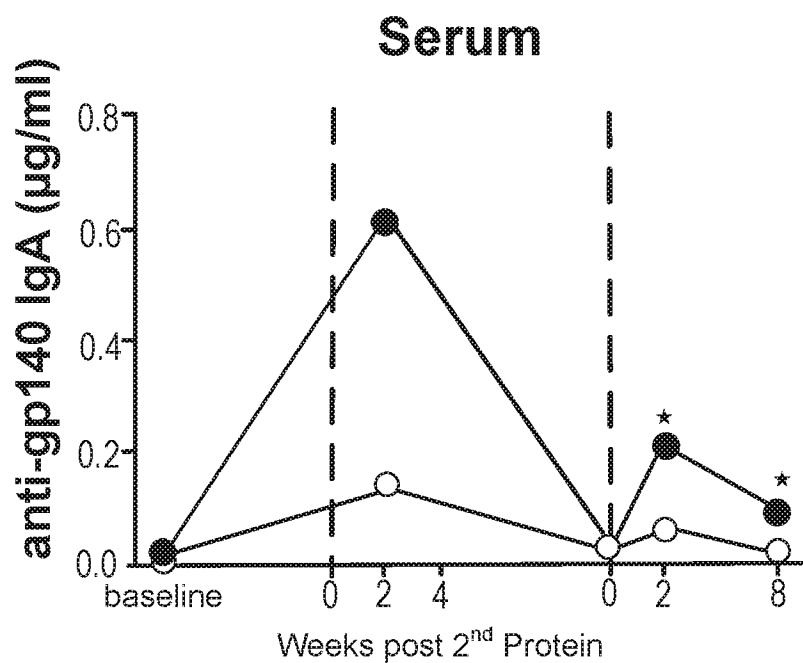
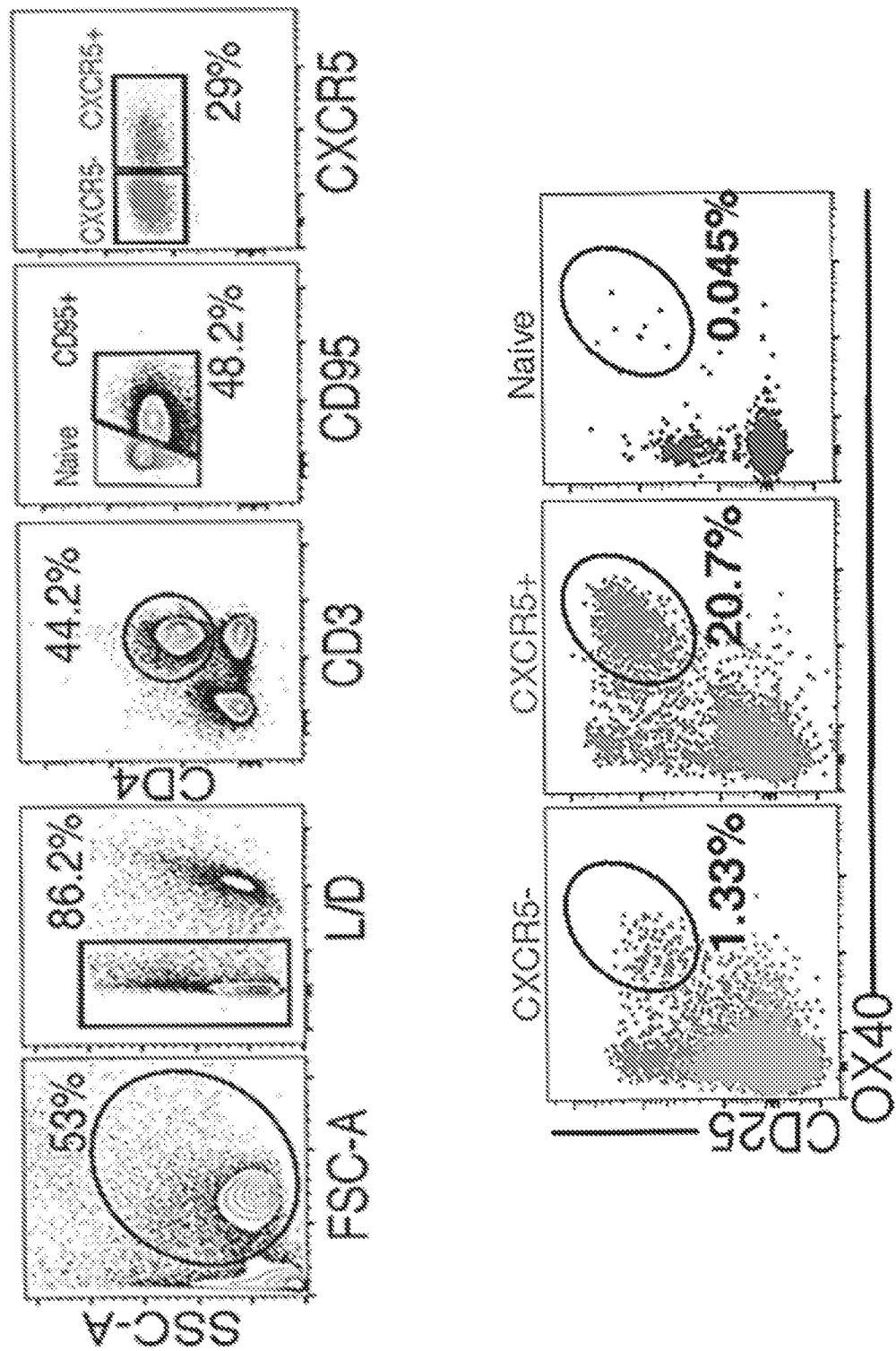


FIG. 5A



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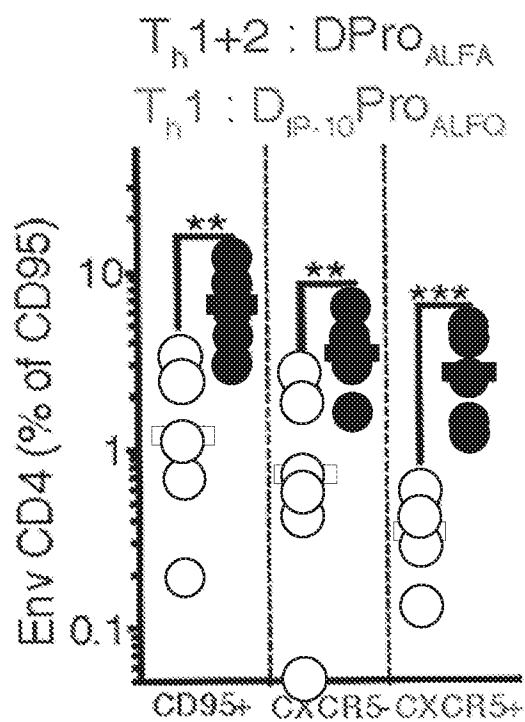


FIG. 5B

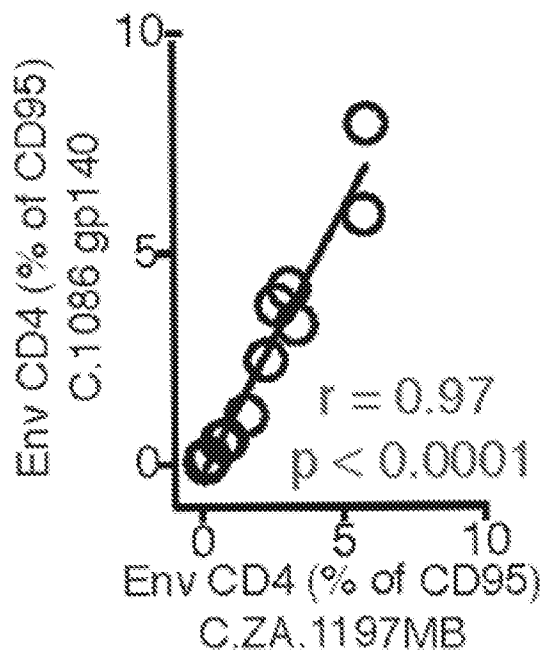


FIG. 5C

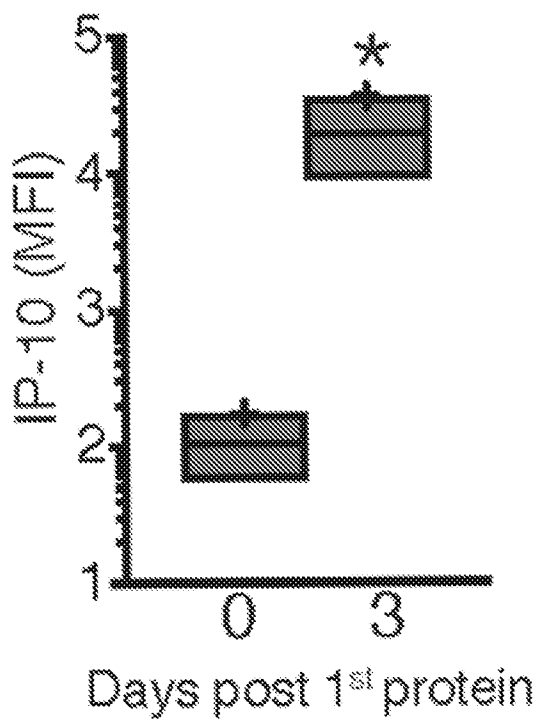
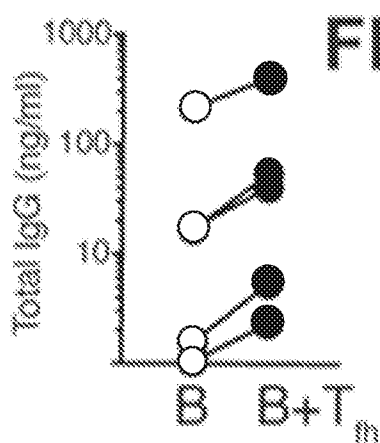
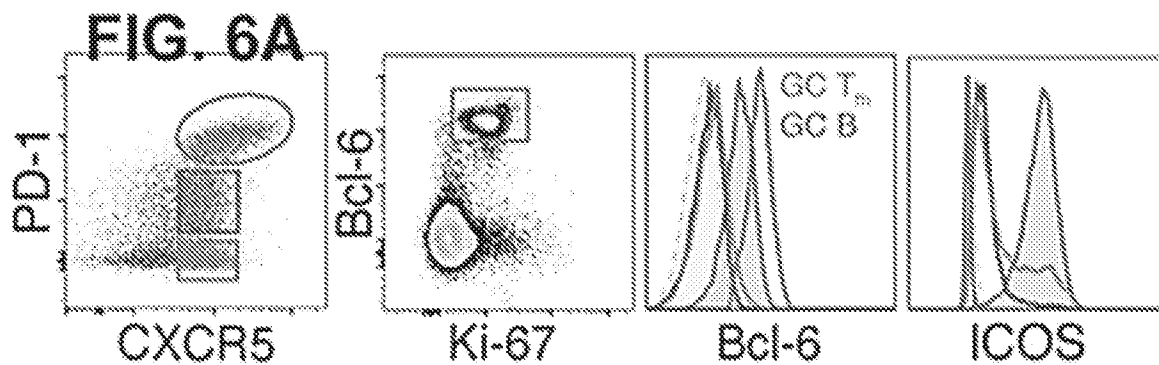
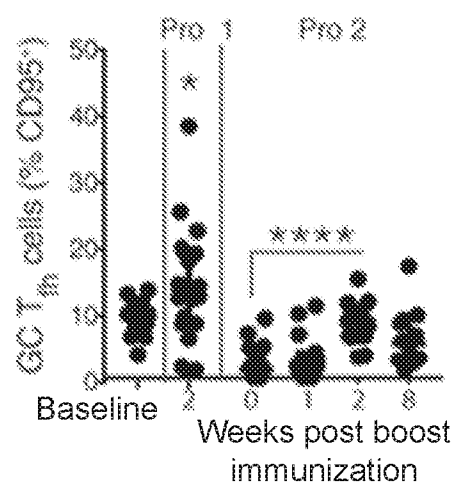
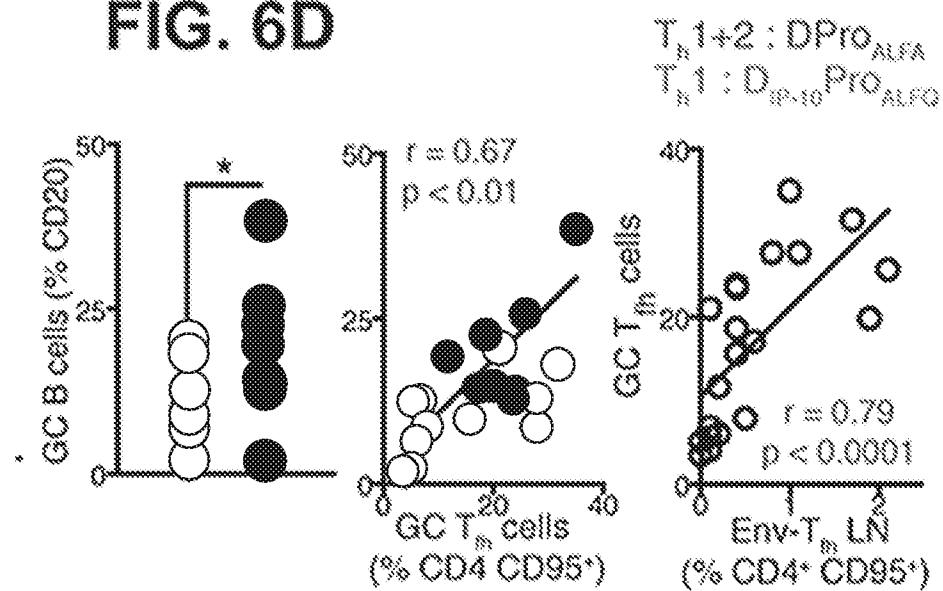
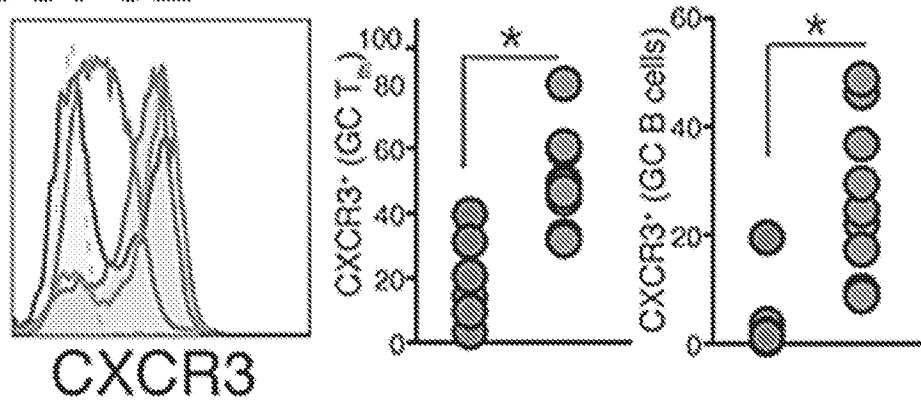
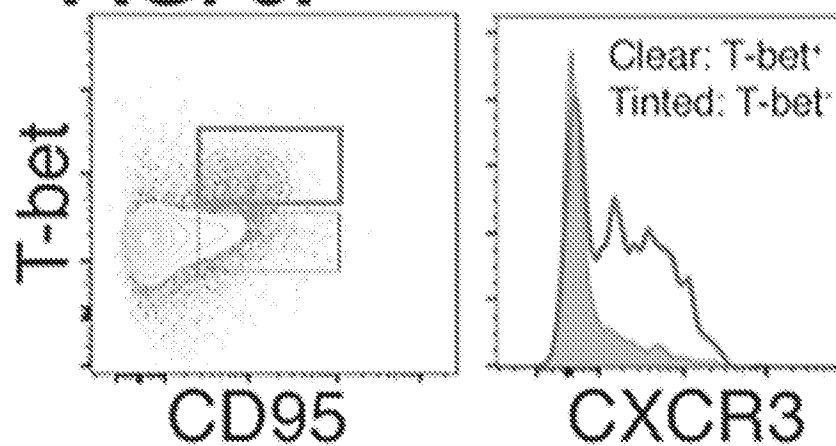
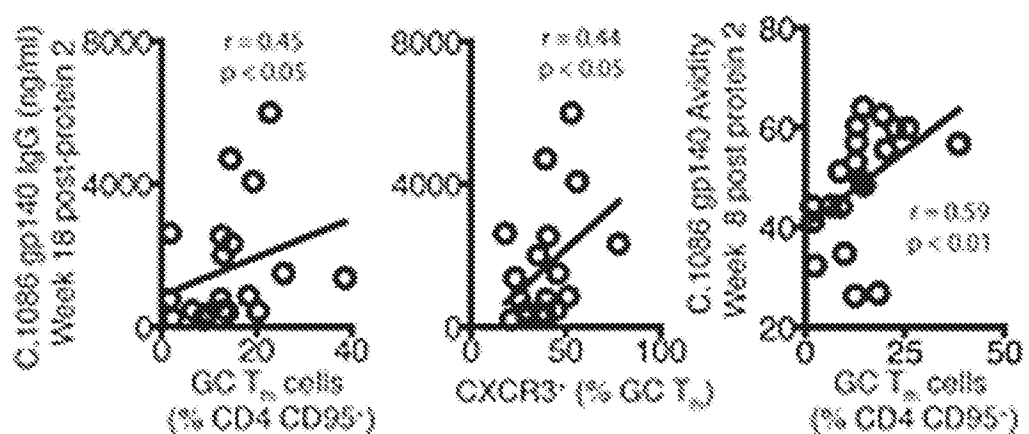


FIG. 5D

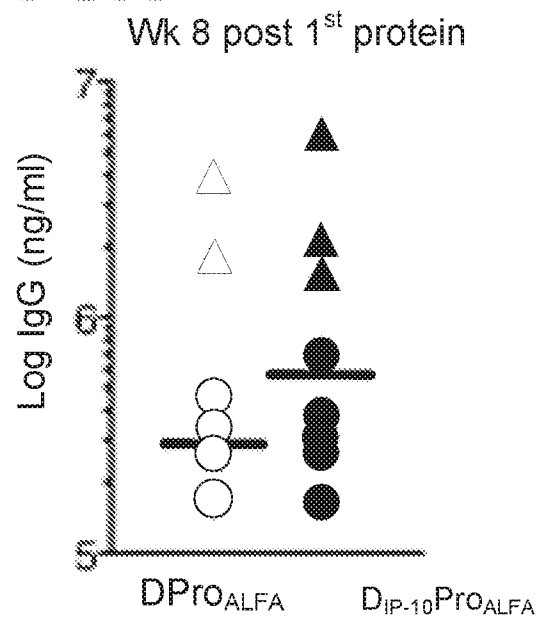
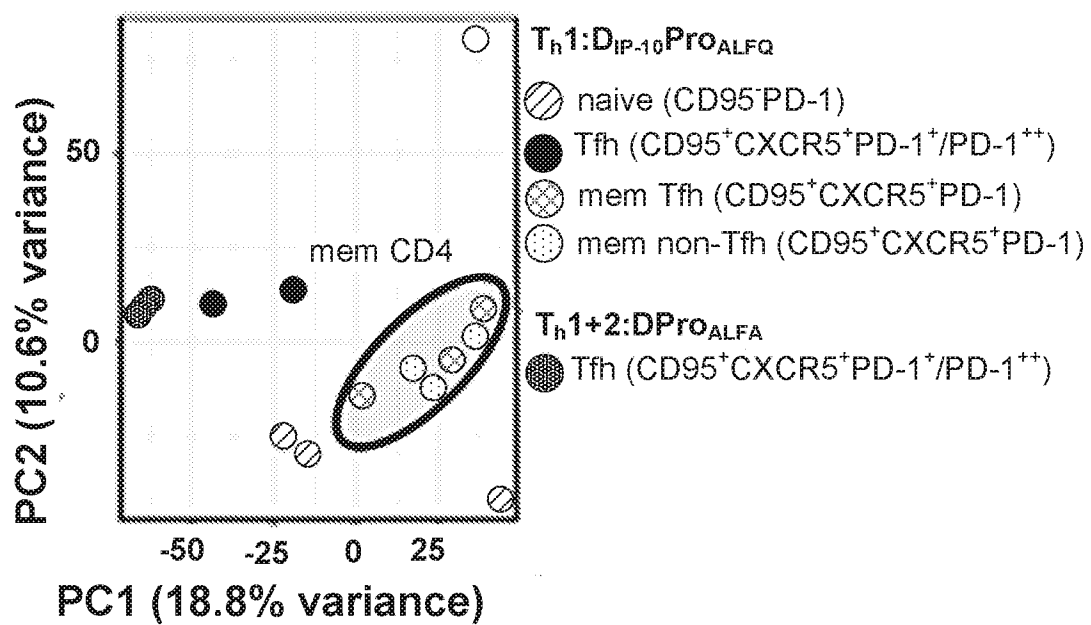
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**FIG. 6C****FIG. 6D**

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FIG. 6E**FIG. 6F****FIG. 6G**

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FIG. 6H**FIG. 6I**

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FIG. 6J

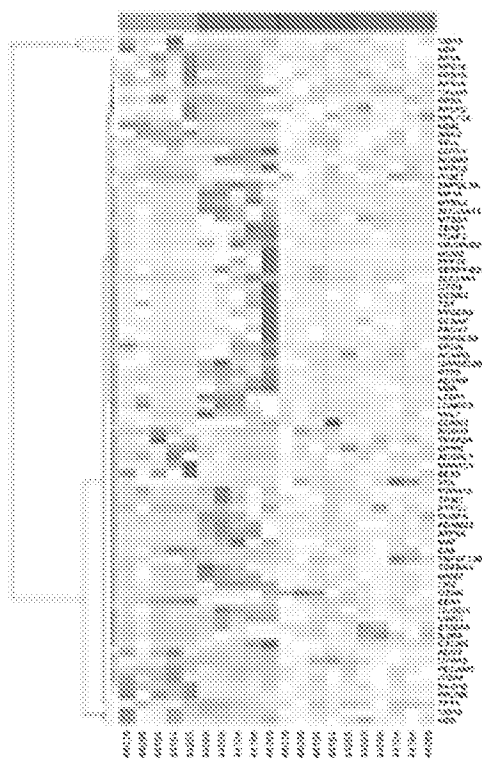


FIG. 6K

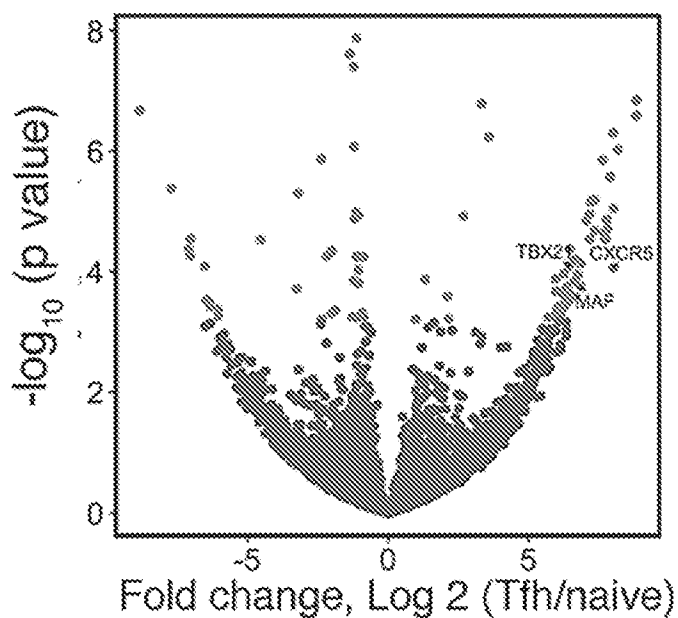
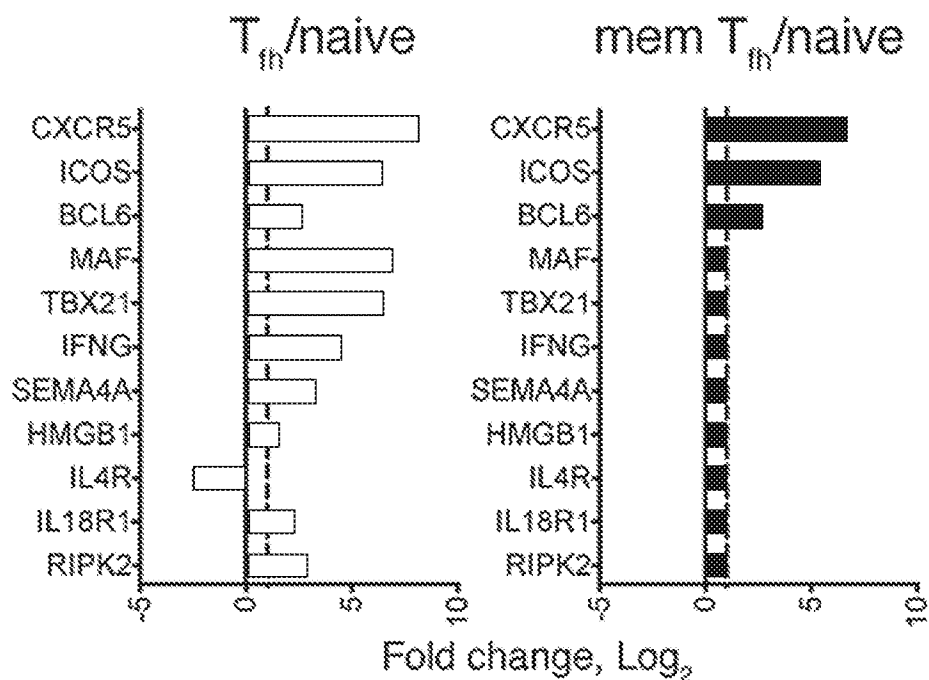


FIG. 6L



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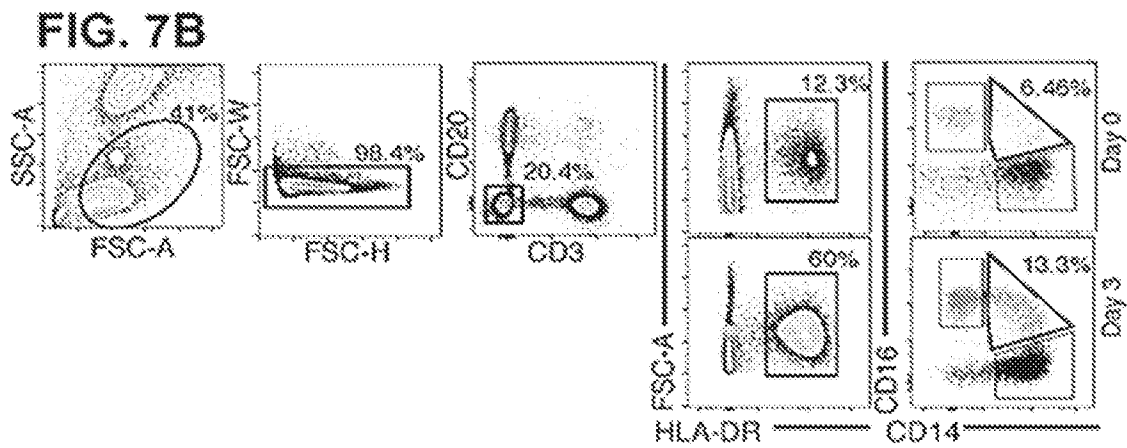
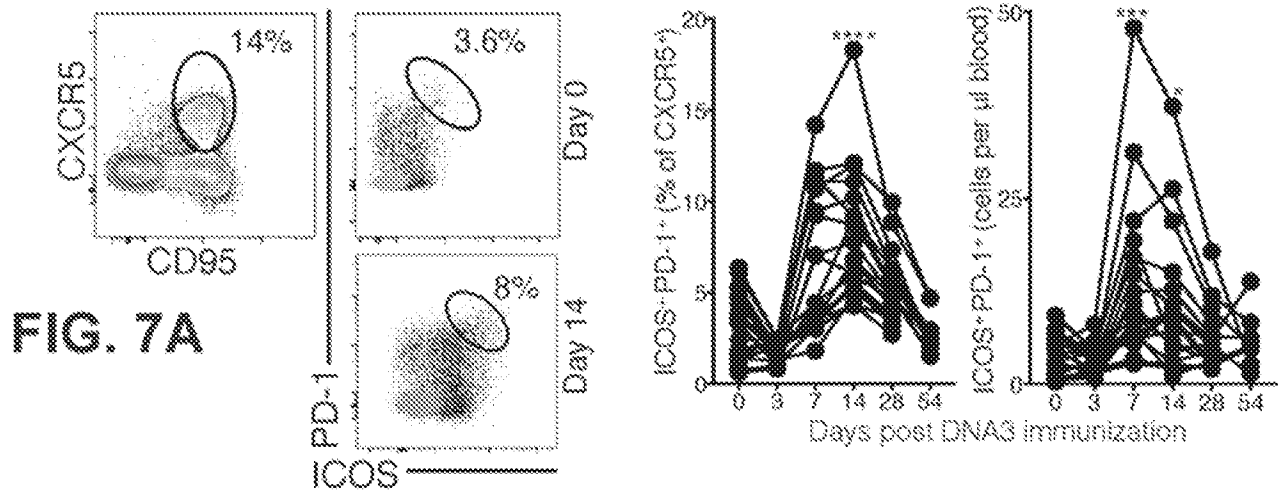
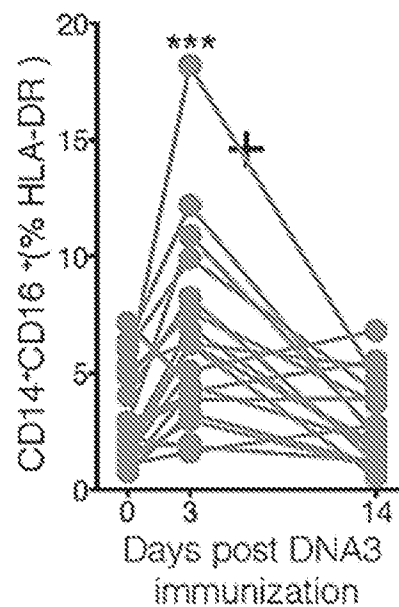


FIG. 7C



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FIG. 7D



FIG. 7E

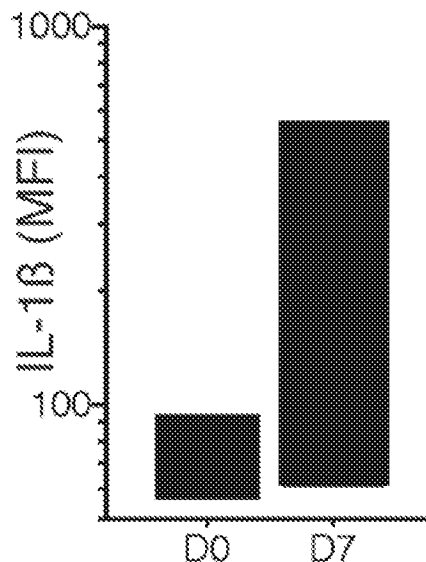


FIG. 7F

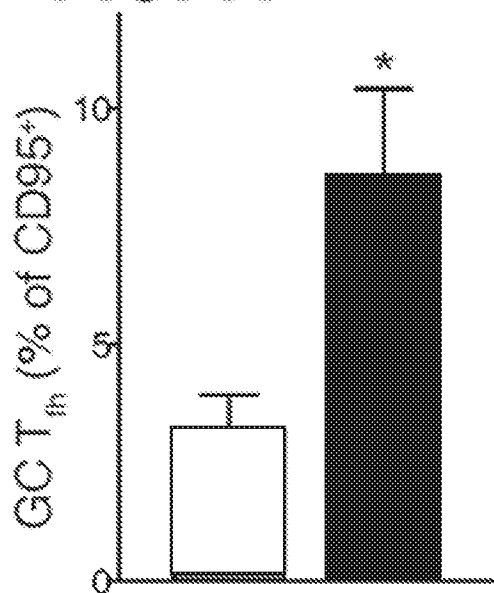
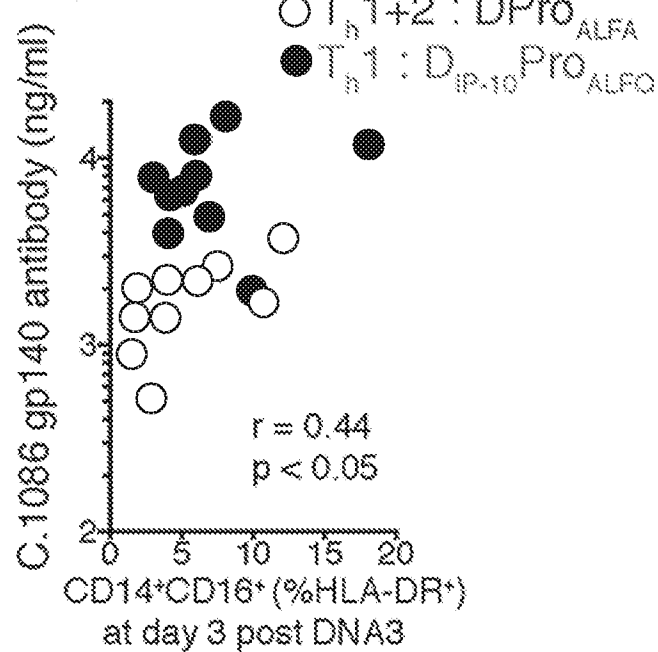


FIG. 7G



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FIG. 8A

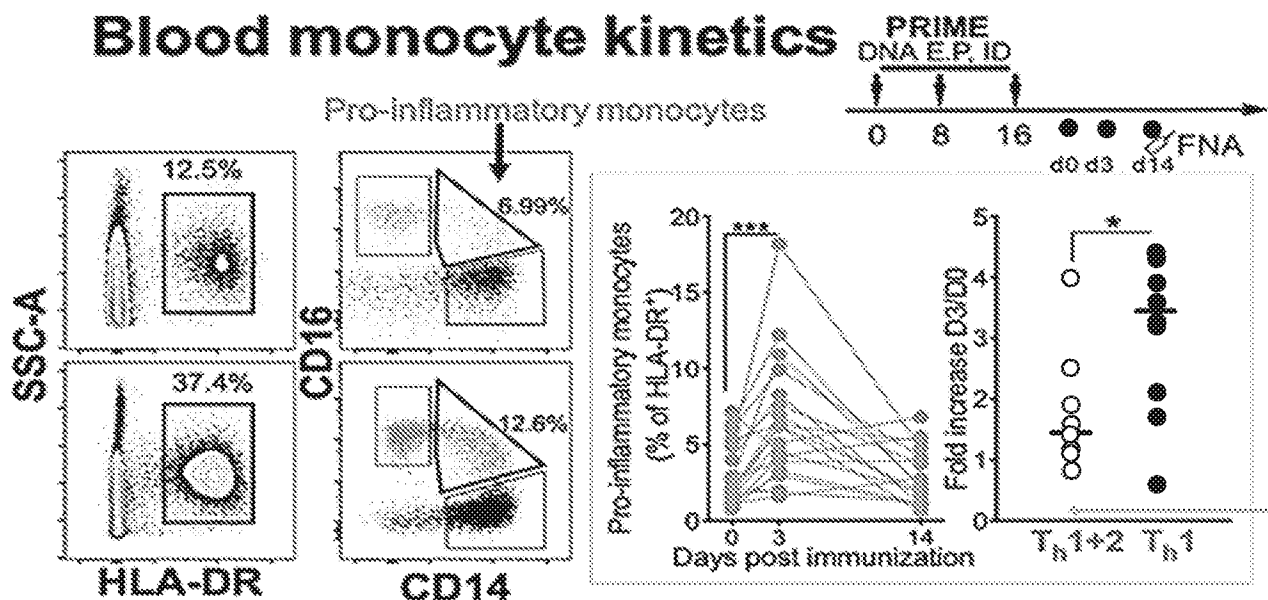


FIG. 8B

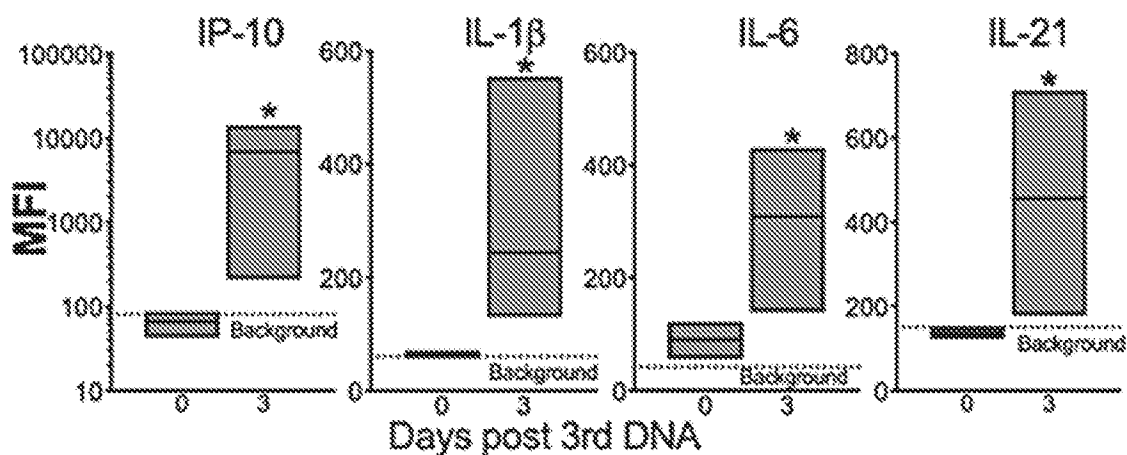


FIG. 8C

Fine needle aspirate (FNA)

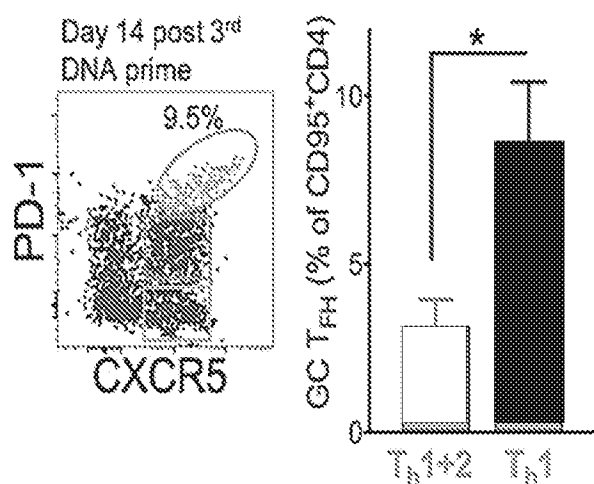
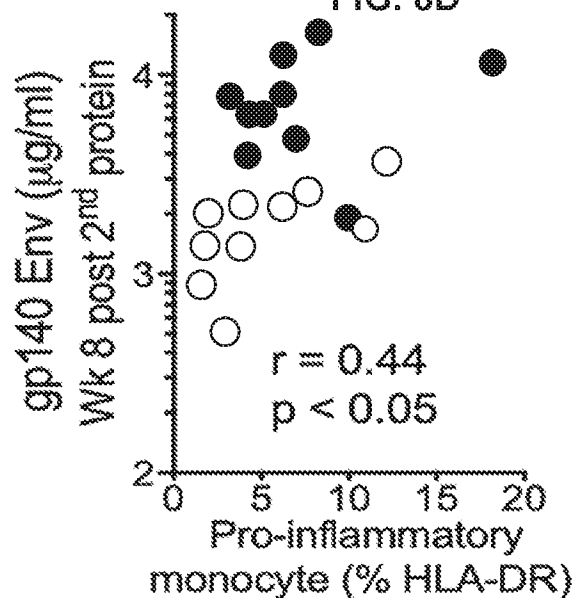


FIG. 8D



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FIG. 9A

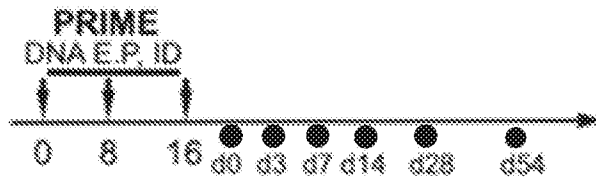


FIG. 9B

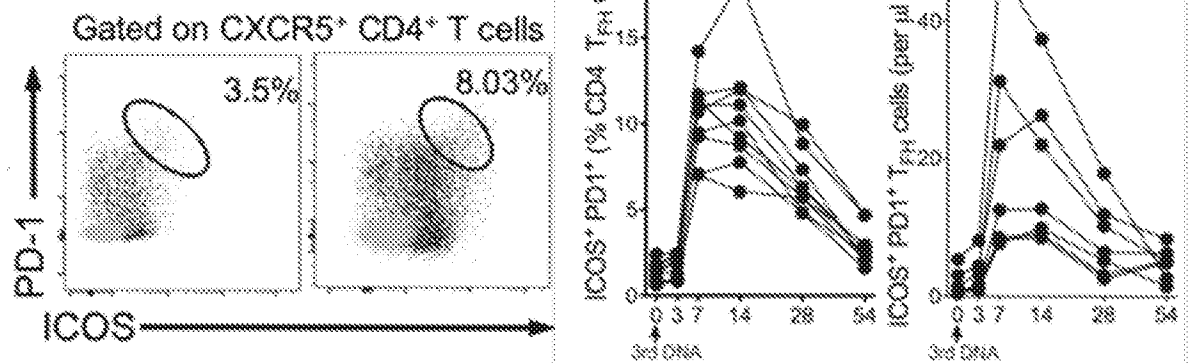


FIG. 9C

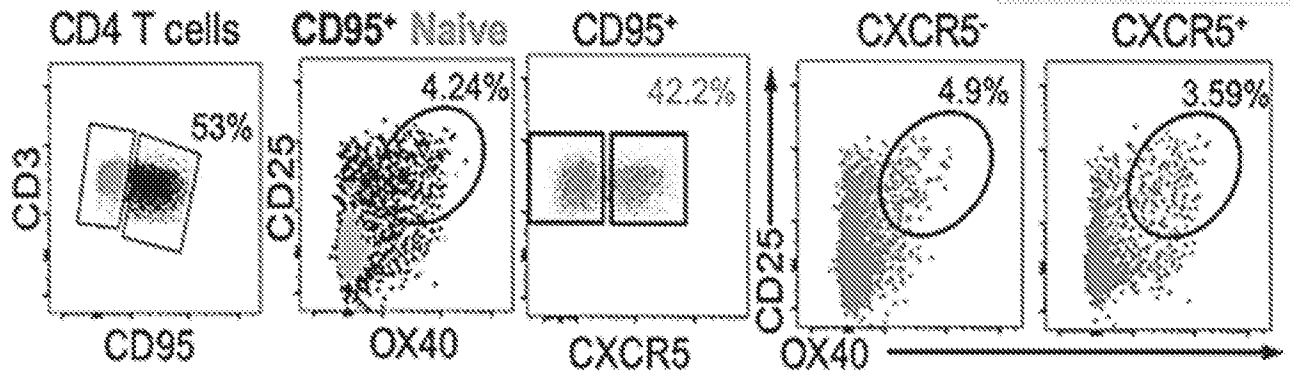
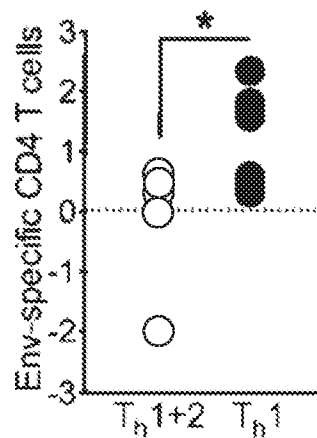


FIG. 9D



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FIG. 10A

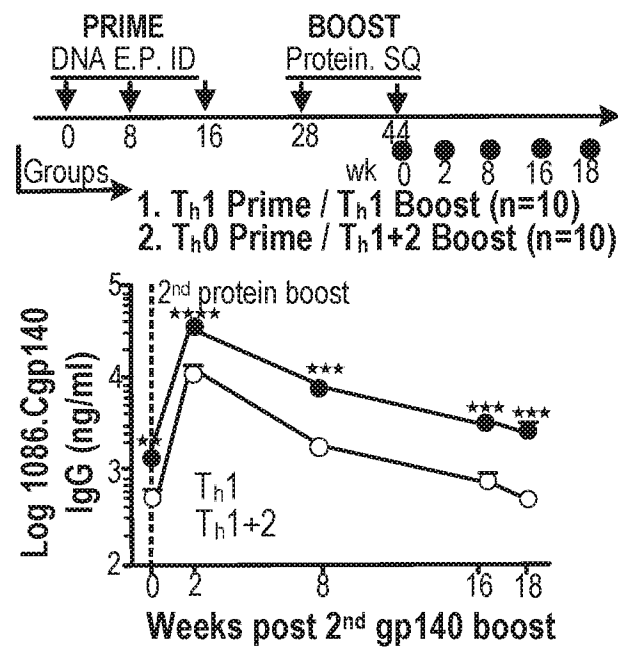
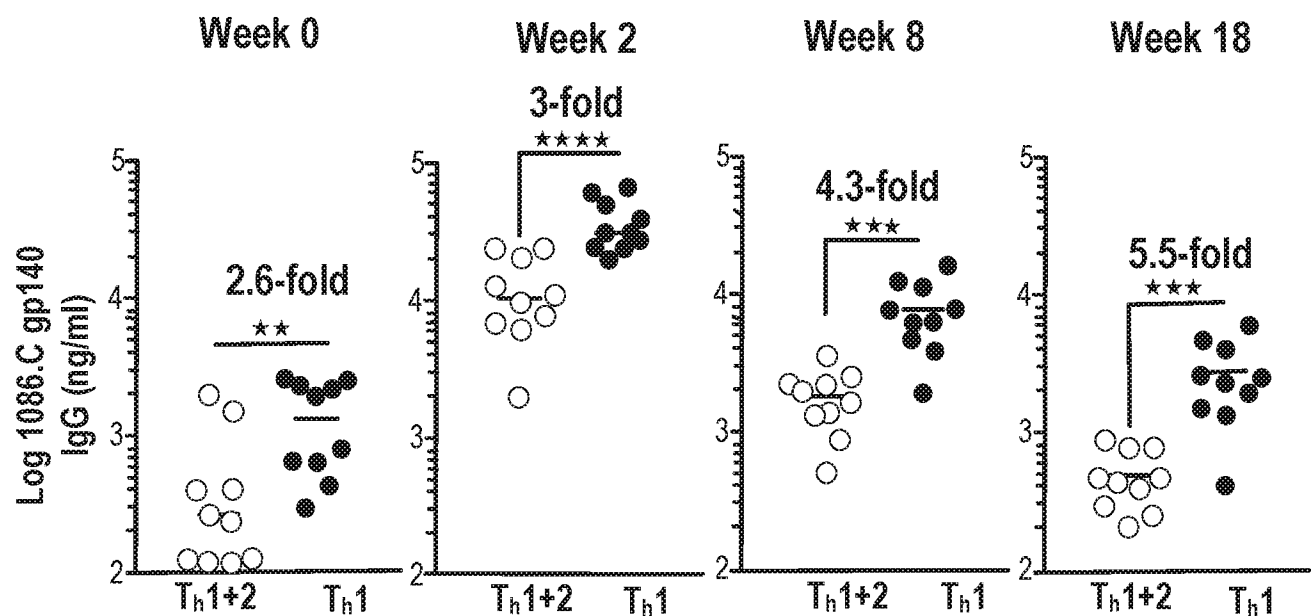


FIG. 10B



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FIG. 11A

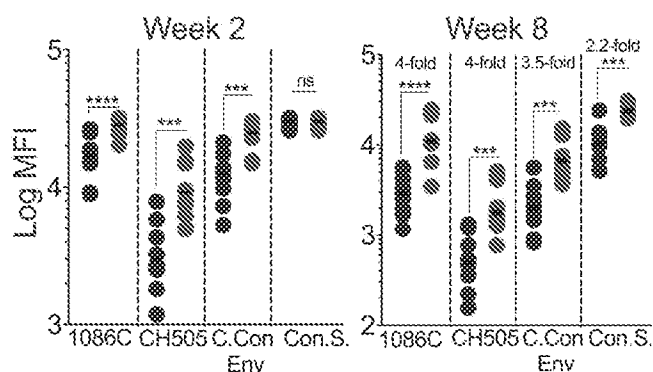


FIG. 11B

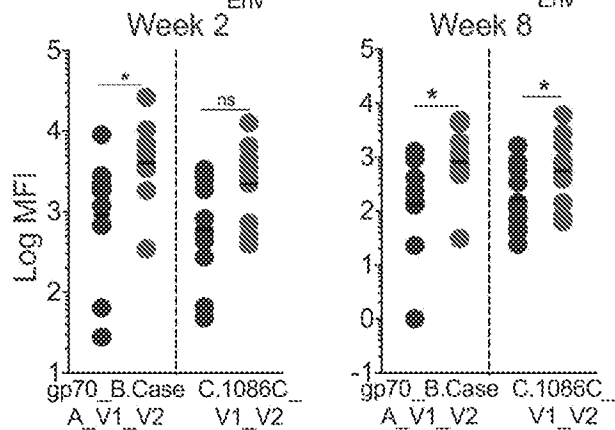
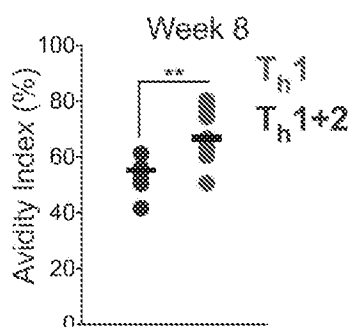


FIG. 11C

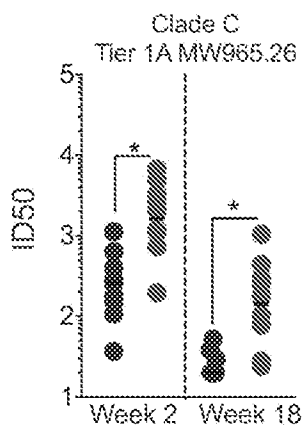
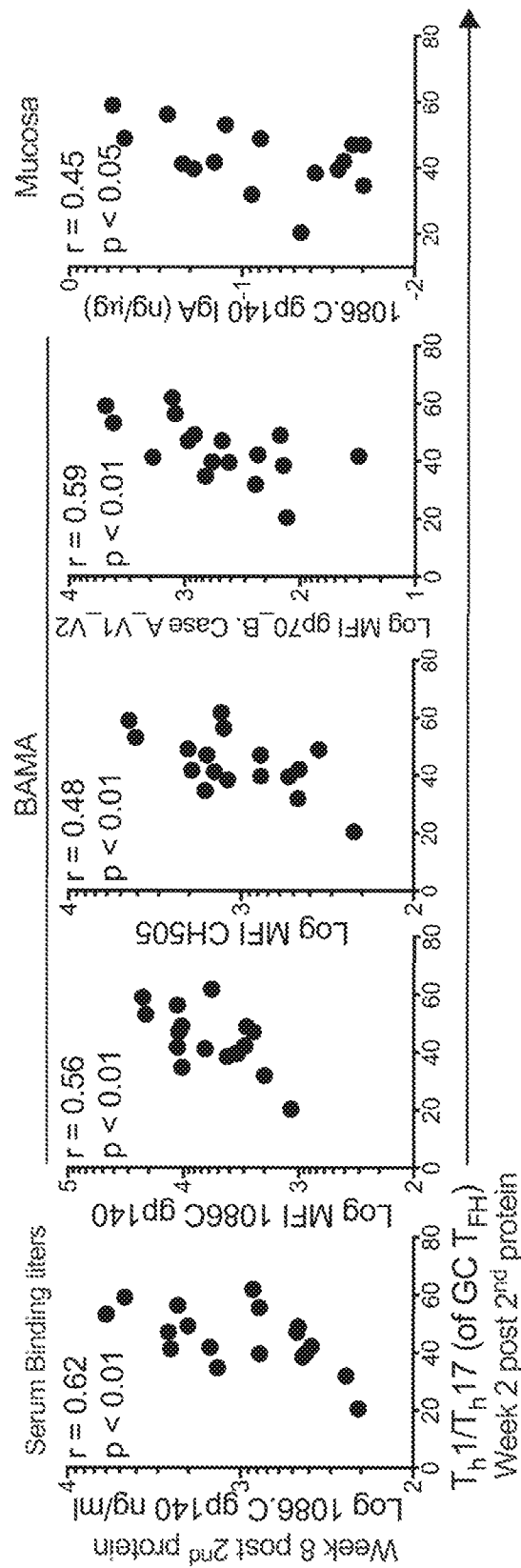


FIG. 11D

FIG. 12



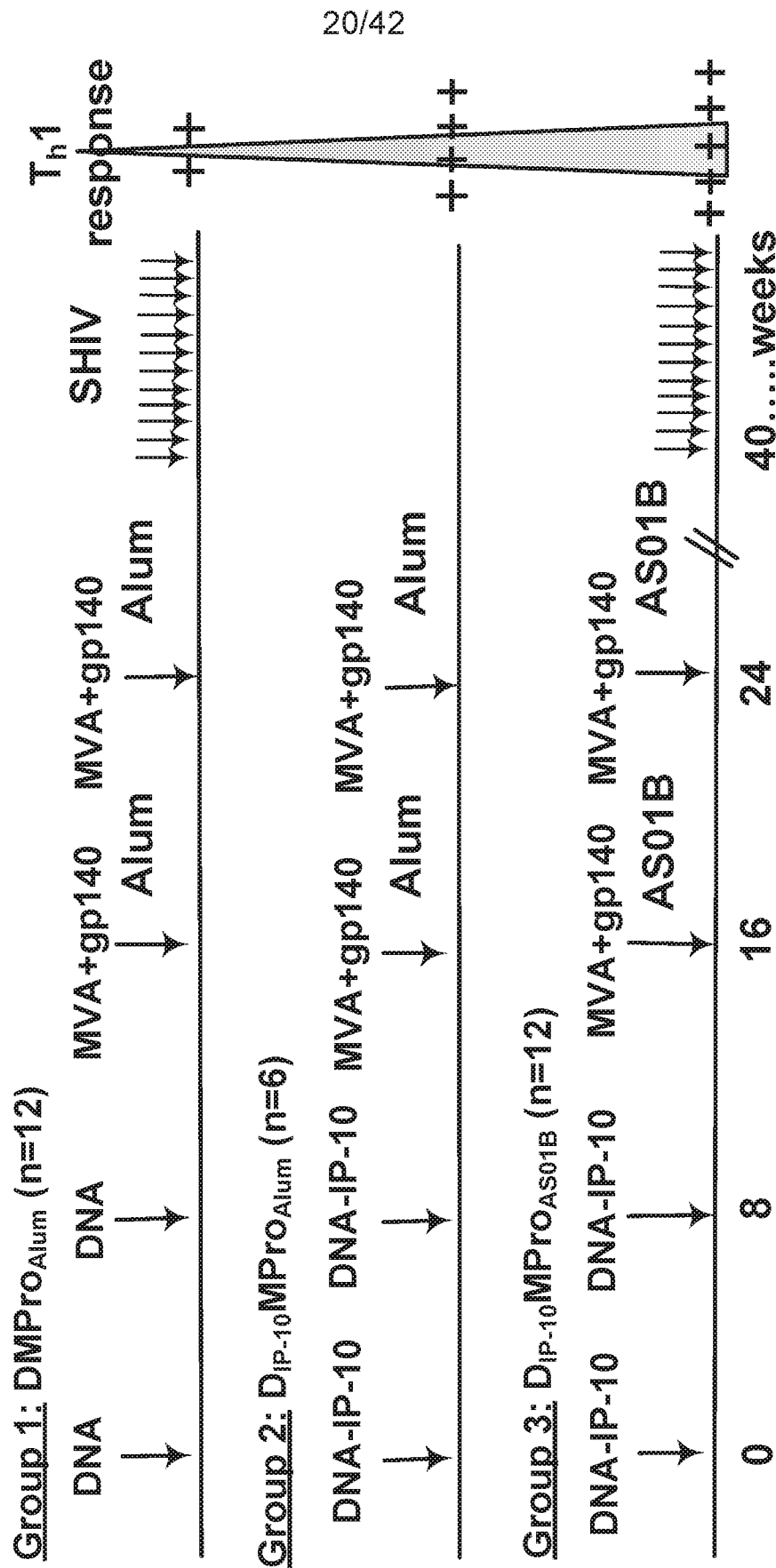


FIG. 13

FIG. 14A

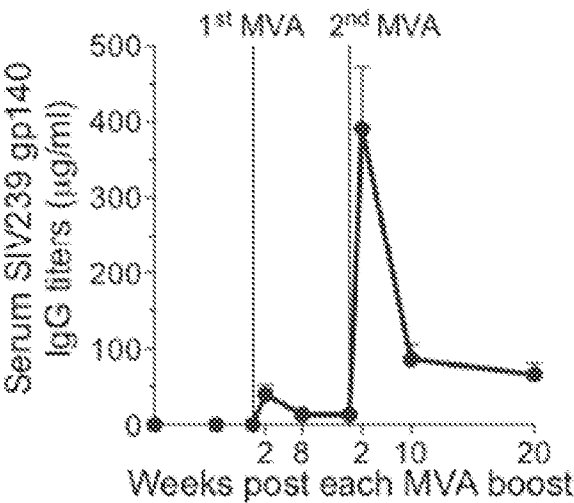


FIG. 14B

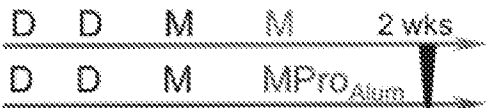


FIG. 14C

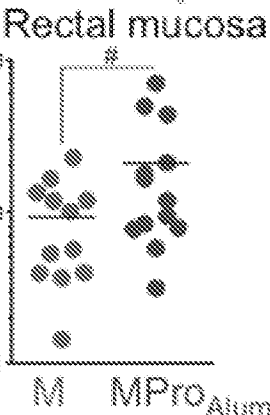
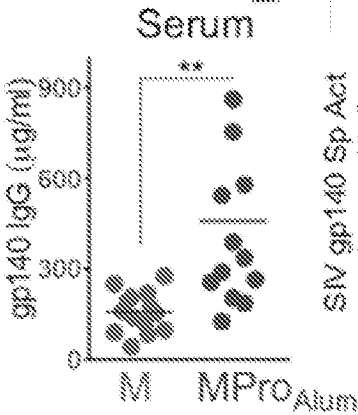


FIG. 14D

FIG. 14E

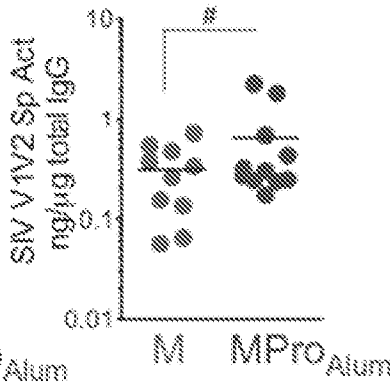
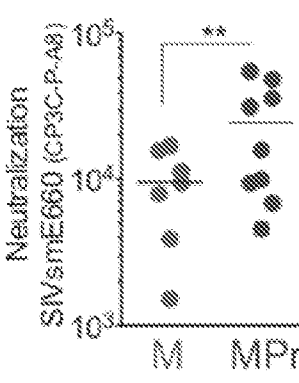


FIG. 14F

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FIG. 15A

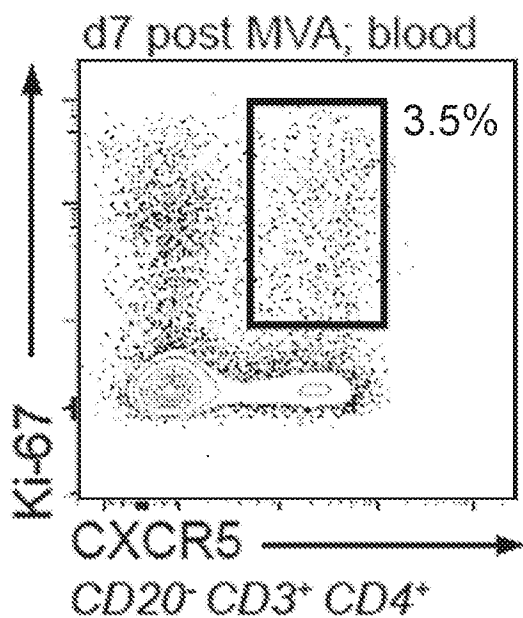


FIG. 15D

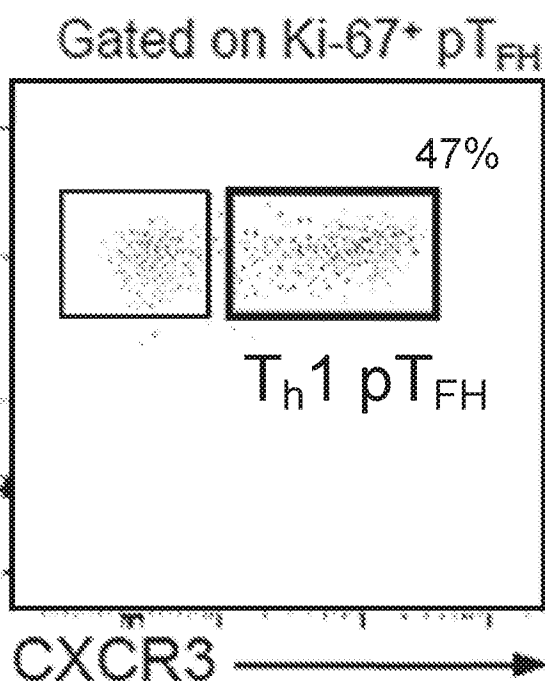


FIG. 15B

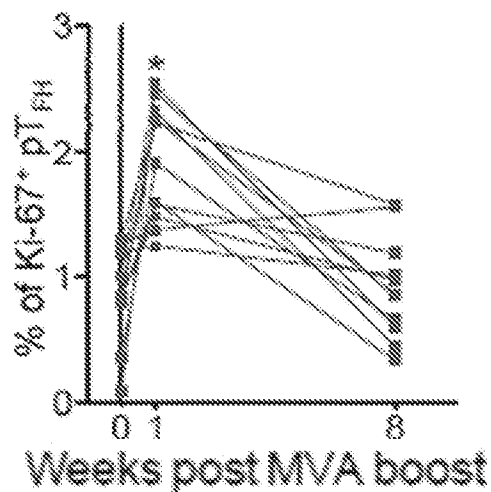


FIG. 15C

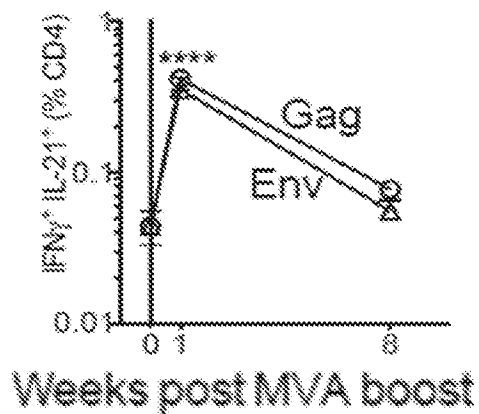
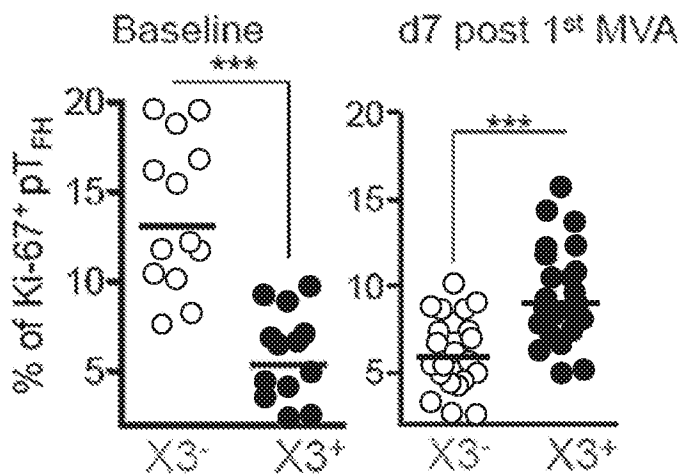


FIG. 15E



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FIG. 16A

2 wks post 2nd MVA; lymph node
 Clear: M
 Filled: MPro_{Alum}

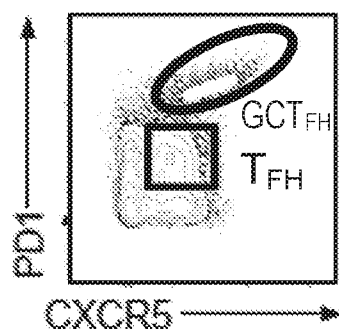
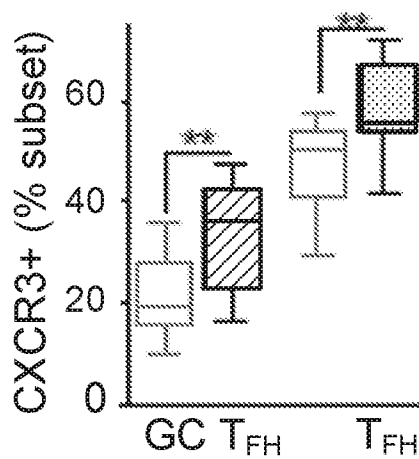
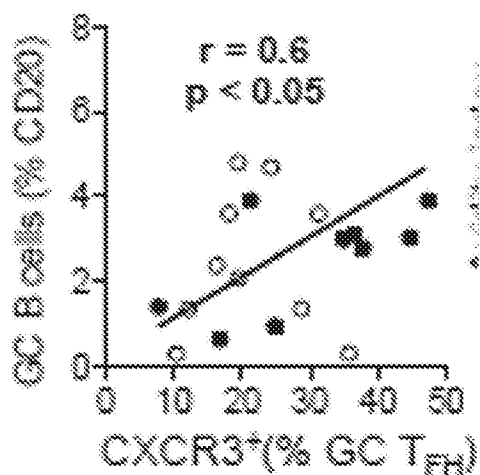
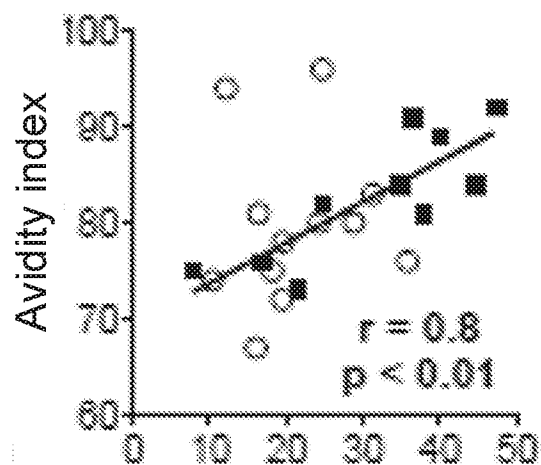
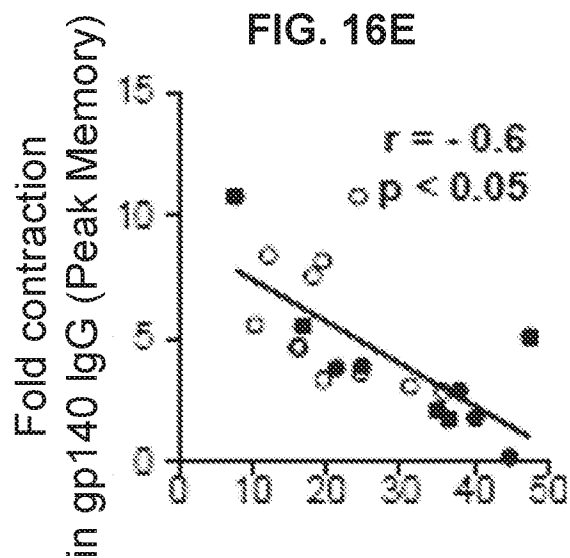
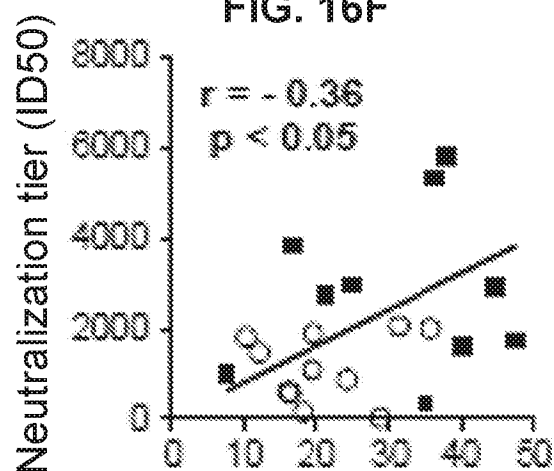
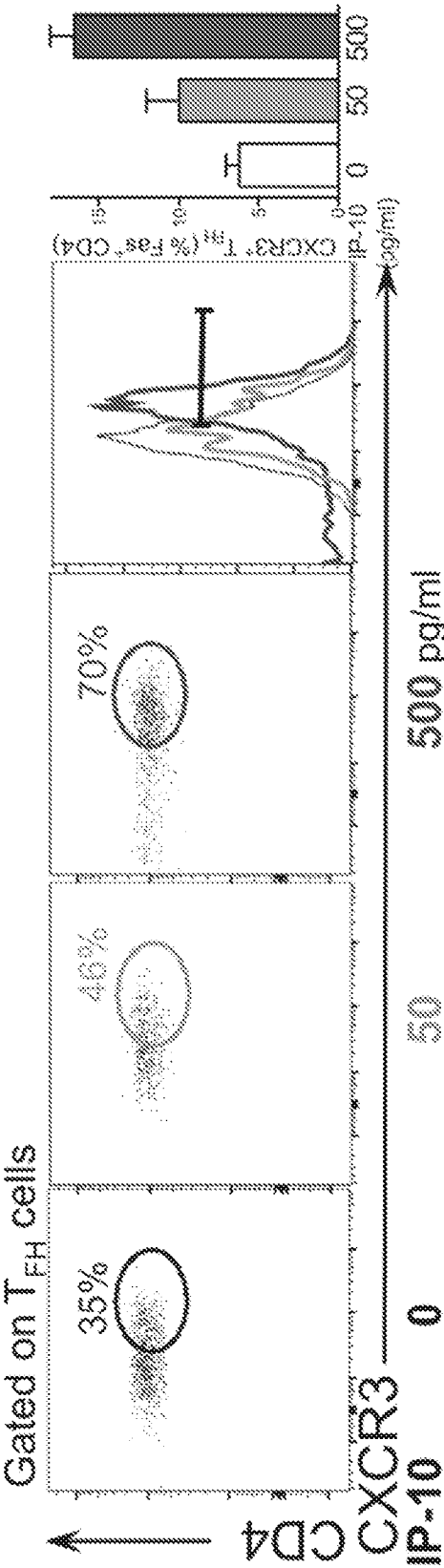
**FIG. 16B****FIG. 16C****FIG. 16D****FIG. 16E****FIG. 16F**

FIG. 17A

FIG. 17B

FIG. 17C



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FIG. 18A

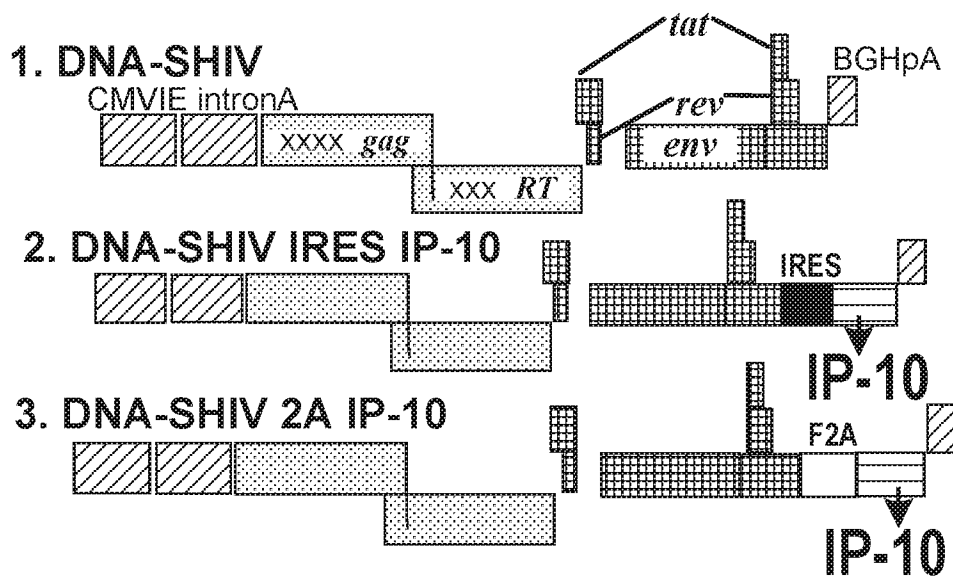


FIG. 18B

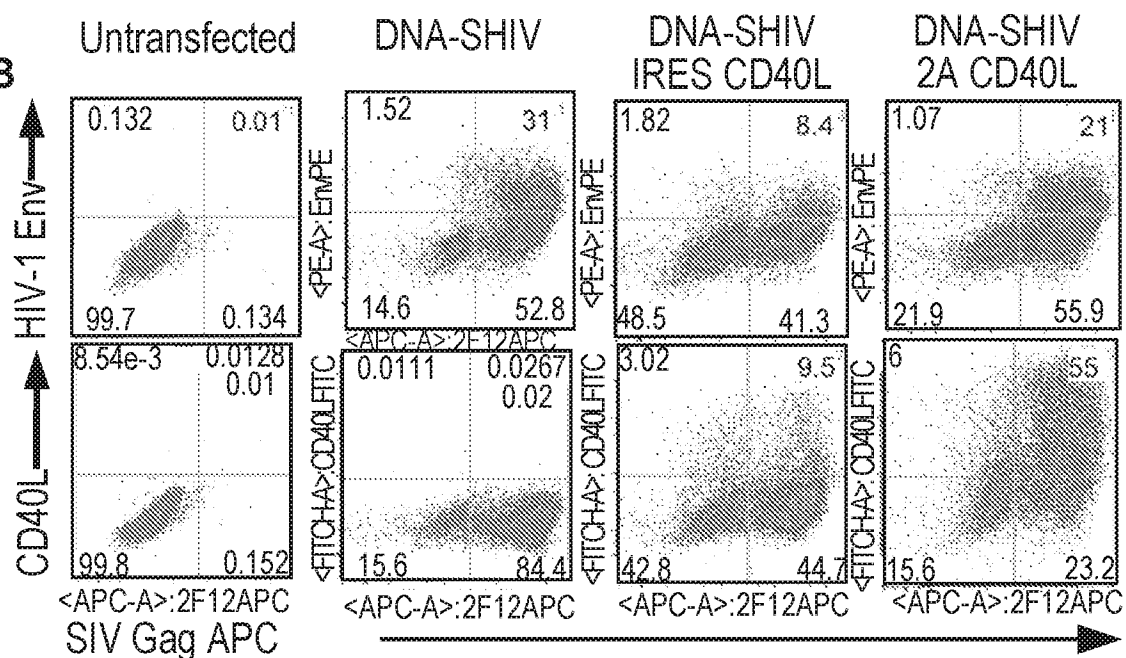
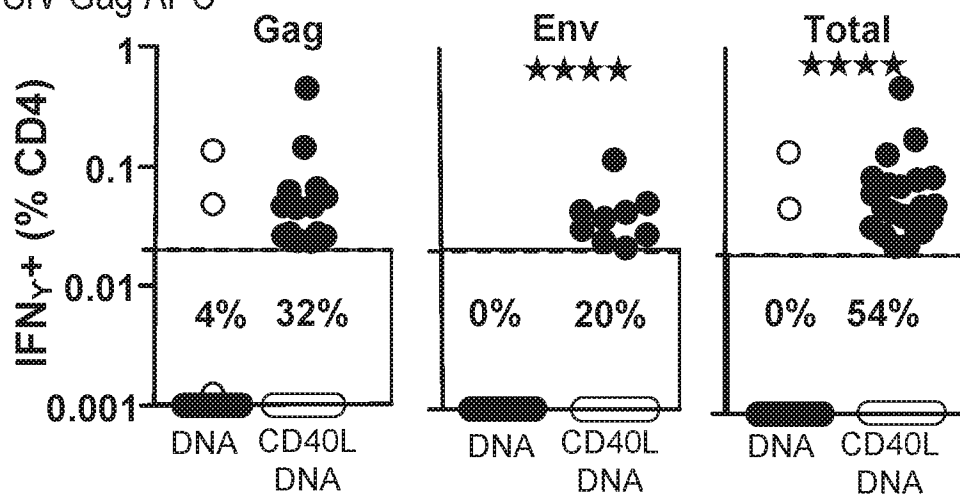
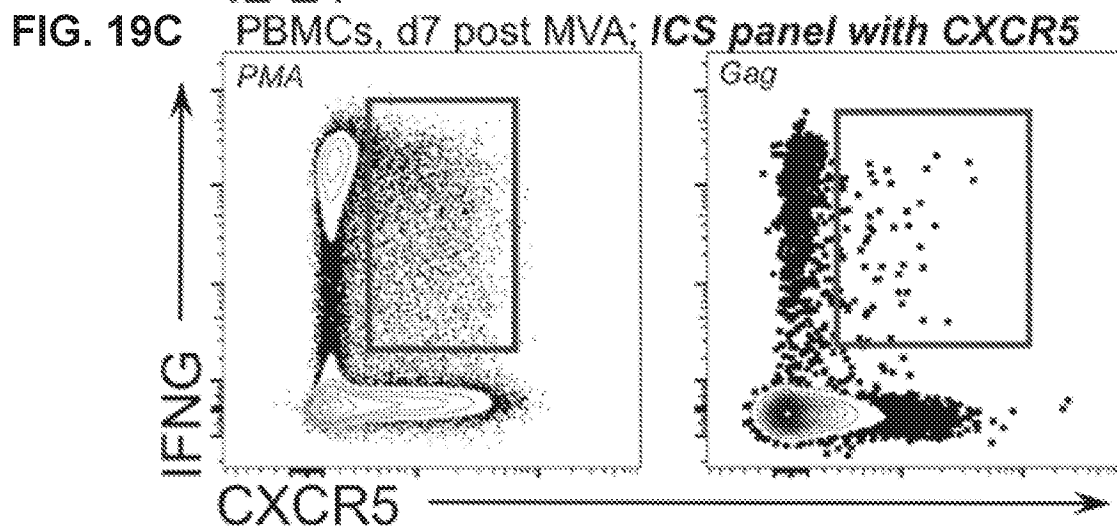
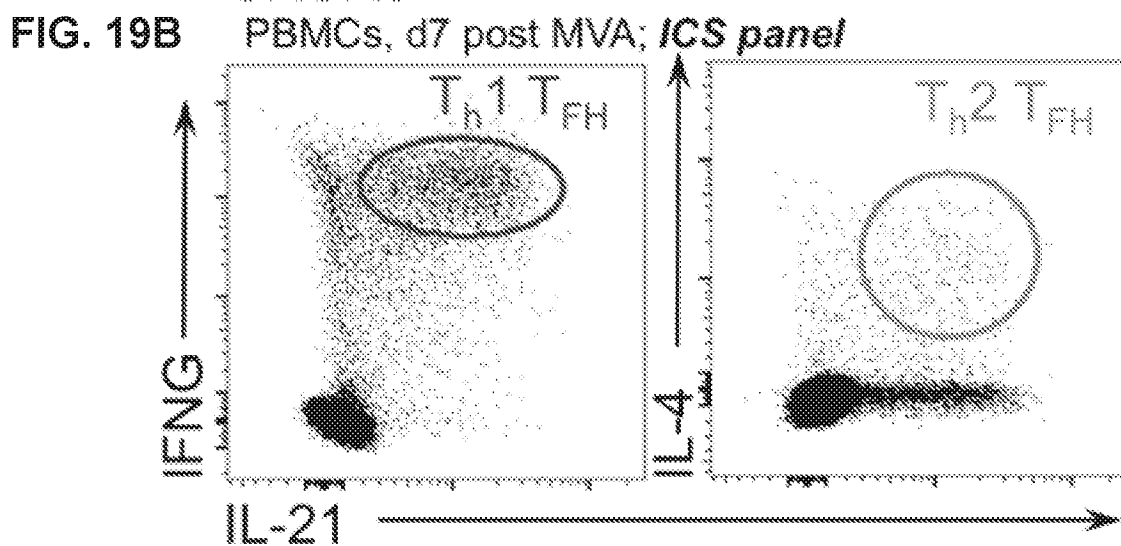
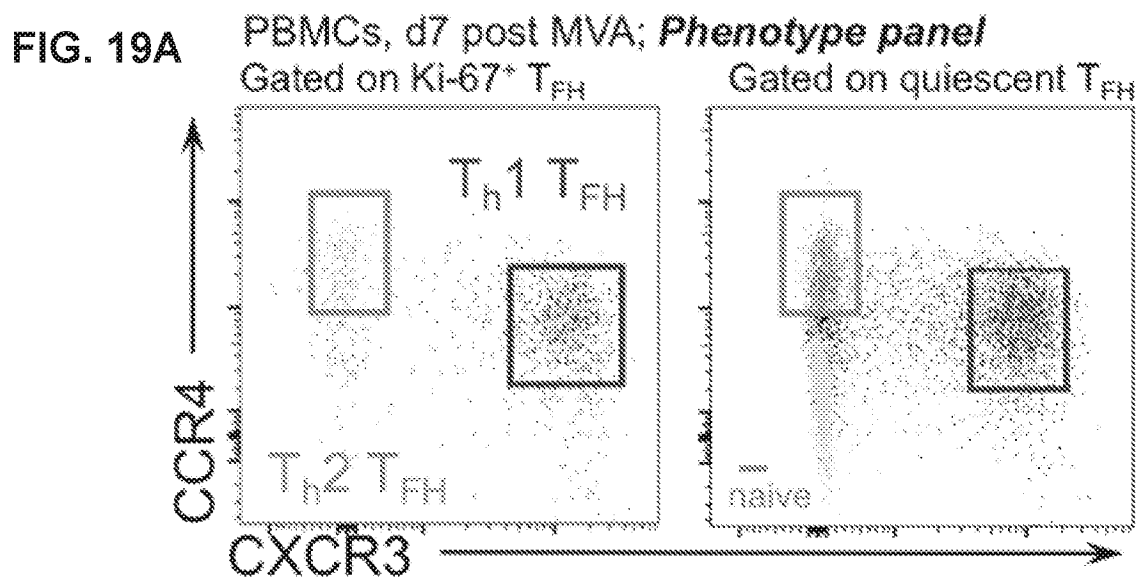


FIG. 18C

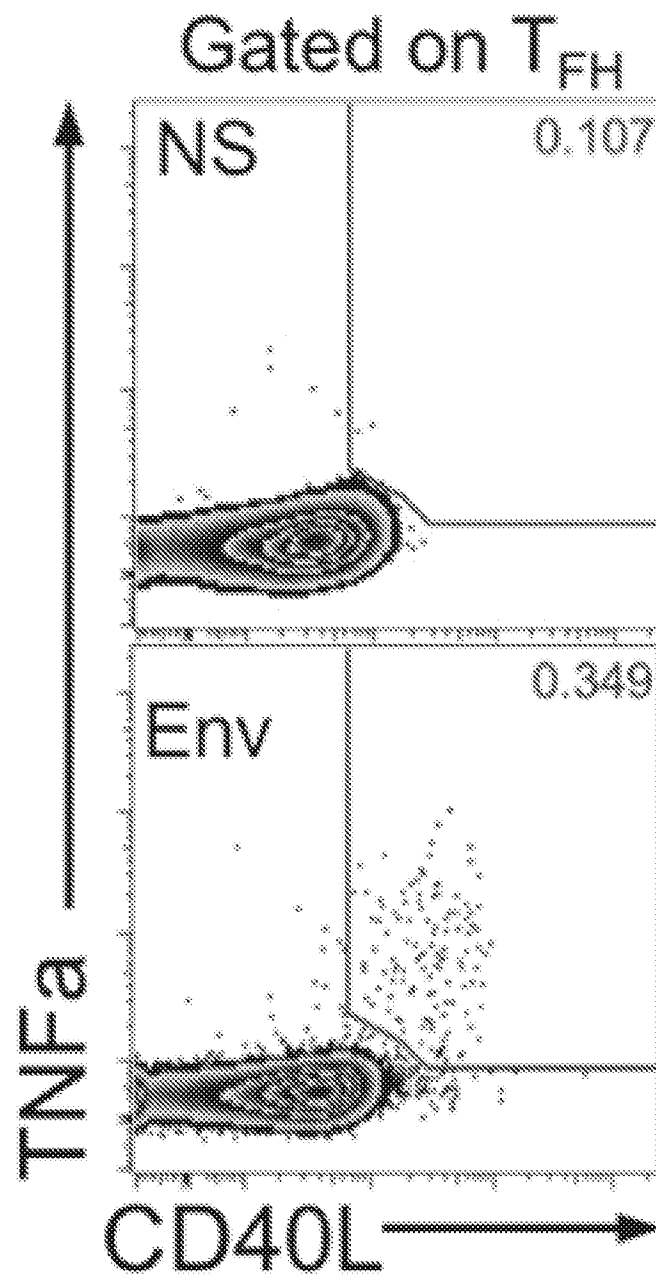


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FIG. 20



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FIG. 21A

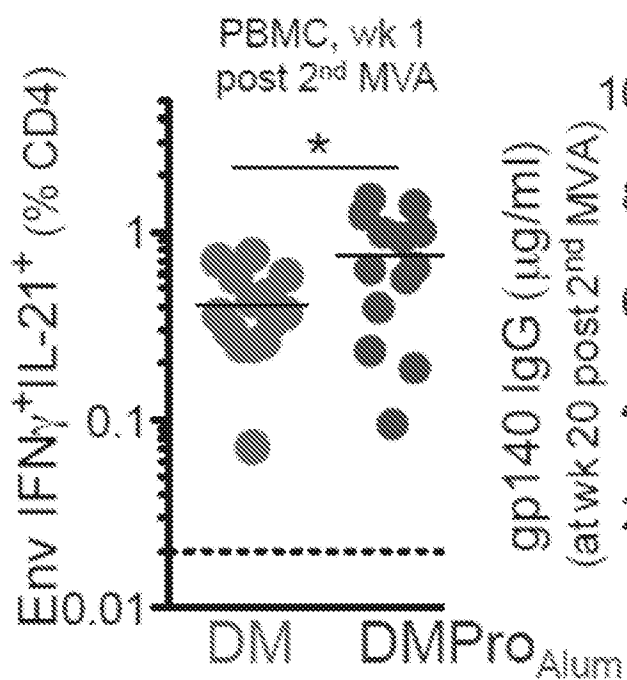


FIG. 21B

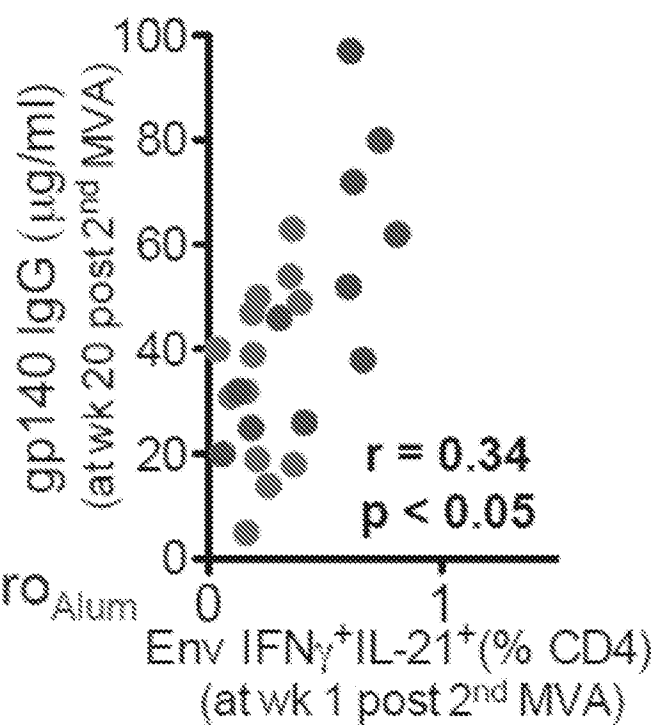


FIG. 22B

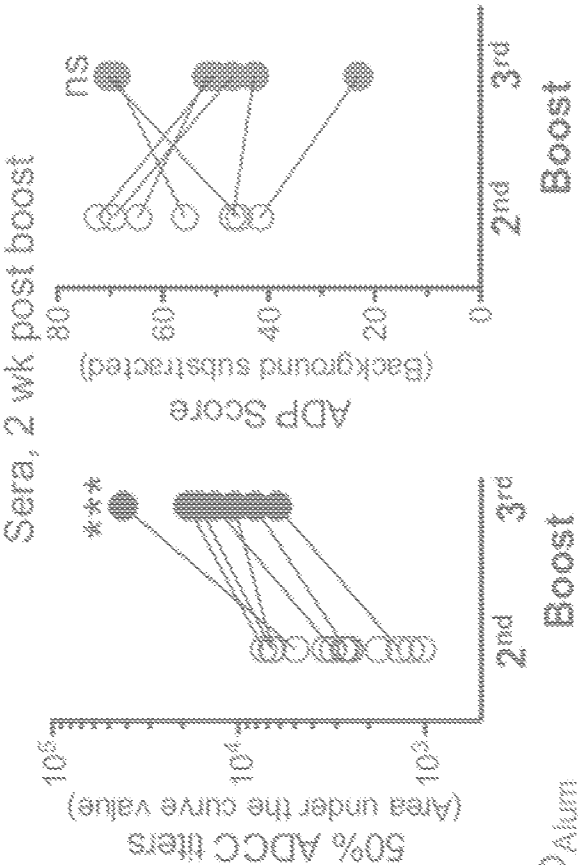


FIG. 22A

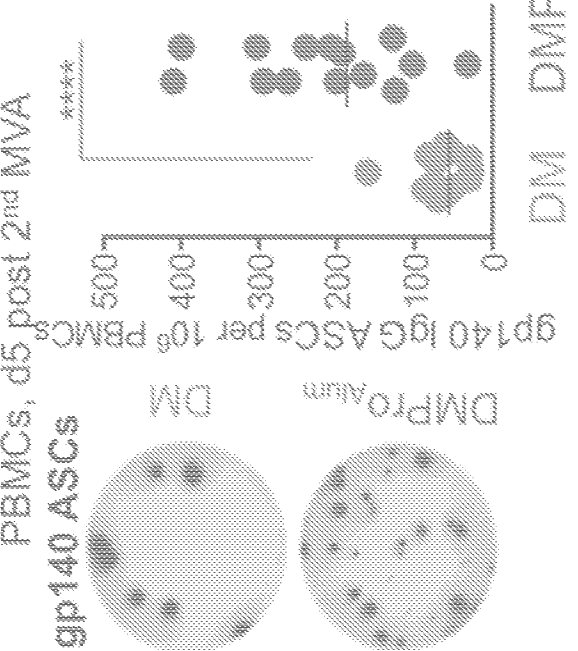


FIG. 23B

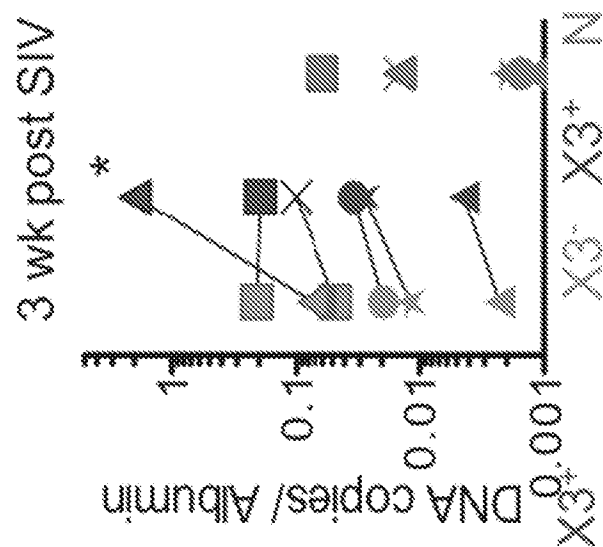
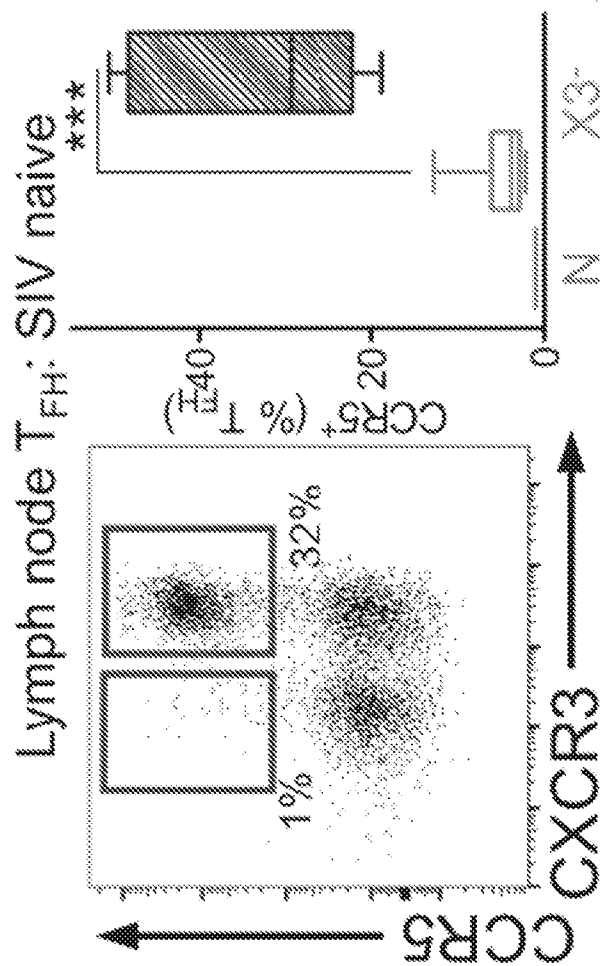


FIG. 23A



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FIG. 24A

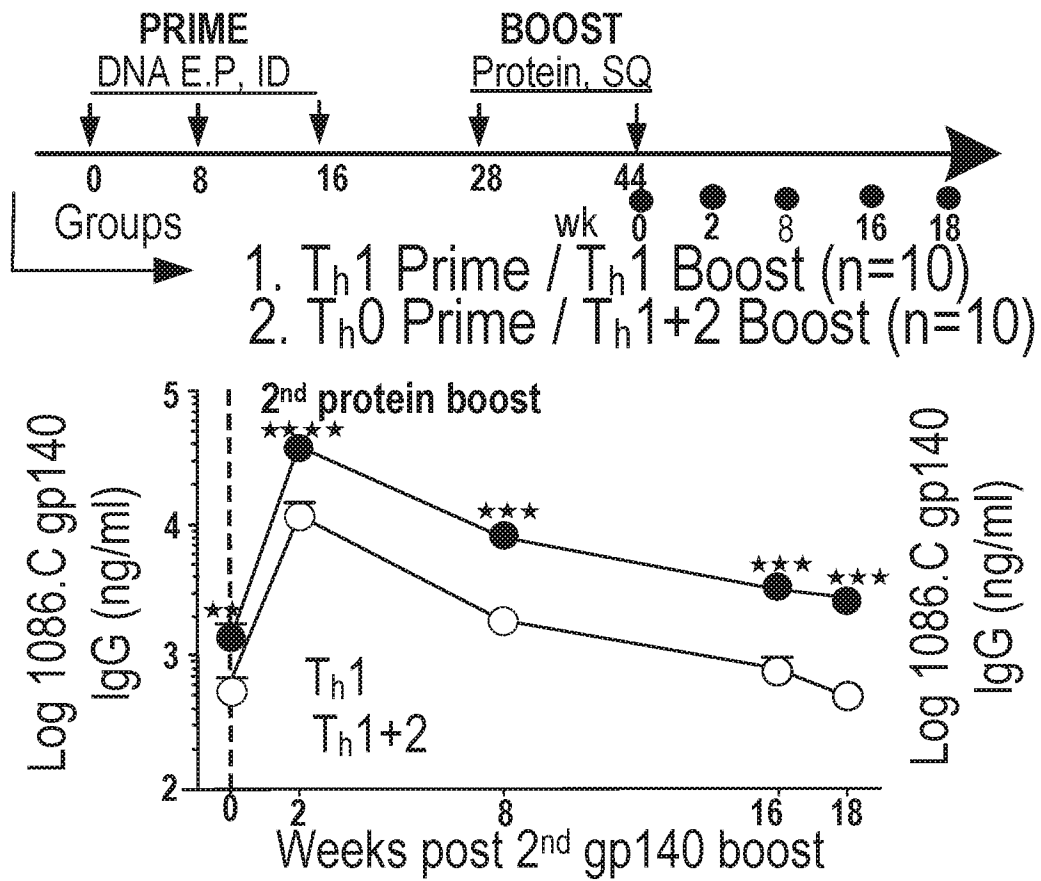
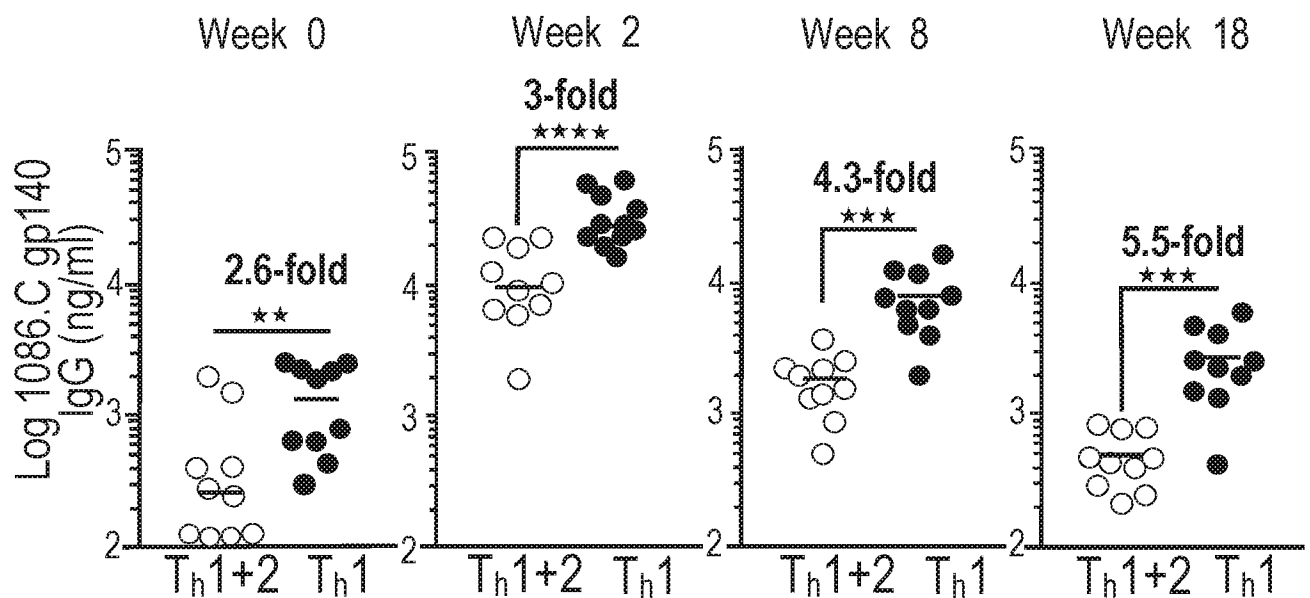


FIG. 24B



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FIG. 25A

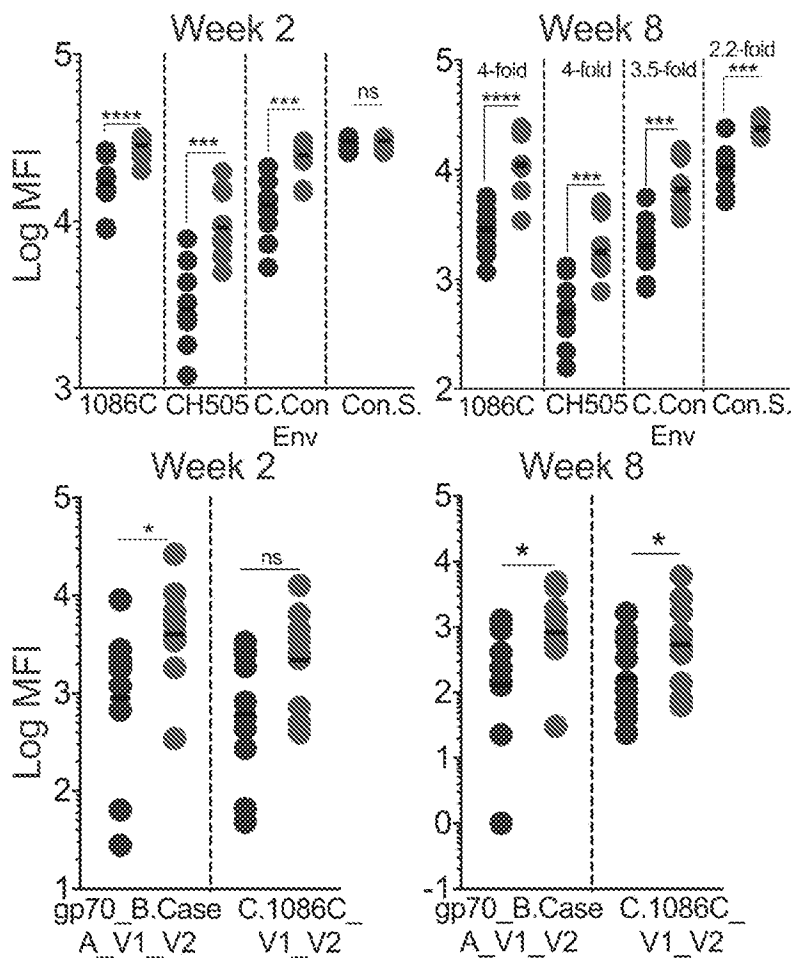


FIG. 25B

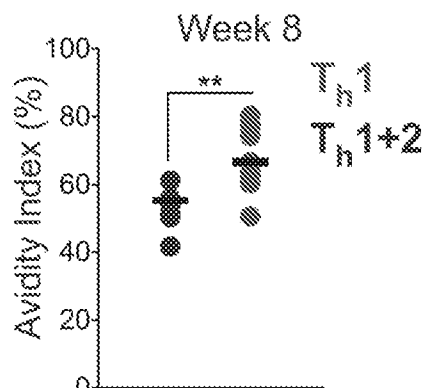


FIG. 25C

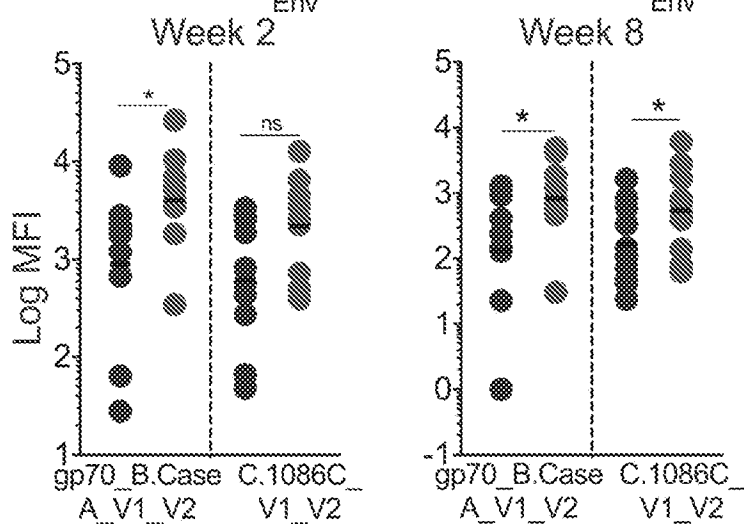
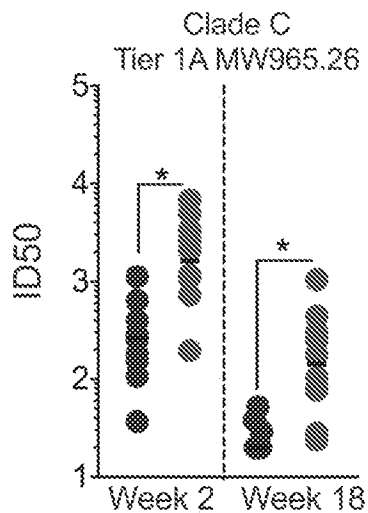
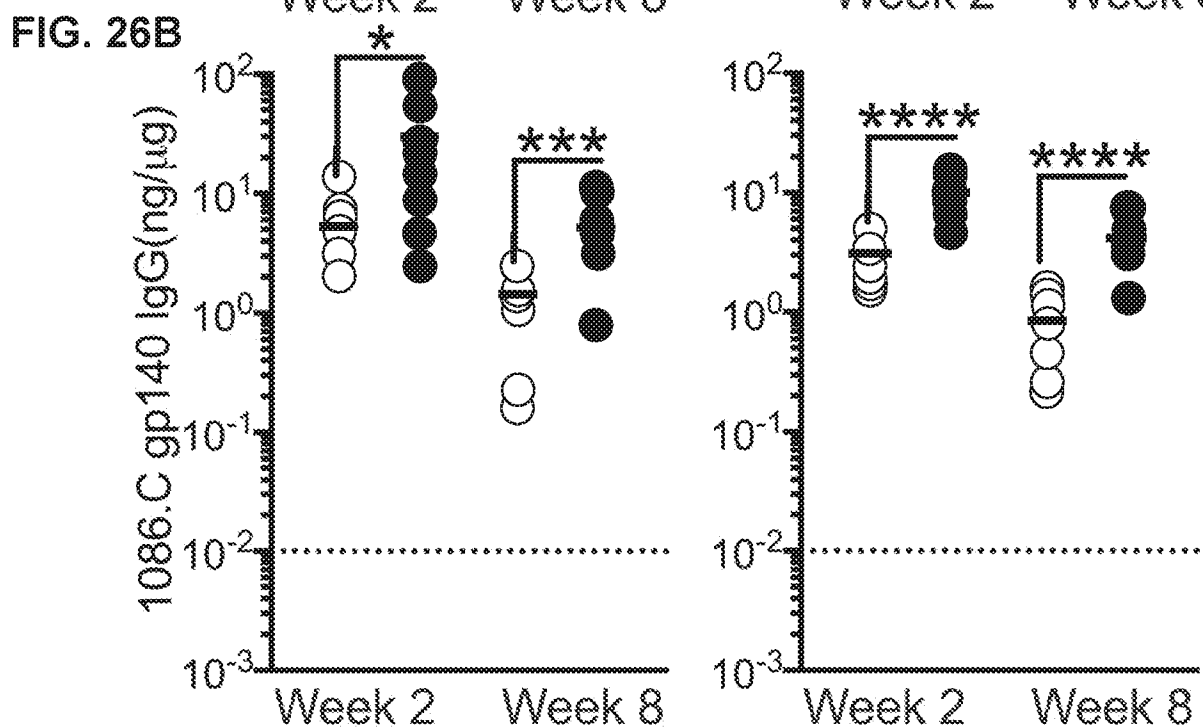
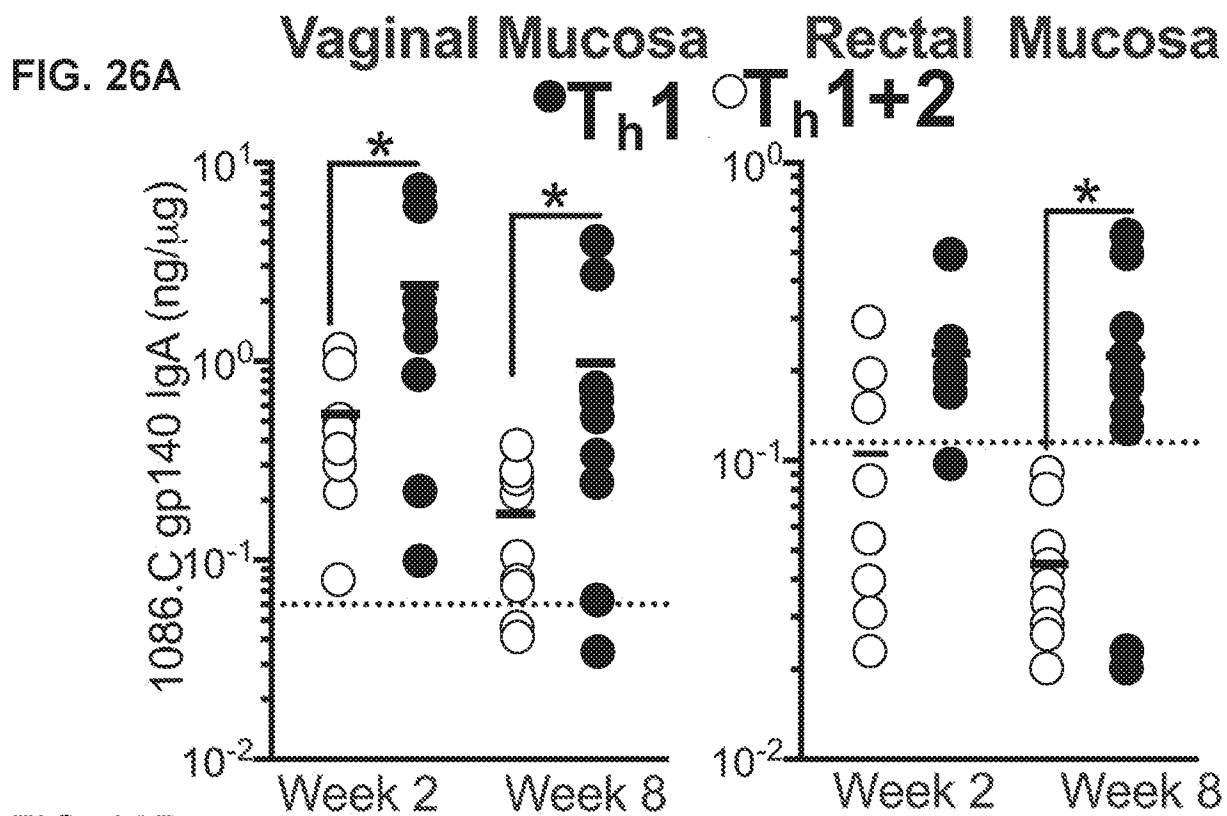


FIG. 25D



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FIG. 27A

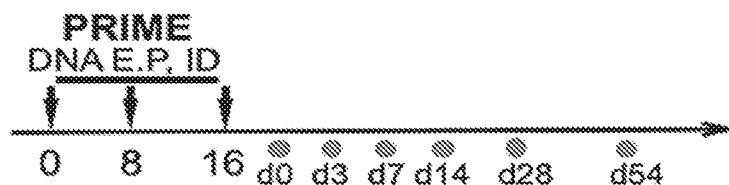


FIG. 27B

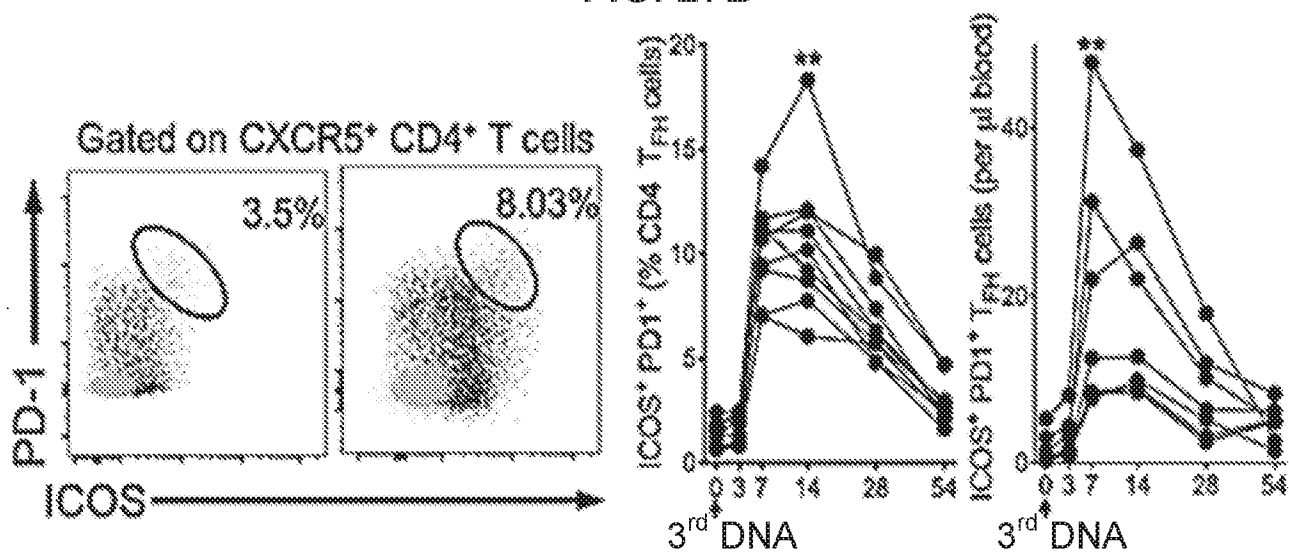


FIG. 27C

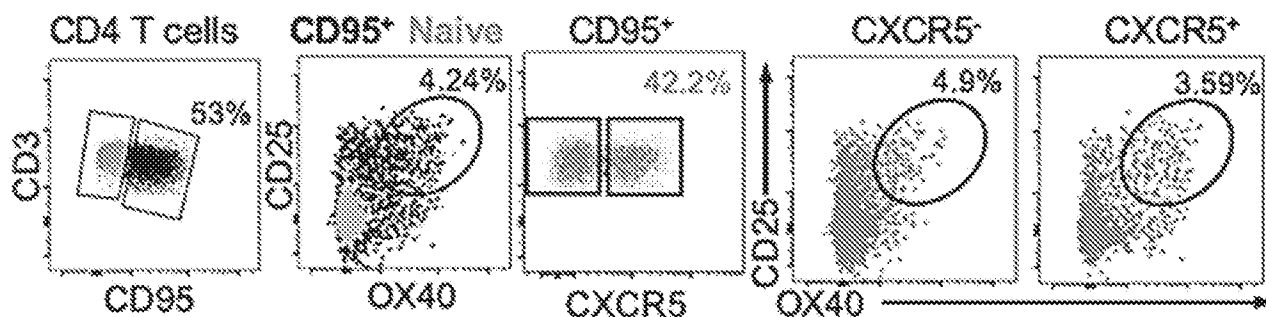
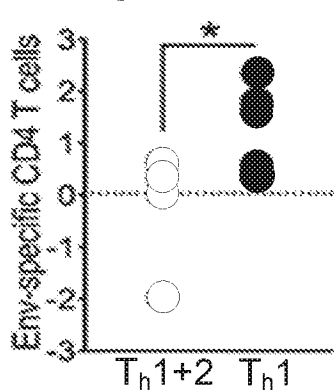


FIG. 27D



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FIG. 28A

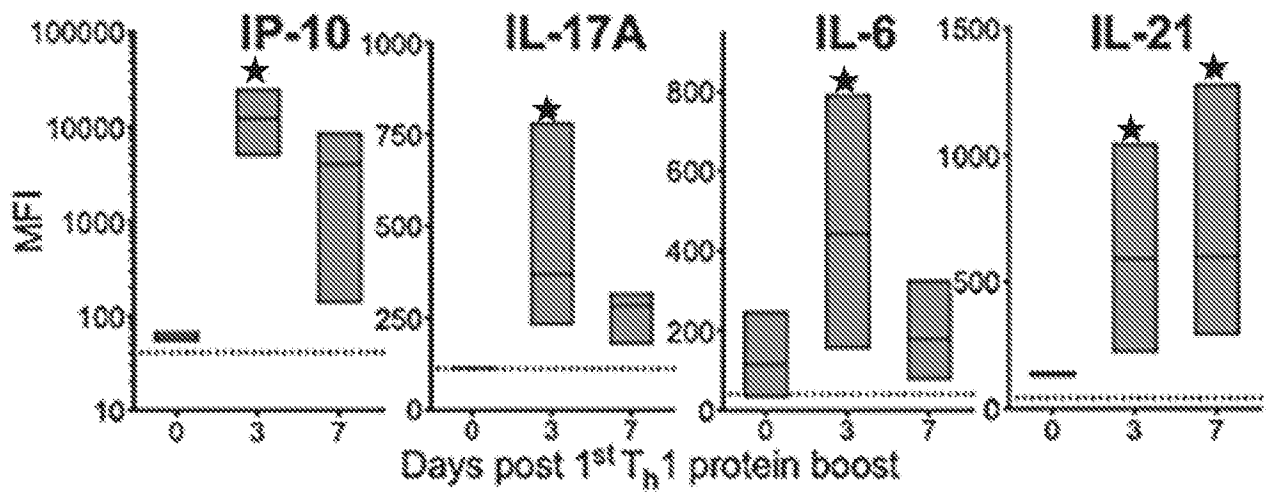


FIG. 28B

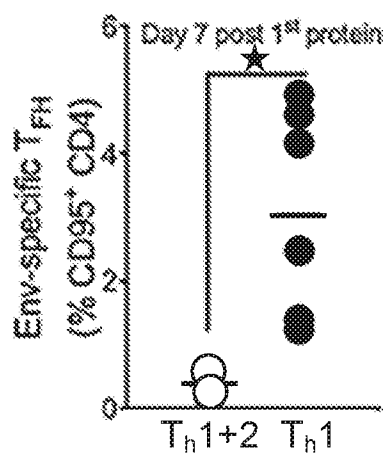


FIG. 28C

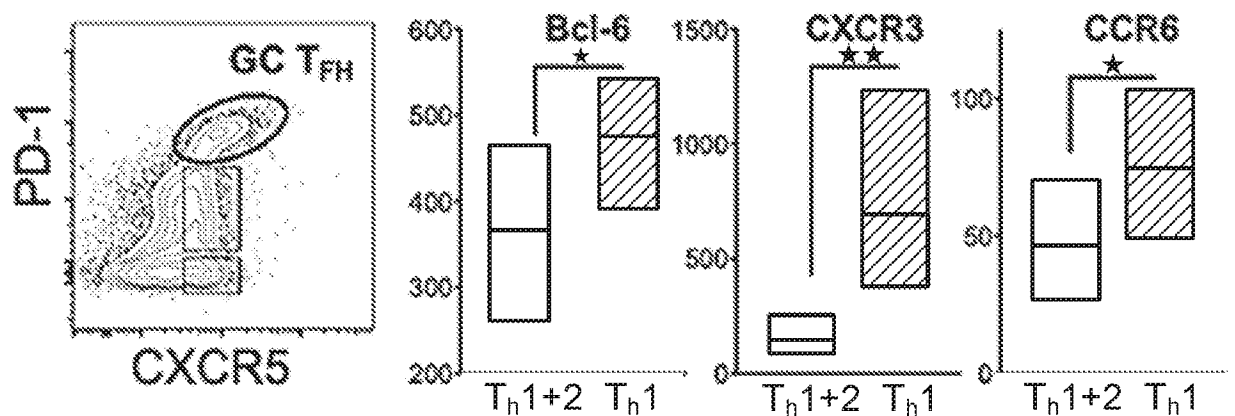
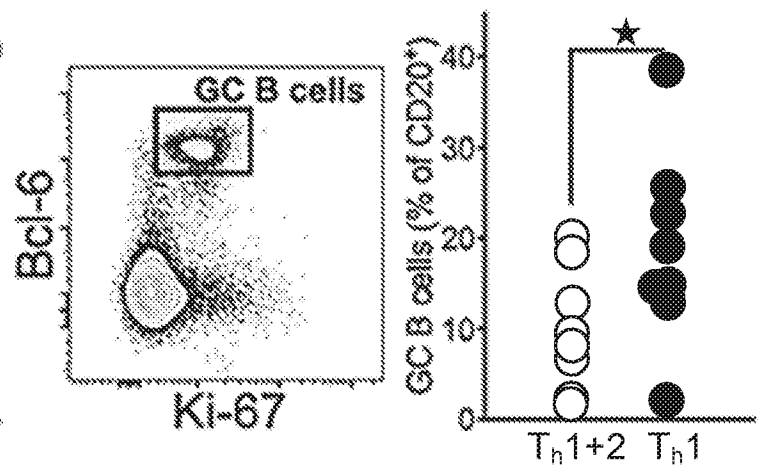


FIG. 28D

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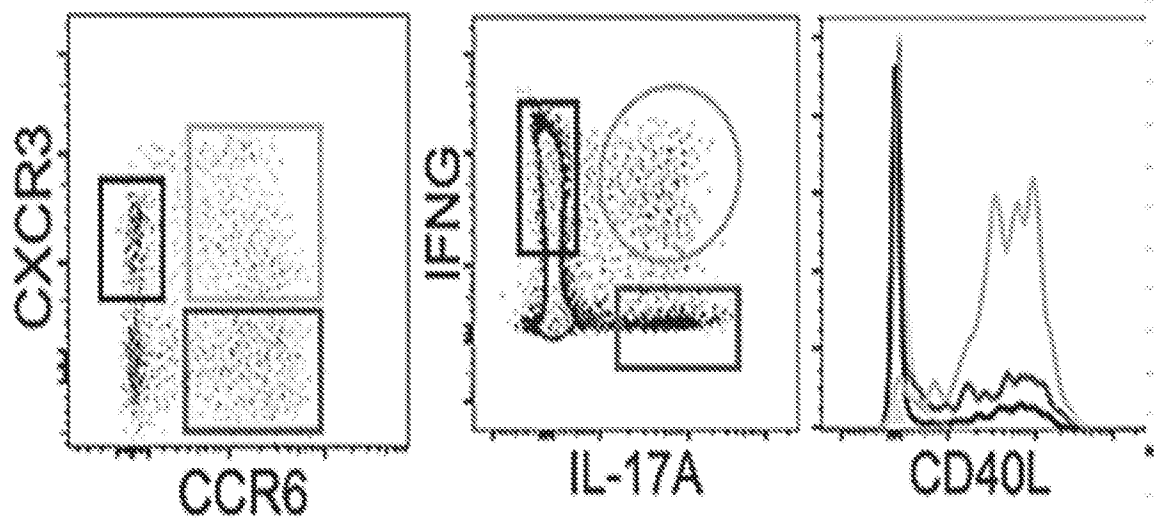


FIG. 28E

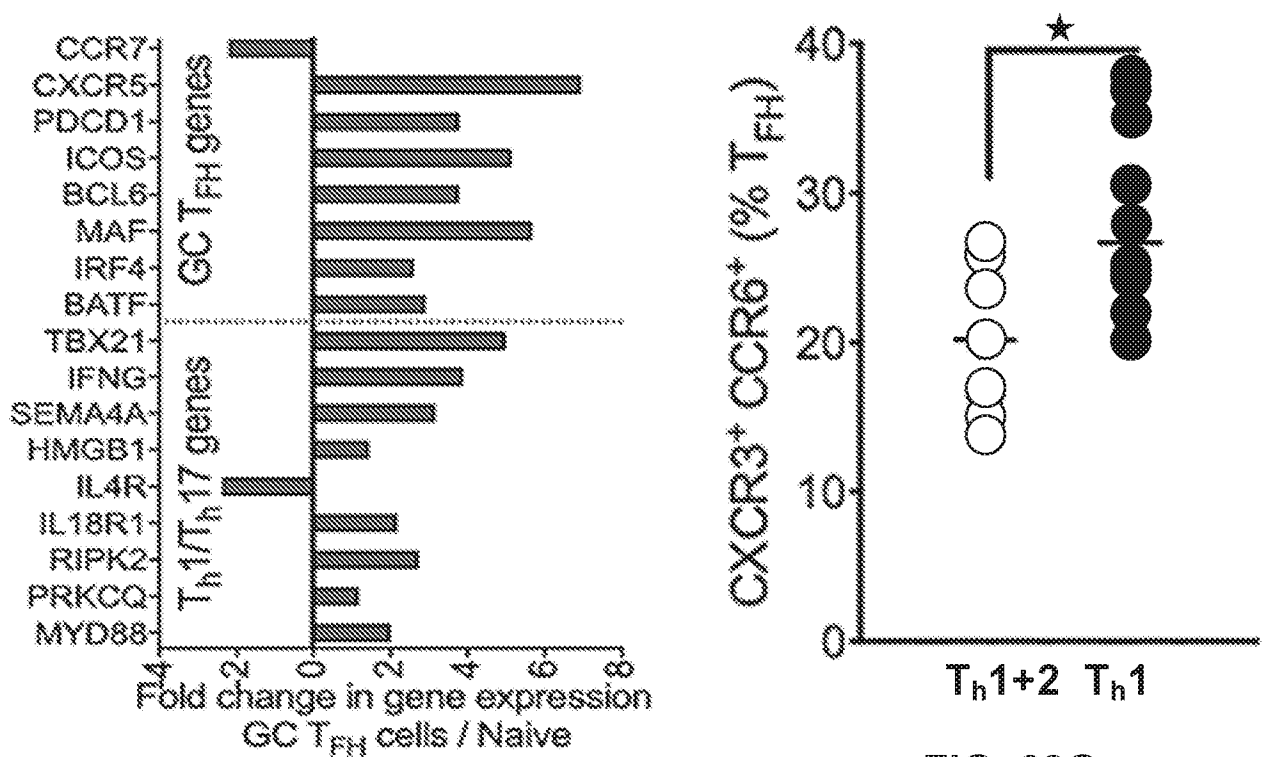


FIG. 28F

FIG. 28G

FIG. 29

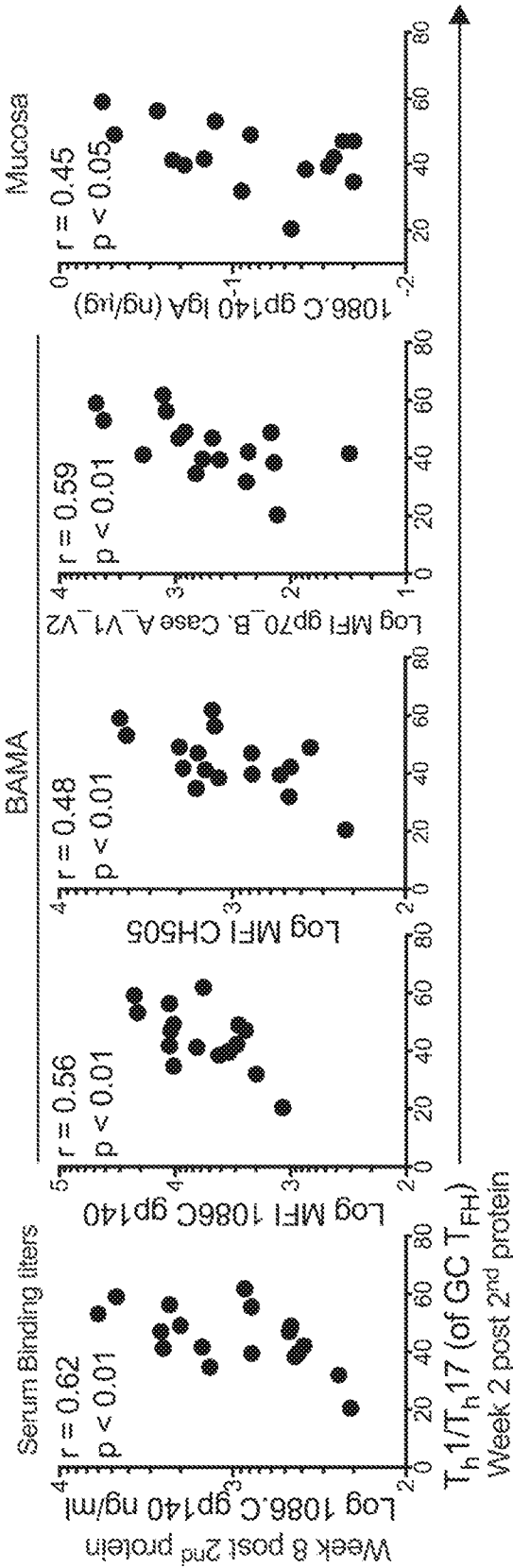


FIG. 30A

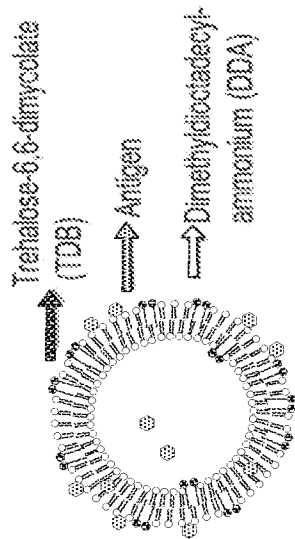


FIG. 30B

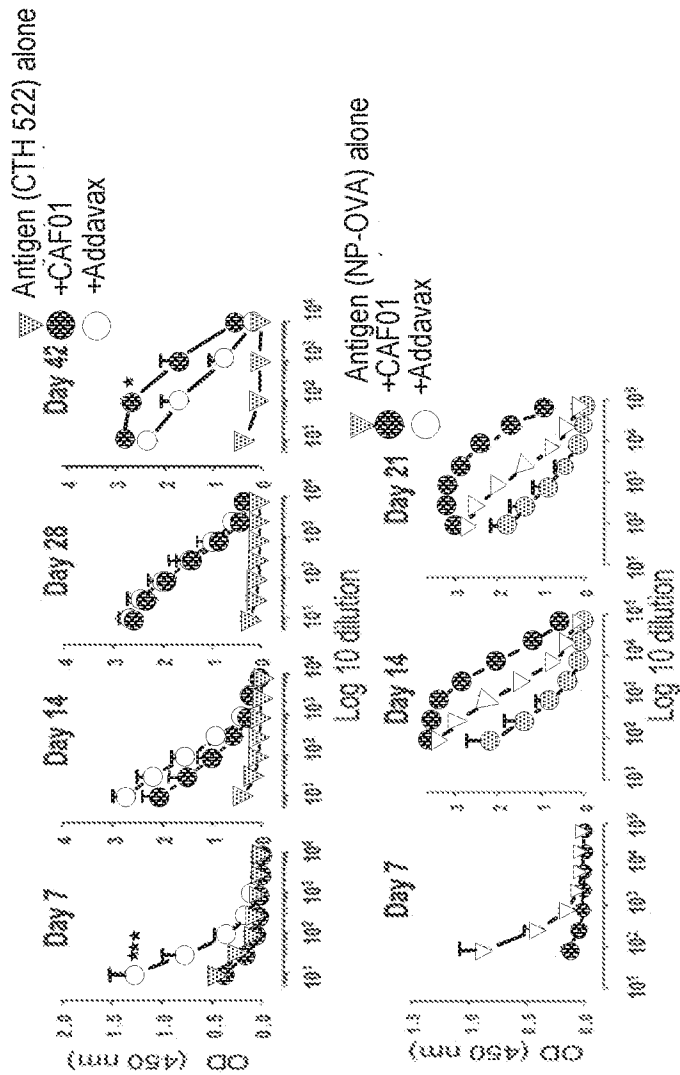


FIG. 30C

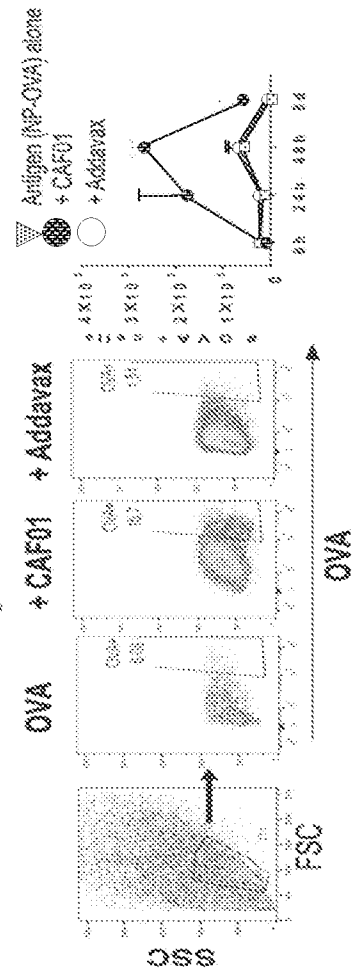


FIG. 30D

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FIG. 31

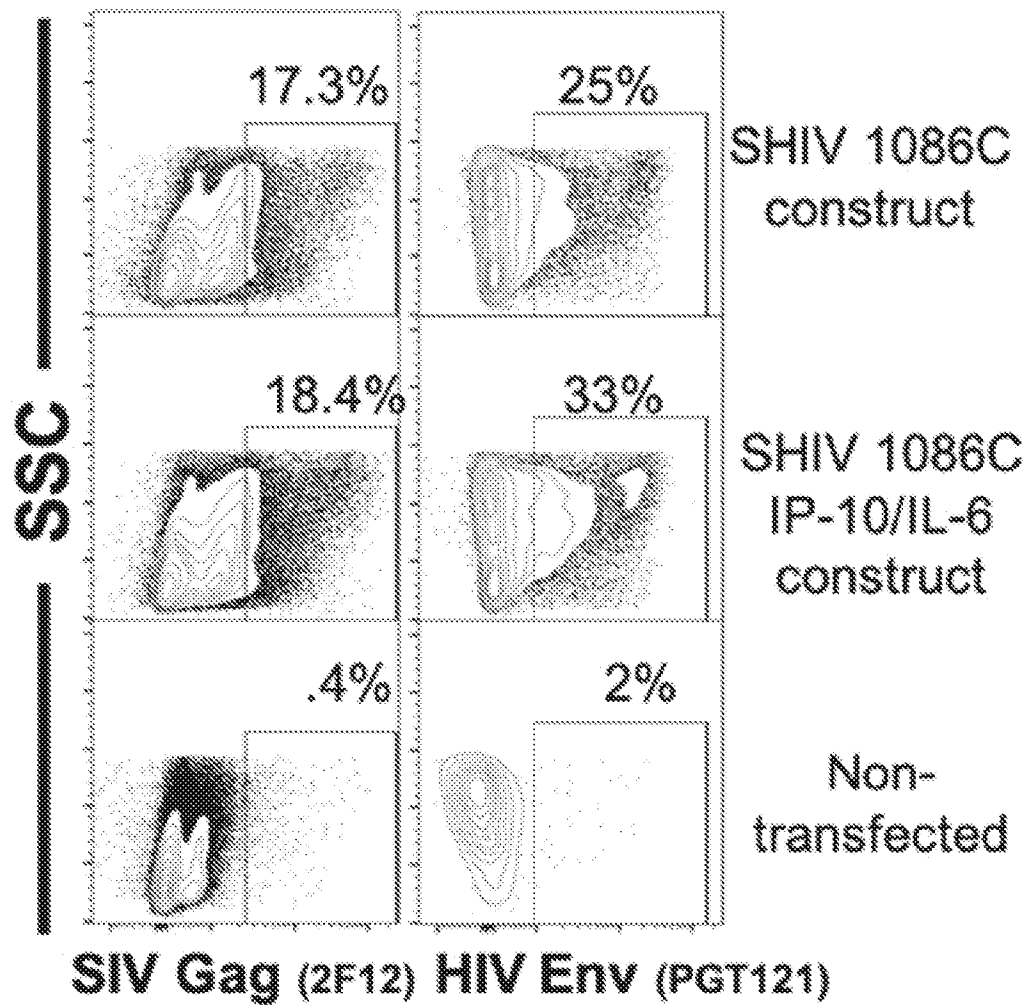


FIG. 32C

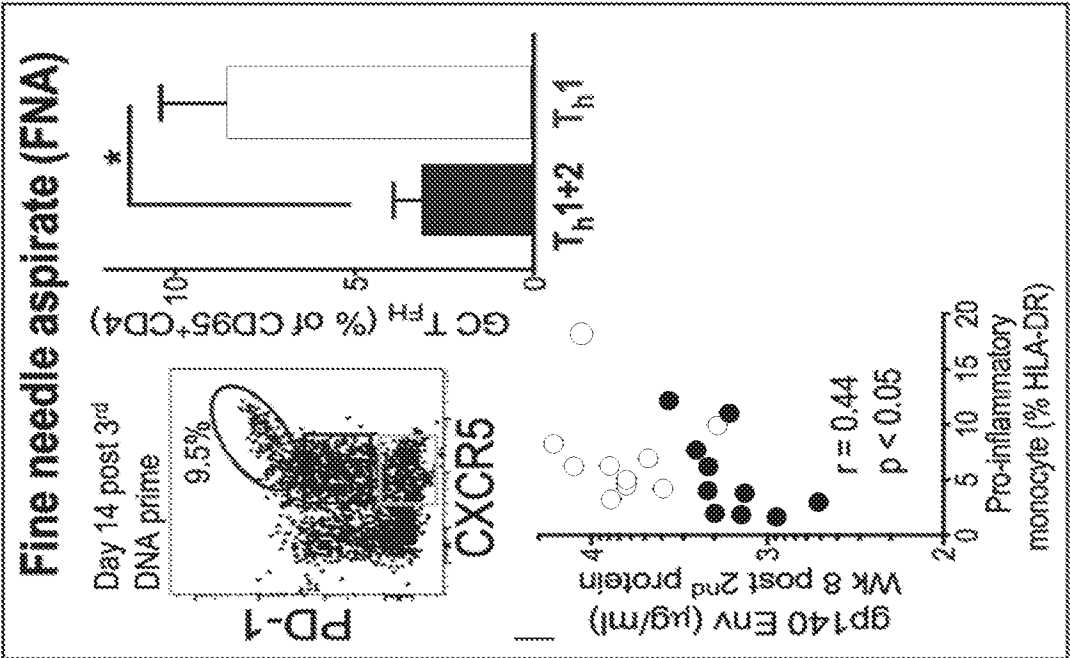


FIG. 32D

FIG. 32A

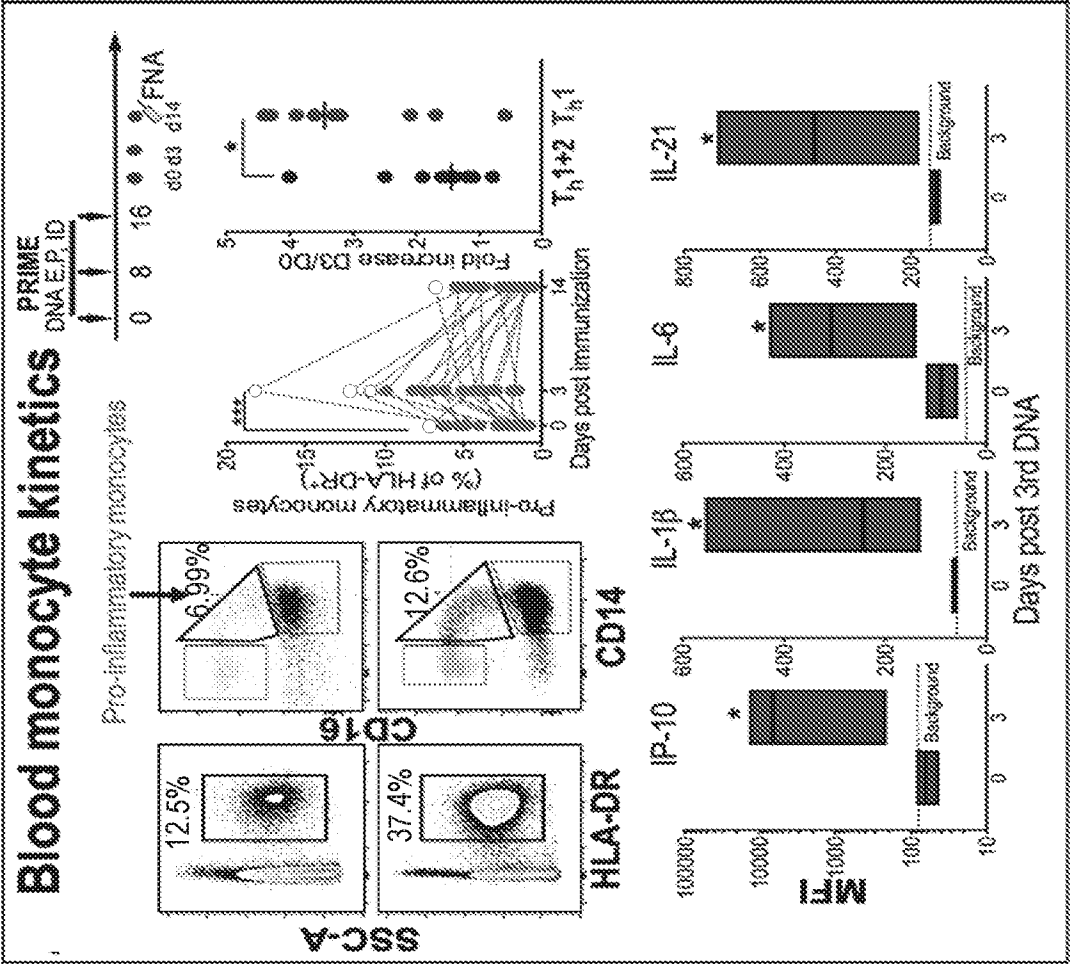


FIG. 32B

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FIG. 33A

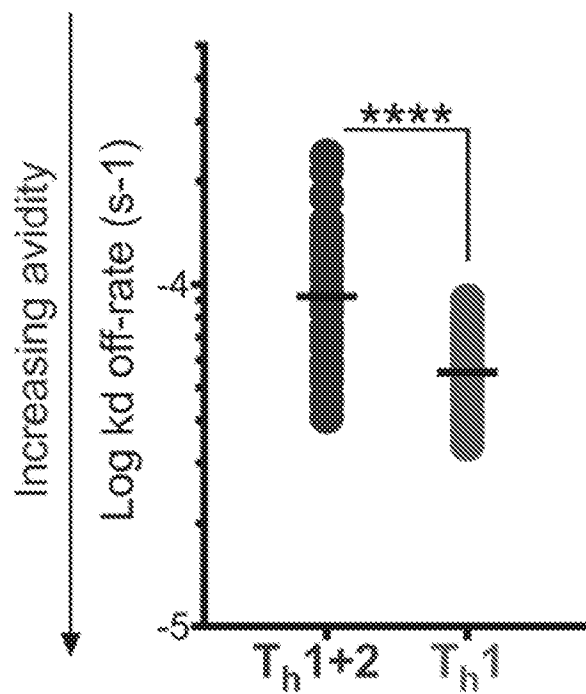
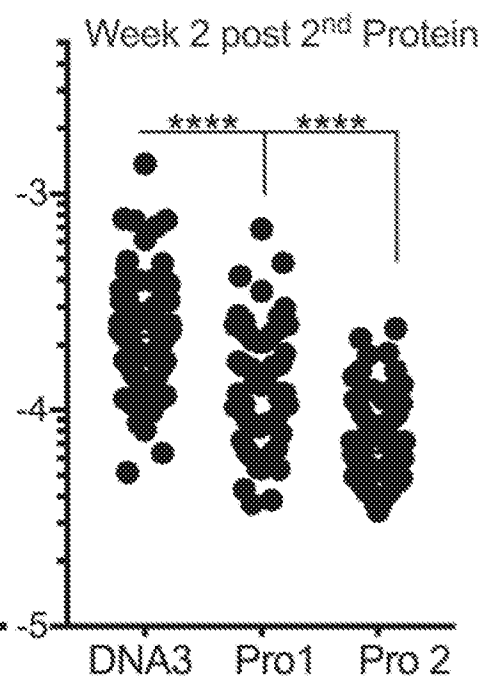


FIG. 33B



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FIG. 34A

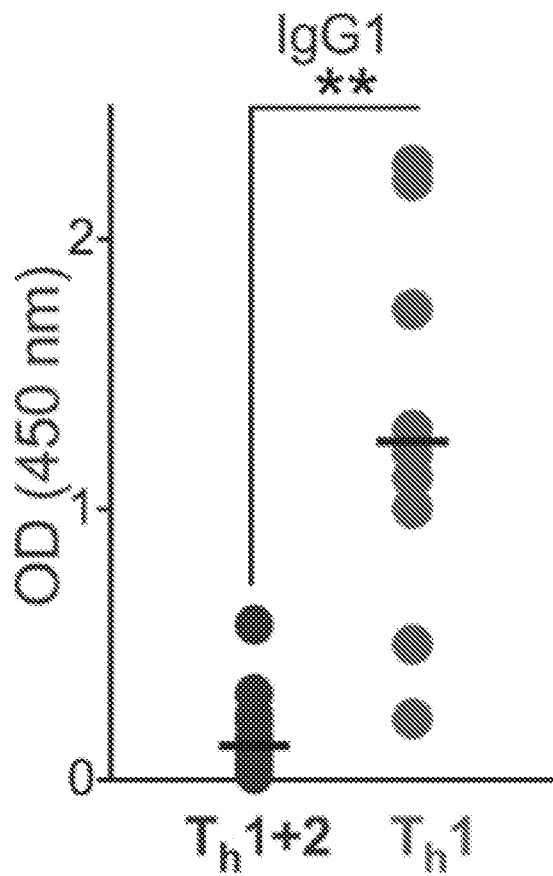
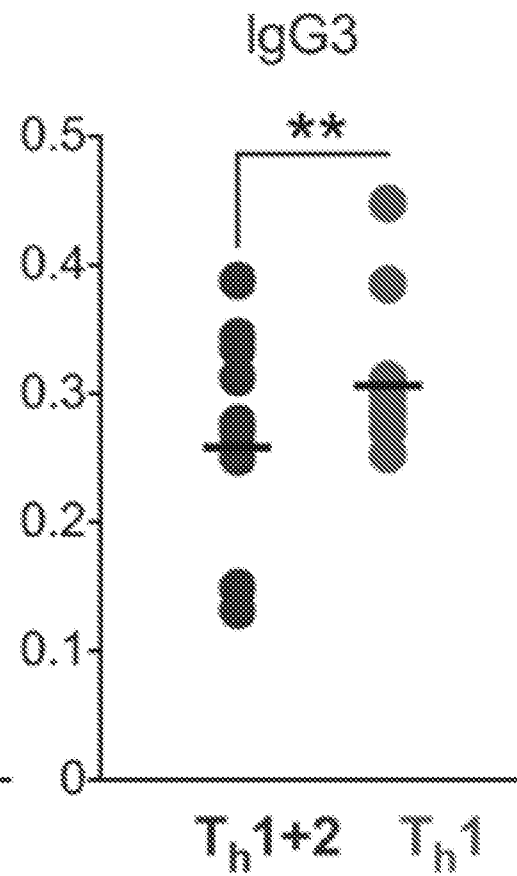


FIG. 34B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/34688

A. CLASSIFICATION OF SUBJECT MATTER
 IPC - A61K 39/12, A61K 39/21, A61K 39/39 (2020.01)
 CPC - A61K 39/12, C12N 7/00, A61K 39/21

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
 See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
 See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2005/0089526 A1 (MOORE et al.) 28 April 2005 (28.04.2005) para [0016], [0034], [0040], [0051], [0117], [0158]-[0159], [0165]-[0167], [0173], [0249], [0260]-[0261], [0306], [0339]	1-23
Y	US 2008/0199492 A1 (SRIVASTAVA et al.) 21 August 2008 (21.08.2008) para [0015], [0030], [0135], [0199], [0206], [0277], [0286], [0316]-[0317]	1-23
Y	US 2017/0182152 A1 (THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY OF THE ARMY) 29 June 2017 (29.06.2017) para [0013], [0060], [0073], Abstract, Claim 1, Claim 16	6, 8, 14, 18-19, 22
Y	US 2012/0177597 A1 (UENO et al.) 12 July 2012 (12.07.2012) para [0050], [0055], [0084], Claim 2	13
Y	US 2016/0032245 A1 (PRESIDENT AND FELLOWS OF HARVARD COLLEGE) 04 February 2016 (04.02.2016) para [0020], [0021], Abstract	13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"D" document cited by the applicant in the international application	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search
 23 September 2020

Date of mailing of the international search report

15 OCT 2020

Name and mailing address of the ISA/US
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/34688

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-23, directed to a method of enhancing the anti-Env antibody response during HIV vaccination in a subject.

Group II, claims 24-29, directed to a pharmaceutical composition for vaccinating a subject against HIV.

Group III, claims 30-36, directed to a pharmaceutical composition for boosting vaccination against HIV.

--continued on next sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-23

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/US 20/34688

Continuation of Box No. III. Observations where unity of invention is lacking.

The inventions listed as Groups I-III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special technical features:

Group I has the special technical feature of a treatment method comprising administering a first and second Th1-polarizing adjuvant to a subject, that is not required by Group II or III.

Group II has the special technical feature of a pharmaceutical composition for vaccinating a subject against HIV, the composition comprising a DNA vaccine and a polynucleotide encoding interferon-induced protein (IP)-10, that is not required by Group I or III.

Group III has the special technical feature of a pharmaceutical composition for boosting vaccination against HIV, the composition comprising QS-21, that is not required by Group I or II.

Common technical features:

Groups I and II share the common technical feature of a DNA vaccine for vaccinating a subject against HIV comprising a polynucleotide encoding an HIV Env polypeptide, and a polynucleotide encoding interferon-induced protein (IP)-10.

Groups I and III share the common technical feature of a composition for boosting vaccination against HIV comprising an HIV Env polypeptide, and QS-21.

Groups II and III share the common technical feature of a pharmaceutical composition for an immune response against HIV, the composition comprising an HIV Env polypeptide, or polynucleotide encoding an HIV Env polypeptide, an adjuvant, and a pharmaceutically acceptable carrier.

However, these shared technical features do not represent a contribution over prior art, because these shared technical features are previously disclosed or made obvious by US 2005/0089526 A1 to Moore et al. (hereinafter 'Moore').

Moore discloses a DNA vaccine for vaccinating a subject against HIV (abstract; para [0040]-[0041] "a method for eliciting an immune response in a subject against HIV-1 or an HIV-1 infected"; para [0129] "Antigenic structure analysis of gp140 proteins"; para [0271]-[0272] "Another example of a boosting vaccine is a bacterial recombinant vector containing the DNA sequence encoding the antigen") comprising a polynucleotide encoding an HIV Env polypeptide (abstract "stable HIV-1 pre-fusion envelope glycoprotein trimeric complexes"; para [0033]-[0034] "This invention also provides a first polypeptide comprising the amino acid sequence of HIV-1 gp120 and HIV-1 gp41"; para [0129] "Antigenic structure analysis of gp140 proteins"), and an interferon-induced protein (IP)-10 (para [0165] "composition further comprises a cytokine and/or a chemokine. ... The chemokine can be ... IP-10"). Moore does not specifically teach the IP-10 is present as a polynucleotide encoding IP-10. However, Moore does teach the HIV envelope polypeptides can be supplied as a polynucleotide encoding the polypeptides on a vector (para [0034] - "a nucleic acid which encodes the instant polypeptide. This invention further provides a vector comprising the instant nucleic acid."; [0174] "the vaccine comprises a therapeutically effective amount of the protein encoded by the above nucleic acid...the vaccine comprises a combination of the recombinant nucleic acid molecule and the mutant viral envelope protein...a DNA plasmid"; [0257]-[0258]). It would have been obvious to one of ordinary skill in the art to have supplied the IP-10 of Moore as a polynucleotide encoding IP-10 in an expression vector, in order to provide all of the vaccine elements in a single vector for ease of use.

Moore discloses a composition for boosting vaccination against HIV comprising an HIV Env polypeptide, and QS-21 (abstract "stable HIV-1 pre-fusion envelope glycoprotein trimeric complexes"; para [0167]-[0168] "a third composition comprising the first trimeric complex or the first particle composition, and an adjuvant. The adjuvant can be...QS-21...a method for eliciting an immune response in a subject against HIV-1 or an HIV-1 infected cell comprising administering to the subject a prophylactically or therapeutically effective amount of the first trimeric complex or first particle composition").

Moore discloses a pharmaceutical composition for an immune response against HIV, the composition comprising an HIV Env polypeptide, or polynucleotide encoding an HIV Env polypeptide (abstract "stable HIV-1 pre-fusion envelope glycoprotein trimeric complexes"; para [0167]-[0168] "a third composition comprising the first trimeric complex...a method for eliciting an immune response in a subject against HIV-1 or an HIV-1 infected cell comprising administering to the subject a prophylactically or therapeutically effective amount of the first trimeric complex or first particle composition"), an adjuvant (para [0167] "and an adjuvant"), and a pharmaceutically acceptable carrier (para [0273] "These vaccines may further contain pharmaceutically suitable or physiologically acceptable carriers").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Group I-III inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.