



## IL-33 enhances the kinetics and quality of the antibody response to a DNA and protein-based HIV-1 Env vaccine



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### ABSTRACT

Induction of a sustained and broad antibody (Ab) response is a major goal in developing a protective HIV-1 vaccine. DNA priming alone shows reduced levels of immunogenicity; however, when combined with protein boosting is an attractive vaccination strategy for induction of humoral responses. Using the VC10014 DNA and protein-based vaccine consisting of HIV-1 envelope (Env) gp160 plasmids and trimeric gp140 proteins derived from an HIV-1 clade B infected subject who developed broadly neutralizing serum Abs, and which has been previously demonstrated to induce Tier 2 heterologous neutralizing Abs in rhesus macaques, we evaluated whether MPLA and IL-33 when administered during the DNA priming phase enhances the humoral response in mice. The addition of IL-33 during the gp160 DNA priming phase resulted in high titer gp120-specific plasma IgG after the first immunization. The IL-33 treated mice had higher plasma IgG Ab avidity, breadth, and durability after DNA and protein co-immunization with alum adjuvant as compared to MPLA and alum only treated mice. IL-33 was also associated with a significant IgM Env-specific response and expansion of peritoneal and splenic B-1b B cells. These results indicate that DNA priming in the presence of exogenous IL-33 qualitatively alters the HIV-1 Env-specific humoral response, improving the kinetics and breadth of potentially protective Ab.

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### 1. Introduction

HIV-1 infection remains a serious health issue worldwide with 36.9 million people living with HIV-1 and 1.8 million estimated new infections in 2017 [1]. Although with increased access to anti-retroviral therapy, particularly in sub-Saharan Africa, the incidence of new infections world-wide is decreasing, it is becoming evident that there are numerous recalcitrant regions and high-risk groups in which rates of new infections are stable or even increasing [2] underscoring the need for an effective preventive HIV-1 vaccine.

Induction of a sustained broadly neutralizing antibody response to HIV-1 is a primary goal of HIV-1 vaccine development, however, despite the development HIV broadly neutralizing antibodies in a subset of HIV-infected patients, their induction through immunization has been difficult to achieve. Elegant technological advancements have been made in obtaining and characterizing broadly

neutralizing antibodies (bNAbs) isolated from HIV-infected subjects [3] and this work has provided rich insight into the key sites of vulnerability on HIV-1 Envelope (Env), the molecular commonalities of these Abs, and their evolution during the infectious process. In turn this has led to the design and pre-clinical evaluation of immunogens that attempt to recapitulate the evolutionary process of bNAb development [4,5]. Such rational immunogen design will likely need to be coupled with proper conditioning to ensure robust B cell engagement gives rise to persistent long-lived humoral immunity to confer durable protection.

Priming conditions, mainly determined by adjuvant and platform (e.g. protein, DNA, viral vector) can substantially influence quality of the B cell response, including breadth and durability. DNA is an attractive priming strategy when combined with protein or viral vector boosting; however, DNA priming alone typically fails to induce substantial Ab, limiting its potential as a stand-alone immunogen [6,7]. The microenvironment in which B cell priming occurs, defined in large part by the local composition of cytokines and growth factors, is likely to influence the quality of the B cell memory and antibody response. This microenvironment can be conditioned through the delivery of exogenous factors [8].

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One such factor is IL-33, which remains under-explored in the context of B cell responses, and several lines of observation suggest it could be advantageous in enhancing the HIV-1 Env-specific response. IL-33 is an alarmin, expressed by numerous cell types, particularly initiated during host defenses and or tissue damage [9,10]. IL-33 has pleiotropic cytokine activities including mediating diverse pro-inflammatory responses [11], activation and recruitment of antigen-presenting cells [12], and enhancing adaptive immunity [13]. A few studies have demonstrated its adjuvant-like ability to enhance antibody and T cell responses [14,15]. IL-33, an IL-1 family member mediates its biological effects via ST2 complexed with the IL-1R accessory protein (IL1RAcP) notably expressed on Th2 CD4+ T cells, ILC2, basophils, and mast cells [16–19]. IL-33 has been shown to enhance B-1 B cell proliferation, and IL-5 and IgM secretion [20]. IL-33 exposure of B-1b B cells also increases production of IL-13, a potent Th2 cytokine [21]. IL-33 is a potent inducer mucosal IgM+ IL-10-producing regulatory B cells [22] in mice, and correlates with increased plasma total IgM and auto-reactive antibodies in rheumatoid arthritis patients [23].

Numerous features of IgM memory suggest that with adequate engagement it could be a valuable contributor to an effective B cell response to HIV. These features include their rapid response, unique and polyreactive immunoglobulin (Ig) repertoire with intrinsic Env reactivity, neutralizing activity, expansive mucosal distribution, strong complement activation, and enhanced antigen presentation abilities [24–26]. Additionally, IgM has been shown to contribute to neutralization of other viruses including dengue, influenza, VZV, and smallpox [27–30]. The ability of IgM memory to differentiate upon antigen-stimulation into IgG and IgA memory and antibody secreting cell (ASC) populations [31] suggest they may contribute to qualitatively distinct Env-specific Ab responses.

Various studies in animal models and humans have shown the ability of the adjuvant, MPLA (3-*O*-desacyl-4'-monophosphoryl lipid A), a TLR4 agonist, and non-toxic derivative of LPS (lipopolysaccharide), to enhance both antibody and cellular immune response to vaccination [32]. MPLA upregulates innate immune responses including activating antigen-presenting cells and inducing pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-12 which in turn enhances features of the adaptive immune response, inducing T helper cells and B cell responses [33–35]. After immunization with MPLA as an adjuvant, higher antibody persistence, enhanced immunological memory and a superior anamnestic response has been observed [36,37]. Priming in the absence of inflammation (antigen only) and boosting with MPLA [36,38] results in the induction and long-term persistence of antigen-specific B-1 B cell and IgM Ab responses in mice [38].

In this study, we used the VC10014 DNA and protein-based HIV-1 vaccine which consists of gp160 plasmids and trimeric gp140 proteins derived from an HIV-1 clade B infected subject who developed broadly neutralizing Abs [39,40], and which has been previously shown to induce Tier 2 heterologous neutralizing Abs in rabbits [41] and rhesus macaques [42]. Using this immunogen platform, we evaluated the ability of IL-33 and MPLA provided during the DNA priming phase to impact the kinetics and quality of the antibody and B cell response.

## 2. Materials and methods:

### 2.1. Mice

Young (6 to 8 weeks of age) C57BL/6J female mice were obtained from The Jackson Laboratories. All animal experiments were approved by the University of Rochester University Committee on Animal Resources and performed in compliance with guidelines defined by National Institutes of Health guide for the care and

use of Laboratory animals (NIH Publications No. 8023, revised 1978).

### 2.2. HIV envelope DNA and protein VC10014 immunogen

Six HIV envelope DNA (F8, G6a, E5a, C6a, H10 and G10a clade B gp160 DNA) and 2 trimeric proteins (F8 and C6a clade B gp140 protein) from VC10014 were a kind gift from Haigwood Laboratory (OHSU, OR). The trimeric gp140 proteins were prepared as previously described, briefly the gp140 DNA was derived from the gp160 env sequence by site-directed mutagenesis (Stratagene, La Jolla, CA) to insert the previously described mutations [5,43] in the primary and secondary protease cleavage sites respectively: REKR  $\rightarrow$  RSKS and KAKRR  $\rightarrow$  KAISS. A large-scale endotoxin-free plasmid preparation (Qiagen, Valencia, CA) was used for stable expression in 293F cells for protein production as described previously [44]. Epitope exposure and antigenicity of gp140 trimeric protein immunogens was assessed by ELISA, biolayer interferometry (BLI), and surface plasmon resonance for binding of multiple bNmAbs as described previously [41]. The HIV envelope DNA obtained from Haigwood laboratories were first used to transform *E. coli* DH5 $\alpha$  and developed into permanent stocks. Plasmid purification for DNA immunizations was performed using endotoxin free mega prep kits (Qiagen# 12381, Germantown, MD) according to manufacturer's protocol.

### 2.3. Immunizations

C57BL/6J mice were injected intramuscularly (i.m.) and intraperitoneally (i.p.) either with MPLA (20  $\mu$ g) (InvivoGen # vac-mpls, San Diego, CA) or recombinant mature IL-33 protein (17.9 kDa) (2.5  $\mu$ g) (Peptotech, Rocky Hill, NJ, #210–33) at one week prior to and at the time of priming (week 0 and week 3) with VC10014 envelope DNA plasmids (F8, G6a, E5a, C6a, H10 and G10a Clade B gp160 DNA). Following priming phase, mice were co-immunized with DNA plasmids and gp140 proteins (C6a and F8 gp140) at week 7 and week 11. A total of 30  $\mu$ g of DNA (5  $\mu$ g of each DNA plasmid) were given intramuscularly along with 25  $\mu$ g of recombinant gp140 trimeric protein (12.5  $\mu$ g each recombinant protein) were delivered intramuscularly by needle injection with alum (aluminium hydroxide, Alhydrogel adjuvant, Invivogen vac-alu-250) as the adjuvant. Non-immunized mice did not receive any injections. Blood was collected by submandibular bleed into EDTA containing tubes; plasma was separated and stored at  $-80^{\circ}\text{C}$  until the assays were performed. C57BL/6J mice were injected intramuscularly (i.m.) and intraperitoneally (i.p.) with IL-33 (2.5  $\mu$ g) at one week prior to and at the time of priming (week 0 and week 3) with 100  $\mu$ g of ovalbumin (Invivogen # vac-pova, San Diego, CA) and alum.

### 2.4. Flow cytometry

Peripheral blood mononuclear cells (PBMCs), peritoneal cells (PerC), and splenocytes were collected from mice and were stained for 1 h with anti-IgG-FITC (Biolegend-406001, San Diego, CA), anti-CD95-PerCpefluor710 (Ebioscience-46-0951-82), anti-CD21-Pacific Blue (Biolegend-123414, San Diego, CA), anti-CD14-DyLight405LS (Novus Biologicals, Centennial, CO), anti-CD11b-BV570 (Biolegend-101233, San Diego, CA), anti-CD4-Qdot605 (Invitrogen-Q10092), anti-CD23-BV786 (BD Horizon-563988, San Jose, CA), anti-CD19-BV711 (Biolegend-115555, San Diego, CA), anti-CD23-BV650 (BD-Horizon-563545, San Jose, CA), anti-GL7-AlexaFluor647 (Biolegend-144606, San Diego, CA), anti-CD45R-AlexaFluor700 (Biolegend-103232), anti-IgD-APC-Cy7 (Biolegend-405716), anti-CD43-PE (Biolegend-143206, San Diego, CA), anti-IgM-PE-Dazzle594 (Biolegend-406530, San Diego, CA),

anti-CD5-PE-Cy5 (Biolegend-100610, San Diego, CA), and anti-CD1d-PE-Vio77 (Miltenyi Biotec-130-105-157, San Diego, CA). Cells were washed twice and stained with live/dead fixable yellow (Invitrogen) for 20 min. One-to-five million total events per sample were recorded on an LSRII instrument (BD Biosciences) and analysis was performed using FlowJo software (Treestar, Inc, Ashland, OR).

## 2.5. ELISA

The binding antibody response to multiple HIV-1 Env proteins was measured by enzyme-linked immunosorbent assay (ELISA). To measure HIV-1 Env-specific or OVA-specific IgG or IgM, 96 well flat-bottom immunoplates (Thermo Scientific) were coated overnight either with Clade A 92RW020 gp120 (Immune Technology Corp. # IT-001-001p), Clade B gp41 (Prospec # hiv-112-a), or proteins obtained from the NIH AIDS reagent repository: Clade B MN gp120 (#12570), Clade C 96ZM651 gp120 (#10080), Clade C CN54 gp120 (#7749), Clade B gp140 SF162 (#12026), RSC 3 Clade B gp120 (#12042), Clade B F8 gp140, or Clade B C6a gp140 produced in the Haigwood laboratory, or ovalbumin protein. Proteins were coated at a concentration of 0.5 µg/ml in PBS, blocked with 3% BSA in PBS for 1 h, then washed with 0.05% Tween 20 in PBS. Samples were diluted to 1:500 and 1:2500 in PBS containing 0.05% Tween 20 and added in duplicate to plate and incubated for 1 h. Plates were washed and binding was detected using anti-mouse IgG-HRP (Jackson ImmunoResearch #115-035-003, West Grove, PA), anti-mouse IgM-HRP (Jackson ImmunoResearch #115-035-075, West Grove, PA), anti-mouse IgG1-HRP (Southern Biotech #1071-05, Birmingham, AL), or anti-mouse IgG2b-HRP (Southern Biotech #1091-05, Birmingham, AL) at a dilution of 1:2000 and developed by KPL SureBlue TMB Substrate. OD values of test plasma sample were divided by assay plate-specific PBST only control wells to obtain relative units (RU), and subsequent area under the curve values across the dilutions determined, unless otherwise noted.

For avidity ELISA, assay was performed similar to previously described [45–47], briefly plates were coated with CN54 gp120 (Acro Biosystems, # GP4-V15227-100ug Newark), coated plates were blocked with 3% BSA and washed thrice with PBS. Plasma samples were diluted to 1:100 and added to plates and incubated for 1 h, washed, and then 8 M urea was added for 30 min. Plates were washed and anti-mouse IgG-HRP or anti-mouse IgM-HRP were added and developed by KPL SureBlue (Sera care, Milford, MA) TMB Substrate.

## 2.6. Statistical analysis

Data were analyzed using GraphPad Prism (version 5.0d, GraphPad Software Inc, La Jolla, CA) to calculate area under the curve values and statistical significance. A minimum group size of  $n = 5$  mice was used for experiments. For two group comparisons, a two-tailed unpaired Mann-Whitney test was used.

## 3. Results

### 3.1. IL-33 influences the kinetics and isotype of the antibody response

Using the clade B VC10014 HIV-1 Env-based immunogens, C57BL/6 mice were primed with VC10014 gp160 plasmid DNA at wk 0 and wk 3. To specifically influence the conditions in which priming occurs, mice were treated with exogenous conditioning agents, either IL-33 or MPLA, one week prior to and at time of plasmid DNA immunizations. Following this priming phase, mice were boosted with co-immunizations of VC10014 gp160 DNA plasmids and gp140 proteins in alum at wk 7 and wk 11 (Fig. 1A). Examining

the plasma binding antibody response at one week following the final immunization (wk 12), treatment with IL-33 or MPLA enhanced the Env-specific IgG response compared to immunization with alum only, with IL-33 treatment resulting in significantly increased ( $p < 0.05$ ) autologous (C6a and F8) gp140 and heterologous clade C CN54 gp120 Env-specific IgG compared to alum only, and the CN54 gp120 IgG response was also significantly greater ( $p < 0.05$ ) compared to MPLA treatment (Fig. 1B), suggesting IL-33 is increasing the breadth of the Env-specific IgG response. IL-33 treatment resulted in ~2–3-fold greater Env-specific IgM that was significant ( $p < 0.05$ ) compared to both alum only and MPLA treated mice.

Examining the longitudinal plasma heterologous CN54 gp120 Ab response; as soon as week 2, which is just after the first VC10014 DNA immunization, significant levels ( $p < 0.05$ ) of Env-specific IgM and IgG are evident in the IL-33 group, and to a lesser degree in the MPLA group compared to the alum only group (Fig. 1C). The CN54 IgG titers in all the immunized groups further increased after the DNA and protein co-immunizations. The CN54 IgM titers were consistently highest in the IL-33 group following week 2. Together, these results indicate IL-33 influences the kinetics of the plasma Ab response and promotes the development of Env-specific IgM.

### 3.2. IL-33 enhances the quality of the antibody response

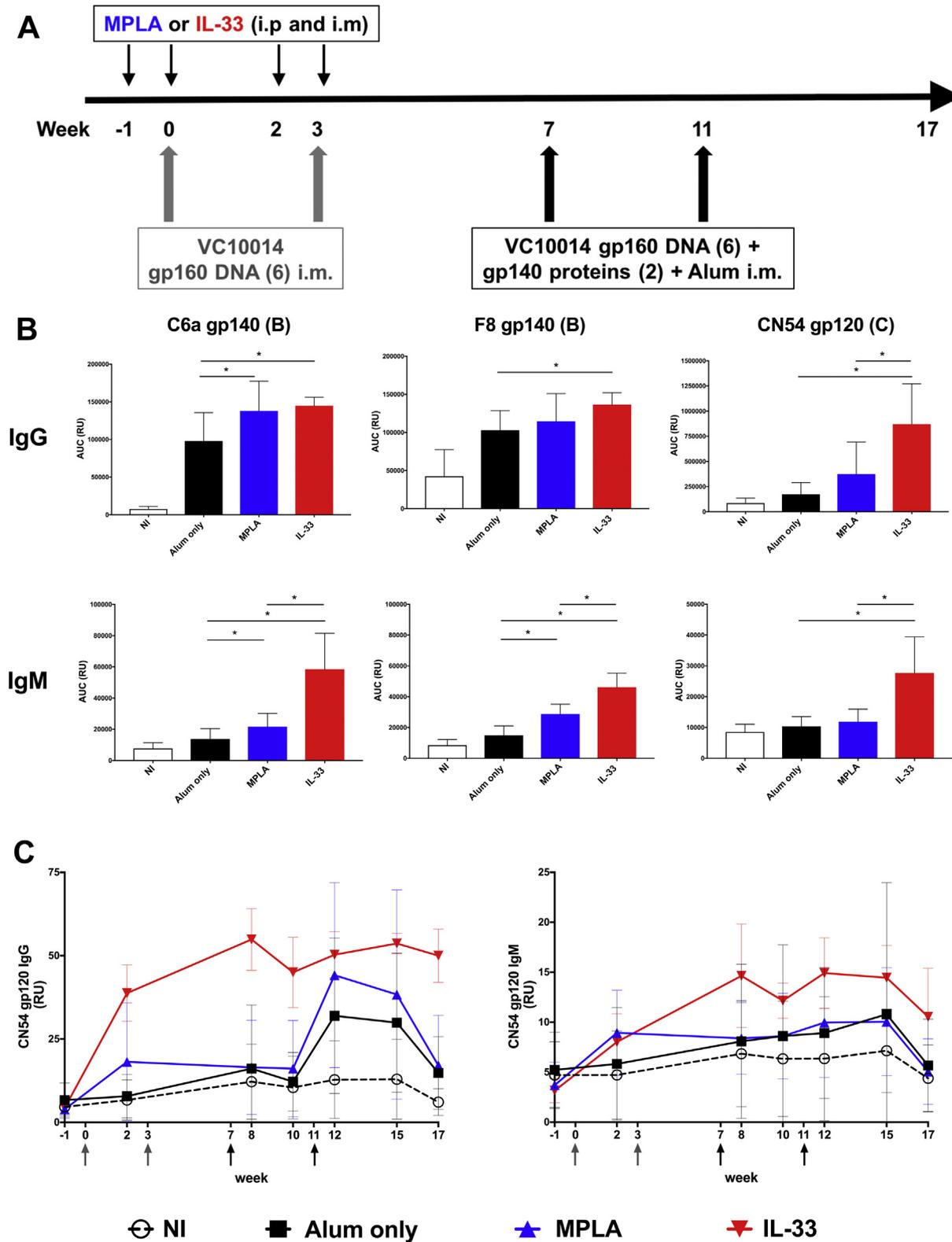
The influence of the priming in the presence of IL-33 or MPLA on the HIV Env-specific IgG subclass utilization was evaluated. While the early gp120 response at wk 2 is dominated by IgG2b in MPLA treated mice, IL-33 treatment results in an IgG1 dominated response (~10:1 IgG1:IgG2b ratio) (Fig. 2A). After the last immunization (wk 12), IgG1 dominated the gp120-specific response in all the immunized groups, with the IL-33 group having significantly higher IgG1 compared to all groups, and significantly higher IgG2b compared to alum only.

Next, we assessed the influence of IL-33 and MPLA on the strength of binding of the HIV Env-specific IgG response by measuring the amount of binding that is maintained following treatment with a chaotropic agent (avidity index). The presence of IL-33 during the priming phase resulted in significantly enhanced avidity compared to the alum only and MPLA groups, with nearly all IgG binding activity maintained following 8 M urea treatment for both the peak plasma Ab response (wk 12) and the durable (wk 17) plasma Ab (Fig. 2B).

To assess the breadth of the plasma Ab response the IgM and IgG binding Ab at wk 12 that was induced to various Env proteins was measured. The IL-33 group demonstrated high titer IgM and IgG binding against the various clade A, B, and C gp120 proteins, including Resurfaced Core 3 (RSC3) gp120, which lacks the V1-V3 variable loops and is indicative of Abs targeting the more conserved core region which contains the CD4 binding site [48]. Increased titers to gp41 were also evident in the IL-33 group (Fig. 2C). Overall these results indicate that IL-33 treatment during priming results in greater Env-specific IgG1, avidity, and breadth.

### 3.3. The influence of IL-33 on the early phase of the B cell response

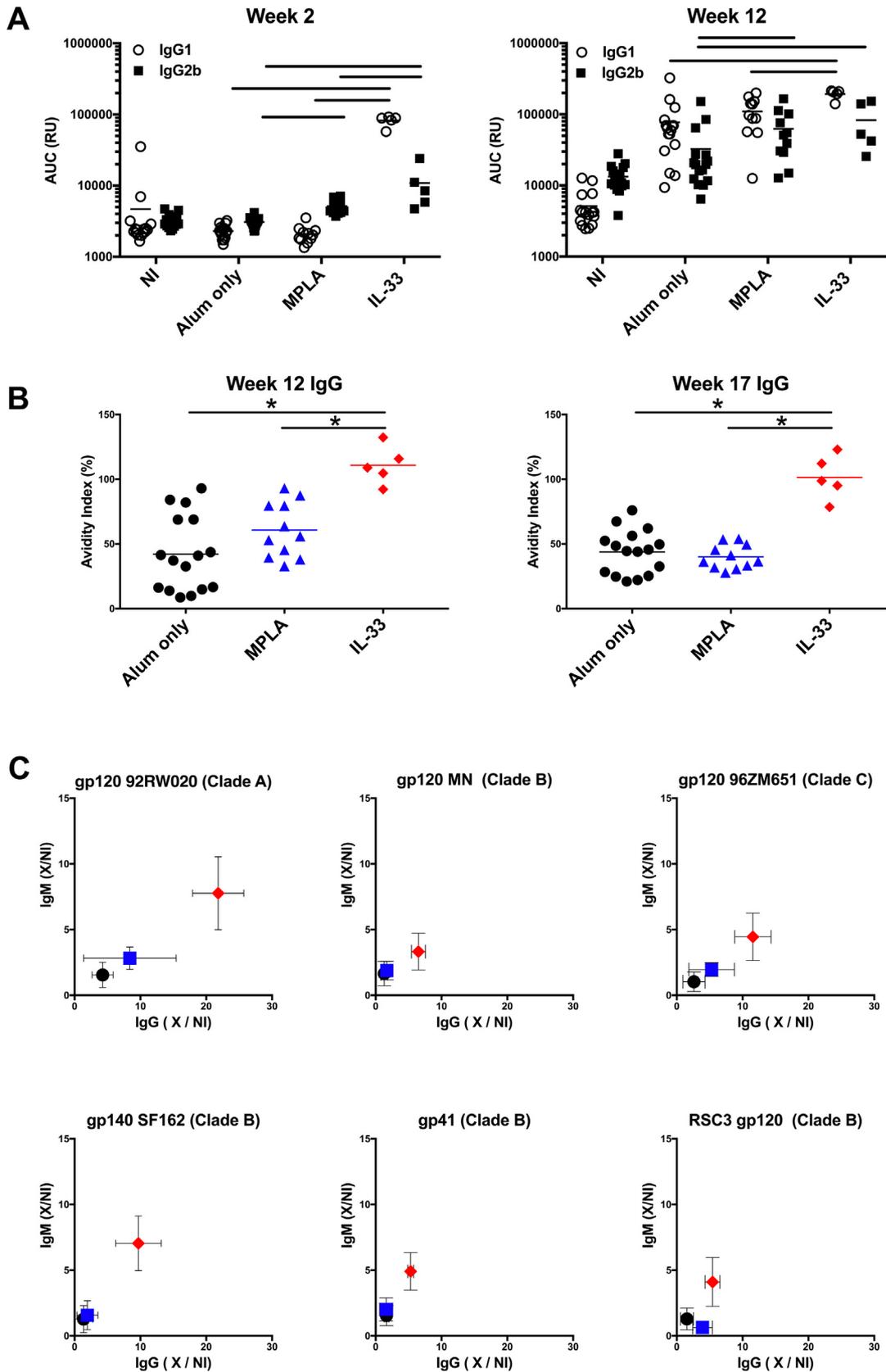
Observing the pronounced influence of IL-33 when present during the priming phase, we sought to better resolve the cellular impacts of IL-33 during the early phase of the response. For this we used ovalbumin protein (OVA), which would enable the assessment of impact of IL-33 beyond HIV-1 and DNA immunogens. For this, again IL-33 was administered one week before and at time of immunization with OVA in alum, and mice were euthanized at wk 4, the peak of the priming phase (Fig. 3A). A single experiment utilizing a group size of 6 mice, maintaining individual mice as



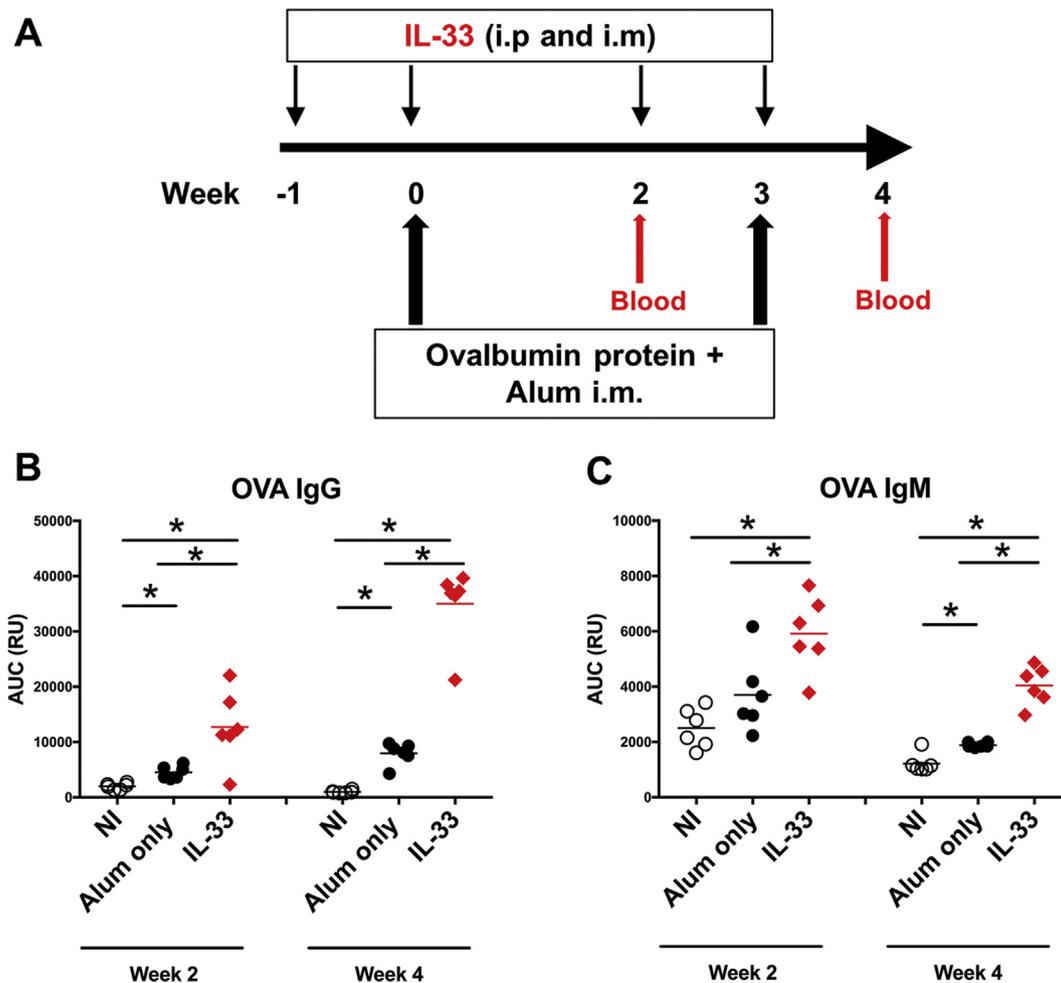
**Fig. 1.** IL-33 improves the Env-specific IgG and IgM response. (A) Immunization schedule. Mice received MPLA (20 µg) or IL-33 (2.5 µg) i.p. and i.m. one week prior to and at time of i.m. priming immunizations with VC10014 DNA plasmids and were then boosted i.m. with co-immunization of VC10014 DNA plasmids and proteins in alum. (B) Plasma Env-specific IgG and IgM determined by ELISA at week 12. (C) Longitudinal plasma (1:500) CN54 gp120-specific IgG and IgM. Symbols represent group mean ± SD. NI: n = 16, Alum only: n = 16, MPLA: n = 11, IL-33: n = 5. \* indicates significant difference (p < 0.05) as determined by two-tailed Mann-Whitney test. Data are representative of three independent experiments.

replicates was performed to evaluate the serological OVA-specific Ab response and the B cell dynamics. The IL-33 group had a significantly higher IgG plasma Ab response (p < 0.05) than the alum

only group after the first OVA immunization that further increased after the second OVA immunization (Fig. 3B). A significantly higher IgM plasma Ab response after each immunization was apparent in



**Fig. 2.** IL-33 impacts the quality of the Env-specific antibody response. (A) CN54 gp120-specific plasma IgG1 and IgG2b was determined by ELISA. Symbols represent individual mice. Lines indicate significant difference ( $p < 0.05$ ) between groups. (B) Avidity index of CN54 gp120-specific plasma IgG determine by ELISA in the absence or presence of 8 M urea treatment. Symbols represent individual mice. \* indicates significant difference ( $p < 0.05$ ) between groups as determined by two-tailed Mann-Whitney test. (C) Plasma (wk 12) was evaluated for IgM and IgG binding to indicated Env by ELISA. Symbols represent group mean  $\pm$  SD fold increase in titer compared to the non-immunized group (X/Ni). NI:  $n = 16$ , Alum only:  $n = 16$ , MPLA:  $n = 11$ , IL-33:  $n = 5$ . Data are representative of three independent experiments.



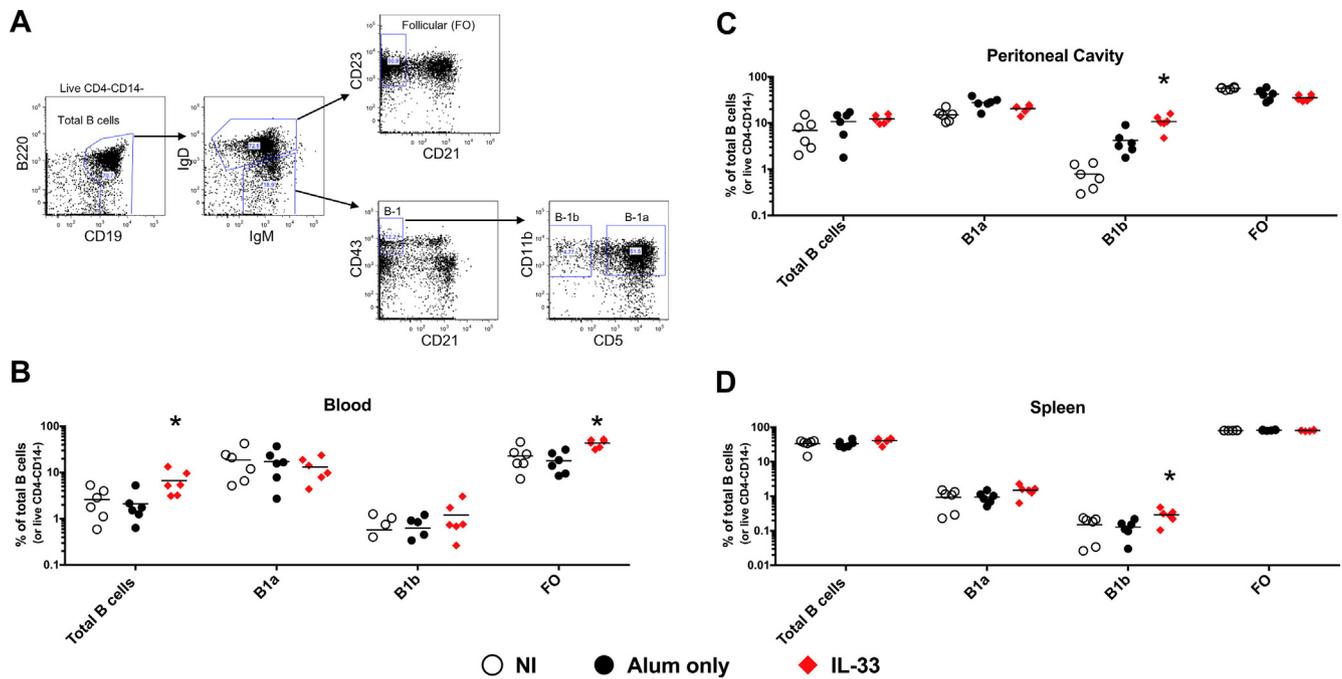
**Fig. 3.** IL-33 increases OVA-specific IgG and IgM. (A) Immunization schedule. Mice ( $n = 6$  per group) received IL-33 (2.5  $\mu\text{g}$ ) i.p. and i.m. one week prior to and at time of i.m. immunization with ovalbumin protein (100  $\mu\text{g}$ ) in alum. Plasma OVA-specific IgG (B) and IgM (C) determined by ELISA. Symbols represent individual mice. \* indicates significant difference ( $p < 0.05$ ) between groups as determined by two-tailed Mann-Whitney test. This experiment was conducted once.

the IL-33 group (Fig. 3C), which is consistent with the results using VC10014 (Fig. 1). The IgM plasma Ab response did not increase following the second immunization, which may suggest class-switching.

To determine if IL-33 impacts the dynamics of B cells flow cytometry phenotyping (Fig. 4A) was performed at wk 4, one week after the final IL-33 treatment. The IL-33 group had a significant 3-fold higher frequency of total CD19<sup>+</sup> peripheral blood B cells compared to the alum only group (2.1 $\pm$ 1.6% vs 6.7 $\pm$ 4.1%,  $p < 0.05$ ) (Fig. 4B). The frequency of B-1a (CD19<sup>+</sup>IgD<sup>low</sup>IgM<sup>hi</sup>CD21<sup>-</sup>CD43<sup>+</sup>CD11b<sup>+</sup>CD5<sup>+</sup>) and B-1b (CD19<sup>+</sup>IgD<sup>low</sup>IgM<sup>hi</sup>CD21<sup>-</sup>CD43<sup>+</sup>CD11b<sup>+</sup>CD5<sup>-</sup>) peripheral blood B cells did not differ significantly between groups, however, the IL-33 group had a 2-fold higher frequency of follicular (FO: CD19<sup>+</sup>IgD<sup>+</sup>CD21<sup>-</sup>CD23<sup>+</sup>) peripheral blood B cells (18.0 $\pm$ 9.2% vs 43.6 $\pm$ 8.4%,  $p < 0.005$ ) compared to the alum only group. In the peritoneal cavity, the IL-33 group had a ~10-fold and 2-fold higher frequency of B-1b cells (10.8 $\pm$ 3.8%) compared to non-immunized (0.8 $\pm$ 0.5%,  $p < 0.005$ ) and alum only (4.2 $\pm$ 2.6%,  $p < 0.005$ ) groups, respectively (Fig. 4C). A similar ~2-fold increase in B-1b cells in the IL-33 group (0.30 $\pm$ 0.1%) was observed in the spleen, compared to the non-immunized (0.15 $\pm$ 0.09%,  $p < 0.05$ ) and alum only (0.13 $\pm$ 0.06%,  $p < 0.05$ ) groups, respectively (Fig. 4D). Together, these results suggest that peripheral FO, and peritoneal and splenic B-1b B cells are responding to IL-33 treatment.

#### 4. Discussion

Treatment with IL-33 during the initial priming immunization with gp160 DNA plasmids resulted in a significant induction of plasma Env-specific IgG within two weeks that was not evident in the alum only group, and only minimal in the MPL treated group (Fig. 1C). This rapid IgG response was dominated by IgG1, but also included substantial IgG2b Env-specific antibody (Fig. 2A). Intramuscular immunization with DNA plasmids alone is poorly immunogenic, however, a few studies have described increased Ab responses when combined with an adjuvant such as GM-CSF or QS-21 [49–52] yet these were primarily only evident following repeated DNA plasmid immunizations. IL-33 may be acting in multi-faceted manner to increase DNA immunogenicity, such as through increasing Env expression, enhancing CD4<sup>+</sup> T cell help, as IL-33 itself has been indicated in promoting Th1, Th2, and Th9 differentiation [18,53,54]. Further, the direct impact of IL-33 on B cells may also be contributing. As the clinical development of plasmid DNA-based immunogens continue, combining IL-33 with other delivery modalities which increase the humoral response such as electroporation and intradermal injection should be evaluated. IL-33 has been previously demonstrated to increase the plasma and mucosal IgG response when delivered intranasally in combination with influenza HA protein in mice [14], and interestingly it has been reported that alum delivered i.p. induces the



**Fig. 4.** IL-33 increases B1-b B cells. (A) Gating strategy. Mice ( $n = 6$  per group) were euthanized at wk 4 following IL-33 treatment and immunizations and B cell subsets defined by flow cytometry from the peripheral blood (B), peritoneal cavity (C), and spleen (D). Frequency of total B cells are presented as % of live CD4-CD14- cells. Symbols represent individual mice. \* indicates significant difference ( $p < 0.05$ ) as compared to alum only group as determined by two-tailed Mann-Whitney test. This experiment was conducted once.

release of endogenous IL-33 and in-part mediates the adjuvant effect of alum [55]. This study exclusively delivered IL-33 via i.p., however delivery via traditional i.m. route should be evaluated to adequately determine the translational feasibility of IL-33 as an adjuvant. Similarly, it remains to be determined if pre-treatment with IL-33 in advance of immunization is a requirement for its optimal adjuvant-like activity.

The autologous clade B IgG response to F8 and C6a gp140 at the peak timepoint (wk 12) was modestly increased compared to the alum only and MPLA treated groups, however, the superiority of the IL-33 treatment in promoting a heterologous IgG response to the clade C CN54 gp120 and the other representative Envs was most apparent (Figs. 1 and 2). Additionally, the predominant IgG1 bias and increased IgG avidity resulting from IL-33 treatment further indicate that IL-33 during the priming phase is qualitatively altering the Env-specific IgG response in a manner that remains evident even following boosting immunizations. The consistent induction of Env-specific IgM was a dominant feature of the IL-33 treatment, frequently at least two-fold greater than that observed with MPLA or alum only (Figs. 1B and 2C), further substantiating the qualitative uniqueness associated with IL-33 treatment, and may be an integral mechanistic component to the development of the enhanced kinetics and breadth of the IgG response.

Although the cellular source of the Env-specific plasma IgM was not defined in this study, the increase in B-1b B cells observed following IL-33 treatment (Fig. 4) suggests they should be considered as a potential contributor to the Env-specific humoral response. B-1 B cells are commonly associated with their preferential anatomic distribution in the peritoneal and pleural cavities and omentum, providing a first line of defense against pathogens through the constitutive production of natural IgM [56,57] and are the primary antibody producers along with marginal zone B cells in response to T cell independent antigens [58,59]. Although bacterial capsular polysaccharides are the most well described B-1 antigens, B-1

responses to viruses particularly influenza have been suggested [30,60,61]. Among B-1 B cells, B-1a are thought to be the primary source of steady state natural IgM and B-1b are associated with a greater degree of antigen-specific responsiveness, including memory and recall responses [62–64]. Although IgM production dominates B-1b Ab secretion, they can also give rise to IgG and IgA expressing plasma cells at mucosal sites and in the bone marrow [65–67]. Follow-up studies to determine if pre-treatment with IL-33 prior to immunization, enhances B cell localization and/or responsiveness to the immunization are warranted. Additionally, experiments to define the precise cellular source of the Env-specific Ab that develops with IL-33 treatment and the modulation or potential contribution of CD4<sup>+</sup> T cell help that might be occurring should be pursued and may resolve a unique developmental pathway for HIV-1 specific B cell responses.

Our results in mice substantiate the previous findings in rabbits [41] and rhesus macaques [42] that the VC10014 HIV-1 Env-based immunogen platform can induce a broad cross-clade Ab response. The trimeric gp140 proteins used in this study are not of the recently developed native-like trimer formats, and generating and testing the VC10014 HIV-1 Envs as native-like trimers [68,69] may further increase their ability to induce broad and potent Ab responses. Our results do indicate that the addition of IL-33 to the current VC10014 immunization regimen can further increase the magnitude, avidity, and breadth of the IgG response and also promote the expansion of B-1b B cells and a robust Env-specific IgM response which are not apparent in the absence of IL-33 treatment. These findings provide justification for evaluating the ability of IL-33 in combination with VC10014 to induce protective immunity to HIV-1 infection.

#### Conflict of interest

The authors declare no conflict of interest.

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