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Regulatory T cell abundance and activation status before and after priming with HIVIS-DNA and boosting with MVA-HIV/rgp140/GLA-AF may impact the magnitude of the vaccine-induced immune responses

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ABSTRACT

Background: Little is known about regulatory CD4 T cells (Tregs) in the context of HIV vaccines. Tregs can be differentiated into resting ($FoxP3^{+}CD45RA^{+}$ – rTregs), activated ($FoxP3^{High}CD45RA^{-}$ – aTregs) and memory ($FoxP3^{Low}CD45RA^{-}$ – mTregs). Tregs, as CD4 T cells, are also frequent targets for HIV infection. We studied how the abundance and phenotypes of Tregs in terms of activation status and expression of HIV-1 binding molecules would have changed during vaccination in healthy volunteers participating in a phase IIa HIV vaccine clinical trial. Subjects were primed three times with HIVIS-DNA and boosted twice with MVA-CMDR-HIV alone (n = 12) or MVA-CMDR combined with protein CN54rgp140 (n = 13). The proportions of β7 integrin in all CD4 T cells and in the Tregs subset decreased moderately after the final vaccination (p = 0.001 and p = 0.033, respectively) and the rTregs proportion within the total Tregs were also decreased after the final vaccination (p = 0.038). All these proportions returned to normal values within the three months after the final vaccination. The magnitude of HIV-Envelope-specific IFNγ + T cells after vaccination (r = 0.66; p = 0.021) correlated directly with the proportion of Tregs, and correlated inversely correlated with ratios of Th17/Tregs (r = −0.75; p = 0.0057) and Th17/mTregs (r = −0.78; p = 0.0065). Higher titers of IgG gp140 antibodies were observed in subjects with higher mTregs proportions (r = 0.52; p = 0.022). Interestingly, pre-vaccination levels of mTregs correlated with vaccine-induced Env-binding antibodies (r = 0.57; p = 0.01) and presence of neutralizing antibodies (r = 0.61; p = 0.01), while the pre-vaccination Th17/mTregs ratio correlated inversely with the magnitude of cellular IFN-γ ELISpot responses (r = −0.9; p = 0.002). Taken together, these results suggest that pre- and post-vaccination Tregs, their activation status, the Th17/Tregs ratio and other host factors affecting Treg abundance, have an impact on the magnitude of HIV vaccine-induced immune responses. Moreover, the DNA-HIVIS/MVA-HIV regimen, alone or in combination with CN54rgp140 induced moderate and temporary alterations of the Tregs activation status. We also show a decrease in expression of the HIV-1 ligand β7 integrin on Tregs and all CD4 T cells.

Abbreviations: AIDS, acquired immunodeficiency syndrome; aTregs, activated regulatory T cells; CNBS, Mozambican National Committee on Bioethics in Health (Comité Nacional de Biotética em Saúde de Moçambique); DMSO, dimethyl sulfoxide; FCS, fetal calf serum; Foxp3, (transcription factor) *forkhead box P3*; mTregs, memory regulatory T cells; PBMC, peripheral blood mononuclear cells; Tregs, regulatory CD4+ T cells; rTregs, resting regulatory T cells; TaMoVac, Tanzania Mozambique vaccine trial

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1. Introduction

Regulatory T cells (Tregs) are a CD4 T cell subset with potential to control the extension of immune responses by several mechanisms, including suppression of antigen presenting cells (APC), B and T cells (Wing and Sakaguchi, 2012). Tregs are typically defined by high expression of the IL2 receptor (CD25) and expression of the transcription factor forkhead box P3 (Foxp3) (Hartigan-O'Connor et al., 2007). Furthermore, based on their activation status, determined by expression levels of FoxP3 and CD45RA, Tregs can be subdivided in three functionally distinct populations, (a) resting ($FoxP3^{+}CD45RA^{+}$), (b) activated ($FoxP3^{High}CD45RA^{-}$) and (c) memory ($FoxP3^{Low}CD45RA^{-}$) Tregs (Miyara et al., 2009). The resting Tregs (rTregs) and activated Tregs (aTregs) are both highly suppressive while memory Tregs (mTregs) are less suppressive and secrete non-regulatory cytokines such as IL-2, IFN-γ and potentially IL-17 (Miyara et al., 2009; van der Veeken et al., 2016).

Under normal physiological conditions, Tregs maintain peripheral tolerance, prevent autoimmunity and regulate immunity to commensal pathogens (Pereira et al., 2017; Sakaguchi, 2000). During infection, they prevent exacerbated immune responses and immunopathology (Sehrawat and Rouse, 2017; Stephen-Victor et al., 2017). However, excessive suppressive Tregs function might be undesirable favoring the development of cancers and chronicity of infections (Takeuchi and Nishikawa, 2016; Nishikawa and Sakaguchi, 2014; Veiga-Parga et al., 2013). In certain pathologic conditions, such as during HIV infection, Tregs might contribute in controlling the chronic and systemic immune activation sequel of HIV infection, yet may also suppress protective anti-HIV immune responses (Kinter et al., 2007; Rueda et al., 2013; Karlsson et al., 2011).

Tregs share some differentiation pathways with Th17 cells, the IL17 producing CD4 T cells counterparts, and reciprocal development between these two CD4 T cells populations, has been demonstrated (Omenetti and Pizarro, 2015). Thus, the balance between Tregs and Th17 cells has been found to be critical for immune homeostasis (Omenetti and Pizarro, 2015; Diller et al., 2016).

Beyond their role as suppressors of the immune response, there is accumulating evidence that Tregs are also involved in mechanisms, which guide the generation of immunological memory (Bhattacharyya and Penaloza-MacMaster, 2017; Sage et al., 2016; Espinoza Mora et al., 2014). Despite the fact that one of the main goals of vaccination is to induce durable immune responses, little is known about the contribution of Tregs in the induction of memory T and B responses in humans, particularly in HIV vaccine studies.

We hypothesized that Tregs might influence vaccine-induced adaptive immune responses. Their abundance and phenotype can have an impact on anti-HIV vaccine-induced T and B responses. In order to verify this, we assessed whether Tregs abundance, their activation status and the Tregs/Th17 ratio, before and after priming healthy subjects with HIVIS-DNA and boosting with Modified Vaccinia Ankara - Chang Mai recombinant (MVA-CMDR)-HIV alone or in combination with CN54rgp140 protein, correlated with the profile of vaccine induced immune responses.

Tregs are frequent targets for HIV infection (Chachage et al., 2016). Lessons learned from previous HIV-1 vaccine efficacy trials show that certain vaccination regimens, such as those based on adenovirus type 5, in spite of their immunogenic potential, may increase the susceptibility for HIV-1 acquisition in some participants, due to factors such as increased levels of immune activation and higher levels of β7 expression, a HIV-1 ligand (Arthos et al., 2008) on CD4 T subsets (Ondondo, 2014; Benlahrech et al., 2009). Thus, we also assessed if the aforementioned vaccination regimen, incorporating recombinant modified vaccinia virus Ankara vector, induced significant systemic phenotypic alterations in Tregs and in the whole CD4 T cell population.

2. Materials and methods

2.1. Study design and population

A randomized phase IIa clinical trial (the Tanzania and Mozambique HIV Vaccine Program – TaMoVac – II trial) for assessment of a prophylactic HIV vaccine was conducted in Maputo at Instituto Nacional de Saúde of Mozambique between October 2013 and November 2015. A total of 40 participants were enrolled in Maputo as shown in Fig. 1. Twenty-five vaccinees were selected for this sub-study, based on availability of biological samples, represented in Table 1. Briefly, the

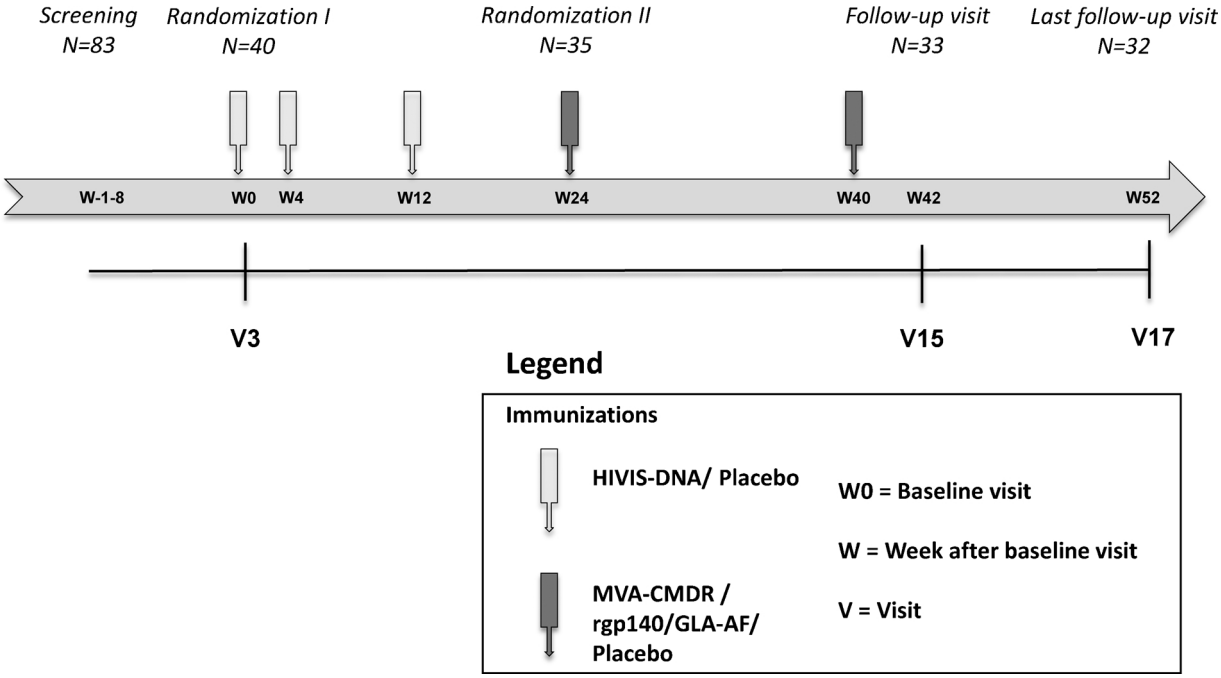

Fig. 1. Diagram showing the vaccination schedule, the number of study volunteers included in the present study and the study weeks corresponding to the immunogenicity points of analysis.

participants were primed three times with HIVIS-DNA, administrated by intradermal injection by Zetajet® device with or without electroporation using the Derma Vax™ electroporation device, using a dose of 0.6 mg in either a concentration of 3 mg/ml or in a concentration of 6 mg/ml. For the DNA immunizations, participants were randomized in three different groups as represented in Table 1. In a second randomization step, participants were boosted intramuscularly with two doses of MVA-CMDR-HIV (10^8pfu per ml) given either alone (n = 12) or concurrent with IM 100 µg CN54 rgp140 adjuvanted with 5ug GLA-AF (n = 13) or just with saline solution. Placebo recipients received saline solution, but were not part of the current study since immune responses to vaccinations were not observed in this study group.

Blood samples were collected pre-immunization and two, four and twelve weeks after the final immunization.

2.2. Vaccine and vaccination schedule

The HIVIS-DNA vaccine was composed of seven plasmids covering HIV subtypes A, B and C, namely, pKCMVgp160 A, pKCMVgp160B, pKCMVgp160C, pKCMVrevB, pKCMVp37 A, pKCMVp37B, and pKCMVRTB, described elsewhere (Brave et al., 2006, 2005; Nilsson et al., 2015). The vaccine was administrated at weeks 0, 4 and 12, Fig. 1.

The MVA-CMDR-HIV is a construct expressing HIV-1 gp150 (subtype E) and Gag and Pol (subtype A), developed by Laboratory of Viral Disease form the National Institute of Allergy and Infectious Diseases and produced by the Walter Reed Army Institute of Research (USA). The vaccine formulation contains 10^8 plaque forming units (pfu)/mL in sterile PBS, 7.5% lactose, pH7.4 (Earl et al., 2009).

The CN54rgp140 is a recombinant subtype C protein, comprised of 670 amino acids (US GMP). It was given in combination with GLA-AF, a synthetic lipid A adjuvant, formulated in aqueous solution (US GMP) (Joachim et al., 2016). The MVA-CMDR-HIV was given alone or in combination with the CN54rgp140/GLA-AF at weeks 24 and 40 as shown in Fig. 1.

2.3. Blood collection and peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from freshly obtained heparin anti-coagulated blood from each vaccinee, using Ficoll-Paque Plus (GE Healthcare, Sweden) and Leucosep tubes (Greiner Bio-One, German). Fresh PBMC were used for IFN-γ ELISpot experiments and the remaining cells were stored in liquid nitrogen in freezing media (10% dimethyl sulfoxide (DMSO) +90% fetal calf serum (FCS)). Serum and plasma samples were stored at −70 °C. Serum was separated from the cell fraction by centrifugation of whole blood collected in serum separation tubes, and stored at −70 °C until testing.

2.4. Peripheral blood mononuclear cell stimulation

The immunophenotyping experiments were conducted on cryopreserved samples. From each vaccinee, frozen PBMC obtained at baseline, two weeks and three months after the final vaccination were thawed, when available. All samples from the same participant were thawed on the same day in a water bath at 37 °C. PBMC were washed twice in complete RPMI medium supplemented with 20% of FCS followed by 10% of FCS (R20 and R10, respectively) and viable cells were counted using the Nucleocounter (Chemometec, Denmark). After counting, PBMC were allowed to recover overnight in R10 medium at 37 °C in an atmosphere of 7.5% CO_2. Subsequently, 0.5 million PBMC suspended in 100 µL of R10 were mixed with monensin, brefeldin A, and co-stimulatory monoclonal antibodies against CD28 (1 µg/mL) and CD49d (1 µg/mL) diluted in R10. Then, each cell suspension was stimulated with 50 µL of antigen, either HIV-1 vaccine-specific peptide pools corresponding to the MVA-CMDR-HIV boost (Env-CMDR, (1 µg/mL)) (Munseri et al., 2015), or staphylococcus enterotoxin A and B (SEAB, (1 µg/mL)) or just with 0.5% of DMSO in R10 (medium). The cell suspensions were incubated for six hours, in an atmosphere of 7.5% CO_2, and subsequently stored overnight at 2–8 °C, as previously described (Bakari et al., 2011).

2.5. Peripheral blood mononuclear cell immunophenotyping

After treatment with antigen or medium and overnight storage, PBMC were treated with 20 mM EDTA and incubated for 15 min in the dark, at room temperature. Subsequently, cells were washed with phosphate buffered saline (PBS) prior staining with 50 µL of the viability dye (fixable viability stain (FVS) 510 (Becton Dickinson (BD), USA)). Cells were kept in the dark at room temperature, for another 15 min, then washed twice with 5% FCS in PBS. After washing, PBMC were stained with monoclonal antibody cocktails for staining of cell surface markers. For those cells requiring intracellular staining for cytokines and FoxP3, 200 µL of Human FoxP3 Buffer A (BD, USA) were added to fix the cells during 10 min at 4 °C. Subsequently, PMBC were washed with diluted BD Perm/Wash buffer (BD, USA) and permeabilized with Human FoxP3 Buffer C 1X for 30 min in the dark at room temperature. Prior to staining, PBMC were washed twice with BD Perm/Wash buffer (BD, USA). The pre-prepared combinations of monoclonal antibodies were added to the cells and incubated at 2–8 °C. After 30 min, cells were washed twice with Perm/Wash buffer (BD, USA) and finally treated with 200 µL of 1X BD CellFix (BD, USA). Combinations of the following monoclonal antibodies and the viability dye, all from BD, USA, were used: CD195-FITC (2D7/CCR5), IL17-PE (SCPL1362), IFNγ-PE-Cy7 (B27), β7-APC (FIB504), CD3-APC-H7 (SK7), CD4-V450 (RPA-T4), FoxP3-PE (259D/C7), CD25-PE-Cy7(M-A251) CD45RA-PerCP-Cy5.5 (HI100), and CD3-FITC (HIT3a), HLA-DR-PE-Cy7 (L243), CD38-APC (HIT2), CD8-APC-H7(SK1), FVS510.

After 30 min, samples were ready to be analyzed on a flow cytometer. A minimum of 100,000 events were acquired on FACSCanto II (BD, USA) using Diva software version 8 (BD, USA). For quality control purposes, the BD Cytometer Setup & Tracking (CST) beads and BD Comp beads were used to ensure consistency of results over time and for compensation, respectively. The post-acquisition analyses, including compensation, were performed using the FlowJo software, version 10 (FlowJo LLC, USA). The fluorescence minus one (FMO) controls were used for definition of positivity applied to markers with continuous or dim levels of expression during post acquisition analysis. Since Tregs proportions were obtained using two different antibodies panels, run on same day, the mean value of these two observed values was used for subsequent analysis. The samples processing and FlowJo analysis were all performed before the un-blinding process of the main study. The strategy for definition of Tregs is showed in Fig. 2a. The gating

Table 1
Baseline characterization of the study participants and randomization groups.

Characteristic	MVA	MVA + CN54rgp140	All
N	12	13	25
Sex M/F	4/8	6/7	10/15
Median age (years) (V3) (IQR)	20 (19–20.75)	20 (19–23)	20 (19–21)
Median #CD4 (cells/µL) (%) (V3)	882 (63.2%)	665 (55.6%)	707 (61.7%)
Median #CD8 (cells/µL) (%) (V3)	404 (27.4%)	327 (37.4%)	382 (28.2%)
Group IA[a] (n)	4	5	9
Group IIA[b] (n)	4	5	9
Group IIIA[c] (n)	4	3	7

[a] 2 intradermic injections of 0.1 mL with 3 mg/mL DNA (600 µg) given with Zetajet® without electroporation.

[b] 2 intradermic injections of 0.1 mL with 3 mg/mL DNA (600 µg) given with Zetajet DNA Zetajet® followed by intradermic DermaVAx electroporation.

[c] 1 intradermic injection of 0.1 mL with 6 mg/mL DNA (600 µg) given with Zetajet DNA Zetajet® followed by DermaVAx electroporation. V3 – Baseline visit.

strategies for definition of CCR5$^+$ in CD4 T cells and definition CD38$^+$HLA-DR$^+$ in CD4 and CD8 T cells are shown as supplementary information.

2.6. IFN-γ ELISpot assays

IFN-γ ELISpot testing was performed on fresh PBMC on the day of blood collection. Three replicates of 200,000 cells per well were used. The IFN-γ ELISpotPLUS kit, in a 2-step detection system, was used in accordance with the manufacturer's instructions (Mabtech), as previously described (Bakari et al., 2011). A peptide pool specific for MVA-CMDR envelope (Env-CMDR) was used for stimulation (Munseri et al., 2015).

2.7. Enzyme-linked immunosorbent assay

Binding antibodies with specificity for HIV CN54rgp140 were measured using a standardized enzyme linked immunosorbent assay (ELISA) as described elsewhere (Joachim et al., 2016).

2.8. TZM-bl cell neutralization assay

Neutralizing antibodies in sera were measured against subtype C 93MW965 pseudotyped viruses and a luciferase based assay in TZM-bl cells as described previously (Nilsson et al., 2015).

2.9. Statistical analysis

The data were analyzed using the Prism software version 6 h (San Diego, CA). The *Mann-Whitney* test was used for comparisons between different groups. The analysis of correlations between two variables was performed using the non-parametric *Spearman Rank correlation*. For pairwise comparisons, the *Wilcoxon* matched-pair signed rank test was used. Differences or correlations with p-values less than 0.05 were considered statistically significant.

Table 2
Immunogenicity summary of study participants at peak of vaccine-induced immune responses.

Characteristic	MVA	MVA + CN54rgp140	All
N	12	13	25
IFNγ ELISpot (env-CMDR) (V15)			
Frequency of responders	6/7	9/10	15/17
Median magnitude of response (sfc/million PBMC)	180.0	334.5	212
Median anti gp140 IgG titers (V16) (Log10)	3.91	4.86	4.39

V3 – Baseline visit. V15 – Two weeks after the last immunization. V16 – Four weeks after the last immunization.

3. Results

3.1. Proportions of Tregs correlate directly whereas the proportions of Th17 cells correlate inversely with the magnitude of IFN-γ ELISpot responses

The median magnitude of IFN-γ ELISpot to peptides pool specific for MVA-CMDR envelope (Env-CMDR) and median titers of anti-gp140 antibodies in vaccinees are shown in Table 2. Overall, at the peak of the immune response (two weeks after the final vaccination), the proportions of unstimulated Tregs correlated positively with the magnitude of IFN-γ ELISpot responses to Env-CMDR (r = 0.66; p = 0.021) (Fig. 2b), despite the absence of significant variation of Tregs proportions *in vivo* after vaccination. After stratification in groups we found an inverse correlation between proportions of unstimulated Tregs and magnitude of anti-gp140 antibodies (r = −0.69; p = 0.021) in those boosted with MVA only (Fig. 2c). Interestingly, the proportions of Tregs measured in

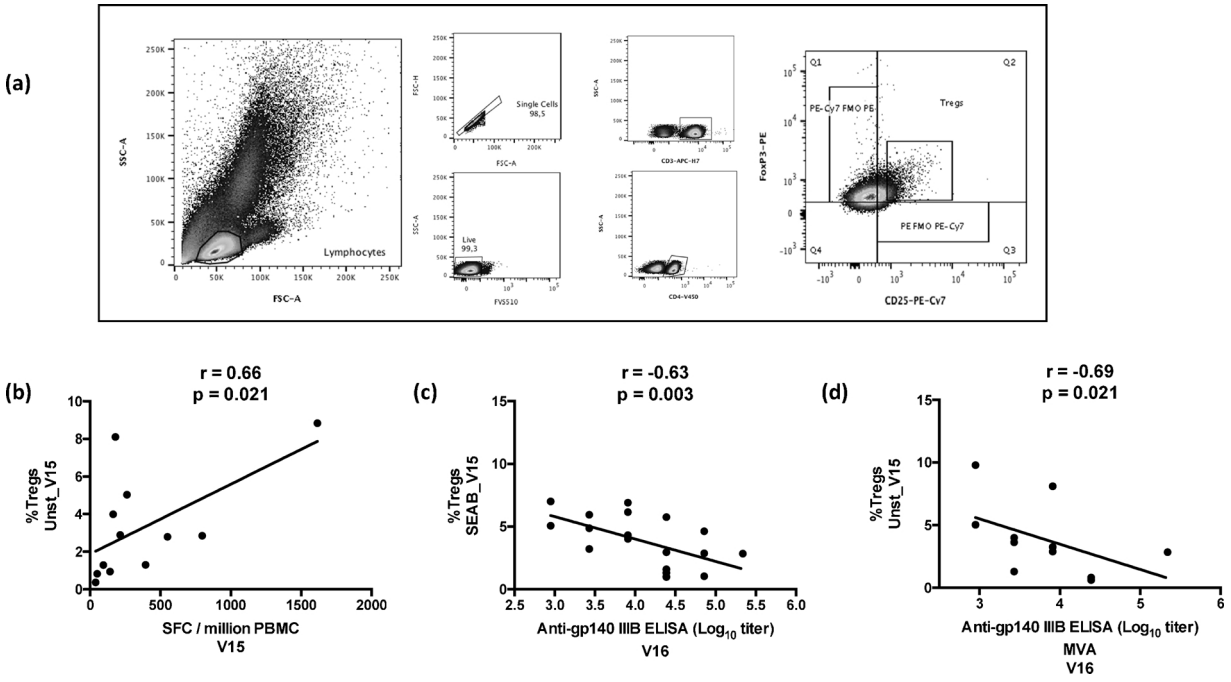

Fig. 2. Correlations between proportions of Tregs (CD4$^+$CD25^{High}FoxP3$^+$) and the magnitude of vaccine induced T and B cell responses. (A) Gating strategy for definition of Tregs by flow cytometry in peripheral blood mononuclear cells (PBMC). (B). Correlation between the proportions of Tregs in unstimulated PBMC and the magnitude of IFNγ ELISpot responses to Env-CMDR, indicated as spot forming cells (SFC)/ million PBMC at two weeks after the final immunization (V15) (n = 12). (C) Correlation between the proportions of Tregs in unstimulated PBMC (V15) and the gp140 IgG antibodies titers at four weeks after the final immunization (V16), in participants boosted with MVA-HIV only (n = 11). (D) Correlation between the proportions of Tregs in PBMC stimulated with staphylococcus enterotoxin A and B measured at V15 and gp140 IgG antibody titers in plasma samples collected at V16, in participants boosted with MVA-HIV alone or in combination with CN54rgp140/GLA-AF (n = 19). Correlation analyses was performed using the Spearman rank test with a p-value < 0.05 considered significant.

PBMC stimulated with SEAB correlated inversely with titers of gp140 binding antibodies (r = −0.63; p = 0.003) (Fig. 2d) and a negative association, although not statically significant, was also observed with neutralizing antibodies (r = −0.39; p = 0.09).

The balance between Tregs and Th17 is critical for immune homeostasis (Voo et al., 2009; Zhou et al., 2008). The Fig. 3a shows the gating strategy for definition of Tregs and Th17 cells. As expected, before the vaccination and two weeks after the final immunization, the proportions of Tregs in unstimulated PBMC were inversely correlated with proportions of total Th17 cells in PBMC stimulated with SEAB ($CD4^+IL17^+$) (r = −0.54, p = 0.031) and (r = −0.52, p = 0.02), respectively (Fig. 3b and c). There was no significant change in the proportions of Th17 cells and ratio of T17/Tregs before the immunization or two weeks after the final vaccination. However, the proportions of Th17 cells and ratio of Th17/Tregs were inversely correlated with the magnitude of IFN-γ ELISpot responses to Env-CMDR (r = −0.71, p = 0,011 and r = −0.75, p = 0.0057, respectively) (Fig. 3c and d) two weeks after the final immunization.

3.2. After immunization, rTregs decrease and mTregs correlate positively with titers of IgG binding antibodies to gp140

Tregs can be differentiated based upon their activation status as represented in Fig. 4a. Both rTregs ($CD4^+CD25^{High}FoxP3^+CD45RA^+$) and aTregs ($CD4^+CD25^{High}FoxP3^{High}CD45RA^-$) are suppressive while mTregs ($CD4^+CD25^{High}FoxP3^{Low}CD45RA^-$) are less suppressive and secrete cytokines such as IL-2 and IFN-γ, and potentially IL-17 (Miyara et al., 2009; van der Veeken et al., 2016).

We found a decreased proportion of rTregs in PBMC stimulated with Env-CMDR, within total Tregs, two weeks after the final immunization (p = 0.038) (Fig. 4b). However, the proportions returned to pre-immunization levels, three months after the final vaccination when compared to baseline levels (p = 0.09). The decrease in rTregs tended to correlate with an increase in magnitude of gp140 IgG binding antibodies (r = −0.46, p = 0.06) (Fig. 4c). In the MVA boosted group, the proportions of rTregs, correlated directly with the magnitude of IFN-γ producing T cells, measured by ELISpot (r = 0.92; p = 0.00067) (Fig. 4d).

The proportions of mTregs in PBMC stimulated with Env-CMDR, within total Tregs, correlated directly with the titers of anti-gp140 antibodies in vaccinated individuals (r = 0.52, p = 0.022) (Fig. 4e). This correlation remained significant when participants, boosted with MVA alone, were considered (r = 0.68; p = 0.02) despite the unaltered proportions of mTregs. Moreover, the ratio of Th17/mTregs correlated inversely with the magnitude of IFN-γ ELISpot responses to Env-CMDR (r = −0,78, p = 0.0065) (Fig. 4f).

Finally, the proportions of aTregs, within total Tregs, correlated directly with the proportions of activated $CD4^+CD38^+HLA\text{-}DR^+$ (r = 0.63; p = 0.038) and $CD8^+CD38^+HLA\text{-}DR^+$ T cells (r = 0.60; p = 0.056), although not statistically significant for CD8 T cells.

3.3. Pre-vaccination levels of Tregs and Th17 cells correlate with vaccine induced immune responses

In most participants, the proportions of Tregs and Th17 cells, were not altered by vaccination. Therefore, we hypothesized that the pre-existing levels of Tregs and Th17 proportions would correlate with the vaccine-induced immune responses after vaccination. Interestingly, the magnitude of IFN-γ ELISpot responses to Env-CMDR at two weeks after the final vaccination correlated inversely with the pre-vaccination levels of Th17 proportions, and with the ratios Th17/Tregs and Th17/mTregs ratios, (r = −0.61, p = 0.067), (r = −0.68, p = 0.050) and (r = −0.9, p = 0.002) (Fig. 5a), respectively, although not statistically significant for the first two. Moreover, the titers of anti-gp140 binding antibodies and of 93MW965 neutralizing antibodies observed four weeks after the final vaccination, correlated with pre-vaccination

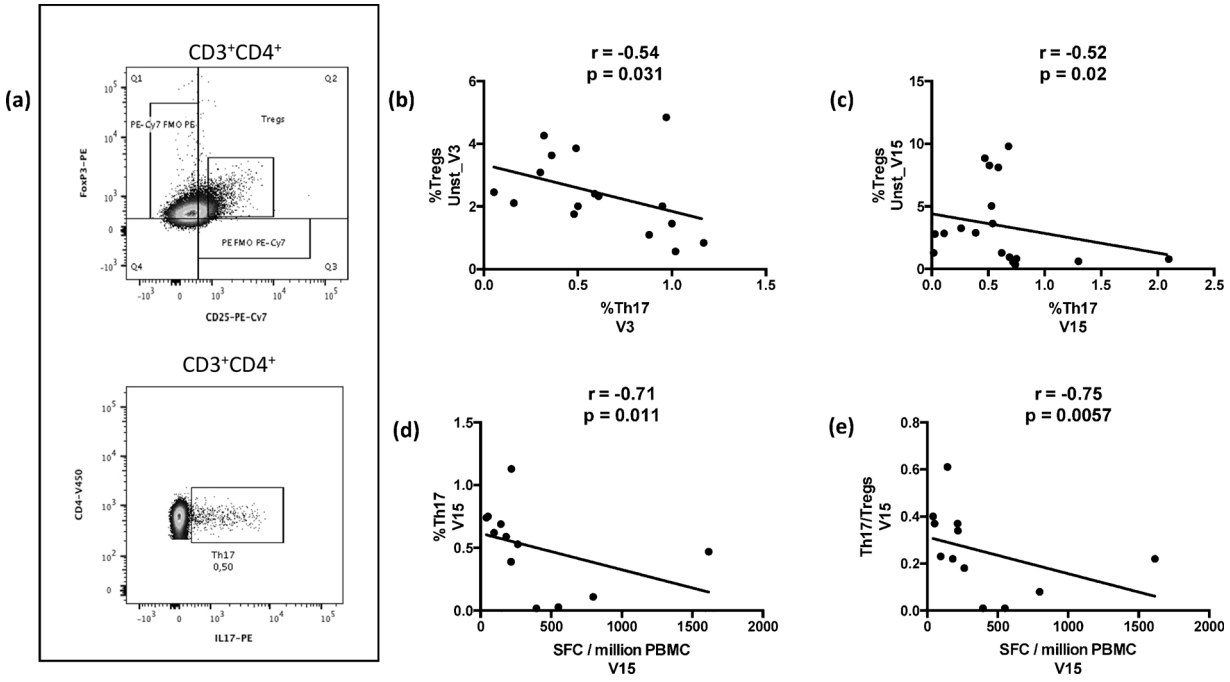

Fig. 3. Correlations between proportions of Th17 cells ($CD4^+IL17^+$) and Tregs ($CD4^+CD25^{High}FoxP3^+$), with the IFN-γ ELISpot response to Env-CMDR antigen. (A) Gates for definition of Tregs and Th17 cells by flow cytometry, in thawed peripheral blood mononuclear cells (PBMC) from vaccinated participants, as described in methods section. Correlation between the proportions of Tregs measured in unstimulated PBMCs and the proportions of CD4 T cells expressing IL-17 (Th17 cells), following stimulation with staphylococcus enterotoxin A and B (SEAB), at baseline (B) and at two weeks (C) after the second boost with MVA-HIV alone or in combination with CN54rgp140/GLA-AF (n = 18). Correlation between proportions of Th17 cells (n = 10) (D) and the Th17/Tregs ratio (n = 12) (E), with the frequency of T cells producing IFNγ, measured by ELISpot indicated as spot forming cells (SFC)/million PBMC at two weeks after the second boost with MVA-HIV alone or in combination with CN54rgp140/GLA-AF. Correlation analyses was performed using the Spearmen rank test. P-values < 0.05 were considered significant.

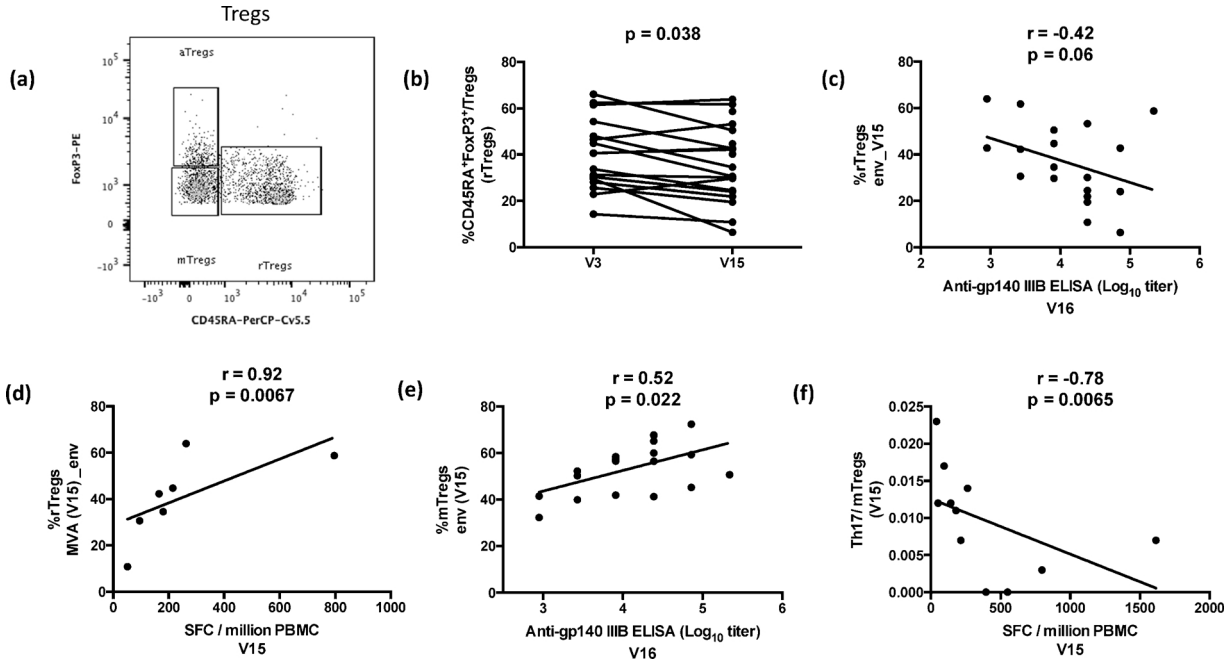

Fig. 4. Correlation between the activation status of Tregs ($CD4^+CD25^{High}FoxP3^+$) and the magnitude of vaccine induced T and B cell responses. (A) Gating strategy for definition of Tregs with resting ($FoxP3^+CD45RA^+$), activated ($FoxP3^{High}CD45RA^-$) and memory ($FoxP3^{Low}CD45RA^-$) phenotype within Tregs population, by flow cytometry, in thawed peripheral blood mononuclear cells (PBMC) from vaccinated participants, as described in methods section. (B) The proportions of rTregs ($FoxP3^+CD45RA^+$/Tregs) measured in unstimulated PBMC observed at baseline (V3) decreased at two weeks after the last immunization (V15) (n = 17). (C) Correlation between proportions of rTregs at V15 in participants boosted with MVA-HIV alone or in combination with CN54rgp140/GLA-AF with the gp140 IgG antibody titers measured in plasma samples collected at four weeks after the last immunization (V16) (n = 19). (D) Correlation between the proportion of rTregs at V15 in participants boosted with MVA-CMDR-HIV alone and their correlation with IFN-γ ELISpot response at same visit (n = 7). (E) Correlation between the proportions of mTregs ($FoxP3^{Low}CD45RA^-$/Tregs) at V15 in participants boosted with MVA-HIV alone or in combination with CN54rgp140/GLA-AF, and gp140 IgG antibody titers at V16 (n = 19). (F) Correlation between the ratio Th17/mTregs and the IFNγ ELISpot response at V15 (n = 11) in participants boosted with MVA-HIV alone or in combination with CN54rgp140/GLA-AF. The correlation was calculated using the Spearmen rank test and statistical significance was set at $p < 0.05$. The *Wilcoxon* matched-pair signed rank test was applied for paired comparisons and differences were considered significant if $p < 0.05$.

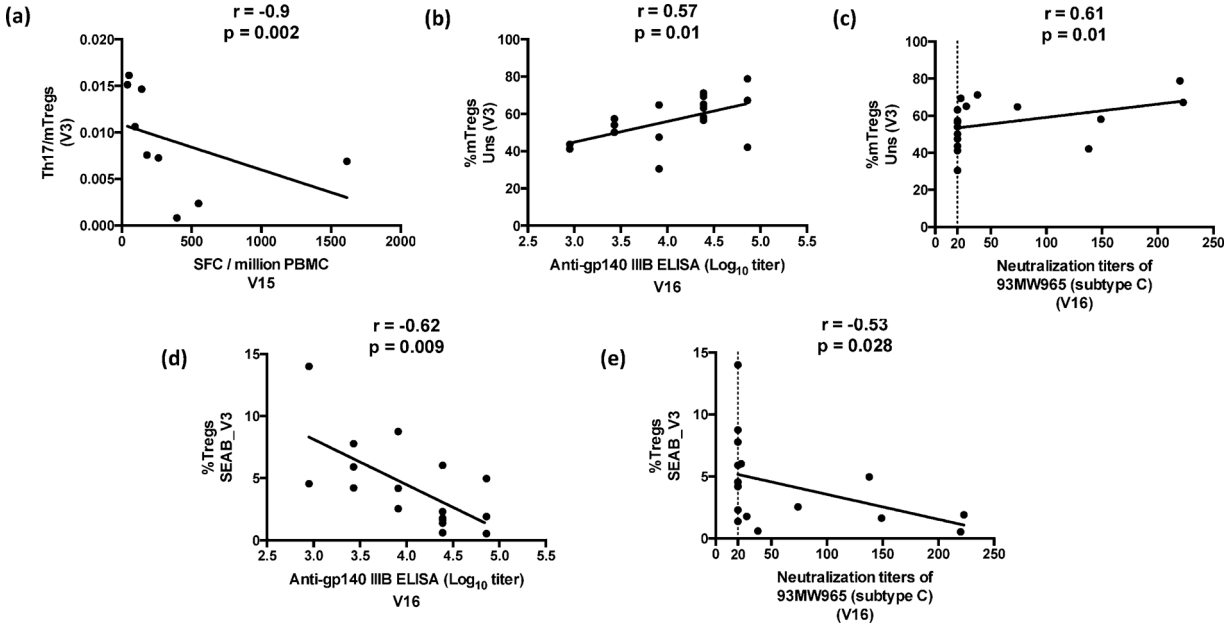

Fig. 5. Correlations between pre-vaccination levels of Tregs ($CD4^+CD25^{High}FoxP3^+$) and the magnitude of vaccine-induced T and B cell responses. (A) Correlation between pre-vaccination ratio of Th17/mTregs and IFNγ ELISpot response to Env-CMDR two weeks after the final boost with MVA-HIV alone or in combination with CN54rgp140/GLA-AF last immunization. Correlation between rTregs prior vaccination, in unstimulated PBMC, and (B) antibody titers of IgG gp140 and (C) titers of neutralizing antibodies against 93MW965 pseudotyped virus, four weeks after the final immunization (V16). Correlation between the proportion of Tregs in PBMC stimulated with SEAB and (D) antibody titers of IgG gp140 and (E) titers of neutralizing antibodies against 93MW965 pseudotyped virus four weeks after the final immunization.

proportions of mTregs when PBMC were not stimulated, and with total Tregs measured in PBMC stimulated with SEAB, ($r = 0.57$, $p = 0.01$ and $r = 0.61$, $p = 0.01$) and ($r = -0.62$, $p = 0.009$ and $r = -0.53$, $p = 0.028$), respectively (Fig. 5b–e).

3.4. Decreased expression of β7 integrin on Tregs correlates with lower IgG anti-gp140 antibodies titers and higher magnitude of IFN-γ ELISpot responses to Env-CMDR

Immunized individuals may have an increased risk of acquiring HIV infection due to increased expression of HIV ligands and increased systemic immune activation (Fauci et al., 2014).

When assessing the proportion of β7-expressing cells within the Tregs gate, as shown in Fig. 6a we found a decrease in $\beta7^+$Tregs two weeks after the final immunization ($p = 0.033$) (Fig. 6b). After stratification, according to boosting regimen, a statistically significant decrease in $\beta7^+$Tregs was only observed in vaccinees boosted with MVA alone ($p = 0.039$). The decrease of $\beta7^+$ Tregs proportions observed in the MVA group correlated with lower antibody titers against HIV-1 gp140 ($r = 0.63$, $p = 0.038$) whereas the correlation was not statistically significant ($r = 0.62$; $p = 0.085$) in participants boosted with MVA combined with CN54rgp140/GLA-AF. We also found an inverse correlation between $\beta7^+$ Tregs and the magnitude of IFN-γ ELISpot responses to Env-CMDR ($r = -0.85$; $p = 0.023$), in participants boosted with MVA combined with CN54rgp140/GLA-AF. However, the observed alterations in $\beta7^+$Tregs reverted to baseline levels three months after the final immunization.

3.5. Decreased expression of β7 integrin and CCR5 in total CD4 T cells with no alteration in T cell activation, two weeks after the final immunization

Analysis of the total population of CD4 T cells showed that at the peak of immune response (two weeks after the final immunization), the proportions of CD4 T cells expressing the β7 integrin *in vitro* decreased significantly in PBMC stimulated with Env-CMDR ($p = 0.001$) (Fig. 6a and c). The decreased expression levels of β7 integrin on CD4 T cells was also observed when participants were stratified according to the boosting regimen, MVA alone ($p = 0.01$) or MVA in combination with CN54 rgp140/ GLA-AF ($p = 0.04$). Nevertheless, the levels of β7 integrin expression on CD4 T cells returned to normal at three months after the final immunization. The decrease in $CD4^+\beta7^+$ T cells did not correlate with any of the measured HIV vaccine-induced immune responses, measured either as IFN-γ spot forming cells (ELISpot) or as titers of anti-gp140 antibodies.

The proportion of HIV co-receptor CCR5 expression was decreased in total CD4 T cells only in those participants boosted with MVA alone ($p = 0.01$), returning to baseline levels at three months after the final immunization. No correlation was observed between CCR5 levels and HIV vaccine-induced immune responses, measured either as IFN-γ ELISpot or titers of anti-gp140 antibodies.

T cell activation measured as the proportion of $CD4^+CD38^+$HLA-DR^+ and $CD8^+CD38^+$HLA-DR^+ T cells remained statistically unchanged after immunization, in both groups.

4. Discussion

Here, for the first time, we show that Tregs may modulate HIV vaccine-induced cellular and antibody responses. We observed significant changes in the activation status and migratory profile of Tregs after vaccination. These changes may have been induced by all vaccine components, or only by the final immunogen. Moreover, we found that both, the pre- and post-vaccination proportions and activation status of Tregs correlated with the magnitude of the vaccine-induced immune responses.

Beyond the contribution of Tregs to HIV vaccine-induced immune responses, Tregs, as other CD4 T cells, are also targets for HIV

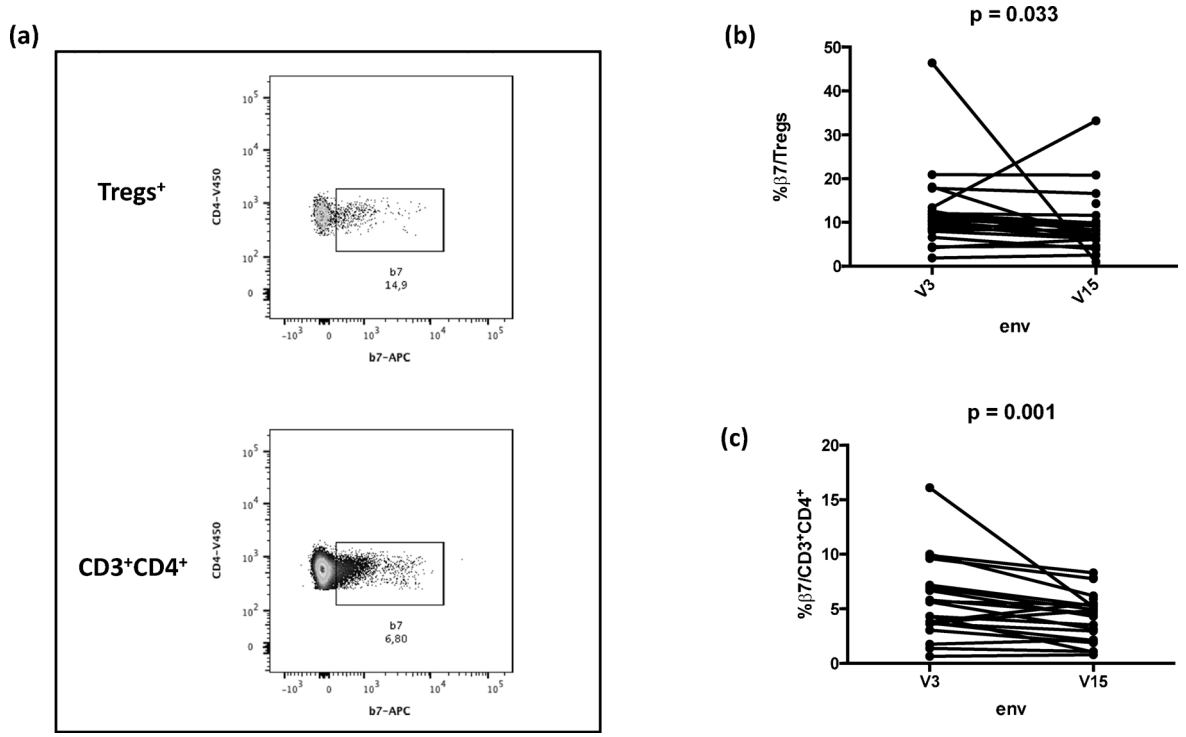

Fig. 6. Proportions of Tregs and total CD4 T cells expressing the β7 integrin was measured by flow cytometry in PBMC stimulated with Env-CMDR antigen at baseline (V3) and two weeks after the last immunization (V15). (A) Representative flow cytometry gates for definition of Tregs ($CD4^+CD25^{High}FoxP3^+$) and CD4 T cells ($CD3^+CD4^+$) populations expressing the $\beta7^+$ integrin. (B) The proportions of β7 integrin in Tregs ($\beta7^+/CD4^+CD25^{High}FoxP3^+$) decreased two weeks after the final vaccination, ($n = 19$). (C) The proportion of CD4 T cells expressing the β7 integrin also decreased two weeks after vaccination ($n = 16$). The *Wilcoxon* matched-pair signed rank test was applied for paired comparison and difference was considered significant with $p < 0.05$.

(Chachage et al., 2016). Adequate T cell priming and systemic dissemination of HIV-specific vaccine-induced CD4 T cells are important factors to confer protection against HIV infection (Perreau et al., 2011; Brenchley and Douek, 2008). However, certain vaccination regimens as those based on replication-defective adenovirus vectors, have been shown to induce increased susceptibility to HIV infection in those subjects who got infected during the vaccine trial (Moodie et al., 2015). The increased susceptibility to HIV was attributed to several factors such as adenovirus-induced immune activation and increased levels of β7 expression, an HIV-1 binding molecule (Arthos et al., 2008), on memory Ad5 specific CD4 T cells (Benlahrech et al., 2009). We found a temporary decrease in expression of β7 on CD4 T cells and on Tregs, at the peak of the immune response. The induction of β7 expression on T cells is mediated by interactions with retinoic acid (RA) producing dendritic cells (Zhu et al., 2013). Recently, it has been shown that a blockade of α4β7 (Byrareddy et al., 2016) or silencing of the catalytic enzyme responsible for synthesis of RA in dendritic cells (Zhu et al., 2013), induces a sustained control of viral load in SIV infected macaques, or decreases the HIV-1 susceptible $\alpha4\beta7^{High}$ CD4 + T cells in vaccinated mice. Thus, the transient and moderate decrease in β7 expression in vaccinees may contribute to prevent HIV infection of target cells at the gut. Further evaluations are required to understand this process and to identify potential molecules or components of the TaMoVac II DNA prime MVA + /- CN54gp140 vaccine regimen, that might influence regulation of β7 expression.

The role of Tregs during HIV infection has been extensively studied and dual outcomes have been observed. Tregs can be beneficial by limiting uncontrolled chronic immune activation, but also unfavorable or even harmful by preventing development of HIV-specific humoral and cellular immune responses (Kinter et al., 2007; Rueda et al., 2013; Karlsson et al., 2011). Little information is available concerning the role of Tregs in the outcome of anti-HIV vaccination. Because Tregs have the potential to suppress immune responses, we expected to find an inverse association between vaccine-induced responses and Tregs frequencies. Unexpectedly, we observed a direct correlation between increased proportions of Tregs and higher IFNγ ELISpot responses to HIV antigens after vaccination. This may be due to the fact that the generation and maturation of memory T cells occurs only in presence of a non-inflammatory environment, that can be favored by Tregs suppressive properties (Bhattacharyya and Penaloza-MacMaster, 2017; Espinoza Mora et al., 2014; Laidlaw et al., 2015; Pace et al., 2012). Moreover, during an immune response, Tregs suppress T cells with low avidity but not those with high avidity, which are important for induction of memory T cells (Pace et al., 2012). In an experimental model of malaria vaccine, depletion of Tregs during the priming or boosting of mice with the malaria vaccine, favored an augmented T effector cell response to liver-stage of malaria but failed to induce memory (Espinoza Mora et al., 2014). Similarly, in the mouse model of lymphocytic choriomeningitis virus, the absence of Tregs during the transition phase, from effector to memory CD8 T-cells, resulted in a permanent impairment of vaccine-induced CD8 T cell memory and function (Bhattacharyya and Penaloza-MacMaster, 2017). Thus, our results suggest that increased Tregs proportions contribute to the generation of vaccine-induced T cell memory in healthy human subjects immunized with a DNA prime-MVA + /-Env protein boost HIV vaccine candidate.

Tregs and Th17 compartments have reciprocal development pathways (Bettelli et al., 2006). Thus, we hypothesized that a change of the Th17/Tregs ratio would have an impact on the development of vaccine-induced memory T cells. At higher proportions of Th17 cells and higher ratios of Th17/Tregs after vaccination we observed lower production of vaccine induced IFN-γ. In addition, the pre-vaccination ratios of Th17/mTregs correlated significantly and inversely with vaccine-induced IFN-γ production. These results corroborate previous findings suggesting that the generation of vaccine-induced memory T cell response might be impaired at a higher pre-vaccination pro-inflammatory environment (Muyanja et al., 2014). Despite the important role of Th17 cells in protecting against microbial translocation during acute HIV infection (Brenchley and Douek, 2012), our results suggest that in HIV negative individuals, the abundance of Th17 cells, and more importantly their abundance in relation to Tregs cells, might predict a lower vaccine-induced T cell response. The balance in Th17/Tregs is regulated by multiple factors including commensal flora and dietary related factors (Omenetti and Pizarro, 2015). Certain commensal microbes such as segmented filamentous bacteria (SFB), a *Clostridia* related species, have been reported as inducers of Th17 cells while others such as *Bacteroides fragilis*, inducers of Tregs. In addition, fat-enriched diets have been implicated in a decrease of Th17 frequencies and in the ability of intestinal APC to generate Th17 cells *in vitro* (Omenetti and Pizarro, 2015).

Besides influencing T cell responses, Tregs can also modulate vaccine-induced B cell responses (de Wolf et al., 2017). Tregs proportions are increased after polyclonal stimulation of PBMC (Taylor and Llewelyn, 2010). In our study, we observed an inverse correlation between pre-and post- vaccination Tregs proportions with the titers of binding and neutralizing antibodies measured after vaccination. The suppressive potential of Tregs is associated with their activation status (Miyara et al., 2009; van der Veeken et al., 2016). Here, we observed a decrease of proportions of highly suppressive rTregs after final vaccination. Similarly, a decrease of rTregs proportions after vaccination was also found in individuals who received live attenuated yellow fever vaccine but not in those immunized with influenza and hepatitis B subunit vaccines (de Wolf et al., 2017), suggesting that the vaccine formulation may modulate Tregs activation status. Thus, live-attenuated or recombinant vaccines might induce alterations in the activation status of Tregs since these vaccines mimic natural infection. However, we cannot rule out the hypothesis of having rTregs migrating from the blood to other tissues. Furthermore, at higher proportions of suppressive rTregs after vaccination, lower titers of gp140 binding antibodies were observed, although not statistically significant. Conversely, at higher proportion of less suppressive mTregs, higher titers of gp140 binding antibodies were measured. Interestingly, participants with higher pre-vaccination proportions of mTregs exhibited higher titers of binding and neutralizing antibodies after vaccination. These observations suggest that inflammation-experienced mTregs are less effective in suppressing the vaccine-induced B cell response. Furthermore, their abundance prior to vaccination might predict a better antibody-mediated immune response.

Thus, the abundance of Tregs and their activation status may also influence the antibody production in HIV vaccine recipients. During early HIV infection, the abundance of Tregs was inversely correlated with the onset of antibodies reactive to different HIV-1 proteins (Matavele Chissumba et al., 2017). It has been reported that Tregs can prevent formation of follicular helper T cells by mechanisms involving interaction with CTLA-4/CD80 and CD86 (Wing et al., 2014).

Collectively, our results suggest that Tregs may impact HIV vaccine-induced T and B cell responses differently. Moreover, our results showed that the TaMoVac II prime boost vaccination regimen, HIVIS-DNA/MVA-CMDR-HIV/gp140-GLA-AF, beyond being safe and immunogenic, may induce a moderate decrease of β7 integrin expression on CD4 T cells, including Tregs, or temporary extravasation of $\beta7^{+}$CD4 T cells from the periphery to the gut.

The main limitations of this study are (1) analysis limited to Mozambican trial participants, (2) the lower detection rate of HIV-specific T cell responses by flow cytometry and (3) absence of functional assays to better unravel the role of Tregs. Finally, the results of this study enforce the importance of evaluating host factors prior to vaccination, such as the microbiome, that might affect Tregs abundance and activation status, which appears to have an impact on magnitude of HIV vaccine-induced immune responses.

Ethics approval and consent to participate

This study used pre-existing de-identified specimens from Mozambican participants of the TaMoVac II clinical trial (NCT01697007). The TaMoVac protocol was approved by all relevant institutions, including the Institutional Comité Instituicional de Bioética from Instituto Nacional de Saúde de Moçambique (INS), Ministry of Health, Mozambique the Comité Nacional de Bioética em Saúde de Moçambique (IRB00002657) and the Pharmaceutical Department, Ministry of Health, Mozambique. All volunteers provided written informed consent for participation in the trial. The study was also reviewed and approved by regulatory authorities in Belgium, Tanzania, Sweden, United Kingdom and United States of America.

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Authors' contributions

RMC, IJ and LK designed and conceived the study. RMC, NB, BW, LK, CG, CN, GS, LK and IJ coordinated the study. RMC, ADL, EN, AB, GS and CN performed the experiments. RMC, CN analyzed the data. RMC and LK drafted the manuscript.

All authors contributed substantially reviewing the manuscript and approved the final version of the manuscript.

Conflicting interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2018.08.006.

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