Journal of
Clinical & Cellular Immunology

Magalhaes et al., J Clin Cell Immunol 2012, S4
DOI: 10.4172/2155-9899.S4-006

Commentary Open Access

Mycobacterium tuberculosis Vaccination Imprints on T-Cell Dynamics Associated with *Mycobacterium tuberculosis* Challenge in Rhesus Monkeys

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Abstract

Non-human primate models aid to design novel vaccine strategies. We analyzed therefore the dynamics of cellular immune responses induced by a bacille Calmette-Guérin (BCG) / recombinant BCG (rBCG, perfringolysin plus Ag85A/B, TB10.4) prime, subsequent adenoviral (Ad35 expressing Ag85A/B and TB10.4) boosts followed by a challenge with *M. tuberculosis* (*Mtb,* Erdman strain) in peripheral blood mononuclear cells from rhesus macaques. T-cell compartment mobilization was defined by CD45RA/CCR7/CD27/CD28 expression on $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T-cells and functional analysis (STAT-5 phosphorylation) was carried out using multicolor flow cytometry. $CD4^+$ as well as $CD8^+$ T-cells showed a marked decrease in IL-7 receptor alpha-chain (IL-7Rα) expression in vaccinated animals (n=12/12) after the first adenoviral boost, but not in control animals (who received saline). BCG- and rBCG- vaccinated animals showed a dramatic decrease of precursor ($CD45RA^+CCR7^+$) and subsequent increase of activated central memory ($CD45RA^-CCR7^+$) T-cells in the $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T-cell compartments 2 weeks after *Mtb* challenge. In contrast, this pattern was observed in control animals only 7 weeks after *Mtb* challenge. Expression of IL-7Rα was markedly reduced in $CD4^+$ T-cell subsets two weeks after *Mtb* challenge in BCG-vaccinated animals, yet not in rBCG-vaccinated animals or control animals. These data show that vaccination profoundly shapes the dynamics of immune memory T-cells associated with *Mtb* infection.

Keywords: Vaccination; Tuberculosis; T-cells; Rhesus monkeys; T-cell differentiation

Background

New tuberculosis (TB) vaccines are needed to improve protection against *M. tuberculosis* (*Mtb*) infection in children and adults. Several approaches are now being developed and some TB vaccine candidates are in Phase I or II clinical trials [1,2]. The concomitant design of 'markers of vaccine take' will aid to escort novel TB vaccines and to monitor patients with TB [3]. A vast body of literature exists concerning T-cell responses induced by bacille Calmette-Guérin (BCG) vaccination in infants [4,5], in adults [6] or in patients with active TB [7,8]. In contrast to the analysis of antigen-specific T-cell responses, the global dynamics of T-cell homing and differentiation after TB vaccination followed by *Mtb* infection have not been studied in detail. Expression analysis of cell surface receptors CD27 and CD28 allows the identification of effector and memory human T-cell subsets [9-12]. These markers can also be tested in rhesus macaques, and they are susceptible to *Mtb* infection [13]. T-cells from non-human primates express, like humans, the cell surface molecules CD45RA, CCR7, CD28 and CD27 and allow the analysis of T-cell homing and differentiation [14-16]. Recent data concerning T-cell subsets sparked new interest in immunological marker analysis, e.g. the immune-modulatory effects of rapamycin are not only due to reduction of IL-2 production, yet to the removal of terminally differentiated T-cells and accumulation of T-cells in the memory T-cell pool [17], the lack of terminally differentiated ($CD45RA^+CCR7^-$) T-cells contributes to the lack of immune-competence [18]. Another piece of evidence that marker profiling is important comes from tumor immunology: antigen experienced, long-lived T-cells with stem-cell like features ($CD95^+$, $c\text{-}kit^+$) reside in the precursor ($CD45RA^+CCR7^+$) T-cell pool [19]; *Mtb* antigen experienced T-cells have been described in a murine model to be able to revert from an memory phenotype to a precursor T-cell phenotype [20].

Thus, the general composition of T-cell subsets impacts on immune-competence. The characterization of T-cell subsets is therefore helpful in order to i) better understand T-cell dynamics as a result of *Mtb* infection and ii) to answer the question whether BCG vaccination impacts on the overall composition of the T-lymphocyte pool in the peripheral circulation.

We evaluated therefore the T-cell subpopulations in peripheral

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Received November 24, 2011; **Accepted** February 21, 2012 **Published** February 24, 2012

Citation: Magalhaes I, Ahmed RK, Vudattu N, Sizemore DR, Weichold F, et al. (2012) *Mycobacterium tuberculosis* Vaccination Imprints on T-Cell Dynamics Associated with *Mycobacterium tuberculosis* Challenge in Rhesus Monkeys. J Clin Cell Immunol S4:006. doi:10.4172/2155-9899.S4-006

blood mononuclear cells (PBMCs) from rhesus macaques following TB vaccination and subsequent challenge with *Mtb* by assessing i) the phenotype of the peripheral T-cells, ii) the presence of regulatory T-cells and iii) the ability of T-cells to respond to IL-7, a key event in T-cell homeostasis and memory formation defined by STAT-5 phosphorylation [21].

Material and Methods

Blood samples

Peripheral blood samples were obtained from female rhesus macaques (*Macaca mulatta*) of Chinese origin with an age range between 3 and 4 years. Animals were captive bred for research purposes and housed in the Astrid Fagraeus laboratory at the Swedish Institute for Infectious Disease Control and at the contained experimental facilities at the Biomedical Primate Research Centre. Animals were tested free of secreting recombinant AERAS-402 prior to infectious challenge. The local Ethical Committee approved all procedures (protocol DNR238/2006-54 and DEC#551, respectively). Peripheral blood mononuclear cells were isolated from freshly obtained, heparinized peripheral blood by Ficoll-Hypaque density gradient centrifugation.

Vaccines and Mycobacterium tuberculosis infection

The vaccine regimen has been described previously [22] (see Supplementary Table S1). Briefly, *Mycobacterium bovis* BCG (SSI1331) was delivered intradermally (i.d.) to 6 animals, and a recombinant (rBCG, AFRO-1) expressing perfringolysin, Ag85A, Ag85B and TB10.4 was delivered i.d. to 6 animals followed by two boosts (delivered intramuscularly) with non-replicating adenovirus 35 (rAd35) AERAS-402, expressing a fusion protein composed of TB antigens Ag85A, Ag85B and TB10.4. Control animals received saline (3 animals). 500 cfu of the *Mtb* Erdman strain were delivered, after sedation, by intratracheal instillation in all animals (vaccinated and control) 39 weeks post-vaccination. Three additional naïve (non-vaccinated) animals were added to the control group prior to *Mtb* challenge (n=6 in all groups at *Mtb* challenge). The study fixed endpoint challenge was 23/24 weeks after challenge, animals that reached a humane endpoint before the fixed study endpoint were euthanised. This was the case for 4 animals in the BCG-vaccinated group, 1 in the rBCG-vaccinated group, and 3 in the control group. Therefore 15 weeks after *Mtb* challenge, 2 animals in the BCG-vaccinated group, 6 animals in the rBCG-vaccinated group, and 3 animals in the control group were available for analysis; 20 weeks after *Mtb* challenge, 2 animals in the BCG15 vaccinated group, 5 animals in the rBCG-vaccinated group, and 3 animals in the control group were available for analysis.

Multicolor flow cytometric analysis

Freshly isolated PBMCs (1 x 10^6) were incubated at 4°C for 15 min with the following antibodies: PE-Cy7 conjugated anti-CCR7 (3D12), PerCP conjugated anti-CD3 (SP-34-2), PE conjugated anti-CD16 (3G8), PE conjugated anti-CD56 (B159), APC-Cy7 conjugated anti-CD8α chain (SK1), Amcyan anti-CD28 (CD28.2) purchased from BD Biosciences (Stockholm, Sweden), APC-Alexa Fluor 700 conjugated anti-CD107a (H4A3), APC conjugated anti-IL-7Rα (CD127) (R34.34), PE-Texas Red conjugated anti-CD45RA (2H4), FITC conjugated anti-CD8β chain (2ST8.5H7) purchased from Beckman Coulter (Marseille, France), and Pacific Blue conjugated anti-CD4 (S3.5) purchased from Caltag Laboratories (Burlingame, CA, USA). Cells were washed in PBS-0.1% FBS, and incubated at 4°C for 15 min with the anti-CD27 (1A4CD27) antibody (Beckman Coulter) labeled with Pacific Orange using the Zenon Pacific Orange Mouse IgG_1 Labeling Kit obtained from Invitrogen (Stockholm, Sweden). Fluorescence intensity was measured using a FACSAria flow cytometer (BD Biosciences) and results were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). Note that anti-CD16 and anti-CD56 antibodies were PE conjugated. Therefore $CD3^-(CD56CD16)^+$ events represent $CD16^+$ and/or $CD56^+$ events.

Characterization of regulatory T-cells

Frozen PBMCs were thawed and incubated at 4°C for 15 min with the following antibodies: PerCP conjugated anti-CD3 (SP34-2), PerCP Cy5.5 conjugated anti-CD4 (L200), PE-Cy7 conjugated anti-CD25 (2A3), APC-Cy7 conjugated anti-CD8α chain (SK1) purchased from BD Biosciences, PE conjugated anti-TSLP-R (1D3) obtained from BioLegend (San Diego, CA, USA), and APC conjugated anti-IL-7Rα (R34.34) purchased from Beckman Coulter. PBMCs were then washed with Stain Buffer (BD Biosciences) and fixed with 1XBioLegend's FOXP3 Fix/Perm solution (BioLegend) at room temperature for 20 min followed by one washing with Stain Buffer and one washing with 1XBioLegend's FOXP3 Perm buffer. PBMCs were resuspended in 1XBioLegend's FOXP3 Perm buffer and incubated at room temperature for 15 min, centrifuged, and resuspended in 1XBioLegend's FOXP3 Perm buffer and incubated at room temperature for 30 min with the Alexa-Fluor 488 conjugated anti-FoxP3 (259D) antibody purchased from Biolegend. Cells are then washed and analysed using a FACSAria flow cytometer (BD Biosciences) and data were analyzed by using FlowJo software (Tree Star Inc.).

IL-7-induced STAT-5 phosphorylation assay

Constitutive and IL-7 induced phosphorylated STAT-5 expression was evaluated in frozen PBMCs as described previously [23]. Briefly, overnight starved thawed PBMCs were incubated with recombinant human IL-7 (rh IL-7-100ng for 10^5 cells, provided by Dr. Michel Morre, Cytheris, Issy-les-Moulineaux, France) for 15 min at 37°C. Lymphocytes were then incubated for 15 min at 4°C with APC conjugated anti-CD4 (SK3), and APC-Cy7 conjugated anti-CD8α chain (SK1) purchased from BD Biosciences and immediately after fixed with 2% paraformaldehyde. The cells were washed with Stain Buffer (BD Biosciences) and permeabilized with 90% methanol for 30 min on ice, followed by two washes with Stain Buffer. Cells were incubated with Alexa-Fluor 488 conjugated anti-phosphorylated STAT-5a antibody (Y694) (BD Biosciences) for 1 hr at room temperature and analysed immediately by flow cytometry using a FACSAria flow cytometer (BD Biosciences) data analysis was performed using FlowJo software (Tree Star Inc.). Human IL-7 shows similar activity as compared to non-human primate (NHP) IL-7 (personal communication, Dr. Michel Morre, Cytheris, Issy-les-Moulineaux, France).

IL-7 and IL-7Rα protein determination

Plasma IL-7 was determined using the commercially available human IL-7 ELISA Kit with a sensitivity of 6.25pg/ml (Diaclone, Stamford, USA). Soluble IL-7Rα was determined using a sandwich ELISA (with a sensitivity of 0.2ng/ml) using a monoclonal capture

antibody anti-IL-7Rα (R34.34, Beckman Coulter) and a biotinylated polyclonal anti-human IL-7Rα antibody obtained from R&D Systems (McKinley, USA). A recombinant human IL-7Rα/Fc chimera protein, obtained from R&D Systems, served as a standard. Streptavidin-horseradish peroxidase (BD) and tetramethylbenzidine substrate (Invitrogen) were used to detect biotinylated bound anti-human IL-7Rα antibody – IL-7Rα complexes; absorbance values were read at 450nm using a Multiskan Ascent ELISA reader (AB Ninolab, Upplands Väsby, Sweden).

Statistical analysis

Mann-Whitney test for unpaired data, and Wilcoxon matched pairs test were performed using GraphPad Software. The paired test was used when comparing the same group of animals over-time and the unpaired test was used when comparing 2 different groups. p values <0.05 were considered significant. In the case of group size n<5 statistical tests were not performed.

Results

Loss of IL-7Rα on T-cells after the first adenoviral boost

We assessed the composition and phenotype of the peripheral T-cell compartment in response to a TB vaccine regimen. We did not observe differences in the frequency of $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T-cells between BCG- and rBCG-vaccinated animals and the control animals (who only received saline) after the prime, the two adenoviral boosts (Figure 1A and supplementary Figure S1).

Expression of the T-cell differentiation and homing markers CD45RA and CCR7, which characterize precursor ($CD45RA^+CCR7^+$) and effector/memory ($CD45RA^{+/-}CCR7^{+/-}$) $CD4^+$ T-cells is shown in Figure 1B for vaccinated and control animals. $CD45RA^+CCR7^+$ T-cells represented the predominant T-cell subset in the $CD4^+$ T-cell compartment (Figure 1B). In BCG-vaccinated animals, $CD45RA^+CCR7^+$ T-cells represented in average (during the course of the vaccination follow-up) 56.26% (ranging from average/time point of

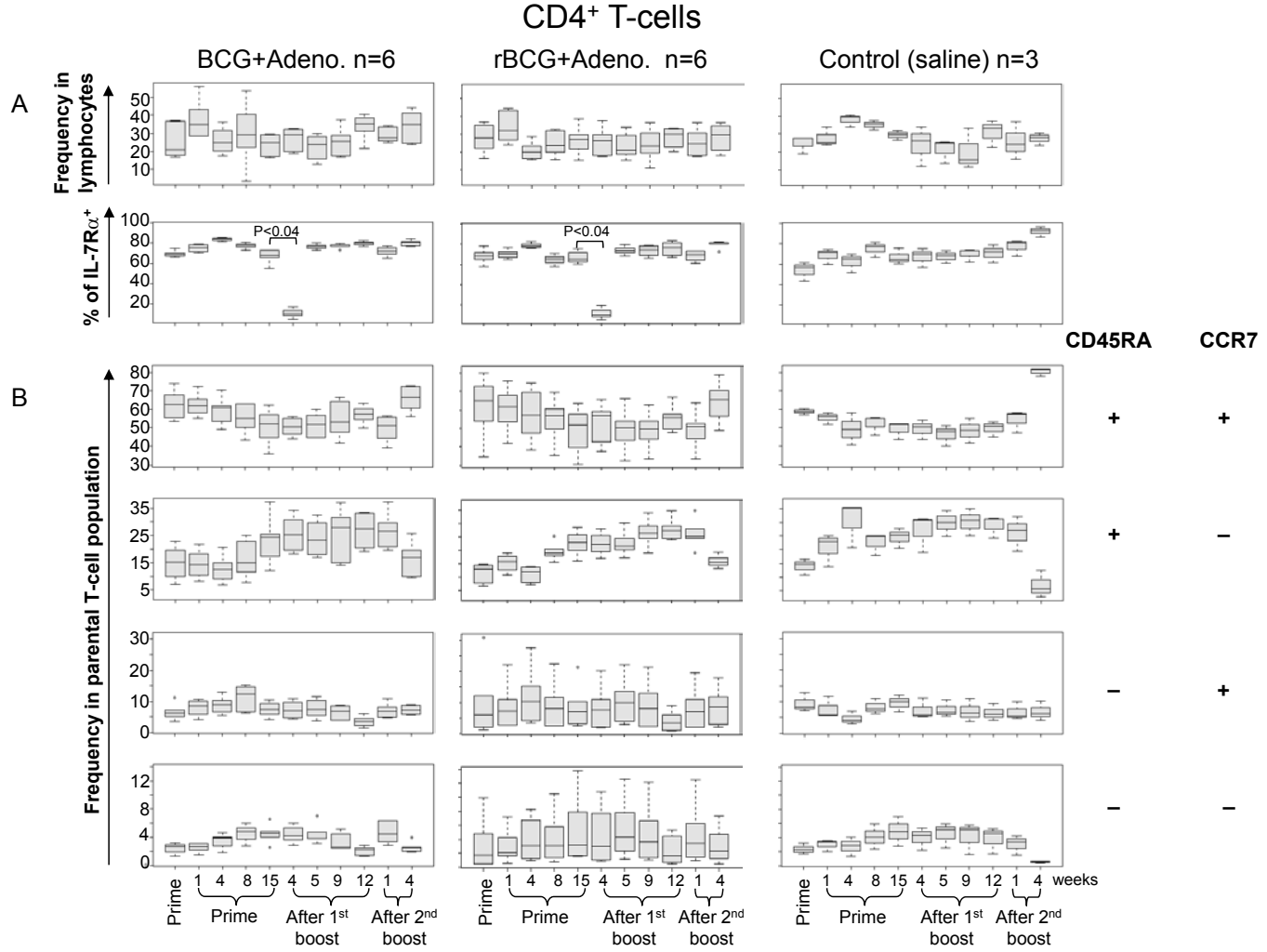

Figure 1: $CD4^+$ T-cell compartment after TB vaccination in BCG-vaccinated and control animals. A. Frequency of $CD4^+$ T-cells in PBMCs, percentage of cell surface expression of IL-7Rα after BCG or rBCG vaccination followed by adenoviral boots. Control animals did not receive any vaccination. **B.** Frequency of CD45RA/CCR7 compartments (from top to bottom: $CD45RA^+CCR7^-$, $CD45RA^+CCR7^-$, $CD45RA^-CCR7^+$, $CD45RA^-CCR7^-$) in $CD4^+$ T-cells after BCG vaccination and in control animals. Except the significant decrease of IL-7Rα cell surface expression 4 weeks after the first adenoviral boost in all vaccinated animals ($p<0.04$), BCG-vaccinated and control animals display similar $CD4^+$ T-cell compartments both in terms of frequency and T-cell phenotype at different time points after vaccination.

49.64% to 65.71%) of CD4⁺ T-cells, 59.01% (ranging from average/time point of 49.35% to 75.04%) of CD8αβ⁺ T-cells. In contrast terminally differentiated CD45RA⁺CCR7⁻ cells were more predominant in the CD8αα⁺ T-cell compartment (average 54.1%, ranging from 40.24% to 62.14% average/time see supplementary Figure S1). The T-cell phenotype, defined by CD45RA/CCR7 and CD27/CD28 expression was stable over time and no differences were observed between the vaccinated (with BCG or rBCG) and control animals (data not shown).

We observed a strong drop in IL7-Rα expression on CD4⁺ and CD8⁺ T-cells 4 weeks after the first adenoviral boost in BCG- and rBCG-vaccinated animals, but not in control animals, i.e. in BCG-vaccinated animals, 67.2% of the CD4⁺ T cells were IL-7Rα⁺ prior to adenoviral injection versus 10.82% 4 weeks after the 1st adenoviral injection ($p<0.04$) (Figure 1A, and supplementary Figure S1 for CD8αα⁺ and CD8αβ⁺ T cells). The loss of IL-7Rα could be detected in all CD4⁺ and CD8⁺ T-cell subsets in all vaccinated animals, defined by CD45RA/CCR7, a more detailed analysis of IL-7Rα expression is provided in the supplementary Figure S2 for CD4⁺ T-cells for BCG- vaccinated and control animals. IL-7Rα cell surface expression levels were restored 5 weeks after the first adenoviral boost (Figure 1 and supplementary Figures S1-2). Plasma IL-7 and soluble IL-7Rα levels were assessed by ELISA, yet we could not detect differences between vaccinated/control animals; the loss of IL-7Rα on peripheral T-cells was not associated

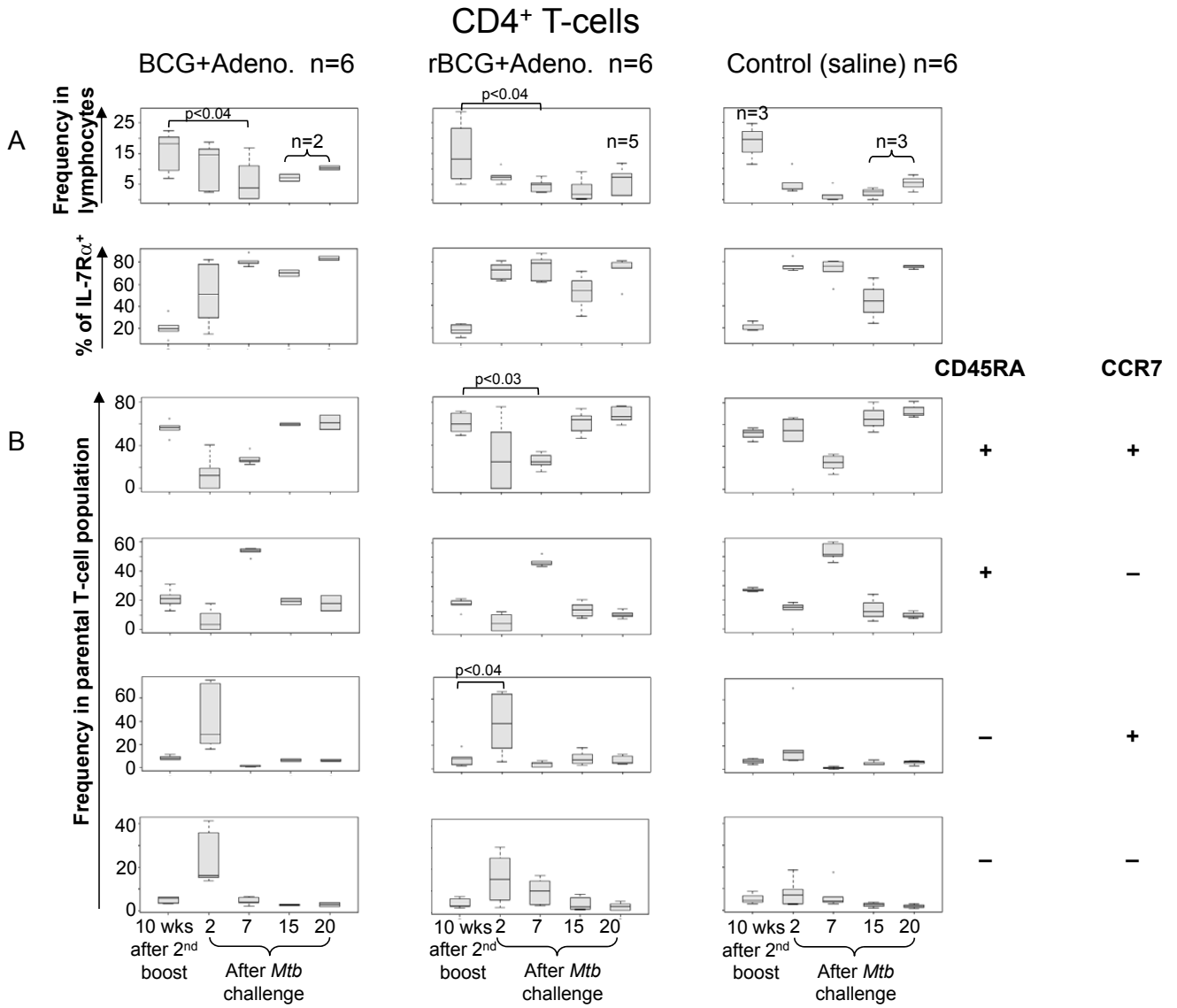

Figure 2: CD4⁺ T-cells dynamics in response to *Mtb* challenge. A. Frequency of CD4⁺ T-cells in lymphocytes and percentage of IL-7Rα⁺ CD4⁺ T-cells after BCG vaccination (and control animals). **B.** Frequency of CD45RA/CCR7 compartments (as described in Figure 1) in CD4⁺ T-cells after BCG or rBCG vaccination followed by adenoviral boots (and control animals). Vaccinated animals showed a trend of decreased frequency of CD45RA⁺CCR7⁺ T-cells ($p<0.04$) and increased frequency of CD45RA⁻CCR7⁺/⁻ T-cells ($p<0.04$ in the rBCG vaccinated group) 2 weeks after *Mtb* challenge. This was absent in control animals. CD45RA⁺CCR7⁺ T-cells constituted the most frequent subset in all NHP groups 20 weeks after *Mtb* challenge. Note, that from 15 weeks after *Mtb* challenge, 2 animals in the BCG-vaccinated group, 3 animals in the control group, and from 20 weeks after *Mtb* challenge, 2 animals in the BCG-vaccinated group were available for analysis since NHPs had to be sacrificed due to TB-associated symptoms.

with increased soluble IL-7Rα levels or alterations in IL-7Rα RNA levels in PBMCs (data not shown). A different alteration in PBMCs after the first adenoviral boost was a transient increase of $(CD56CD16)^{+}$/ $dimCD3^{+/-}CD4^{-}CD8^{-}$ cells in BCG- and in rBCG-vaccinated animals but not in control animals (supplementary Figure S3).

Differential dynamics of the T-cell compartment in vaccinated animals after *Mtb* challenge

We analyzed the phenotype of the T-cell compartment after *Mtb* challenge and observed a decrease of $CD4^{+}$ T-cells frequency (Figure 2A), $CD4^{+}$ T-cells represented 15.9% of the $CD3^{+}$ T-cell population before *Mtb* challenge and 6% by 7 weeks after *Mtb* challenge ($p<0.04$ in vaccinated animals). This was found to be true for $CD8\alpha\alpha^{+}$ and $CD8\alpha\beta^{+}$ T-cells (data not shown), concomitant with a relative increase of $CD3^{low}$ T-cells. T-cells that expres low amounts of CD3 ($CD3^{low}$ T -cells) represented prior to *Mtb* infection less than 20% of the lymphocytes, they increased up to 40% after *Mtb* challenge ($p<0.04$ when comparing time point of prime vaccination and 7 weeks after *Mtb* challenge in vaccinated groups, Figure 3B). We also observed a decrease in the frequency of (precursor) $CD45RA^{+}CCR7^{+}CD4^{+}$ T-cells in vaccinated animals (from 55.8% to 14.5% in BCG-vaccinated animals 2 weeks before and after *Mtb* challenge, respectively) (Figure 2B, with a similar trend for $CD45RA^{+}CCR7^{+}$ $CD8\alpha\beta^{+}$ T-cells, data not shown), but not in control animals. *Mtb* infection resulted in a shift of T-cell subpopulations in the (BCG or rBCG) vaccinated animals, yet not in the control animals. Precursor $CD45RA^{+}CCR7^{+}CD28^{+/-}CD27^{+}$ T-cells were decreased 2 weeks after *Mtb* challenge in vaccinated, yet not in control animals (i.e. the frequency of $CD45RA^{+}CCR7^{+}CD28^{+}CD27^{+}CD4^{+}$ T-cells was reduced from an average of 47% before *Mtb* challenge to 17% after *Mtb* challenge in rBCG-vaccinated animals, $p<0.04$, supplementary Figure S4). The drop of precursor T-cells was accompanied by an increase of central memory and effector memory T-cells, defined by $CD45RA^{-}CCR7^{+/-}$ cell surface expression (Figure 2B) in vaccinated animals. A similar trend of decrease of precursor $CD45RA^{+}CCR7^{+}$ $CD4^{+}$ T-cells was also observed in (non-vaccinated) control animals, but only later, i.e. 7 weeks after infection with *Mtb* and not 2 weeks as

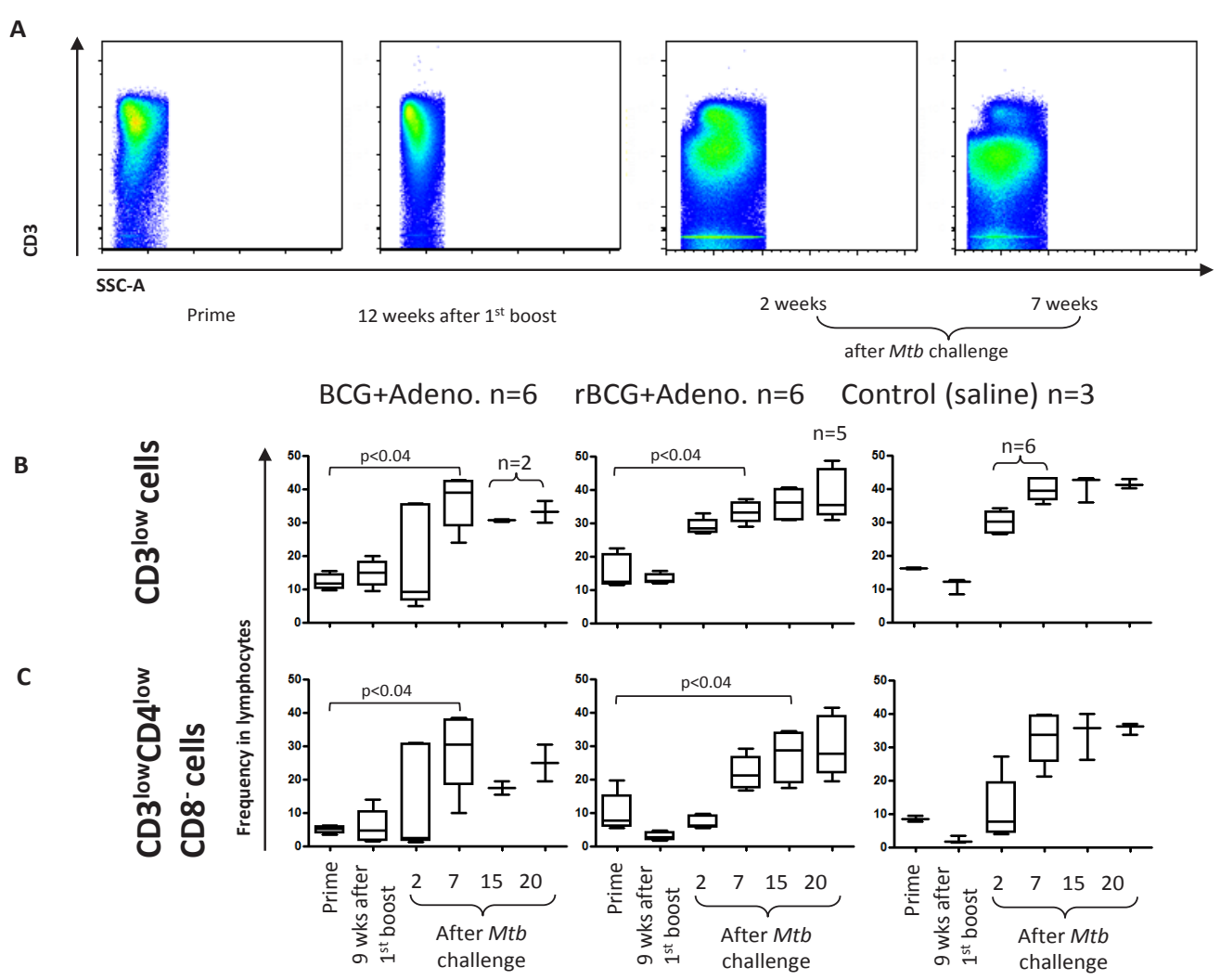

Figure 3: Decreased CD3 expression after *Mtb* challenge. A. Fluorescence intensity of CD3 staining at different time points during TB vaccination and after *Mtb* challenge from a representative animal is demonstrated. Note the increased frequency of $CD3^{low}$ cells after *Mtb* challenge. **B.** The frequency of $CD3^{low}$ cells was increased after *Mtb* challenge in all animals ($p<0.04$ in vaccinated 6 animals). **C.** $CD3^{low}CD4^{low}CD8^{-}$ constituted after *Mtb* challenge the most frequent cell population.

compared to vaccinated animals (Figure 2B), with a parallel increase of CD45RA$^+$CCR7$^-$ cells. In all animals, irrespective of the vaccination status, 7 weeks after *Mtb* challenge CD45RA$^+$CCR7$^-$ cells were the most frequent subset (approximately 50%) in CD4$^+$ T-cells.

We then observed a similar change of T-cell populations in infected animals, irrespective if they received a (r)BCG vaccine or not, during the time frame 7 to 20 weeks after *Mtb* challenge: the frequency of CD45RA$^+$CCR7$^+$ CD4$^+$ T-cells was increased, whereas CD45RA$^+$CCR7$^-$ CD4$^+$ T-cell frequency decreased (from a mean frequency of 47% 7 weeks after *Mtb* challenge to 11% in rBCG-vaccinated animals 20 weeks after *Mtb* challenge, Figure 2B).

We assessed the presence of T-cells expressing CD25 and FoxP3 in response to TB vaccination and after *Mtb* challenge. Similar to humans, CD4$^+$CD25$^+$FoxP3$^+$ regulatory T-cells can be detected in rhesus macaques [24]. A similar situation is true for CD8$^+$ T-cells expressing CD25 and FoxP3 with regulatory properties in humans [25,26] and in cynomolgus macaques [27]. No differences in the frequency of CD4$^+$, CD4$^+$CD8$^+$ or CD8$^+$ T-cells expressing simultaneously CD25^{high} and FoxP3 was observed between vaccinated animals after vaccination and control animals (data not shown) either during vaccination or after *Mtb* challenge.

Differences in IL-7Rα expression after *Mtb* infection in CD4$^+$ T-cells in BCG-vaccinated animals

IL-7Rα expression within the different CD4$^+$ T-cell CD45RA/CCR7 compartments after *Mtb* challenge is shown in Figure 4. In PBMCs from 4/6 BCG-vaccinated animals we observed a decreased expression (not statistically significant) of IL-7Rα expression on CD4$^+$ T-cells 2 weeks after *Mtb* infection: only 20% or less of CD45RA$^-$CCR7$^{+/-}$ CD4$^+$ T-cells expressed the IL-7Rα as compared to >70% in rBCG-vaccinated or control animals. This trend of decreased IL-7Rα expression was particularly accentuated in the CD45RA$^+$CCR7$^-$ CD4$^+$ T-cell compartment (p=0.05) in BCG- vaccinated animals as compared to rBCG-vaccinated animals. IL-7Rα down-regulation was not found in the CD8αα$^+$ or CD8αβ$^+$ T-cell subsets of BCG-vaccinated NHPs (data not shown). Analysis of PBMCs, drawn at 7 and 20 weeks after *Mtb* challenge, revealed that CD4$^+$ T-cells from all animals displayed again similar high levels of IL-7Rα expression (~80%).

Appearance of CD3$^{low/-}$CD4$^+$CD8$^-$ cells after *Mtb* infection

Lastly, we observed 2 weeks after *Mtb* challenge that the frequency of CD3$^{low/-}$ CD4$^+$CD8$^-$ lymphocytes was transiently increased (20-40% of lymphocytes) in all animals. CD3$^{low/-}$CD4$^+$CD8$^-$ cells represented in average 6.4% of lymphocytes 12 weeks after the 1st boost for all animals, and reached in average 32.15% 2 weeks after *Mtb* infection

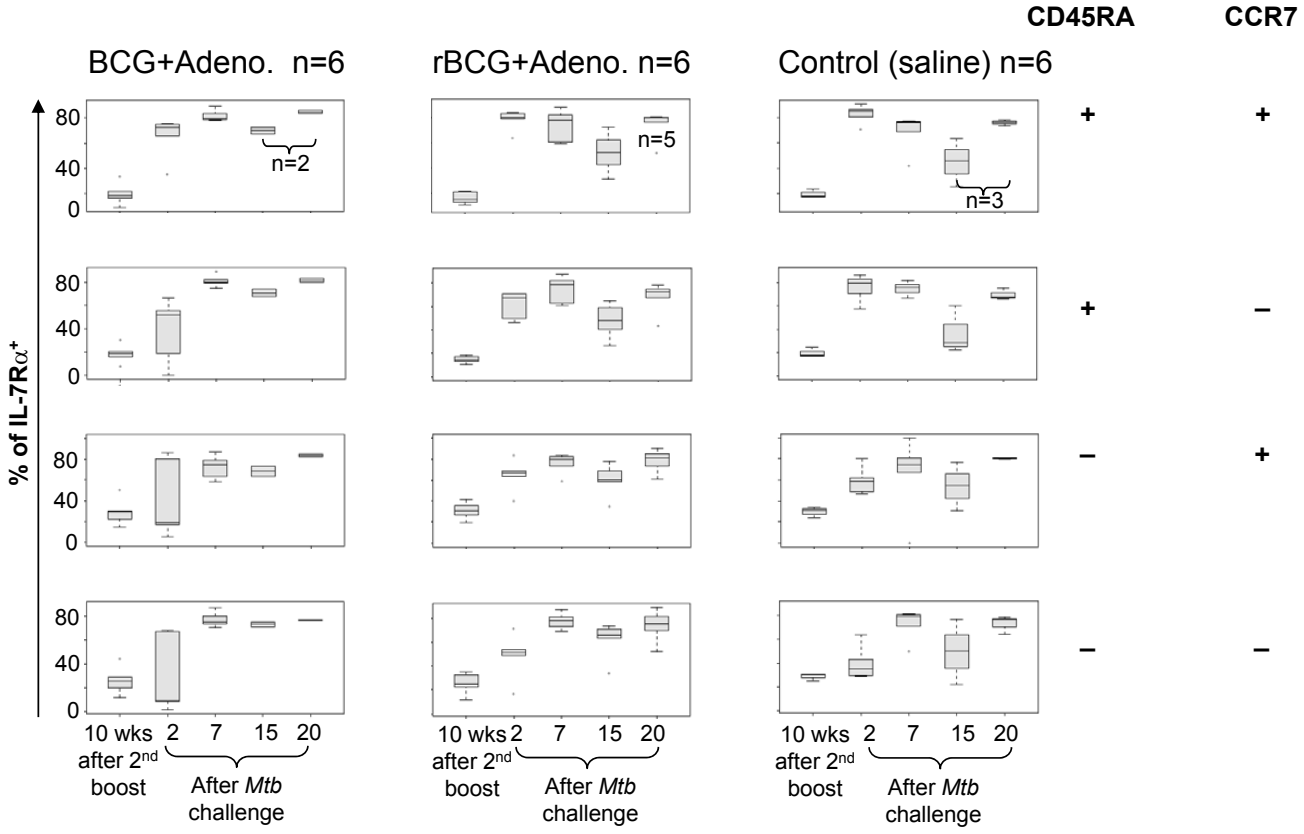

Figure 4: Expression of IL-7Rα in CD4$^+$ T-cells after *Mtb* challenge. The frequency of IL-7Rα cell surface expression within the different CD45RA/CCR7 CD4$^+$ T-cell subsets from vaccinated and control animals (all infected with *Mtb*) is displayed as box plots. 4/6 BCG-vaccinated show a decrease of IL-7Rα$^+$ CD4$^+$ T-cell 2 weeks after *Mtb* challenge, and 2/3 non-vaccinated animals showed a decreased expression of IL-7Rα 15 weeks after *Mtb* challenge.

($p<0.001$). $CD3^{low/-}CD4^{+}CD8^{-}$ cells displayed a $CD45RA^{-}CCR7^{+/-}$ phenotype and co-expressed CD28 and CD27. 20% of these cells stained positive for IL-7Rα. From 7 to 20 weeks after the *Mtb* challenge, the $CD3^{low}CD4^{low}CD8^{-}$ cells constituted the predominant lymphocyte population in the peripheral circulation of all groups of animals exposed to *Mtb* challenge (e.g. 21% of lymphocytes 15 weeks after *Mtb* challenge, in rBCG-vaccinated animals, Figure 3C). $CD3^{low}CD4^{low}CD8^{-}$ cells exhibited a $CD45RA^{-}CCR7^{-}$ phenotype, they did not express CD28 and/or CD27 nor the IL-7Rα (data not shown).

Mtb infection does not impact on STAT-5 phosphorylation

The binding of IL-7, a key cytokine mediating T-cell memory, to its receptor results in phosphorylation of the transcription factor STAT-5 [28]. Defects in STAT-5 T-cell phosphorylation has been associated with impaired T-cell function in combination with decreased cytokine production [23]. Elevated levels of phosphorylated STAT-5 were found in $CD4^{+}$ and $CD8^{+}$ T-cells from patients infected with human immunodeficiency virus (HIV) [29] associated with T-cell activation. We examined therefore in $CD4^{+}$ and $CD8^{+}$ cells the constitutive and IL-7-induced phosphorylated STAT-5 levels in vaccinated and control animals (Figure 5). The constitutive levels of phosphorylated STAT-5 were the same at different time points measured after vaccination and after *Mtb* challenge. IL-7-induced STAT-5 phosphorylation (up to 95% in $CD4^{+}$ cells and up to 80% in $CD8^{+}$ cells) was identical at all time

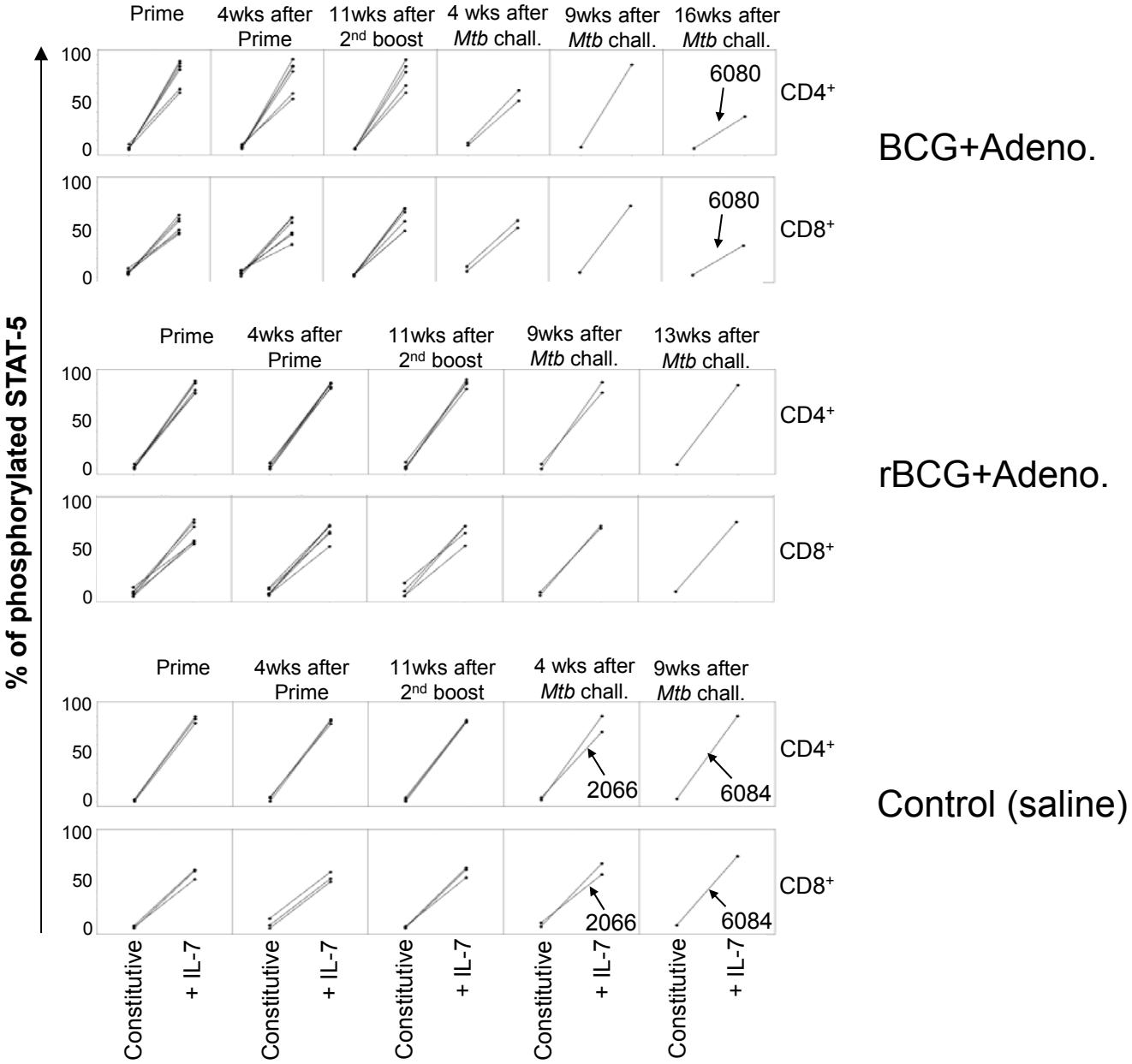

Figure 5: Constitutive and IL-7 induced STAT-5 phosphorylation. Percentage of STAT-5 phosphorylation in response to IL-7 stimulation in BCG- , rBCG-vacinated and control animals. Vaccination and *Mtb* infection did not impact on constitutive or IL-7-induced STAT-5 phosphorylation. Animals that showed an increase or decreased IL-7Rα expression after *Mtb* infection are marked with an arrow.

points before and after *Mtb* challenge, except for one animal (BCG-vaccinated animal ID6080) who showed a reduced IL-7 induced STAT-5 phosphorylation 16 weeks after *Mtb* challenge.

Discussion

Cell mediated immunity plays an important role in the control of *Mtb* infection. Both $CD4^+$ [30] and $CD8^+$ [31] T-cell populations are required to contain *Mtb* along with the production of cytokines, including IFN-γ [32] and TNF-α [33,34]. Simultaneous production of cytokines by polyfunctional T-cells, which have been shown to be more prevalent in HIV^+ patients who control infection compared to 'non-controllers' [35,36], were also identified in (protected) BCG-vaccinated mice after *Mtb* challenge [37]. However, little is known about the global phenotypic changes of peripheral T-cell lymphocytes after *Mtb* infection. The presence or absence of certain T-cell subsets (e.g. precursor T-cells enriched for long-lived antigen-specific T-cells, or depletion of terminally differentiated T-cells by drugs, i.e. rapamycin [17,18] or anti-TNF mAbs [38]) impacts on antigen-specific and on general immune-competence. The aim of this report was therefore to assess the T-cell compartment phenotype in the context of a TB vaccination study comparing the protective efficacy of a BCG or a rBCG prime followed by 2 adenoviral boosts in rhesus macaques challenged with a high dose *Mtb*. After *Mtb* challenge, 4 animals in the BCG-vaccinated group, 1 in the rBCG-vaccinated group, and 3 in the control group developed active TB and reached therefore the study endpoint (survival curves have been previously reported) [39].

No changes in the frequency or expression levels of CD45RA, CCR7, CD27 or CD28 were detected in vaccinated animals compared to control animals during the course of TB vaccination. The only difference was a marked decrease in IL-7Rα on $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T-cells identified in 12/12 vaccinated animals 4 weeks after the adenoviral boost. We have previously shown that TB vaccination (BCG or rBCG prime followed by two adenoviral boosts) of rhesus macaques induced antigen-specific T-cell responses with a peak of IFN-γ production one week after the first adenoviral boost; furthermore a strong memory T-cell proliferation was observed in $CD4^+$ and in $CD8\alpha\alpha^+$ T-cells in rBCG-vaccinated animals one week after the first adenoviral boost [22] and this coincided with reduced cell surface IL-7Rα expression reported in the current report.

Loss of IL-7Rα in $CD8^+$ and $CD4^+$ T-cells in patients with HIV has previously been shown [40,41]: IL-7Rα down-regulation can be induced by T-cell stimulation via the T-cell receptor [42], or it can be mediated directly by IL-7 or other cytokines [43,44]. We did not observe changes in plasma levels of a broad test panel of cytokines including IL-7, IL-2 and IL-15 (which receptors share the common gamma chain), or IL-1β, IL-6, TNF-α and soluble IL-7Rα (data not shown). This suggests that decreased IL-7Rα surface expression was not due to elevated IL-7 (or other cytokine) levels, decreased IL-7Rα mRNA (no evidence by PCR) or IL-7Rα shedding (no changes in soluble IL-7Rα). Whether the decrease of IL-7Rα is due to a direct or an indirect effect of the adenovirus or the vaccine targets (TB antigens) is not known up to now. Other reports suggested that IL-7 and IL-2 receptor downregulation may be associated with the expansion of antigen-specific T-cells: downregulation of CD25 expression in correlation with IL-2 signal transduction has been described after viral infection (with lymphocytic choriomeningitis virus) [45]. Mice, vaccinated with adenoviral vectors exhibited up to fourteen days after vaccination (against *Plasmodium berghei*) IL-7Rα negative T-cells in the majority of antigen-specific $CD8^+$ T-cells [46], which was less accentuated if an pox-virus was used to deliver the same antigens. An explanation might be that IL-7Rα internalization is associated with T-cell activation: the rAd35 was chosen due to the low seroprevalence in humans [47] and low levels of neutralizing antibodies induced by Ad35 in rhesus monkeys [48]. It could very well be that rAd35 induced a strong expression of *Mtb* antigens which resulted in activation of antigen-specific T-cells plus anti-adenoviral cellular immune responses. This effect was transitory in nature since IL-7Rα levels were restored five weeks after the first adenoviral boost in 12/12 animals. We could not observe a loss of IL-7Rα expression after the second adenoviral boost, most likely due to the presence of anti-adenoviral antibodies induced by the first rAd35 application.

A transient increase of $(CD56CD16)^{+/dim}CD3^{+/-}CD4^-CD8^-$ cells was observed four weeks after the first boost. Webster and colleagues have reported that $CD16^+CD3^-CD8\alpha^-$ cells do not express any of the natural killer (NK) markers [49] in rhesus macaques. NK-T cells express CD56 in combination with CD8 or CD4, and the frequency of $CD4^-CD8^-$ cells is very low in NHPs [50]. The appearance of $(CD56CD16)^{+/dim}CD3^{+/-}CD4^-CD8^-$ cells may therefore represent activated T-cells which downregulated CD3 and express CD56 upon activation [51]. This observation may also lent support that the first adenoviral boost induced a strong inflammatory reaction with subsequent T-cell activation.

The frequency and phenotype of the T-cell compartment was altered after *Mtb* challenge, lymphopenia following BCG infection of rhesus macaques [52] and in patients with TB [7,53,54] has been described. We could also detect lymphopenia after *Mtb* challenge and a transient decrease of $CD4^+$, $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T-cells. This is in contrast with a report from Langermans and colleagues who did not observe any change in the $CD4^+$ or $CD8^+$ count after *Mtb* challenge of BCG-vaccinated rhesus and cynomolgus monkeys [55]. However, doses and timing of vaccination/challenge were different than those in our study. Two weeks after *Mtb* challenge, the frequency of $CD45RA^+CCR7^+$ T-cells was dramatically decreased in $CD4^+$ T-cells (and to a lesser extent also in the $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T-cell subsets in BCG and rBCG-vaccinated animals). The concomitant expansion of activated/effector T-cells in these animals suggests that their T-cells lost CD45RA expression. This reflects a remarkable change in the composition of peripheral T-cells in 12/12 vaccinated animals (removal of precursor T-cells and expansion of central memory T-cell subsets).

A decrease of precursor T-cells was shown by Rodrigues and colleagues in patients with active TB as compared to healthy volunteers [54], and BCG-specific $CD45RA^-CCR7^-CD27^+$ effector $CD4^+$ and $CD8^+$ T-cells could be detected in PBMCs from BCG vaccinated children [5]. Our results suggest that vaccination resulted in the expansion of T-cells that rapidly acquired an activated phenotype upon *Mtb* infection, these T-cells were detected two weeks after *Mtb* challenge in vaccinated animals but ***not*** in control animals. Vaccination leads therefore to priming of T-cell populations, which can be quickly mobilized and expanded after *Mtb* challenge. $CD45RA^+CCR7^-$ T-cells were shown to be present seven weeks after *Mtb* challenge in both vaccinated and control animals: control animals eventually mobilized the effector/memory T-cell compartment in response to infection, but

this was delayed as compared to vaccinated animals. Thus, not only the quantity/quality of a cellular immune response directed against *Mtb* may serve as 'markers of successful vaccine take', but also the timing of mobilization of antigen-specific T-cells after encounter with the pathogen.

Interestingly, a transient increase of $CD3^{low/-}CD4^{+}$ cells and a massive increase of $CD3^{low}CD4^{low}$ T-cells appeared seven weeks after *Mtb* challenge that persisted until the end of the study in all animals. $CD3^{low/-}CD4^{+}$ and $CD3^{low}CD4^{low}$ cells displayed characteristics of activated cells: they did not express CD45RA or IL-7Rα. They may have originated from activated $CD3^{+}CD4^{+}$ T-cells which downregulated CD3 and CD4 following *Mtb* infection. Low CD3 expression could result from low and/or dysfunctional TCR zeta chain expression [56] associated with *Mtb* infection, yet the coordinated loss of CD3 and low CD4 expression in response to *Mtb* infection has not been reported to our knowledge.

A decreased frequency of Tregs in peripheral blood has been reported in cynomolgus macaques (concomitant with a migration to the airways) as early as 2 weeks after *Mtb* infection with a low dose *Mtb* [57]; the subsequent development of latent and active TB was associated with Treg frequency. However, we have not been able to detect major changes in the frequency or the phenotype of regulatory $CD4^{+}$ T-cells, $CD4^{+}CD8^{+}$ or $CD8^{+}$ T-cells with potential regulatory properties in PBMCs from the different vaccination groups. We could also not detect differences in Tregs associated with early or late onset of active TB. Several explanations may apply, i.e. the differential susceptibility to TB in NHP strains. Langermans and coworkers showed that rhesus macaques are more susceptible to TB infection as compared to cynomologus macaques [55]. Up to now, a detailed analysis concerning Treg compartments in rhesus and cynomolgus macaques is not available; a lower Treg frequency in rhesus macaques as compared to cynomolgus macaques could play a role in differential susceptibility to develop clinically active TB.

It has been documented that *Mtb* infection impairs cellular immune functions [56], yet it does obviously not lead to impaired IL-7-responsiveness in PBMCs, either in response to vaccination or after *Mtb* infection, defined by STAT-5 phosphorylation in the NHP model described in the current report.

We have previously shown that rBCG-vaccinated animals exhibited increased survival as compared to BCG-vaccinated and control animals [39]. In the current report, the BCG- and rBCG-vaccinated animals displayed a very similar immune marker profile during the course of vaccination and after *Mtb* challenge. Although rBCG vaccination resulted in increased survival as compared to BCG vaccination, both vaccination regimens shaped the phenotype of the total T-cell compartment in a very similar way. Yet, a the immune marker profile analysis in BCG and rBCG-vaccinated animals was found to be different in one point: we observed 2 weeks after *Mtb* challenge a drop of IL-7Rα expression in differentiated $CD4^{+}$ T-cells, particularly in the terminally differentiated ($CD45RA^{+}CCR7^{-}$) compartment in BCG-vaccinated animals, but not in rBCG-vaccinated animals [39]. The massive expansion of $CD4^{+}$ or $CD8^{+}$ T-cells generated during acute infection(s) is ultimately reduced by apoptosis in a process termed 'contraction' [58]. Data from murine models suggest that antigen-specific T-cells, which continue to express IL-7Rα, provide precursors for memory T-cells. Downregulation of IL-7Rα on T-cells may indicate the 'contraction' phase of the *Mtb* induced immune response [59-61] and IL-$7R\alpha^{+}$ T-cells may survive this process.

Although the small number of animals per group may represent a limitation in this study, our data suggest that vaccination-induced changes in the cellular immune system composition shapes profoundly the dynamics of immune responses after *Mtb* challenge: $CD4^{+}$, $CD8\alpha\alpha^{+}$ and $CD8\alpha\beta^{+}$ T-cells are mobilized earlier and they are better 'protected' from loss of the IL-7Rα during subsequent *Mtb* infection. Less pronounced loss of IL-7Rα on T-cells, associated with rBCG vaccination, may aid to preserve a broader T-cell repertoire capable of containing *Mtb*.

Acknowledgements

The study was in part financed by the Aeras foundation, by a Marie-Curie Host Fellowship for Early Stage Researchers Training grant to I.M., from the Söderberg foundation, SIDA, Vetenskapsrådet, HLF and Vinnova to M.M.

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This article was originally published in a special issue, **Vaccine Development and Immune Response** handled by Editor(s). Dr. Janet Plate, Rush University, USA