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Research paper

Minipigs as a neonatal animal model for tuberculosis vaccine efficacy testing

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ABSTRACT

Many vaccines against childhood diseases are administered early after birth, but vaccine development studies frequently test efficacy in adult rather than in neonatal animal models. In countries with endemic tuberculosis (TB), Bacillus Calmette-Guerin (BCG) is administered as part of the neonatal vaccine regimen because it prevents against the disseminated form of TB in children, although it has variable efficacy against pulmonary TB. Several promising new vaccines against TB are currently being tested in adult animal models. Here we evaluated neonatal piglets as an animal model to test vaccine efficacy. For this purpose, minipigs were vaccinated or not with BCG 48 h after birth and their immune response followed longitudinally until adolescence. We characterized the memory and activation phenotype of T cells, cytokine profile, and monocyte activation in response to BCG stimulation from 4 weeks of age into adolescence- age of 24 weeks. Immunological responses in vaccinated and non-vaccinated animals were further monitored upon infection with a low dose exposure to *Mycobacterium tuberculosis* strain HN878 via the aerosol route. Comparing the immunological response elicited by BCG vaccination in minipigs vs similar studies in infants, suggest that minipigs have the potential to serve as an effective neonatal animal model for vaccine development.

1. Introduction

Tuberculosis (TB) is a leading cause of human disease and death in developing countries alongside acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV). Over the last 80 years, many efforts have been made to create a vaccine that protects against pulmonary TB, but none have proven efficacy. Bacillus Calmette-Guerin (BCG) vaccine remains the best option to partially protect infants from disseminated TB disease but has highly variable efficacy against childhood and adulthood pulmonary TB (Tameris et al., 2013; WHO, 2015). New vaccines and boosters have been tested for efficacy in adult animal models of TB with positive results, however, a phase IIb clinical booster study with infants having natural exposure to *Mycobacterium tuberculosis* (Mtb) demonstrated lack of protection (Tameris et al., 2013; WHO, 2015). It is well established that T cell mediated immune response differs in newborns in comparison to adults but the differences are yet to be determined (Marchant and Goldman, 2005). The TB research community has openly admitted a knowledge vacuum for pediatric immunity in TB (WHO, 2015). In particular, there is a lack of knowledge in the kinetics induced by neonatal BCG vaccination and the functional and phenotypic attributes of BCG-induced memory T cell responses (Soares et al., 2013). To reduce the incidence of TB in children, WHO recommends additional research in understanding the host-pathogen interaction, and neonatal and infant immunity to Mtb infection (WHO, 2013). It is therefore essential to study neonatal immunology and test vaccine efficacy against infant and childhood TB in appropriate animal models.

Our goal was to evaluate minipigs as a neonatal model to make progress towards WHO's recommendations. Many human applied studies, including immunity studies, have used the pig as an animal model (Meurens et al., 2012). There is great potential in developing the minipig for future vaccine studies since pigs closely resemble humans for > 80% of immune parameters analyzed (versus 10% for mice) and have wide availability from commercial sources for research purposes (Meurens et al., 2012). In a previously reported study (Ramos et al., 2017), we demonstrated that aerosol infection with a highly virulent clinical strain of Mtb HN878 is possible in Sinclair minipigs. Here, neonatal piglets were vaccinated with BCG 48 h after birth. Thereafter, we followed longitudinal responses to characterize T cells, cytokine profiles, and monocyte activation in response to BCG stimulation from

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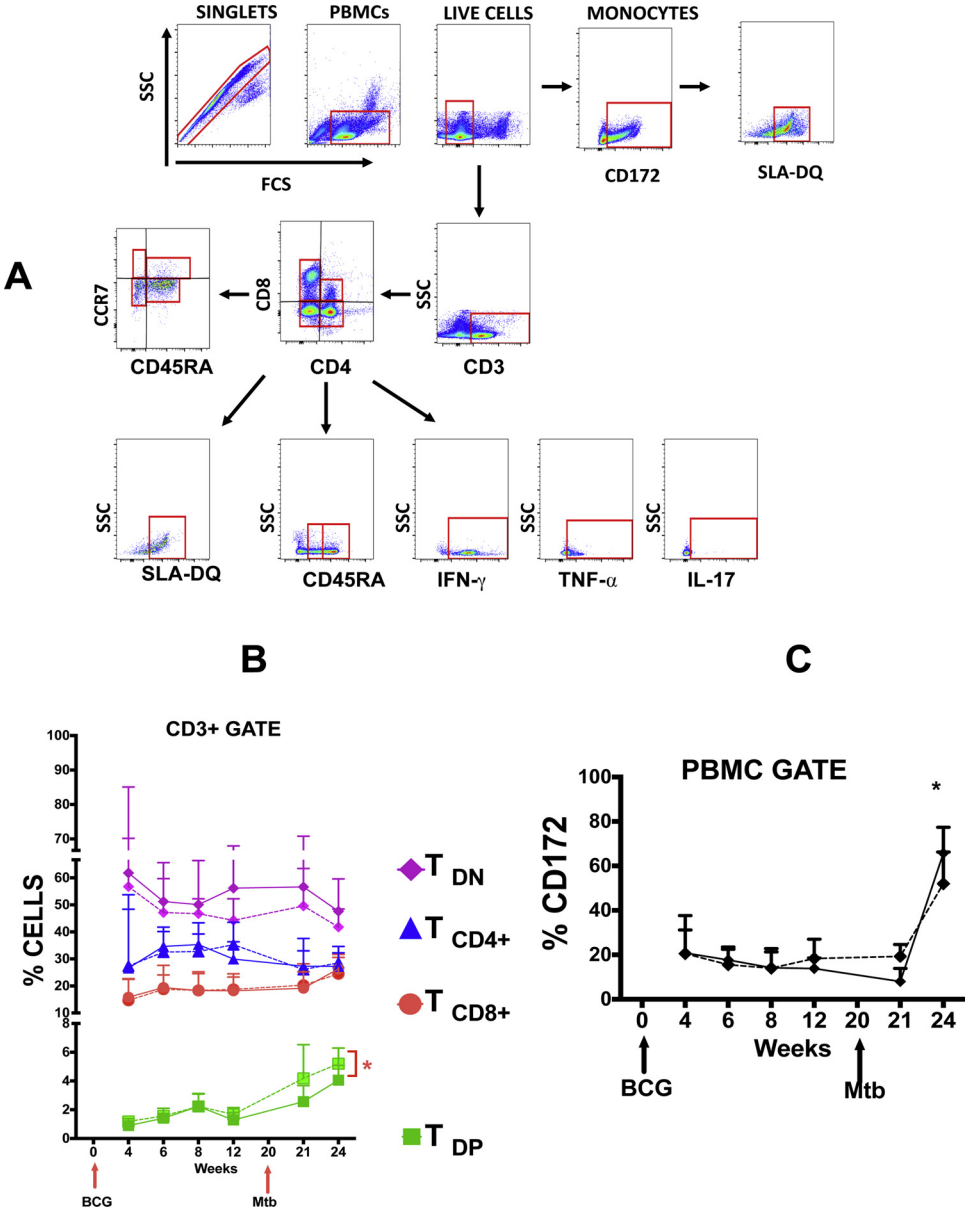

Fig. 1. Longitudinal changes in the abundance of T cell subsets and monocyte cell populations in PBMC obtained from vaccinated (solid line) or unvaccinated (dashed lines) pigs in response to BCG stimulation. Briefly, all PBMC cultures were normalized to 1×10^{6} cells/ml and percentages determined from the live PBMC parent population following the gating strategy shown in (A). Briefly, analysis was performed by selection of singlets on FCS vs SSC followed by selection of the PBMC population in a FCS vs SSC plot. Live PBMCs were extracted from the violet 405 vs SSC plot. Thereafter live PBMCs were interrogated for expression of the CD3 (T cells) or CD172 (monocytes) surface markers. Activation of monocytes was monitored via SLA-DQ expression vs SSC plot. CD4 and CD8 specific antibodies were used to discriminate expression of these markers on CD3 positive cells. Four populations were defined based on co-expression of CD3+ with the CD4 and CD8 markers. Each of those populations was screened for levels (low or high) of expression of the CD45A vs SSC. Alternatively, the memory phonotype for each of the CD3+/CD4/CD8 population was defined by relative co-expression of the CD45A and CCR7 markers. Levels of expression of SLA-DQ cell surface molecule defined the activation stage of the same populations. Finally, the intracellular levels of expression for cytokines IFNγ, TNF-α, and IL-17 on each of the CD3+/CD4/CD8 populations were also determined for each sample. Detailed methods are given in M&M. (B) Relative frequencies of CD3 + T cell subsets in response to BCG stimulation. Four populations of CD3 + T cells were defined based on co-expression of CD4 and CD8 markers; double negative (CD3+CD4-CD8-, T_{DN} pink rhomboids), CD8 + T cells (CD3+CD4-CD8α+; T_{CD8+} red circles), CD4 + T Cells (CD3+CD4+CD8α-, T_{CD4+} blue triangles) and double positive T cells (CD3+CD4+CD8α+, T_{DP} green squares). (C) Frequency of monocyte cells defined by CD172 cell surface expression. Statistical difference at $P < 0.05$ noted with *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4 weeks of age to 24 weeks. Our aim was to determine if neonatal piglets have similar immunological responses to infants vaccinated at birth with BCG. By demonstrating a similar course of TB infection and similar immune response to BCG in comparison to humans, this model can be used for development of vaccines against TB.

2. Material and methods

2.1. Animals

Two pregnant Sinclair minipig sows (Sinclair Bio-Resources, MO) were acquired 2–3 weeks before expected farrowing dates. Pregnant sows were housed (72 ± 6 ^{0}F, 30–70% humidity, 12:12 light-cycle) in separate runs but with visual and tactile contact with each other. Sows had *ad libitum* water access and were fed 1000 g/day of a locally sourced feed (Panepinto Show-Feed, CO). Feed was increased to 1500 g/day during the last week of gestation and was provided *ad libitum* during lactation. Sows were monitored once daily up to the last week of gestation, and then were monitored 5 times a day for labor/farrowing. Farrowing occurred one week apart and, as expected for Sinclair-minipigs, the sows farrowed 7 and 6 piglets each; two runts and one piglet showing neurological abnormalities were euthanized. Neonates were examined, had umbilical cord stumps dipped in iodine, and were provided with a partitioned area containing supplemental heat sources and free choice milk replacer. Needle teeth were trimmed at 1–2 days age, a dose of iron was administered at 1–2 days and 10–12 days age, males were neutered under general anesthesia at 10–12 days age, and all piglets were weaned at 3–4 weeks.

Ten healthy neonatal-piglets were used for the study: 5 piglets (3 and 2 from the first and second litter, respectively; sex: 3 females and 2 males) were vaccinated 48 h after birth by single intradermal injection at the base of tail with 0.05 ml of BCG (Statens Serum Institute, Copenhagen, Denmark) as recommended for children below one year of age. The other 5 piglets served as non-vaccinated controls (3 females and 2 males). Piglets were not vaccinated for any other diseases and were kept under barrier conditions to prevent exposure to natural swine

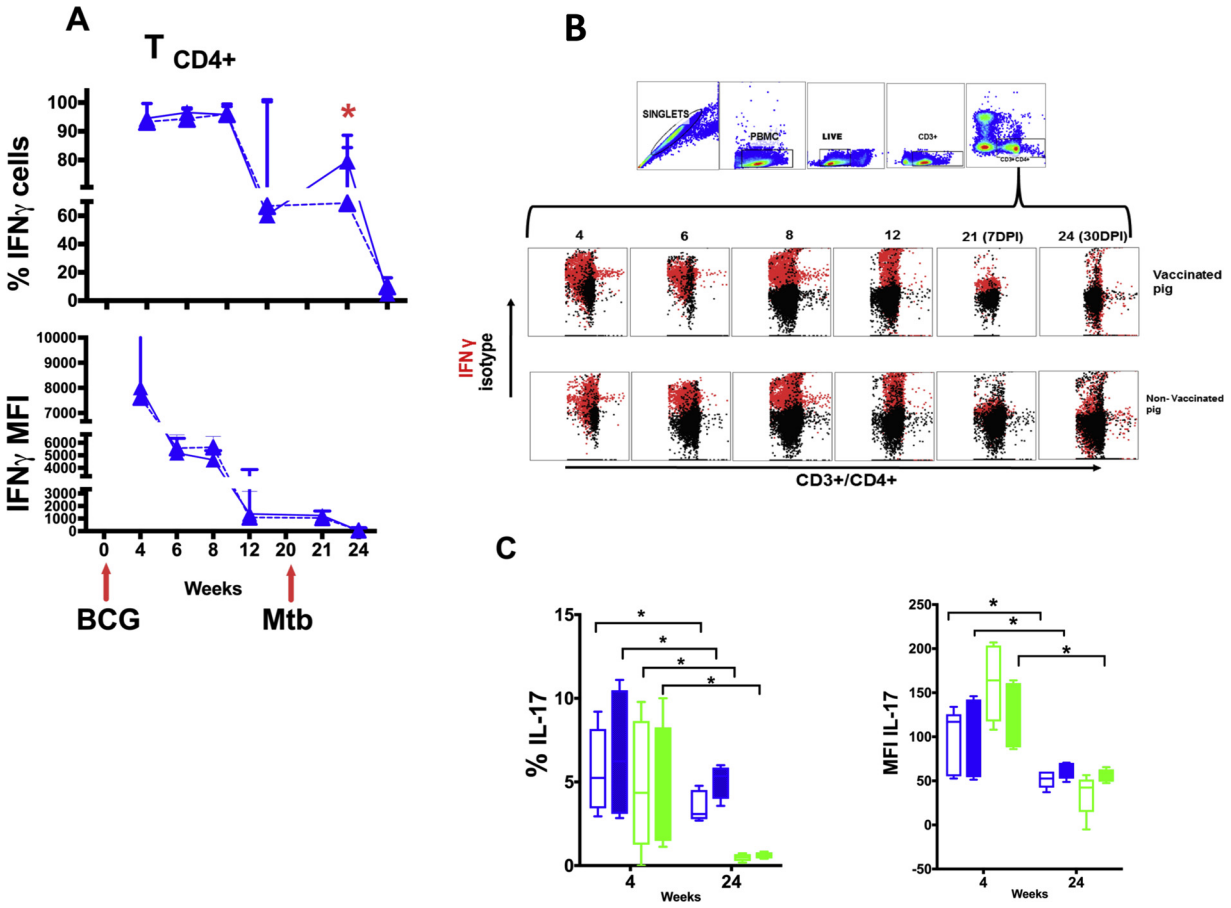

Fig. 2. Longitudinal changes in the expression of cytokines in BCG stimulated PBMC. (A) Frequency (% of positive cells) and mean fluorescence intensity (MFI) of PBMC T_{CD4+} subset expressing IFNγ from vaccinated (solid line) or unvaccinated (dashed lines) pigs after in-vitro stimulation with BCG. (B) gating strategy (as shown in Fig. 1) for selection of CD3+/CD4+ expressing intracellular IFNγ. Lower rows are longitudinal changes from 4 to 24 weeks (numbers on top of dot plots) showing overlaid of dot plots for CD4+/ IFNγ + (red) and CD4+/ mouse IgG1k isotype (black) obtained from representative BCG vaccinated and non-vaccinated piglets. (C) Percentage (left) and MFI (right) expression for IL-17 in T_{CD4+} (blue bars) and T_{DP} (green bars) from BCG vaccinated (solid bars) and unvaccinated (empty bars) piglets at 4 and 24 weeks of age. Statistical difference at $P < 0.05$ noted with *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

diseases. Piglets were bled at various time points and at 5 months of age were challenged with Mtb HN878 as previously described in (Ramos et al., 2017). Studies were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Colorado State University (CSU) Institutional Animal Care and Use Committee.

2.2. Cell cultures

Two milliliters of blood were collected from each animal into heparin at weeks 4, 6, 8, 10, 12, 21 and 24 after birth. Positive reinforcement was provided immediately after blood collection. Peripheral blood mononuclear cells (PBMC) were separated using Lympholyte®Cell-Separation medium (Cedarlane) following manufacturer's recommendations. PBMC were cultivated at 10^6 cells/ml in 96-well plates with RPMI-1640 medium containing supplements and 10% FBS (cRPMI). Thereafter, PBMC were incubated for 18 -hs at 37 °C in 5% CO_2 in the presence of cRPMI without stimulant or cRPMI containing 5 μg/ml phytohemagglutinin (PHA), or 10^6 CFU/ml BCG (SSI). Supernatants were harvested and stored at -80^{0}C until evaluated by ELISA, whereas cells were processed for flow cytometry as detailed below.

2.3. Flow cytometry

Flow cytometry by way of eight-color fluorescence-antibody-panels was used to study cell phenotypes and cytokine expression. Four panels of eight-color including the viability dye (Fixable Viability Stain 510) and seven or six antibodies were used for all time points in the study. Panel 1 and 2 included antibodies to characterize surface and cell activation markers only (panel 1) or cytokines within the T cell subset (panel 2).

The following three antibodies, and three isotype controls, were used for all four panels: Alexa Fluor 700-conjugated anti-CD3 (IgG2a, clone BB23-8E6-8C8, custom-conjugation by BD Biosciences, San Diego, CA, USA), V450-conjugated anti-CD8α (IgG2a, clone 76-2-11, custom-conjugation by BD Biosciences), PerCP-Cy5.5-conjugated anti-CD4 (IgG2b, clone 74-12-4, BD Biosciences), Alexa Fluor 700-conjugated mouse IgG2a isotype control for CD3 (clone MOPC-173, BD Biosciences), V450-conjugated mouse IgG2a isotype control for CD8 (clone MOPC-173, BD Biosciences), and PerCP-Cy5.5-conjugated mouse IgG2b isotype control for CD4 (clone 27-35, BD Biosciences).

For panel 1 the following three additional antibodies were used to measure cellular activation and determine T cell memory phenotype: PE-Cy7-conjugated anti-human CCR7 (IgG2a, clone 3D12, BD Biosciences), PE-conjugated anti-CD45RA (IgG1, clone MIL13, Thermo Fisher, Rockford, IL, USA), Allophycocyanin (APC)-conjugated SLA-DQ (IgG2a, clone TH16, generously provided by Dr. Joan Lunney, USDA).

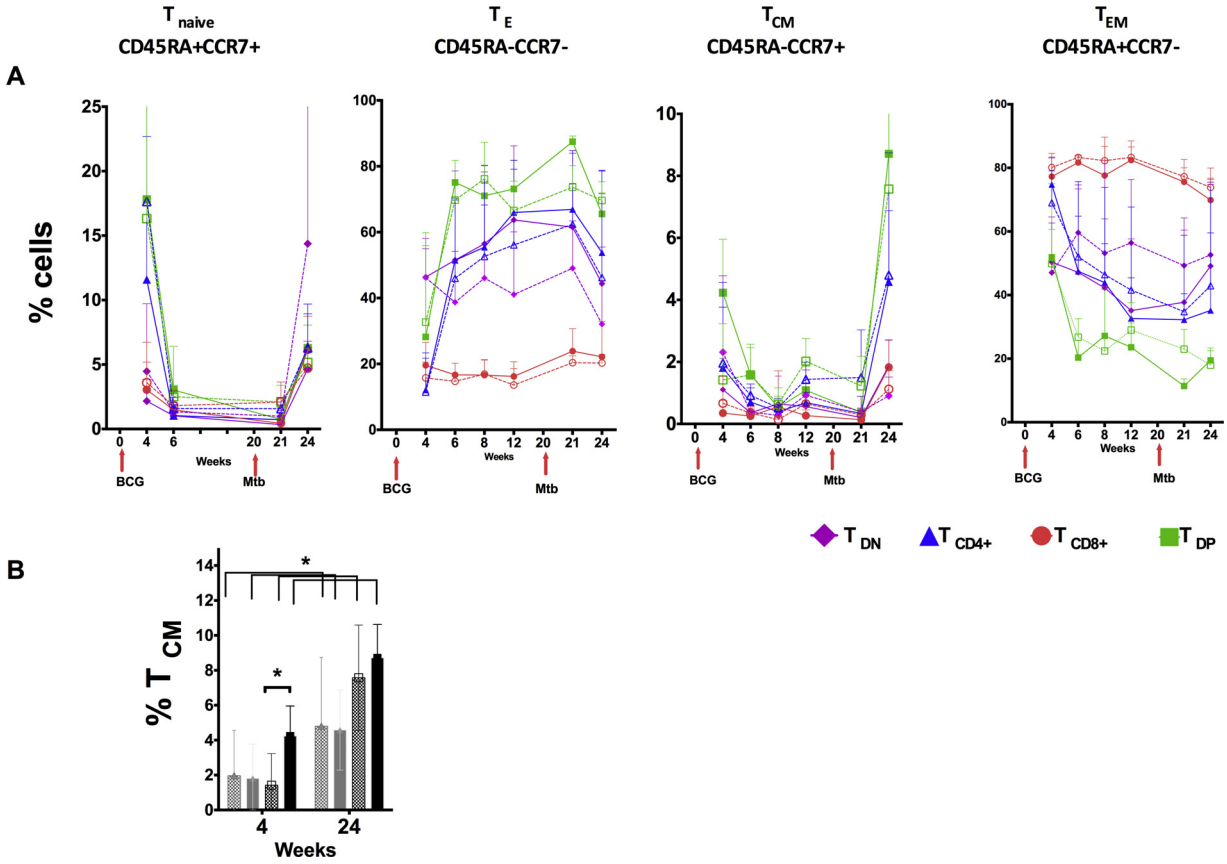

Fig. 3. Longitudinal changes in T cell memory subsets. (A) Four memory T cell subsets (as defined in Fig. 3A) were identified based on their relative expression of the CD45A and CCR7 surface molecules: naïve cells (CD45RA + CCR7+; T_{Naive}), effector cells (CD45RA−CCR7-; T_E), central memory cells (CD45RA−CCR7+; T_{CM}) and effector memory cells (CD45RA + CCR7-; T_{EM}) over time in BCG vaccinated (solid lines) and unvaccinated (dashed lines) piglets after in-vitro stimulation with BCG. (B) Percentage of T_{CM} for T_{CD4+} (blue bars) and T_{DP} (green bars) from unvaccinated (checker bars) and BCG vaccinated (solid bars) and pigs at 4 and 24 weeks of age. Statistical difference at $P < 0.05$ noted with *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

SLA-DQ was custom conjugated in-house according to manufacturer's instructions with Lightning-Link® APC conjugation kit (Innova Biosciences, Babraham, Cambridge, UK). Panel 1 also identified monocytes using FITC-conjugated anti-CD172 (IgG2b, clone 74-22-15A, BD Biosciences). Panel 1 isotype controls contained the following: PE-Cy7-conjugated rat IgG2a isotype (clone R35-95, BD Biosciences) for CCR7, PE-conjugated mouse IgG1 isotype (Thermo Fisher) for CD45RA, APC-conjugated mouse IgG2a isotype (clone G155-178, BD Biosciences) for SLA-DQ and FITC-conjugated mouse IgG2b isotype (clone MPC-11, BD Biosciences) for CD172.

Panel 2 used the following antibodies for intracellular cytokine detection: FITC-conjugated anti-human TNFα (IgG1, clone Mab11, BD Biosciences), PE-conjugated IFNγ (IgG1, clone P2G10, BD Biosciences), and Alexa Fluor 647-conjugated anti-human IL-17A (IgG1, clone SCPL1362, BD Biosciences). Panel 2 isotype controls contained the following: FITC-conjugated mouse IgG1 isotype (clone MOPC-21, BD Biosciences) for TNFα, PE-conjugated mouse IgG1 isotype (clone MOPC-21, BD Biosciences) for IFNγ, and Alexa Fluor 647-conjugated IgG1 isotype (clone MOPC-21, BD Biosciences) for IL-17.

All antibodies were monoclonal. Unless specified all antibodies were anti-swine. and were found by referencing US Veterinary Immune Reagents Network Swine Reagents. Those antibodies which are anti-human have been tested for cross-reactivity (Gerner et al., 2015). The same panels were used for all time points.

Surface staining: PBMCs were stained with V510 Fixable Viability-Dye (Thermo Fisher Scientific) followed by incubation for 30 min at 4^0C with surface antibodies panels indicated above. Thereafter, cells were washed in Stain Buffer (BD Biosciences), fixed in 4% paraformaldehyde overnight and analyzed. **Intracellular cytokine staining:** After 12 -hs stimulation, PBMC were cultured with Brefeldin A (1 μg/ml) for an additional 6 -hs. Thereafter, and following surface staining detailed above, cells were permeabilized with Perm/Wash (BD Biosciences) at 4^0C for 15 min and incubated with anti-cytokine antibodies and corresponding isotypes for 30 min at 4^0C. Data acquisition (100,000 events/sample) was done using a FACS CANTOII instrument equipped with FACSDiva. Data analysis was performed with the FlowJo software (FlowJo, LLC, versions 10.5.0 and 10.5.3).

2.4. Cytokine assay

Interleukin-2 (IL-2) concentrations in culture supernatants were determined using the Swine IL-2 Cytoset™ (Thermo Fisher Scientific). ELISAs were performed following manufacturer's protocols that defined positive IL-2 to be higher than 62 pg/ml.

2.5. Aerosol challenge

At 20 weeks of age, pigs were challenged with the virulent W-Beijing Mtb strain HN878 with an estimated target dose of 25 CFU. Methods for inoculum preparation, aerosol challenge, post-mortem examination were reported previously (Ramos et al., 2017).

2.6. Recovery of Mtb bacilli from lung samples

Recovery of live bacilli from pools of lesions from each animal after challenge was assessed using the BACTEC/MGIT 320 system (BD

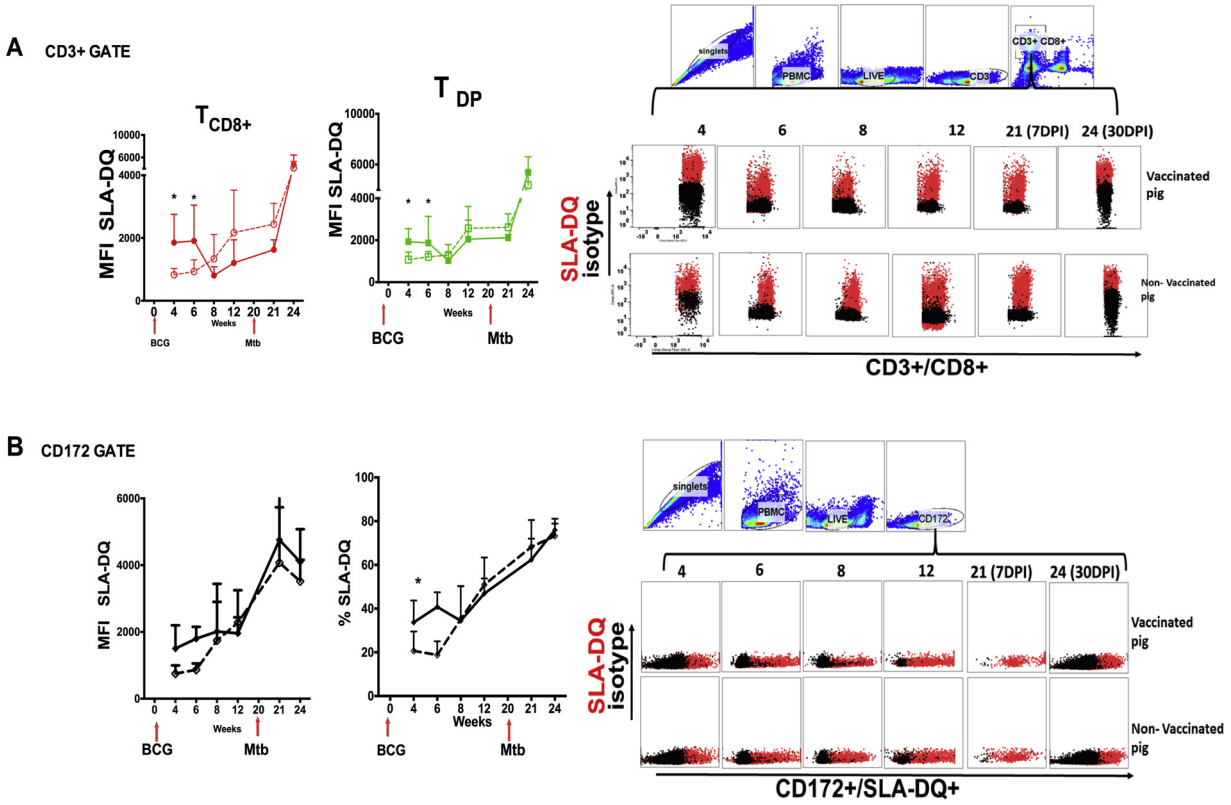

Fig. 4. Cell activation state in T cell subsets and monocytes based on their relative cell surface expression for the SLA-DQ molecule. (A) MFI expression of SLA-DQ in T_{CD8+} and T_{DP} subsets. On the right panel is shown the gating strategy (as shown in Fig. 1) for selection of CD3+/CD8+ cells expressing intracellular SLA-DQ. Lower rows are longitudinal changes from 4 to 24 weeks (numbers on top of dot plots) showing overlaid of dot plots for CD4+/ CD8+ /SLA-DQ (red) and CD4+/ CD8+ / IgG1k isotype (black) obtained from representative BCG vaccinated and non-vaccinated piglets. (B) MFI (left) and frequency of positive cells (right) expression of SLA-DQ monocytes over time in BCG vaccinated (solid lines) and unvaccinated (dashed lines) pigs after in-vitro stimulation with BCG. On the right panel is shown the gating strategy (as shown in Fig. 1) for selection of CD3+/CD172+ cells expressing intracellular SLA-DQ. Lower rows show overlaid of dot plots for CD4+/ CD172+ /SLA-DQ (red) and CD4+/ CD172+ / isotype (black) obtained from representative BCG vaccinated and non-vaccinated piglets. Statistical difference at $P < 0.05$ noted with *. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Diagnostic Systems, Sparks, MD) (Kolibab et al., 2014). Briefly, 0.2 ml of the lung homogenates (from granulomatous and non-granulomatous tissues) from each minipig was added to the BACTEC vials. The BACTEC vials (containing 4.0 ml of 7H9 broth with 0.25% glycerol) were supplemented with 0.1 ml per vial of an antimicrobial mixture containing polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) to prevent the growth of non-mycobacterial contaminants. Additionally, 0.5 ml of OADC (oleic acid, albumin [bovine], dextrose, catalase) was added to each vial. For the CFU assay, 0.2 ml of each BACTEC vial was serially diluted and cultured on Middlebrook 7H11 agar plates. Plates were incubated at 37 °C for 3–4 weeks and the number of CFU visible on each agar plate was counted, allowing for relative enumeration of CFU to be calculated for each lung sample.

2.7. Statistical analysis

Graph-Pad Prismv6 was used for data presentation. Frequencies (% of positive cells) or median fluorescence intensity (MFI) of cell phenotypes from the BCG stimulated samples were used for a repeated measures ANOVA (Rv3.2.3 software) to compare the mean response of unvaccinated versus vaccinated animals; $P < 0.05$ were considered statistically significant.

3. Results

3.1. Cell phenotype kinetics

Longitudinal changes in peripheral blood T cell subsets and monocytes derived from BCG vaccinated and unvaccinated piglets were measured using flow cytometry. Following the gating strategy shown in Fig. 1A, we monitored the frequency of four populations of CD3 + T cells based on co-expression of CD4 and CD8 markers (Fig. 1B) and defined as double negative (T_{DN}; CD3+CD4-CD8-); CD8+(T_{CD8+}; CD3+CD4-CD8α+), CD4+(T_{CD4+}, CD3+CD4+CD8α-) and double positive (T_{DP}; CD3+CD4+CD8α+), as well as, a subset of myeloid monocytes expressing the CD172 marker (Fig. 1C).

The ratio of all T cell subsets was maintained over time with T_{DN} being the most abundant followed by T_{CD4+}, T_{CD8+} and T_{DP} (Fig. 1A). Percentage of T_{DN} in the BCG-vaccinated appeared higher than in unvaccinated animals over time but was not statistically significant (Fig. 3A). T_{CD8+} remained below 25%, while T_{CD4+} were higher at weeks 6–12 but dropped thereafter. T_{DP} previously defined as a subset of T-helper cells with antigen experience in pig and non-human primates (Talker et al., 2013) were the least abundant. Interestingly though, T_{DP} were the only T cell subset to show a statistically significant difference between the unvaccinated and BCG-vaccinated pigs at week-24. The abundance of monocytes remained constant through 4-12-weeks. However, at one-week post-Mtb challenge (21-weeks) the percentage of CD172+ cells showed a slight decrease *($p > 0.05$)* for both groups. Moreover, the monocytes more than tripled in abundance 4-weeks post-Mtb challenge and demonstrated a higher increase in BCG vaccinated compared to unvaccinated animals *($p < 0.05$)* (Fig. 1C).

3.2. Longitudinal changes of cytokine expression

The kinetics of intracellular (IFNγ, TNFα, IL-17) and supernatant IL-

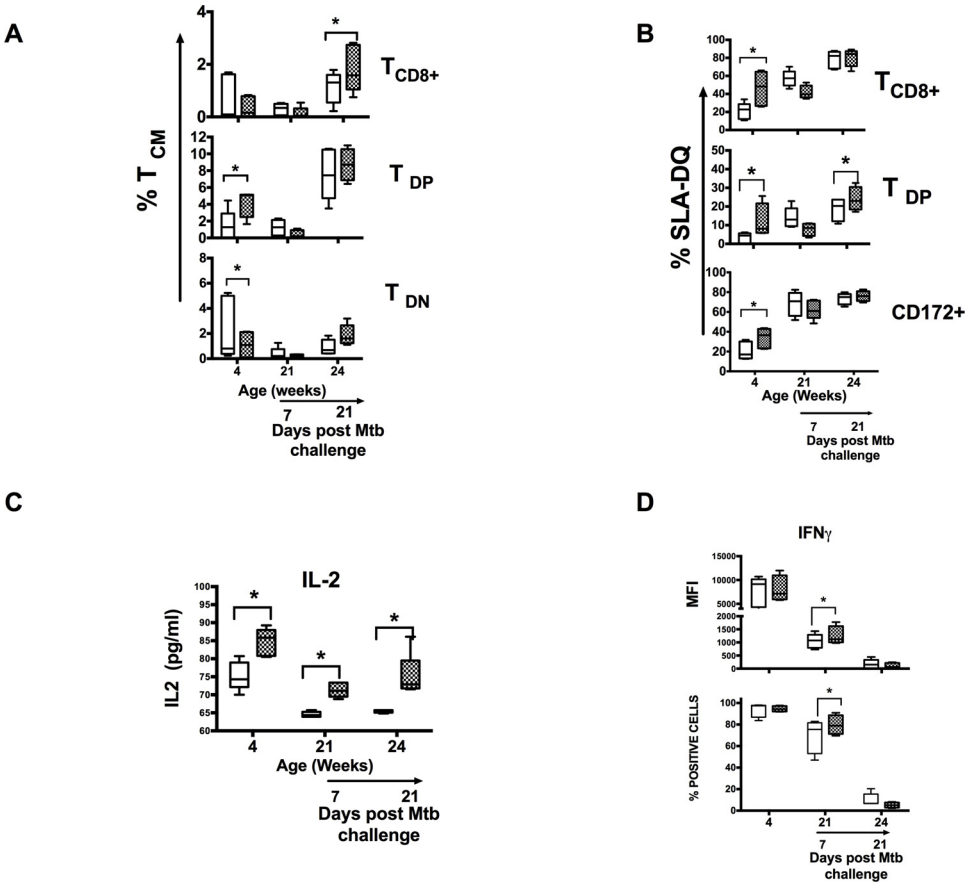

Fig. 5. Summary of the immunological differences between BCG vaccinated and unvaccinated pigs. Statistically significant differences between vaccinated (open bars) and unvaccinated (closed bars) pigs at 4, 21 and 24 weeks of age (corresponding to 4 weeks after BCG vaccination and 1 and 4 weeks after challenge) found (A) in the frequency of T_{CM}; (B) SLA-DQ expression for T_{CD8+} and T_{DP} cells and monocytes; (C) Expression for IL-2 was measured via ELISA and represented as concentration of cytokine (pg/ml) found in the supernatants harvested (prior to Brefeldin treatment) from the same cell cultures used to analyze intracellular cytokines and (D) frequency and MFI in the T_{CD4+} expressing intracellular IFNγ.

2 cytokine produced by BCG-vaccinated and unvaccinated animals after stimulation of PBMCs with BCG was studied. Intracellular expression was monitored via flow cytometry as frequency (%) and median fluorescence intensity (MFI) in T cell subsets expressing the cytokine of interest (Fig. 2A, Fig. S1).

Intracellular expression for IFNγ showed a peculiar constitutively high frequency, ˜60–90%, from 4-12-weeks in all T cell subsets and in all culture systems (stimulated with BCG and PHA mitogen or left unstimulated). Fig. 2A–B only shows the kinetics of intracellular IFNγ expression by T_{CD4+} after BCG stimulation, but similar trends were found for other T cell subsets (Fig. S1). Even more peculiar was the drastic drop (near or below 25%) in expression of IFNγ in all T cell subsets at 1-4-weeks post-Mtb challenge (21 and 24-weeks). Interestingly, IFNγ MFI gradually dropped with time in all subsets (Fig. S1).

Expression of TNFα in all T cell subsets remained between 25–50% through most time points, with similar pattern of expression in MFI (Fig. S1). Frequency and MFI for IL-17 positive cells was notably higher at 4-weeks for all T cell subsets but decreased at 6-weeks (Fig. S1). Thereafter the percentages of T cell subsets expressing IL-17 decreased in the T_{DP} and T_{CD8+} populations, while it increased in the T_{DN} and T_{CD4+} (Fig. S1). The percentage of T_{DP}-IL-17+ and T_{CD4+}-IL-17+ and their corresponding MFI were higher at 4-weeks compared to 24-weeks (4-weeks post-Mtb challenge) (Fig. 2C). At 24-weeks, T_{CD4+} were the dominant subset expressing IL-17 (Fig. 2C).

3.3. Kinetics of emergence of memory T cells

BCG is capable of generating effector memory T cells (Henao-Tamayo et al., 2014; Kagina et al., 2009; Soares et al., 2008; Tena-Coki et al., 2010). Here we defined memory phenotype of the T cells through co-expression of CCR7 and CD45RA as previously reported in infant (Soares et al., 2013) and in pig studies (Hlavova et al., 2014). Four memory phenotypes have been classified: naïve cells (CD45RA + CCR7+; T_{Naive}), effector cells (CD45RA-CCR7-; T_E), central memory cells (CD45RA-CCR7+; T_{CM}) and effector memory cells (CD45RA + CCR7-; T_{EM}).

The frequency of T_{Naive} was highest (10–15%) within T_{CD4+} and T_{DP} at 4-weeks but dropped to 5% to meet frequencies similar to other T cell subsets for the remaining time points. The amount of T_E rose continuously from 4-21-week in T_{CD4+}, T_{DP}, and T_{DN} (Fig. 3A). Changes in frequency of T_{CM} were more pronounced in T_{CD4+} and T_{DP}. Focusing further in these T cell subsets and as shown in Fig. 3B, at 24-weeks (4-weeks post-Mtb challenge) there were statistically significant increases in the frequency of T_{CM}-T_{CD4+} and T_{CM}-T_{DP} when compared to 4-weeks (4-weeks post-BCG vaccination). Interestingly, the T_{CD8+} had the lowest amount of T_E and maintained similar expression through all time points (Fig. 3A). However, the frequency of T_{EM} was the highest in T_{CD8+}, compared to all other subtypes, through all time points, decreasing only after Mtb challenge.

We concluded that at 4-weeks and upon *in vitro* stimulation with BCG, the T_{Naive} subtype is more abundant within the T_{DP} and T_{CD4+} cells. Thereafter, as animals got older the percentages of T_{DP}, T_{CD4+} and T_{DN} decreased while the T_E subtype increased significantly (Fig. 3). The T_{CD8+} display a unique pattern characterized by a highly predominant T_{EM} phenotype. Notably, the T_{DP} show the most dynamic changes within the T_{CM} and T_{Naive} subsets.

3.4. Activated phenotypes

Activation of T cells as well as monocytes (Fig. 4A and B, respectively) was monitored through SLA-DQ expression (Dillender and Lunney, 1993; Ladinig et al., 2014), an ortholog of HLA-DQ (Chardon et al., 1999; Lunney et al., 2009; Pennington et al., 1981). In this instance, changes in expression of SLA-DQ (% of cells and MFI) were

Table 1
Summary of results obtained from BCG vaccinated at birth (Pig ID = 1–5) or unvaccinated (Pig ID 6–10) minipigs for gross pathology and viable bacilli recovered from lung (NG = non-granuloma; GR = granuloma) or mediastinal lymph nodes (NL) samples at necropsy after 2 month challenge with Mtb HN878. Bacterial growth was determined via Time to Positivity (TTP in days) results that were measured using the BACTEC/MGIT system. Bacterial viability in BACTEC vials was also determined via enumeration of colony forming units (CFU//ml) on Middlebrook 7H11 solid media.

Pig ID	Lung	Lymph Nodes	*Mtb* GROWTH			
(BCG Vaccinated)			TTP (days)		CFU/ml	
1	Small lesions	No abnormalities noted	Lung (NG)		Lung (NG)	
			Lung (GR)		Lung (GR)	**145**
			LN		LN	
2	Small disseminated lesions in upper lobe	No abnormalities noted	Lung (NG)		Lung (NG)	
			Lung (GR)		Lung (GR)	
			LN	**8**	LN	
3	1 small lesion in lower left lobe	1 lesion in mediastinal LN	Lung (NG)		Lung (NG)	
			Lung (GR)		Lung (GR)	
			LN		LN	
4	No abnormalities noted	No abnormalities noted	Lung (NG)		Lung (NG)	
			Lung (GR)	**3**	Lung (GR)	
			LN		LN	
5	Small disseminated lesions	No abnormalities noted	Lung (NG)		Lung (NG)	
			Lung (GR)	**7**	Lung (GR)	
			LN		LN	
6	Small lesions in apical lobe only	• No abnormalities noted	Lung (NG)	**15**	Lung (NG)	**1033**
			Lung (GR)		Lung (GR)	
			LN		LN	
7	Small disseminated lesions	• Hard lesions present in submandibular and mediastinal LN	Lung (NG)		Lung (NG)	
			Lung (GR)	**15**	Lung (GR)	**4233**
			LN		LN	
8	1 lesion in top right lobe Lesions near bronchial tree	• Hardened mediastinal and submandibular LN	Lung (NG)		Lung (NG)	
			Lung (GR)	**16**	Lung (GR)	**3983**
			LN	**9**	LN	
9	Small lesions in apical lobe only	• No abnormalities noted	Lung (NG)		Lung (NG)	
			Lung (GR)		Lung (GR)	
			LN	**7**	LN	
10	Small lesions in apical lobe only	• No abnormalities noted	Lung (NG)		Lung (NG)	
			Lung (GR)	**16**	Lung (GR)	**50**
			LN		LN	

Table 2
Comparisons between BCG studies in infants and BCG neonatal minipigs and infants.

T cell phenotypes and cytokine profiles in response to BCG in-vitro stimulation	Minipigs	Infants	Reference
Predominant CD4 + T cell memory phenotype	effector T cells	effector T cells	10,11,12
Predominant cytokines expressed by CD4 + T cells	IFNγ, TNFα	IFNγ, TNFα	10
Frequency of central memory cells	10%	30-80%	10
Up-regulation of SLA-DQ in CD4 + T cells	Increased over 6 time points measured	Decreased over 2 time points measured	10
Predominant CD8 + T cell memory phenotype	Memory and effector T cells	Memory and effector T cells	10,11

noticeable in the T_{CD8+} and T_{DP}. Upregulation of SLA-DQ was higher post-Mtb challenge for both T cell subsets (Fig. 4A). In monocytes, the MFI for SLA-DQ gradually increased from 4-12-weeks, with a peak one-week post-Mtb challenge (Fig. 4B), while the number of monocytes expressing surface SLA-DQ increased after 6-weeks (Fig. 4B). As animals became older there was a trend showing increased percentages of SLA-DQ positive cells and greater up regulation of SLA-DQ on monocytes.

3.5. Variances of immunity between BCG vaccinated and unvaccinated pigs

Fig. 5 summarizes immune differences between BCG-vaccinated and unvaccinated animals after PBMC stimulation with BCG. Significant changes in PBMC characteristics were observed only at 4 and 24-weeks (in one instance at 21-weeks), corresponding to 4-weeks after BCG vaccination or 4-weeks post-Mtb challenge respectively. More precisely, differences were found in the frequency of T_{CM} (Fig. 5A), SLA-DQ expression in $T_{CD8+}T_{DP}$, and monocytes (Fig. 5B), as well as IL-2 extracellular concentrations (Fig. 5C). There was also a higher frequency, and MFI, in the $T_{CD4+}IFN\gamma+$ at 21-weeks (7-days post-Mtb challenge) in the vaccinated animals (Fig. 5D). However overall, differences observed at 4-weeks were not manifested at 21 or 24-weeks, except for increased percentages of T_{DP} expressing SLA-DQ (Fig. 5B) and higher levels of IL-2 as shown by ELISA (Fig. 5C). These data suggest that with age and upon challenge, PBMC show limited evidence of induced "memory" following BCG vaccination.

Similar to our previous pilot study (7), no clinical symptoms were observed during the two-month observation period following Mtb challenge, although small lesions were observed at necropsy in all animals (Table 1). Viable bacilli and Mtb growth in lung samples from each minipig was assessed using the Mycobacteria Growth Indicator Tube (BACTEC/MGIT 320) liquid culture system measuring time to positivity (TTP) in days, followed by plating on agar to enumerate CFUs (Table 1). Viable Mtb were detected by both techniques in pulmonary non-granulomatous tissue and granulomas from one and three unvaccinated piglets, respectively. In vaccinated animals, detection of viable Mtb was discordant between both techniques: positive CFUs but no growth in the BACTEC system were detected in pulmonary granulomas from one animal, whereas three samples from mediastinal lymph nodes were positive using BACTEC but no CFUs were recovered. Collectively, these results roughly suggest that BCG protected the vaccinated piglets to some extent and reduced Mtb burden. However, our

assays only tested small fragments from the whole lung of each animal and furthermore, the PANTA cocktail is known to affect mycobacterial growth (Heifets et al., 2000).

4. Discussion

This study monitored the longitudinal response to BCG for T cells and monocytes obtained from BCG-vaccinated and unvaccinated neonatal pigs from 4-weeks of age until adolescence (24-weeks of age). The experimental design herein purposefully resembled the longitudinal study by SATVI on healthy infants vaccinated with BCG SSI 48 h after birth in Cape Town, South Africa. In that study, 73 infants were enrolled and PBMC were collected and monitored for an extensive array of immune parameters (Soares et al., 2013). Remarkably, the results of this study in pigs are similar to the SATVI infant study (Soares et al., 2013), as well as other BCG studies in infants (Kagina et al., 2009, 2010; Soares et al., 2008; Tena-Coki et al., 2010) (Table 2). This supports further development of the minipig model for assessing efficacy of vaccines targeted at neonates or adolescents.

Pulmonary findings compatible with TB lesions after two months of infection were found in all pigs and we were able to recover live bacilli from granulomas of four animals (three in unvaccinated group and one from the BCG vaccinated group). Interestingly non-granulomatous lung sample from one animal in the unvaccinated group had positive CFU after MGIT culture. BCG provided some degree of protection in the vaccinated piglets but the magnitude of such protection remains to be determined.

Notably, the predominant memory T cell subset elicited by BCG was T_{E}, including the T-helper population defined as T_{CD4+} and T_{DP}. This finding agrees with three other studies measuring T cell kinetics in response to BCG in infants (Kagina et al., 2009; Soares et al., 2008; Tena-Coki et al., 2010). In the SATVI study, infants were found to have a peak response of T_{CD4+} at 10-weeks after BCG vaccination with a predominant T_{CM} subset along with changes in T_{E} cells (Soares et al., 2013). We conclude that BCG vaccination in pigs induces T_{CD4+} cells predominantly T_{E}, expressing constant levels of TNFα, as wells as high intracellular of IFNγ, which declined in PBMC of post-Mtb challenge. There were fewer than 10% $T_{CD4+}T_{CM}$ cells in the pigs, in comparison to T_{E} ranging from 40 to 80% in the four T cell subtypes. In the infant study (Soares et al., 2013), $T_{CD4+}T_{CM}$ were reported at 30–80%, however only two time points were compared.

There is a sparse amount of data about T_{CD8+} responses after BCG vaccination in infants. Most studies report T_{CD8+} producing undetectable or low frequency of IFNγ, TNFα and IL-2 cytokines (Kagina et al., 2009, 2010; Soares et al., 2008). However, two studies reported a predominant $T_{CD8+}T_{EM}$ supportive of the results observed in pigs (Soares et al., 2008; Tena-Coki et al., 2010). Our studies demonstrated that, in response to BCG, pig $T_{CD8+}T_{EM}$ cells uniquely upregulated SLA-DQ, which was not seen in the T_{DN} and T_{CD4+} subsets. Thus, future characterization of the T_{CD8+} subset will be necessary.

Surprisingly, no studies were found in the literature evaluating T_{DP}-helper cells in response to BCG in infants. Our study showed that T_{DP} cells may have a role in the establishment of T_{CM} after BCG vaccination, both at 4-weeks after vaccination and post- Mtb challenge. In addition, T_{DP} cells were the most prominent T_{E} phenotype. It is generally believed that co-expression of CD4 and CD8 only occurs transiently in the thymus (Koch and Radtke, 2011). However, both pigs and non-human primates have shown large populations of T_{DP} in lymphoid tissues and blood, also defined as T-helper cells (Gerner et al., 2015; Saalmuller et al., 1987). T_{DP} cells were first described in humans (Blue et al., 1986) with the capability of expressing memory markers, while maintaining co-expression of CD4 and CD8 for one year in culture (Overgaard et al., 2015). Further, in ontogeny-development studies, T_{DP} cells continuously expand in the blood from birth to adulthood and proliferate with antigenic stimulation and tumor like environments (Gerner et al., 2015).

Although controversial, IFNγ has been used as a correlate of protection in TB vaccine efficacy (Kagina et al., 2010). Most studies on TB vaccine efficacy measured IFNγ using ELISA or ELISPOT assays but studies of the kinetics of intracellular IFNγ expression from neonates to adulthood are very scarce. Therefore, it was striking to observe high intracellular IFNγ levels in neonates. More striking was to find that the kinetics of intracellular IFNγ production by T cells in minipigs highly resemble those reported by the SATVI infant study (Soares et al., 2013). In the latter, the response to BCG by infant PBMCs showed very high intracellular levels of IFNγ at an early age with a peak at 10 weeks after birth (Soares et al., 2013). In the infant studies, it was reasoned that this study was performed in a community with high Mtb burden, thus high levels of intracellular IFNγ by T cells were possible due to continuous exposure of infants to Mtb. However, prior to Mtb challenge, pigs also showed high frequencies of IFNγ similar to infants. As reported for BCG vaccination in mice (Kaveh et al., 2014), it is also possible that BCG bacilli remain in circulation during the first weeks after vaccination of infants and minipigs and thus might explain high levels of intracellular IFNγ by T cells. However, a recent study (Smith et al., 2016) where infants were BCG vaccinated at 6 weeks of age instead of 48 h after birth (as in SATVI and in this study) reported low levels of intracellular IFNγ by T cells in PBMC collected from infants at 4 months of age (Smith et al., 2016). Differences in the age of vaccination (48 h versus 6 weeks) and sampling times may explain outcomes for each study. Altogether, our studies and infant studies support the need for future studies to investigate BCG vaccination, expression of IFNγ from neonatal to adolescent T cells.

Our study only measured expression of cytokines and immune phenotypes in peripheral blood. Thus, we speculate that the lower frequencies of IFNγ+ cells after challenge were due to recruitment of these cells to the site of infection, as the response to PHA stimulation by PBMC remained stable. We also reasoned that the high levels of IFNγ in the piglets PBMCs should have induced higher up regulation of SLA-DQ in monocytes early on. However, monocytes only up regulated SLA-DQ when animals were older. Recent neonatal studies have proposed an impaired ability of monocytes to respond adequately to TLR stimulation (Kollmann et al., 2009). The immaturity of innate cells in infants decreases the capacity of MHC class II antigen presentation and subsequent stimulation of antigen specific T cell memory (Dowling and Levy, 2014). Future studies should investigate why piglet monocytes did not show high frequencies nor up-regulated SLA-DQ despite the presence of high percentages of IFNγ cells at an early age (Butler et al., 2009; Sinkora and Butler, 2009).

Regarding TNFα expression by infant leukocytes, the SATVI study reported a steady state ranging from 50 to 60% in T_{CD4+} cells obtained at 6–40 weeks postnatally, which decreased at 1 year of age. A steady range was observed in the piglets as well (Soares et al., 2013). As for IL-17, this cytokine was less frequent in both infants and piglet cells and our data showed that IL-17 expression changes as pigs age. Interestingly it appears that at 4-weeks of age and after Mtb challenge, T_{CD4+} cells produced the highest amounts of IL-17. In regards to IL-2 cytokine, we can only conclude that PBMCs, from vaccinated pigs after BCG stimulation in vitro, appeared to produce higher amounts of IL-2 than PBMCs from unvaccinated animals.

There were three main benefits to our study that were not possible to evaluate in the SATVI infants study. First, we were able to study T_{CD8+}, T_{DP} and T_{DN} longitudinally. Second, we were able to compare vaccinated animals to unvaccinated control animals. Third, we were able to challenge all pigs with a highly virulent clinical strain of Mtb and determine whether BCG vaccination resulted in a difference in disease outcome. In comparison to other animal models used to study BCG effects, our study may be the only one to emulate the current BCG vaccination regimen in endemic countries. As a pilot study, our animal care protocol approved bleedings to be started at 4-weeks of age. To further develop this model and learn about the neonatal response, it would be necessary to start assessing the immune response earlier than

four weeks of age. Our study demonstrates that pigs are capable of producing similar immune cell phenotypes as human infants and thus should be considered as a valuable intermediate animal model before trials in NHP for future TB vaccine studies. Future studies with pigs can be expanded to identify longitudinal immune correlates between immune cells from blood, lungs and lymphoid tissue. This is an added benefit of the neonatal pig model as for ethical reasons, studies in infants are limited to evaluating immunity in peripheral blood cells.

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Declaration of Competing Interest

Authors declare no conflict of interest with research presented in this manuscript.

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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.vetimm.2019.109884.

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