

# Comparative proteomics identified immune response proteins involved in response to vaccination with heat-inactivated *Mycobacterium bovis* and mycobacterial challenge in cattle

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## ABSTRACT

There is an imperative need for effective control of bovine tuberculosis (BTB) on a global scale and vaccination of cattle may prove to be pivotal in achieving this. The oral and parenteral use of a heat-inactivated *Mycobacterium bovis* (*M. bovis*) vaccine has previously been found to confer partial protection against BTB in several species. A role for complement factor C3 has been suggested in wild boar, but the exact mechanism by which this vaccine provides protection remains unclear. In the present study, a quantitative proteomics approach was used to analyze the white blood cell proteome of vaccinated cattle in comparison to unvaccinated controls, prior (T0) and in response to vaccination, skin test and challenge (T9 and T12). The Fisher's exact test was used to compare the proportion of positive reactors to standard immunological assays for BTB (the BOVIGAM<sup>®</sup> assay, IDEXX TB ELISA and skin test) between the vaccinated and control groups. Using reverse-phase liquid-chromatography tandem mass spectrometry (RP-LC-MS/MS), a total of 12,346 proteins were identified with at least two peptides per protein and the Chi<sup>2</sup>-test ( $P = 0.05$ ) determined 1,222 to be differentially represented at the key time point comparisons. Gene ontology (GO) analysis was performed in order to determine the biological processes (BPs), molecular functions (MFs) and cell components (CCs) the proteins formed part of. The analysis was focused on immune system BPs, specifically. GO analysis revealed that the most overrepresented proteins in immune system BPs, were kinase activity and receptor activity molecular functions and extracellular, Golgi apparatus and endosome cell components and included complement factor C8 $\alpha$  and C8 $\beta$  as well as toll-like receptors 4 (TLR4) and 9 (TLR9). Proteins of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) (JAK-STAT) and protein kinase C (PKC) signaling pathways were furthermore found to potentially be involved in the immune response elicited by the inactivated vaccine. In conclusion, this study provides a first indication of the role of several immune system pathways in response to the heat-inactivated *M. bovis* vaccine and mycobacterial challenge.

**Abbreviations:** ATP, adenosine triphosphate; BTB, bovine tuberculosis; BPs, biological processes; C(x), control group at time-point x; CCs, cell components; FDR, false discovery rate; GO, gene ontology; HRP, horseradish peroxidase; IREC, Instituto de Investigación en Recursos Cinegéticos; JAK-STAT, Janus kinase (JAK)-signal transducer and activator of transcription; MAC, membrane attack complex; MFs, molecular functions; MTBC, *Mycobacterium tuberculosis* complex; MYD88, myeloid differentiation protein 88; NF $\kappa$ B1, nuclear factor  $\kappa$ B 1; OD, optical density; O/U, overrepresented/underrepresented; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptors; PSM, peptide spectrum match; RP-LC-MS/MS, reverse-phase liquid-chromatography tandem mass spectrometry; RT, room temperature; SOCS, suppressor of cytokine signal; T(x), time-point x (week); TB, tuberculosis; TBS, tris-buffered saline; TLR, toll-like receptor; TRIF, toll-interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN; UPBRC, University of Pretoria Biomedical Research Centre; V(x), vaccinated group at time-point x

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## 1. Introduction

Tuberculosis (TB) is one of the world's most common causes of human deaths from infectious diseases (World Health Organization, 2015). It is caused by mycobacteria of the *Mycobacterium tuberculosis* complex (MTBC) which contains, amongst others, *Mycobacterium tuberculosis* (*M. tuberculosis*), infecting humans predominantly, and *Mycobacterium bovis* (*M. bovis*), the causative pathogen of bovine TB (BTB). Bovine tuberculosis affects the cattle industry worldwide, infecting approximately 50 million cattle and inflicting costs of around \$3 billion every year (Garnier et al., 2003). Although test-and-slaughter policies have been successful in the past (Wedlock et al., 2002), eradication schemes often fail to effectively control BTB in domestic cattle (Schiller et al., 2011). Apart from cattle, the main reservoir of BTB in industrialized countries (Gortazar et al., 2012), humans and many other domestic and wild mammalian species can be affected (Kidane et al., 2002; Michel et al., 2006; Hlavsa et al., 2008; Rodriguez et al., 2009; Pesciaroli et al., 2014). In developing countries, the presence of such a wildlife reservoir (Corner, 2006; Fitzgerald and Kaneene, 2013) in combination with budgetary and/or ethical constraints (Michel, 2014) may further compromise control. In sub-Saharan Africa, there is a vast human-livestock-wildlife-interface and veterinary public health surveillance is often sub-optimal or non-existing (Ayele et al., 2004); an epidemiological setting beneficial for effective propagation of mycobacteria. In South Africa, specifically, spillback from wildlife to domestic cattle has been established by Musoke et al. (2015), raising specific concerns for rural livelihoods and public health in marginalized communities. It has furthermore been shown that there is increasing intra- and inter-species transmission of *M. bovis* (Hlokwe et al., 2014), highlighting the need for more effective control of BTB. The *M. bovis* Bacillus Calmette-Guérin (BCG) vaccine widely-used in humans may be effective against BTB to a certain extent (Garrido et al., 2011), but it is known to interfere with TB diagnosis in eradication programs in cattle. Moreover, BCG has previously shown variable efficacy, both in humans (Ottenhoff and Kaufmann, 2012) and in cattle (Ameni et al., 2010). This could be linked to prior exposure to non-tuberculous mycobacteria (NTM) (Arbelaez et al., 2000) which are highly prevalent in many countries (Buddle et al., 2002), including South Africa (Gcebe et al., 2013). Lastly, as suggested by van der Heijden et al. (2017), the use of BCG as a live vaccine preparation in regions where the HIV prevalence is particularly high (UNAIDS, 2016), might be unwarranted. Hence, new, safe and effective vaccines are urgently needed for the control of BTB.

Recently, in Spain, parenteral and oral immunization with the heat-inactivated *M. bovis* vaccine resulted in partial protection of wild boars against BTB, more specifically a reduction in thoracic lesions was seen (Garrido et al., 2011; Beltran-Beck et al., 2014). Analyses of the white blood cell proteome suggested that complement factor C3 may play a role in protection against TB after oral immunization with the inactivated vaccine in the wild boar model (Beltran-Beck et al., 2014; de la Fuente et al., 2016). Furthermore, recent results showed an increased lytic efficiency of bovine macrophages ex vivo, trained with heat-inactivated *M. bovis* (Juste et al., 2016), suggesting the recently proposed concept of trained immunity as potential mechanism of vaccine protection (Netea et al., 2011). Finally, oral administration of the inactivated vaccine in farmed red deer elicited elevated levels of C3 but reduced the response to avian and bovine tuberculin compared to control animals (Lopez et al., 2016). Although the influence of the administration route is currently unknown, the advances made in these studies in other species (Garrido et al., 2011; Beltran-Beck et al., 2014; Lopez et al., 2016), indicated that the parenteral use of the inactivated vaccine in cattle might induce protection against *M. bovis* without the risk of transmission of the vaccine strain. To address this hypothesis, cattle were vaccinated subcutaneously with a heat-inactivated *Mycobacterium bovis* vaccine, skin tested and challenged with live *M. bovis* BCG. The immune response profiles of cattle to the vaccination have

recently been determined (van der Heijden et al., 2017) and cattle vaccinated with the inactivated vaccine showed strong cell-mediated as well as early and sustained humoral immune responses and bovine tuberculin reactivity as determined by the BOVIGAM<sup>®</sup>, IDEXX TB ELISA and skin test, respectively. In the present study, a quantitative proteomics analysis was conducted to compare the white blood cell proteome of these vaccinated as well as unvaccinated control cattle at key time points before and after vaccination, skin test and challenge. Immune system related proteins (C8 $\alpha$ , C8 $\beta$ , Toll-like receptor 4 (TLR4), and TLR9) were associated with the effect of vaccination after challenge.

## 2. Materials & methods

### 2.1. Ethics statement

This study was carried out in accordance with the recommendations of the South African National Standard 10386 “The Care and Use of Animal for Scientific Purposes”. The protocol was approved, under project number V086-14, by the Animal Ethics committee of the University of Pretoria.

### 2.2. Experimental design and sample processing

Experimental design and sampling were described previously by van der Heijden et al. (2017). In short, twelve calves of 4–6 months of age with no prior sensitization to *M. bovis*, as demonstrated by the BOVIGAM<sup>®</sup> assay and IDEXX TB ELISA, were selected for this study. The animals were held in class II biological containment holding facilities of the University of Pretoria Biomedical Research Centre (UPBRC), Onderstepoort campus, Pretoria. Calves were randomly assigned to either the heat-inactivated *M. bovis* group or the control group ( $n = 6$  per group). The vaccine consisted of an emulsion of heat-inactivated *M. bovis* (Neiker strain) and Montanide<sup>™</sup> ISA 50 V 2 (SEPPIC, France) adjuvant to a concentration of  $1 \times 10^7$  CFU/ml. The control inoculum consisted of an emulsion of phosphate buffered saline (Sigma-Aldrich, South Africa) and 50% Montanide<sup>™</sup> ISA 50 V 2 (SEPPIC, France) adjuvant (v/v). The animals were vaccinated subcutaneously in the left mid cervical area with 1 ml of the inactivated vaccine or the control inoculum at T0, respectively. Animals in the vaccinated group received a booster vaccination at T3. At T9, all animals were skin tested using avian and bovine tuberculin as well as two previously described protein cocktails (Jones et al., 2012). After reading of the skin test (T9 + 3 days), all animals were challenged with live *M. bovis* BCG through intranodular injection (Villarreal-Ramos et al., 2014) into the right pre-scaphular lymph node. Whole blood samples were collected from the jugular vein of vaccinated (V) and control (C) cattle, in EDTA coated tubes, using a vacutainer system, at time points T0 (C0 and V0; before vaccination), T9 (C9 and V9; 9 weeks after vaccination) and T12 (C12 and V12; 3 weeks after skin test and challenge). After centrifugation of whole blood for 10 min at  $1500 \times g$ , buffy coats were recovered using a sterile Pasteur pipette and transferred to sterile 2 ml Cryo.s<sup>™</sup> cryovials (Greiner Bio One) containing 0.5 ml RNeasy<sup>®</sup> (Thermo Fisher Scientific). Under maintenance of the cold chain (4 °C), preserved buffy coats were transferred from the Department of Veterinary Tropical Diseases of the University of Pretoria to the Instituto de Investigación en Recursos Cinegéticos (IREC) and subsequently stored at 4 °C for a maximum of 5 days prior to protein extraction.

### 2.3. Characterization of immune response profiles

Results from the BOVIGAM<sup>®</sup> assay, IDEXX TB ELISA and skin test in vaccinated animals and unvaccinated controls were described in detail in earlier work (van der Heijden et al., 2017). For this study, test results obtained by van der Heijden et al. (2017) were interpreted according to the manufacturers' protocols (Thermo Fisher Scientific (BOVIGAM<sup>®</sup>))

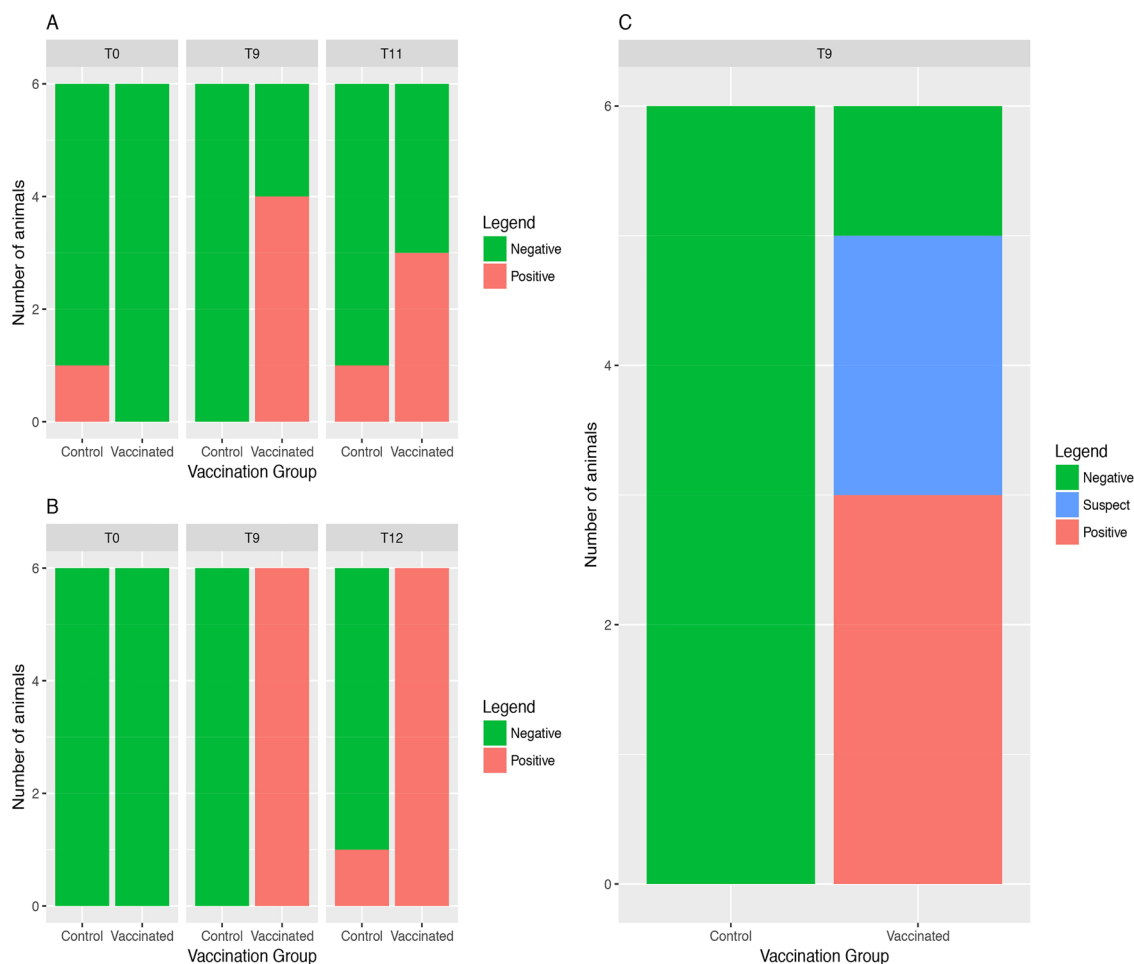
and IDEXX (TB ELISA)) to obtain a qualitative measure for the outcomes. These results and those of the skin test, interpreted according to OIE standards by [van der Heijden et al. \(2017\)](#), were collated for the present paper (Fig. 1 and S1 Table). To compare the proportion of positive reactors in vaccinated and control animals for all tests at time points relevant for the present proteomic analysis (T0, T9 and T12), the Fisher's exact test from The R Stats package ([R Core Team and contributors worldwide, 2017](#)) was used in R ([R Core Team, 2016](#)). For the comparison of the BOVIGAM® assay results, T11 was used instead of T12 as no data were available for T12.

#### 2.4. Protein extraction and quantification

Proteins were extracted from buffy coat samples of individual vaccinated and control cattle at T0, T9 and T12 using the AllPrep DNA/RNA/ProteinMini Kit (Qiagen, Inc. Valencia, CA, USA) according to manufacturer's instructions. Proteins from the six individual samples of each group were resuspended in PBS supplemented with 0.5% Triton X-100 and protein concentration was determined using the BCA Protein Assay (Thermo Scientific, San Jose, CA, USA) using bovine serum albumin (BSA) as a standard. Pools of 150 µg protein were made by mixing equal amounts of proteins from two samples of each group to obtain three biological replicates for each group, per time point. Proteins were then methanol/chloroform precipitated and stored at −20 °C until analysis.

#### 2.5. Protein digestion and proteomics analysis

Samples were dissolved in urea buffer (8 M urea, 25 mM ammonium bicarbonate, pH 8), reduced with 10 mM dithiothreitol for 1 h at 37 °C and then alkylated with 50 mM iodoacetamide for 1 h at room temperature (RT) in darkness. The mixture was diluted fourfold to reduce urea concentration and then in-solution digested overnight at 37 °C with 60 ng/µl sequencing grade trypsin (Promega, Madison, WI, USA) (1:20 protease to protein ratio). Digestion was stopped by the addition of 1% final concentration of trifluoroacetic acid and the samples were then lyophilized. The peptides were finally desalted on OMIX Pipette tips C18 (Agilent Technologies, Santa Clara, CA, USA), dried-down and stored at −20 °C until mass spectrometry analysis. The desalted peptides were resuspended in Solvent A (0.1% formic acid in water) and analyzed by reverse-phase liquid-chromatography tandem mass spectrometry (RP-LC-MS/MS) using an Easy-nLC II system coupled to an ion trap LTQ mass spectrometer (Thermo Scientific). The peptides were concentrated (on-line) by reverse phase chromatography using a 0.1 × 20 mm C18 RP precolumn (Thermo Scientific) and then separated using a 0.075 × 100 mm C18 RP column (Thermo Scientific) operating at 0.3 ml/min. Peptides were eluted using a 60-min gradient from 5 to 40% solvent B (0.1% formic acid in acetonitrile). ESI ionization was done using a Fused-silica Pico-Tip Emitter ID 10 mm (New Objective, Woburn, MA, USA) interface. Peptides were detected in survey scans from 400 to 1600 amu (1 mscan), followed by fifteen data dependent MS/MS scans (Top 15), using an isolation width of 2 mass-



**Fig. 1.** Characterization of immune response profiles. (A) Results of the BOVIGAM® assay carried out at T0, T9 and T11 in vaccinated animals and unvaccinated controls. (B) Results of the IDEXX TB ELISA carried out at T0, T9 and T12 in vaccinated animals and unvaccinated controls. (C) Results of the skin test carried out at T9 in vaccinated animals and unvaccinated controls. Produced using the ggplot2 ([Wickam, 2009](#)) package in R ([R Core Team, 2016](#)).

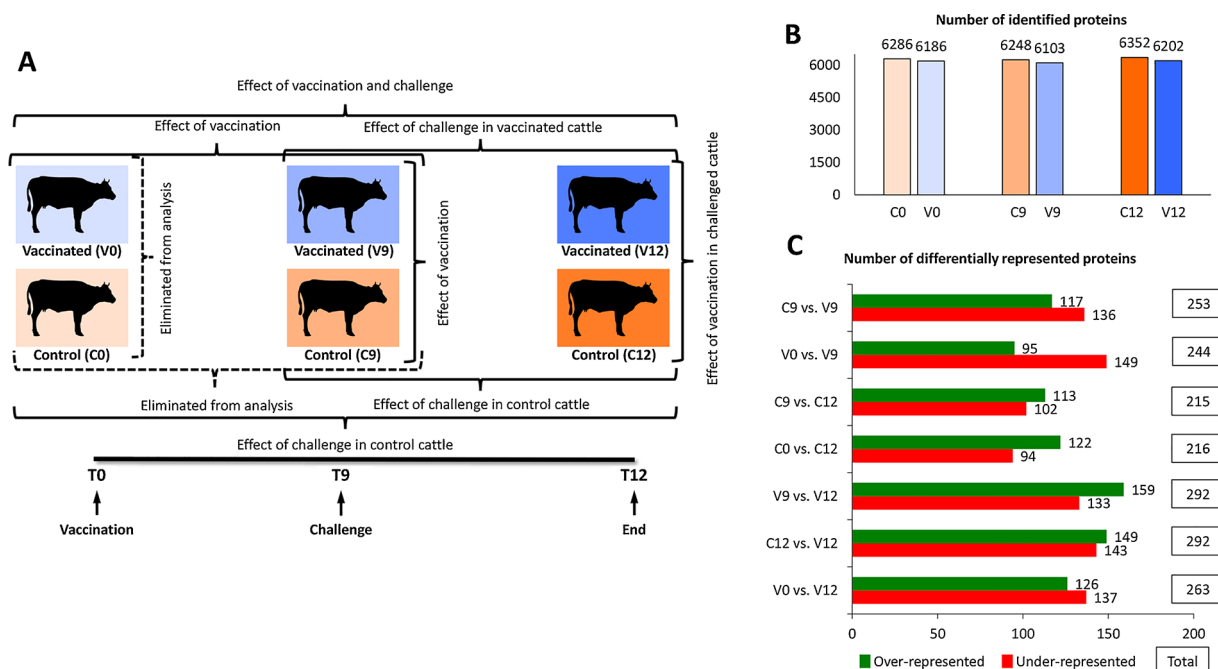
to-charge ratio units, normalized collision energy of 35% and dynamic exclusion applied during 30 s periods. The MS/MS raw files were searched against the Uniprot *Bos taurus* database (32,127 entries in April 2016) (The UniProt, 2017) using the SEQUEST algorithm (Proteome Discoverer 1.4, Thermo Scientific). The following constraints were used for the searches: tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.8 Da for MS/MS fragment ions and the searches were performed allowing optional Met oxidation and Cys carbamidomethylation. A false discovery rate (FDR) < 0.05 was considered as condition for successful peptide assignments and at least two peptides per protein were the necessary condition for protein identification (S2 Table). The total number of peptide-spectrum matches (PSMs) for each protein were normalized against the total number of PSMs on each sample and the mean of three replicates was compared between vaccinated and control groups at the different time points by Chi<sup>2</sup>-test ( $P = 0.05$ ). Comparative proteomics analyses were conducted using data from samples V0 vs. V9 and C9 vs. V9 (effect of vaccination), C0 vs. C12 and C9 vs. C12 (effect of skin test and challenge in control animals), V0 vs. V12 (effect of vaccination, skin test and challenge in vaccinated animals), V9 vs. V12 (effect of skin test and challenge in vaccinated animals) and C12 vs. V12 (effect of vaccination in challenged animals) (Fig. 2A). The statistically significant differentially represented proteins in the comparative analyses of C0 vs. V0 and C0 vs. C9 were not included in further analysis as they represent differences not related to vaccination and/or infection such as individual-to-individual variations as determined by gene ontology analysis (Fig. 2A). Gene ontology (GO) analysis for biological processes (BPs), molecular functions (MFs) and cell components (CCs) of the differentially represented proteins between groups was performed by Blast2GO software (version 3.0) (Gotz et al., 2008). Proteins were grouped according to BP, MF and CC GO. Venn diagrams were constructed using the Bioinformatics & Evolutionary Genomics tool (VIB-Ugent, 2016). Pathway maps were

constructed using GO annotations and the Kyoto Encyclopedia of Genes and Genomes pathway maps (Kanehisa and Goto, 2000). The mass spectrometry proteomics data have been deposited at the PeptideAtlas repository with the identifier number PASS01083 (<http://www.peptideatlas.org/PASS/PASS01083>).

## 2.6. Verification of proteomics results by western blot analysis and ELISA

Rabbit, unconjugated, polyclonal primary antibodies against selected differentially represented immune response proteins TLR4 (Bioss Antibodies, Boston, MA, USA), TLR9 (antibodies-online GmbH, Aachen, Germany), C8 $\alpha$  (Sigma-Aldrich, St. Louis, MO, USA) and C8 $\beta$  (antibodies-online GmbH) were used in western blot and ELISA. During verification steps, protein extracts from human promyelocytic HL60 cells and human buffy coat were added as positive controls, whereas protein extracts from *Ixodes scapularis* embryo-derived cells (ISE6) were used as negative controls.

For western blot analysis, 15  $\mu$ g total proteins from pooled samples from each group at T0, T9 and T12 were separated by electrophoresis in a SDS-10% polyacrylamide gel (Life Science, Hercules, CA, USA) and transferred to a nitrocellulose membrane using a wet electroblotting system (Mini-Protean Tetra hand cast system, Bio-Rad, Hercules, CA, USA). The membrane was blocked with 3% BSA (Sigma-Aldrich) for 1 h at RT and washed three times with Tris-buffered saline (TBS) (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween 20). The membrane was incubated with primary antibodies (at 1:1000 dilution in TBS), incubated for 2 h at RT and washed three times with TBS. The membrane was then incubated for 2 h at RT with an anti-rabbit horseradish peroxidase (HRP) conjugate (Sigma-Aldrich) diluted 1:2000 in TBS with 3% BSA and washed three times with TBS and finally developed with Pierce ECL western blotting substrate (Thermo Scientific) according to the manufacturer's recommendations. The intensity of protein bands in the western blot membrane was determined by densitometric analysis



**Fig. 2.** Proteomics experimental design and global analysis. (A) A quantitative proteomics analysis was conducted to compare the white blood cell proteome of vaccinated and unvaccinated control cattle that were skin tested and challenged with *M. bovis* BCG. The peptide spectrum matches (PSMs) for each protein were normalized against the total number of PSMs on each sample and the mean of three replicates was compared between vaccinated and control groups at the different time points by Chi<sup>2</sup>-test ( $P = 0.05$ ). Comparative proteomics analyses were conducted using data from samples V0 vs. V9 and C9 vs. V9 (effect of vaccination), C0 vs. C12 and C9 vs. C12 (effect of skin test and challenge in control animals), V0 vs. V12 (effect of vaccination, skin test and challenge in vaccinated animals), V9 vs. V12 (effect of skin test and challenge in vaccinated animals) and C12 vs. V12 (effect of vaccination in challenged animals). (B) Total number of identified proteins in control (C) and vaccinated (V) cattle at different time points T0, T9 and T12. (C) Number of differentially represented proteins in different comparative analyses.



using ImageJ 1.44p (National institute of Health, USA). The intensity of protein bands was normalized against the intensity of the control  $\beta$ -Actin band developed using an anti- $\beta$ -Actin (Sigma-Aldrich) primary antibody.

An indirect ELISA was conducted to quantitate the levels of the selected immune response proteins, using 0.1  $\mu$ g total protein/well of individual samples from vaccinated and control cattle at T0, T9 and T12. Each sample was analyzed in duplicate. The plates were incubated with 0.1  $\mu$ g/well of buffy coat protein extract overnight at 4 °C, blocked with 100  $\mu$ l/well of PBS/3% BSA for 1 h at RT and then washed three times with 300  $\mu$ l/well PBS/0.05% Tween 20. The primary antibodies were diluted 1:500 and 100  $\mu$ l/well incubated for 2 h at RT and wells were washed three times with 300  $\mu$ l/well PBS/0.05% Tween 20. The secondary anti-rabbit Ig HRP conjugate (Sigma-Aldrich) was diluted 1:1500 and 100  $\mu$ l/well incubated for 1 h at RT and then washed four times with 300  $\mu$ l/well PBS/0.05% Tween 20. The 3,3',5,5'-Tetramethylbenzidine one solution (Promega) (100  $\mu$ l/well) was added and incubated for 15 min at RT. The reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) was measured at 450 nm and compared between groups at each time point by two-sample paired Student's *t*-test with unequal variance (*P* = 0.05).

### 3. Results

#### 3.1. Characterization of immune response profiles

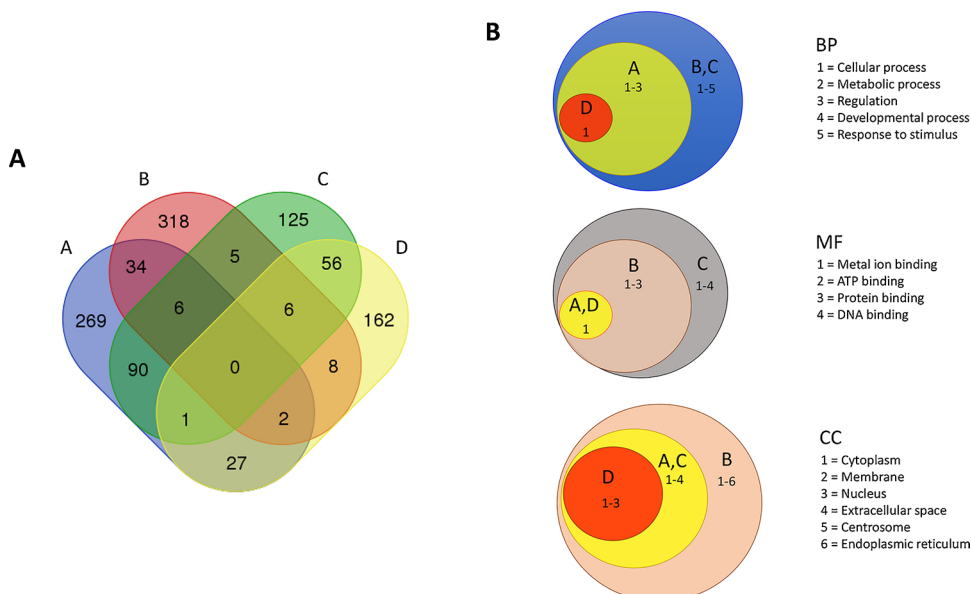
The detailed host immune response profiles were analyzed and discussed in depth by van der Heijden et al. (2017). In short, the heat-inactivated *M. bovis* vaccine induced strong and sustained cell-mediated and humoral immune responses profiles, reflecting the remarkable immunogenicity of the vaccine, as demonstrated by the BOVIGAM® assay and skin test, and the IDEXX TB ELISA, respectively (van der Heijden et al., 2017). The control group showed no reactivity prior to challenge and only a moderate response post-challenge, which was ascribed to the immunogenicity of the challenge strain, which proved to be low (van der Heijden et al., 2017). The results of the immunological tests employed to characterize the host immune response profiles in vaccinated cattle and unvaccinated controls obtained in the previous study (van der Heijden et al., 2017) were collated for this paper and are presented in Fig. 1. Using the BOVIGAM®, 1/6 animals in the control group tested positive on T0 and T11, but not on T9 (0/6) (Fig. 1A). In the vaccinated group, 0/6 animals tested positive on T0, but 4/6 and 3/

6 animals tested positive on T9 and T11, respectively (Fig. 1A). No significant difference to the control group was found in the BOVIGAM® assay at these time points. Using the IDEXX TB ELISA, 0/6 animals tested positive in the control group at T0 and T9, but 1/6 animals tested positive at T12 (Fig. 1B). In the vaccinated group, 0/6 animals tested positive on T0, but 6/6 animals tested positive on T9 and T12 (Fig. 1B). The proportion of positive reactors was significantly higher in the vaccinated group compared to the control group at T9 (*p* < 0.01) and T12 (*p* < 0.05). No animals (0/6) in the control group showed reactivity in the skin test (Fig. 1C) (van der Heijden et al., 2017). In the vaccinated group, 3/6 animals tested positive ( $\Delta$ mm between the bovine and avian injection site  $\geq 4$  mm) and 2/6 showed suspect ( $\Delta$ mm between the bovine and avian injection site:  $4\text{mm} \geq \Delta\text{mm} > 2\text{mm}$ ) reactions in the skin test (Fig. 1C) (van der Heijden et al., 2017). The proportion of reactors was significantly higher in the vaccinated group compared to the control group (*p* < 0.05).

#### 3.2. Quantitative comparative proteomics reveals differences between vaccinated and control cattle in response to vaccination, skin test and challenge with *M. bovis* BCG

A quantitative proteomics analysis was conducted to compare the white blood cell proteome of vaccinated and unvaccinated control cattle skin tested at T9 and subsequently challenged with *M. bovis* BCG (Fig. 2A). The goal of the study was to identify proteins that may be associated with the effect of vaccination (V0 vs. V9 and C9 vs. V9), effect of skin test and challenge in control animals (C0 vs. C12 and C9 vs. C12), effect of vaccination, skin test and challenge in vaccinated animals (V0 vs. V12), effect of skin test and challenge in vaccinated animals (V9 vs. V12) and effect of vaccination in challenged animals (C12 vs. V12) (Fig. 2A). A total of 12,346 proteins were identified with two or more peptides per protein in at least one of the three analyzed biological replicates per group (S2 Table). The number of identified proteins was similar between different samples (per time point and group) (Fig. 2B). After statistical analysis, over-represented and under-represented proteins were identified in all comparisons, with a similar number of differentially represented proteins (Fig. 2C). Using a Venn diagram, the differentially represented proteins were grouped according to the different time point and group comparisons (Fig. 3A).

The GO analysis first focused on BPs, MFs and CCs affected by vaccination and the combination of skin test and challenge, which revealed that proteins from a multitude of BPs, MFs and CCs were



**Fig. 3.** Differences between vaccinated and control cattle in response to vaccination, skin test and challenge with *M. bovis* BCG. (A) Venn diagram showing the number of differentially represented proteins grouped according to different comparisons. (B) Venn diagrams for the characterization of the most represented BPs, MFs and CCs (≥ 40 differentially represented proteins). Code: A, Effect of vaccination (V0 vs. V9 and V9 vs. C9); B, Effect of skin test and challenge in control animals (C0 vs. C12 and C9 vs. C12); C, Effect of skin test and challenge in vaccinated animals (V9 vs. V12); D, Effect of vaccination and skin test and challenge in vaccinated animals (V0 vs. V12). BPs = biological processes; MFs = molecular functions; CCs = cell components.

differentially represented (S1 Figure). The characterization of the most represented ( $\geq 40$  differentially represented proteins) BPs, MFs and CCs showed that developmental process and response to stimulus BPs, and adenosine triphosphate (ATP) binding and protein binding MFs were associated with response to skin test and challenge in both vaccinated (V0 vs. V12) and control (C0 vs. C12) cattle (Fig. 3B). Distinctive BPs, MFs or CCs were not found associated with response to vaccination alone, but DNA binding MF was associated with skin test and challenge in vaccinated cattle alone, whereas centrosome and endoplasmic reticulum CCs were associated with skin test and challenge in control cattle (Fig. 3B).

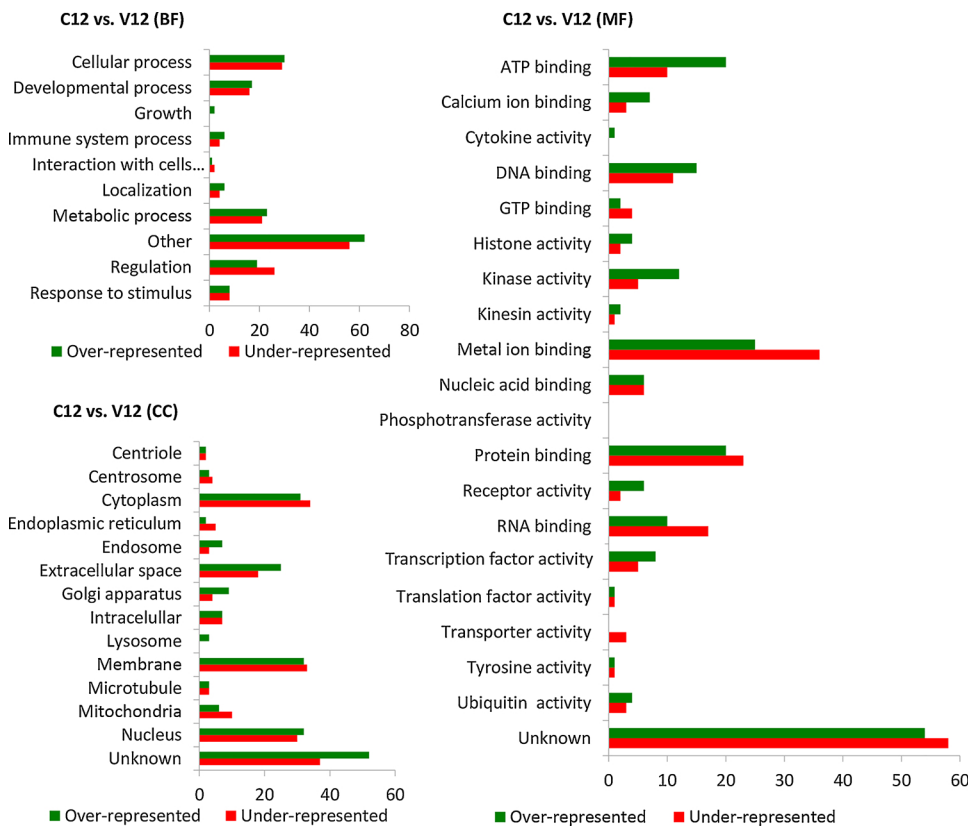
### 3.3. Quantitative comparative proteomics identifies immune response proteins associated with the effect of vaccination in cattle vaccinated, skin tested and challenged with *M. bovis* BCG

The analysis was then focused on characterization of the immune response in vaccinated and skin tested and challenged cattle by specifically targeting the immune system proteins (Fig. 4). A total of 35 immune system proteins were identified of which representation was affected in different ways in response to vaccination, skin test and challenge (Fig. 4), similarly as the effect of these interventions on the immune system BP (S1 A Figure). A few proteins that form part of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway (JAK-STAT) and protein kinase C (PKC) pathways were differentially represented at several comparisons, including ADAM metalloproteinase domain 15, interferon- $\kappa$ , protein kinase C beta type and tyrosine-kinase protein (Fig. 4), in animals which were vaccinated, skin tested and challenged. To identify the proteins

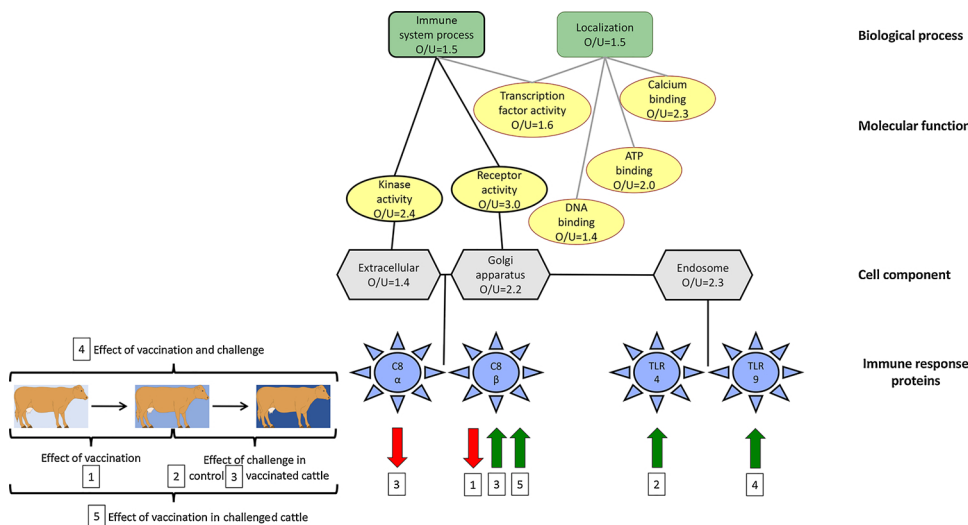
associated with the effect of vaccination in challenged animals, the BPs, MFs and CCs containing the differentially represented proteins after comparing vaccinated and control cattle at T12 (C12 vs. V12; Fig. 2A) were characterized (Fig. 5). As shown before for other comparisons (Fig. 3B), the most represented BPs, MFs and CCs ( $\geq 40$  differentially represented proteins) at C12 vs. V12 were cellular process, metabolic process and regulation BPs, metal ion binding and protein binding MFs, and cytoplasm, membrane, nucleus and extracellular space CCs (Fig. 5). However, the most upregulated BPs, MFs and CCs (over-represented/under-represented proteins  $\geq 1.4$ ) in the C12 vs. V12 comparative analysis were immune system process and localization BPs, kinase activity, receptor activity, transcription factor activity, DNA binding, ATP binding and calcium binding MFs, and extracellular, Golgi apparatus and endosome CCs (Fig. 6). The regulatory pathways connecting the most upregulated BPs, MFs and CCs at C12 vs. V12 were then mapped to predict the differentially represented immune system proteins that best fitted into the immune system process pathways (Fig. 6). These proteins were selected for further analysis and included C8 $\alpha$ , C8 $\beta$ , TLR4 and TLR9 (Fig. 6). The analysis of these proteins' differential representation in response to vaccination, skin test and challenge with *M. bovis* BCG showed that vaccination resulted in the under-representation of C8 $\beta$  (V0 vs. V9), while skin test and challenge (V9 vs. V12) led to over-representation of C8 $\beta$  but under-representation of C8 $\alpha$  (Fig. 6). Skin test and challenge furthermore resulted in the over-representation of the two identified TLR proteins in both vaccinated (V9 vs. V12) and control (C9 vs. C12) cattle (Fig. 6). The C12 vs. V12 comparison evidenced the over-representation of C8 $\beta$ , which suggested that this protein might be associated with the response to vaccination in cattle (Fig. 6).

ID	Name	C9 vs. V9	V0 vs. V9	C9 vs. C12	V9 vs. V12	C12 vs. V12	C0 vs. C12	V0 vs. V12
F1N102	8B complement component 8, beta							
Q2KIH5	Complement component 8, alpha polypeptide							
B5T268	Toll-like receptor 2							
Q6WCD4	Toll-like receptor 4							
Q56GY1	Toll-like receptor 9							
C1ITJ8	Major histocompatibility complex class I-related gene protein							
E1B9H1	TINAGL1 tubulointerstitial nephritis antigen like 1							
E1B8U4	ADAM15 ADAM metalloproteinase domain 15							
E1BGY0	DROSHA drosha ribonuclease III							
E1BJ91	IFNK interferon kappa							
F1MBI8	SYNGAP1 synaptic Ras GTPase activating protein 1							
F1MBU9	PIK3C2B phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta							
F1MCX4	TYK2 tyrosine kinase 2							
F1MEX6	RNF19B ring finger protein 19B							
F1MJT4	Prf1 perforin 1 (pore forming protein)							
F1MQ78	ALOX5 arachidonate 5-lipoxygenase							
F1MQA9	Tyrosine-protein kinase							
F1MUM9	ADAR adenosine deaminase, RNA specific							
F1MV80	MPO myeloperoxidase							
F1MVZ6	P2X purinoceptor							
F1N1Q0	Tyrosine-protein kinase							
F1N3B8	2'-5'-oligoadenylate synthase 2							
F1N3K3	IL1RL2 interleukin 1 receptor like 2							
P05126	Protein kinase C beta type							
P08037	Beta-1,4-galactosyltransferase 1							
Q0II78	Chemokine C-C motif receptor-like 2							
Q27956	Leukemia inhibitory factor							
Q28178	Thrombospondin-1							
Q32PG1	AP-3 complex subunit beta-1; adaptor-related protein complex 3, beta 1							
Q3SYW5	5-azacytidine-induced protein 2							
Q3ZCM0	Tyrosine-protein kinase							
A5PIQ7	PXK protein							
Q5D0K3	MHC class II antigen							
A7Z055	PLAA protein							
Q8SQB1	C-C motif chemokine 20							

**Fig. 4.** Differential representation of immune system proteins. The differential representation of immune system proteins is shown as under-represented (red) or over-represented (green) proteins in latest time point when compared to earliest time point for the same group, or in vaccinated animals when compared to control animals. Comparative proteomics analyses were conducted using data from samples V0 vs. V9 and C9 vs. V9 (effect of vaccination), C0 vs. C12 and C9 vs. C12 (effect of skin test and challenge in control animals), V0 vs. V12 (effect of vaccination, skin test and challenge in vaccinated animals), V9 vs. V12 (effect of skin test and challenge in vaccinated animals) and C12 vs. V12 (effect of vaccination in challenged animals). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).



**Fig. 5.** GO analysis of differentially represented proteins for the characterization of the effect of vaccination in challenged animals. Over-represented and under-represented proteins in the C12 vs. V12 comparative analysis were grouped according to BP, MF and CC. Protein differential representation is shown in vaccinated cattle when compared to control animals.



**Fig. 6.** Regulatory pathways connecting the most upregulated BPs, MFs and CCs in the C12 vs. V12 comparative analysis. The most upregulated BPs, MFs and CCs (over-represented/under-represented proteins, O/U  $\geq 1.4$ ) at C12 vs. V12 were identified and mapped to identify the immune response proteins involved in these pathways. Protein under-representation (red) or over-representation (green) is shown in relation to the effect of vaccination, skin test and challenge in vaccinated and control animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

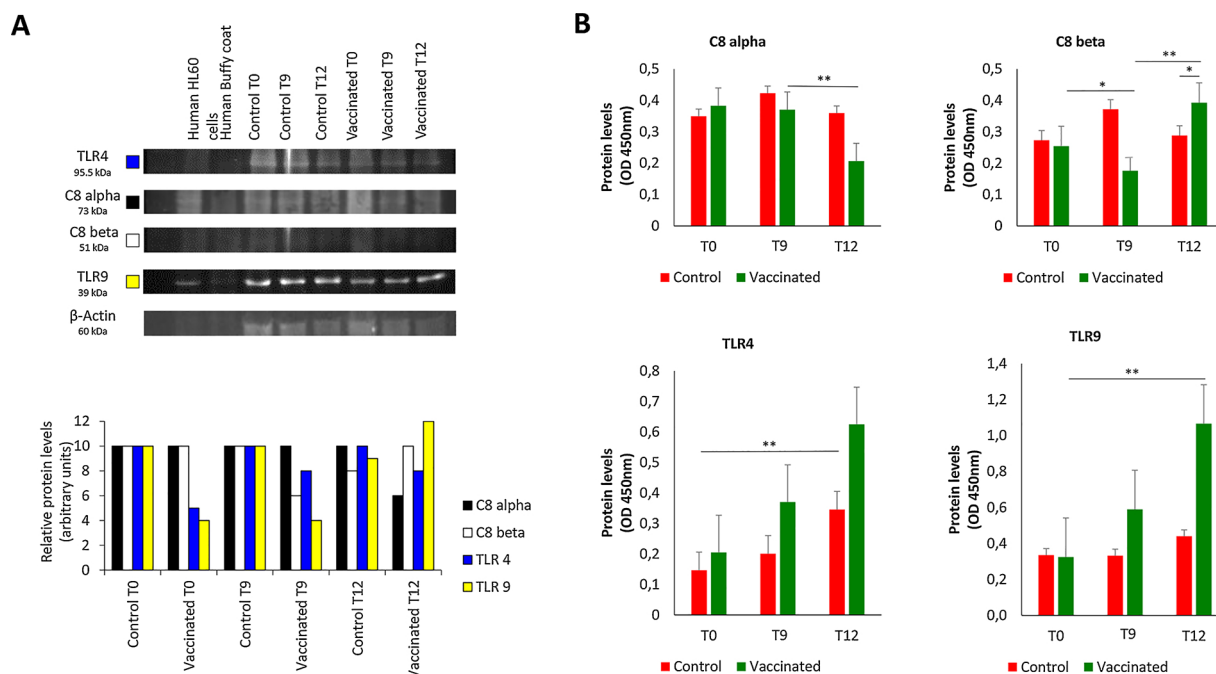
### 3.4. Western blot and ELISA analyses verified the proteomics results for selected immune system process proteins

Antibodies against selected immune system process proteins (C8 $\alpha$ , C8 $\beta$ , TLR4 and TLR9) and reactive against multiple species were selected for analysis by western blot and ELISA. Western blot analysis of the pooled samples of vaccinated and control cattle, from key time points (T0, T9 and T12), detected the presence of the selected cattle proteins (Fig. 7A and S2 Figure). A semi-quantitative analysis of relative protein levels after normalization against  $\beta$ -Actin showed a pattern similar to that obtained in proteomics (Figs. 4 and 7A and S2 Figure). Subsequently, an ELISA was used to quantitate protein levels in individual cattle samples (Fig. 7B). The results showed statistically significant differences between the time point comparisons that verified

the results of the comparative proteomics approach, thus providing additional support for the results of the study (Figs. 4 and 7B).

### 4. Discussion

The aim of the current study was to investigate the white blood cell proteome of vaccinated and unvaccinated control cattle in an attempt to characterize immune response proteins and pathways which play a role in mycobacterial disease. The host immune response profiles to the heat-inactivated *M. bovis* vaccine in a broader sense were recently studied in cattle (van der Heijden et al., 2017) and the results were collated in this study. A clear cell-mediated response to the inactivated vaccine was demonstrated by the BOVIGAM<sup>®</sup> assay (Fig. 1A) and this response was further corroborated by the reaction to the skin test at T9



**Fig. 7.** Verification of proteomics results. Antibodies against selected immune system process proteins were selected for analysis by western blot and ELISA. **(A)** Western blot analysis results. The intensity of protein bands in the membrane (upper panel) was determined by densitometric analysis, normalized against the intensity of the control  $\beta$ -Actin band developed using an anti- $\beta$ -Actin primary antibody and represented as relative protein levels in arbitrary units (lower panel). **(B)** Indirect ELISA results. The optical density (OD) was measured at 450 nm, represented as average + SD, and compared between groups at each time point by two-sample paired Student's *t*-test with unequal variance ( $P = 0.05$ ;  $N = 6$  biological replicates).

(Fig. 1C). Furthermore, a strong humoral response was detected in response to the inactivated vaccine (T9) (Fig. 1B). These results indicate that the vaccine is able to elicit distinct immune responses in cattle generally accepted as correlates of protection against BTB. To further elucidate the immune response profiles in vaccinated animals in more depth, a quantitative proteomics analysis was carried out. In total, 35 differentially represented immune system proteins associated with vaccination (C9 vs. V9 and V0 vs. V9), response to skin test and challenge in control animals (C0 vs. C12 and C9 vs. C12), response to skin test and challenge in vaccinated animals (V9 vs. V12), vaccination, skin test and challenge (V0 vs. V12) or effect of vaccination in challenged animals (C12 vs. V12) were identified (Fig. 4). Four of these differentially represented immune proteins were found to best fit the pathways connecting the most upregulated BPs, MFs and CCs; C8 $\alpha$ , C8 $\beta$ , TLR4 and TLR9 (Fig. 6).

#### 4.1. Differential representation of kinase activity and receptor activity molecular functions in immune systems biological processes

The four identified differentially represented proteins form part of immune system processes, have molecular functions in kinase activity and receptor activity pathways and are associated with the extracellular space, Golgi apparatus and endosome cell components (Fig. 6). Several proteins involved in essential signaling pathways of the host immune system, which are activated through the interaction of pathogen-associated molecular patterns (PAMPs) with pattern recognition receptors (PRRs) and may lead to inflammatory responses and antimicrobial action (Mogensen, 2009), were identified to be differentially represented at various time point comparisons in vaccinated, skin tested and challenged animals (Fig. 5). In these immune signaling cascades, a multitude of kinases and receptors are involved (Mogensen, 2009). Thus, it is not surprising that kinase and receptor activity MFs were upregulated after challenge, as this could be indicative of recognition of the pathogen by the innate immune system. This in turn may activate downstream immune mechanisms of the host, such as a myriad of

immunological signaling pathways or the complement system as well as adaptive immunity. Furthermore, differential representation of proteins involved in kinase activity which are closely related to JAK-STAT and PKC signaling were detected at several comparisons (Fig. 4). The cytokine-activated JAK-STAT pathway is known to be involved with downstream signaling through the receptor for interferon-gamma (IFN- $\gamma$ ) (Darnell et al., 1994; Shuai and Liu, 2003), a crucial cytokine in the control of mycobacterial infection (Flynn and Chan, 2001). Imai et al. (2003) demonstrated that infection with BCG in murine macrophages was associated with increased suppressor of cytokine signal (SOCS)-mediated negative regulation of the IFN-JAK-STAT pathway. Furthermore, deficiency or inhibition of STAT1 has shown to lead to increased susceptibility to mycobacterial infection (Imai et al., 2003; Shuai and Liu, 2003). The PKC pathway is involved in a wide variety of interactions with T cells (Isakov and Altman, 2013) and is inextricably linked to the JAK-STAT pathway as it is known to play a role in the phosphorylation of STATs (Shuai and Liu, 2003). It has previously been shown that regulation of proteins of the PKC family differs between infections with pathogenic or non-pathogenic mycobacteria (Chaurasiya and Srivastava, 2008), implying that members of the MTBC might alter the PKC pathway to evade the immune system of the host. Therefore, the findings of the present study could reflect an interaction, modulated by the immune system of the host, between signaling pathways in response to vaccination, skin test and mycobacterial challenge. The exact mechanism by which JAK-STAT and PKC family proteins exert their functions in mycobacterial infections was beyond the scope of this study and remains to be elucidated.

#### 4.2. Complement

In the current study, complement factor C8 $\alpha$  and C8 $\beta$ , were differentially represented compared to the control group at several time points (Fig. 4) and C8 $\beta$  was associated with the effect of vaccination in challenged animals (Fig. 6). The role of the complement system, which sets a coordinated enzyme cascade against foreign cells through



pathogen recognition, opsonization and pathogen elimination (Nesargikar et al., 2012; Merle et al., 2015), in mycobacterial infection has been studied quite extensively. Carroll et al. (2009) showed that mycobacteria use the host's innate immune system through activation of the complement system to facilitate uptake into macrophages and avoid killing. In contrast, vaccination triggered activation of the complement system using surface TLR signaling (Beltran-Beck et al., 2014), resulting in partial protection against BTB. Complement factor C3, specifically, has been reported to play a role in protection against BTB in wild boar (Beltran-Beck et al., 2014) vaccinated with the inactivated vaccine. However, complement factor C8 seemed to be involved in the response to the vaccine in cattle in the current study. Complement factor C8 is known to form an integral part of the membrane attack complex (MAC) which comprises complement components C5b-9 and is involved in direct killing through pore formation in the cell membrane and subsequent lysis, as well as activation of transcription factors and downstream immune signaling (Nesargikar et al., 2012). Therefore, the observed under-representation of C8 $\beta$  prior to challenge (V0 vs. V9) on the one hand, and the over-representation of C8 $\beta$  after skin test and challenge (V9 vs. V12 and C12 vs. V12) on the other hand suggests that the vaccine was able to initiate immune responsiveness ultimately leading to direct killing through the MAC. This might be induced in vaccinated animals only after challenge. Interestingly, C8 $\gamma$ , which is another C8 subunit (Bubeck et al., 2011), or any of the other complement factors that form part of the MAC, were not differentially represented in the current study. However, it is important to note that C8 $\alpha$  and C8 $\beta$  specifically are known to be most crucial to final MAC assembly (Bubeck et al., 2011). It is therefore speculated that over-representation of these C8 subunits exclusively, might facilitate more rapid and effective MAC formation. It is unclear, however, what could explain the discrepancy between C8 $\alpha$  and C8 $\beta$  differential representation at V9 vs. V12 (Fig. 4).

### 4.3. Toll-like receptors

The TLR family proteins are part of the innate immune response and are involved in pathogen recognition. In this study, TLR9 was found to be over-represented after vaccination, skin test and challenge in the proteomes of vaccinated animals (V0 vs. V12), whereas TLR4 was over-represented after skin test and challenge in control animals (C0 vs. C12) (Figs. 4 and 6). Toll-like receptor 4 has previously been found to play a role in immunity against mycobacteria (Quesniaux et al., 2004; Wang et al., 2011), and it is suggested to be important for a Th1 bias of the adaptive immune response (Heldwein et al., 2003). Meade et al. (2007) have found that expression of TLR4 genes is suppressed in peripheral blood mononuclear cells of *M. bovis* infected animals, implying that the pathogen may inhibit the immune response in this way to aid its survival in the host. Another PRR, that has been studied to a much lesser extent in relation to TB, is TLR9. Bafica et al. (2005) found that TLR9 indeed plays a role in the innate as well as adaptive immune response against TB in the murine model, through stimulation of pro-inflammatory cytokine release by dendritic cells and macrophages and mediation of a Th1 response, respectively. Interestingly, 'synergistic activity' between TLR2 and TLR9 was demonstrated, leading to increased resistance to mycobacterial infection (Bafica et al., 2005). Several other important immune mediators downstream of TLR signaling pathways, such as myeloid differentiation protein 88 (MYD88) (Quesniaux et al., 2004), toll-interleukin-1 receptor (TIR) domain-containing adaptor inducing IFN $\beta$  (TRIF) (Beltran-Beck et al., 2014), Nuclear factor  $\kappa$ B 1 (NFKB1) (Meade et al., 2008) and IL1 $\beta$  in combination with IL1R1 (Wang et al., 2011) have also been implicated in TB, emphasizing the importance of TLRs in TB immunity (Kleinnijenhuis et al., 2011). In the present study, the finding of an over-representation of TLR9 in response to vaccination, skin test and challenge in the proteome of vaccinated animals (V0 vs. V12) (Fig. 4) might suggest a protective signature in these animals. In fact, not only TLR9 but also

TLR2 was found to be overrepresented in animals after vaccination, skin test and challenge (V0 vs. V12) (Fig. 4) and these results should be validated in order to discern whether a similar mechanism as described by Bafica et al. (2005) might play a role here. Co-representation of these TLRs in the vaccinated animals could be indicative of a cooperative action against mycobacteria. Of interest is the finding by Kiemer et al. (2009) that attenuated mycobacterial strains are more capable of activating TLR9 signaling as compared to virulent strains. This could explain why animals vaccinated with the inactivated vaccine, showed increased TLR9 representation after skin test and challenge (V0 vs. V12). TLR4 has been shown to be capable of recognizing mycobacterial antigens (Heldwein et al., 2003; Quesniaux et al., 2004), but the exact role of TLR4 remains unclear as studies with TLR4 deficient mice have shown conflicting results (Kleinnijenhuis et al., 2011). Means et al. (1999) confirmed that TLR4 recognizes mycobacteria, but further demonstrated that it is unable to recognize heat-inactivated *M. tuberculosis*. Similarly, in the study presented here, an over-representation of TLR4 in control animals, but not vaccinated animals, in response to skin test and challenge (C0 vs. C12), was found (Fig. 4). This result might suggest that, in unvaccinated controls, live BCG is readily processed by TLR4. In contrast, in vaccinated animals, pathways through PRRs other than TLR4 might take over.

### 4.4. Limitations of the study

Animals were challenged with live *M. bovis* BCG rather than a virulent *M. bovis* due to practical constraints (van der Heijden et al., 2017). Therefore, future efficacy studies of the heat-killed *M. bovis* vaccine should include a virulent challenge coupled with assessment of pathological changes, in order to determine the true protective capacity of the vaccine.

In the current study, samples of two animals per group were pooled, bringing the total number of biological replicates per group to three. Pooling of samples is common practice in several "omics" fields (Peng et al., 2003; Kendziorowski et al., 2005), including that of proteomics (Diz et al., 2009). It is a useful approach when the available material per biological replicate is limited, to reduce biological variation, or when there are financial constraints (Karp and Lilley, 2007; Diz et al., 2009; Oberg and Vitek, 2009), as was the case in this study. Karp and Lilley (2007) have described that the use of several small pools of samples, as employed in this study, is an acceptable method which will still allow for estimation of the variance within treatment groups as well as the identification of outliers. As a result, since the purpose of this study was to evaluate and compare the proteome of vaccinated animals versus control animals on a group-level, the effect of pooling should be minimal.

Recently, van der Heijden et al. (2017) described the immune response profiles of cattle to the inactivated vaccine used in the present study, as well as to live *M. bovis* BCG and a formalin-inactivated BCG vaccine. The latter candidates were left out in this experiment, in part on account of financial considerations, but more importantly due to a lack of merit: The formalin-inactivated vaccine candidate showed very limited reactivity in cattle (van der Heijden et al., 2017) and was deemed ineffective. Principally, the BCG group was excluded from further evaluation because the epidemiological setting of southern Africa strongly favours the use of an inactivated vaccine. Furthermore, although BCG has been shown to convey protection with variable efficacy (Hewinson et al., 2003; Buddle et al., 2006), its immunogenicity appeared poor in the previous study and van der Heijden et al. (2017) demonstrated that the inactivated *M. bovis* candidate was more immunogenic.

## 5. Conclusion

In conclusion, this study provides a first glance at alterations to the bovine proteome in response to vaccination with heat-inactivated

*Mycobacterium bovis* and mycobacterial challenge. The GO analysis of the differentially represented proteins identified in this study provides insights into the role of regulatory immune system pathways in response to vaccination and mycobacterial challenge. Further studies into the exact role of these immune response proteins and the specific mechanism by which these regulatory pathways are activated is necessary and could potentially help identify novel biomarkers or correlates of protection against bovine tuberculosis in cattle.

## Declarations of interest

None.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vetimm.2018.10.013>.

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