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Humoral and cellular immune responses in sheep following administration of different doses of an inactivated phase I vaccine against *Coxiella burnetii*

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ABSTRACT

An inactivated *Coxiella burnetii* Phase I (PhI) vaccine (Coxevac®) is licensed in several European countries for goats and cattle to prevent coxiellosis. The vaccine is also applied to sheep, although detailed information about the ovine immune response and vaccine dose is missing. Eighteen gimmers from a *C. burnetii* unsuspected flock were randomly divided into three groups of six. Group 1 (Cox1) and 2 (Cox2) were vaccinated twice with 1 ml and 2 ml Coxevac®, respectively, three weeks apart (primary vaccination). The same procedure was applied with Cox3 (2 ml sodium chloride, control group). A third injection (booster) was performed after nine months. Potential side effects were determined by measuring the rectal body temperature and skin thickness at the injection site. Blood samples were collected to detect phase-specific IgM and IgG antibodies and interferon-γ (IFN-γ) release by immunofluorescence assay and ELISAs, respectively. Moreover, a cell infection neutralization assay determined the appearance of neutralizing sera. Body temperatures increased for one day post vaccination, and the skin swelled only slightly. Regardless of the vaccine volume, immunized sheep reacted first with an IgM and IgG PhII response. Ten weeks after the primary vaccination, IgG PhI antibodies predominated. Boosting eight months after primary vaccination resulted in a robust IgG PhI increase and strong IFN-γ response. In the vaccinated animals, the neutralizing effect is more widespread after the administration of 1 ml than after the treatment with 2 ml. In summary, differences between 1 and 2 ml Coxevac® are minor, and a vaccine volume of 1 ml seems to be sufficient. A booster after the primary vaccination is apparently necessary to stimulate the cell-mediated immune response in naïve sheep.

1. Introduction

Coxiella burnetii (C. burnetii) is an obligate, intracellular bacterium with high zoonotic potential. Ruminants are the main reservoir of the pathogen. They shed *C. burnetii* mainly during abortion or physiological parturition with birth products but also through feces and milk. Infected ruminants may suffer from coxiellosis, which includes reproductive disorders such as abortion, stillbirth, and weak offspring [1]. Especially in sheep, the health impact appears minor compared to other domestic ruminants, and healthy lambs are born [2–4]. Therefore, *C. burnetii* can remain unrecognized in sheep due to missing clinical signs [4,5]. Inhalation of *C. burnetii*-contaminated dusts and aerosols causes Q fever in about 40 % of infected people, and they develop flu-like symptoms [6]. Chronic manifestation of Q fever, particularly endocarditis, was reported in up to 5 % of patients [7,8]. Besides goats, sheep have caused many small-scale human Q fever epidemics throughout Europe, and infected flocks pose a risk to public health [9,10].

In recent years, the immune response to *C. burnetii* has been studied much more in goats than sheep. The phase-specific IgM and IgG Phase II (PhII) antibodies rise two weeks after experimental infection with *C. burnetii* Phase I (PhI) antigen in goats [11]. At the same time, IgM PhI also increases to a lesser extent, and IgG PhI rises after six weeks post infection. The first response of IgG

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PhII compared to IgG PhI was also observed in naturally infected goats and was associated with acute infection [12,13]. In sheep, the IgG response to a *C. burnetii* PhI-vaccine highly depended on the infection status of the animals. Sheep from the field with low-grade *C. burnetii* infection and almost no measurable antibodies initially showed an IgG PhII response [14]. Moreover, antibodies waned after more than one year. In contrast, pre-infected sheep with high phase-specific IgG levels still had elevated IgG PhI and PhII more than one year after immunization [3,14].

The cell-mediated immune response is essential for the protective immunity against natural *C. burnetii* infections, and IFN-ɣ plays a crucial role in controlling *C. burnetii* replication [15,16]. The T cell-mediated Th1 immunity seems critical for eliminating *C. burnetii* at later stages of infection [17,18]. Less is known about the cell-mediated immune response to *C. burnetii* in ruminants. After the experimental *C. burnetii* infection of goats, the release of IFN-ɣ increased after six weeks but seemed mainly dependent on parturition as on infection [11]. A subsequent study used an IFN-ɣ assay to measure the cell-mediated immune response in a naturally infected flock of sheep over ten years to assess the level of infection [19].

Vaccines have been developed for several decades to control *C. burnetii* in ruminants [20–26]. Many of them were formaldehyde-inactivated whole-cell vaccines (WCVs). Vaccines based on the PhI-antigen were more protective than vaccines containing PhII-antigens [22,27]. These results suggest that the PhI-antigen is an essential protective component, but it remains unclear if there are specific protective antigenic epitopes in PhI-antigen [28].

Since 2010, an inactivated *C. burnetii* PhI-vaccine (Coxevac®, Ceva Santé Animale, Libourne, France) has been licensed in many European countries for cattle and goats. This vaccine has also been widely used in sheep to control *C. burnetii* infections [14]. According to former product information, a dose of 1 ml is sufficient to vaccinate sheep, which is half of the recommended volume for goats. The manufacturer no longer maintains this recommendation, but it is widely used to immunize sheep throughout Europe [14,29]. An effective vaccine against *C. burnetii* requires the induction of long-lasting humoral and cell-mediated immune responses [16]. Therefore, the present study aimed to investigate potential differences of the Coxevac® volume, 1 ml, and 2 ml, on possible side effects and the humoral and cell-mediated immune response in sheep.

2. Material and methods

2.1. Animals

Eighteen healthy yearling sheep (Landrace of Bentheim) from a *C. burnetii* unsuspected sheep farm were tested for antibodies against *C. burnetii* by two phase-specific ELISAs (Euroimmun, Lübeck, Germany) and one commercial ELISA (IDEXX Q Fever AB Test, IDEXX B. V., Hoofddorp, The Netherlands). Afterward, the seronegative sheep were randomly divided into three groups, with six animals in each group.

Sheep were housed separately from all other animals in the Clinic for Swine and Small Ruminants. To prove the *C. burnetii* unsuspicious environment of the animal housing, six dust samples (Sarstedt AG & Co. KG, Nümbrecht, Germany) were collected by rolling the swab over 1 m on the barriers within the animal housing every six weeks as previously described [2]. These samples were pooled into two specimens and analyzed by a real-time PCR (IS*1111*; VetMAX™ *C. burnetii* Absolute Quant Kit, Thermo Fisher Scientific, Dreieich, Germany) following the manufacturer, and cycle quantification (Cq) values of ≤45 were assessed as positive. All dust samples from the animal housing tested *C. burnetii* negative during the one-year study period.

Procedures on sheep were licensed by the federal state government of Lower Saxony (Az. 33.8–42502-05-19A476). They were conducted in accordance with German animal welfare legislation and the EU Directive 2010/63/EU for animal experiments. All animals were handled according to high ethical standards and national legislation.

2.2. Skin thickness and body temperature

The first and third injections were applied on the left chest wall subcutaneously, whereas the second dose was injected into the right chest wall. Before sheep were vaccinated, an area of 15x15 cm on the lateral chest wall was shorn-free and regularly shorn during the entire study period. The position of the wool-free area was approximately 15 cm behind the shoulder and 20 cm below the backbone of each animal. This procedure was performed for all three injections. However, the third vaccination was placed 5 cm behind the first injection. On the day of vaccination, the skin thickness was measured by forming a skinfold at the injection site, and a manual readout with a skinfold caliper (Hauptner, "Analog", Solingen, Germany) was applied. After the vaccine was administered, the skin thickness was determined at weekly intervals for four weeks, followed by monthly intervals up to four months post vaccination. The value of the pre-injected skin measurement from every sheep was used as baseline levels to determine the increase in skin thickness.

Rectal body temperature was determined with a digital thermometer (digi-vet SC 12, WDT, Garbsen, Germany) after each vaccination once a day at 8 a.m. for seven days.

2.3. Vaccination and blood sampling

Sheep were vaccinated twice at intervals of three weeks with an inactivated *C. burnetii* PhI-vaccine (Coxevac®, Ceva Santé Animale, Libourne, France, Ch.-B.: 0405HG1D), and thereby the primary vaccination was completed as recommended by the manufacturer for cattle and goats. Group 1 (Cox1) and 2 (Cox2) received 1 ml and 2 ml, respectively. The volume of 1 ml contains 72 Q fever units (relative potency of PhI-antigen measured by ELISA compared to a reference item). It is approximately equivalent to 100 mg of inactivated corpuscular PhI-antigen of *C. burnetii,* according to the manufacturer. The third group (Cox3) received 2 ml of sodium chloride solution (NaCl 0.9 % ad us. vet., WDT, Garbsen, Germany; Ch.-B.: 18S1387) subcutaneously. A third injection (booster) was applied nine months after the first immunization to all animals with the same vaccine or sodium chloride volume and with the same vaccine charge. The period of nine months was derived from the manufacturer's recommendation for revaccination of cattle and was therefore also chosen for sheep. The injection was always performed with a new needle (Hypodermic Needle, 20Gx1½", WDT, Garbsen, Germany) for every sheep. The injection sites were regularly shorn and marked with a circle using a black skin marker (Edding, Wunstorf, Germany).

Blood samples with serum and lithium heparin tubes (Vacuette®, Greiner Bio-One, Frickenhausen, Germany) were taken from the *Vena jugularis*. Serum samples were centrifuged at 2000 g for 15 min, and the supernatant was stored for further examination at −20 °C. Lithium heparin blood was further processed as described below.

An illustrated overview of the use of Coxevac®/sodium chloride solution and sampling dates is presented in Fig. 1.

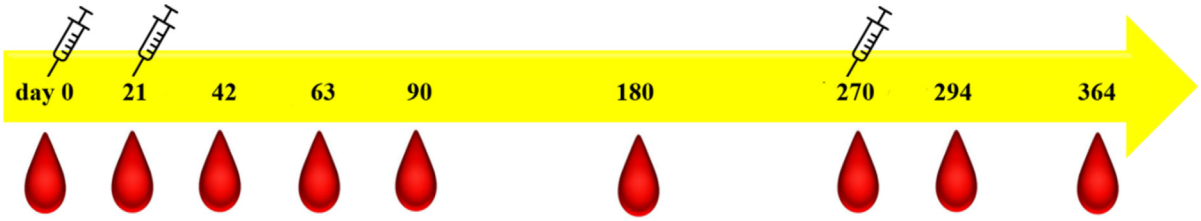

Fig. 1. Injection (syringes) and blood sampling (droplets) dates of 18 sheep.

2.4. IgM detection via immunofluorescence

The phase-specific IgM responses were measured with a modified indirect immunofluorescence assay. Firstly, sera (20 µl) were pre-incubated with 60 µl rabbit anti-sheep IgG (Thermo Fisher, Osterode, Germany) for 15 min at room temperature in Eppendorf tubes (Eppendorf, Hamburg, Germany) and then centrifuged for 10 min at 500 g. The supernatant was used to make the following dilutions: 1:4, 1:8, 1:16, 1:64, and 1:128. As controls, ovine precolostral serum (negative) and a positive serum from naturally infected sheep were used [14]. Dilutions, negative and positive controls were applied to *C. burnetii* PhI and PhII coated slides (Vircell, Granada, Spain). The slides were incubated for 90 min at 37 °C. Unbound serum was removed by washing with PBS (pH = 7.2) and distilled water. After drying off, 5 µl anti-sheep-IgM solution (MegaFLUO®Vet, Megacor, Hörbranz, Austria) was applied on the slides and incubated for 30 min at 37 °C followed by a washing step with PBS (pH = 7.2) and distilled water. Subsequently, each diagnostic field on the slide was fixed with a mounting medium and a cover slide. The slides were examined using UV light microscopy (Leitz, Labolux S, Wetzlar, Germany) at ×400 magnification. IgM seropositive samples were determined by fluorescence, whereas samples with no fluorescence were considered negative. The IgM titers were converted by $\log_2$ to illustrate the development of every study group [30].

2.5. IgG detection via ELISA

The IgG PhI and PhII immune responses were determined separately with two phase-specific ELISAs (Euroimmun, Lübeck, Germany). These ELISAs were applied according to the manufacturer's instructions and have been recently described in detail [14]. The test results were presented quantitatively in relative units (RU) determined by a standard curve.

Based on our long-standing experience and the reliable reproducibility of the assays, we used the ELISAs for IgG and the IFA for IgM detection.

2.6. IFN-γ detection assay

Every Lithium-heparin sample (280 µl) was stimulated within 2 h after collection with *C. burnetii* PhI (dilution 1:90) and PhII (1:10) antigen (Virion/Serion GmbH, Würzburg, Germany) within a 96-well plate (Greiner Bio-One, Frickenhausen, Germany). Moreover, control antigens of PhI and PhII (dilution 1:50, Virion/Serion GmbH, Würzburg, Germany) were applied additionally with every specimen. Pokeweed-mitogen (Sigma-Aldrich, Taufkirchen, Germany, 0.55 µg/ml) was used as stimulation control. After an incubation period of 16 to 18 h at 37 °C, stimulated samples were centrifuged for 5 min at 500 g. The supernatants were removed for further analysis. Subsequently, the IFN-γ release after T-cell stimulation was measured by an ELISA (ID Screen® Ruminant IFN-γ-Test, IDvet, Grabels, France). This ELISA was applied according to the manufacturer's instructions. Briefly, 10 µl of the stimulated serum samples were diluted with 90 µl buffer. The stimulated samples and the negative and positive control included in the test kit were incubated for 60 min at 37 °C. Six washing steps with 300 µl wash buffer followed. Afterward, 100 µl of the enzyme conjugate was added to each well, followed by an incubation time of again 60 min at 37 °C. Another wash step, as described previously, followed. After adding 100 µl of substrate solution, incubation for 15 min, and adding 100 µl of stop solution, the results were measured by photometer (BioTek, Bad Friedrichshall, Germany) using a wavelength of 450 nm. The level of interferon production was expressed in S/P % and determined by a standard curve.

2.7. Serum neutralization assay

Due to the methodological complexity of the newly established neutralization assay, ten sheep (four selected animals from Cox1 and four from Cox2, each with pronounced anti-*Coxiella* titers and two control sheep) were chosen for the analysis. In addition, blank samples (day 0), samples eight months after completion of primary vaccination (day 270), and three months after booster vaccination (day 364) were considered for this test.

In each of the three parallel approaches, the individual sheep sera were centrifuged for 30 min at 10,000 g and 4 °C to remove cell debris. Subsequently, *C. burnetii* (MOI 10) was added to 100 µl of sera and incubated for 6 h at 4 °C on a rotator. PBS/FBS were included as controls. The *C. burnetii*/serum suspension (100 µl) was then added to a six-well plate with 1 x 10^5 L929 reporter cells per well. *C. burnetii*-infected and non-infected reporter cells were used as positive and negative controls, respectively. After an incubation of 72 h, the samples were probed with anti-*Coxiella* antiserum to analyze the proportion of *C. burnetii*-positive cells by flow cytometry [31]. This procedure included cell trypsinization stopped with FBS. Subsequently, cells were centrifuged for 5 min at 1,000 g and washed with 200 µl washing buffer (10 % FBS in PBS). The reporter cells were fixed with 2 % paraformaldehyde in PBS for 10 min and washed/permeabilized with 200 µl incubation buffer (0.5 % BSA and 0.5 % Saponin in PBS). The permeabilized cell suspension was then incubated for 30 min at room temperature and treated with primary CoxII antiserum [31], kindly provided by Prof. A. Lührmann (Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany), in the incubation buffer for a further 60 min. Afterward, the reporter cells were incubated with a specific secondary antibody (goat anti-rabbit, Alexa488, CellSignaling) in incubation buffer for 30 min at 4 °C. Finally, the reporter cells were resuspended in 200 µl PBS and analyzed for *Coxiella*-infection using MACSQuant flow cytometry (Miltenyi Biotec, Bergisch Gladbach, Germany) [31].

The results of the blank sample (animals before vaccination) from every sheep were used as baseline levels. The proportion of infected cells was expressed in percentage of total cells.

2.8. Statistical analyses

Normal distribution was assessed by the Shapiro-Wilk test with all data. The rectal body temperature and the increase in skin thickness at each measurement time point were analyzed using

ordinary one-way analysis of variance (ANOVA) in conjunction with the Tukey test for multiple comparison with $\alpha = 0.05$ or Kruskal-Wallis test followed by Dunn's multiple comparison test with $\alpha = 0.05$.

In previous studies, Coxevac® induced an immune response in pre-infected sheep [3,14]. As the controls did not show any immune response against *C. burnetii* during the entire study period, only outcomes from IgM, IgG, and IFN-ɣ assays obtained from groups Cox1 and Cox2 were included in further statistical analyses. Data from each sampling date were analyzed by unpaired *t*-test or Mann-Whitney test.

Outcomes from the neutralization experiments were analyzed using analysis of variance (ANOVA) in conjunction with the Tukey test for multiple comparisons with $\alpha = 0.05$.

P-values < 0.05 were considered significant. All analyses were made using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Body temperature after vaccination

After the first vaccination, all treated sheep developed a body temperature of ≥40 °C after 24 h. The mean body temperature of both vaccinated groups was significantly higher than that of the non-vaccinated group (Fig. 2). Differences between Cox1 and Cox2 were not significant. Only one sheep from Cox2 developed pyrexia 24 h after the second immunization, but there were no significant differences between all three groups after the second vaccination. The booster vaccination (third injection) increased body temperatures mainly in the Cox2 group on day 1 post vaccination, which was significantly higher than in Cox3. In general, body temperature reached physiological levels 48 h after Coxevac® administration on all three vaccination dates.

3.2. Increase in skin thickness at the injection site

After the first immunization, the skin at the injection site was significantly thicker in group Cox2 compared to Cox1 and Cox3 at four measurement time points (Fig. 3). A vaccine volume of 1 ml did not increase the skin swelling significantly compared to animals that received sodium chloride.

The second vaccination of both study groups resulted in a significant difference in skin response between Cox1 and the control group only one week after vaccination. In summary, the skin swellings in group Cox1 were most of the time larger compared to group Cox2 and Cox3.

After booster vaccination, no significant differences were found in the increase in skin thickness among the three study groups.

3.3. IgM appearance during vaccination

Three weeks after the first vaccination, both vaccinated groups developed *C. burnetii* IgM PhI and PhII antibodies (Fig. 4A, B). The second immunization did not result in a stronger stimulation of IgM, and animals tested IgM negative after three months. The third vaccination (booster) again elicited a short IgM response (day 294). However, there were no significant differences in the IgM response between groups Cox1 and Cox2. The control group, Cox3, did not show any IgM reaction during the entire study period.

3.4. IgG appearance during vaccination

The first vaccination stimulated the IgG PhII response in both groups (Fig. 4C). Three weeks after the second vaccination, the

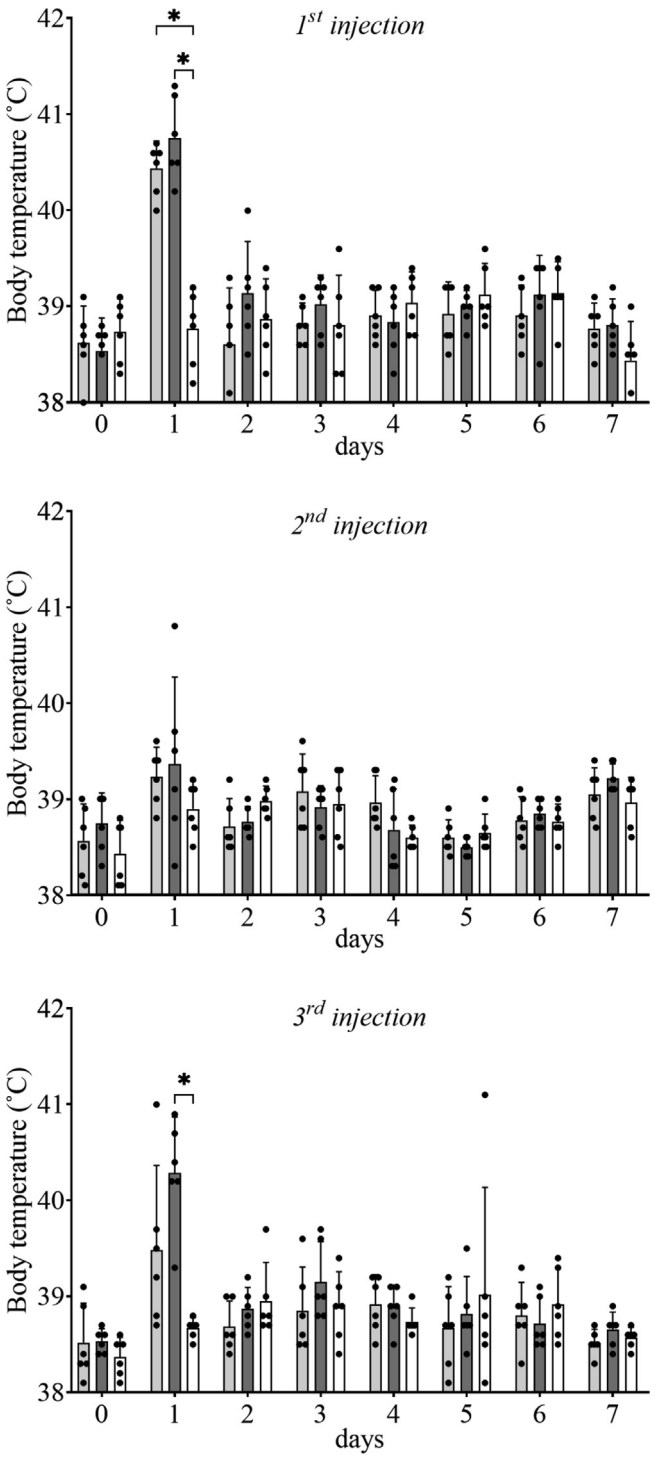

Fig. 2. Rectal body temperature (mean with 95% CI) of sheep within seven days after vaccination with 1 ml (Cox1, light grey) and 2 ml (Cox2, dark grey) Coxevac® at three different vaccination dates. One group (Cox3, white) served as a control. Dots represent individual values.

IgG PhI levels also increased, but the response was lower than for IgG PhII (Fig. 4D). Approximately two months after the second vaccine injection (day 90), the first peak of IgG PhI levels was reached, whose mean values were detectably higher than those of IgG PhII levels. Afterward, both phase-specific IgG levels decreased. The booster vaccination induced a strong IgG PhI and PhII response with a dominance of IgG PhI. A significant difference between the Cox1 and Cox2 groups was not observed until day 90

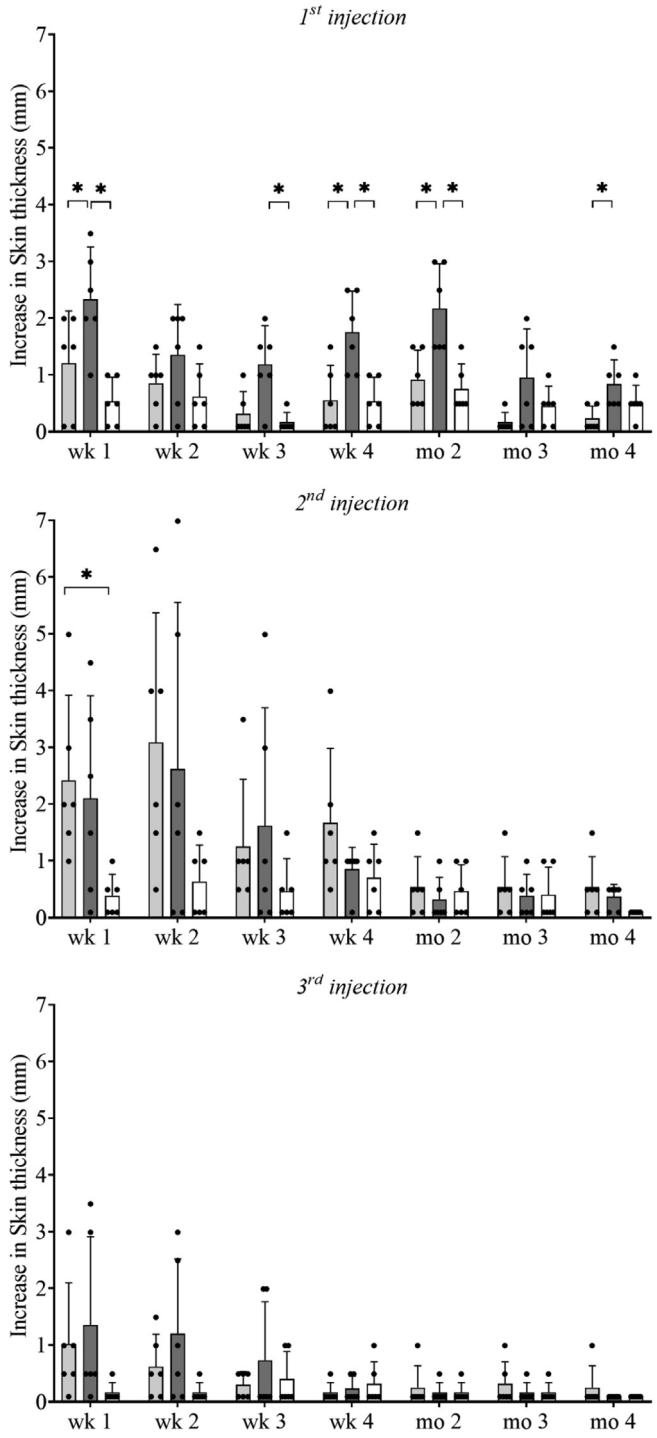

Fig. 3. Increase in skin thickness (mean with 95% CI) in sheep weeks and months after vaccination with 1 ml (Cox1, light grey) and 2 ml (Cox2, dark grey) Coxevac® at three different vaccination dates. One group (Cox3, white) served as a control. Dots represent individual values.

(69 days after second vaccination), with IgG PhI levels significantly higher in sheep vaccinated with 2 ml than in sheep vaccinated with 1 ml. The control group (Cox3) developed no *Coxiella*-specific IgG antibodies.

3.5. Interferon-ɣ appearance during vaccination

The primary vaccination induced only a weak IFN-ɣ response (day 42, PhI and PhII IFN-ɣ) in both study groups (Fig. 4E, F). About six weeks after the second injection, the IFN-ɣ response stimulated by *C. burnetii* PhI-antigen (PhI IFN-ɣ) was still feeble in both groups but significantly higher in Cox2 than in Cox1. In contrast, the booster dramatically stimulated the cell-mediated immune response in both study groups, with slightly higher levels for Cox2. Thirteen weeks after the booster vaccination, the IFN-ɣ levels decreased. This phenomenon could be observed for the PhI as well as the PhII IFN-ɣ assay (Fig. 4E, F).

3.6. Neutralization potential of sera against Coxiella infection

Eight months after the primary vaccination (day 270; day of third injection) and three months after the booster (day 364), the vaccine stimulated the production of neutralizing antibodies for Cox1 and Cox2, respectively, and less *Coxiella*-infected cells were determined. Thus, neutralizing *Coxiella* antibodies were detectably present in at least three sheep of Cox1 and one of Cox2 (Fig. 5), which may indicate a possible difference in vaccine efficacy between the two study groups. The serum of the two control sheep did not contain neutralizing antibodies.

4. Discussion

Although the inactivated *C. burnetii* PhI-vaccine, Coxevac®, has been used off-label in sheep for several years in Europe, little information is available on its effects on sheep health and immune response. In addition, there are conflicting recommendations for vaccine doses in sheep [19]. Therefore, the present study provides new insights into vaccine response in sheep and supports preventing Q fever cases in sheep and humans.

In our present study, the side effects of the three vaccinations were minor. A rise of rectal body temperature above 40 °C lasted for 24 h without affecting the general well-being of the sheep, which is in line with findings from Archard and Rodolakis [32]. The skin reaction in the present study is much less than that reported by the vaccine manufacturer for goats (3–4 cm) and cattle (9–10 cm). Only after the first vaccination, there was a significant difference in skin thickness between Cox1 and Cox2. This is possibly associated with the higher applied *C. burnetii* PhI-antigen in group Cox2. A repeated application of the vaccine resulted in a severe increase in skin thickness in goats, especially when the goats have been naturally infected with *C. burnetii* (the authors' observation). In our study, the sheep were *C. burnetii* naïve, which probably reduced the risk of skin swellings. Adverse skin reactions after vaccination were also not observed in naturally infected sheep [2,14]. Taken together, sheep seem to develop fewer side effects after Coxevac® application than goats. This suggests that the immunological reaction to the *C. burnetii* antigen is different compared to goats. Differences between sheep and goats in response to *C. burnetii* were hypothesized in previous studies and need further clarification to support reliable risk assessments [2,33,34].

Phase-specific ELISA showed a PhII-dominated immune response in sheep immediately after vaccination in both groups. This is in line with seronegative sheep from the field after immunization with Coxevac® [14]. The vaccine could also contain components of PhII-antigens corresponding to natural infection [35]. Another explanation might be a possible emergence of the PhII-antigens from PhI surface proteins during the production of the vaccine because the PhI *Coxiella* are inactivated and purified with formaldehyde to obtain the surface antigens contained in Coxevac®.

Both phase-specific IgM levels increased sharply after the first injection but showed a decreasing trend after second vaccination. The decay of IgM antibodies ten weeks (day 90) after the primary vaccination occurred independently of the amount of vaccine,

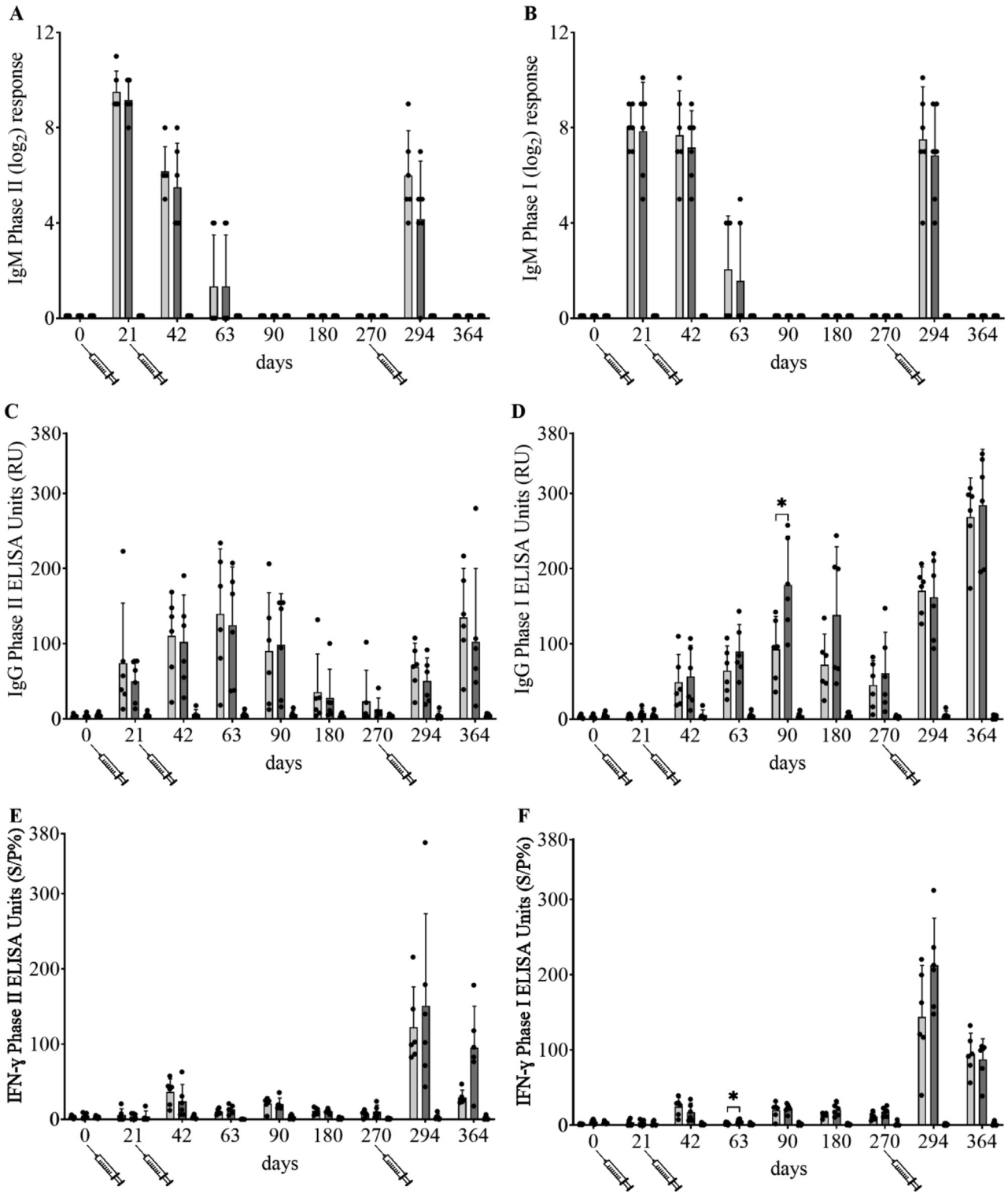

Fig. 4. IgM, IgG, and IFN-γ PhI and PhII response (mean with 95% CI) after triple vaccination (day 0, 21, and 270, syringes) with Coxevac®: 1 ml = light grey (Cox1), 2 ml = dark grey (Cox2), control = white (Cox3). Dots represent individual values.

which is consistent with results from vaccinated goats [20]. Moreover, similar results were obtained from goats after nasal *C. burnetii* inoculation indicating that the *C. burnetii* PhI-vaccine induces the same IgM response as an infection [11].

Three months after the start of the vaccination procedure, IgG-PhI antibodies outweighed humoral anti-*Coxiella* immunity in the vaccinated sheep. The rise of IgG PhII antibodies followed by a switch to IgG PhI dominance has already been observed in goats during acute infection [11,12] and in sheep after vaccination [14]. This phase change is thus characteristic of both natural infections and after vaccination. In naturally pre-infected sheep, the dominant IgG PhI immune response lasted more than one year after primary vaccination [3,14]. In contrast, IgG PhI levels in the present study decreased continuously after two administrations

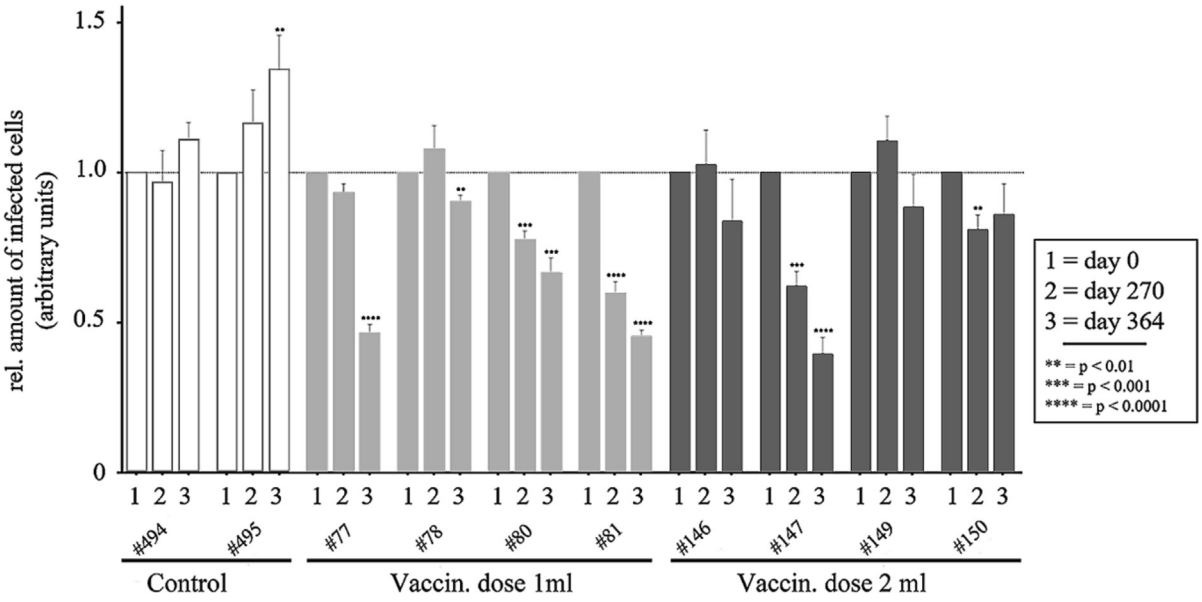

Fig. 5. *Coxiella*-infected cells after application of two different volumes of an inactivated *Coxiella burnetii* PhI-vaccine (Coxevac®), indicating the production of neutralizing antibodies. Vaccine dose: 1 ml = light grey (Cox1), 2 ml = dark grey (Cox2), control = white (Cox3). Day 0 = first injection, day 270 = third injection (booster), day 364 = 3 months post booster.

of the vaccine. These findings support the notion that the inactivated *C. burnetii* PhI-vaccine strongly stimulates the naturally acquired immunity in sheep and that a booster vaccination might be unnecessary for pre-infected sheep.

Recently, different approaches were applied to measure the IFN-ɣ release in small ruminants after experimental or natural *C. burnetii* infection [11,19]. Different assays and protocols were used, hampering direct comparison with our findings. After *C. burnetii* infection in sheep and goats, the IFN-ɣ release was low or delayed [11,19]. Similar outcomes were obtained after the primary vaccination in the current study and are in line with findings from Coxevac® vaccinated goats, which also displayed weak IFN-ɣ production after primary vaccination [36]. The lack of IL-10 neutralization in the lithium-heparin serum samples may be a "contributing factor" to the poor IFN-ɣ release after primary vaccination because IL-10 has an immunosuppressive effect. [19].

Interestingly, a strong release of IFN-ɣ occurs in samples after the third vaccination by stimulation with *C. burnetii* antigens (PhI or PhII). The appearance of IFN-ɣ after the third vaccination (and its previous absence) might be directly linked to IgG subclass changes controlling different antibody effector functions during the immune response. Thus, during the vaccination experiment, the humoral immune response could initially involve the synthesis of IgG1 (Th2 profile), while the production of IgG2 (Th1 profile) accompanies the observed cell-mediated immune response [37,38]. Our future studies will help to clarify this interesting and important point.

The neutralization assay used showed a tendency for more Cox1 animals to have protective antibodies after immunization than Cox2 animals. This apparent inverse dose-dependent effect when using 1 ml versus 2 ml could be caused by aggregation and/or precipitation of antigens at the injection site. The larger injection volume possibly increases the risk of such a phenomenon. As a likely result, antigen-presenting cells, such as dendritic cells and macrophages, may present fewer antigens, and the immune response may be correspondingly lower. The cytotoxic effect of the preservative thiomersal used in Coxevac® could also contribute to the reverse impact of the vaccine dose. Thiomersal is degraded in the body via thiosalicylate to ethyl mercury, which reacts particularly with thiol (SH) groups. Thus, there is an antimicrobial effect but also a cytotoxic effect. Clearly, the limited number of animals studied with the neutralization test also limits our assumptions. However, based on our preliminary results of the newly established neutralization assay, more sheep should be studied in future trials to provide more information about neutralizing antibodies as they complement the crucial T cell immunity. Overall, our results suggest an equivalent, if not a better neutralizing effect of the sera from vaccinated sheep after the application of 1 ml compared to 2 ml.

The immunity provided by inactivated vaccines is generally not as long-lasting as that obtained from live/attenuated vaccines. Multiple doses over time are needed to get ongoing immune protection [39,40]. Typically, the first dose does not produce protective immunity but "primes" the immune system. A protective immune response develops after the second and/or third dose [39,40]. Moreover, in contrast to live vaccines, the immune response to an inactivated vaccine consists mainly of antibodies [39,40]. Usually, there is little or sometimes also no cellular immunity. Nevertheless, Coxevac® induces a T cell response with well-detectable IFN-ɣ release after the boost (Fig. 4E, F), suggesting that vaccine-mediated immunity becomes fully effective only at this point in treatment. This indicates that Coxevac®, in addition to humoral immunity, also enhances the formation of a sustained cellular immune response after three vaccinations. This critical finding of our study may open the door to a link between inactivated vaccines and cellular immunity. It will be helpful for the development/improvement of inactivated vaccines. However, the mechanism by which Coxevac® induces cellular immunity upon an additional boost remains to be studied in more detail. A shorter period between the primary vaccination and the booster and more measurement time points could provide deeper insights into the cell-mediated immune response of Coxevac® in the future.

B cell/antibody- and T cell-mediated cellular immunity are required for an effective vaccine [39,40]. However, inactivated vaccines usually escape T cell immunity because the antigens do not persist in the organism for a substantial period. Vaccines must endure in the organism long enough for antigen presentation to T cells to activate this critical arm of the immune system. It is well

known that the immune system can not only discriminate self from non-self via pathogen-associated molecular patterns (PAMPs) [41], but it can also distinguish between live and dead pathogens through a distinct set of PAMPs called *vita*-PAMPs (e.g., mRNA detected by TLR8), which induce follicular T helper cell (T_{FH}) responses [42]. However, inactivated vaccines are not very good at producing T_{FH} [43]. Only using additional *vita*-PAMPs (like bacterial mRNA) in these vaccines could help improve their immunological effects [42,43]. In the context of our observations in Fig. 4, it is interesting that previous studies revealed that initially produced antibodies exert a significant immunomodulatory effect on the downstream T cell immunity [44]. Thus, a robust and protective cellular/interferon immune response against bacterial pathogens usually requires an effective primary humoral immune reaction characterized by antigen-specific antibodies (Fig. 4). Their role includes modulating Th1 activation via Fc receptors (FcR) [44] by facilitating a rapid uptake, processing, and presentation of pathogen-derived antigens by antigen-presenting cells (APCs) for an enhanced T cell response [44]. In this way, the different components of a combined immune response triggered by a pathogen or vaccine could cooperate to maximize their effector mechanism.

IFN-ɣ is an important mediator of endotoxin/LPS-induced immune responses [45]. The cellular and immunological factors regulating LPS-induced IFN-ɣ production are not fully understood. Nevertheless, it seems that the majority of IFN-ɣ-producing immune cells after LPS challenge are natural killer (NK) cells [45] which might also play a crucial role in the defense against *C. burnetii* [46]. As a PhI-vaccine, Coxevac® contains large amounts of PhI-LPS, which, as a bacterial virulence factor, is known to subvert activation of cellular responses via Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR 4) [47] and thus cannot act as "natural adjuvant" of the vaccine in cellular immunity. However, since Coxevac® might also contain, to some extent, the TLR-stimulating PhII-LPS, this could support the observed production of IFN-ɣ after the boost vaccination [48]. Indeed, activation of TLR2 and/or TLR4 is known to lead to the production and secretion of IFN-ɣ [49]. Clearly, further intensive work is needed to elucidate the immunological mechanisms of how Coxevac® as an inactivated vaccine can elicit a specific and potent T cell/ IFN-ɣ immune response after late boost vaccinations.

5. Conclusions

Overall, no significant differences were detected between the immune response to 1 ml and 2 ml vaccine volume (Fig. 6). Little side effects and the immune response indicate that Coxevac® is a safe and low-risk vaccine for sheep. In the context of the One Health concept, large-scale and consistent vaccination programs with appropriate vaccination schedules are desirable [50]. A lower vaccine volume of 1 ml for sheep compared to 2 ml in goats and 4 ml in cattle may lead to a higher acceptance by sheep farmers due to the reduced costs.

Based on the results of this study, a vaccination dose of 1 ml Coxevac® has no disadvantages compared to a vaccination dose of 2 ml Coxevac® in sheep. However, a booster is required to stimulate the cell-mediated immune response sufficiently. The current study did not determine the optimal time for the booster vaccination. An early booster vaccination e.g., after six months could avoid an immunological gap based on the waned IgG PhI response. This hypothesis should be investigated in further follow-up trials.

The vaccination of a larger number of animals and a subsequent challenge with *C. burnetii* is necessary to give more detailed recommendations regarding vaccine volume in sheep and its protective effect. An immunological IgG subclass test (e.g., IFT or ELISA) should be performed to analyze IgG subclass switching during vaccination. Novel vaccine approaches for Q fever should be focused on the cooperative stimulation/activation of humoral and cell-mediated immune responses to *Coxiella* [28].

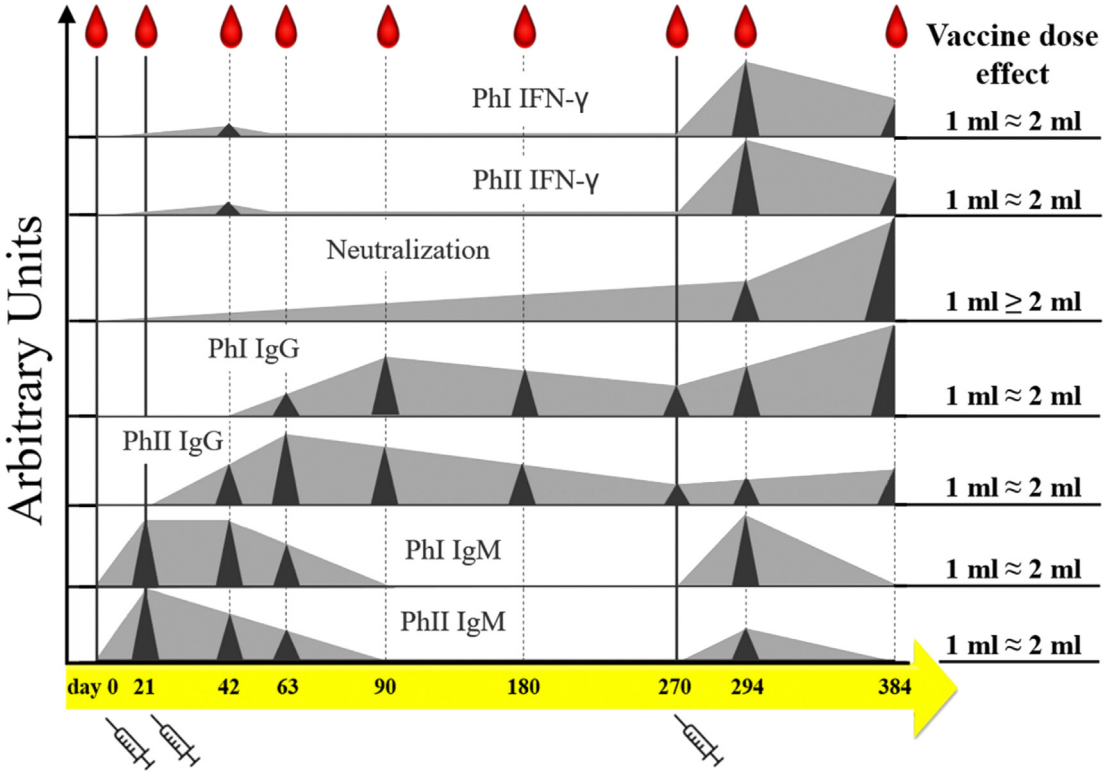

Fig. 6. Overview of sheep's humoral and cellular immune response after triple application of two doses (1 and 2 ml) of an inactivated *Coxiella burnetii* PhI-vaccine (Coxevac®).

CRediT authorship contribution statement

Benjamin U. Bauer: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Kay M. Schwecht:** Formal analysis, Investigation, Data curation, Writing – original draft. **Rico Jahnke:** Formal analysis, Investigation, Data curation, Writing – review & editing. **Svea Matthiesen:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Martin Ganter:** Methodology, Validation, Resources, Writing – review & editing. **Michael R. Knittler:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft, Writing – review & editing, Visualization.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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