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Early cytokine response after vaccination with *Coxiella burnetii* phase I in an infected herd of dairy cattle

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Abstract

Introduction: *Coxiella* (*C.*) *burnetii*, the aetiological agent of Q fever, is able to modulate the macrophage/T-lymphocyte axis in an infected organism and impair synthesis of monokines and lymphokines. **Material and Methods:** The purpose of this research was to determine the levels of the cytokines that play a key role in the response to *C. burnetii* antigens (IL-1β, IL-2, IL-6, IL-10, IFN-γ and TNF-α) in the serum of animals originating from an infected herd prior to vaccination (day 0) and at 1, 7, and 21 days afterwards. **Results:** The vaccination of animals did not affect the production of IL-6, IL-1β, or IL-2. The serum levels of these cytokines were too low to measure in most of the samples. The initial levels of TNFα, IFNγ, and IL-10 were higher in seropositive than in seronegative animals, although significant differences between seropositive shedders and seropositive nonshedders appeared only in the levels of IFNγ and IL-10. Additionally, the course of the post-vaccination response concerning these two cytokines was different among seronegative nonshedders, seropositive nonshedders, and seropositive shedders. **Conclusion:** It seems that analysis of the IFNγ and IL-10 concentrations in animal blood serum may have some practical value in an assessment of the health status of seropositive animals and post-vaccination response.

Keywords: dairy cattle, *Coxiella burnetii*, vaccine, cytokines.

Introduction

Q fever caused by *Coxiella burnetii* is a worldwide zoonotic disease that affects livestock, companion animals, and humans (1). *C. burnetii* is an obligate intracellular bacterium, replicating exclusively in the phagolysosomes of the host's cells (14, 17). The pathogen occurs in two antigenic phases: highly virulent phase I and avirulent phase II (1, 9, 16, 17). Ruminants are the most important reservoir of this pathogen, and cattle, sheep, and goats facilitate transmission as they may shed large quantities of *C. burnetii* with birth products, milk, urine, and faeces (7). Two strategies are implemented to control *C. burnetii* infection in ruminants. The non-medical approach advocates hygiene, and the medical approach applies treatments including vaccines and tetracycline therapy. Antibiotic therapy is not very effective while vaccination might significantly reduce the level of *C. burnetii* shedding in infected animals (16).

Although vaccination induces a Th1-type immunological response, which is responsible for post-vaccine protective immunity (14), it is mainly the concentration of circulating antibodies that is considered in an assessment of the post-vaccination response. The only test applied thus far to evaluate the cellular response is the skin test method (for delayed-type hypersensitivity) (11). In a cohort study on human patients conducted by Schoffelen *et al.* (11, 13), the determination of IFNγ-specific production by patient blood leukocytes was proven to have clinical value in diagnosing both infections and post-vaccine responses.

Additionally, the production of IL-2, pro-inflammatory IL-1β, TNFα, and IL-6, as well as anti-inflammatory IL-10, distinguished seropositive from seronegative patients (11).

There is only one report, published by Sobotta *et al.*, (15) on cytokine response to *C. burnetii* in cattle describing the *in vitro* infection model utilising bovine macrophages. In this study the inflammatory response of primary monocyte-derived macrophages resulted in very fast but transient upregulation of IL-1β, IL-12, and TNFα gene expression. This response declined within as short a time as 24 h, which correlated with the pathogen intracellular replication. In the present study we decided to determine the levels of the cytokines that play a key role in the response to *C. burnetii* antigens *i.e.*, IL-1β, IL-2, IL-6, IL-10, IFN-γ, and TNF-α in the serum of animals originating from a herd infected prior to vaccination (day 0) and at 1, 7, and 21 days afterwards.

Material and Methods

Animals used in the study. The investigations used a *C. burnetii*-infected herd of Holstein-Friesian dairy cows (n = 68) dwelling in north-eastern Poland. Animals were fed TMR (total mixed ration) and kept in a loose-housing system with free access to a feeding table and rubber mats, without the possibility of daily exercise. The cows were in the second or third lactation cycle. The herd was maintained in appropriate sanitary conditions. Bronchopneumonia, the first clinical symptom of the disease, began about four weeks after introducing new individuals into the herd. Other infections, *e.g.* with bovine herpesvirus type 1 (BHV1), bovine viral diarrhoea virus (BVDV), bovine respiratory syncytial virus (BRSV), and parainfluenza type 3 virus (PI3), were ruled out based on laboratory tests. A few months later the first spontaneous abortions occurred (n = 9). There were also other reproductive disorders observed in almost half of the animals (placenta retention, endometritis, and drastic drop in insemination effectiveness). On the grounds of serological results, qPCR tests, genotyping by multi-locus sequence typing (MLST), and multiple-locus-variable number tandem repeat analysis (MLVA), *C. burnetii* infection was confirmed. The National Reference Laboratory (NRL) for Q fever of the National Veterinary Research Institute in Pulawy, Poland performed these assays and substantiated the infection. Two cows were eliminated from the herd before vaccination. All other cows (n = 66) were immunised with the Coxevac commercial vaccine (CEVA Santé Animale, France) according to the manufacturer's instructions. Prior to the vaccination, the presence of serum antibodies and shedding of bacteria with cow's milk had been determined in all cows.

Serological and qPCR examinations before vaccination. The serum samples for serological tests (n = 66), individual milk samples (n = 66), and vaginal swabs (n = 66) were taken from the cows. The presence of serum *C. burnetii* antibodies was determined with the IDEXX Q Fever Ab Test (IDEXX, Switzerland) according to the manufacturer's instructions. The test served to detect antibodies specific to antigens of phase I and II *C. burnetii*. The shedding of bacteria was verified based on an analysis of milk samples and vaginal swabs with the qPCR method, which detected the insertion fragment IS*1111* of the transposase gene. This part of the research was carried out in compliance with the guidelines by the diagnostic kit's producer, the kit being an ADIAVET™ Cox Real Time (Biomérieux, France) performed on a 7500 Fast Real-Time PCR apparatus with 7500 v2.3 software (Applied Biosystems, USA). Additionally, the qPCR samples in order to confirm *C. burnetii* identification were subjected to genotyping using MLST and MLVA methods. Phylogenetical analysis revealed that the strain infecting this herd belonged to a new sequence type (ST61) and represented genotype I (data in press). The ELISA, qPCR tests, and genotyping were conducted at the NRL for Q fever.

Sample collection after vaccination. Based on results of pre-vaccination qPCR and ELISA, the animals were divided into four groups: seronegative nonshedders (n = 28), seropositive nonshedders (n = 28), seropositive shedders (n = 7), and seronegative shedders (n = 3). Blood samples were taken from all 66 milking cows prior to vaccination (day 0) and at 1, 7, and 21 days afterwards. Blood samples were collected using plastic vacuum tubes and transported within a 90-min period to the laboratory. According to the manufacturer's instructions, all animals in the herd must be vaccinated, therefore creation of a control group without vaccination was not possible.

Determination of cytokines in blood serum. The sampled blood was left for 2 h at room temperature until it clotted, after which it was centrifuged for 20 min at 1,000 × g. Samples in aliquot were stored at −80°C. After the final sampling, the levels of cytokines were determined in all collected samples. The serum levels of IL-1β, IL-2, IL-6, IL-10, TNFα, and IFNγ were determined using commercial immunoassay (ELISA) kits (Uscn Life Science Inc., USA) according to the manufacturer's protocol. All serum samples were tested in duplicate.

Statistical analysis. Data regarding the cytokine response to the vaccine within the groups were subjected to one-way analysis of variance (ANOVA). The Bonferroni post-hoc test was used to determine differences between day 0 and the subsequent samplings within each group. Because of the unequal number of animals in the groups, differences between seronegative nonshedders (n = 28), seropositive nonshedders (n = 28), and seropositive shedders (n = 7) on particular sampling days were assessed with the non-parametric Kruskall-Wallis test followed by Dunn's post-hoc test. Due to the small number of seronegative shedders (n = 3), the results obtained from this group were not statistically analysed or compared to results from the other groups.

Statistical evaluation of the results was performed using GraphPad Prism software (GraphPad Software, USA).

Results

Determination of serum IL-6 in tested animals was unsuccessful, therefore evaluation of vaccination impact on IL-6 production was unworkable. Likewise, the levels of IL-1β and IL-2 in most samples were undetectable. In seronegative nonshedders, the levels of these two cytokines individually exceeded the detectability threshold in only 16% (IL-2) and 19% (IL-1β) of the measurements. In the seropositive shedders, IL-2 was detectable in 28.5% of the group, while IL-1β could be determined in 25% of the cows. It was only in the seropositive shedders that both cytokines together reached detectable levels in serum, which was evident in as many as 42.8% of the animals (data not shown).

The group of seronegative shedders (n = 3) was characterised by a very weak cytokine response with regard to the cytokines determined. No measurable concentrations of IL-6, IL-1β, IL-2, or IFN-γ were achieved in any of the samplings, while low levels of IL-10 were detected in the first three samplings. It was only TNFα that remained at a higher level in the seronegative shedders throughout the whole experiment (Fig. 1).

Early cytokine response to vaccination with Coxevac in seronegative nonshedders, seropositive nonshedders, and seropositive shedders (differences between day 0 and the subsequent samplings within groups). The serum levels of IFN-γ, TNFα, and IL-10 in seronegative nonshedders were detectable throughout the entire experiment. Levels of these cytokines increased after vaccination. IFNγ and TNFα production peaks appeared 24 h post-vaccination, and significantly elevated levels of both cytokines lasted for seven days after the injection (IFN-γ at $P < 0.05$, and TNFα at $P < 0.001$). By the last sampling date, three weeks after the vaccination, both cytokine levels had declined to the pre-vaccination values (Fig. 1). IL-10 responded differently. The production of this cytokine increased significantly ($P < 0.05$) within just a day of vaccination but did not peak until day seven post vaccination and stayed at a high level until the experiment terminated ($P < 0.05$) (Fig. 1).

In the seropositive nonshedders, levels of IFN-γ, TNFα, and IL-10 also increased after vaccination. The peak production of TNFα occurred 24 h post vaccination ($P < 0.001$) and remained at a higher level until day seven ($P < 0.05$), after which it declined, returning to the initial value by day 21 after vaccination (Fig. 1). IFNγ responded more slowly. A considerable increase in the production of this cytokine was observed as early as 24 h after vaccination ($P < 0.05$), but the peak production did not occur until day seven. Three weeks after vaccination, the level of IFNγ had returned to the base value (Fig. 1). Finally, the production of IL-10 began to increase no earlier than on day seven after vaccination, and the increasing trend continued until the end of the experiment, meaning that the highest level of this cytokine was measured in serum from the last sampling ($P < 0.05$) (Fig. 1).

In the seropositive shedders, the levels of all cytokines tested were high before vaccination, with IL-10 reaching the highest content (Fig. 1). Vaccination did not have a significant effect on the production of IFNγ and IL-10. The serum concentrations of both cytokines remained high throughout the whole experiment, slightly decreasing no earlier than 21 days post vaccination, although the differences were not statistically significant (Fig. 1). It was only the level of TNFα that showed a transient increase after vaccination. Twenty-four hours after the injection, the level of this cytokine was considerably higher than before the treatment ($P < 0.05$), although seven days after the vaccination, it fell to the basis value (Fig. 1).

Comparison of the course of individual cytokine responses between the seronegative and seropositive animals (differences between groups). The initial level of IFNγ was much higher in the serum of seropositive shedders than in the serum of cows from the other groups ($P < 0.001$). The dynamics of the post-vaccine response regarding this cytokine were similar in both groups of seropositive cows and differed from the response of the seronegative animals. The peak of IFNγ production in the seronegative nonshedders was noted 24 h after vaccination, while the response of the seropositive animals was delayed, peaking on day seven post vaccination. The final level of this cytokine in the seropositive shedders was much higher than in the other groups ($P < 0.001$) (Fig. 2A).

The post-vaccination response of TNFα followed a similar course in all the groups. Although the basis level of this cytokine was much higher in the seropositive than in the seronegative animals ($P < 0.001$), the dynamics of its response to the vaccine were similar, with peak production occurring 24 h after vaccination and the eventual equalisation of its serum content in all the groups ensuing three weeks after vaccination (Fig. 2B).

The most diverse response to the vaccine, where the groups of animals having a different status were distinguished, appeared with respect to IL-10. The basis concentration of this cytokine was considerably higher in the seropositive than in the seronegative animals ($P < 0.001$), and the differences persisted at a similar level throughout the whole experiment. In seronegative animals, the IL-10 production peak appeared seven days after vaccination, after which a notable decrease in its serum concentration took place. In the seropositive nonshedders, the vaccine caused a slow but persistent increase in the production of IL-10 until the end of the experiment. Finally, in the seropositive shedders, a high level of this cytokine persisted over the course of the entire study. Although a gradual and slight decrease in the level of IL-10 was observed, it was not statistically significant (Fig. 2C).

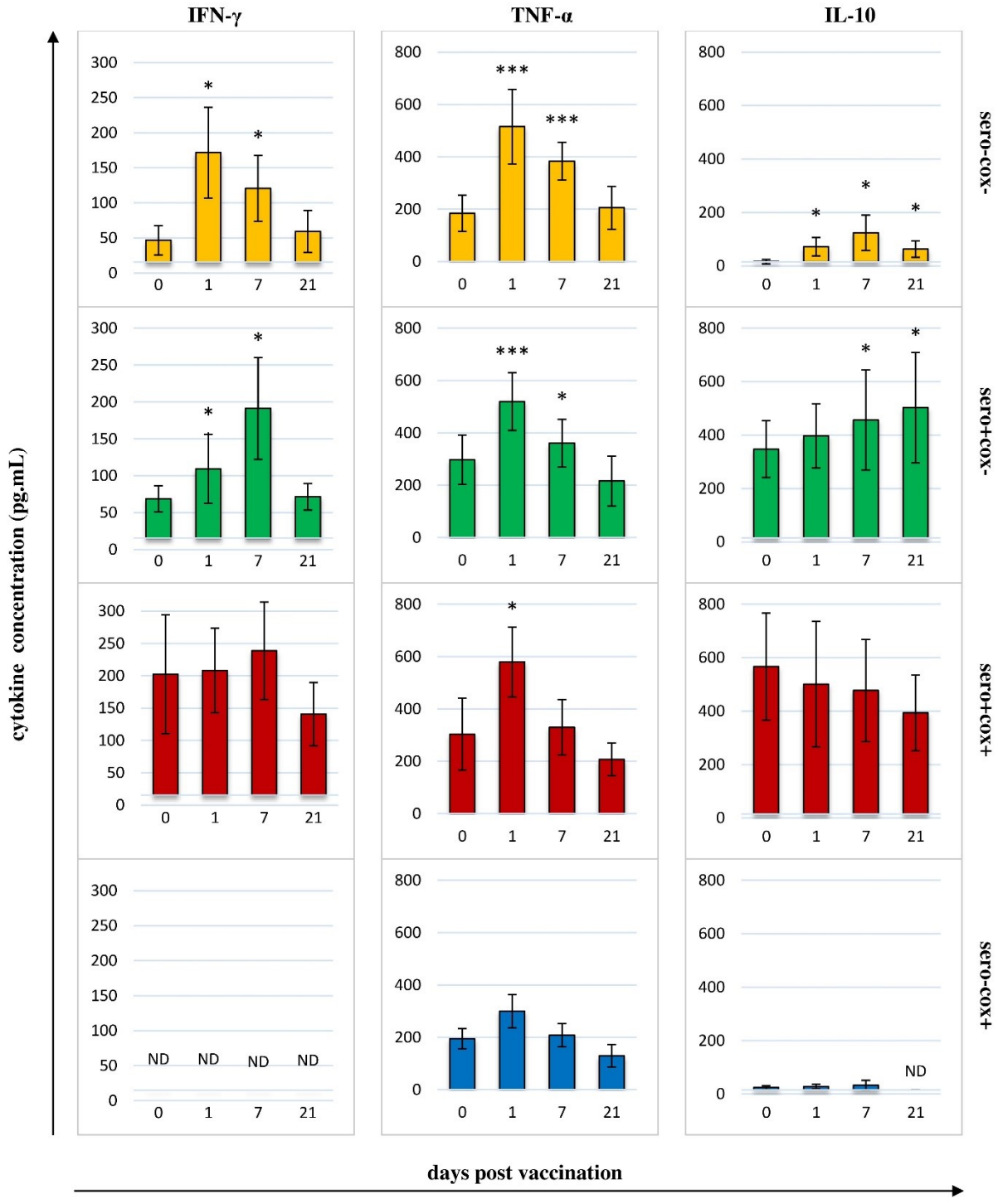

Fig. 1. Early cytokine response to vaccination with Coxevac in dairy cattle. Sero−cox− – seronegative nonshedders, n = 28; sero+cox− – seropositive nonshedders, n = 28; sero+cox+ – seropositive shedders, n = 7; sero−cox+ – seronegative shedders, n = 3. All data expressed as mean values ±standard deviation. Cytokine response to the vaccine within the groups was subjected to one-way analysis of variance (ANOVA). The Bonferroni post-hoc test was used to determine differences between day 0 and the subsequent samplings within each group. Asterisks refer to statistically significant differences at: * $P < 0.05$, *** $P < 0.001$. ND – not detectable cytokine level

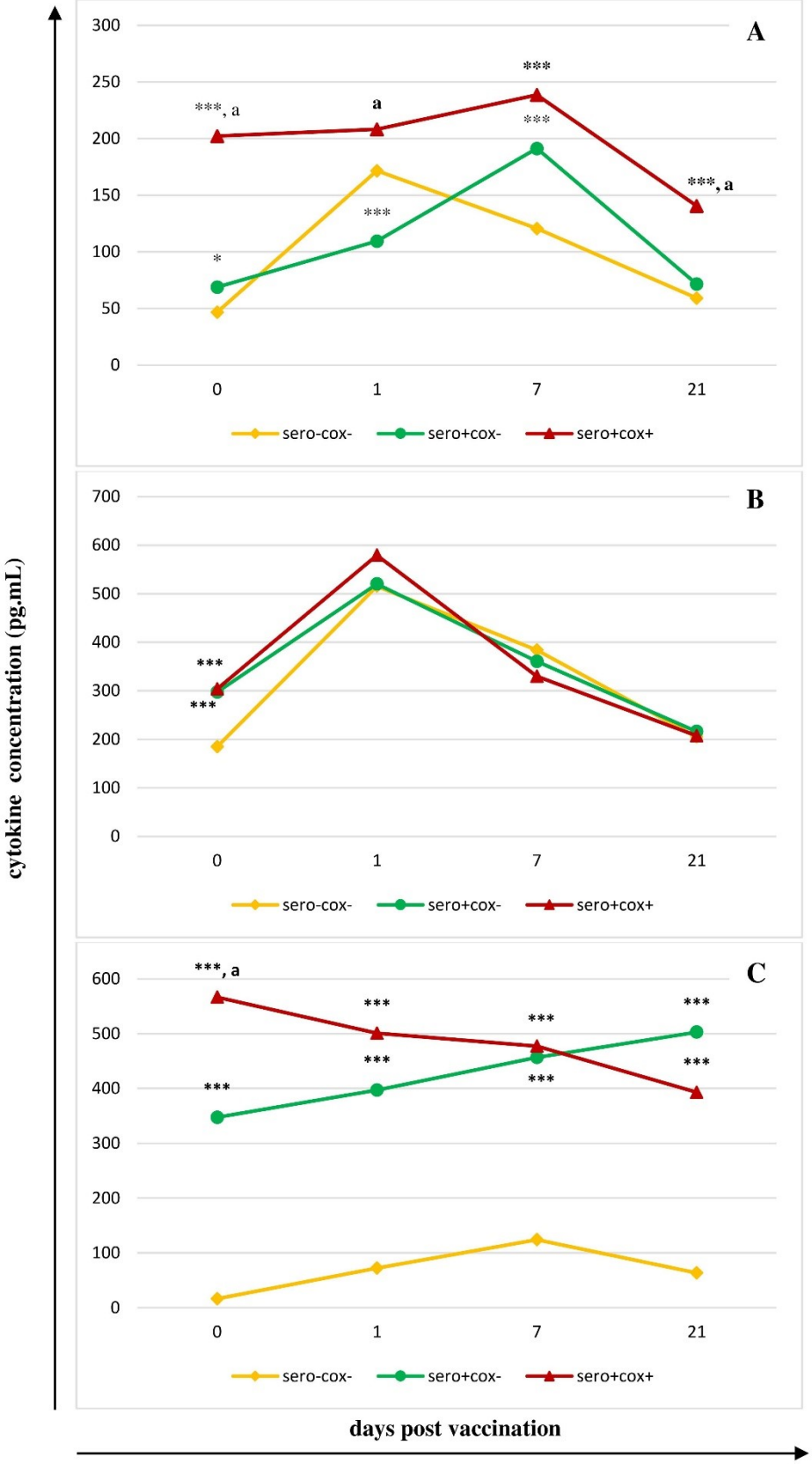

Fig. 2. Comparison of the course of individual cytokine responses between the seronegative and seropositive animals. A – IFNγ, B – TNFα, C – IL-10. Sero−cox− – seronegative nonshedders, n = 28; sero+cox− – seropositive nonshedders, n = 28; sero+cox+ – seropositive shedders, n = 7. All data expressed as mean values. Because of the unequal number of animals in the groups, differences between the groups on particular sampling days were assessed with the non-parametric Kruskall-Wallis test followed by Dunn's post-hoc test. Asterisks refer to statistically significant differences between seronegative and seropositive animals at: * $P < 0.05$, *** $P < 0.001$; a – refers to statistically significant differences between seropositive nonshedders and seropositive shedders at $P < 0.001$

Discussion

Commercial vaccines against Q fever administered to human patients, *e.g.* Q-Vax, and to ruminants, *e.g.* Coxevac, contain inactivated phase I *C. burnetii* because the protective action of the vaccines requires the presence of the bacterium's full phase I lipopolysaccharide (LPS) (9). The human vaccine is used only to protect people at risk of occupational exposure, but is not available in Poland or most other European countries. Human vaccination sensitises T lymphocytes, which occurs 10 to 15 days after injection and lasts for at least 5 years, while the antibody response is modest and transient (6). No revaccination is carried out in human patients before the five-year period elapses. In ruminants, the main aim of vaccination is reduction of pathogen shedding by infected animals, which results in a significant reduction in abortion rates. Annual vaccine recall doses are recommended in veterinary practice (9, 17), however according to Rodolakis *et al.* (9) not all individuals in a herd require annual recall vaccines. This was proven by the detection of immunological markers (levels of antibodies and positive results in a skin test). However, the cytokine response of an organism after vaccination had not been tested in cattle prior to writing. This is the first study to monitor the efficacy of cattle herd vaccination with a phase I *C. burnetii* vaccine by measuring the cytokine levels. It brings new insights into the field of early cytokine response in vaccinated dairy cattle and supplements the *in vitro* results obtained by Sobotta *et al.* (15).

The evident post-vaccination response of IFNγ, TNFα, and IL-10 observed in our study was predictable, as these are central cytokines engaged in both the immunological response to *C. burnetii* antigens and in the pathogenesis of chronic Q fever. However, the weak response of IL-1β and IL-2 and the lack of any response of IL-6 may be surprising because these results are divergent from the findings reported by others (4, 8, 10, 12, 13). In human research on Q fever, Schoffelen *et al.* (13) demonstrated a considerably higher specific production of IL-1β, IL-2, and IL-6 by whole blood leukocytes in seropositive patients than in seronegative patients. Overproduction of IL-6 by the patients' peripheral blood mononuclear cells (PBMC) stimulated by *Coxiella* antigens was observed both in acute (4) and chronic Q fever (8). In both cases, a correlation between the IL-6 concentration and the clinical manifestation of the disease was observed. In another *in vitro* experiment, splenocytes of mice infected with *C. burnetii* produced IL-6 at an early stage of infection, while IL-1β remained undetectable (12). Also in an *in vitro* experiment by Sobotta *et al.* (15) bovine macrophages infected with *C. burnetii* did not release detectable levels of IL-1β protein, despite upregulated IL-1β gene expression. However, all these studies are based on *in vitro* stimulation of a patient's cells. Only one study was performed to detect these cytokines in serum in the course of acute infection in mice and guinea pigs. As in our experiment, the researchers were unable to detect measurable levels of IL-1β and IL-2; however, they detected IL-6 (10). The differences between our data and the results reported by the researchers may be caused by species-specific characteristics (cattle *vs.* humans or rodents) or by different research models (serum *vs. in vitro* cultured cells from a patient). Serum concentrations of some locally produced, macrophage-derived pro-inflammatory cytokines (IL-1β and IL-6) might be too low to be detected. However, it is highly likely that the local cytokine response at the site of vaccination would have been more pronounced, but its assessment at whole herd level was not possible.

The initial pre-vaccination levels of IFNγ, TNFα, and IL-10 in our study were much higher in the seropositive than seronegative animals, and the seropositive shedders produced much higher amounts of IFNγ and IL-10 than the seropositive nonshedders. It has long been known that IFNγ plays a critical role in the development of the protective Th1 immune response against intracellular pathogens such as *C. burnetii*. In an *in vitro* study on human THP-1 monocytes infected with *C. burnetii*, exogenous IFNγ stimulated TNFα production, decreased the viability of bacteria, inhibited their intracellular growth, and caused apoptosis of infected cells (2). In an experiment on mice infected with different strains of the bacterium, high serum levels of IFNγ correlated with the control of the bacterium's growth and the stimulation of phagocyte functions (10). Schoffelen *et al.* (11) demonstrated the clinical value of the determination of specific IFNγ production by *in vitro* stimulation of a patients' blood with *C. burnetii* antigens for confirmation of previous infection. The specific production of IFNγ was considerably higher in seropositive than in seronegative patients. In the study carried out by Penttila *et al.* (8), the PBMC of patients with chronic Q fever released much higher amounts of not only IFNγ but also IL-10 following *C. burnetii* stimulation when compared with healthy subjects.

Ghigo *et al.* (3), who studied the role of IL-10 in the replication of *C. burnetii* in monocytes, confirmed that IL-10 promotes bacterial replication by inhibiting production of TNFα through an autocrine loop, which contributes to the chronic course of the disease. Monocytes of patients with Q fever endocarditis are unable to kill pathogens, unlike monocytes of patients with acute Q fever or healthy subjects. Endocarditis patient monocytes are characterised by overproduction of IL-10, both spontaneously and in response to pathogen antigens, while the monocytes of patients with acute Q fever release small quantities of IL-10. These observations coincide with our results, such as a much higher level of IL-10 in the seropositive shedders than in the seropositive nonshedders.

To verify the prognostic values of IL-10 and TNFα in humans, their levels were measured in patients with different courses of the disease over a period of three years. In patients with an uncomplicated Q fever course, the levels of these cytokines gradually decreased to the

levels noted in healthy people. In patients with Q fever endocarditis, the level of TNFα remained unchanged or increased significantly, while that of IL-10 decreased. According to Honstettre *et al.* (4), a persistently high level of TNFα could serve as a marker of the chronic form of Q fever. In the light of these results, eventual equalisation of TNFα serum levels may confirm the protective effect of the vaccine, despite the initial differences between seropositive and seronegative animals in our experiment.

The course of the post-vaccine response in our experiment with respect to IFNγ and IL-10 enabled us to distinguish between the seropositive and seronegative individuals, while the TNFα response dynamics were similar in all animals, and these results partly reflect the literature data. After vaccination of a human patient, CD4 and CD8 cells produce IFNγ, which may be responsible for the strong protective effect of the vaccine. On the other hand, through an interaction with monocytes and macrophages, the phase I LPS in naturally infected subjects decreases both the lymphoproliferative response and synthesis of IFNγ and IL-2 by sensitised T lymphocytes (the immunosuppressive action), which causes the different IFNγ responses of lymphocytes to stimulation with *C. burnetii* antigens in vaccinated and infected persons (6). These observations explain, in part, the results of our experiment. The IFNγ production peak seven days after the vaccination treatment in the seropositive individuals could have actually been the result of the activation of sensitised T lymphocytes, and the much weaker response of the seropositive shedders might point to the suppressive effect of the pathogen itself. In turn, the early TNFα production peak noted in all groups of animals in our study, regardless of their immunological status, must have been connected with the efficient response of monocytes/macrophages to the vaccine antigen, as production of this early pro-inflammatory cytokine is largely their work. In an *in vitro* study by Sobotta *et al.* (15) the primary bovine macrophages responded to *C. burnetii* infection with upregulated TNFα gene expression as rapidly as within three hours post infection.

In our study, the high levels of IL-10 persisting in the seropositive cows throughout the whole experiment are rather worrying, because a high level of this cytokine can promote the replication of *C. burnetii* and is linked to a defect in phagocyte killing activity. As reported by Honstettre *et al.* (5), a decrease in the production of IL-10 in conjunction with the persistent production of IFNγ supports the bactericidal activity of macrophages by weakening the inhibitory effect of IL-10. The weakly decreasing trend in the level of this cytokine observed in our study in the seropositive shedder group may indicate the slowly developing protective effect of the vaccine. This group of animals was characterised by the weakest post-vaccine cytokine response, despite the highest initial levels of IFNγ and IL-10, which seems to confirm a certain level of immunosuppression and may indicate an increased risk of the development of the chronic form of the disease.

In conclusion, the results obtained in our experiment suggest that cytokines TNFα, IFNγ, and IL-10 might have some diagnostic/prognostic value in a dairy cattle herd infected with Q fever. Due to large differences in the initial concentrations of IFNγ and IL-10 between seropositive shedders and seropositive nonshedders, and due also to the different course of post-vaccination response with respect to these cytokines, analysis of their concentrations seems to have a greater practical value than the determination of TNFα levels, both for an assessment of the health status of seropositive individuals and for monitoring the post-vaccination response course. Naturally, justification of the usefulness of analyses of cattle serum to determine the cytokines mentioned above would require further verification, supported by broader-scale research, lasting much longer and conducted on a higher number of infected animal herds.

Conflict of Interests Statement: The authors declare there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with laws and regulations of the Local Ethical Committee for Animal Experiments in Olsztyn as regards care and use of experimental animals (permission no. 25/2013). The consent of the herd owner was obtained before sampling.

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