Research paper

Evidence for transfer of maternal antigen specific cellular immunity against *Mycobacterium avium* ssp. *paratuberculosis* via colostrum in a goat twin model

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ABSTRACT

Colostrum intake is one of the most important factors in neonatal health in ruminants, mainly because of its unique immunological properties. Both in practice as well as in research, the attention of lactogenic immunity is focused on the importance of colostral antibodies and less attention is given to the functional role of maternal cells in colostrum. Here we study the transfer of maternal leukocytes *via* colostrum and the functionality in goat kids. In experiment 1, twenty twin pairs of goat kids from dams previously immunized with an inactivated *Mycobacterium avium* subsp. *paratuberculosis* (MAP) vaccine were fed maternal colostrum from their dam (kid 1) or pasteurized and frozen/thawed bovine colostrum (kid 2). The presence of cell mediated immune response (CMIR) against *Mycobacterium avium* antigens in the kids was assessed using intradermal skin testing with PPD-A tuberculin. Linear mixed effect models showed an increase in skin thickness in response to intradermal PPD-A injection in maternal colostrum fed kids compared to bovine colostrum fed kids. After intradermal PPD-A application, serum concentration of MAP specific antibodies increased in kids fed maternal colostrum, indicating antigen specific activation of the adaptive immune system. We did not detect a similar increase in antibodies in the kids fed bovine colostrum.

In experiment 2, a more reductionistic approach was applied to specifically study the effects of the transfer of maternal colostral leukocytes on CMIR in goat kids. Similar to experiment 1, twin kids from MAP immunized dams were randomly divided over two groups. The experimental group received colostrum replacer supplemented with fluorescently labelled colostral cells of the dam and the control group received colostrum replacer only. No difference in skin response following intradermal PPD-A injection was observed between both groups of kids. Histologic examination of the skin at the intradermal injection site did not show fluorescently labelled cells.

In conclusion, in our initial experiment we observed an antigen specific CMIR in goat kids fed fresh colostrum with colostral leukocytes from vaccinated dams. The lack of a DTH response in kids fed colostrum replacer supplemented with maternal colostrum derived leukocytes indicated that the complete colostral matrix is probably required for colostrum leukocytes to transfer across the intestinal epithelial barrier and modulate the neonatal immune response. In line with earlier studies, our results indicate that caprine maternal leukocytes present in colostrum can functionally contribute to the newborns' early adaptive immune responses adding to the importance of colostrum feeding in ruminant neonates.

1. Introduction

High neonatal mortality rates in the bovine and caprine dairy industries illustrate that the neonatal period in ruminants is not easily managed. Colostrum intake is one of the most important factors in neonatal health in ruminants, mainly because of its unique

immunological properties. At birth, ruminants are immunologically naïve as there is no transfer of maternal immunity such as antibodies across the placenta (Barrington and Parish, 2001). This makes the young ruminants highly susceptible to a variety of pathogens, unless there is sufficient maternal immunity transferred *via* colostrum. Both in practice as well as in research, the attention of lactogenic immunity is focused on the importance of antibodies absorbed from colostrum by the neonate. The importance of the transfer of maternal immunoglobulins by providing a sufficient amount of high quality colostrum directly post-partum is widely recognized (Robbers et al., 2021). Less attention is paid to the transfer of cellular immune components through colostrum (Gonzalez and Dus Santos, 2017). Research indicates that maternal leukocytes from colostrum can migrate intercellularly across the offspring's gut wall (Liebler-Tenorio et al., 2002). Maternal cells are able to reach the neonatal circulation until at least three days postpartum in sheep lambs (Schnorr and Pearson, 1984), which seems longer than the period until the so-called gut closure for maternal antibodies (24−36 h) (Weaver et al., 2000). It has been demonstrated that the colostral environment induces phenotypical changes in leukocytes, thereby enabling them to enter the systemic circulation a few hours after ingestion to a point where 1% of circulating cells are maternal leukocytes (Reber et al., 2006). Data from several studies suggests that transferred maternal cells can fulfill protective functions or enhance the neonatal immune response. Donovan and colleagues found that in the first few days after colostrum feeding, leukocytes from newborn calves fed whole colostrum showed enhanced proliferative responses *in vitro* against antigens to which their dams were previously immunized (Donovan et al., 2007). Others suggested that maternal leukocytes enhance the antigen-presenting capacity of the neonatal immune system (Reber et al., 2005). In addition, (Reber et al., 2008) proposed that presence of maternal leukocytes from colostrum was correlated with faster development of neonatal lymphocytes in the first week of life. Another study showed that calves receiving cell-free colostrum displayed higher incidence of overall disease compared to calves receiving whole colostrum (Novo et al., 2017). Together, these studies all demonstrate an important role for colostral leukocytes in the newborn.

Intradermal skin tests are frequently used to determine *in vivo* cell mediated immune responses (CMIR) in many species (Allen Black, 1999; Bandrick et al., 2008; Hernández et al., 2005; Zhu et al., 1999). Whenever such a cell mediated immune response is provoked in response to an intradermally injected antigen and the response develops over time, it is called a delayed type hypersensitivity (DTH) response, also known as a type IV immune response. This secondary immune response is characterized by activation and proliferation of mainly $CD4^+$ lymphocytes, followed by infiltration of the skin, resulting in a local, inflammatory response (Vohr, 2005). A widely used example of such an immune response is the tuberculin skin test used for clinical diagnosis of (bovine) tuberculosis. Bovine tuberculin (PPD-B) is injected intradermally and in an individual previously exposed to *Mycobacterium bovis* this leads to local inflammation and swelling of the skin caused by infiltrating antigen experienced T lymphocytes and macrophages. *Mycobacterium avium* and closely related subspecies, such as *Mycobacterium avium* ssp *paratuberculosis* (MAP), are common enteric pathogens of ruminants. Infection with *M. avium* and/or related subspecies can lead to false positive skin test reactions to PPD-B, due to the presence of conserved antigens in tuberculins. For this reason a comparative skin test using PPD-B and PPD-A, a *Mycobacterium avium* derived tuberculin, can be performed. The increase in skin thickness 72 h post injection is used as a diagnostic indicator whether the test subject has been exposed to *Mycobacteria* (OIE, 2018).

While many studies demonstrated an important role for colostral leukocytes in newborn ruminants, *in vivo* mechanisms of action and antigen specific functionality are not clear. We therefore designed two experiments in goat twins in which CMIR was studied *in vivo* with the use of an intradermal skin test. We used a MAP vaccine registered for use in goats to prime MAP specific CMIR in nulliparous goats prior to breeding

and evaluated transfer of antigen specific CMIR to the offspring. In the first experiment, twin kids received either maternal colostrum from MAP vaccinated dams or pasteurized frozen/thawed bovine colostrum. In a second experiment, twin kids were fed colostrum replacer supplemented with or without isolated maternal colostral cells (from MAP vaccinated dams). In both experiments intradermal skin testing with PPD-A, a *Mycobacterium avium* derived tuberculin, was used to measure MAP specific CMIR in the goat kids.

2. Materials and methods

2.1. Ethics

All studies were reviewed and approved by the Utrecht University Animal Ethics Committee and the Dutch Central Committee for Animal Experiments under permit № AVD1080020185064. The animal experiments were conducted in accordance with the Dutch law on Animal Experimentations (Wet op de Dierproeven) and the European regulations on the protection of animals used for scientific purposes (EU directive 2010/63/EU).

2.2. Animals and treatments

2.2.1. Experiment 1

Sample size was calculated prior to commencing the study. Because expected means and standard deviations were uncertain and because of the paired design of the experiments, sample size was determined for comparing paired proportions. We aimed to identify a difference of 45 % between the pairs of kids with a 95 % precision and a power of 80 %. For this, a sample size of 20 pairs was required. Considering a potential fallout percentage of 20 % (*e.g.* due to unexpected singletons), we aimed to include 24 twins in this experiment. Dams were selected at a certified CAE/CL free commercial dairy goat farm. There were no indications of endemic paratuberculosis based on farm history and the farmers declaration. In total 40 clinically healthy nulliparous goats were vaccinated against *M. avium* subsp. *paratuberculosis* (Gudair® vaccine) for the purpose of this study approximately two weeks prior to the three-week mating period. Goats were checked twice by transabdominal ultrasound scanning at approximately 9 and 10 weeks of gestation to select goats with twin pregnancies. All twin-carrying goats were subjected to a PPD-A tuberculin skin test at approximately 100 days post vaccination to verify that the Gudair® vaccination induced a DTH response against PPD-A. The 24 dams which showed the highest skin test response were selected. At three weeks before the expected kidding, the animals arrived at the research facilities of the Farm Animal Health division of the Department of Population Health sciences, Faculty of Veterinary Medicine, Utrecht University. The dams were placed in four different compartments of the research facility, each containing a deep-litter pen for six goats. The goats were provided with *ad libitum* straw and restricted amount of concentrate three times a day according to a commercial feeding scheme and had free access to water. Dams were monitored 24/7 by CCTV with care takers on site to detect kidding and to ensure that kids were immediately separated from the dam at birth. Dams were briefly restrained at parturition and were released back into the group immediately following parturition. Kids were directly moved to individual pens next to the adult goats, which allowed visual, auditory and nose-to-nose contact between kids and dams, however suckling was not possible.

The newborn twins were allotted over two treatment regimens using a predefined pseudo-random block randomization scheme to ensure that 1st/2nd born kids and male/female kids were equally distributed between the two treatments. Group 1 received colostrum and milk derived from their dam (Caprine colostrum, CC). Colostrum was obtained by milking the goats by hand and kids were fed a volume corresponding with 5% of the kid's bodyweight. At 2−4 hours postpartum, goats were machine-milked completely for the first time. Kids in the CC group were

again fed 5% of their bodyweight. The kids in group 2 (Bovine colostrum, BC) received pasteurized and frozen/thawed bovine colostrum and milk replacer (Denkamilk Capriplus, 22 % crude protein) (28.5 g dissolved in 150 mL water) in similar amount as the kids in the CC group. The bovine colostrum was obtained from a certified paratuberculosis-free dairy farm. After feeding the kids in both treatment groups a volume of colostrum equal to 10 % of their body weight within 4 h postpartum, all kids were fed similar quantities of milk following a commercial schedule at 07:00, 12:00 and 19:00, starting at 200 mL/feeding at the first day to 500 mL/feeding at the end of week two. Kids in the CC group were fed dam's milk, while kids in the BC group received equal amounts of the commercial milk replacer. Dams were milked twice a day (at 6:00 and 18:00). The required amount was directly fed to the corresponding offspring and surplus milk from the morning milking was stored in a fridge until the afternoon feeding at 12:00. Milk and milk replacer were heated with a water bath and fed at 40 °C. Kids were euthanized by intravenous injection of pentobarbital sodium (100 mg/kg BW, Euthanimal 20 %) at 27–33 days of age and subjected to necropsy. After completion of the experiment dams were returned to a commercial Dutch goat farm. For a graphical visualization of the experimental design, see Fig. 1A.

2.2.2. Experiment 2

Sample size was calculated prior to commencing the study. In 15 out of 20 twins from the first experiment, the highest DTH response was measured in the group that received maternal colostrum. Therefore we aimed to identify a difference of 70 % between the pairs of kids with a 95 % precision and a power of 80 %. For this, a sample size of 10 pairs was required. Considering a potential fallout percentage of 20 % (*e.g.* due to unexpected singletons), we aimed to include 12 twins in this experiment. Similarly to the selection procedure described in experiment 1, 44 clinically healthy nulliparous goats were vaccinated against paratuberculosis (Gudair® vaccine). Approximately 9 and 11 weeks after the start of the mating period goats were checked twice for twin pregnancy by transabdominal ultrasound scanning. Twelve clinically healthy and pregnant dams showing the highest skin test response to intradermal skin testing with PPD-A were selected. Housing and procedures around parturition were similar to experiment 1. Kids were housed in similar
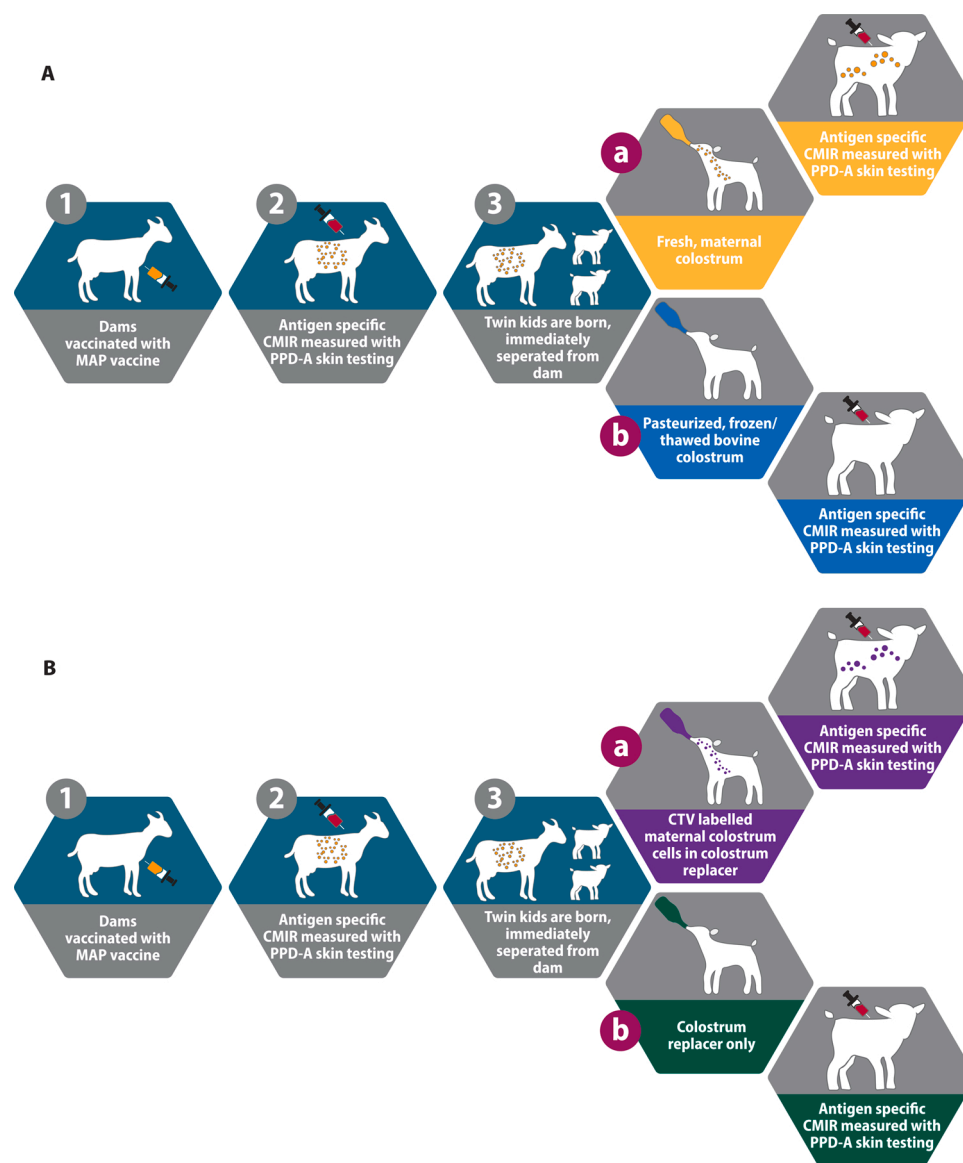

Fig. 1. Graphic visualization for experiment 1 (A) and experiment 2 (B). During step 1, dams were vaccinated with MAP vaccine, and this resulted in a CMIR against MAP as confirmed by PPD-A skin testing in step 2. After twin kids were born (step 3), kids were immediately separated from the dam and divided over treatments "a" and "b". Afterwards, in both kids the CMIR against MAP was assessed by PPD-A skin testing.

conditions as in experiment 1. Since sex and order of birth were not related to the outcome parameters in experiment 1, but birthweight was, the newborn twins were allotted over two treatment regimens using a predefined pseudo-random block randomization scheme in which the kids were equally distributed among the treatment groups according to their birthweight (BW). The first group received colostrum replacer (Capracol®, Arts Food Products BV, 's Hertogenbosch, Netherlands) supplemented with CellTrace Violet (CTV) (CellTrace™ Violet Cell Proliferation Kit, Life Technologies, Paisley, UK,) -labelled maternal cells derived from their dams' first milking colostrum (Cells, C). The kids in the second group (No cells, NC) received colostrum replacer in similar quantity as the C group. A detailed description of the cell isolation and preparations can be found in a subsequent paragraph (***Cell isolation and CTV staining***). Both groups received their first colostrum feeding at 2 h after birth, followed by a second feeding of milk replacer (Denkamilk Capriplus, 22 % crude protein) at four hours post-partum. When the first feeding was not voluntarily ingested by bottle feeding, an esophageal tube was used to ensure adequate colostrum intake. After colostrum feeding all kids were fed similar quantities of milk replacer, following the schedule as described in experiment 1. Dams were milked twice a day, at 06:00 and at 18:00. Kids were euthanized by intravenous injection of pentobarbital sodium (100 mg/kg BW, Euthanimal 20 %) at 8–10 days of age. After completion of the experiment dams were returned to a commercial Dutch goat farm. For a graphical visualization of the experimental design, see Fig. 1B.

2.3. Intradermal skin tests in goats and kids

In **experiment 1** the intradermal skin test in goats and kids consisted of a single intradermal injection at the left side of the neck at day 7 postpartum with 0.1 mL PPD-A 2500 IE (obtained from *M. avium* subsp. *avium* strain D4ER) (Thermo Fisher Scientific). Before injection, the injection site was shaved and double fold skin thickness was measured with a Hauptner tuberculin caliper. Injection was performed using a disposable 1 mL plastic syringe with a 0.45 × 16 mm needle. In adult goats, the injection site was assessed at 72 h post injection using a Hauptner caliper. In the kids, the assessment was performed at 24, 48 and 72 h post injection. The assessment of the reaction in the kids consisted of three separate measurements. First, the diameter of the swelling a the injection site was measured using a Vernier caliper. This measurement included all clinical alterations of the skin, taking induration as well as erythema into account. Second, the double fold skin thickness at the injection site was measured using a Hauptner tuberculin caliper. Third, the reaction site was checked for the presence of edema. All measurements were performed unblinded by the same person.

Intradermal skin tests in the kids in **experiment 2** consisted of 2 bilateral intradermal injections at day 7–9 post-partum: one with 0.1 mL PPD-A 2500 IE (obtained from *M. avium* subsp. *avium* strain D4ER) (Thermo Fisher Scientific) and one with 0.1 mL sterile PBS. Randomization was applied to correct for the side of injection. Since in experiment 1 the skin reaction of the kids peaked at 24 h post injection, the assessment in **experiment 2** was only performed at 24 h post injection. The assessment of the reaction in the kids consisted of two separate measurements. First, the double fold skin thickness at the injection site was measured using a Hauptner tuberculin caliper. Second, the reaction site was checked for the presence of edema. Measuring the double fold skin thickness was performed unblinded by the same person that performed the intradermal injection. Checking for edema was performed blinded by another researcher.

2.4. Cell isolation and CTV staining

In **experiment 2** goats were milked by hand to obtain a volume corresponding with 10 % of the kids' BW, from which colostral cells were isolated. Colostrum was equally divided over 16 50 mL tubes (Falcon) and diluted with PBS to 50 mL per tube. Tubes were centrifuged

at 400 *g* at room temperature (RT) for 20 min. Supernatant was discarded and pellets were resuspended, pooled in 20 mL PBS and centrifuged for 10 min at 400 *g* (RT). After discarding supernatant, the pooled pellet was resuspended in 30 mL PBS. Cell Trace Violet (CTV) was added to a final concentration of 5 μM and the solution was incubated for 20 min in the dark at RT. After incubation, 200 μl of the cell suspension was added to 1 mL of FACS buffer (PBS, 2% FCS, 0.01 % Na-azide) and refrigerated until further analysis. Another 1.5 mL of cellular concentrate was used for antigen-specific IFN-γ release assays. The remaining cell suspension was centrifuged and the supernatant with leftover staining without the cells was added to 170 mL colostrum replacer, resulting in a solution of 75 g of colostrum replacer into 200 mL of liquids. This colostrum was used for feeding the NC group. The CTV stained cell pellet was resuspended in 30 mL of PBS and added to another 170 mL of colostrum replacer and fed to the C group kids.

2.5. Sample collection

During **experiment 1** jugular blood samples (serum vacutainer tube) were collected from all goat kids prior to colostral feeding. Additionally, blood samples were collected on days 1, 7, 14, 21 and the day of euthanasia (day 27–33). Furthermore, independent of colostrum fed to goat kids, 20 mL of collected colostrum samples were collected in 25 mL tubes to determine total and MAP specific IgG levels.

For **experiment 2**, from all goat kids jugular blood samples were collected before first colostrum feeding, at 24 after birth and at the day of euthanasia (d7−10). Two vacutainer blood tubes were collected, a serum sample for antibody measurement and a heparin anticoagulated sample for *in vitro* stimulation with PPD-A. Immediately following parturition, two blood samples were collected from dams for antibody measurement and *in vitro* stimulation with PPD-A as well.

2.6. Antibody measurement

In **experiment 1**, fresh colostrum was diluted with PBS (1:2) and centrifuged at 2000 G for 20 min at 4 °C. 2 mL supernatant was collected and divided over 2 micronic tubes and stored at −20 °C until further analyses. In **both experiments**, jugular blood samples were centrifuged at 2500 *g* and 4 °C for 15 min, after which the serum was stored at −20 °C. For **both experiments**, caprine and bovine IgG concentrations were analyzed using Bethyl "Goat IgG ELISA Quantitation Set" (Cat. No. E50−104) and Bethyl "Bovine IgG ELISA quantitation Set" (Cat. No. E10−118) according to instructions provided by the manufacturer. For **experiment 1**, the level of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) specific antibodies were analyzed using the Paratuberculosis Screening Ab Test (Cat. No. 06-07,130-27, IDEXX) and S/P ratios were calculated using OD450 values subtracted by the negative control samples, with the negative control as the lower limit, according to the manufacturer's instructions.

2.7. Antigen-specific interferon (IFN)-gamma release assays

In **experiment 2**, 1.5 mL whole colostrum, isolated (non-stained) colostral cells incubated in RPMI supplemented with 10 % FCS and 1% Antibiotic-Antimycotic (Gibco), and whole heparinized blood samples of dams and kids were stimulated *in vitro* with PPD-A (250 IU/mL), and a mixture of PMA (50 ng/mL)/Ionomycin (2 μg/mL) as positive control and PBS as negative control. Samples and their stimulation were incubated for 24 h at 37 °C. Samples were centrifuged and supernatant was collected and stored at −20 °C until further analysis. For IFNγ detection, the BOVIGAM assay (Applied Biosystems, Foster City CA, USA) was used according to manufacturer's protocol.

2.8. Flow cytometry

In **experiment 1** 125 mL colostrum of 3 individual goats was

obtained for immune cell phenotyping. The colostrum was diluted 1:1 with PBS and centrifuged at 400 *g* for 20′ at 4 °C. Per goat, the cell pellets were washed twice with 10 mL PBS and divided in two. One part was stained with CD4 (Clone 44.38, MCA2213A647, BioRad), CD8 (Clone CC63, MCA837PE, BioRad) and WC1 (Clone CC15, MCA838 F, BioRad) in FACSbuffer. The other half of the milk sample was stained with CD68 (Clone ED1, MA5−16654, Invitrogen) after fixation with BD Fix and Perm (BD Cytofix/Cytoperm™ Kit (Cat. No. 554,714), according to the manufacturers protocol. Flowcytometry was performed on a BD FACS-Verse™ Flow Cytometer.

In **experiment 2**, cell viability of isolated, CTV stained maternal colostral cells was assessed using Fixable Viability Dye ViaKrome 808 (Beckman Coulter, Brea, CA) according to the manufacturer's protocol. In short; cells were harvested, washed with PBS, and stained with 1 μL dye per 5×10^5 cells for 20 min at RT in the dark. Stained cells were washed with PBS, and fixed in 1% paraformaldehyde (PFA). Cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter) and data were analyzed with FlowJo software (FlowJo LCC, Ashland, OR). Gating strategy can be found in Figure S1.

2.9. Post mortem examinations of kids

In a pilot experiment cell culture derived cells were stained using the CTV staining protocol and were subjected to fixation using 4% neutral buffered formaldehyde and checked for presence of CTV derived fluorescent signal. Subsequently fresh CTV stained cells were injected intradermally in fresh goat skin obtained from a slaughterhouse and subjected to fixation in 4% neutral buffered formaldehyde, paraffin embedded and processed for HE staining using routine protocols and checked for the presence of CTV fluorescent signal. In both procedures cells could be visualized using an Olympus BX51 (fluorescence) microscope. In **both experiments** post mortem examination was performed to check for gross pathology and in addition **in experiment 2** both injection sites were examined and a sample was fixed in 4% neutral buffered formaldehyde for 48 h for histology. Formaldehyde-fixed samples were processed routinely into paraffin blocks. Paraffin-embedded tissues were cut into 4 μm sections, collected on silane-coated glass sides and dried for at least 48 h at 37 °C. After deparaffinization and rehydration in graded alcohols, sections were either stained routinely with haematoxylin and eosin (HE) or examined without further staining for the presence of CTV stained cells using an Olympus BX51 (fluorescence) microscope.

2.10. Statistical analysis

Statistical analyses were performed using R (R Core Team, 2019). When data was normally distributed (Shapiro-Wilks normality test), mean outcome variables of continuous data were compared using paired sample T-tests. For not normally distributed values, the Friedman test was applied using the PMCMRplus package (Pohlert, 2021). For the latter, post-hoc tests were applied (Dunn's many-to-one) with adjustments for multiple testing (Bonferroni).

Univariable regression models were performed to check for crude associations between increase in skin thickness and possible explanatory variables. Based on the outcome of the univariable models, associative linear mixed effects models were developed using the lme4 package (Bates et al., 2015). Residuals of the full models were checked for normality using a normal probability (Q-Q) plot. Backward model reduction based on Akaike Information Criterion (AIC) was performed to determine which variables contributed to a well-fitting model, using the drop1 function (Bates et al., 2015). A variable was dropped from the model when the removal resulted in a reduction in AIC of 2 or more.

Experiment 1. Three linear mixed effect models were fitted: one to evaluate the treatment effect (CC *vs* BC) and potential confounders, followed by another model to assess which parameters relate to the response observed in the CC group only. A third model was constructed

to assess the temporal dynamics of MAP specific antibodies after intradermal injection with PPD-A in the kids.

For the first model, the dependent variable was the increase in skin thickness. The main variables of interest were treatment group (CC *vs* BC) and time. These variables were included in the model as fixed effects, and the interaction between treatment group and time as well. Two other fixed effects were introduced to check for confounding, being birthweight and the housing compartment of the kids were located. Random intercepts were included for the individual kids, nested in their corresponding mother in order to account for the paired study design. After model reduction, the final model was: Increase ~ factor(group) + factor(time) + birthweight + (1|Dam/KidID).

We used another model to further assess which variables were associated with the increase in skin thickness observed in kids in the CC group. The dependent variable here was the increase in skin thickness in the CC group. The fixed factors time, birthweight, and the compartment of the facility in which the kids were located were included, as well as total and MAP specific antibody levels at the day of kids' intradermal skin testing, and the dams' increase in skin thickness. Since we expected a correlation between total antibody level and MAP specific antibody level, we inserted this as an interaction. A random factor 'Kid ID' was included to adjust for the repeated measures of skin thickness within the kids. After model reduction, the final model was: Increase ~ factor(time) + birthweight + total_antibodies + MAP_specific antibodies + total_antibodies*MAP_specific antibodies + reaction dam + (1|KidID).

The third model looked at the temporal dynamics of MAP specific antibodies within and between treatment groups after intradermal injection with PPD-A. The model with the interaction time*group had the lowest AIC, and the final model was: MAP_specific_antibodies ~ factor (group) + factor(time) + factor(Group):factor(Time) + (1|Kid_ID).

Experiment 2. One linear mixed effects model was fitted to assess the treatment effects. We included group (C *vs* NC), birthweight and age of the kids as fixed effects. Random intercepts were included for the individual kids, nested in their corresponding mother in order to account for the paired study design. Based on these results, no additional modelling was done.

3. Results

3.1. Experiment 1

3.1.1. MAP specific immune response in dams
Approximately 100 days after MAP vaccination dams were intradermally challenged with PPD-A to assess the MAP specific immune response. At 72 h post intradermal PPD-A injection, the dams showed a classical DTH response at the site of injection. Average increase in skin thickness following intradermal PPD-A injection for the dams used in our study was 7.52 mm (± 2.82 mm) at 72 h post injection (Fig. 2A). First and second colostrum milkings contained IgG (Fig. 2B) and both dams' first and second colostrum milkings and serum contained MAP specific antibodies (Fig. 2C).

3.1.2. In vivo assessment of transfer and functionality of maternal leukocytes in goat kids
The 24 dams gave birth to 18 healthy twins, 2 triplets and the remaining 4 goats gave birth to a stillborn twin, a singleton, and two pairs from which one kid was stillborn. The 18 healthy twins and the two heaviest kids of the triplets were included in the study. Mann-Whitney-Wilcoxon test showed no correlation between sex or order of birth and increase in skin thickness, distributions in Supplementary Table S1. All kids ingested colostrum equal to approximately 10 % of their birthweight and this resulted in the rapid increase of blood immunoglobulin concentrations (caprine in CC and bovine in BC groups) 24 h postpartum (Fig. 3). To check for the presence of maternal lymphocytes, colostrum of three dams was collected to assess the phenotype of colostrum cells using flow cytometry. In all three samples, CD8⁺
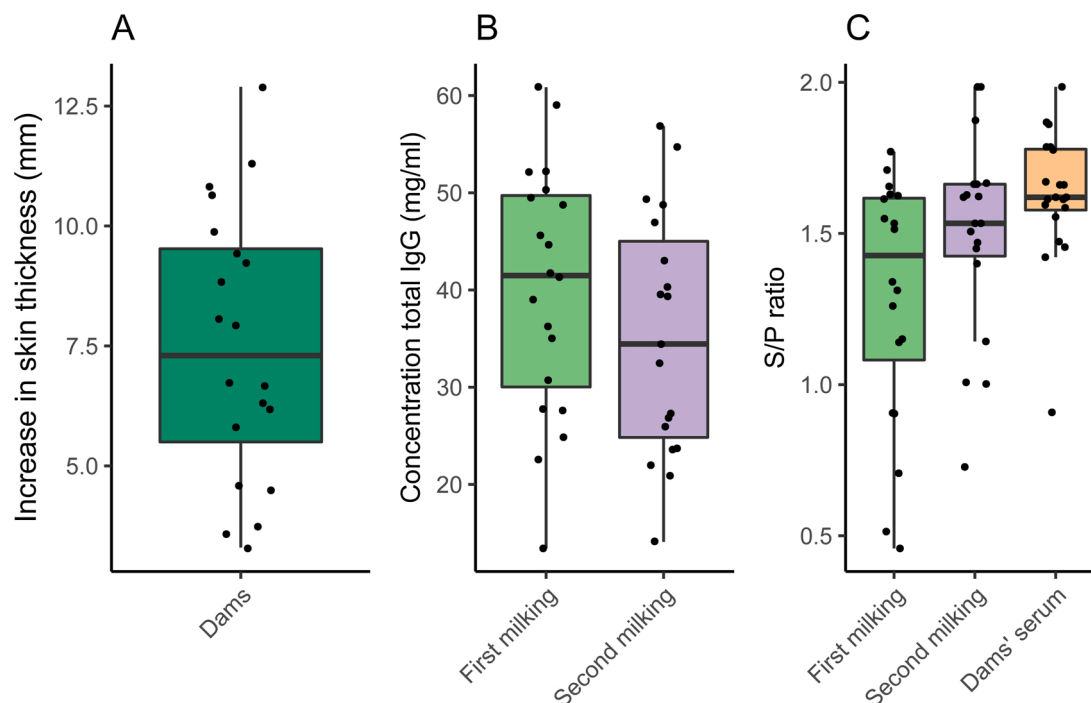
Fig. 2. Dams' (n = 20) increase in skin thickness (mm) in response to intradermal PPD-A injection (A), concentration of total IgG in colostrum milkings 1 and 2 mg/ml (B) and S/P ratio of MAP specific antibodies in colostrum milkings and serum (C). Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.
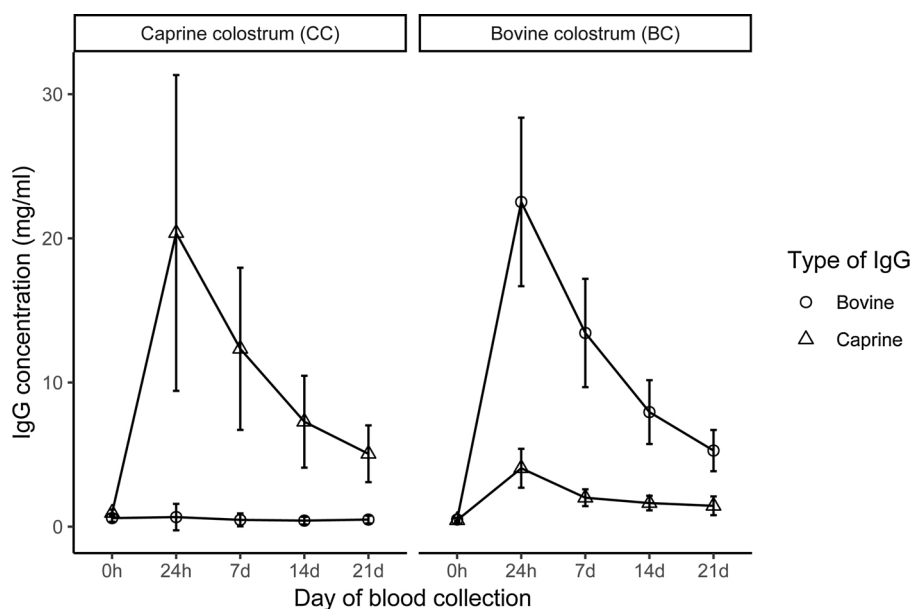

Fig. 3. Serum IgG concentration for kids receiving caprine colostrum (CC, n = 20) and bovine colostrum (BC, n = 20). Circles depict averages of bovine IgG and triangles depict caprine IgG in mg/ml ± 1 x SD per indicated time point.

lymphocytes appeared most abundant (Supplementary Table S2).

At seven days of age, all kids were subjected to intradermal injection with PPD-A to assess their MAP specific immune response. Mean increase in skin thickness, diameter of the response and proportion displaying edema in both groups are shown in Supplementary Table S3 and Supplementary Figures S2 and S3, respectively. Fig. 4 shows the increase in skin thickness in response to challenge with PPD-A for both CC and BC group at several time points post-injection, while data per pair of kids can be found in Figure S4. Backward model reduction was performed to determine variables associated with skin thickness. Our main variable of interest, group, stayed in the linear mixed effect model as an explanatory factor (Table 1). Skin thickness increase in the CC group was 0.20 mm more than in the BC group (95 % CI 0.06 mm – 0.35 mm). In addition to the group effect, factors "time" and "birthweight" remained in the final model for illustration purposes as estimates' confidence intervals contained "zero" and are therefore not considered significant. The estimates showed that the mean increase at 24 h was 0.02 mm higher (CI: -0.07 mm – 0.12 mm) than at 48 h. Additionally, it was 0.09 mm (CI: -0.01 mm – 0.18 mm) higher when compared to the mean increase at 72 h (Table 2).
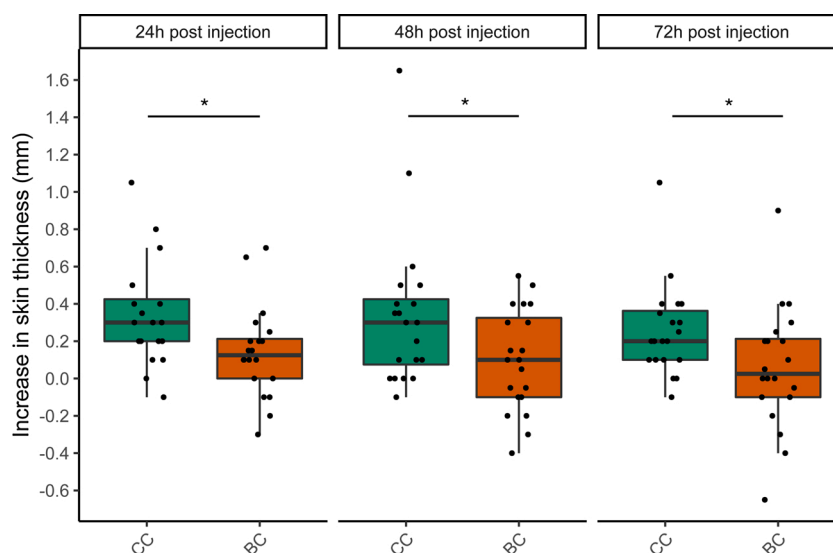
Fig. 4. Kids' increase in skin thickness (mm) at 24h, 48h and 72h post intradermal PPD-A injection. Treatment groups are indicated with CC (n = 20) as receiving maternal (caprine) colostrum and BC (n = 20) as receiving bovine colostrum. Significance (p < 0.05) between groups within each time point is indicated with an asterisk (Paired sample T-Test). Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

Table 1

Estimates, standard errors and confidence intervals for the reduced linear mixed effect model for explaining the observed difference in skin thickness between treatment group (CC) and control group (BC).

Variable	Level	β	S.E. (β)	95 % CI (β)	Variance (S.D.)
Intercept		0.2190	0.2726	0.2300; 0.4708	
Group	Treatment (CC)	0.2045	0.0741	0.0558; 0.3500	
Time	48 h post injection	−0.0225	0.0488	−0.1182; 0.0732	
	72 h post injection	−0.0863	0.0488	−0.1819; 0.0094	
Birthweight		0.0357	0.0720	−0.1081; 0.1766	
Kid/Dam					*0.036 (0.19)*
Dam					*0.010 (0.10)*

Variances of the random effects are in *cursive*.

Table 2

Estimates, standard errors and confidence intervals for the reduced linear mixed effect model for associations with skin thickness in treatment group (CC).

Variable	Level	B	S.E. (β)	95 % CI (β)	Variance (S.D.)
Intercept		0.0125	0.9702	−1.6581; 1.6830	
Time	48 h post injection	−0.0025	0.0711	−0.1417; 0.1367	
	72 h post injection	−0.0900	0.0711	−0.2292; 0.0492	
Birthweight		0.0888	0.1056	−0.0930; 0.2707	
Total antibodies		0.0753	0.1079	−0.1105; 0.2611	
MAP antibodies		−0.0261	−0.5395	−0.9549; 0.9027	
Total antibodies * MAP antibodies		−0.0290	0.0616	−0.1351; 0.0771	
Reaction dam		−0.0360	0.0247	−0.0784; 0.007	
Kid ID					*0.054 (0.23)*

Variances of the random effects are in *cursive*.

3.1.3. Variables affecting increase in skin thickness in the CC group

A second linear mixed effects model was fitted to define which variables were associated with an increase in skin thickness in the CC group. Estimates and corresponding standard errors and confidence intervals are displayed in Table 2. A larger increase in skin thickness and higher concentration of MAP specific antibodies in the mother were associated with a smaller increase in skin thickness observed in the kid. A higher birthweight and a higher concentration of total antibodies were positively associated with a larger increase in skin thickness in the kids. Interaction between total amount of antibodies and MAP specific antibodies in serum of the kids remained in the model as well, which was associated with less increase in skin thickness in kids. For all of these associations, the confidence intervals of the estimates contained "zero" and are therefore not considered significant.

3.1.4. Adaptive immune response following intradermal PPD-A skin testing

MAP specific IgG concentrations increased rapidly after colostrum feeding and remained similar up until seven days of age. After intradermal injection with PPD-A at day seven an increase in the MAP specific antibody levels was observed in the kids fed maternal colostrum

(Fig. 5). At experimental day 21, approximately 14 days post intradermal injection there is a peak in the antigen specific antibody response in the kids fed maternal colostrum (CC) only, after which the MAP specific antibody levels decline. According to the linear mixed effects model CC kids showed significantly higher MAP specific antibody levels compared to BC kids at 7, 14 and 21 days after intradermal injection of PPD-A (Supplementary Table S4). Because the residuals were not randomly distributed, we performed non-parametric tests as well, which revealed a significant increase in MAP specific antibodies at 7, 14 and 21 days after intradermal injection in the CC group. In the BC group there was a significant decrease compared to the day of intradermal injection, however almost all values were below the detection limit of the MAP antibody ELISA.
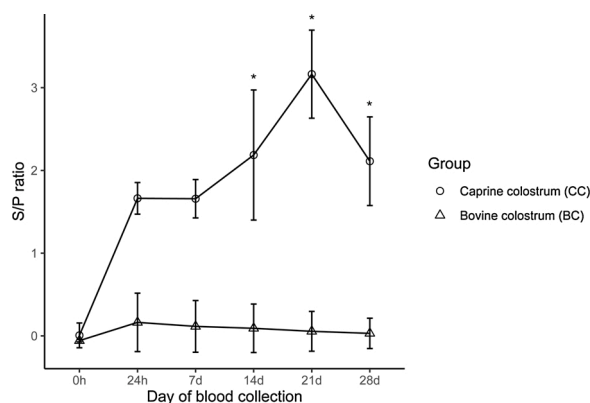
Fig. 5. S/P ratio of MAP specific antibodies over time. Asterisks indicate a significant difference (*p < 0.05) between time points 14 days, 21 days and 28 days, and the day of intradermal injection at day 7 (Friedman test, Dunn's many to one comparison, Bonferroni adjustment)) in mg/ml ± 1 x SD per indicated time point.

3.2. Experiment 2

3.2.1. MAP specific immune response in dams

Approximately 100 days after vaccination with MAP, dams were tuberculinated with PPD-A to assess the MAP specific immune response. At 72 h post intradermal injection, the dams showed a classical DTH response following intradermal PPD-A injection. Average increase in skin thickness following intradermal injection of PPD-A for the dams used in experiment 2 (n = 12) was 8.28 mm (± 3.8 mm) at 72 h post injection.

3.2.2. In vivo assessment of transfer and functionality of maternal leukocytes in goat kids

The 12 dams gave birth to 11 healthy twins, the remaining dam gave birth to two stillborn kids and 1 healthy kid. Eleven twin pairs were included in the analyses. Viability of isolated and stained colostral cells was assessed when this was possible within 16 h after the staining procedure (Figure S1). Mean viability was 91 % (± 6.07, n = 5). All kids ingested the intended amount of bovine derived colostrum replacer (200 mL) with or without CTV labelled maternal colostrum cells. Colostrum

ingestion by the kids was confirmed by increased bovine IgG concentrations in serum (C and NC groups) 24 h post ingestion (Fig. 6). At 7–9 days of age, all kids were intradermally injected with PPD-A to assess their MAP specific immune response. Mean increase in skin thickness resulting from PPD-A intradermal injection did not differ significantly between groups, nor did it differ from the increase in skin thickness as a result from injection with PBS (Paired sample T test, Fig. 7). A linear mixed effects model was fitted to further explore the effect of treatment group and other factors on the increase in skin thickness. However, treatment was dropped from the model as it did not have an effect on the skin thickness following intradermal PPD-A injection. Furthermore, examination of the skin at the site of injection using fluorescent microscopy did not show cells that were stained with CTV.

3.2.3. Stimulation assays

Whole blood, whole colostrum and isolated cells were stimulated with PPD-A and IFNγ secretion was assessed by ELISA. Whole blood samples of four of the 11 dams responded to stimulation with PPD-A above the threshold (O.D. > 0.1), while all of the samples responded to the positive control (Fig. 8A). Both whole colostrum samples and isolated colostral cells did not produce IFNγ above the threshold (O.D. > 0.1) following stimulation with either PPD-A or the positive control (Fig. 8B,C). None of the stimulations of kids' whole blood with PPD-A resulted in production of IFNγ, however these samples did respond to stimulation with the positive control (Fig. 8D-F). We found no differences in IFNγ production at any of the time points between the two treatment groups. No associations were found between the four dams with blood samples that showed IFNγ production in response to PPD-A stimulation *in vitro* and other outcome parameters, such as increase in skin thickness in either the dams or their kids.

4. Discussion

In the first animal experiment we provide evidence for the transfer of a functional and PPD-A specific CMIR from MAP vaccinated dams to the twin receiving the dam's colostrum as opposed to the twin receiving colostrum replacer. We hypothesize that this was due to a direct, antigen specific action of transferred maternal colostral leukocytes contributing to a neonatal CMIR. In the second experiment we isolated the dam's colostral cells and fed kids a bovine colostrum replacer supplemented
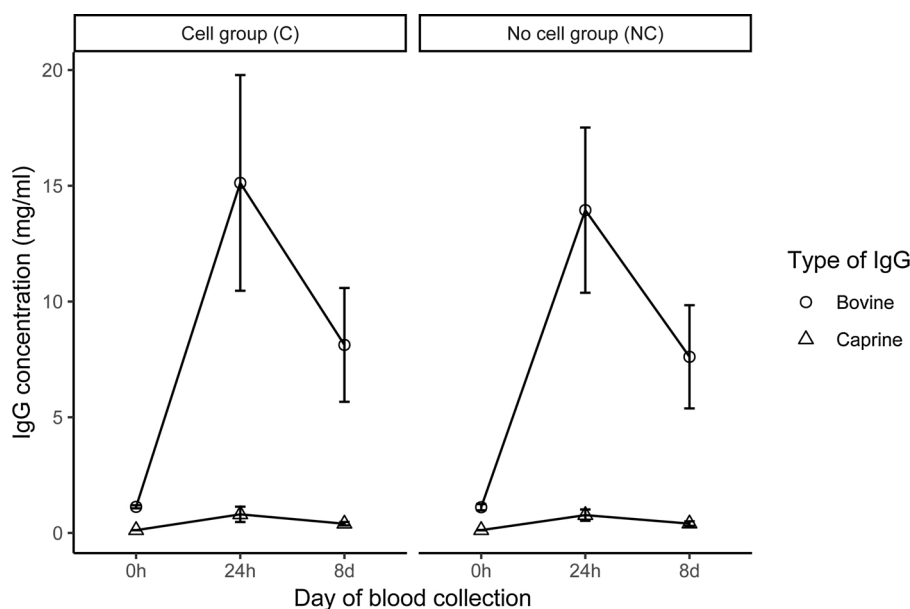

Fig. 6. Serum IgG concentration for kids receiving maternal cells (C, n = 11) and no maternal cells (NC, n = 11). Circles depict presence of bovine IgG and triangles depict caprine IgG in mg/ml ± 1 x SD per indicated time point.
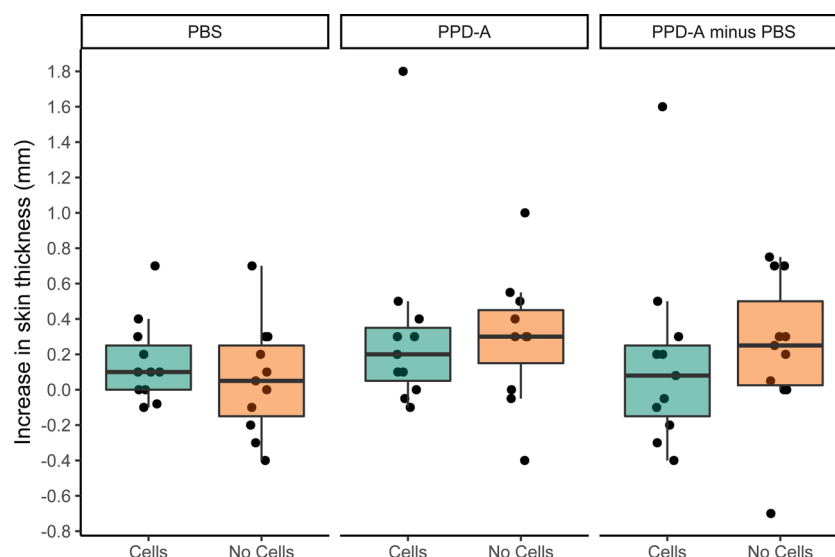
Fig. 7. Kids' increase in skin thickness in response to intradermal injection with PBS and PPD-A at 24h post injection. (Cells group n = 11; No Cells group n = 11). No significant differences were detected (Paired sample T-test). Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

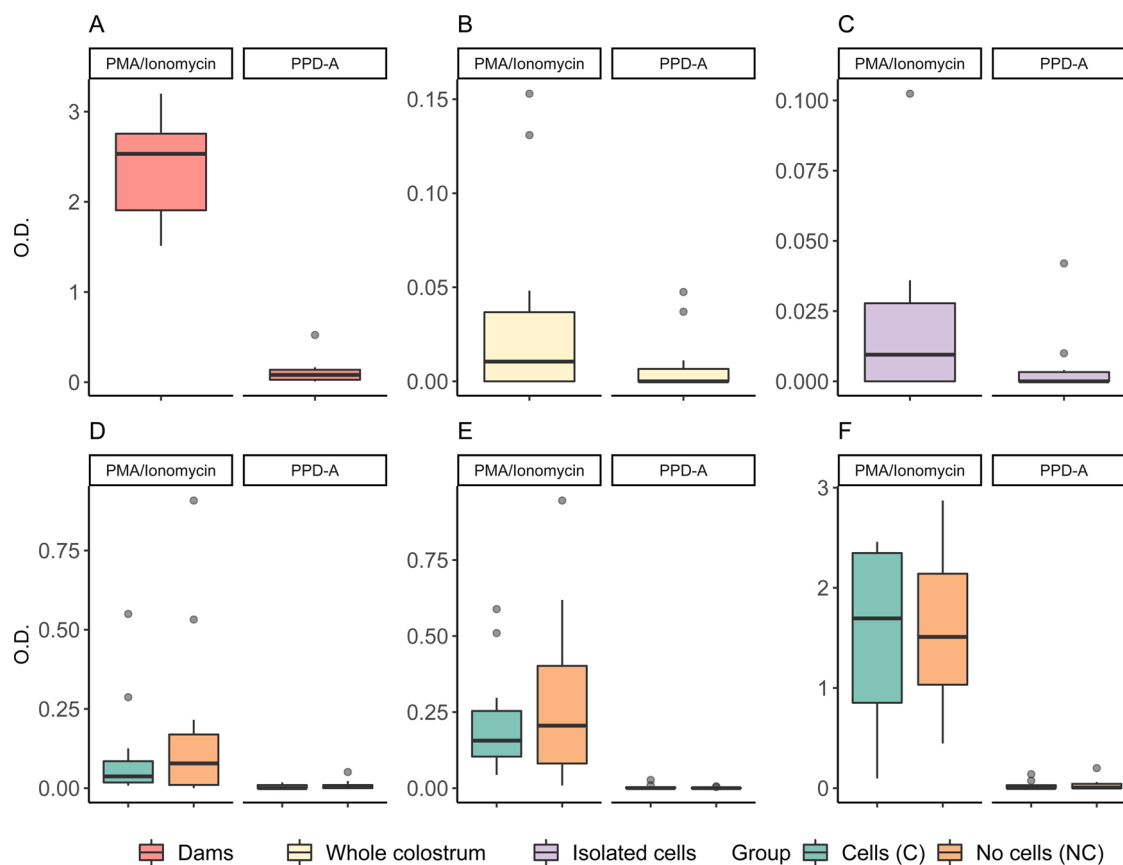**Fig. 8.** Interferon γ release assays. Top row represents O.D. values for PPD-A stimulation of dams' whole blood samples (A), whole colostrum samples (B) and isolated colostral leukocytes (C). Bottom row shows O.D. values for kids' whole blood stimulations at day 0 (D), 24h (E) and at day 8-10 (F). PMA/Ionomycin was used as positive control. Box and whisker represent Q1-Q3 and 1.5 x IQR, respectively, with the median indicated by horizontal bar.

with labelled maternal colostrum cells. We hypothesize that the lack of a DTH response in kids fed colostrum replacer supplemented with leukocytes derived from maternal colostrum indicates that the complete colostral matrix is required for colostrum leukocytes to transfer across the epithelial barrier and modulate the neonatal immune response.

The aim of the first experiment was to investigate the functionality of colostral leukocytes in goat kids with the use of an intradermal skin test. Kids received maternal colostrum from MAP vaccinated dams (caprine colostrum, CC) or freeze/thawed pasteurized bovine colostrum (BC). The MAP vaccination in the dams led to a strong DTH response following

intradermal injection with PPD-A. *In vivo* DTH functionality of maternal cells transferred to kids *via* colostrum was demonstrated by a statistically significant higher increase in skin thickness in response to intradermal PPD-A injection in the CC group compared to the BC group. Similar to the study by (Fernández et al., 2006) the observed difference between groups was greatest at 24 h post injection. Our results are similar to those of (Bandrick et al., 2008) who showed that antigen specific maternal leukocytes transferred with colostrum of pigs vaccinated against *Mycoplasma hyopneumoniae* evoke a DTH response in piglets upon intradermal skin injection with *Mycoplasma hyopneumoniae antigens*. With respect to our model to investigate variables contributing to the increase in skin thickness observed in the CC group, we found that antibody concentrations of the dam, antibody concentrations in the kids and birthweight of the kids were not significantly associated with the increase in skin thickness.

Delayed-type hypersensitivity (DTH) is characterized by early (6−12 h post injection) activation of antigen-specific memory T lymphocytes upon a second encounter with an antigen, and is therefore also referred to as cell-mediated hypersensitivity. When soluble antigens of *M. tuberculosis* are introduced in the skin, the response typically peaks at 24–72 h after antigen contact (Vohr, 2005). We used this type of DTH response in order to determine functionality of maternal T lymphocytes in the antigen unexperienced kids. The somewhat early peak in response observed in the kids in our study matches with the observation that there is considerable variation in DTH response between different animal models. In a Guinea Pig Potency Assay (GPPA), the reaction is read 24 h after intradermal skin testing by measuring the resulting erythema at the site of injection (Steadham et al., 2002). In contrast, when the same tuberculin is applied in cattle for TB diagnosis, the reaction is measured 72 h post injection by measuring the increase in skin fold thickness (OIE, 2018). Both types of reaction to PPD-A are considered DTH type reactions. In the current study the reactions observed in the adult goat dams followed the reaction type observed in cattle TB diagnostics. On the other hand, in the kids the timing of the reaction resembled those in sensitized guinea pigs with a peak response at 24 h post injection, and even though significant, increase in skin thickness was rather small in the kids. These differences can be explained by a number of factors such as skin anatomy, maturity of the immune system and number of antigen specific T cells. These characteristics change with age as the dams have a completely developed adaptive immune response including both effector and memory T-cells, while newborns solely depend on their pool of specific but naïve (and thus not yet clonally expanded) T-cells, which are potentially supplemented with some maternally derived activated mycobacterial antigen-specific T-cells and antibodies.

A linear mixed effects model shows that intradermal injection with PPD-A resulted in an increase of MAP specific antibodies in the goat colostrum fed kids. Despite several attempts to transform the data (squared, square root, log transformations), we did not obtain a normal distribution of the residuals, leading to less reliable predicted values of this model. Based on the data we observed, this is likely caused by the lack of variation in some data points in the model. For the majority of the kids in the BC group the concentration of MAP specific antibodies was zero and the corresponding residuals remained close to zero as well. In contrast, the concentration of MAP specific antibodies in the CC group significantly increased following PPD-A injection, and therefore an increase in variance was to be expected. As a consequence, the combined data results in not normally distributed residuals of the model. To tackle this, we performed some additional testing and found an increase in MAP specific antibodies up to 14 days after intradermal injection with PPD-A in the CC group. This indicates endogenous production of antigen specific antibodies in the kids, suggesting that the adaptive arm of the immune system is activated upon intradermal challenge with PPD-A. Since this is only seen in the kids fed maternal colostrum, we hypothesize that this adaptive immune response is the result of the stimulation of maternal PPD-A specific B and/or T cells (Bandrick et al., 2008; Tuboly et al., 1988).

Our most important finding of the first experiment was that feeding fresh maternal goat colostrum to goat kids increased their skin thickness after intradermal injection with PPD-A compared to feeding pasteurized and frozen/thawed bovine colostrum. The increased skin thickness in maternal colostrum fed kids is indicative of a DTH cell mediated immune response. The increase in MAP specific antibodies in the CC group two weeks after intradermal injection with PPD-A adds to the likelihood that the adaptive arm of the neonatal immune system is activated in the CC kids. We hypothesize that the activation of the adaptive arm of the immune system could be caused or stimulated by maternally derived antigen specific helper T and/or B cells.

In the second experiment we aimed to investigate the role of the cellular fraction of colostrum separate from the other colostrum components including maternal antibodies. Colostral leukocytes were isolated and labelled with a fluorescent dye suitable for tracking viable cells with the aim to track these cells in the kids. The isolated and CTV stained cells of the tested samples were viable at the time of feeding, however this is no indication of functionality. In contrast to the results in the first experiment, we found no difference in skin thickness increase in response to intradermal injection with PPD-A between kids receiving bovine colostrum replacer with maternal colostral cells and kids receiving colostrum replacer only. As a delayed type hypersensitivity response is characterized by lymphocyte infiltration (Hernández et al., 2005), a histopathological evaluation of the injection site was performed to study whether labelled maternal cells were present and involved in the local skin response. None of the skin injection sites at 24 h post injection showed CTV staining, hence we concluded that no dermal infiltration of labelled maternal leukocytes took place. To determine the transfer and functionality of antigen specific maternal lymphocytes, an antigen stimulation assay measuring IFNγ production was performed on whole colostrum, isolated colostral cells and blood from both dams and kids. However as we were unable to show consistent antigen specific IFNγ production in both dams and kids in all matrices, these tests were not useful for assessing the transfer of antigen specific T cells.

We hypothesize that the lack of a detectable, antigen specific cellular immune response in the second experiment might be explained by the failure of the transfer of maternal immune cells as a result of the absence of the complete colostral matrix. The importance of the colostral matrix was earlier for the transfer of maternal colostral cells was described by (Reber et al., 2006). In their study, they showed that the colostral environment is essential for colostral cells to pass into the neonatal bloodstream by inducing phenotypical changes on the cellular membrane. We suggest that by isolating colostral cells from their natural environment, trafficking markers on the outer membranes might not have been properly expressed or have been lost and cells were not able to transfer across the epithelial barrier of the neonatal gut.

In both groups of the second experiment, the increase in skin thickness resulting from intradermal PBS (control) injection did not differ significantly from the increase in skin thickness caused by PPD-A intradermal skin testing. Also, the response to intradermal PBS injection did not differ between groups. This implies that the response to PPD-A observed in the second experiment is likely caused by a non-specific and possibly innate immune response, while an antigen specific adaptive response failed to appear. Taken together with the results from experiment 1, in which we did observe a difference in DTH response between the two groups of kids, we infer that the response observed in the CC group in the first experiment was antigen specific and caused by the difference in colostrum feeding regiment.

In conclusion, in our initial experiment we observed a likely direct, antigen specific action of maternally derived colostral leukocytes contributing to a neonatal CMIR. Given the results of the subsequent experiment we hypothesize that the complete colostral matrix is probably essential for maternally derived colostral cells to transfer across the epithelial barrier as viable leukocytes functional in the neonatal environment. In line with earlier studies, our results indicate that caprine maternal leukocytes present in colostrum can functionally contribute to

the newborns' early adaptive immune responses.

Data availability

Data will be made available on request.
The data that has been used is confidential.

Funding

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.

Acknowledgements

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2022.110402.

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