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Interactions between *Mycoplasma mycoides* subsp. *mycoides* and bovine macrophages under physiological conditions

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Abstract

We investigated the interactions of unopsonized and opsonized *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) with bovine macrophages *in vitro*. *Mmm* survived and proliferated extracellularly on bovine macrophage cell layers in the absence of *Mmm*-specific antisera. Bovine complement used at non-bactericidal concentrations did neither have opsonizing effect nor promoted intracellular survival, whereas *Mmm*-specific antisera substantially increased phagocytosis and *Mmm* killing. A phagocytosis-independent uptake of *Mmm* by macrophages occurred at high MOIs, also found to induce the production of TNF, and both responses were unaffected by non-bactericidal doses of bovine complement. Bovine complement used at higher doses killed *Mmm* in cell-free cultures and completely abrogated TNF responses by macrophages. These results provide a framework to identify *Mmm* antigens involved in interactions with macrophages and targeted by potentially protective antibodies and point towards a pivotal role of complement in the control of inflammatory responses in contagious bovine pleuropneumonia.

Keywords: Contagious bovine pleuropneumonia, *Mycoplasma mycoides*, macrophages, complement, TNF

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Introduction

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is a wall-less bacterium and the causative agent of contagious bovine pleuropneumonia (CBPP), a highly contagious respiratory disease of cattle notifiable to the World Organization for Animal Health (Woah, 2015). CBPP lung lesions and chronic carrier state are suggestive of an uncontrolled inflammatory response by the host's immune system and immune evasion mechanisms developed by *Mmm* respectively (Provost et al., 1987). Although macrophages patrolling the surface of respiratory mucosae represent the first line of defense against pathogens invading the lung, little is known of the interactions of these cells with *Mmm*. Macrophages containing *Mmm* antigens are found in the lungs of infected animals (Scanziani et al., 1997) and they produce the archetypal pro-inflammatory cytokine TNF upon infection *in vitro* (Jungi et al., 1996). However, studies investigating the fate of *Mmm* in the presence of macrophages are lacking.

Another major contributor to the host defense against infection is the complement system. Complement proteins are present in extracellular fluids allowing direct killing and non-specific opsonization, followed by increased phagocytosis of pathogens (Volanakis, 1998). Non-specific opsonization can result in killing but also in intracellular survival if pathogens are able to prevent fusion of phagosomes with lysosomes (Cosma et al., 2003; Horwitz, 1983). Upon activation, the complement promotes inflammation through the induction of pro-inflammatory cytokines (Okusawa et al., 1988; Laudisi et al., 2013). These pleiotropic effects of complement are not exclusive and may be dose-dependent. For example, the complement membrane attack complex responsible for the pathogen killing activity of complement can also induce the production of pro-inflammatory cytokines by phagocytes when used at non-bactericidal doses (Laudisi at al., 2013). Thus, in CBPP, interactions between *Mmm*, macrophages and complement may either benefit the host, through direct or opsonization-mediated killing of *Mmm* or: paradoxically; harm the host, through opsonization-mediated intracellular survival of *Mmm* and amplification of inflammatory responses.

Although previous work suggests that mycoplasmas are generally phagocytosed by macrophages only in the presence of specific antiserum (Marshall et al., 1995), the question remains open for *Mmm*. Also, effects of complement other than opsonization, such as direct killing and cytokine production, may impact disease outcome in CBPP. Therefore, the following study was undertaken to investigate the interaction of bovine macrophages with *Mmm* under physiological conditions, *i.e.*, in the presence of complement prepared from the same cell donor. Firstly, the effect of bovine complement on the viability of *Mmm* was analyzed to determine non-bactericidal concentrations. Secondly, the uptake of *Mmm* by macrophages and subsequent extra- and intracellular survival in the presence of bovine complement at non-bactericidal doses was investigated. Finally, we addressed the possibility that complement acts as a potentiator of *Mmm*-induced TNF responses of macrophages.

Methods

Bacterial strains

Non-fluorescent *Mmm* strain 8740-Rita and *Mycoplasma bovis* strain Oger2, and fluorescent mNeonGreen-expressing *Mmm* mutant strain 8740-Rita clone 6 (RN6), were grown in PPLO-based medium as described previously (Bonnefois et al., 2016). Mycoplasmas obtained from log-phase cultures were washed once in PBS by centrifugation at 10000 x *g* for 10 min before resuspension in macrophage culture medium (see below) at 1-2x10^9 colony forming units per mL, or CFU/ml, as determined through dilution and plating on PPLO-based media supplemented with 1 % Noble Agar (Difco, USA). Briefly, serial 10-fold dilutions in PBS were prepared in a final volume of 500µL with a change of tip and vortexing between each tube. 20µL from each tube were spotted onto PPLO agar plates and incubated at 37°C for four days before counting the colonies under a light microscope.

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Bovine complement and antiserum

Non-decomplemented bovine sera obtained from 3 adult Jersey cattle housed at Cirad's animal facility served as a source of complement. These animals were purchased in France were CBPP has been eradicated and were kept outdoor after approval by the Languedoc-Roussillon regional ethics committee (French CE-LR#36) in the authorized project using animals for scientific purposesAPAFIS#27628. Sera were aseptically collected from whole blood tubes after clotting for 30 min at room temperature at 19-23°C, followed by centrifugation at 800 x g for 20 min. Single-use aliquots of non-decomplemented sera were kept in liquid nitrogen until use after quick thawing at 37°C. Bactericidal capacities of bovine complement were assessed by incubation of $1x10^6$-$1x10^8$ viable *Mmm* with different concentrations of non-decomplemented bovine sera at 100 µL/well in 96-well flat-bottom-plates for 1 h at 37°C and 5% CO_2 before vortexing and CFU titration. That range of viable *Mmm* corresponds to the lowest and highest number of bacteria used to infect macrophages. The specific *Mmm* antiserum was obtained by pooling sera from two-years old Zebu cattle recovering from an experimental infection with *Mmm* strain 8740-Rita (Yaya et al., 1999). These animals were selected on the basis of their positivity using a CBPP-specific cELISA (LeGoff et al., 1998) and a record of clinical signs typical of CBPP. Prior to use, *Mmm* antisera were decomplemented by heating at 56°C for 30min.

Macrophages and bacterial infection

Macrophages were derived from monocytes obtained from the same three animals used to prepare non-decomplemented bovine sera. Briefly, monocytes were purified from PBMC by positive selection of CD14+ cells using anti-human CD14 magnetic beads (Miltenyi, Germany) according to the manufacturer's instructions. Monocytes were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 0.4 mg/mL ampicillin and 10% heat-inactivated fetal calf serum (FCS; Eurobio AbCys, France), seeded at 3 x 10^5cells/well of 96-well flat-bottom-plates in 200 µl, and incubated for 6 days at 37°C and 5% CO_2 with a change of half of the medium at day 3. Before infection, the medium was removed and replaced with IMDM lacking FCS to which the following stimuli were added to reach a final volume of 100 µl/well: i) appropriate amounts of mycoplasmas to obtain MOIs of 1, 10 and 100; and ii) non-decomplemented autologous bovine sera or antiserum or FCS. Non-decomplemented bovine sera were used at non-bactericidal doses ranging from 5 to 10% v/v depending on the donor, whereas antiserum was used at 20% v/v and FCS at 10% v/v. After 1 h incubation, the medium was replaced and 100µl of complete IMDM medium was added to the wells before harvesting or further incubation. For harvesting macrophages, monolayers were scrapped off using 200µl tips, pelleted by centrifugation at 500 x g for 5 min, resuspended in 200 µL PBS, and passaged 5 times through a 27gauge needle, which lysed more than 80% of cells as shown by Trypan blue viability counts. *Mmm* CFU titers were determined as described above. For gentamycin assays, an MOI of 10 was used, as it was shown in preliminary macrophage-free assays that addition of 400 µg/ml of gentamicin to the culture for 3 hours reliably killed 100% of mycoplasmas. Cultures infected with *M. bovis* strain Oger2 were used as positive controls of intracellular survival in the presence of gentamycin (Suleman et al., 2016).

Flow cytometry

Macrophages were infected with fluorescent and non-fluorescent *Mmm* at MOIs of 500-1000 as described above. Due to the high MOI, non-decomplemented bovine sera could be used at up to 40% v/v without affecting *Mmm* viability. Before harvesting, the medium was replaced by 100 µl/well of fresh medium and cells were scraped off, homogenized by pipetting up and down, and transferred to 5 ml cytometry tubes. After addition of 10 µL/tube of propidium iodide (PI, BD Biosciences, USA) and 10min incubation at 4°C in the dark, 100 µL of sheath fluid was added to each tube. Preliminary macrophage-free tests showed that 100% of *Mmm* took up PI in these conditions. Since PI and mNeonGreen fluoresce in different wavelength, it was possible to gate out PI positive macrophages to exclude dead cells (i.e., with loss of membrane integrity) and extracellular *Mmm*. This allowed analysis of strictly intracellular mNeonGreen-dependent fluorescence. CytochalasinD (Sigma, France), an inhibitor of actin-dependent phagocytosis, was used at 10 µg/ml. At least 5000 events were analyzed within the PI negative gate with a FacsCanto flow cytometer (BD Biosciences) equipped with the FacsDiva software (BD Biosciences). Gates and quadrants were used to delineate populations of interest

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and calculate statistics respectively. Results were expressed in percentages of cells fluorescing above background (*i.e.*, fluorescence produced by macrophages infected with non-fluorescent *Mmm*) and geometric mean fluorescence intensity (MFI), which is proportionally related to numbers of mNeonGreen+ *Mmm* per cell.

ELISA

Supernatants were collected from macrophage cultures infected as above for 24 h with *Mmm* at MOIs ranging from 100 to 1000 and stored at -20°C until use. Non-decomplemented bovine sera were used at both bactericidal and non-bactericidal concentrations and the highest dose was also used for FCS. Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 (Invivogen, France), used at 2 µg/ml, served as a positive control. To quantify bovine TNF, a sandwich ELISA was performed using a matched antibody pair (BioRad, France) as previously described (Totté et al., 2015). Supernatants were tested in duplicates and results obtained from a labsystems multiskan MS ELISA reader were expressed in mean optical densities.

Statistics

A non-parametric Mann–Whitney U-test (https://www.socscistatistics.com/tests/mannwhitney/) was used to analyze differences between responses obtained with various stimuli. Single and double asterisks represent p values of <0.05 and <0.01 respectively. A difference was considered to be significant at a p value of <0.05.

Results

Bactericidal effect of bovine complement on *Mmm* viability in the absence of macrophages

A dose-dependent effect of non-decomplemented bovine sera, containing bovine complement, on *Mmm* CFU titers was observed *in vitro* (Fig1a). Depending on the dose and animals used the decrease in CFU titers varied between 0 and 3 logs, indicating a potent effect of bovine sera and a high variability among animals. There was no sign of *Mmm* aggregation induced by non-decomplemented serum when cultures were checked under a light microscope, and passage through a 27G needle had no impact on CFU titers, indicating that reduced viability occurred through direct cytotoxicity. The killing effect was due to complement since it was neutralized by heating sera at 56°C for 30 min (Fig1b) a method known to inactivate complement (Volanakis, 1998). The mycoplasmacidal activity and inter-animal variability of bovine sera was stable over the 3 years study period (Supplementary Fig1).

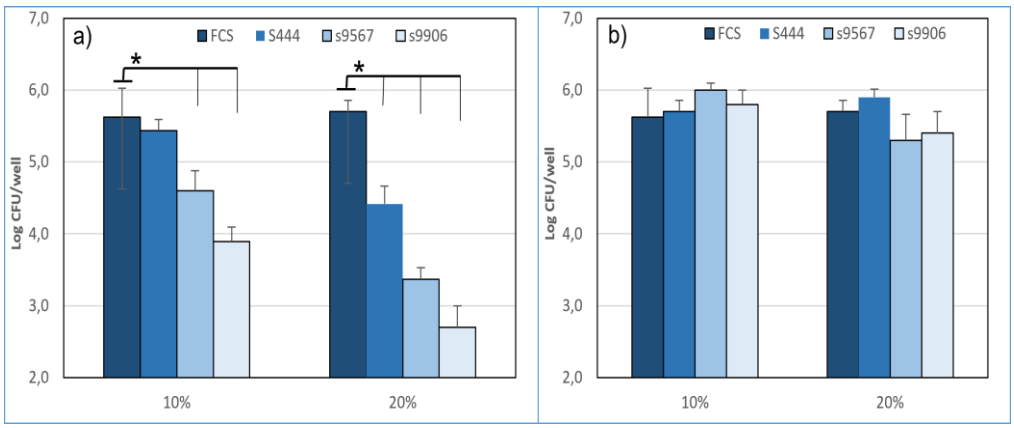

Figure1: Cytotoxic effect of bovine complement on *Mmm* in vitro before a) and after b) heating at 56°C. *Mmm* was incubated for 1 h with decomplemented sera (FCS) or different concentrations (10%, 20%) of bovine sera from 3 different animals (s+animal number). Results from 3 different experiments are shown as mean+/-SD of CFU expressed in log10. Asterisks and bars indicate statistically significant differences with FCS.

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Effect of bovine complement on *Mmm* opsonization and survival in the presence of macrophages

A protocol was used to focus on *Mmm* associated to macrophages and to exclude non-adherent and plastic-adherent mycoplasmas from the analysis (see methods). Furthermore, no cytopathological effects of *Mmm* on macrophage monolayers were detected at any MOI used. In the absence of bovine complement and antiserum, complete survival of *Mmm* occurred at 24 h and growth of up to 1 log was observed at 48h, indicating no killing activity by macrophages (Fig2). Similar trends were observed at MOIs of 1 and 100 whereas only survival of *Mmm* without growth (i.e., CFU titers remained constant) was observed in the absence of macrophages. The addition of non-decomplemented bovine sera at non-bactericidal concentrations had no effect on *Mmm* viability at any time-points (Fig2). As expected, addition of *Mmm*-specific antiserum induced significant killing activity after 24 h and up to 2.5 logs decrease in CFU titers at 48h (Fig2).

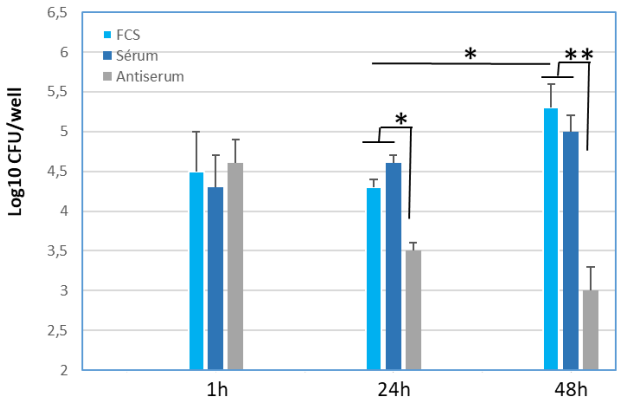

Figure 2: Macrophages do not kill *Mmm* even in the presence of bovine complement. Macrophages were infected with *Mmm* at an MOI of 10 in the absence (FCS) or presence (serum) of non-decomplemented bovine sera at non-bactericidal concentrations and incubated for 1, 24, and 48h before assessment of macrophages-associated *Mmm* titers. Anti-*Mmm* antiserum (antiserum) was added to additional wells as positive controls of bactericidal activity. Results from 3 different experiments using 3 animals are shown as mean+/-SD of CFU expressed in log10. Asterisks indicate statistically significant differences with FCS.

In order to analyze intracellular survival of *Mmm* experiments were repeated in the presence of gentamicin to kill all extracellular mycoplasmas. This was performed only at an MOI of 10, which represented a concentration of $3x10^6$ CFU/ml of *Mmm*, since gentamicin did not kill 100% extracellular mycoplasmas at higher *Mmm* concentrations. As seen in Fig.3, gentamicin assays indicated that the overwhelming majority of *Mmm* survival at 24 h was due to extracellular mycoplasmas and that bovine complement had no effect on *Mmm* survival inside macrophages. This was not due to a bias in gentamicin assay since significant survival was observed with *M. bovis* Oger2 (Fig3).

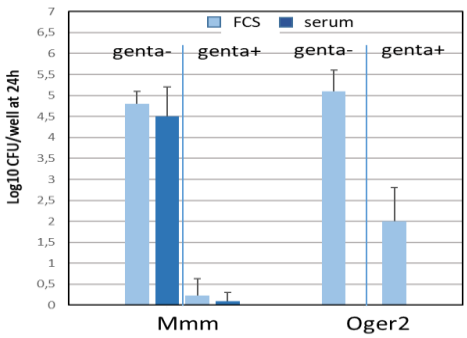

Figure 3: Bovine complement has no impact on *Mmm* survival inside macrophages. Macrophages were infected with *Mmm* at an MOI of 10 in the absence (FCS) or presence (serum) of bovine complement at non-bactericidal concentrations and incubated for 24 h. Cells were then treated for 4-hours with gentamycin before harvesting and assessment of macrophage-associated *Mmm* titers.

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Wells containing macrophages infected with *M. bovis* (Oger2) were used as positive controls of intracellular survival in the presence of gentamycin. Results from 3 different experiments using 3 animals are shown as mean+/-SD of CFU expressed in log10.

Effect of bovine complement on the uptake of fluorescent *Mmm* by macrophages

We were able to analyze *Mmm*-dependent intracellular fluorescence by gating out extracellular fluorescence as explained in "Methods". As seen in Fig4, in the absence of bovine complement or antiserum, more than 90% of macrophages had engulfed fluorescent *Mmm* but at low levels as indicated by low FITC fluorescence intensity ("Li" in Fig4a). Indeed, FITC signals did not exceed one log above background level and could not be detected when MOIs of 100 or less were used (data not shown). No differences were detected between 1 and 24 h PI in both percentages of positive cells and MFI (Fig4a and Fig4b). Non-decomplemented bovine sera used at non-bactericidal concentrations had no effect on FITC fluorescent levels at any time-points (Fig4a and Fig4b). Data at 48h were not exploitable due to high background fluorescence. The low-level uptake was not affected by cytochalasinD ("Li" in Fig4c) indicating that it was not dependent on phagocytosis. A substantially stronger FITC fluorescence intensity was observed in the presence of antiserum indicating a strong increase in *Mmm* uptake by macrophages ("Hi" in Fig4a). At 24 h PI, the % of cells within the "Hi" gate increased while the mean fluorescence intensity decreased (gate P5 and MIF in Fig4b) suggesting partial destruction of fluorescent *Mmm* within macrophages. Moreover, the vast majority of highly fluorescent cells disappeared in the presence of cytochalasinD (FIg4c) indicating that the uptake of *Mmm* by macrophages in the presence of antiserum was dependent on phagocytosis.

The lack of effect of bovine complement on the uptake of *Mmm* by macrophages was confirmed for all three animals tested (Table1). Indeed, the addition of non-decomplemented bovine sera at non-bactericidal concentrations had no impact on numbers of positive cells and MFI when compared to FCS at 1 h and 24 h PI. In addition, the positive effect of antiserum on *Mmm* uptake was confirmed as well as the increase in percentage of positive cells at 24 h PI accompanied by a decrease in MIF, indicating progressive destruction of phagocytized fluorescent *Mmm*.

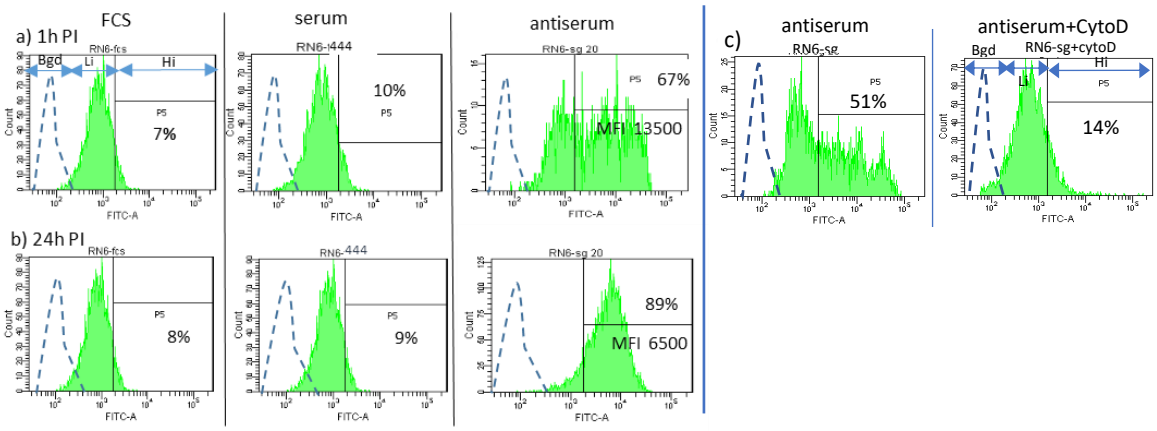

Figure 4: Bovine complement does not impact *Mmm* uptake by macrophages. Macrophages were infected with either non-fluorescent or fluorescent *Mmm* at an MOI of 500-1000, and either in the absence (FCS) or in the presence (serum) of non-decomplemented bovine sera at non-bactericidal concentrations. They were incubated for 1 h (a) and 24 h (b) before analysis of intracellular FITC fluorescence by flow cytometry (see Methods). Anti-*Mmm* antiserum (antiserum) was added to additional wells as positive control of specific phagocytosis. Typical histogram plots are shown for one representative animal as an example. Histograms and arrows display background (bgd, hatched line), and low (Li, green line) and high (Hi, green line) fluorescence intensities produced by macrophages infected with non-fluorescent and fluorescent *Mmm* respectively. The effect of cytochalasinD, an inhibitor of phagocytosis, on *Mmm* uptake by macrophages is shown in c). Percentages of highly fluorescent cells and MFI are given by the P5 interval.

Table 1: Effect of bovine complement (serum) from three different animals (mean+/-SD) on the uptake of fluorescent *Mmm* by autologous macrophages as measured by flow cytometry 1 h and 24 h post infection

Stimuli	Low fluorescence intensity[a]				High fluorescence intensity[a]			
	% pos [b]		MFI[c]		% pos [b]		MFI[c]	
	1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h
FCS	93+/-2	96+/-5	1013+/-33	995+/-33	7+/-3	10+/-1	NA	NA
Serum	92+/-3	99+/-3	1008+/-52	1050+/-42	8+/-4	9+/-2	NA	NA
Antiserum[d]	NA	NA	NA	NA	63+/-10	94+/-5	14518 +/-1313	6905 +/-948

(a): low and high fluorescence intensities above background fluorescence (see also Figure 4)
(b): Percentages of positive cells
(c): Mean fluorescence intensity
(d): sera from CBPP convalescent animals
NA: not applicable. The effect of antiserum is measurable only within the high fluorescence intensity whereas, in the presence of complement, the vast majority of positive cells were found in the low fluorescence intensity range.

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Effect of bovine complement on *Mmm*-induced TNF production by macrophages

As shown in Fig5, in the absence of bovine sera and antiserum, *Mmm* induced the production of TNF by macrophages when MOIs of 500-1000 were used. At an MOI of 100, TNF was not detected (not shown). There was no effect of bovine complement at non-bactericidal doses (Fig.5, serum1). In order to analyze the effect of non-decomplemented bovine sera at bactericidal doses, concentrations of both non-decomplemented bovine sera and FCS had to be increased. For one animal, a substantial increase in background (*i.e.*, TNF induced by FCS alone in the absence of *Mmm*) prevented its use. Nevertheless, non-decomplemented bovine sera from the remaining two animals, used at bactericidal concentrations inducing at least 1log decrease in *Mmm* viability, almost completely abrogated the production of TNF induced by *Mmm* (Fig5, serum2).

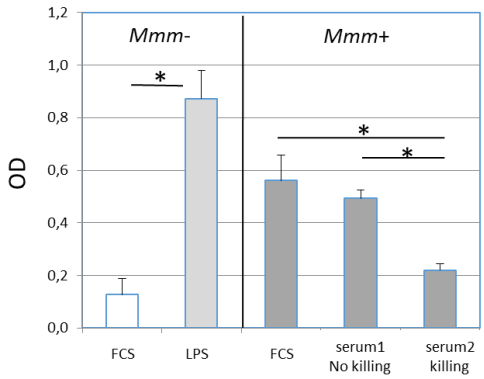

Figure 5 : Effect of bovine complement on *Mmm*-induced release of TNF by autologous macrophages. Macrophages were either left uninfected (*Mmm*-) or infected (*Mmm*+) with *Mmm* at an MOI of 500-1000 in either the absence (FCS) or presence of non-decomplemented bovine sera at non-bactericidal (serum1) and bactericidal (serum2) concentrations and incubated for 24 h before TNF titration of supernatants. Lipopolysaccharide (LPS) was added to additional wells as positive controls. Results from 3 different experiments using complement from two animals are shown as mean+/-SD of optical densities (OD). Asterisks indicate statistically significant differences.

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Discussion

Given their pivotal role in first-line immune defenses against mucosal pathogens, we have investigated the interactions between macrophages and *Mmm*, the causal agent of CBPP. Non-decomplemented bovine sera were used as a source of complement to mimic physiological conditions present at infected sites and cryopreservation in liquid nitrogen was instrumental in overcoming the instability of complement. The strong bactericidal effect of some non-decomplemented bovine sera on *Mmm* viability that we observed in this study is in conflict with previous work (Gaurivaud et al., 2014). One likely explanation is that we used bovine sera instead of guinea pig sera. Similarly, non-decomplemented caprine sera was found to reduce the viability of *Mycoplasma mycoides* subsp. *capri* (*Mmc*), *Mmm*'s closest relative (Schieck et al., 2016). Given its stability over time *in vivo* and variable potency between animals, it would be interesting to assess whether a correlation exists between the anti-*Mmm* effect of bovine sera and the resistance of animals to CBPP. Considering the pleiotropic effects of complement on immune responses to pathogens, we have explored the possible impact of bovine complement, used at non-bactericidal concentrations, on important functions of macrophages such as non-specific opsonization-mediated phagocytosis and production of pro-inflammatory TNF.

In the first place, we analyzed the kinetics of *Mmm* survival in the presence of bovine macrophages and in the absence of complement and antiserum. Lysis of macrophages prior to *Mmm* titration allowed taking into account both intracellular and membrane-associated mycoplasmas. *Mmm* CFU titers remained stable after 24 h and increased by up to 1log after 48h, indicating the absence of measurable killing by macrophages. Little or negligible numbers of macrophage-associated *Mmm* survived intracellularly in the presence of gentamicin, suggesting that viable *Mmm* were mainly located on cell membranes. Absence of killing by macrophages or even growth on macrophages in the absence of specific antiserum and complement has been reported previously for other mycoplasmas (Marshall et al., 1995; Howard et al., 1976). We found no measurable effect of bovine complement on *Mmm* CFU titers, suggesting that neither non-specific opsonization-mediated killing nor intracellular survival occurred under our experimental conditions. It should be noted that pre-incubation of *Mmm* with bovine complement for 30 min at 37°C before infecting macrophages rather than concomitantly did not change the results. However, we cannot exclude the possibility that the presence of *Mmm* growing on macrophages masks a low-level killing activity. On the other hand, *Mmm* titers substantially decreased over time when anti-*Mmm* antiserum was added to the medium indicating that intrinsic killing capacities of macrophages were not altered. Again, absence of killing by macrophages, even in the presence of complement, has been shown previously for several mycoplasmas (Marshall et al., 1995). It is possible that the presence of a polysaccharide capsule (Buttery, 1970) protects non-opsonized and non-specifically opsonized *Mmm* from phagocytosis (Weis, 1985) which may be further investigated using non capsulated variants (Gaurivaud et al., 2014).

In order to gain a better insight on the interactions of *Mmm* with bovine macrophages, we used a more direct approach based on flow cytometry-assisted monitoring of intracellular fluorescence of phagocytes after infection with fluorescent *Mmm*. Our results show that, in the absence of complement and antiserum, more than 90% of macrophages take up fluorescent *Mmm* at low levels, as indicated by low mean fluorescence intensities. Unfortunately, gentamicin was not 100% bactericidal at MOIs used for flow cytometry, thus preventing assessment of the viability of intracellular *Mmm*. The lack of decrease in fluorescence over time suggests that either no killing occurred or that it was not sufficient to cope with ongoing growth of *Mmm* on macrophages. CytochalasinD had no effect on the number of cells harboring low intracellular fluorescence, suggesting that the uptake of *Mmm* by macrophages was independent of phagocytosis. Interestingly, it has recently been shown that *Mmc* interacts with C-type lectin receptors (CTLRs) (Lindenwald et al., 2020) some of which mediate endocytosis. Several CTLRs are present on macrophages, which warrants further studies on their potential role in CBPP. Moreover, pathogen-associated molecular patterns (PAMPs) of *Mmm* recognized by these receptors have potential as virulent factors. Bovine complement used at non-bactericidal concentrations had no effect on the uptake of *Mmm* by macrophages at any time points, confirming viability experiments using CFU titration. On the other hand, anti-*Mmm* antiserum induced a strong and phagocytosis-dependent increase in *Mmm* uptake leading to more than 90% of cells located in the high fluorescence intensity gate at 24

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hpi. Moreover, the mean fluorescence intensity within that gate decreased at 24 hpi confirming the progressive destruction of *Mmm* as seen with CFU titration. These last results are intriguing since *Mmm* and related mycoplasmas possess a two-protein system capable of cleaving immunoglobulins G (IgG) (Arfi et al., 2016). Decomplementation of anti-*Mmm* antisera did not affect their opsonizing potency indicating that IgGs are most likely involved.

Our results confirm previous work demonstrating the capacity of *Mmm* to induce TNF production by macrophages in the absence of either complement or antiserum (Jungi at al., 1996). Interestingly, TNF was induced only at high MOIs (i.e., >500) also shown by flow cytometry to be associated with phagocytosis-independent engulfment of *Mmm*. This suggests a possible involvement of autophagy in the pro-inflammatory response of macrophages to *Mmm*. There was no beneficial effect of bovine complement on the TNF response of infected macrophages when used at non-bactericidal concentrations. The effect of bovine complement at bactericidal doses was also analyzed, considering the possible release of pro-inflammatory molecules from *Mmm* undergoing lysis. We found that, when used at bactericidal concentrations, bovine complement efficiently abrogated the production of TNF by macrophages in response to *Mmm* infection.

In summary, using a combination of CFU titration, gentamicin assays, fluorescent *Mmm*, and TNF ELISA, we were able to show that: i) macrophages were not capable of killing *Mmm* efficiently in the absence of antiserum; ii) bovine complement used at non-bactericidal doses neither had opsonizing effect nor did it promote intracellular survival; iii) bovine complement did not impact the phagocytosis-independent *Mmm* uptake by macrophages that occurred in the absence of antiserum; iv) bovine complement used at non bactericidal concentrations did not amplify *Mmm*-induced TNF responses of macrophages, and even abrogated that response when used at concentrations inducing bactericidal activity. These results provide a framework to identify PAMPs as well as *Mmm* antigens recognized by CTLRs and protective antibodies. In addition, they point towards a role for complement in the control of TNF-dependent inflammatory responses in CBPP.

Data, scripts, code, and supplementary information availability

Data are available online at https://zenodo.org/record/7442582#.ZAiYrHbMJOC. Supplementary Fig1 is available online at https://www.biorxiv.org/content/10.1101/2022.12.06.519279v1.supplementary-material

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no conflicts of interest in relation to the content of the article.

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