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To cite this version:

Laurence Dedieu, Valerie Balcer-Rodrigues, Ousmane Cisse, Mahamadou Diallo, Mamadou Niang. Characterisation of the lymph node immune response following Mycoplasma mycoides subsp. Mycoides SC infection in cattle. Veterinary Research, 2006, 37 (4), pp.579-591. 10.1051/vetres:2006020 . hal-00903042

HAL Id: hal-00903042

https://hal.science/hal-00903042

Submitted on 11 May 2020

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Vet. Res. 37 (2006) 579–591
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DOI: 10.1051/vetres:2006020

Original article

Characterisation of the lymph node immune response following *Mycoplasma mycoides* subsp. *Mycoides* SC infection in cattle

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(Received 9 August 2005; accepted 30 January 2006)

Abstract – Contagious bovine pleuropneumonia (CBPP), caused by *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (*Mmm*SC), is still a major cattle disease in Africa. Development of long-term protective vaccines, the only relevant strategy to achieve CBPP eradication, requires the characterisation of the protective immune mechanism. To this aim, the present study investigated the cellular immune response persisting in the lymph nodes of cattle infected naturally and experimentally by contact, one year post exposure. The lymph node cell composition, *Mmm*SC responsiveness and phenotype of the *Mmm*SC-responding lymphocytes were compared between animals according to the different outcomes of the infection. To unravel the protective mechanism, the study focussed on the *Mmm*SC-specific memory immune response generated in recovered cattle, known to develop long-term immunity and to be resistant to reinfection. An *Mmm*SC-specific immune response, mediated by IFNγ-secreting CD4 T-cells, was detected in the lymph nodes of all recovered cattle. Furthermore, the magnitude of this immune response was significantly higher in animals with complete recovery than in recovered animals presenting lung sequestra. The findings suggest that, in recovered cattle, a subset of *Mmm*SC-primed IFNγ-secreting CD4 T-cells homed to the regional lymph nodes as *Mmm*SC-specific memory T-cells, likely responsible for the protective anamnestic response. Induction and expansion of this subset of *Mmm*SC-specific CD4 memory T-cells might be a major goal to develop efficient long term protective vaccines against CBPP.

contagious bovine pleuropneumonia / *Mycoplasma mycoides* subsp. *mycoides* SC / vaccine / cell-mediated immunity / CD4 T-cell response

1. INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (*Mmm*SC), still remains a major cattle disease in Africa [31]. CBPP is the only bacterial disease included in the list A of the Office International des Epizooties (OIE), leading to the exclusion of infected countries from international trade. The disease is responsible for heavy economic losses due

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Article published by EDP Sciences and available at http://www.edpsciences.org/vetres or http://dx.doi.org/10.1051/vetres:2006020

to mortality, loss of weight, reduced working ability or fertility. Based on a combination of a stamping out policy, control of cattle movement and quarantine, CBPP has been eradicated from numerous countries such as Australia, the USA and Europe. However, these measures are impracticable in Africa where nomadism and transhumance are a necessity. In Africa, the only realistic prophylaxis has to rely on vaccination. However, the live attenuated vaccines currently in use, based on the T1 strain, are of low efficacy, requiring annual, costly, vaccination campaigns, and still retain some virulence [23, 31]. The development of more efficient vaccines is therefore an important goal to achieve CBPP eradication from Africa. To this aim, understanding the parameters of the *Mmm*SC-specific host immune protection is an important prerequisite.

Several observations point to a critical role of cell-mediated immunity in the pathogenesis and control of *Mmm*SC infections [24, 25, 32]. Furthermore, a recent study of the *Mmm*SC-induced peripheral blood response revealed the predominant contribution of *Mmm*SC-specific IFNγ-secreting CD4 T-cells to protection against CBPP [5].

The present study focused on the *Mmm*SC-specific immune response persisting in the lymph nodes of cattle recovered from an *Mmm*SC infection transmitted by the natural route, one year post exposure. The objective was to characterise the *Mmm*SC-specific acquired memory. Indeed, after infection, a pool of antigen-experienced lymphocytes homes to the draining lymph nodes as memory cells [10–12, 34]. Memory cells play a critical role in long-term immunity and mediate protective immunity against a new challenge [13, 34]. The characterisation of the *Mmm*SC-specific memory immune cell subsets in recovered animals, known to be refractory to new infection, will help identifying the protective mechanism. The development of vaccines expanding these *Mmm*SC-specific memory immune cells will, therefore, provide efficient vaccinal strategy against CBPP.

2. MATERIALS AND METHODS

2.1. Experimental infection

The experimental infection protocol was designed according to the French national legislation for animal experimentation and was performed, in accordance with the same guidelines, at the Central Veterinary Laboratory in Bamako (Mali), as already described [20]. The objective was to reproduce a natural in-contact *Mmm*SC infection. Eleven naive Peulh zebus were taken from CBPP-free areas where they had never been vaccinated against CBPP. These animals were 3 to 6 years old and the sex ratio was 3 females for 8 males (Tab. I). These naïve cattle, identified as the "contact" group, were placed in contact with 8 *Mmm*SC-infected N'dama cattle taken from a field outbreak, 3 to 4 weeks after its onset. These 8 cattle, constituting the "infected" group, were 5 to 7 years old with a sex ratio of 5 females for 3 males (Tab. I). All animals were selected for their negative status for foot and mouth disease, tuberculosis and brucellosis and were dewormed before use. The animals were housed in close contact for 12 months, outdoors during the day and indoors at night.

Temperature and clinical signs were recorded daily. A serological follow up of the *Mmm*SC infection was carried out by the complement fixation test (CFT), recommended by the OIE as the standard serological test for CBPP diagnosis [4]. Animals with respiratory distress were directly sacrificed to reduce suffering, and the remaining animals were post-mortemed at the end of the experiment. During the post mortem (PM) analysis, two lymph nodes (LN), a mediastinal and a tracheobronchic draining the infected lung region, were taken from each animal of the "contact" group and from the two recovered cattle from the "infected" group. Only one LN was taken from all other cattle of the "infected" group.

Table I. Results of the experimental transmission of an *Mmm*SC infection. Eleven naive cattle ("contact" group) were naturally infected by close contact housing with 8 *Mmm*SC-infected cattle ("infected" group) taken from the field as described in Materials and Methods. The animals were assigned to three groups according to the outcome of the *Mmm*SC infection and intensity of the lung lesions. The sex (f: female; m: male) and age (in years) was indicated for each animal.

Animals (sex; age)	Clinical form	Outcome	Post mortem analysis	Groups
Contact group				
C3 (m; 4)	Acute	Recovery	Sequestra (5 × 10 cm)	Group 3
C4 (f; 5)	Subclinic / chronic	Recovery	Resolved lesions	Group 1
C5 (f; 5)	Subclinic / chronic	Recovery	Small sequestra (0.5 × 1cm)	Group 3
C6 (m; 3)	Subclinic / chronic	Recovery	Small sequestra (3 × 5 cm)	Group 3
C7 (f; 5)	Subclinic / chronic	Recovery	Small sequestra (1 × 2 cm)	Group 3
C8 (m; 4)	Subclinic / chronic	Recovery	Resolved lesions	Group 1
C9 (m; 6)	Acute	Recovery	Sequestra (3 × 10 cm)	Group 3
C11 (m; 6)	Subclinic / chronic	Recovery	Small sequestra (2 × 2 cm)	Group 3
C12 (m; 3)	Acute	Death (26 wpc)	Lung hepatization and pleural fluid	Not retained
C13 (m; 4)	Subclinic / chronic	Recovery	Resolved lesions	Group 1
C14 (m; 3)	Acute	Death (19 wpc)	Lung hepatization and pleural fluid	Not retained
Infected group				
I1 (f; 7)	Acute	Death (3 wpc)	Lung hepatization and pleural fluid	Group 2
I2 (m; 6)	Acute	Death (6 wpc)	Lung hepatization and pleural fluid	Group 2
I4 (f; 6)	Acute	Death (9 wpc)	Large sequestra (15 × 20 cm)	Group 2
I5 (f; 7)	Acute	Death (8 wpc)	Large sequestra (15 × 20 cm)	Group 2
I6 (f; 6)	Acute	Death (6 wpc)	Multiple sequestras	Group 2
I11 (f; 6)	Acute	Death (7 wpc)	Large sequestra (15 × 20 cm)	Group 2
I9 (m; 7)	Subclinic / chronic	Recovery	Resolved lesions	Group 1
I12 (m; 5)	Subclinic / chronic	Recovery	Resolved lesions	Group 1

wpc: weeks post contact.

2.2. Lymph node cell preparation

LN were collected in Hanks balanced salt solution (Eurobio, Les Ulis, France) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B (Sigma, St. Quentin, France) and maintained on ice during transport to the laboratory. The cells were released from the LN, in warm RPMI-1640 culture medium (Eurobio), by slicing the tissues in small pieces with a scalpel. Cells were filtered on gauze, centrifuged and washed with Hanks balanced salt solution without calcium and magnesium (Eurobio), supplemented as above. The LN cells were numbered, resuspended in foetal calf serum (Eurobio) supplemented with 10%DMSO (Sigma), and stored frozen at 2×10^7 cells/mL in liquid nitrogen. The cells were maintained frozen in liquid nitrogen during shipment to France.

To assess the non-specific immune response, two LN (mediastinal and tracheobronchic) from four healthy cattle, identified as the "negative control" group, were taken at a slaughterhouse in Montpellier and prepared as described above.

2.3. *Mmm*SC strain and growth conditions and antigen preparation

The *Mmm*SC T1 attenuated strain was used for all in vitro stimulation for practical reasons (level 2 confinements). Cultures were as previously described [5]. Heat-inactivated *Mmm*SC were obtained after one hour incubation at 60 °C. The *Mmm*SC total proteins in the crude cell suspension were titrated by the bicinchoninic acid method [30].

2.4. Lymphoproliferation assays and cell phenotyping

LN cells were prepared as previously described [5]. One millilitre (2.5×10^6 cells/mL) was then distributed into each well of a 24-well microtitre plate and incubated with heat-inactivated *Mmm*SC (5 µg/mL), concanavalin A (2.5 µg/mL; Sigma) as a positive control to verify cell viability or in RPMI media (negative control), for 5 days at 37 °C with 5% CO_2.

The cell phenotyping was performed by immunofluorescence staining, as previously described [5], using specific mouse monoclonal antibodies (MAb) to the following bovine leukocyte antigens: WC1 (CC15, SEROTEC, Cergy St. Christophe, France), CD2 (IL-A26), CD4 (IL-A12), CD8 (IL-A105), major histocompatibility complex class II (MHCII) molecules (J11), interleukin-2 receptor CD25 (IL-A111) [1, 14, 16, 18]. Except for the former, all other MAbs were from the International Livestock Research Institute (ILRI, Nairobi, Kenya). MAb DU_2-104, kindly provided by W. Hein, was used to detect the B-cells [17]. Flow cytometry analysis, measurement of blastogenesis, of the various cell subsets and of their state of activation (CD25 expression) have already been described [5].

The *Mmm*SC-responsiveness was investigated by measuring the *Mmm*SC-induced (1) blastogenesis, (2) percentage of $CD25^+$cells within the total population, (3) percentage of $CD25^+$ cells within the CD4 T-cell subset and (4) IFNγ production. The relative activation of the CD4 T-cells was also evaluated by measuring the median of fluorescence intensity (MdFI) of the gated CD25+CD4 T-cells. The *Mmm*SC-specific responsiveness was validated if at least 2 of the 4 immune parameters were above cut off values determined from the "negative control" group. These cut off points were defined for each immune parameter as the mean value of *Mmm*SC-induced minus unstimulated data plus 2 standard deviations (SD) among negative control cattle.

2.5. IFNγ ELISA

Lymphoproliferation assay supernatants were collected on day 5 to quantify gamma interferon (IFNγ) production using a commercially available enzyme-linked immunosorbent assay (Bovigam γ interferon test, BioCore, Omaha, NE, USA) according to the manufacturer's instructions. Data were expressed as the mean OD units (± SD) of duplicate assays.

2.6. Statistical analysis

The unpaired Student *t*-Test was used for comparative analysis between the "negative control" group and the various groups of "*Mmm*SC-infected" cattle and to compare these groups between themselves. The Paired Student *t*-Test was used for comparison between in vitro unstimulated and *Mmm*SC-stimulated cells. *P*-values less than 0.05 were considered significant. *P*-values less than 0.01 were considered highly significant.

3. RESULTS

3.1. Experimental infection

The results of the experiment are presented in Table I (clinical form, outcome and post mortem (PM) analysis). All animals from either the "contact" or the "infected" group presented clinical signs of an *Mmm*SC infection (temperature, cough, nasal discharge, dyspnoea) accompanied

Table II. Ex vivo phenotypic analysis of the lymph node cells. The mean percentage and standard deviation of each cell subset are presented for the different groups of animals: "negative control" cattle ($n = 4$), animals with complete recovery (group 1; $n = 5$), animals with acute infection leading to death ($n = 6$), animals recovered with persisting lung lesions ($n = 6$).

	Lymphocyte subsets				
Groups of animals	CD4 (%)	CD8 (%)	WC1 (%)	B (%)	CD25 (%)
"Negative control"	27.06 ± 5.14	8.08 ± 1.11	1.52 ± 0.59	49.39 ± 4.2	9.62 ± 3.75
Group 1: complete recovery	35.49 ± 5.92** © ¤	17.74 ± 3.02** © ¤	0.98 ± 0.48*	43.87 ± 6.20* © ¤¤	10.2 ± 2.98
Group 2: death	26.98 ± 6.53	12.42 ± 6.41	0.52 ± 0.24** # ¤¤	53.97 ± 11.75	9.08 ± 4.20
Group 3: recovery + sequestra	26.17 ± 3.37	14.77 ± 3.30**	1.3 ± 0.46	53.09 ± 4.53	5.85 ± 1.87** ## ©

* Significant difference ($p < 0.05$) and ** highly significant difference ($p < 0.01$) with the "negative control" group. # Significant ($p < 0.05$) and ## highly significant difference ($p < 0.01$) with group 1. © Significant ($p < 0.05$) and ©© highly significant difference ($p < 0.01$) with group 2. ¤ Significant ($p < 0.05$) and ¤¤ highly significant difference ($p < 0.01$) with group 3.

by a significant *Mmm*SC-specific serological response measured by the CFT (data not shown). As shown in Table I, two "contact" cattle died from acute infection (C12, C14) while nine recovered either from a subclinic/chronic form or after an acute clinical period. Unfortunately, the death of the two acutely-infected zebus occurred at night, thus no viable cell samples could be taken. Therefore, in order to study animals succumbing from the infection, six cattle from the "infected" group with acute infection leading to death were included in the study as well as two recovered animals. Although several parameters were different between both groups of cattle, the comparative analysis, according to the epidemiological data discussed below, was assumed to be relevant. The animals were assigned to three groups according to the outcome of the infection and intensity of the lung lesions: group 1: animals with complete recovery (C4, C8, C13, I9, I12), group 2: animals with acute infection leading to death (I1, I2, I4, I5, I6, I11), group 3: recovered animals presenting lung sequestras (C3, C5, C6, C7, C9, C11).

The PM analysis revealed that animals with acute infection were characterised by typical CBPP lesions such as lung hepatisation and large quantities of pleural fluid (C12, C14, I1, I2), large (I4, I5, I11) or multiple sequestra (I6) involving almost completely one lung lobe. Instead recovered animals presented either fibrotic scars (C4, C8, C13, I9, I12) or small sequestra (C5, C6, C7, C11), although 2 cattle showed a medium size sequestra (C3 and C9).

3.2. Ex vivo phenotypic analysis of the lymph node cells

The percentage of each cell subset (CD4 and CD8 T-cells, $WC1^+$ $\gamma\delta$ T-cells and B-cells) as well as the percentage of activated ($CD25^+$) cells was monitored for each LN from each animal. Table II presents the results (mean percentage (± SD) obtained for the three groups compared to the "negative control" group. It shows that animals from group 1 were characterised by a significant increase of both the CD4 and CD8 T-cells compared to the "negative control" group ($p < 0.01$) but also to groups 2 ($p < 0.05$) and 3 ($p < 0.05$). Group 3 was also characterised by a highly significant increase of the CD8 T-cells compared to the "negative control" group ($p < 0.01$). In parallel, a significant decrease of the B-cells was

Table III. Analysis of the *Mmm*SC non-specific responsiveness. Lymph node cells from the "negative control" group cattle were stimulated for 5 days in vitro with *Mmm*SC and the percentage of blastic cells, CD25$^+$cells, CD4$^+$CD25$^+$cells and IFNγ production were determined by flow cytometry and ELISA, respectively. The percentage of CD4$^+$CD25$^+$cells was calculated within the CD4 T-cell gate and expressed as a percentage of the CD4 T-cell subset. The ConA responsiveness was indicated as a control. Data are the mean percentage or OD Units (± SD) obtained after stimulation minus the unstimulated control. The cut off values for *Mmm*SC-specific responsiveness were calculated by the mean value obtained for the "control" cattle + 2 SD.

	ConA stimulation (% of cells)	*Mmm*SC stimulation (% of cells)	Cut off (mean + 2 SD)
Blasts	16.3 ± 6.7	1.24 ± 1.5	4.23
CD25	26.5 ± 6.1	1.62 ± 1.23	4.16
CD25$^+$CD4 T-cells	42.91± 8.87	5.48 ± 4.55	14.58
IFNγ	1.24 ± 1.07	0.03 ± 0.06	0.15

observed in group 1 compared to the "negative control" group ($p < 0.05$) and to group 2 ($p < 0.05$) and 3 ($p < 0.01$). A significant decrease of the γδ T-cells was also noticed in group 1 animals compared to the "negative control" group ($p < 0.05$). However, group 2 animals were characterised by the lowest percentage of γδ T-cells compared to either the "negative control" group ($p < 0.01$), group 1 ($p < 0.05$) or group 3 ($p < 0.01$). Nevertheless, the very low proportion of the γδ T-cell subset in the cattle lymph nodes has to be noticed. No other difference was measured either between groups 2 or 3 and the "negative control" group or between groups 2 and 3.

The measurement of the CD25-expressing cells revealed a significant decrease in group 3 compared to the "negative control" group ($p < 0.01$), to group 1 ($p < 0.01$) and group 2 ($p < 0.05$).

3.3. *Mmm*SC responsiveness of lymph node cells

The *Mmm*SC responsiveness of the LN cells was then compared between the 3 groups of animals. For accurate discrimination between *Mmm*SC-specific and non specific response, cut off values were first determined (Tab. III) with LN cells from the "negative control" group, as described in Materials and methods.

A preliminary analysis was performed on 10 *Mmm*SC-infected cattle to assess whether variability in the *Mmm*SC-specific immune response occurred between the mediastinal and the tracheobronchic LN. The results, shown in Table IV, revealed no significant difference for any of the immune parameter tested. Accordingly, the data from both LN were treated regardless of their origin.

The first finding of the study was that all the *Mmm*SC-infected cattle presented an *Mmm*SC-specific cellular responsiveness except two animals. Indeed, I1 and I2 were unable to develop an in vitro response to *Mmm*SC stimulation, likely due to the severe lung lesions. Both animals were characterised after 5 days of culture by a very low percentage of CD4 T-cells, 6.10 ± 3.73%, while a mean percentage of 25.20 ± 7.26% of CD4 T-cells was observed in all other animals.

Figure 1 presents the mean value (+ SD) of each parameter obtained after *Mmm*SC stimulation minus unstimulated control for all the *Mmm*SC-responding samples in each group of animals, after excluding I1 and I2 from group 2. One LN (mediastinal) from animal C8 was also excluded in

Table IV. Comparative analysis of the *Mmm*SC-specific immune response between the mediastinal and the tracheobronchic lymph nodes. Lymph node cells from the 10 *Mmm*SC-infected cattle, for which both lymph nodes were available, were stimulated for 5 days in vitro with *Mmm*SC and the percentage of blastic cells, $CD25^+$ cells, $CD4^+CD25^+$ cells and IFNγ production were determined by flow cytometry and ELISA, respectively. The percentage of $CD4^+CD25^+$ cells was calculated within the CD4 T-cell gate and expressed as a percentage of the CD4 T-cell subset. Data are the mean percentage or OD Units (± SD) obtained after *Mmm*SC stimulation minus the unstimulated control.

	Mediastinal lymph node (% of cells)	Tracheobronchic lymph node (% of cells)
Blasts	11.53 ± 5.40	11.35 ± 8.05
CD25	12.76 ± 5.01	10.16 ± 6.29
$CD4^+$ $CD25^+$	26.51 ± 9.35	24.36 ± 9.09
IFNγ	1.68 ± 1.04	1.53 ± 1.01

group 1 from analysis since *Mmm*SC only increased one parameter (IFNγ secretion) above cutoff levels.

Figure 1 shows that *Mmm*SC exposure induced in all the three groups an *Mmm*SC-specific blastogenesis, CD25 expression and IFNγ production. However, the highest level of *Mmm*SC responsiveness was observed in group 1 animals with mean values for each parameter exceeding their respective values in the other two groups. The major finding was the significant increase in the percentage of *Mmm*SC-specific CD25-expressing cells within the CD4 T-cell subset observed in group 1 (30.80 ± 6.98%) compared to the other groups (20.12 ± 4.54% in group 2 ($p < 0.05$) and 20.01 ± 8.95% in group 3 ($p < 0.01$)). Measured within the total population, the percentage of *Mmm*SC-specific $CD25^+$-cells showed no significant difference between the three groups. The increase in blastic cells after *Mmm*SC stimulation was highly significant ($p < 0.01$) when comparing group 1 (16.77 ± 7.56%) to group 3 (9.06 ± 2.86%) while non significant compared to group 2 (11.29 ± 5.23%). A strong IFNγ production was triggered by *Mmm*SC in all three groups and, although the mean level of production was higher in group 1 (2.01 ± 1.06%), this was not statistically significant compared to group 2 (1.35 ± 0.61%) or group 3 (1.41 ± 0.841%).

3.4. Phenotyping of the *Mmm*SC-responding lymphocytes

A phenotypic study of the *Mmm*SC-responding lymphocytes was then performed on 4 *Mmm*SC-responding lymph nodes within each group. Figure 2 presents the results (mean value ± SD) obtained after *Mmm*SC stimulation compared to unstimulated cultures for the three groups of animals. Before comparing the results between the three groups, a first analysis was performed with the paired Student *t*-Test to assess the statistical significance of the *Mmm*SC-induced data compared to unstimulated cells. This analysis demonstrated that exposure to *Mmm*SC triggered, in group 1, a significant ($p < 0.05$) increase of MHCII-bearing cells (74.13 ± 3.36% with *Mmm*SC versus 61.8 ± 8.32% without stimulation). As shown in Figure 2, this increase might be due in part to B-cells, since all B-cells were expressing MHC II (data not shown), although the *Mmm*SC-induced B-cell proliferation and increase of CD25+B-cells were not statistically significant compared to unstimulated cells. Instead, the significant CD4 T-cell activation observed after *Mmm*SC exposure indicated that these cells were the main *Mmm*SC-responding lymphocytes. Indeed, the CD4 T-cell subpopulation was the only subset presenting a highly significant ($p < 0.01$) increase in the percentage of $CD25^+$cells in response to

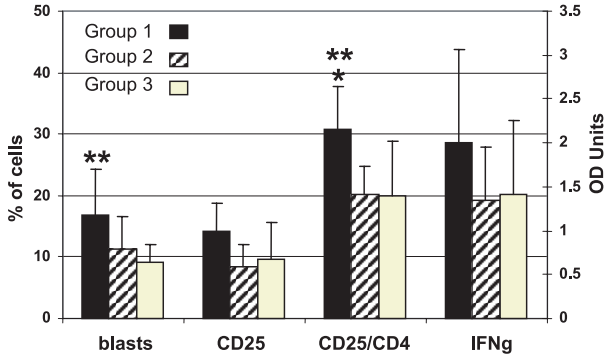

Figure 1. Comparative analysis of the lymph node cell *Mmm*SC responsiveness. Lymph node cells from the 3 groups of *Mmm*SC-infected cattle were stimulated for 5 days in vitro with *Mmm*SC and the percentage of blastic cells, $CD25^+$ cells, $CD4^+CD25^+$ cells and IFNγ production were determined by flow cytometry and ELISA, respectively. The percentage of $CD4^+CD25^+$ cells was calculated within the CD4 T-cell gate and expressed as a percentage of the CD4 T-cell subset. Group 1: animals with complete recovery, $n = 5$; Group 2: animals with acute infection leading to death, $n = 4$; Group 3: animals recovered with persisting lung lesions, $n = 6$. Data are the mean percentage or OD Units (± SD) obtained after *Mmm*SC stimulation minus unstimulated control. * Significant difference ($p < 0.05$) between groups 1 and 2; ** highly significant difference ($p < 0.01$) between groups 1 and 3.

*Mmm*SC stimulation (60.96 ± 12.37% versus 26.74 ± 7.07% in unstimulated cells). Furthermore, not only this percentage increased but also did the relative level of CD25 expression per CD4 T-cell as shown by the significant ($p < 0.05$) rise of the MdFI after *Mmm*SC stimulation (474.23 ± 190.51 fluorescent units) compared to unstimulated cells (116.53 ± 25.62 fluorescent units). However, *Mmm*SC did not trigger any CD4 T-cell proliferation. The proportion of $CD25^+\gamma\delta$ T-cells was constantly negligible (below 2% of the total cells) and thus not included in Figure 2. A similar result was observed for the $CD25^+CD8$ T-cells in 3 samples while the 4th sample showed a strong increase in the $CD25^+CD8$ T-cell percentage after exposure to *Mmm*SC.

For group 2, except for the significant CD4 T-cell activation elicited by *Mmm*SC, no other change was noticed compared to unstimulated cultures. Exposure to *Mmm*SC led to a significant ($p < 0.05$) increase of the percentage (36.61 ± 11.76% versus 16.50 ± 7.72% in unstimulated cells) and MdFI (112.04 ± 31.70 versus 53.28 ± 10.32 fluorescent units in unstimulated cells) of CD25+CD4 T-cells without cell proliferation. The percentage of CD25+CD8 T-cells was always below the cut off. In contrast to group 1, a decrease of the mean percentage of MHCII-bearing cells, although non significant, was observed after *Mmm*SC stimulation. Moreover, a decrease of the fluorescence intensity of the MHC II cell surface expression, after exposure to *Mmm*SC, was also noticed during flow cytometry analysis (data not shown).

In group 3, *Mmm*SC triggered a significant ($p < 0.05$) increase of the percentage of MHCII-expressing cells (66.19 ± 3.33%) and CD25+ cells within the B-cell subset (4.79 ± 1.83%), CD4 (37.78 ± 8.93%) and CD8 (31.67 ± 3.81%) T-cell subsets, compared to unstimulated cells (57.98 ± 2.68%, 0.4 ± 0.63%, 24.5 ± 6.76%, 14.9 ± 2.35%, respectively). *Mmm*SC stimulation also led to a significant ($p < 0.05$) increase of the MdFI of the CD25+CD4 subset (211.24 ± 77.54 fluorescent units versus 135 ± 59.66 fluorescent units without stimulation). As noticed above for groups 1 and 2, no T-cell

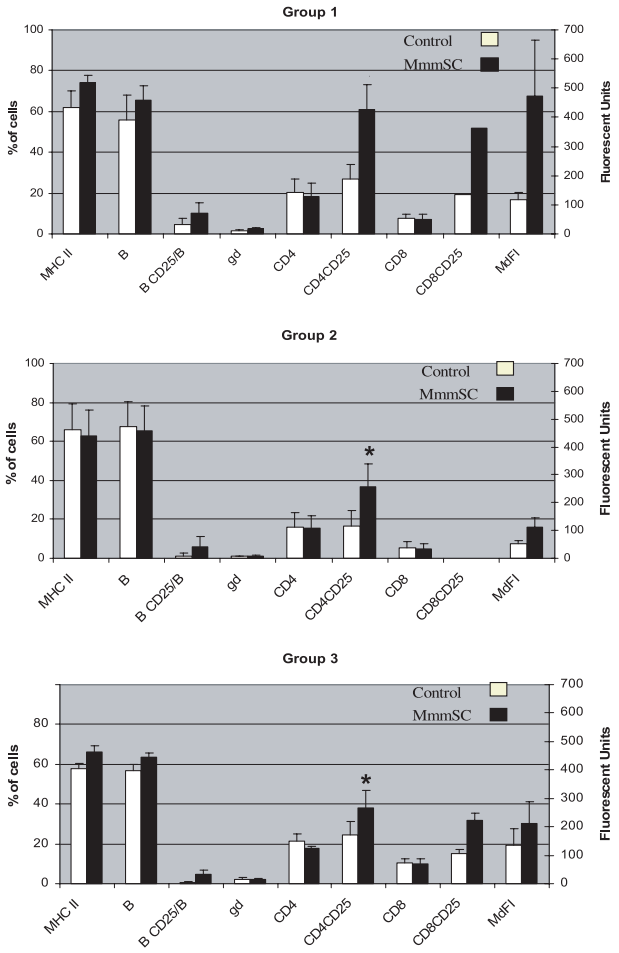

Figure 2. Phenotypic characterisation of the *Mmm*SC-responding lymph node lymphocytes. Lymph node cells from four cattle among each group were cultured for 5 days with either medium alone (control) or with *Mmm*SC and analysed by flow cytometry for measurement of the percentage of B-cells, γδ, CD4 and CD8 T-cells, MHC II and CD25 expression. The percentage of $CD25^+$ cells was calculated within each gated cell subset and expressed as a percentage of this subset. The MdFI of CD25 expression was measured for the gated $CD4^+CD25^+$ T-cells. Data represented are the mean percentage (± SD). Only one result for $CD25^+CD8$ T-cells was significant in group 1 (no SD) and none in group 2. * Significant difference ($p < 0.05$) with group 1.

proliferation was triggered by *Mmm*SC while a significant ($p < 0.05$) B-cell proliferation occurred (63.61 ± 2.11% versus 56.66 ± 3.48% in unstimulated cells).

A comparative analysis of the *Mmm*SC-specific CD4 T-cell activation was then performed between the three groups. The results confirmed that the percentage of CD4 T-cells responding to *Mmm*SC stimulation (CD25-expressing) as well as the relative level of activation of these CD4 T-cells (MdFI) was significantly higher in group 1 ($p < 0.05$) compared to groups 2 and 3. No significant difference was measured between groups 2 and 3.

4. DISCUSSION

The aim of the study was to unravel the protective immune mechanism elicited in cattle recovered from an *Mmm*SC infection and known to develop a long-term immunity [15, 21]. Antigen recognition in the context of infection generates two subsets of immune cells, the effector cells remaining in the infected tissues to enable immediate protection and the memory cells homing to the draining lymph nodes and responsible for the anamnestic response [9, 11, 22]. The *Mmm*SC-specific immune mechanism generated, therefore, during the primary immune response and persisting in the regional lymph nodes should represent the *Mmm*SC-specific memory response, basis of the protective secondary response.

An experimental infection was thus implemented by natural transmission of the *Mmm*SC infection from infected cattle (infected group) to a group of naive animals (contact group). For immunological relevance, a natural infection (respiratory route) of the natural host (cattle) was used, although CBPP is described as difficult to reproduce. The *Mmm*SC-specific immune response of the regional lymph nodes was then analysed.

This study demonstrated that an *Mmm*SC-specific cell-mediated immune response was, indeed, persisting in the mediastinal and tracheobronchic lymph nodes of all recovered animals, several months after the *Mmm*SC infection. A significant proportion of lymph node cells underwent blast transformation, expressed a $CD25^+$ phenotype (a correlate of cell activation) and produced IFNγ, after in vitro re-exposure to *Mmm*SC. Phenotyping indicated that the main *Mmm*SC-responding lymphocytes were CD4 T-cells. Similar results were obtained from the study of *Mmm*SC-specific peripheral immune responses where IFNγ-secreting CD4 T-cells were detected in the blood of all recovered cattle [5]. These findings suggest that the *Mmm*SC-specific IFNγ-secreting CD4 T-cell responses detected in the blood of all recovered cattle and persisting in their regional lymph nodes play a significant role in the protective mechanism against CBPP. Nevertheless, the absence of CD4 T-cell proliferation in response to *Mmm*SC, observed in both studies, is striking and might suggest an immunosuppressive mechanism (regulatory T-cells, IL-10, TGF-β). Further analysis of the cytokine response will determine their involvement; instead, the role of Treg in cattle still requires relevant reagents. However, the significant IFNγ production, confirming T-cell activation, and an ongoing study showing that T-cell proliferation was measurable eight days after *Mmm*SC stimulation[1] do not support an immunosuppressive process. The present study, instead, seems to demonstrate that following the primary immune response, a subset of in vivo *Mmm*SC-primed IFNγ-secreting CD4 T-cells homed to the draining lymph nodes. This suggests that these cells are *Mmm*SC-specific memory T-cells, likely responsible for the protective anamnestic response.

A main role for the CD4 T-cells was predictable since *Mmm*SC, like the majority of mycoplasma, is an extracellular pathogen [27]. The CD4 T-cell response is thus initiated by "antigen presenting cell" engulfing and processing *Mmm*SC and MHC class II presentation of *Mmm*SC epitopes. We assume that protection relies on the role of IFNγ to promote vigorous phagocyte response and thus *Mmm*SC capture. A protective role for the IFNγ-induced IgG2 antibody [3], only able to promote killing by neutrophils in cattle [8, 19], is also expected since neutrophil recruitment has been observed in CBPP lung lesions[2] [26]. However, confirmation of a typical Th1-like T-cell response requires the assessment of the Th2 cytokines. This study will be implemented since immunological reagents are now commercially available to develop ELISA for bovine Th2 cytokines. In contrast, the role of the CD8 T-cells, generally linked to intracellular pathogens observed

[1] Totte P., personal communication.
[2] Ferronha M.H., personal communication.

in animals with lung sequestras is not clear. Although *Mmm*SC have been immunohistochemically detected in macrophages, data are lacking to confirm its viability in any host cell [27, 29]. A by-stander effect of the *Mmm*SC-specific CD4 T-cell response was rather suggested [5]. Further studies are required to assess the cytokine environment and determine whether these cells play a role in protection. Significant γδ T-cell activation was observed in the *Mmm*SC-specific peripheral immune response [5] while it could not be detected in this study. Indeed, while γδ T-cells are a significant component of the afferent and efferent lymph and, in ruminants, may constitute up to 50% of the PBMC in young animals, they are only a minor population within the lymph nodes, suggesting that they re-circulate preferentially through non lymphoid tissues [33].

Recovery from an *Mmm*SC infection and protection requires thus an *Mmm*SC-specific IFNγ-secreting CD4 T-cell response. Its onset and magnitude but also other immune parameters such as the local immune response, since CBPP is a mucosal disease, are likely to play a role. A recent study demonstrated, indeed, a significant correlation between the presence of *Mmm*SC-specific IgA and an attenuation of the clinical impact of the *Mmm*SC infection[3]. The significantly lower magnitude of the *Mmm*SC-specific IFNγ-secreting CD4 T-cell response detected in the group of cattle recovered but with sequestras might explain this outcome. While likely involved in the process of recovery, this response was not sufficient to efficiently control the *Mmm*SC infection. Nevertheless, sequestras, as granulomas, develop as part of the protective host response to block the deleterious effects of the pathogen [21, 28, 29]. The intensity of the immune response detected in the lymph nodes of some animals succumbing from infection was also significantly lower than in animals with complete recovery. The presence of viable *Mmm*SC in the respiratory lymph nodes, described in acute cases of CBPP [2, 29], might also have played a role. Indeed, although not proved in vivo, the in vitro ability of viable *Mmm*SC to trigger apoptosis in bovine leucocytes has been demonstrated [6]. The *Mmm*SC unresponsiveness, observed in two cattle with strong acute lung lesions, might represent the final step of this immunopathological process. A correlation between progression of CBPP and a decreased ability of the PBMC to produce IFNγ was previously observed [5] while in this study the lymph node CD4 T-cells were able to produce IFNγ. Unfortunately, data on the kinetic of the blood immune response, but also on the Th1/Th2 balance, are lacking for this group of cattle in order to further unravel the immunopathological mechanism leading to a fatal issue.

The comparative analysis of these groups of animals might be limited by the introduction of different parameters (breeds, age, sex ratio…), however several epidemiological data support this comparison: (1) both breeds, N'Dama cattle and Peulh zebus, belong to the genus *Bos* and are thus susceptible to naturally occurring CBPP, (2) a full susceptibility to *Mmm*SC infection is described for animals over two years of age and (3) variation in the susceptibility according to the sex of the animals has not been reported [7, 15, 21]. According to these authors, the difference in the ratio of acute versus chronic CBPP formed between both groups might be due to (1) the fact that only cattle with evident clinical signs of CBPP were selected in the former group while in a newly infected herd, nearly a third of the animals developed an acute form, (2) geographical difference in the susceptibility of N'Dama cattle observed between some African countries and (3) a decline of infection pressure on the "contact" group resulting from the rapid death of cattle from the "infected" group but also from the time-dependent decrease of the *Mmm*SC strain virulence already described within a herd, although no molecular tools are available to assess this parameter.

[3] Niang M., paper in preparation.

Therefore, animals with total recovery were characterised by the highest magnitude of the *Mmm*SC-specific CD4 T-cell response, likely responsible for a more efficient control of the *Mmm*SC infection before the development of lung lesions. The long-term protection developed in these animals should thus rely on the ability of these memory T-cells, after a new antigen encounter, to rapidly adopt an effector phenotype, migrate to the site of infection and trigger a faster and stronger response [11, 12]. Indeed, memory cells, in contrast to naive cells, are activated at lower antigen concentration and are less dependent on costimulation factors leading to higher response even at the early onset of infection [13]. Future studies will focus on the anamnestic response triggered by a second *Mmm*SC challenge to help confirm the protective mechanism. Nevertheless, the induction and expansion of this subset of *Mmm*SC-specific CD4 memory T-cells appear to be one of the required goals in order to develop efficient long-term protective vaccines against CBPP.

ACKNOWLEDGEMENTS

This work was part of an INCO project entitled "Development of an improved vaccine against contagious bovine pleuropneumonia" supported by a European Commission research grant (ICA4-CT-2000-30015). The authors would like to dedicate this work to the memory of Mahamadou Diallo for his strong participation in this project.

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