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Original article

Analysis of cellular responses to *Mycoplasma mycoides* subsp. *mycoides* small colony biotype associated with control of contagious bovine pleuropneumonia

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Abstract – A better understanding of protective immune memory against contagious bovine pleuropneumonia (CBPP) is needed in order to facilitate the development of safer vaccines based on selected components of the pathogen. For this purpose, cells collected from lymph nodes draining the lungs of *Mycoplasma mycoides* subsp. *mycoides* small colony biotype (*Mmm*SC)-infected cattle were stimulated with the pathogen in vitro and evaluated concurrently for proliferation (CFSE based method), expression of activation, memory markers and cytokine production. Direct evidence is presented for a major contribution of $CD4^+$ T cells to the vigorous proliferative and T1 biased cytokine recall responses observed in cattle that have recovered from infection but not in animals developing the acute form of the disease. Two different phenotypes of *Mmm*SC-specific memory CD4 were observed based on CD62L expression and proliferative capacities. Furthermore, recall proliferation of B cells also occurred but was strictly dependent on the presence of CD4. The information provided in this study will facilitate the search for *Mmm*SC antigens that have potential for the development of subunit vaccines against CBPP.

contagious bovine pleuropneumonia / *Mycoplasma mycoides* subsp. *mycoides* SC / vaccine / memory / CD4

1. INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* small colony biotype (*Mmm*SC) is the etiological agent of contagious bovine pleuropneumoniae (CBPP), an economically important bacterial epizootic classified in the A-list of contagious animal diseases by the World Organization for Animal Health[1]. CBPP is an infectious pulmonary disease of cattle characterized by low mortality and high morbidity in endemic areas. Acute inflammatory lesions in the lungs may lead to death due to respiratory distress but in most cases chronic infection occurs causing weight loss and reduced fertility [19]. CBPP is widespread in Africa and present in some parts of Middle East and Asia, and poses a threat to disease-free countries [11].

Current vaccination strategy is based on the administration, via the subcutaneous route, of partially attenuated strains of *Mmm*SC. Major drawbacks of this method include the following: (i) the need for costly annual vaccination campaigns because of short duration of protection [24]; (ii) residual virulence provoking abscesses at the site of vaccination and, in some cases, invasive edema [20]; (iii) risks of reversion to increased virulence. For these reasons, improved "next-generation" vaccines against CBPP are urgently needed [10]. One attractive option is the development of a safer

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subunit vaccine based on selected components of *Mmm*SC. An important goal of a CBPP subunit vaccine is to induce long-term immunological memory. Protective memory cells must be capable of expansion and rapid effector functions upon reencounter with the pathogen [21, 28]. Previous work has shown that activation of $CD4^+$ T cells and IFN-γ production are detected in animals that have recovered from CBPP, thus suggesting a role for these responses in protection [7, 8]. However, direct evidence of proliferation, expression of memory markers, and IFN-γ production by $CD4^+$ T cells or any other lymphocyte subsets in response to *Mmm*SC is lacking.

The aim of this study was to further characterize cellular memory responses associated with the control of CBPP. For this purpose, cattle that have completely recovered from infection and acquired life-long immunity were compared with animals developing acute CBPP. The presence and characteristics of *Mmm*SC-driven memory recall responses were analyzed in draining lymph nodes because of their central role in the generation of memory responses to infection. Initial experiments focused on the kinetics of the in vitro recall response to *Mmm*SC by analyzing changes in frequencies and numbers of major lymphocyte subsets. In addition, the effect of antigenic stimulation on the expression of CD25 and CD62L, two indicators of pathogen-driven lymphocyte activation in cattle [13, 25], and on the expression of the memory marker CD45RO, was evaluated. Proliferation among major lymphocyte subsets was analyzed further by the CFSE based method on optimal time-points. Finally, production of IFN-γ and IL-4 was measured by several methods including direct intracellular staining to determine the type of cytokine response and which lymphocyte subsets were involved.

2. MATERIALS AND METHODS

2.1. Animals and experimental infections

The first experiment took place in Cameroon at the National Veterinary Laboratory (Garoua). Naïve zebu cattle over two years of age were endobronchially inoculated with local virulent isolates of *Mmm*SC as previously described [26]. Briefly, animals were sedated prior to intubation and inoculation with 50 mL of a fresh mycoplasma culture collected in log phase growth, followed by 30 mL of 1% suspension of agar in normal saline. In the second experiment, carried out in Mali at the Central Veterinary Laboratory (Bamako), a natural in-contact infection was reproduced [17]. Three to 6-year-old zebu cattle purchased from a CBPP-free area and negative for antibodies to *Mmm*SC were put in contact with animals taken from a CBPP field outbreak. The experiments were carried out according to the guidelines in the guide to the Care and Use of Experimental Animals, provided by the French Ministry of Agriculture. Postmortem analysis was performed one month and one year after infection in the first and second experiment, respectively. The animals were classified into three groups according to disease outcome and postmortem analysis: (i) acute CBPP characterized by a marbled appearance of the lungs due to hepatisation and absence of sequestra (see below) – these animals developed a respiratory distress and were euthanized before the end of the experiment to limit suffering; (ii) chronic CBPP characterized by the presence of various-sized capsules composed of fibrous connective material surrounding infected tissues (i.e., sequestra); (iii) chronic CBPP characterized by fully resolved lesions in the form of fibrotic scars and only observed in animals slaughtered one year post-infection (i.e., experiment 2).

2.2. Preparation of cells from lymph nodes and CFSE staining

Mediastinal lymph nodes draining the lungs of infected ($n = 11$) and noninfected ($n = 3$) cattle were collected at necropsy in ice-cold Hanks balanced salt solution (HBSS, Eurobio, Les Ulis, France) supplemented with antibiotics. Cells collected from sliced lymph nodes were filtered through sterile gauze and washed with HBSS. Cells were shipped to Cirad cryopreserved in liquid nitrogen at 2×10^7 viable cells/mL in fetal calf serum (Eurobio) supplemented with 10% DMSO (Sigma, Saint-Quentin, France). Upon thawing, dead cells and debris were removed by centrifugation on Ficoll density gradient (Eurobio) prior to use in stimulation assays. Loading of cells with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Cergy-Pontoise, France) was performed as described previously [22] with minor modifications. Briefly, cells were resuspended at 2×10^7 cells/mL in HBSS containing CFSE at a final concentration of 0.5 μM. After incubation for 10 min at 37 °C, the

reaction was stopped by adding an equal volume of ice-cold heat-inactivated fetal bovine serum (FCS) and the cells were washed three times in RPMI medium containing 10% (vol/vol) FCS.

2.3. Cell culture and cytokine ELISA

Cells purified from lymph nodes were resuspended in complete RPMI (RPMI-1640 supplemented with 10% FCS (Eurobio), 2mM glutamine, antibiotics and 2-mercaptoethanol (Sigma)). The cells were seeded at a density of 3×10^5 cells/well in 96-well flat-bottomed plates for stimulation prior to surface phenotyping by flow cytometry and at 2×10^6 cells/well in 24-well plates for intracellular staining (see flow cytometry), and cultured at 37 °C in 5% CO_2 in air. Heat inactivated whole *Mmm*SC (T1/44 strain) was prepared as previously described and added at a final concentration of 5 μg/mL [7]. Concanavalin A (ConA, 5 μg/mL) stimulation was used as a positive control. For intracellular cytokine staining, Brefeldine A (Sigma) was added at 5 μg/mL for the last 15 h of incubation to block cytokine secretion. Supernatants were collected at different incubation times and after centrifugation of cells and stored at –20 °C until use. Commercially available ELISA were used according to the manufacturer's instructions to detect secreted IFN-γ (BioCore, Omaha, NE, USA) and IL-4 (Perbio, Brebieres, France). The results are expressed as optical densities and are means of duplicate assays.

2.4. Flow cytometry and intracellular cytokine staining

For cell surface phenotyping, expression of bovine differentiation antigens and activation/memory markers was analyzed by indirect immunofluorescence staining using the following specific mouse monoclonal antibodies (Mab): Mab GC50 (IgM) for CD4, Mab BAQ11A (IgM) for CD8, Mab GB21A (IgG2b) specific for the γ chain of the T-cell receptor (TCR) of all γδ T cells, Mabs CACT116A (IgG1) or CACT108A (IgG2) which recognize the α chain of bovine CD25, and Mab BAQ92A (IgG1) for CD62L (all obtained from VMRD, Pullman, Washington, USA); Mab DU2-104 (IgM) reacts with B-cells and was a kind gift of W. Hein (Basel Institute for Immunology, Basel, Switzerland, [15]); and Mab ILA150 (IgG3) for CD45RO obtained from the International Livestock Research Institute (ILRI, Nairobi, Kenya, [2]). After washing, the cells were stained with a cocktail of fluorochrome-conjugated, isotype-specific antibodies (Tebu, Le Perray, France). Normal mouse serum (Sigma) was used to evaluate nonspecific binding and to set gates delineating positive populations. Single fluorescence-stained samples were used to optimize compensation of signals. Two or three-color analyses were performed on a FACscan flow cytometer equipped with the CellQuest 3.01 software (Becton Dickinson, San Jose, CA, USA) after acquisition of at least 5 000 events within a typical forward and side scatter gate set to exclude dead cells and debris. The level of proliferation of a given subset was estimated by calculating a growth index using the following formulae: [(number of viable cells at day 8 × % subset at day 8)/100] / [(number of viable cells at day 0 × % subset at day 0)/100], where numbers of viable cells were determined using the trypan blue exclusion assay (Sigma).

For analysis of cell division, CFSE content (green fluorescence, FL1) was measured in total cells or within gated cell subsets when combined with surface staining. Cell division is associated with decrease in fluorescence intensity. Frequencies of a given cell subset within each division cycle was determined by drawing regions that represented increments of 2-fold reduction in CFSE intensity relative to that in the undivided cells.

Intracellular cytokine staining was performed, as previously described, with some modifications [27]. Briefly, the cells were first labelled with Mabs against surface differentiation antigens as above, fixed in PBS containing 1% formalin and 0.1% NaN_3, and permeabilized in PBS, 10% FCS, 0.1% saponin. Cytokines were stained with Mab 7B6 (IgG1) and 10H8 (IgG2b) against bovine IFN-γ and IL-4, respectively, kindly given by K. Walravens (CERVA, Brussels, Belgium, [27]). After washing, appropriate fluorochrome-conjugated isotype-specific antibodies were used as secondary reagents (Tebu, France). At least 10 000 events were acquired within a CD4 gate. Staining with isotype-matched control antibodies did not increase background fluorescence. On some occasions, an amplification procedure was used prior to intracellular cytokine staining. Briefly, both unstimulated and *Mmm*SC-stimulated cultures were treated with ConA and BrefeldinA (5 μg/mL) for the last 15 h of incubation. Because of the limited amount of frozen material available and the high cell number required for these experiments, they could not all be performed on each and every animal of the chronic CBPP group. However, care was taken as to use

animals with sequestra and animals with fully resolved lesions in each experiment.

2.5. Depletion studies

Cells were depleted of $CD4^+$ T cells by positive selection using a magnetic activated cell sorting system (Macs, Miltenyi Biotec, Paris, France) and according to the manufacturer's instructions. Briefly, cells were stained with Mab ILA12 (IgG2a) against bovine CD4 (ILRI, Nairobi, Kenya, [16]) and incubated with Macs microbeads conjugated to goat anti-mouse IgG (H+L) prior to separation on MS columns. Unbound cell fractions collected from the columns repeatedly contained less than 1% $CD4^+$ T cells as confirmed by flow cytometry. To exclude the possible impact of the technique on the reactivity of cells to in vitro antigenic stimulation, control cells, termed "undepleted" cells thereafter, were stained with an irrelevant Mab ILA110 (IgG1) which binds to neutrophils [16] and ran through the whole procedure.

2.6. Statistical analysis

Nonparametric Mann-Whitney *U*-test and Wilcoxon matched pairs rank test were used to analyze differences between infected and non-infected animals, and between different T-cell subsets, respectively. A difference was considered to be significant at $P = 0.05$. Statistical analysis was performed using a standard statistical package (StatView 5-0 for Windows; SAS Institute Inc.).

3. RESULTS

3.1. Characterization of the proliferative recall response to *Mmm*SC in draining lymph nodes

Cells isolated from lymph nodes draining the lungs of infected and uninfected cattle were stimulated in vitro with *Mmm*SC Ag and harvested after 0, 4, 6, 8, and 10 days of culture for flow cytometric analysis. Changes in frequencies and in numbers of major lymphocyte subsets and in the expression of activation and memory markers were monitored. Differences between animals exhibiting sequestra ($n = 3$, experiment 1) and those with fully resolved lesions ($n = 3$, experiment 2) were not significant; thus, data from these animals were pooled in one chronic CBPP group. In addition, animals developing acute CBPP in both experiments showed similar patterns of response and were pooled in one group.

As shown in Figure 1, an increase in the percentages of $CD4^+$ T cells and the proliferation (i.e., growth index above 2) was detected but only in stimulated cultures prepared from animals developing chronic CBPP. Naïve animals and those developing acute CBPP did not show any significant changes in these parameters at any time-points despite comparable responses to the mitogen ConA (not shown). A strong increase in percentages of $CD4^+$ cells expressing the activation marker CD25 and the peripheral lymph node homing receptor CD62L was also observed in animals from the chronic CBPP group (Fig. 1). Three-color flow cytometric analysis of CD62L expression on double positive $CD4^+CD25^+$ cells revealed a mixed population at day 4 ($45 \pm 10\%$ $CD62L^+$) and confirmed the strong increase at day 6 ($80 \pm 15\%$ $CD62L^+$). In contrast, in animals undergoing acute CBPP, expression of CD25 on $CD4^+$ T cells was much lower ($P = 0.007$) and CD62L expression was either unaffected or down regulated (Fig. 1). There was no increase in percentages and numbers of B cells, $CD8^+$ T cells and $\gamma\delta TCR^+$ T cells (data not shown).

Vigorous recall proliferation of $CD4^+$ T cells from animals controlling CBPP was confirmed by the CFSE method. As shown in Figure 2, substantial numbers of stimulated $CD4^+$ T cells had undergone proliferation at day 4 as shown by decreased CFSE staining intensity, but without any significant increase in their frequency in comparison to non-stimulated cells. This was likely due to the concomitant proliferation of a $CD4^-$ population (Fig. 2). However, by day 6, percentages of $CD4^+$ T cells had increased by more than 3-fold in stimulated cultures (Fig. 2). Similar observations were made for all 4 animals tested, with small differences in the magnitude of the response, most likely due to the random bred nature of these animals (Tab. I).

Further analysis of the phenotype of proliferating cells (i.e., CFSElow fraction) indicated that B cells represent the dominant population at day 4 while $CD4^+$ cells largely outnumbered all other subsets by day 6 (Fig. 3).

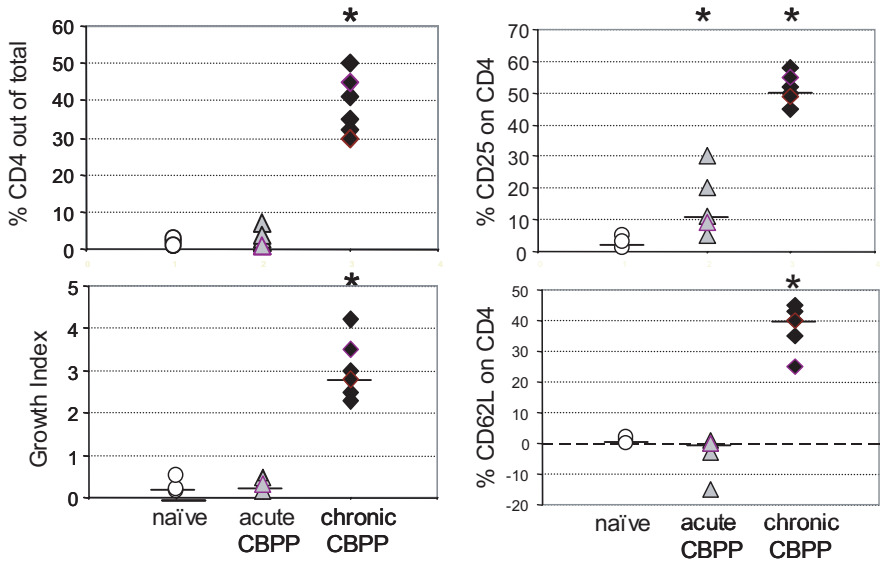

Figure 1. Comparison of *Mmm*SC-driven recall responses by $CD4^+$ T cells between naïve (white, $n = 3$) and infected cattle developing acute (grey, $n = 5$) and chronic (black, $n = 6$) forms of CBPP. Cells from mediastinal lymph nodes draining the lungs were purified and incubated with or without heat-inactivated *Mmm*SC. Results represent the net effect of *Mmm*SC (i.e., stimulated cultures minus non-stimulated cultures) from a kinetic study that is representative of three experiments. Only the maximal values are shown (i.e., day 8 for animals with chronic CBPP and day 10 for the others). The growth index was obtained (see also Materials and methods) by the following formulae: (number of viable CD4 at day 8/number of viable CD4 at day 0). Bars show median values and asterisks indicate significant difference between infected and naïve groups.

Table I. Association between increase in percent of CD4 and increase in CD62L expression upon *Mmm*SC stimulation of cells obtained from draining lymph nodes of cattle that controlled CBPP[a].

Animal number	CD4 of total cells (%)		CFSElow cells[b] among CD4 (%)		CD62L on (%)			
					CFSElow CD4		Total CD4	
	Day 4	Day 6	Day 4	Day 6	Day 4	Day 6	Day 4	Day 6
1	11	54	56	95	51	80	46	75
2	12	46	70	95	73	96	70	94
3	19	66	52	96	52	90	54	86
4	7	43	60	94	49	86	57	85

[a] Cattle #1 and #2 exhibited well formed sequestra at post mortem while animals #3 and #4 presented fully resolved lesions (i.e., fibrotic scars).

[b] Proliferative fraction within the stimulated CD4 population.

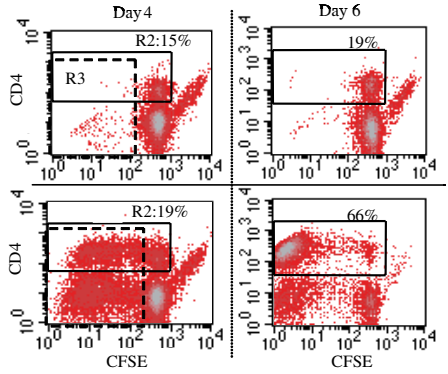

Figure 2. Both $CD4^+$ and $CD4^-$ populations proliferate in response to *Mmm*SC in vitro. As an example, results of a typical two-color flow cytometric analysis are shown in density plots for one animal representative of the chronic CBPP group and for one experiment representative of three. Cells were loaded with CFSE and incubated for 4 (left panel) and 6 days (right panel) with (lower panel) or without (upper panel) inactivated *Mmm*SC. Cell division is represented by decreasing CFSE fluorescence intensity (*X* axis, R3 gate, dotted lines) and frequencies of $CD4^+$ T cells are given by the gate R2 (solid lines) and indicated on the plots.

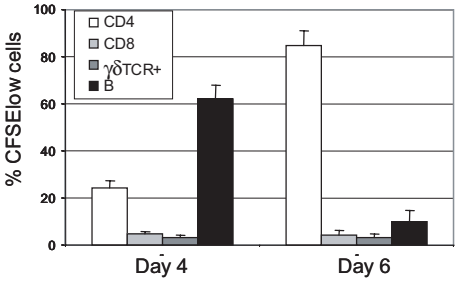

Figure 3. Proportions of lymphocyte subsets among cells that proliferate in vitro in response to *Mmm*SC. An R3 gate was set as shown in Figure 2 to analyze cells with CFSE content lower than that of non proliferating stimulated cells (i.e., CFSElow corresponding to the proliferative fraction). Mean percentages (± standard deviation) of $CD4^+$, $CD8^+$, $\gamma\delta TCR^+$ T cells and B cells among CFSElow cells are given for three animals from the chronic CBPP group.

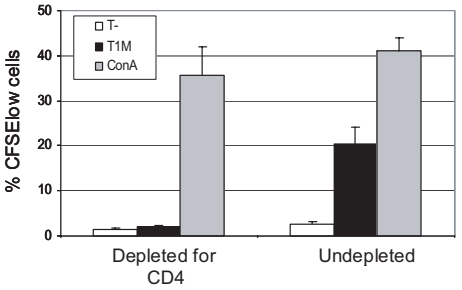

Figure 4. Effect of CD4 depletion on *Mmm*SC-induced proliferation. Cells were incubated for 4 days in the absence (T^-) or presence of *Mmm*SC Ags (T1M) or the mitogen Concanavalin A (ConA) as a positive control for proliferation. Mean percentages (± standard deviation) of CFSElow cells are given for three animals from the chronic CBPP group.

Proliferation was also observed among $CD8^+$ and $\gamma\delta TCR^+$ T cells but these subsets comprised less than 10% of proliferating cells at all time points (Fig. 3). Finally, depletion of cultures for $CD4^+$ T cells completely abrogated the proliferative response to *Mmm*SC Ags (Fig. 4) indicating that B cells and $CD8^+$ and $\gamma\delta TCR^+$ T cells are strictly dependent on CD4 for proliferation.

3.2. Both $CD4^+$ $CD62L^+$ and $CD4^+$ $CD62L^-$ memory cells proliferate in response to *Mmm*SC

Strong and sustained recall proliferation of $CD4^+$ T cells occurred in vitro in response to *Mmm*SC Ags as shown by the large increase in the proportion of $CD4^+$ T cells that had undergone more than 4 divisions between day 4 and day 6 (Fig. 5a). Both $CD4^+CD62L^+$ and $CD4^+CD62L^-$ cells proliferated to a similar extent at day 4 but the first subset was largely dominant by day 6 and comprised more than 80% of the total $CD4^+$ stimulated population (Fig. 5b) versus 50% in unstimulated cultures (not shown). Similar trends were observed in all four animals tested (Tab. I). Finally, up regulation of the memory marker CD45RO on $CD4^+$ T cells was consistently observed after 6 days of culture with increase in both percentage positive and fluorescence intensity (Fig. 5c).

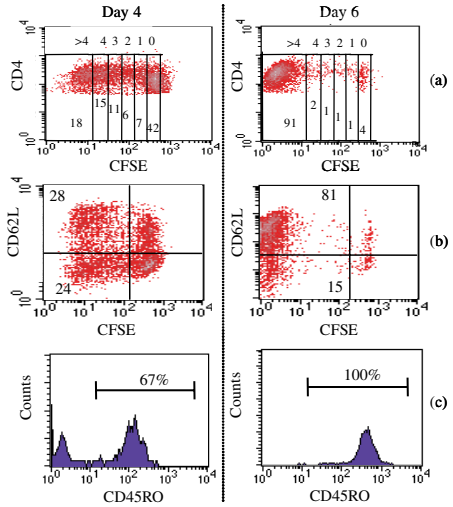

Figure 5. Kinetics of cell division (a) and CD62L (b) and CD45RO (c) expression among $CD4^+$ T cells in response to *Mmm*SC Ags. An R2 gate was set as shown in Figure 2 to analyze stimulated $CD4^+$ T cells. As an example, results from one animal of the chronic CBPP group and for one representative experiment of two are shown. The numbers above rectangular regions indicate the number of divisions (see also Material and methods) and percentages of cells within each region are given on the plots (a). Percentages of $CD62L^+$ (upper left quadrant) and $CD62L^-$ (lower left quadrant) with decreased CFSE content are given on plots (b). Finally, numbers shown on plots (c) indicate the percentages of $CD45RO^+$ cells among $CD4^+$ T cells.

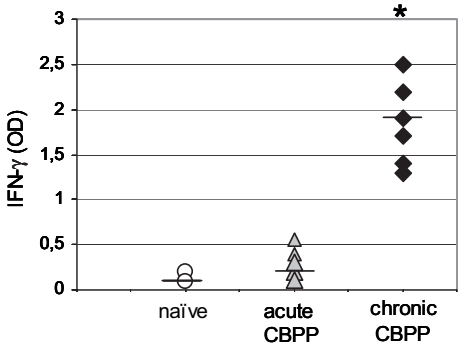

Figure 6. IFN-γ recall response to *Mmm*SC Ags by cells collected from draining lymph nodes of naïve (white, $n = 3$) and infected cattle developing acute (grey, $n = 5$) and chronic (black, $n = 6$) CBPP. The results are expressed as optical densities (OD) and represent the net effect of *Mmm*SC (i.e., stimulated cultures minus non-stimulated cultures). Only maximal values from a 10-day kinetics study that is representative of three experiments are shown. Bars and asterisks indicate median values and significant differences ($p < 0.05$) with uninfected animals respectively.

3.3. Characterization of the IFN-γ response to *Mmm*SC in draining lymph nodes

Secreted IFN-γ was detected in cultures prepared from all animals developing chronic CBPP with a progressive increase until day 4–6 of stimulation with *Mmm*SC Ags. In contrast, out of the five animals developing acute CBPP, only two produced IFN-γ but later (day 8–10) and at much lower levels ($P = 0.0062$, Fig. 6).

Dual staining for intracellular IFN-γ and surface phenotype indicated that the majority of cells producing IFN-γ in response to *Mmm*SC also expressed the CD4 T- cell lineage marker (Fig. 7). However, IFN-γ production by other subsets was also evident although to a lesser extent since both number of positive cells and fluorescence intensity were lower (Fig. 7). The major contribution of $CD4^+$ T cells to the IFN-γ recall response was confirmed in four animals of the chronic CBPP group (Fig. 8). Although the difference between numbers of $CD4^+$IFN-γ^+ and $CD4^-$IFN-γ^+ stimulated cells was not significant at the group level ($P = 0.06$), the first subset systematically outnumbered the second at the individual level.

3.4. The response of $CD4^+$ T cells to *Mmm*SC is Th1 biased

Using an ELISA based method, IL-4 secretion could not be detected in response to *Mmm*SC Ags in supernatants collected every 2 days for up to 10 days after stimulation whereas it was produced in the presence of ConA (not shown). Also, *Mmm*SC-stimulated $CD4^+$ T cells were negative for cytoplasmic IL-4 as analyzed by direct intracellular staining without amplification (not shown). Finally, even after amplification, IL-4^+CD4^+ cells were detected in only two out of three

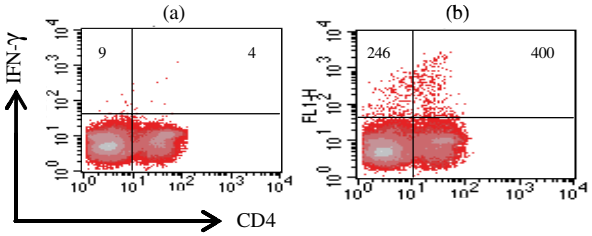

Figure 7. Both CD4$^+$ and CD4$^-$ populations produce IFN-γ in response to *Mmm*SC in vitro. The cells were incubated for 2 days in the absence (a) or presence (b) of *Mmm*SC Ags before analysis of intracellular IFN-γ (*Y* axis) and surface CD4 (*X* axis) expression by flow cytometry. As an example, the results of a typical two-color flow cytometric analysis are shown in density plots for one animal of the chronic CBPP group and for one representative experiment of three. Numbers of CD4$^-$ IFN-γ+ cells (upper left panel) and CD4$^+$ IFN-γ+ cells (upper right panel) are shown on the plots.

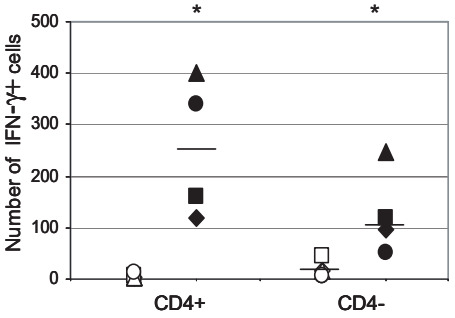

Figure 8. *Mmm*SC-induced production of cytoplasmic IFN-γ in CD4$^+$ and CD4$^-$ T cells collected from four animals of the chronic CBPP group. Cells were incubated with (black) or without (white) *Mmm*SC Ags for 2 days and immediately processed for intracellular staining of IFN-γ (no amplification step). Each symbol represents one animal and indicates the number of IFN-γ+ cells within the given population. Bars and asterisks indicate median values and significant differences ($P < 0.05$) with unstimulated cells respectively.

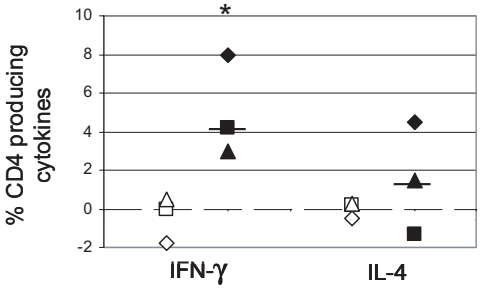

Figure 9. Production of IFN-γ and IL-4 by CD4$^+$ T cells in response to *Mmm*SC. Cells from uninfected animals (white, $n = 3$) and infected animals developing chronic CBPP (black, $n = 3$) were incubated with or without *Mmm*SC Ags for 5 days and subjected to intracellular cytokine detection after amplification as described in material and methods. Each symbol represents one animal and indicates background corrected frequencies of cytokine+ cells among 10 000 CD4 (response from cells with *Mmm*SC Ags minus response of cells without Ags). Bars and asterisks indicate median values and significant differences ($P < 0.05$) with uninfected animals respectively.

chronically infected animals tested and at low frequencies in comparison to IFN-γ$^+$CD4$^+$ cells (Fig. 9).

4. DISCUSSION

A better understanding of the basis of protective immunity against *Mmm*SC is needed in order to facilitate the development of improved vaccines against CBPP. Here, we provide direct evidence for the generation of *Mmm*SC-specific memory CD4$^+$ T cells capable of strong recall proliferation and IFN-γ production in animals controlling CBPP (i.e., sequestra) and in animals that have completely recovered from the disease (i.e., resolved lesions).

CFSE based analysis indicated that both B lymphocytes and CD4$^+$ T lymphocytes proliferate in vitro in response to *Mmm*SC. However, even though B cells comprised the

majority of the proliferating population (i.e., CFSElow) until day 6 of culture, no enrichment for that subset was observed at any time point. In contrast, after 6 days in vitro, $CD4^+$ T cells largely outnumbered all other subsets among CFSElow cells, which resulted in a substantial enrichment of cultures for CD4. These results provide an explanation to previous studies reporting a lack of enrichment for CD4 despite strong activation (i.e., up-regulation of CD25) in 5-day-old stimulated cultures [8] and underline the benefits of using CFSE loading in analysis of recall responses. Because of concomitant B-cell proliferation, changes in frequencies of $CD4^+$ T cells was not detected before day 6 while proliferation of these cells was measured as early as day 4 using the CFSE method. Other T-cell subsets also showed some degree of proliferation in response to *Mmm*SC but their contribution to the overall proliferative response was negligible. Depletion studies clearly showed that the *Mmm*SC-driven proliferation was under the strict control of $CD4^+$ T cells.

As anticipated, a strong increase in CD25 expression was observed on $CD4^+$ T cells upon incubation with *Mmm*SC Ags. More surprisingly, a strong up regulation of the peripheral lymph node homing receptor CD62L also occurred on $CD4^+$ T cells. It is generally accepted that extravasation and trafficking of activated lymphocytes to infected tissues require down regulation of CD62L expression [6]. Accordingly, loss of CD62L expression on T-cell upon activation was demonstrated in cattle infected with *Mycobacterium bovis* and respiratory syncytial virus both in vivo and in vitro after antigenic stimulation [13, 25]. In CBPP, CFSE loading indicated that in most animals both $CD62L^+$ and $CD62L^-$ cells are present in similar proportions within the proliferative fraction of *Mmm*SC-stimulated $CD4^+$ T cells at day 4 (Fig. 5, Tab. I). However, further proliferation among CD4 was associated with a strong increase in frequencies of $CD4^+CD62L^+$ cells. Analysis of CD45RO expression indicated that both subsets are memory CD4. In view of the recent observations on human and mouse lymphocytes [21, 29], the *Mmm*SC-specific $CD4^+CD45RO^+CD62L^+$ cells described in this study may be central memory T cells (Tcm) while $CD4^+CD45RO^+CD62L^-$ cells would be effector memory T cells (Tem). There is very limited information available on pathogen-derived T(cm) cells in ruminants. To the best of our knowledge, only *Babesia* and *Fasciola*-specific bovine $CD4^+CD45RO^+CD62L^+$ T cell clones have been reported so far [3, 4] but the authors suggested that high CD62L expression on these cells might be due to repeated stimulation in vitro. In our CBPP model, we found comparable levels of both $CD62L^+$ and $CD62L^-$ phenotypes among proliferating CD4 as early as day 2 after a single stimulation in vitro (results not shown). In addition, the memory/effector CD4 population (i.e., $CD4^+CD45RO^+$) present in the lymph node at day 0 comprised both $CD62L^-$ and $CD62L^+$ cells. These observations are consistent with two different subsets proliferating in parallel at the beginning of the culture rather than one subset differentiating into the other. Nevertheless, additional experiments are needed to better characterize these cells.

Secretion of IFN-γ upon stimulation with *Mmm*SC Ags was detected in culture supernatants prepared from all animals controlling CBPP. Intracellular staining of cytoplasmic IFN-γ provided direct evidence that CD4 are the main producers although production by other cell type was also evident. Finally, depletion of $CD4^+$ T cells completely abrogated the response (not shown), thus, suggesting that other subsets required the help of CD4 and/or respond in a bystander manner.

Because polarized cytokine expression profiles by T cells are rarely seen in ruminants, ratios of IL-4 and IFN-γ amounts are currently used to determine Th1 versus Th2 responses [5, 14]. In this study, both *Mmm*SC-induced secreted IL-4 and cytoplasmic IL-4 production in stimulated $CD4^+$ T cells were monitored by ELISA and flow cytometry, respectively. Our results indicated that no or little IL-4 was produced after *Mmm*SC stimulation in comparison to IFN-γ, suggesting a strong bias towards a Th1 type of response.

Live mycoplasmas have been detected in draining lymph nodes of *Mmm*SC-infected animals [1, 23] and have shown, recently, to induce apoptosis of lymphocytes in vitro [9]. In this study, impaired in vitro responses of animals developing acute CBPP was not due to an intrinsic lack of responsiveness of their lymphocytes as indicated by control experiments with the mitogen ConA (not shown). Also, although a role for regulatory CD4 (i.e., Treg) can not be completely excluded, the proportions of ex vivo $CD4^+$ T cells expressing CD25 were not significantly different between groups (not shown) and up regulation of that marker after *Mmm*SC stimulation in vitro was not detected in three out of five animals developing acute CBPP. The possibility that live *Mmm*SC dysregulates antigen uptake/presentation by macrophages and dendritic cells is currently under investigation.

In summary, the results presented here extend previous results [7, 8] and further support the idea that Th1 memory lymphocytes are involved in the control of CBPP. Vigorous proliferative and T1 biased cytokine recall responses, with a predominant contribution of $CD4^+$ T cells, were observed in cattle that have recovered from CBPP, but not in animals developing the acute form of the disease. Depletion studies confirmed that these responses are under the strict control of $CD4^+$ T cells. Putative protective immune mechanisms include the following: (i) production of IFN-γ, which potentiates phagocytic and bactericidal activity of macrophages and stimulates opsonization by bovine neutrophils through its IgG2 promoting effect [12], and (ii) CD4-dependent proliferation of B cells (this study). Interestingly, IgA levels in bronchial lavage fluids have recently been correlated with reduced severity of CBPP [18]. Additionally, we have described for the first time two phenotypes of *Mmm*SC-specific memory $CD4^+$ T cells that differ in CD62L expression and proliferative capacities. The population expressing high level of CD62L was associated with sustained recall proliferation in vitro. Thus, the *Mmm*SC-specific memory CD4 described in this study have strong effector and proliferative recall capacities and will be valuable tools to identify components of the pathogen that have potential for the development of a subunit vaccine against CBPP.

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