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Timing of activation of CD4$^+$ memory cells as a possible marker to establish the efficacy of vaccines against contagious agalactia in sheep

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ABSTRACT

Mycoplasma agalactiae is a major pathogen of sheep and goats in many areas of the world and particularly in Mediterranean countries. It causes contagious agalactia, an infectious disease primarily affecting mammary glands. Many vaccines against the pathogen are currently under development. The aim of the study was to investigate the involvement of T cell-mediated immunity during vaccination and challenge experiments against *Mycoplasma agalactiae*. A comparison of the antigen-specific expansion of interferon gamma positive T cell memory and naïve subsets was performed between vaccinated and non-vaccinated sheep to identify cellular subsets whose activation was different between protected and non-protected sheep. Data reported in this manuscript demonstrated that two out of the three vaccines used in this study protected sheep from the disease. In the protected groups CD4$^+$ memory interferon-γ$^+$ T cells underwent an early expansion ($p < 0.05$ when compared to unprotected groups), whilst memory CD8$^+$ Interferon-γ$^+$ T cells increased in non-protected animals 7 days after infection ($p < 0.05$). γδ$^+$ Interferon-γ$^+$ T cells reached peaks of expansion in infected and in two vaccinated groups thus indicating that these cells are not preferentially involved in protection or pathogenesis ($p < 0.05$).

Hereby we propose that the early activation of CD4$^+$ memory Interferon-γ$^+$ T cells could be considered as a marker of protection from the disease as well as a tool to establish vaccine efficacy.

1. Introduction

Mycoplasma agalactiae is the major pathogen causing contagious agalactia (CA), a severe syndrome of small ruminants characterized by mastitis, arthritis and kerato-conjunctivitis (Bergonier et al., 1997). The disease is a real priority in Mediterranean livestock and it is included in the list of notifiable diseases by the OIE (World Organization for Animal Health) because of its economic impact and widespread distribution (OIE, 2008).

In Italy currently available vaccines consist of *M. Agalactiae* inactivated with formalin or saponin and autogenous products (so called "farm vaccines") supplied by the Italian Experimental Zooprophylactic Institutes (IZSs) which are limited in their use to officially notified infected farms. Some vaccine formulations (saponin or ethanol inactivated vaccines) seem to be more effective than others (OIE, 2008). However, a live attenuated vaccine, which is prohibited in the EU, has been used in Turkey since the 1930s, with good clinical results. As a consequence, no standard vaccine against CA has been recommended in spite of its severe

Abbreviations: CA, contagious agalactia; MA, *Mycoplasma agalactiae*; dpi, days post infection; PBMC, peripheral blood mononuclear cells.

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economic losses (Nicholas and Ayling, 2003; Nicholas et al., 2009). The control of CA in endemic areas is still a controversial issue (Buonavoglia et al., 2009).

Little is known about the immunity acquired during infection or vaccination against *M. agalactiae*. There is some data on humoral immunity including the stimulation of *Mycoplasma*-specific immunoglobulins and T cells (Buonavoglia et al., 2008; La Manna et al., 2011). In particular, our group described an increase of antigen-specific IFN-γ positive lymphocytes in infected sheep peripheral blood, initially sustained by $CD4^+$ T cells at day 15 after infection, followed by CD8/IFN-γ double positive cells (La Manna et al., 2011).

The antigenic variation of *M. agalactiae* surface antigens (Bergonier et al., 1996; de la Fe et al., 1996; Nouvel et al., 2009) could reasonably be one of the causes of the loss of efficacy of host immune response, but there is still lack of information regarding the immune response itself elicited by *M. agalactiae* such as the identity of the related lymphocyte subsets and cytokines involved (Buonavoglia et al., 2008; Castro-Alonso et al., 2010; La Manna et al., 2011).

It is well known that upon stimulation by specific antigen, naive T cells lose CD45RA, acquire CD45RO antigen and are finally recruited into the peripheral pool of memory T cells (Akbar et al., 1988; Uehara et al., 1992).

The rationale of the present study was to investigate the involvement of T cell subsets in sheep treated with different vaccine formulations followed by an experimental challenge with *M. agalactiae*, in order to find a marker of the efficacy of the host immune response and/or of the protection conferred by the vaccine. The attention was then focused both on the memory cell populations ($CD45RO^+$T lymphocytes), whose timing of expansion was considered a consequence of the efficiency of vaccination, and in the naïve cell populations ($CD45RA^+$T lymphocytes) that could be considered the reservoir of memory cells (Sprent and Surh, 2011).

2. Materials and methods

2.1. Experimental design

Twenty ewes, belonging to Comisana breed, aged from 2 to 4 years, were microbiologically and serologically confirmed as negative for *Mycoplasma* spp. and any other aetiological agents of mastitis. All animals were subjected to synchronization of their estrum. Forty days after parturition (to allow newborn sheep to receive natural colostrum from their mothers) the herd was divided into 4 groups of 5 animals. Three groups were subcutaneously injected with the approved dose of vaccine, as recommended by manufacturer instruction, whereas five non-infected sheep were maintained as the control group. Four weeks after the first vaccination, all the vaccinated groups were boosted with the same vaccine that they have received before. Vaccines tested in this experiment were supplied by different sources (Table 1): farm vaccine (inactivated with formalin and combined with Quil A as adjuvant) from the Vaccine laboratory of Istituto Zooprofilattico Sperimentale della Sicilia (hereafter called "farm" vaccine); commercial vaccine (inactivated with formalin and combined with aluminium hydroxide as adjuvant) that was bought from a local veterinary pharmacy (hereafter called "commercial" vaccine); a novel saponin-inactivated vaccine, in which saponin acts as inactivant and adjuvant in the same time (hereafter called "Saponin" vaccine).

Thirty days after the treatment all vaccinated ewes developed an antibody response against *M. agalactiae* as detected by commercial ELISA (IDEXX-Institut Pourquier, Montpellier, France). All the animals, including the control group, were then challenged introducing 10^5 CFU of a live *M. agalactiae* strain in both teats by intracanalicular route (Tola et al., 1999). The challenge day was considered as day 0 of the experiment.

Immunoglobulin levels and *Mycoplasma* excretion were weekly monitored for the whole duration of the experiment (manuscript submitted), while microbiological and biomolecular analyses demonstrated that the CA induced in these sheep was exclusively due to *M. agalactiae*. Heparinized blood samples for the flow cytometric analysis were collected at days 0, 7, 15, 30 and 90 after challenge.

2.2. Challenge

For inoculum preparation a field strain of *M. agalactiae* (IZS PA131) – recently isolated from a Sicilian outbreak of CA and confirmed by biochemical and PCR analysis – was utilized. After isolation the strain was cultured in modified Hayflick's broth (500 ml) (Miles and Nicholas, 1998) for 72 h incubation at 37 °C, before the culture was harvested at 10.000 g for 30 min and re-suspended in the same volume of phosphate buffered saline (PBS) solution, pH 7.2. *M. agalactiae* antigen suspension was further stored in 1.5 ml cryovials and frozen at −80 °C. Viable *M. agalactiae* antigen was quantified before inoculation by the method described elsewhere (Miles and Nicholas, 1998). A 1.5 ml-inoculum containing 10^5 CFU of *M. agalactiae* was injected intracanalicularly into both halves of all sheep. Milk samples collected from these ewes were tested in order to confirm and quantify the excretion of *M. agalactiae* and the absence of any other mastitis agents (Miles and Nicholas, 1998). The experiment was carried out in accordance with

Table 1
Overview of the vaccines formulations used in this experiment.

Group #	Vaccine	Producer	Inactivation procedure	Adjuvants
1	None	–	–	–
2	Farm vaccine	IZS Sicilia, Italy	0.4% formalin	200 μl/ml Quil A
3	Saponin vaccine	IZO	2 mg/ml saponin	–
4	Commercial	Aglovax	0.2% formalin	Aluminium hydroxide

The group 1 was treated with PBS only in place of the vaccine injections. Groups 2, 3 and 4 were treated with a proper dose of vaccine, and boosted following the manufacturer instruction, as described in Section 2.

the Code of Practice for Housing and Care of Animal used in Scientific Procedures (EU Directive 2010/63/EU) and with the authorization of the Italian Ministry of Health (Decreto Ministeriale No. 101/2006-A).

2.3. Data and samples collection

Blood, serum, milk, ocular and nasal swabs samples and clinical data about the presence or absence of mastitis, arthritis and keratoconjunctivitis were recorded weekly for 26 weeks after challenge, while flow cytometric analyses were performed at days 0, 15, 30 and 90 after infection.

2.4. Serological and microbiological tests

Screening for anti-*M. agalactiae* antibodies was performed utilizing a commercial ELISA kit (Institut Pourquier, France) following the manufacturer's instructions. Antibodies concentrations, measured as optical density values, were analyzed with a statistical model of analysis of variance (ANOVA) for repeated measures. The confidence value was fixed at 0.05. The presence of *M. agalactiae* from animals was confirmed by specific PCR and culturing milk and swabs samples as described elsewhere (McAuliffe et al., 2003; Poveda and Nicholas, 1998). Microbiological tests also included screening for conventional mastitis agents (data not shown).

2.5. Antigen preparation and flow cytometric analysis

A selected *M. agalactiae* strain was isolated from cultures in semi-solid medium (Mycoplasma Experience, UK; Mycoplasma broth and agar, Oxoid, USA) and irradiated to be used as a source of antigenic *in vitro* stimulus for lymphocytes (La Manna et al., 2011). Peripheral blood mononuclear cells (PBMC) were collected from whole blood and cultured as described elsewhere (La Manna et al., 2011). Cells were thus collected after 48 h of *in vitro* antigen exposure and flow-cytometric analyses were assessed. These assays were performed staining the cells with anti-surface-FITC-labelled mAbs and anti-IFN-γ PE-labelled mAbs at the concentration suggested by the suppliers in 100 μl PBS/0.1% sodium azide. Surface immunofluorescence was detected by anti-CD8 (FITC-labelled, clone CC63, mouse IgG2a, Serotec, U.K.), anti-CD4 (FITC-labelled, clone 44.38, mouse IgG2a, Serotec, U.K.), anti-WC1 (FITC-labelled, clone 19.19, mouse IgG1, Serotec, U.K.), anti-CD45R0 (unlabelled mouse IgG1, clone GC42A1) and by anti-CD45RA (unlabelled mouse IgG1, clone 73B1). Staining were assessed by indirect labelling using a rat anti mouse IgG1 (PerCP-labelled, cat.n. 340272, rat IgG). After three washes, cells were fixed and permeabilized by Leucoperm™ reagent set (Serotec, U.K.) and then incubated with anti-IFN-γ mAb PE-labelled (clone CC302, mouse IgG1, Serotec, U.K.) for 15 min at 4 °C. After three washes, cells were collected using a FACS-CAN cytometer (Becton Dickinson, U.S.A.) and analyzed by CELL-QUEST PRO program (Becton Dickinson, U.S.A.). The data related to the cellular subsets of $CD4^+$ $CD45R0^+$, $CD4^+$ $CD45RA^+$, $CD8^+$ $CD45R0^+$, $CD8^+$ $CD45RA^+$ and $\gamma\delta^+$ T-lymphocytes were analyzed and the values of the fluorescence were determined subtracting those of the corresponding negative controls, *i.e.* the fluorescence of PBMC cultured with media alone and treated with the same reagents for staining. Each analysis was performed collecting 10,000 events of live lymphocytes previously physically gated.

2.6. Statistics

The Mann–Whitney test was used to analyze the significance between values of different groups. The confidence value was fixed at 0.05.

3. Results

3.1. Microbiological monitoring

Microbiological analyses confirmed that *M. agalactiae* was the only cause of CA in the animals, and that no other mastitis agents were detected in the affected animals for the whole duration of experiments. *Mycoplasma* pathogen is mainly excreted by milk, and secondary by nasal fluids. The amount of *M. agalactiae* spread by ocular fluids was not statistically significant (data not shown). The amount of *Mycoplasma* secreted by different groups was positively related to the severity of clinical symptoms, and consequently to the vaccine efficacy (manuscript submitted).

3.2. Comparison of clinical signs among groups treated with different vaccines

Intracanalicular infected ewes belonging to control group showed classical signs of CA seven days post infection. The presence of *M. agalactiae* in all secreting sheep was confirmed both by specific PCR and by cultural techniques.

The percentages of sheep affected by mastitis in control and/or vaccinated groups during the period of observation are shown in Fig. 1. The clinical and immunological data were collected from day 0 to day 90 after infection. Clinical

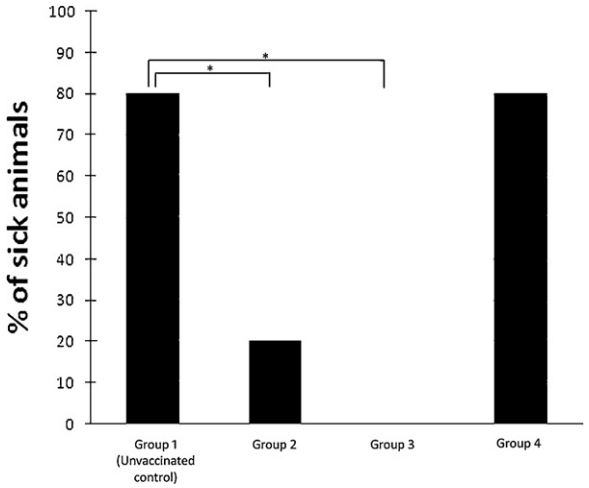

Fig. 1. Clinical trend of infected sheep.
The Figure shows the incidence of mastitis in the four groups during the monitoring period of 90 days. $^*p < 0.05$ when groups 2 and 3 were compared to control group 1 data.

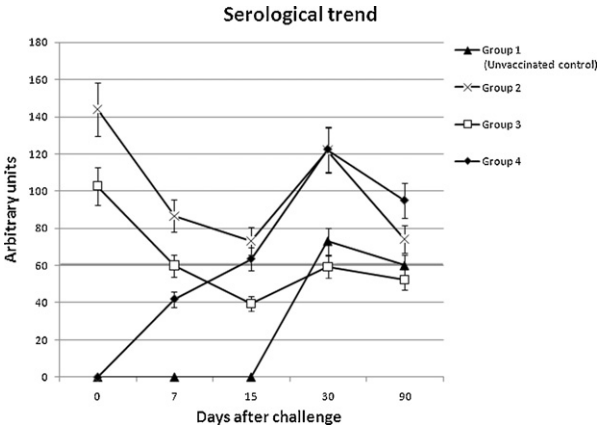

Fig. 2. Anti-*M. agalactiae* IgG trend in infected and control animals. The presence of specific IgG against *M. agalactiae* was detected by commercial ELISA kit. Values are calculated following manufacturer instructions. Even if data were collected weekly, only days 0, 7, 15, 30 and 90 after challenges are shown, in order to compare the serological trend with the flow cytometric data.

signs of CA in the naturally infected and vaccinated animals arose only after the second week post challenge.

The highest value of prevalence of mastitis was recorded four weeks after challenge. A total of 9 sheep were affected by mastitis: 4 sheep belonging to group 1 (infected control group), 4 belonging to group 4 (sheep treated with commercial vaccine), 1 belonging to group 2 (farm vaccine) and none belonging to group 3 (saponin vaccine). Only two ewes of the total amount of treated sheep (8%) showed other CA signs: one was affected by arthritis (group 3) and one by monolateral conjunctivitis (control group) that overlapped with mastitis. The difference between control and vaccinated groups 2 and 3 was statistically significant ($p < 0.05$).

3.3. Immunoglobulin detection in protected and unprotected animals

The level of specific anti-*Mycoplasma agalactiae* IgG antibodies was evaluated in order to determine if there was a significant difference between the various groups in the time-span of 26 weeks. The presence of immunoglobulin was thus monitored with a qualitative ELISA from one week before vaccination and them continued on a weekly basis for 26 weeks. The day before challenge about 75% of vaccinated sheep were positive for anti-*Mycoplasma* IgG. In the control group, anti-*Mycoplasma agalactiae* IgG was not detectable until 4 weeks after challenge. The statistical analysis confirmed as not significant the variation of OD values among the groups over time (Fig. 2).

3.4. $CD4^+$-IFN-γ^+ T-cell naïve and memory subsets detection

The monitoring of T cell naïve ($CD4^+$ $CD45RA^+$) and memory ($CD4^+$ $CD45RO^+$) subsets was assessed comparing the vaccinated groups with the non-vaccinated group in terms of *in vitro* expansion of cellular subsets specifically activated by an irradiated *M. agalactiae*; IFN-γ was considered as a marker of cellular activation, as previously published (Dedieu et al., 2010; La Manna et al., 2011). The cellular trend was analyzed showing the percentage of antigen-specific IFN-γ^+ cells and distinguishing between $CD4^+$ $CD45RO^+$ or $CD4^+$ $CD45RA^+$.

Data in Fig. 3 shows the ratio (expressed in percentages) of CD45RO (dark columns)/CD45RA (grey columns) in gated $CD4^+$ IFN-γ^+ cells in each group at different time points. Animals treated with vaccines used in the group 2 and 3 showed an early cellular expansion at day 7, followed by a more relevant expansion of $CD4^+$ $CD45R0^+$ IFN-γ^+ at day 30 in the group 3, and 15 dpi in the group 2, maintaining high percentages of memory cells at day 90 after infection in both groups.

In the group 4, this subset shows a mild expansion not earlier than day 15, reaching and maintaining a plateau until the day 90 after the infection.

In the control group there was a slight increase of the value of antigen-specific $CD4^+$ $CD45R0^+$ IFN-γ^+ T-lymphocytes, reaching a plateau from day 30 on, even if the percentages reached a value lower than those of the same time points of clinically protected vaccine groups.

The Control and commercial vaccine treated groups show higher percentages of naïve $CD4^+$ T cells 7 dpi when compared with the same time points of protected groups.

Fig. 3B describes the absolute percentages of $CD4^+$ $CD45RO^+$ IFN-γ^+ cells during the time of observation. Data obtained from animals of groups 1 and 4 does not reveal any significant modifications of the absolute percentage of the memory subset in all time points studied. The expansion of $CD4^+$ memory IFN-γ^+ in the group 2 became significant only from day 30 post infection when compared with data obtained from animals of Group 1 ($p < 0.05$). Maximal absolute percentages of $CD4^+$ $CD45RO^+$ IFN-γ^+ cells were observed 30 and 90 dpi in the group 3 ($p < 0.05$).

Percentages of $CD8^+$ IFN-γ^+ naïve and memory T-lymphocytes in sick and healthy animals

Fig. 4A shows the percentages of $CD45RO^+$ (dark columns) and $CD45RA^+$ (grey columns) in gated $CD8^+$ IFN-γ^+ cells. In group 1 high percentages of CD8 memory cells from day 15 to day 90 were detected.

High percentages of $CD8^+$ $CD45R0^+$ IFN-γ^+ T-lymphocytes were detected from day 0 to day 30 in the group 4. In the group 3, two peaks of $CD8^+$ memory IFN-γ^+ cells were observed at days 15 and 90. The higher percentages of $CD8^+$ $CD45R0^+$ IFN-γ^+ T cells in the group 2 were observed in two time points of immunological monitoring: one occurred at day 7 and the other reached a plateau from day 30 to day 90.

Fig. 4B shows the absolute percentages of CD8 memory IFN-γ^+T cells during the observation period. Cells from animals of Group 1 increased significantly, when compared to group 3which was largely protected, from day 15 to day 90. In the unprotected group 4 $CD8^+$ $CD45RO^+$ IFN-γ^+ cells increased only at day 30 in absolute percentages.

3.5. $\gamma\delta^+$ IFN-γ^+ T lymphocytes during the observation period

In group 4 no significant expansion of $\gamma\delta^+$ IFN-γ^+ T lymphocytes was detected during all the time points of

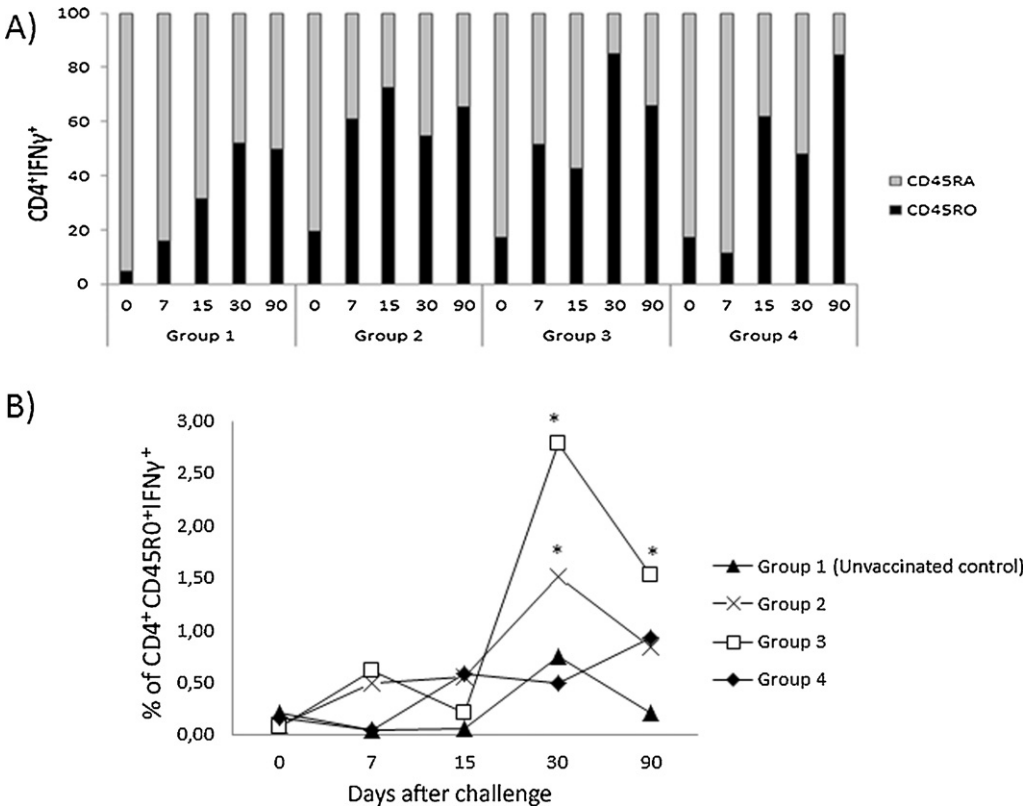

Fig. 3. CD4 memory and naïve IFN-γ^+ T cells during the observation period. (A) The relative frequencies, expressed in percentages, of $CD4^+CD45R0^+$IFN-γ^+ and $CD4^+CD45RA^+$IFN-γ^+ T-lymphocytes in $CD4^+$IFN-γ^+ cells. In panel B thepercentages of $CD4^+CD45R0^+$IFN-γ^+ are shown. It can be noted that the comparison of the percentage of activated $CD4^+CD45R0^+$ T-lymphocyte subset shows a significant difference between groups 3 and 2 and control group ($p < 0.05$) at days 30 and 90 post-challenge.

observation (Fig. 5). The $\gamma\delta^+$ IFN-γ^+ T lymphocytes showed an early, statistically significant expansion, at day 7 in the control group ($p < 0.05$), followed by a new expansion 30 dpi when compared to the same time points of group 4. They subsided to lower levels again at day 90. $\gamma\delta^+$ IFN-γ^+ T lymphocytes rose in the group 2 30 dpi ($p < 0.05$). A relevant peak of this cellular subset was also detected in the group 3 at day 15 after infection.

4. Discussion

The study of the antigen-specific cellular subsets activated in response to *M. agalactiae*, was aimed at developing a broader awareness of the protective mechanisms conferred by the different vaccine formulations to the natural host of CA. Even if the data reported concern a limited number of animals, they represent a step forward in our knowledge of small ruminant *Mycoplasma* infections.

From the data recorded, it was possible to conclude that the best vaccine involved in protection from clinical signs was the saponin vaccine, followed by the farm vaccine. The commercial vaccine was not able to confer protection in 80% of animals (Fig. 1). The time of detection of antigen-specific IgG in our model was consistent with those found in other reports (Buonavoglia et al., 2009; de la Fe et al., 2007). Nevertheless the choice of a test to detect specific antibody should be carefully considered, due to the intrinsic variability and geographical origin of strains. A very recent work (Poumarat et al., 2012) compares two different ELISA tests and the conclusion reached by authors is that a variety of factor influence the test performance. These data, together with the statistical analysis of the results, encourage to consider not significant the antibody level hereby detected.

The T cell IFN-γ^+ lymphocytes response to *M. agalactiae* infection detected in the control group during this experiment confirmed our previous results describing, in infected sheep, an initial increase of $CD4^+$IFN-γ^+T cells followed by an expansion of $CD8^+$ IFN-γ^+ T lymphocytes after a short *in vitro* re-exposure to irradiated *M. agalactiae* (La Manna et al., 2011). The same method, improved with the detection of CD45RO or CD45RA, could give an idea of the establishment of immunological memory. Since memory cells are involved in an effective immune response induced by a vaccination, as reported in other systems (Addae et al., 2006), the contribution of this population could be considered a goal for a good vaccine.

The results have emphasized how in the group treated with the effective vaccines (saponin and farm), the most represented T cell subset is $CD4^+$ $CD45RO^+$ IFN-γ^+, which is able to increase from day 7 after infection to day 90. The early increase of $CD4^+$ $CD45RO^+$ IFN-γ^+ is not traceable in the control group and in the group inoculated with the commercial vaccine. We think that the timing of expansion

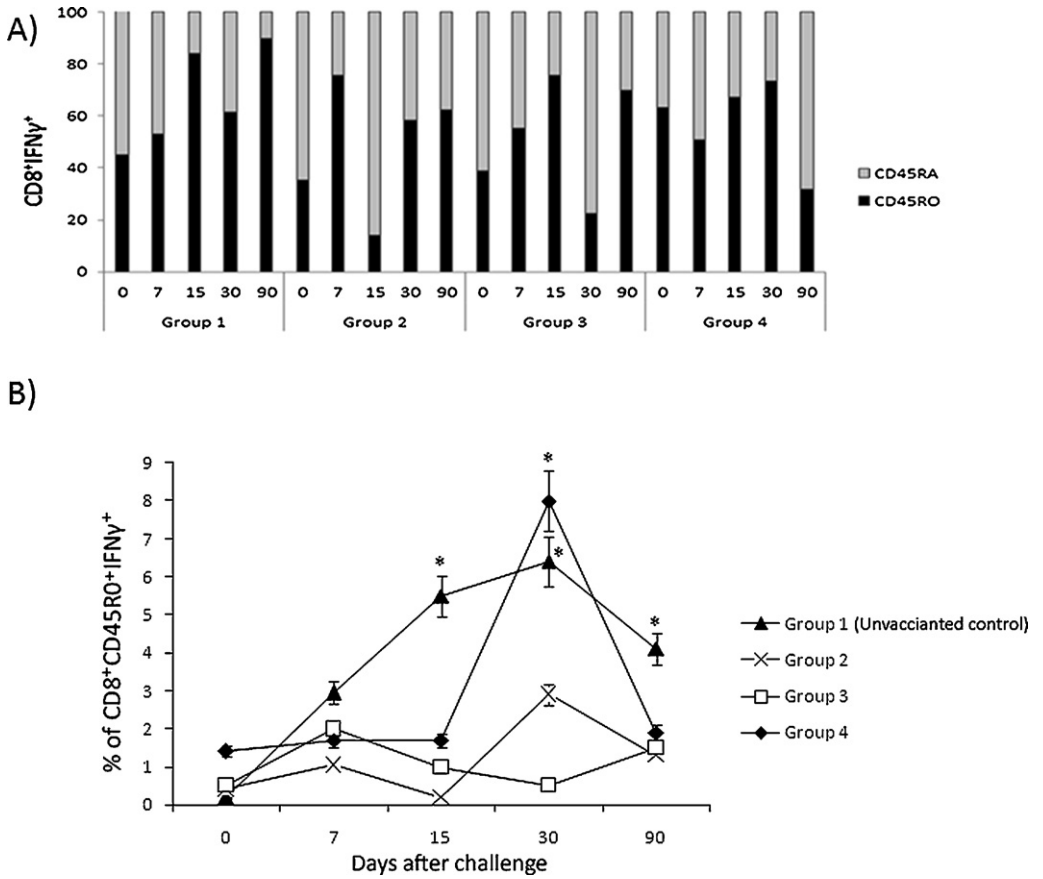

Fig. 4. CD8 memory cells increase in sick animals. (A) The relative frequencies, expressed in percentages, of $CD8^+CD45RO^+IFN\text{-}\gamma^+$ and $CD8^+CD45RA^+IFN\text{-}\gamma^+$ T-lymphocytes among $CD8^+IFN\text{-}\gamma^+$ cells. The comparison of the percentages of $CD8^+CD45RO^+IFN\text{-}\gamma^+$ T-lymphocyte subsets among groups, illustrated in panel B, shows the significant difference between non-protected groups (groups 4 and 1) and protected groups (2 and 3, $p < 0.05$) at days 7 and 30 post-challenge.

of this subset could be considered as a correlate of protection against CA. Further studies are necessary to univocally establish a correlation between the early activation of antigen specific $CD4^+CD45R0^+$ IFN-γ^+ T-lymphocytes and the protection against CA, as well as if the correlation is mechanistic or non-mechanistic (Plotkin and Gilbert, 2012). A correlation between the protection from the disease and the increase of this cellular subset could be dissected with more accurate analyses that at date are not feasible due to the lack of specific reagents. In the control group and in group 4, its activation undergoes a consistent delay, which could be the cause, together with the expansion of $CD8^+CD45R0^+IFN\text{-}\gamma^+$T-lymphocytes, of the disease signs in the animals.

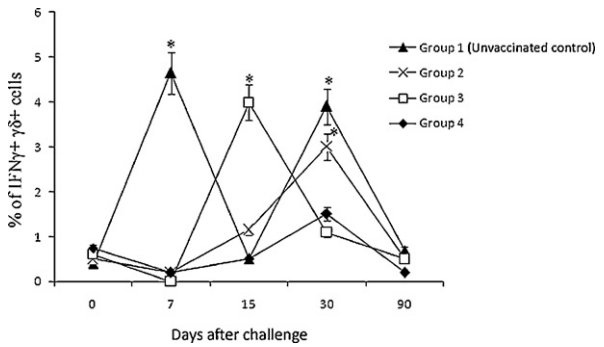

Fig. 5. IFN-γ positive γδ T-cells trend in vaccinated and control groups. The mean percentages of IFN-γ positive γδ T-cells in the time course of the experiment in the different groups of sheep is showed.

A certain number of CD8 memory T cells responding *in vitro* to *M. agalactiae* is also detectable on day 0. The reason why it happens is not univocally definable. Animals were not stabled under sterile conditions, it cannot be ruled out that it is a consequence of some other less-common microorganism, antigenically similar to *M. agalactiae*, not detected by our microbiological test that cross-reacts with some epitope of *M. agalactiae*. It would be interesting to perform a deeper analysis in order to classify memory cells, as shown in other systems (Caccamo et al., 2006), but unfortunately some of these reagents are not available for sheep. In all cases, $CD4^+$ $CD45RO^+$ IFN-γ^+ T cells are a well-known correlates of protection, as suggested by other models of infectious diseases in humans (Naess et al., 1998)and in cattle (Endsley et al., 2007). In order to assess an effective subunit vaccine against *Mycoplasma mycoides* subsp. *mycoides*, researchers are studying molecules able

to activate central and effector memory $CD4^+$cells, since it seems they are the most suitable peptides to be included in such vaccines (Totté et al., 2008).

In the group that was unprotected by vaccine, the infection caused an increase of the $CD8^+CD45RO^+$ IFN-γ^+ T-lymphocytes at day 30 after infection. This group suffered from a delay in cellular response, due to the absence of any previous contact with the pathogen (both natural or by vaccination). The unavailability of data around day 60 after infection does not allow us to confirm or to exclude the expansion, also in this group, of a memory subset effective in the protection against *M. agalactiae*. An analogous trend could have happened in the group treated with the commercial vaccine where, until 30 days after infection, protection against the disease was detected. In this case, the strain would not be able to stimulate any cellular immune response, with respect to the wild type strains that are circulating nowadays, maybe because of *M. agalactiae* antigenic variation.

It would be interesting to study the pattern of production of whole chemokines and cytokines during natural infection in order to determine if, together with the presence of $CD4^+CD45R0^+$ IFN-γ^+ T-lymphocytes, the absence of clinical signs can be related with other subsets (Th-2, Th-17, Th-9, Th-22, *etc.*). Even if the cytokine IFN-γ was used as a marker of the activation state in other *Mycoplasma* models of infection (Nicholas and Ayling, 2003; Scacchia et al., 2007), it should not be discounted as being involved during the *M. agalactiae* infection. However, antigen-specific IFN-γ^+ T cell subsets could be useful to monitor the T-cell mediated immune response during the infection.

In this study the fluctuating trend of $\gamma\delta^+$ IFN-γ^+ T cells cannot be related with the expansion of one of the T cell subset hereby considered. Many other cell types (NK, NKT, $CD4^-CD8^-$ T cells), that would be interesting to analyze in our model, could be responsible for the activation of $\gamma\delta^+$ IFN-γ^+T cells through different mechanisms including transactivation induced by cytokines released NK, NKT, $CD4^-CD8^-$ T cells.

The detection of T lymphocyte memory subsets represents an important tool in evaluating the protection against CA elicited by available and experimental vaccines, and could be also useful in selecting the antigens with potential for incorporation in a subunit vaccine against CA.

Competing interests

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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