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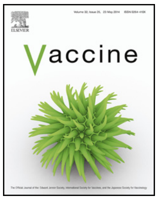

Phenotypic characterization of bovine memory cells responding to mycobacteria in IFNγ enzyme linked immunospot assays

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ABSTRACT

Bovine tuberculosis (bTB) remains a globally significant veterinary health problem. Defining correlates of protection can accelerate the development of novel vaccines against TB. As the cultured IFNγ ELISPOT (cELISPOT) assay has been shown to predict protection and duration of immunity in vaccinated cattle, we sought to characterize the phenotype of the responding T-cells. Using expression of CD45RO and CD62L we purified by cytometric cell sorting four distinct CD4^{+} populations: CD45RO^{+}CD62L^{hi}, CD45RO^{+}CD62L^{lo}, CD45RO^{-}CD62L^{hi} and CD45RO^{-}CD62L^{lo} (although due to low and inconsistent cell recovery, this population was not considered further in this study), in BCG vaccinated and *Mycobacterium bovis* infected cattle. These populations were then tested in the cELISPOT assay. The main populations contributing to production of IFNγ in the cELISPOT were of the CD45RO^{+}CD62L^{hi} and CD45RO^{+}CD62L^{lo} phenotypes. These cell populations have been described in other species as central and effector memory cells, respectively. Following *in vitro* culture and flow cytometry we observed plasticity within the bovine CD4^{+} T-cell phenotype. Populations switched phenotype, increasing or decreasing expression of CD45RO and CD62L within 24 h of *in vitro* stimulation. After 14 days all IFNγ producing CD4^{+} T cells expressed CD45RO regardless of the original phenotype of the sorted population. No differences were detected in behavior of cells derived from BCG-vaccinated animals compared to cells derived from naturally infected animals. In conclusion, although multiple populations of CD4^{+} T memory cells from both BCG vaccinated and *M. bovis* infected animals contributed to cELISPOT responses, the dominant contributing population consists of central-memory-like T cells (CD45RO^{+}CD62L^{hi}).

1. Introduction

Bovine tuberculosis (bTB) has cost the British taxpayer £500 million over the last decade and it is expected to cost over £1 billion in the next decade [1], making it one of the most significant veterinary health problems in the U.K. Development of efficacious vaccines against TB necessitates a better understanding of protective immune responses to this disease. Defining correlates of immunity can assist in the development of novel vaccines and vaccination strategies. The *ex vivo* ELISPOT measures the frequency of IFNγ producing cells in PBMCs without prior culture *in vitro* and it is considered to be an indicator of the frequency of effector cells. The cultured IFNγ ELISPOT (cELISPOT) assay measures long lived memory T-cell populations, mainly considered to be central memory cells, believed to be important for the induction of protection [2].

In cattle, memory CD4 T-cells have been defined according to cell surface expression of CD45RO [3–6] and CD62L [5] and more recently by the expression of CCR7 [7], which is similar to the definition of central and effector memory T-cells in humans [8]. CD62L is required for entry into lymph nodes *via* high endothelial venules [8] and central memory T-cells are thought to be CD62L^{hi} to allow migration to the lymph nodes. In humans, CD45RO^{+} cells have shorter telomeres than naïve or CD45RO^{-} T-cells and divide frequently [9]. Thus, in a generalized simplified model, central memory T-cells would be CD45RO^{+} CD62L^{hi}; effector memory cells would be CD45RO^{+} CD62L^{lo} and naïve cells would be both CD45RO^{-} CD62L$^{lo/hi}$. A human study showed that PPD-B specific cELISPOT responses were primarily cells expressing surface CCR7 or CD62L, as depletion of these cells dramatically reduced the cELISPOT response [10]. These data are supported by other studies that have linked cELISPOT responses to central memory T-cells [11,12].

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The cELISPOT has been used to show enhanced Ag85A specific memory responses after priming with bacille Calmette-Guérin (BCG) and boosting with rAd85A intranasally [13]. cELISPOT responses at week 14 post vaccination were predictors of protection, correlating negatively with the pathology score after infection with *Mycobacterium bovis* [14,15]. Furthermore, cELISPOT responses were shown to correlate with duration of protection against *M. bovis* in calves vaccinated with BCG [16]. When measured 12 months post-BCG vaccination, the number of PPD-B-specific IFNγ spot forming units (SFU) was greater in vaccinated than in control animals. In contrast, after 24 months post vaccination, no significant difference in PPD-B-specific IFNγ SFU was observed in vaccinated and control animals, which correlated with a lack of protection in vaccinated animals.

Depletion assays have shown that the main producers of IFNγ, following stimulation of peripheral blood mononuclear cells from *M. bovis* infected cattle with mycobacterial antigens, are CD4$^+$ T-cells [17]; therefore, in this work we have concentrated on studying the CD4 T-cell response. It has been shown that CD4CD45RO$^+$ and/or CD4$^+$CD62L$^+$ are capable of producing IFNγ [4]. However, the phenotype of the cells producing IFNγ in terms of central *versus* effector memory remains to be elucidated. In this work we characterized the CD45RO and CD62L phenotype of bovine antigen-specific CD4$^+$ T-cells responding in the *ex vivo* and cELISPOT systems. We also sought to determine whether the phenotype of the IFNγ$^+$ cells remained constant in the culture conditions compared to the originally sorted phenotype. Identification of the cells responsible for the production of IFNγ in the cELISPOT will permit focusing of efforts to further characterize these cells with the aim of identifying correlates of protection.

2. Materials and methods

2.1. Cattle

Animal work was carried out according to the UK Animal (Scientific Procedures) Act 1986. The study protocol was approved by the AHVLA Animal Use Ethics Committee (UK Home Office PCD number 70/6905).

Six Holstein–Friesian cross cattle of between 8 and 26 months of age were used for these experiments; three of these animals were vaccinated as neonates with BCG and three were reactors to the tuberculin skin test and considered naturally infected with *M. bovis*. The aim of these experiments was to determine potential differences in detectable responses in the *ex vivo* and the cultured IFNγ ELISPOT between vaccinated and infected animals. The responses in naïve animals are negligible and therefore were not included in these studies.

2.2. Preparation of CD4$^+$ cells for sorting

Cattle peripheral blood mononuclear cells (PBMC) were prepared from peripheral blood by gradient density centrifugation and CD4$^+$ T cells were enriched by paramagnetic bead isolation (MACs) (Miltenyi, UK) using monoclonal antibody (mAb) CC8 (IgG$_{2a}$) to bovine CD4 (Serotec, UK) as previously described [4]. CD4$^+$ T cells were stained with mAbs CC32 (IgG$_1$) to bovine CD62L (AbD Serotec) and IL-A116 (IgG$_3$) to CD45RO [3] (a kind gift from Dr Jan Naessens, ILRI), followed by staining with rat mAb to mouse IgG$_1$: Brilliant violet 421 (BD Pharmingen), goat anti-mouse IgG$_{2a}$: FITC (Southern Biotech), and goat anti-mouse IgG$_3$: R-phycoerythrin (R-PE) (Southern Biotech). After incubation, cells were washed, resuspended in RPMI1640 (Gibco) and passed through a 30 μm filter for sorting.

Cells were sorted on a Moflo Astrios Flow Cytometer (Beckman Coulter, USA) at 60 psi with a 70 μm nozzle at up to 20,000 events per second, according to the gating strategy shown in Supplementary Fig. 1 into four populations: CD45RO$^+$ CD62L^{hi}, CD45RO$^+$ CD62L^{lo}, CD45RO$^-$ CD62L^{hi} and CD45RO$^-$ CD62L^{lo} based on expression of CD45RO and CD62L. After sorting, individual cell populations were shown to be >98% pure by flow cytometry.

2.3. Preparation of antigen-presenting cells

CD14 cells were isolated from peripheral blood mononuclear cells using human CD14$^+$ paramagnetic beads (Miltenyi) according to manufacturer recommendations. CD14$^+$ cells were used as antigen presenting cells (APC) in ELISPOT assays at a ratio of 1 CD14$^+$ to 10 CD4$^+$ T cells. The purity of the isolated cells ranged from 85% to 98% (Supplementary Fig. 2)

2.4. *Ex vivo IFNγ ELISPOT*

The *ex vivo* IFNγ ELISPOT protocol was followed as published elsewhere [2]. The number of sorted cells was variable and this in part dictated the number of cells used for the assay; generally, 1–2 × 10^5 sorted cells were added to each well with APC in a final volume of 200 μl in a 96-well round bottom plate. Cells were cultured with medium alone, PPD-B (10 μg/ml; APHA, Weybridge) or pokeweed mitogen (PWM) (5 μg/ml; Sigma) in triplicate, depending on the number of cells available. Background SFU (unstimulated cells) was subtracted and data expressed as mean SFU per 10^6 cells.

Given the different proportions in which these sub-populations are represented in PBMC, we quantified the contribution of each subpopulation to *ex vivo* ELISPOT. SFU were multiplied by the ratio of originally sorted cells (O) divided by the number of cells put into culture (d0) to obtain eSO$_1$ to eSO$_4$ *i.e.* eSO1. . .4 = eSFU1. . .4 * O/d0. This figure was added for all populations, eSO$_1$ + eSO$_2$ + eSO$_3$ + cSO$_4$, and used as total potential SFU (eSO$_t$) of the original CD4 population *i.e.* eSO$_t$ = eSO$_1$ + eSO$_2$ + eSO$_3$ + eSO$_4$. To obtain the proportion of SFU that each individual cell population would have contributed (eSP), each individual SO was divided by the SO$_t$ and multiplied by 100 *i.e.* eSP$_{1.4}$ = (eSO$_{1.4}$/eSO$_t$) * 100.

2.5. Evaluation of cytokine responses by intracellular staining (ICS)

ICS was performed as previously described [18]. Cells were surface stained for CD4, CD45RO (IL-A116) and CD62L (CC32) and intracellularly for IFNγ (CC302). Cells were acquired using a Cyan ADP analyser and Summit 4.3 software (Beckman Coulter, USA). Data were analyzed using FlowJo v7.6.5 software (Treestar, USA).

2.6. Cultured IFNγ ELISPOT

Sorted T-cell populations were cultured for 2 weeks as described elsewhere [2], using PPD-B and fed IL-2 at days 3 and 7 and medium replaced on days 10 and 12. After 14 days, intracellular staining and ELISPOT assays were performed as above. In the cELISPOT, to avoid saturation, 1 × 10^3 cells were used per well in the assay.

To determine the contribution of each cell population to the cELISPOT the number of SFU (S) was multiplied by the ratio of cells obtained at 14 days and divided by the number of cells with which the cultures were started (d14/d0) *i.e.* cSO$_{1...4}$ = (cSFU$_{1.4}$ * d14/d0) * O/dO; this would provide the SFU related to the originally cultured cells. To obtain a relationship of this figure to the number of cells sorted for each population this figure was multiplied by the ratio of the total number of cells sorted for each population (O) divided by the number of cells placed into culture at day 0 (d0). This figure would provide

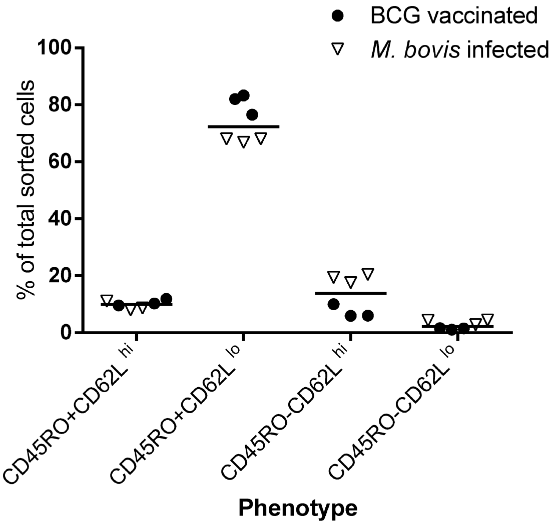

Fig. 1. The majority of peripheral blood CD4⁺ T-cells exhibit a CD45RO⁺CD62L^{lo} phenotype. CD4⁺ cell populations were sorted according to their expression of cell surface CD45RO and CD62L using the gating strategy described in Supplementary Fig. 1. CD4⁺ T-cells were sorted into CD45RO⁺CD62L^{hi}, CD45RO⁺CD62L^{lo}CD45RO⁻CD62L^{hi} and CD45RO⁻CD62L^{lo}. The largest proportion of CD4⁺ T-cells in all animals expressed the phenotype CD45RO⁺ CD62L^{lo}. No significant difference between BCG vaccinated (dark symbols) and *M. bovis* infected (clear symbols) animals was observed in the proportions of CD4⁺ T-cell as defined by CD45RO and CD62L.

the number of SFU formed had all originally sorted cells been placed into culture (cSO). This figure was added for all populations ($cSO_1 + cSO_2 + cSO_3 + cSO_4$) and used as total potential SFU (cSO_t) of the original CD4 population, *i.e.* $cSO_t = cSO_1 + cSO_2 + cSO_3 + cSO_4$. To obtain the proportion of SFU that each individual cell population would have contributed (cSP), each individual SO was divided by the SO_t and multiplied by 100 (*i.e.* $cSP_{1.4} = (cSO_{1.4}/cSO_t) * 100$).

2.7. Statistical analysis

Statistical analysis and graph creation were carried out using GraphPad Prism 5.02 (GraphPad Software, CA. USA) employing 2-way Anova or Student's *t*-test analysis. *p* values <0.05 were considered significant.

3. Results

3.1. The proportion of peripheral blood CD4⁺ cells expressing the memory associated markers CD45RO⁺ CD62L^{lo} is higher than the proportion of CD45RO⁺ CD62L^{hi}, CD45RO⁻CD62L^{hi} and CD45RO⁻CD62L^{lo}

Flow cytometry analysis of CD4⁺ populations prior to sorting revealed that the majority of cells expressed cell surface CD45RO. Supplementary Fig. 1 shows the gating strategy used for the sorting of the four different CD4⁺ T-cell populations, based on expression of cell surface markers CD45RO and CD62L. These sorted cell populations were used for all subsequent experiments. Fig. 1 shows the proportions of the different cell populations found in the BCG vaccinated and *M. bovis* infected animals after sorting. The majority (mean of 74.13%) of *ex vivo* CD4⁺ T-cells were CD45RO⁺ CD62L^{lo} in both BCG vaccinated and TB reactor animals (Fig. 1). The rarest population was the CD45RO⁻ CD62^{lo} cells. As a proportion of the total sorted CD4⁺ T-cells, the different cell populations were: CD45RO⁻ CD62^{lo} cells 1.14–4.46%; CD45RO⁺ CD62L^{lo} T-cells 66.85–83.29%; CD45RO⁺ CD62L^{hi} T-cells 8.05–11.84%; and CD45RO⁻ CD62L^{hi} T-cells 5.94–20.45%. There was no significant difference of the different CD4⁺ T-cells subpopulations between BCG vaccinated and naturally *M. bovis* infected cattle. Due to the small number of CD45RO⁻CD62L^{lo} cells isolated, it was not possible to analyze their responses in the studies described below.

3.2. CD45R0⁺CD62L^{hi}, CD45R0⁺CD62L^{lo} and CD45R0⁻CD62L^{hi}, all contain IFNγ precursors, although their contribution is variable in the ex vivo and cELISPOT

Sorted cells were stimulated *in vitro* to determine the frequency of IFNγ secreting cells in the *ex vivo* ELISPOT and cELISPOT assays, after 1 or 14 days of *in vitro* culture, respectively. Fig. 2 shows that cells within all populations are capable of secreting IFNγ following antigen specific stimulation for 24 h (Fig. 2A) or 14 days (Fig. 2B) *in vitro*, from both BCG vaccinated and *M. bovis* infected cattle.

In the *ex vivo* ELISPOT, *M. bovis* infected animals contained the highest frequency of IFNγ producing cells in all populations compared to BCG vaccinated animals. The highest number of IFNγ producing cells was detected in the CD45RO⁺CD62L^{hi} population followed by the CD45RO⁺CD62L^{lo} and then the CD45RO⁻CD62L^{hi} populations.

In the cELISPOT the differences in the frequencies of IFNγ secreting cells in the different populations between *M. bovis* infected and BCG vaccinated animals were less evident. All cell populations contained similar frequencies of IFNγ⁺ cells from *M. bovis* infected and BCG vaccinated animals. In conclusion, all three sub-populations studied were able to respond in both assays on a qualitative, cell-by-cell, basis.

Given the different proportions in which these three subpopulations are present in PBMC, we quantified the subset contribution of each subpopulation to the *ex vivo* ELISPOT and cELISPOT. The contribution of each subpopulation to the overall *ex vivo* and cELISPOT responses is shown in Fig. 3A and B. The CD4⁺CD45RO⁺CD62L^{hi} T cell subpopulation expanded on average 3.52 fold (±4.14) during the 14 day culture (expansion meaning the net effect of cell growth and cell death during the culture phase), whilst the other two populations contracted CD45RO⁺CD62L^{lo} (0.88 ± 0.63) and CD45RO⁻CD62L^{hi} (0.62 ± 0.38) during culture.

The highest response in the *ex vivo* ELISPOT resided within the CD45RO⁺CD62L^{lo} population in both *M. bovis* infected and BCG vaccinated animals, followed by the CD45RO⁺CD62L^{hi} population (Fig. 3A); the proportion of IFNγ secreting cells in the CD45RO⁻CD62L^{hi} cells was minimal. In contrast, in the cELISPOT, the highest response resided within the CD45RO⁺CD62L^{hi} population (Fig. 3B); both the CD45RO⁺CD62L^{lo} and CD45RO⁻CD62L^{hi} populations, respectively, contained fewer IFN secreting cells.

3.3. The expression of the CD45RO and CD62L phenotype of CD4⁺ T cells exhibits a degree of plasticity during in vitro culture

We also investigated the *in vitro* dynamics of CD45RO and CD62L expression after short (24 h) and long term (14 d) culture. After culture, cells were re-stimulated and cell surface antigen expression and IFNγ production determined by ICS-cytometry. Fig. 4 presents the results from a representative BCG vaccinated animal. As early as 24 h in culture, the expression of both CD45RO and CD62L changed in all three populations, with a proportion of CD45RO⁺CD62L^{hi} cells acquiring a CD62L^{lo} phenotype, whilst a proportion of CD45RO⁺CD62L^{lo} cells acquired a CD62L^{hi} phenotype. A small proportion of these cells also became CD45RO⁻CD62L^{hi}. Finally, small proportions of CD45RO⁻CD62L^{hi} cells became CD45RO⁺CD62L^{hi} or CD45RO⁺CD62L^{lo} (Fig. 4).

Greater plasticity in the expression of these markers was observed after a longer culture period. All cells producing IFNγ after antigenic re-stimulation after 14 day culture, regardless of the input cell population phenotypes at the beginning

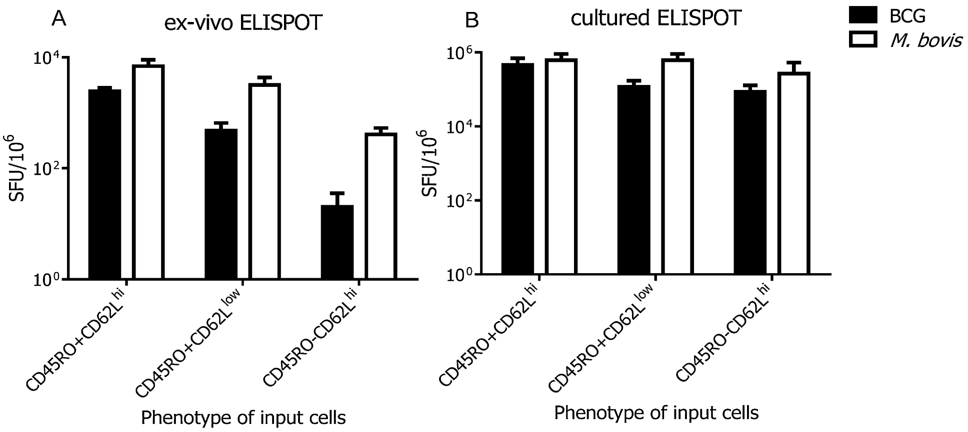

Fig. 2. All, *CD45R0*$^+$*CD62L*^{hi}, *CD45R0*$^+$*CD62L*^{lo} and *CD45R0*$^-$*CD62L*^{hi}, CD4 populations contain IFNγ secreting precursors. The frequency of IFNγ secreting cells from each sorted phenotype was evaluated in IFNγ *ex vivo* ELISPOT after 24 h (A) or cELISPOT after 14 days in culture (B) in BCG vaccinated (dark columns) and *M. bovis* infected (white columns) animals. CD45RO$^-$CD62L^{lo} sorted cells were excluded from the analysis due to an insufficient number of cells. Data are representative of three experiments with CD4$^+$ T-cells from three cattle. Results are expressed as the mean SFU/10^6 cells ±SEM.

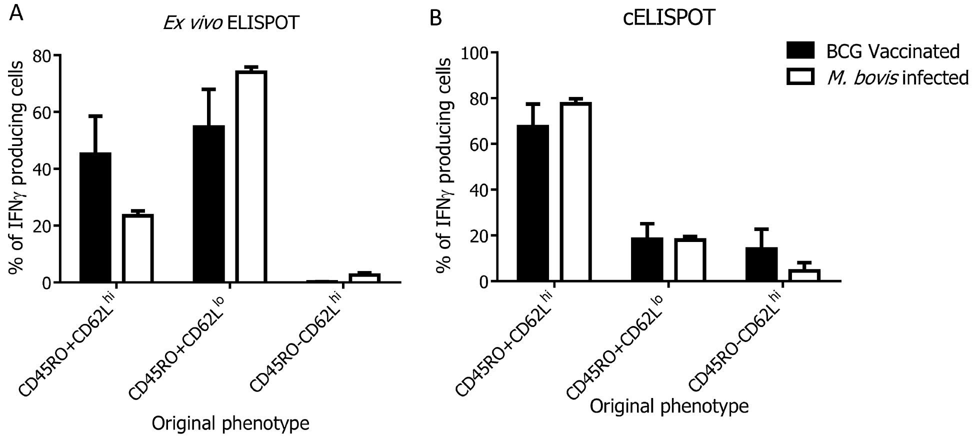

Fig. 3. The main contributors to the *ex vivo* ELISPOT are CD45RO$^+$CD62^{lo}, whilst CD45RO$^+$CD62L^{hi} are the main contributors to the cELISPOT. Using the data from Fig. 2, the proportion of cells contributing to the IFNγ secreting cells was calculated using the formulas described in materials and methods and plotted to show the extent to which different CD4$^+$ T-cells subsets contributed to the population secreting IFNγ in the *ex vivo* ELISPOT (A) and cELISPOT (B) for BCG vaccinated (black columns) and *M. bovis* infected (white columns) animals.

of *in vitro* culture (CD45RO$^+$CD62L^{hi}, CD45RO$^+$CD62L^{lo}, or CD45RO$^-$CD62L^{hi}), expressed CD45RO. Whilst the majority of CD45RO$^+$CD62L^{hi} and CD45RO$^+$CD62L^{lo} retained stable CD62L expression, substantial proportions of the CD45RO$^+$CD62L^{hi} decreased expression to become CD62L^{lo}, whilst a substantial proportion of CD45RO$^+$CD62L^{lo} increased expression to become CD62L^{hi} (Fig. 5). The most dramatic *in vitro* dynamics in CD45RO and CD62L expression were displayed by the antigen-specific IFNγ$^+$ CD45RO$^-$CD62L^{hi} subpopulation Not only did these cells express CD45RO after 2 weeks in culture, but the majority also decreased expression of CD62L to become CD62L^{lo} (Fig. 5). These results clearly demonstrate that it is difficult to draw conclusions on the nature of memory cell responses using expression of the markers CD45RO and CD62L after *in vitro* culture.

4. Discussion

The IFNγ cELISPOT has been proposed as a predictor of protection against bTB and duration of immunity in BCG or BCG/subunit vaccine heterologous prime-boost vaccination of cattle [15]. However, the nature of the cells producing IFNγ in this assay has not been defined. The phenotype of memory T-cell responses to mycobacteria in cattle has previously been defined in terms of CD45RO and CD62L [15] and we therefore wanted to characterize the cells responding in the cELISPOT using these markers. The results presented in this study indicate that CD4$^+$ T cells with both the central memory-associated phenotype CD45RO$^+$CD62L^{hi}, and effector memory-associated phenotype CD45RO$^+$CD62L^{lo} are involved in the IFNγ *ex vivo* ELISPOT and cELISPOT response in cattle

In humans, cells responding in the cELISPOT to hepatitis C virus have been defined as predominantly CD4$^+$CCR7$^+$CD62L^{hi} T cells and less frequent CD4$^+$CCR7$^-$CD62L^{lo} populations [10] [11]. CD62L and CCR7 expression is required for cell extravasation through high endothelial venules (HEV) to enable migration from blood to secondary lymphoid organs, which is thought to be a characteristic of central memory cells [8,19,20]. We have not evaluated CCR7 expression in the present study. However, a recent study has described that the populations of CD4$^+$ T cells expressing high levels of CD62L (CD62L^{hi}) largely overlap with those expressing CCR7 (Waters et al., pers. comm.).

On a cell by cell basis, CD45RO$^+$CD62L^{hi} and CD45RO$^+$CD62L^{lo} cells contribute to a similar extent to the *ex vivo* and cELISPOT; however, as a proportion of overall responses, CD45RO$^+$CD62L^{hi} cells dominate the cELISPOT compared to CD45RO$^+$CD62L^{lo} cells. This would be consistent with central memory functionality of cELISPOT IFNγ secreting cells. In contrast, CD45RO$^+$CD62L^{lo} contributed to a greater extent to the *ex vivo* ELISPOT compared to CD45RO$^+$CD62L^{hi}

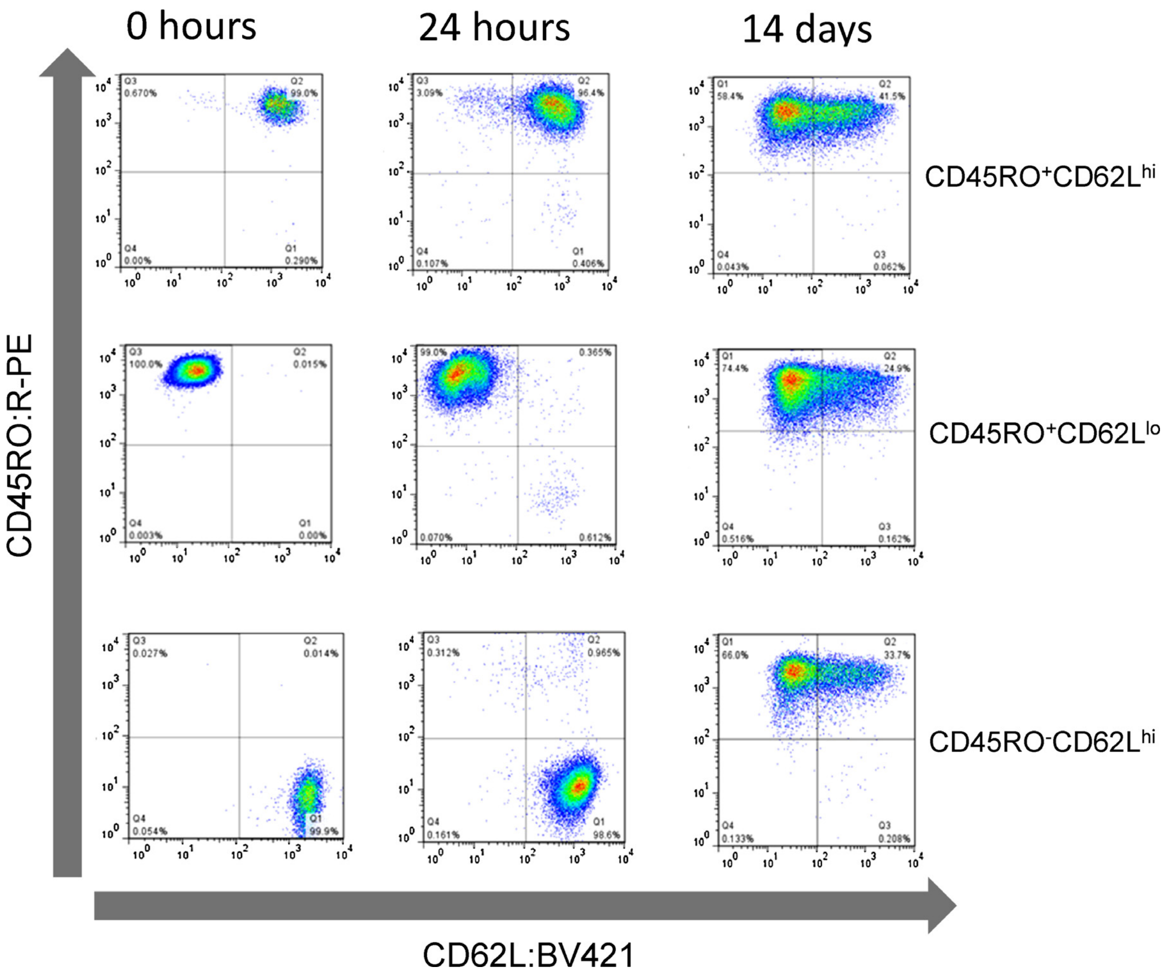

Fig. 4. All, *CD45R0*$^+$*CD62L*^{hi}, *CD45R0*$^+$*CD62L*^{lo} and *CD45R0*$^-$*CD62L*^{hi}, CD4 populations exhibit plasticity on their expression of their originally defining cell surface markers. Representative flow cytometric analysis of the different cell populations after sorting (0 h) or after culture for 24 h or 14 days. CD4$^+$ T-cells expressing CD62L and CD45RO were stained as described in materials and methods.

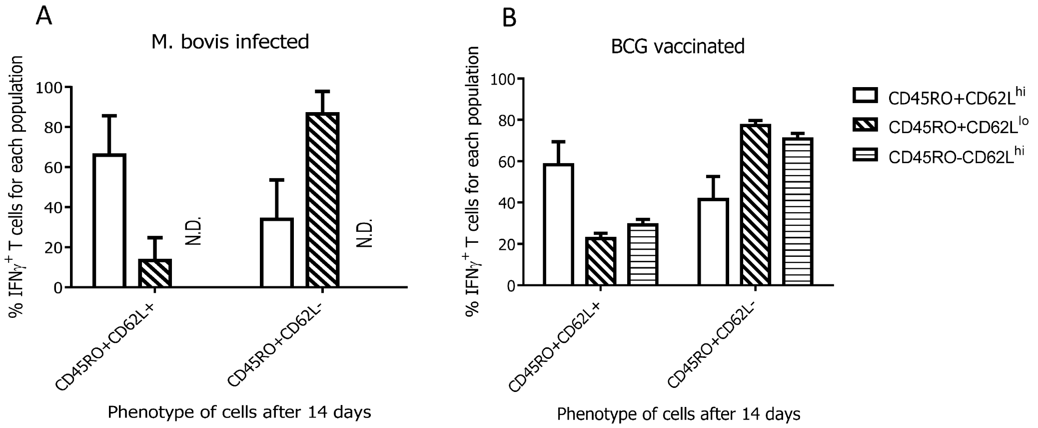

Fig. 5. All IFNγ producing cells, after 14 days culture are CD45RO$^+$, regardless of the starting population. The proportion of IFNγ secreting cells which switched to CD45RO$^+$CD62L^{lo} or CD45RO$^+$CD62L^{hi} after 14 days *in vitro* culture in *M. bovis*-infected (A) and BCG vaccinated (B) cattle were measured by ICS as indicated in materials and methods. The individual bars denote the phenotype of input cells and data are representative of three experiments with CD4$^+$ T-cells from three cattle. Results are the mean percentage of CD4$^+$ cells producing IFNγ ± SEM. N.D. There were not sufficient number of CD45RO$^-$CD62L^{hi} cells in *M. bovis* infected animals to carry out this assay.

cells, which would be consistent with effector function. Therefore, the current data indicate that CD45RO and CD62L could contribute in the definition and characterization of memory cell populations.

In this work we have also shown that within the different CD4$^+$ cell populations some cells show a degree of plasticity as they switch phenotype by up- or down-regulating CD45RO and CD62L within 24 h of *in vitro* stimulation. Such plasticity has been observed in other CD4 T cell subsets, such as the ability of Tregs to become Th17 cells [21,22] and of Th17 cells to exhibit Th1 characteristics *i.e.* to switch to dominant IFNγ expression from dominant IL-17 expression [23].

After 14 days culture, the majority of CD4$^+$ cells from all animals were CD45RO$^+$ regardless of CD62L expression status. Therefore, it is difficult to undertake phenotype-functionality evaluation of *ex vivo* sorted subsets by phenotyping cells after even a short *in vitro* stimulation period; this demonstrates that to determine the contribution of *in vivo* generated T cell memory subsets to the outcome of functional assays, these subsets need to be purified prior to culture.

Production of IFNγ was observed mainly in CD45RO$^+$ cells, which is supported by another study which found that sorted CD4$^+$ CD45RO$^+$, but not CD4$^+$ CD45RO$^-$ T-cells produced IL-4 and IFNγ transcripts as well as biologically active IFNγ in bovine T-cells [24]. This further supports the view that CD45RO expression relates to an activation/memory state for T-cells [25].

In this study, no difference was detected in the ability of cells derived from *M. bovis* infected animals or from BCG-vaccinated animals to respond in the IFNγ *ex vivo* ELISPOT or cELISPOT assays. This is not surprising and needs to be viewed in the context of timing. Vaccine-induced responses were measured before infection and thus reflect the effect of vaccination, whilst the responses in the infected animals were measured after the infection had been established. Responses to infection in naïve animals would be delayed compared to responses in vaccinated and infected animals [26]; responses in naïve animals after infection would be less likely to control or clear the infection, whilst responses in vaccinated animals would be more likely to control mycobacteria. Further, the *M. bovis* infected cattle in our study were without clinical manifestations of the disease and can therefore be viewed as latent or with very slow progressing disease and with low bacterial loads that could allow central memory development. It is likely that disease progression leading to increased bacillary/antigen load would lead to exhaustion of this memory pool due to constant stimulation without antigenic clearance. Several vaccine studies with cattle have demonstrated that memory responses are positive correlates of protection as they negatively correlate with TB-associated pathology [15,16] and mycobacterial burden [27]. The cELISPOT response correlates with slow disease progression in natural infection [28]. It has been shown that during active replication, effector T-cells are expanded and memory cells are detectable after control or eradication [28,29].

In summary, the central or effector memory function of bovine CD4$^+$ T-cells, as evaluated in the *ex vivo* ELISPOT and cELISPOT can be defined by the surface markers CD45RO and CD62L. Furthermore, due to the plasticity in the expression of these cell surface markers, it would be difficult to directly correlate what is observed *ex vivo* in blood or peripheral blood mononuclear cells directly to what is observed after a period of culture. This correlation can only be achieved by defining the *ex vivo* population prior to culture.

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Conflict of interest: The authors declare no conflict of interests.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.10.113.

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