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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 consisted 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 hi 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].
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 (IgG2a) to bovine CD4 (Serotec, UK) as previously described [4]. CD4+ T cells were stained with mAbs CC32 (IgG1) to bovine CD62L (AbD Serotec) and IL-A16 (IgG3) to CD45RO [3] (a kind gift from Dr Jan Naessens, ILIRI), followed by staining with rat mAb to mouse IgG1: Brilliant violet 421 (BD Pharmingen), goat anti-mouse IgG2a: FITC (Southern Biotech), and goat anti-mouse IgG3: R-phycocerythin (R-PE) (Southern Biotech). After incubation, cells were washed, re-suspended 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+CD62Llo, CD45RO+CD62Llo, CD45RO+CD62Lhi and CD45RO−CD62Lhi 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.

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 BD 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 × 10^5 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. cSO1,4= (cSFU1,4 * d14/d0) O/d0; 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 plated into culture at day 0 (d0). This figure would provide
CD45RO+ CD62Llo CD45RO (clear in 7278 were:

CD45RO+ CD62Lhi CD45RO+CD45RO−CD62Llo CD45RO−CD62Lhi. The largest proportion of CD4+ T-cells in all animals expressed the phenotype CD45RO+ CD62Llo. 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 (cSO1 + cSO2 + cSO3 + cSO4) and used as total potential SFU (cSO) of the original CD4 population, i.e. cSO = cSO1 + cSO2 + cSO3 + cSO4. To obtain the proportion of SFU that each individual cell population would have contributed (cSP), each individual SO was divided by the SO, and multiplied by 100 (i.e. cSP = (cSO14/cSO1) * 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+ CD62Llo is higher than the proportion of CD45RO+ CD62Lhi, CD45RO−CD62Llo and CD45RO− CD62Lhi.

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+ CD62Llo in both BCG vaccinated and TB reactor animals (Fig. 1). The rarest population was the CD45RO−CD62Llo cells. As a proportion of the total sorted CD4+ T-cells, the different cell populations were: CD45RO− CD62Llo cells 1.14–4.46%; CD45RO+ CD62Llo T-cells 66.85–83.29%; CD45RO− CD62Lhi T-cells 8.05–11.84%; and CD45RO− CD62Lhi 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− CD62Llo cells isolated, it was not possible to analyze their responses in the studies described below.

3.2. CD45RO+CD62Llo, CD45RO−CD62Lhi and CD45RO−CD62Lhi, 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−CD62Llo population followed by the CD45RO−CD62Lhi and then the CD45RO+CD62Lhi 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 sub-populations are present in PBMC, we quantiﬁed the subset proportion 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+CD62Llo 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−CD62Llo (0.88 ± 0.63) and CD45RO−CD62Lhi (0.62 ± 0.38) during culture.

The highest response in the ex vivo ELISPOT resided within the CD45RO+CD62Llo population in both M. bovis infected and BCG vaccinated animals, followed by the CD45RO−CD62Llo population (Fig. 3A); the proportion of IFNγ secreting cells in the CD45RO−CD62Lhi cells was minimal. In contrast, in the cELISPOT, the highest response resided within the CD45RO+CD62Lhi population (Fig. 3B); both the CD45RO+CD62Llo and CD45RO−CD62Lhi populations, respectively, contained fewer IFNγ 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+CD62Lhi cells acquiring a CD62Llo phenotype, whilst a proportion of CD45RO−CD62Llo cells acquired a CD62Lhi phenotype. A small proportion of these cells also became CD45RO−CD62Lhi. Finally, small proportions of CD45RO−CD62Lhi cells became CD45RO−CD62Llo or CD45RO+CD62Llo (Fig. 4).

Greater plasticity in the expression of these markers was observed after a longer culture period. All cells producing IFNγ after antigen re-stimulation after 14 day culture, regardless of the input cell population phenotypes at the beginning.
of in vitro culture (CD45RO⁺CD62Lhi, CD45RO⁺CD62Llo, or CD45RO⁻CD62Lhi), expressed CD45RO. Whilst the majority of CD45RO⁺CD62Lhi and CD45RO⁻CD62Llo retained stable CD62L expression, substantial proportions of the CD45RO⁺CD62Lhi decreased expression to become CD62Llo, whilst a substantial proportion of CD45RO⁻CD62Lhi increased expression to become CD62Lhi (Fig. 5). The most dramatic in vitro dynamics in CD45RO and CD62L expression were displayed by the antigen-specific IFNγ⁺ CD45RO⁺CD62Lhi subpopulation. Not only did these cells express CD45RO after 2 weeks in culture, but the majority also decreased expression of CD62L to become CD62Llo (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⁺CD62Lhi and effector memory-associated phenotype CD45RO⁻CD62Llo 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⁺CD62Lhi T cells and less frequent CD4⁺CCR7⁻CD62Llo 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 (CD62Lhi) largely overlap with those expressing CCR7 (Waters et al., pers. comm.).

On a cell by cell basis, CD45RO⁺CD62Lhi and CD45RO⁺CD62Llo cells contribute to a similar extent to the ex vivo and cELISPOT; however, as a proportion of overall responses, CD45RO⁺CD62Lhi cells dominate the cELISPOT compared to CD45RO⁺CD62Llo cells. This would be consistent with central memory functionality of cELISPOT IFNγ secreting cells. In contrast, CD45RO⁺CD62Llo contributed to a greater extent to the ex vivo ELISPOT compared to CD45RO⁺CD62Lhi.
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 vitro 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 naive animals would be delayed compared to responses in vaccinated and infected animals [26]; responses in naive 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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