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Potential of the dual IFN-γ/IL-2 fluorescence-immunospot assay to distinguish different stages in bovine tuberculosis

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ABSTRACT

Human studies have identified the potential of measuring *Mycobacterium tuberculosis* specific IFN-γ and/or IL-2 secreting T cell subsets to distinguish different clinical stages of human tuberculosis (TB). To assess these functional T cell subsets in different states of bovine TB we have established a bovine dual IFN-γ/IL-2 fluorescence-immunospot (FluoroSpot) assay and analysed the frequencies of *Mycobacterium bovis* (*M. bovis*) specific IL-2 and/or IFN-γ producing cells in PBMC from 30 cattle naturally infected with *M. bovis*. Depending on their post mortem results the animals were grouped in 22 cattle with visible lesions (VL) and 8 cattle without visible lesions (NVL). In response to bovine tuberculin purified protein derivative (PPD-B) the frequencies of cytokine producing cells and proportions of IL-2 single producers were significantly higher in VL compared to NVL while PWM-induced cytokine responses were similar between the two groups. Dual IL-2$^+$IFN-γ$^+$ T cells could be identified as the largest PPD-B responsive T cell subset in both cattle groups. In conclusion, our FluoroSpot is a valid method to enumerate individual antigen-specific IFN-γ$^+$ and IL-2$^+$ T cell subsets *ex vivo*. The greater levels of single IL-2 producing T cells associated with the presence of pathology could be a potential biomarker for active TB in cattle.

1. Introduction

Human infection with *Mycobacterium tuberculosis* (*M. tb*) results in a spectrum of clinical outcomes, including individuals that progress to active disease with clinical symptoms [active TB infection (ATBI)] and individuals that maintain a persistent latent stage of infection in the absence of clinical symptoms [latent tuberculosis infection (LTBI)]. However, current diagnostic tests based on *ex vivo* interferon-gamma (IFNγ) release assays are limited in their ability to discriminate between ATBI and LTBI (McNerney et al., 2012). Thus, measurement of additional biomarkers may be required to enable this distinction. One such biomarker that has shown promise in humans is the cytokine interleukin-2 (IL-2). Data from analysis of virus-associated diseases indicate that functional T cell signatures are directed by antigen load, with high IFN-γ/low IL-2 responses dominating in situations of high antigen load and antigen persistence, while the converse is true (*i.e.*low IFN-γ/high IL2 responses) in situations involving antigen clearance (Harari et al., 2005; Pantaleo and Harari, 2006). Studies investigating ATBI patients with or without anti-mycobacterial therapy have expanded on these findings to demonstrate that these cytokine signatures associated with high/low antigen load have also be observed in mycobacterial infection (Millington et al., 2007; Casey et al., 2010). Furthermore, simultaneous analysis of mycobacterial antigen-induced IFN-γ and IL-2 production in ATBI and LTBI/non-ATBI individuals revealed a bias towards IL-2 only and/or IL-2/IFN-γ dual responses in LTBI/non-ATBI, and IFNγ only responses in ATBI (Casey et al., 2010; Sester et al., 2011; Essone et al., 2014; Zhang et al., 2017).

Mycobacterium bovis (*M. bovis*) is a member of the *M. tb* complex and is the main causative agent of bovine TB, a disease that represents a significant economic animal health problem (Waters et al., 2012). In Great Britain, diagnosis of bovine TB is based on the results of the single intradermal comparative cervical tuberculin (SICCT) skin test and/or the whole-blood IFN-γ release assay. Unlike human infection, it remains unclear whether distinct stages of ATBI and LTBI occur in *M. bovis*-infected cattle (Pollock and Neill, 2002; Cassidy, 2006; Alvarez et al., 2009), although it has been suggested that skin test or IFNγ test positive animals with no visible lesions may represent latently infected cattle (Alvarez et al., 2009). Given that mycobacterial antigen-induced IFN-γ and IL-2 production have both been detected in *M. bovis* infected cattle (Whelan et al., 2011; Rhodes et al., 2014), we sought to determine whether the functional signatures described above for ATBI and LTBI in humans were also observed in bovine TB. To this end, we established a bovine dual IFN-γ/IL-2 fluorescence-immunospot (FluoroSpot) assay and analysed the frequency of bovine tuberculin purified protein

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derivative (PPD-B) specific IL-2 and/or IFN-γ producing cells in *M. bovis* infected cattle presenting with visible lesions or no visible lesions as proxies for ATBI and LTBI respectively.

2. Material and methods

2.1. Cattle

All animals were housed at the Animal and Plant Health Agency at the time of blood sampling, and procedures were conducted within the limits of a United Kingdom Home office license under the Animal (Scientific Procedures) Act 1986, which were approved by the APHA Animal Welfare and Ethical Review Body (AWERB) committee. Heparinised blood samples were obtained from naturally *M. bovis* infected cattle sourced from GB herds known to have bovine TB. All animals tested positive to both the SICCT test and the whole blood IFN-γ assay. At post mortem examination, eight lung lobes and the following lymph nodes (LN) were assessed for the presence of gross visible lesions: left and right mandibular LN; left and right medial retropharyngeal LN; cranial mediastinal LN; caudal mediastinal LN, left and right bronchial LN; and cranial tracheobronchial LN. Based on post mortem analysis, the cattle were grouped into 22 animals with visible lesions (VL) and 8 animals with no visible lesions (NVL). There was no significant difference in the age of the animals between the two groups, and both VL and NVL groups contained a mix of cattle breeds.

2.2. PBMC isolation

Peripheral blood mononuclear cells (PBMC) were isolated from heparinised cattle blood by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich, UK). Purified PBMC were cryopreserved in fetal calf serum (Sigma-Aldrich, UK) containing 10% DMSO (Sigma-Aldrich, UK) before using the cells in the FluoroSpot assay.

2.3. IFN-γ/IL-2 FluoroSpot assay

Coating and incubation with cells were carried out under sterile conditions. Low fluorescent clear 96-well PVDF filter plates (Multiscreen$_{HTS}$ IP-FL, Merck Millipore, UK) were pre-wetted with 15 µl/well 35% ethanol for one minute and washed before coating with 50 µl/well of 15 µg/ml anti-bovine IFN-γ (clone MT17.1, Mabtech, Sweden) and with 15 µg/ml anti-bovine IL-2 (customised polyclonal rabbit antibody, bovine IL-2 affinity purified, Abcore, USA) in PBS overnight at 4 °C. To investigate the effect of anti-bovine IL-2 on numbers of IFN-γ secreting cells, and anti-bovine IFN-γ on numbers of IL-2 secreting cells, additional wells were coated only with anti-bovine IFN-γ or anti-IL-2 as described before. On the next day plates were washed with PBS and blocked with 200 µl/well complete cell culture medium [RPMI 1640 containing 2 mM GlutaMax, 25 mM HEPES, 0.1 mM NEAA, 5×10^{-5} M βmercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin (all from Gibco Life Technologies, UK) and 10% fetal calf serum (Sigma-Aldrich, UK)]. Complete medium with or without bovine tuberculin purified protein derivative (PPD-B, 300 U/ml, Prionics, Switzerland), avian tuberculin purified protein derivative (PPD-A, 250 U/ml, Prionics, Switzerland) and pokeweed mitogen (PWM, 10 µg/ml; Sigma, UK) as a positive control were added to duplicate wells before culturing with PBMC (10^5 cells/well) in a final volume of 200 µl/well complete medium. To enhance antigen-specific responses and to minimise the attenuating effect of IL-2 capture on IFN-γ responses, soluble anti-CD28 (clone EC4 or CD10 at 5 µg/ml and 1 µg/ml) was added with antigen and medium control in some of the experiments for the development of the FluoroSpot. After 22–24 h at 37 °C and 5% CO_2 plates were washed with PBS and developed for 2 h with 50 µl/well of 2 µg/ml BAM-conjugated anti-bovine IFN-γ (clone MT307, Mabtech) and 2.5 µg/ml biotinylated anti-bovine IL-2 (polyclonal goat antibody, R&D, UK) in 0.1% BSA/PBS. After washing, plates were incubated for 1 h with 50 µl/well fluorophore-labelled secondary antibodies, anti-BAM-490 (Mabtech) and streptavidin550 (Mabtech), diluted 1:200 in 0.1% BSA/PBS at room temperature. Plates were washed with PBS and incubated for 15 min with 50 µl/well fluorescence enhancer (Mabtech) at room temperature before being emptied and air-dried. Plates were read and analysed in an ELISpot/FluoroSpot reader system (iSpot Spectrum, AID, Strassberg, Germany) with software version 7.0, where fluorescent spots were counted utilizing separate filters for FITC and Cy3. Camera settings (exposure and gain) were adapted for each filter and threshold for counting spots were defined by intensity and size. The same spot settings were used throughout. Cytokine-producing cells were counted as spot-forming cells (SFC).

2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 7.03 (GraphPad Software, USA). Spearman's rank correlation was used to measure correlation of IFN-γ SFC between anti-IFN-γ and anti-IFN-γ/IL-2 dual coated FluoroSpot plates and also to measure correlation of IL-2 SFC between anti-IL-2 and anti-IFN-γ/IL-2 dual coated FluoroSpot plates with 95% confidence intervals. Mann-Whitney 2-tailed U test for nonparametric data analysis was used to identify statistically significant differences between groups. The D'Agostino & Peason omnibus normality test was used to assess Gaussian distribution, and parametric or non-parametric tests used where appropriate. Non-parametric Wilcoxon matched-pairs signed rank test was used to identify statistically significant differences between pairs.

3. Results and discussion

To study the frequency of *M. bovis* specific IFN-γ and/or IL-2 secreting cells in cattle we developed a bovine dual colour IFN-γ/IL-2 FluoroSpot assay using in-house anti-IL-2 and commercial anti-IFN-γ antibodies. The combination of these antibodies allowed the detection of individual cytokine producing cells as well-defined spots (Fig. 1), an important requirement for the reproducible digital co-localisation of red and green SFCs to identify IFN-γ/IL-2 co-producers. Comparison of the dual FluoroSpot and single IL-2 FluoroSpot results showed similar IL-2 SFC for antigen/mitogen-induced responses (Fig. 2A). However, the dual IFN-γ/IL-2 FluoroSpot underestimated the IFN-γ SFC detection compared to the FluoroSpot results from only anti-IFN-γ coated wells (Fig. 2B). Further analysis of the data revealed high values for the Spearman's rank correlation between the dual and single readout systems ($r_s = 0.964$ for PPD-B and $r_s = 0.946$ for PWM), suggesting that this underestimation in IFN-γ SFC detection was consistent across the animals studied. Quast and colleagues (2005) could show that this is a consequence of IL-2 absorption by membrane-bound anti-IL-2 capture antibodies. To counterbalance this effect, we have used co-stimulatory anti-CD28 antibodies (Hogg et al., 2011; clone EC4 and CD10) as recommended for the human IFN-γ/IL2 FluoroSpot assay (Quast et al., 2005). Although showing a clear effect on IL-2 and IFN-γ secretion with purified cattle $CD4^+$ T cells, addition of anti-CD28 did not increase the numbers of IFN-γ SFC to the level detected in wells without anti-IL-2 capture antibody when cattle PBMC were used in the FluoroSpot assay (data not shown). In another attempt to address the issue, we pre-cultured cells with antigen but in the absence of coated antibodies before transferring them to anti-IFN-γ/IL-2 coated FluoroSpot plates. However, this was also unsuccessful in compensating the effect of anti-IL-2. These results highlight that care must be taken when comparing frequencies of IFN-γ producing cells derived from the dual colour FluoroSpot assay with historical data derived from the single cytokine ELISpot assay.

We used this bovine dual-colour IFN-γ/IL-2 FluoroSpot assay to enumerate *M. bovis* specific functional T cell subsets in PBMC from 30 cattle, naturally infected with *M. bovis* at different stages of disease progression. Depending on their post mortem results the animals were

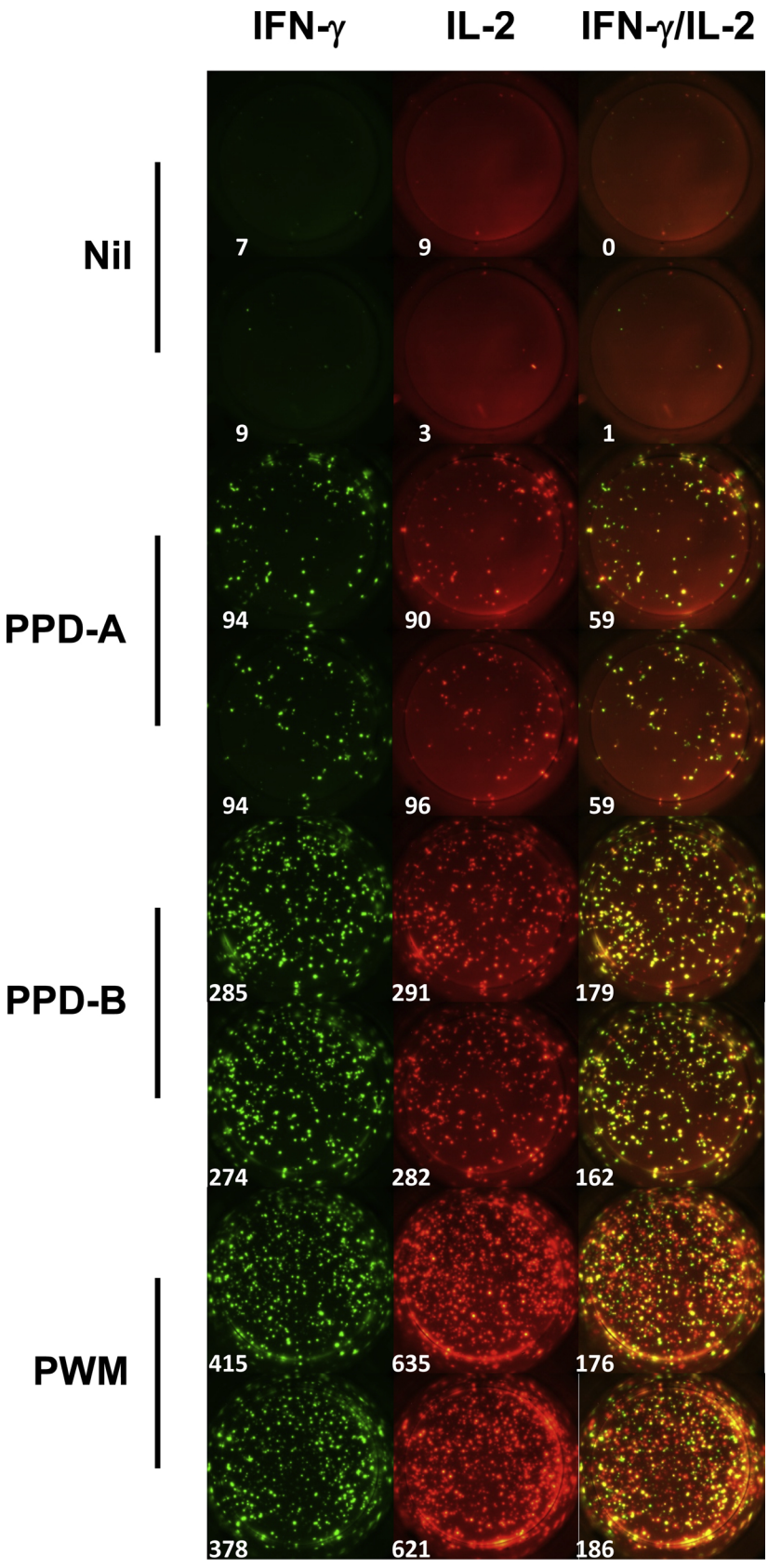


Fig. 1. Images of the in-house dual-colour IFN-γ/IL-2 FluoroSpot assay from a representative *M. bovis* infected cattle. 1×10^5 PBMC/well from a naturally *M. bovis* infected cattle were stimulated with PPD-A, PPD-B or PWM (each in duplicate wells) and the cytokine secreting cells detected by FluoroSpot assay. The fluorescence reader generated two images: FITC image of total IFN-γ secreting cells and Cy3 image of total IL-2 secreting cells. The third dual-stained image for enumeration of dual IFN-γ/IL2 cytokine secreting cells is generated by digital overlay of the single stained images applying a spot centre location algorithm. SFC counts are shown as white numbers.

grouped in 22 V L and 8 NVL cattle. For analysis of mycobacterial-specific responses, our investigations focused on the PPD-B and B-A (PPD-B minus PPD-A) readouts after *in vitro* stimulation with PPD-B and PPD-A. Assessment of PPD-B and the comparison of PPD-B and PPD-A responses (B-A) are used in routine diagnostic tests and have a high sensitivity in detecting *M. bovis* infection. Similar numbers of IFN-γ only, IL-2 only and dual IFN-γ/IL-2 producing cells were observed between VL and NVL animals following PWM stimulation, suggesting that

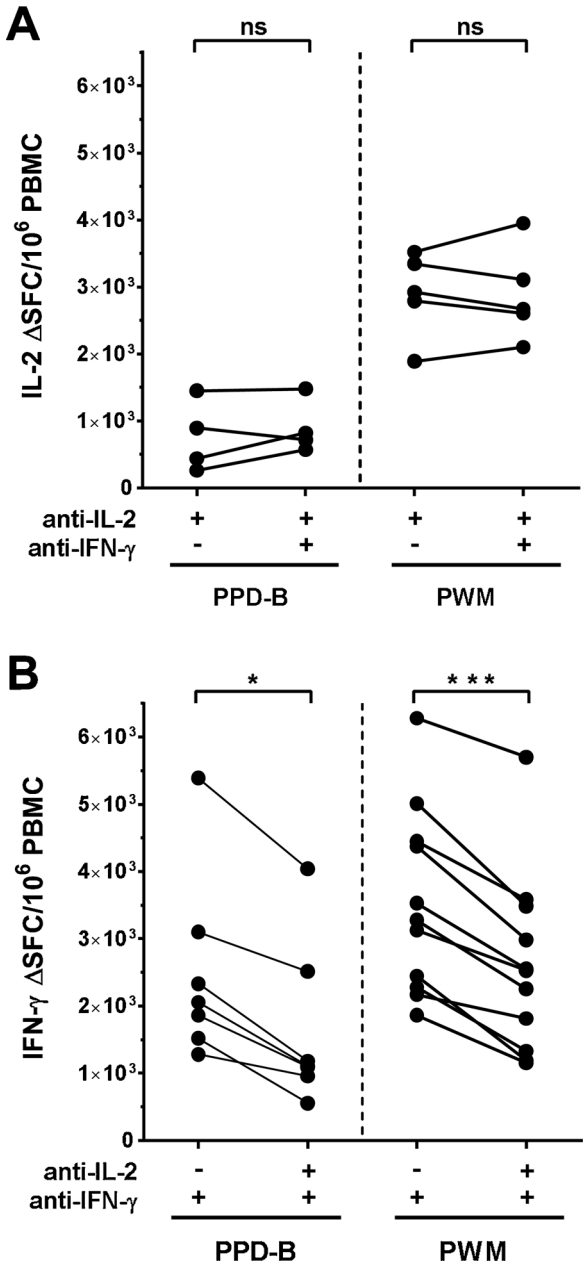
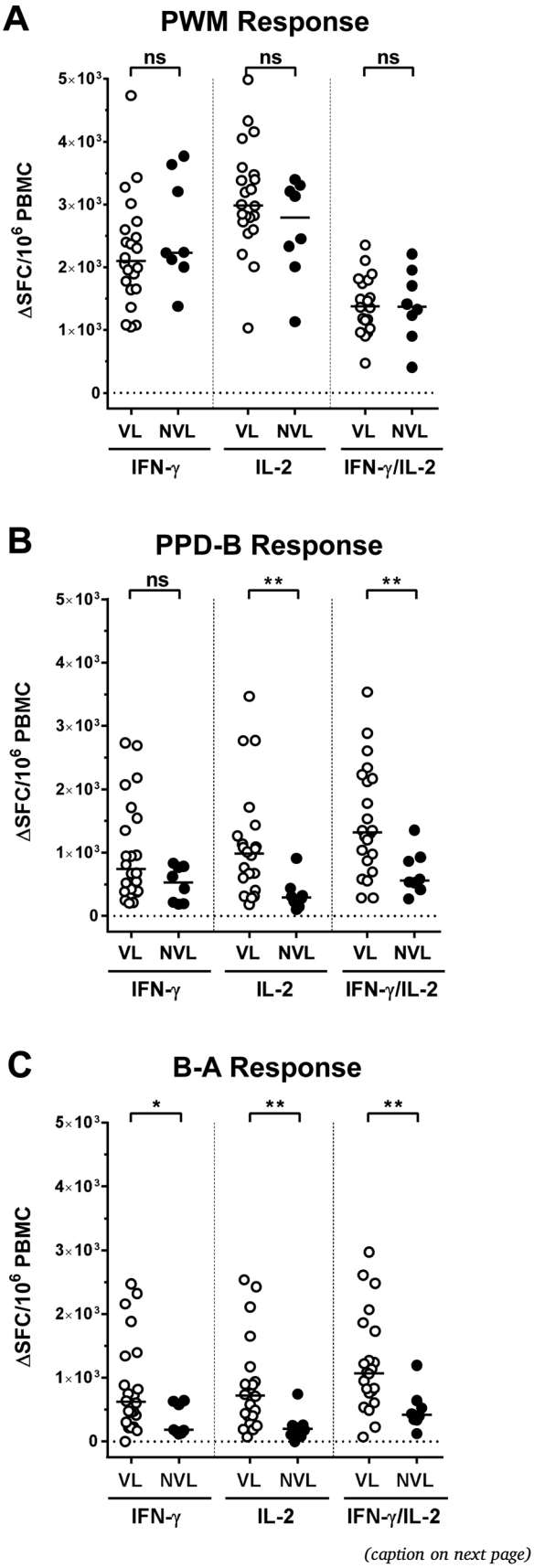


Fig. 2. Underestimation of IFN-γ SFC in the presence of anti-IL-2 antibodies. 1×10^5PBMC/well from naturally *M. bovis* infected cattle were stimulated (A) with PPD-B (4 cattle) or PWM (5 cattle) in the presence of coated anti-IL-2 only or a combination of anti-IFN-γ and anti-IL-2 capture antibodies and (B) with PPD-B (7 cattle) or PWM (11 cattle) in the presence of coated anti-IFN-γ only or a combination of anti-IFN-γ and anti-IL-2 capture antibodies. Numbers of total IL-2 or IFN-γ secreting cells were determined by FluoroSpot. Each symbol represents background subtracted data for an individual animal. Nonparametric Wilcoxon matched-pairs signed rank test. *$p < 0.05$, ***$p < 0.001$.

both groups of animals were capable of producing these cytokines (Fig. 3A). In contrast, frequencies of all 3 cytokine-producing cell subsets were higher in PBMC of VL compared to NVL animals following stimulation with mycobacterial antigens (Fig. 3B and C), and this difference reached statistical significance for IL-2 only and IFN-γ/IL-2 dual producers (Fig. 3B, $p_{IL\text{-}2} = 0.0024$, $p_{IL\text{-}2/IFN\text{-}\gamma} = 0.0087$; Fig. 3C, $p_{IL\text{-}2} = 0.0024$, $p_{IL\text{-}2/IFN\text{-}\gamma} = 0.0052$) and IFN-γ only producers (Fig. 3C, $p = 0.0237$). These results were consistent with our previous study showing higher IL-2 responses in VL compared to NVL cattle after PPDB stimulation using ELISA to determine cytokine production (Rhodes et al., 2014). Similar results have also been demonstrated in human studies. For example, using flow cytometry, a higher percentage of total PPD-B reactive $CD4^+$ T cells (cells producing IFN-γ and/or IL2) were



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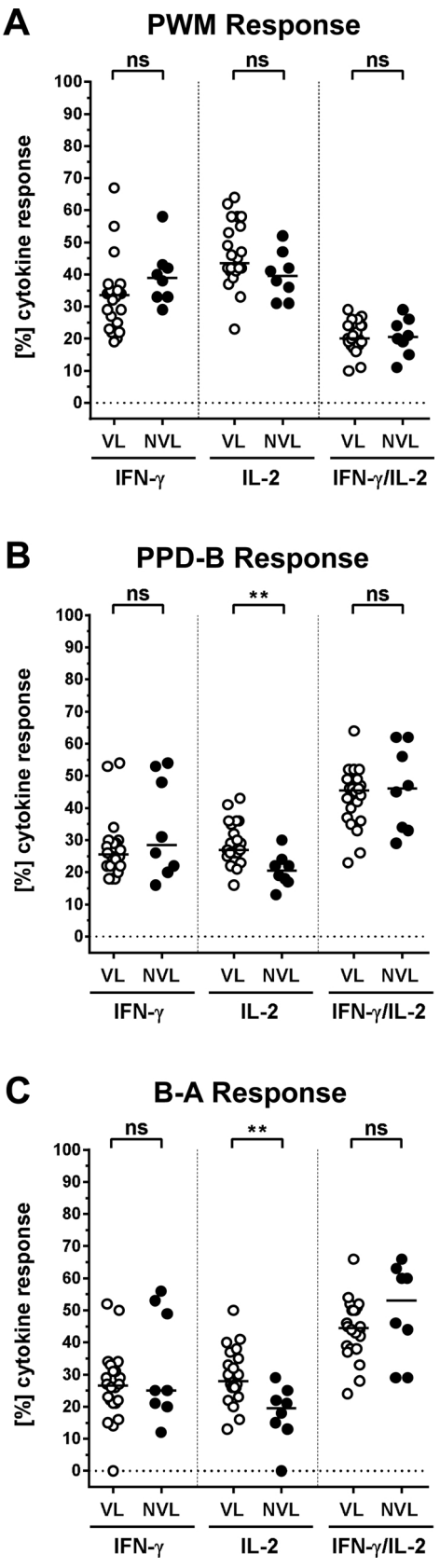

Fig. 3. Frequencies of IFN-γ only, IL-2 only and dual IFN-γ/IL-2 producing cells in VL and NVL cattle. 1×10^5PBMC/well from naturally *M. bovis* infected cattle with VL (22 cattle) and NVL (8 cattle) were stimulated with mitogen (A) and mycobacterial antigens (B and C). Numbers of IFN-γ only, IL-2 only and IFN-γ/IL2 dual spot forming cells (SFC) were quantified by FluoroSpot. Each symbol represents background subtracted data for an individual animal while horizontal bars represent the median value. Nonparametric Mann-Whitney *U test* *p < 0.05, **p < 0.01.

observed in ATBI patients compared to those successfully treated for the disease (Sester et al., 2011). Furthermore, human studies using dual FluoroSpot techniques have shown greater frequencies of IFN-γ only, IL-2 only and dual IFN-γ/IL-2 secreting T cells in ATBI compared to non-ATBI patients in response to stimulation with the specific mycobacterial antigens ESAT-6 or CFP10 (Zhang et al., 2017). However, other human studies have shown conflicting results, with greater frequencies in ATBI patients seen solely in the IFN-γ only producing cells in response to ESAT-6 or ESAT-6/CFP10 stimulation (Essone et al., 2014; Chesov et al., 2015), while other studies report no significant differences between ATBI patients and LTBI or treated patients in the absolute frequencies of total cytokine-secreting T cells specific for PPDB (Casey et al., 2010; Essone et al., 2014) or ESAT-6/CFP10 (Casey et al., 2010).

In order to distinguish VL and NVL animals independently from their absolute numbers of antigen-specific cells and to adjust for the high level of inter-individual variations of SFC responses, we compared proportions of PPD-B-specific IFN-γ and/or IL-2 T cell subsets to the overall cytokine response per individual cattle (Fig. 4). In contrast to the mitogen induced response (Fig. 4A), the main PPD-B responding T cell subset in both cattle groups was the dual IFN-γ/IL-2 secreting T cell population. Analysis of the PPD-B only data (Fig. 4B) demonstrated similar proportions of this T cell subset in VL and NVL animals. Although there was a greater proportion of this dual-producing subset in NVL animals after subtracting the unspecific response to PPD-A, this did not reach statistical significance (Fig. 4C, $p = 0.2706$; $median_{VL} = 45\%$, IQR 38–50; $median_{NVL} = 53\%$, IQR 33–62). For the other functional subsets, a significantly greater proportion of IL-2 only secreting T cells was observed in VL compared to NVL animals following analysis of both the PPD-B data (Fig. 4B, $p = 0.0025$; $median_{VL} = 27\%$, IQR 26–35; $median_{NVL} = 21\%$, IQR 17–24) and the B-A data (Fig. 4C, $p = 0.0026$; $median_{VL} = 28\%$, IQR 25–36; $median_{NVL} = 20\%$, IQR 14–24). In contrast, there was no significant difference in the median percentage of the IFN-γ only secreting T cells between the two groups. A higher proportion of IFN-γ/IL-2 co-producing TB-specific cells in individuals with a lower degree of pathology has been reported in human studies. Using dual IFN-γ/IL-2 FluoroSpot technique, an increased number/percentage of antigen-specific IFN-γ/IL2 co-producing cells in LTBI individuals and TB patients after treatment (Millington et al., 2007; Casey et al., 2010; Essone et al., 2014) could be identified in comparison with non-treated ATBI patients. Similar to FluoroSpot data, triple IFN-γ/IL-2/TNF-α flow cytometry found a higher percentage of antigen-specific $CD4^+$ T cells that were polyfunctional triple IFN-γ/IL-2/TNF-α or dual IFN-γ/IL2 producers in PBMC of LTBIs compared with ATB (Caccamo et al., 2010; Harari et al., 2011; Sester et al., 2011; Sallin et al., 2018). However, as mentioned earlier, not all of the results reported from the different human studies are consistent. Some groups demonstrated the dominance of IL-2 only and dual IL-2/IFN-γ secreting T cells subsets in LTBI when using ESAT-6/CFP-10 as antigen but not after stimulation with PPD-B (Casey et al., 2010; Essone et al., 2014) whilst another reported an association of dual IL-2/IFN-γ secreting T cells with latent disease only when stimulated with PPD-B (Sester et al., 2011). Lastly, one study failed to confirm any differences in proportions of ESAT-6 or CFP-10 antigen-specific IFN-γ and IL-2 producing T cell subsets in LTBI and ATBI (Chesov et al., 2015). These results highlight that differences in many factors, including assay methods, antigen preparations and study participants, may influence the outcomes of these studies.

Our results did not show a significantly higher percentage of IFN-γ/

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Fig. 4. Proportions of IFN-γ only, IL-2 only and dual IFN-γ/IL-2 producing cells in VL and NVL cattle. 1×10^5PBMC/well from naturally *M. bovis* infected cattle with VL (22 cattle) and NVL (8 cattle) were stimulated with mitogen (A) and mycobacterial antigens (B and C). Data are expressed as the percentage of the total number of cytokine secreting cells (IFN-γ only, IL-2 only and IFN-γ/IL2 dual producers) per individual cattle. Each symbol represents background subtracted data for an individual animal while horizontal bars represent the median value. Nonparametric Mann-Whitney *U test* $^{**}p < 0.01$.

IL-2 dual producers in cattle with no pathology (Fig. 4C), although the numbers of NVL animals studied were low. Interestingly, and in contrast to what has been assumed to be associated with a protective $CD4^+$ T cell response (Millington et al., 2007; Casey et al., 2010; Sester et al., 2011; Essone et al., 2014; Zhang et al., 2017), numbers and proportions of antigen-specific single IL-2 producing cells were greater in cattle with pathology. This is consistent with our previous findings of higher IL-2 responses in VL compared to NVL cattle (Rhodes et al., 2014) and could suggest that antigen-specific T cell subsets in cattle with high (VL) or no (NVL) pathology are different compared to their human equivalent.

In conclusion, the FluoroSpot described herein is a valid method to enumerate individual antigen-specific IFN-γ^+ and IL-2^+ T cell subsets *ex vivo*. The greater levels of single IL-2 producing T cells associated with the presence of pathology could be a potential biomarker for active TB in cattle.

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