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Hannah J. Metcalfe [a,b,1], Sabine Steinbach [a,1], Gareth J. Jones [a], Tim Connelley [b], W. Ivan Morrison [b], Martin Vordermeier [a,2], Bernardo Villarreal-Ramos [a,*,2]

[a] TB Immunology and Vaccinology Team, Department of Bacteriology, Animal and Plant Health Agency, Weybridge, Surrey KT15 3NB, UK
[b] Immunity Division, The Roslin Institute, Royal (Dick) School of Veterinary Studies, University of Edinburgh, Easter Bush, Midlothian EH25 9RG, UK

ABSTRACT

There is a need to improve the efficacy of Bacille Calmette-Guérin (BCG) vaccination against tuberculosis in humans and cattle. Previously, we found boosting BCG-primed cows with recombinant human type 5 adenovirus expressing antigen 85A (Ad5-85A) increased protection against *Mycobacterium bovis* infection compared to BCG vaccination alone. The aim of this study was to decipher aspects of the immune response associated with this enhanced protection. We compared BCG-primed Ad5-85A-boosted cattle with BCG-vaccinated cattle. Polyclonal CD4+ T cell libraries were generated from pre-boost and post-boost peripheral blood mononuclear cells – using a method adapted from Geiger et al. (2009) – and screened for antigen 85A (Ag85A) specificity. Ag85A-specific CD4+ T cell lines were analysed for their avidity for Ag85A and their Ag85A epitope specificity was defined. Boosting BCG with Ad5-85A increased the frequencies of post-boost Ag85A-specific CD4+ T cells which correlated with protection (reduced pathology). Boosting Ag85A-specific CD4+ T cell responses did not increase their avidity. The epitope specificity was variable between animals and we found no clear evidence for a post-boost epitope spreading. In conclusion, the protection associated with boosting BCG with Ad5-85A is linked with increased frequencies of Ag85A-specific CD4+ T cells without increasing avidity or widening of the Ag85A-specific CD4+ T cell repertoire.

1. Introduction

Mycobacterium bovis, the main cause of bovine tuberculosis (bTB), is a world-wide problem and is endemic in the south west of England and parts of Wales [1–3]. As well as having a significant economic impact, infection of cattle with *M. bovis* presents a risk for human health. The incidence of bTB may be exacerbated by wild-life reservoirs which are not easy to monitor accurately and therefore there is a need to implement supplementary measures to the current control methods [1,2,4,5]. Vaccination of cattle would be a logistically convenient measure for controlling bTB [6].

To date, the live attenuated *M. bovis* Bacille Calmette-Guérin (BCG) is the only available vaccine against TB but it has variable

efficacy in both humans and cattle (reviewed elsewhere [7–9]). Nevertheless, strategies which boost BCG protection are being explored because of the efficacy of BCG against non-pulmonary infections [10]. One such strategy is boosting BCG with viral vectors expressing mycobacterial proteins such as antigen 85A (Ag85A).

Ag85A is one of a family of three secreted and membrane retained enzyme isoforms, highly conserved across mycobacteria and involved in the synthesis of important constituents for the cell wall [11–13]. These proteins are classed as virulence factors because they are thought to promote bacterial survival in macrophages along with adhesion/invasion and dissemination in host cells [13]. Ag85A is also an immunogenic Ag that is recognised by T cells and it is considered a potential candidate for boosting cellular immunity primed by BCG [14,15].

Protection against mycobacteria has been shown to be mediated mainly by T cells [16]. CD4+ T cells are thought to be particularly important; depletion of CD4+ T cells in small animal models has been shown to reduce protection against infection with mycobacteria to a greater extent than depletion of other T cell types [17].

* Corresponding author at: Animal and Plant Health Agency (APHA), TB Immunology and Vaccinology Team, New Haw, Addlestone, Surrey KT15 3NB, UK.
 E-mail address: Bernardo.Villarreal-Ramos@apha.gsi.gov.uk (B. Villarreal-Ramos).
 [1] These authors are joint first authors.
 [2] These authors are joint senior authors.

Previously, we showed that boosting BCG with viral vectors such as human type 5 adenovirus (Ad5) or Modified Vaccinia Ankara virus expressing Ag85A reduced the number of animals presenting TB lesions/granulomas in cattle infected with *M. bovis* [18,19]. However, there is a need to understand the immune mechanisms associated with the observed protection to devise means of improving future vaccine development. We hypothesised that enhanced immunity is associated with the magnitude, avidity and specificity of the CD4+ T cell response to Ag85A. To test this hypothesis, CD4+ T cell libraries were generated from BCG primed and Ad5-85A boosted animals using a method adapted from Geiger et al. [20] and compared to CD4+ T cell libraries generated from BCG control cattle. Ag85A-specific CD4+ T cell lines were selected for avidity studies and analysis of T cell receptor epitope specificity.

2. Materials and methods

2.1. Vaccine experiment

The biological materials used in this study were derived from a vaccine experiment described previously by Dean et al. [19]. Animal work was carried out according to the UK Animal (Scientific Procedures) Act 1986. The study protocol was approved by the APHA Animal Use Ethics Committee (UK Home Office PCD number 70/6905). Briefly, all animals were vaccinated with 1×10^6 colony forming units (CFU) *M. bovis* BCG Danish 1331 subcutaneously at week (wk) 0; Ad5-85A boosted cows were inoculated at wk 8 with 1×10^9 infectious units of Ad5-85A by intradermal injection on the shoulder; all animals were challenged endobronchially with 2×10^3 CFU *M. bovis* AF2122/97 strain at wk 12. Total pathology score was determined at post-mortem examination at wk 26 [19]. The present study utilised cryopreserved PBMC collected from nine BCG-primed Ad5-85A-boosted cows and six BCG-vaccinated control cows.

2.2. CD4+/CD14+ cell sorting and culture

CD4+ and CD14+ T-cells were isolated as previously described [21]. Briefly, CD4+ T cells from PBMC were labelled with mAb CC8 to bovine CD4 (Bio-Rad AbD Serotec) and isolated using paramagnetic beads conjugated to rat anti-mouse IgG2 (Miltenyi) as described by the manufacturer's instructions. CD14 cells from PBMC were isolated using paramagnetic beads conjugated to antibodies against human CD14 (Miltenyi) as described by the manufacturer's instructions. CD4+ and CD14+ cell purities were assessed by flow cytometry and found to be >95%. Cells were cultured in RPMI 1640 containing 2 mM GlutaMax, 25 mM HEPES, 0.1 mM NEAA, 5×10^{-5} M β-mercaptoethanol, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Gibco Life Technologies, UK) and 10% FCS (Sigma-Aldrich, UK).

2.3. CD4+ T cell libraries

Polyclonal CD4+ T cell libraries were generated from pre-boost (wk 8) and post-boost (wk 11) PBMC using a method adapted from Geiger et al. [20]. Autologous feeder cells were prepared by treating wk 0 PBMC with 50 µg/ml mitomycin C (Sigma-Aldrich) in PBS, at a density of 10^7 PBMC/ml, for 30 min at 37 °C. Polyclonal libraries were generated in 96-well flat-bottom plates using 6×10^4 autologous feeder cells/well and 4×10^3–1×10^4 CD4+ T cells/well from BCG-vaccinated control samples or 1×10^3–5×10^3 CD4+ T cells/well from post-Ad5-85A boost samples. CD4+ T cells were stimulated with 1 µg/ml lectin from *Phaseolus vulgaris* leucoagglutinin (PHA) (Sigma-Aldrich) in the presence of 10 U/ml recombinant human interleukin 2 (IL-2) (Gentaur, Belgium). Libraries were fed with 10 U/ml IL-2 on day 5, expanded and fed where necessary between days 6–8, and half the medium was replaced with cytokine-free medium on day 9. 1×10^5–2×10^5 CD4+ T cells per library well were screened for Ag85A-specificity on day 11 using antigen presenting cells (APC) adhered to flat-bottom 96-well plates (2 h adhesion of 1×10^5 PBMC/well, washed 3 × with HBSS (Gibco)) either unpulsed (negative control) or pulsed with 5 µg/ml Ag85A (Lionex, Germany) for 3 days.

2.4. Antigen-specific proliferation assay

Proliferation was determined on day 4 after radiolabelling cells for 16 h with [Methyl-³H]-thymidine (³H-TdR) (1 µCi/well, 24.7 Ci/mmol, Perkin Elmer, USA). Cells were harvested onto 96-well glass filter plates (UniFilter-96, GF/C – PerkinElmer, USA) using a Harvester 96 Mach III (TomTec Inc., USA) and analysed for radioactivity using a MicroBeta² 2450 (Perkin Elmer) plate counter.

Positive Ag85A-responses in the 96-well CD4+ T cell libraries had to pass the following criteria:

(1) Ag85A counts per min (cpm)/negative control cpm $\geqslant 2$
And:
(2) Ag85A cpm – negative control cpm $\geqslant$ the mean of all the negative controls in the 96-well library plate.

Ag85A-specific CD4+ T cell line frequencies were calculated based on >37% negative wells according to the Poisson distribution and expressed per million CD4+ T cells [22].

2.5. Ag85A-specific CD4+ T cell line maintenance

Ag85A-specific CD4+ T cells were selected from 1×10^5–2×10^5 CD4+ T cells using 5 µg/ml (initial screen) or 10 µg/ml (after selection) Ag85A and 5×10^3 CD14+ APC per well of 96-well U-bottom plates; this was carried out in parallel to the proliferation assays for 11 days. Cells were fed with 10 U/ml IL-2 on day 4/7; replacing half the medium with cytokine-free medium on day 9. Ag85A-specific CD4+ T cell lines were expanded after each selection using PHA/IL2 (plus CD14+ APC) for 9 days and cryopreserved. Ag85A-specific CD4+ T cell line avidity was determined after the first cryopreservation (3rd passage: PHA-Ag85A-PHA) and epitope specificity was determined after the second cryopreservation (5th passage: PHA-Ag85A-PHA-Ag85A-PHA).

2.6. Ag85A avidity and peptide assays

The avidity of each Ag85A-specific CD4+ T cell line (1×10^5 cells/well in a 96-well U-bottom plate) was assessed using 5×10^3 CD14+ APC/well in an Ag85A-dose dependent ³H-TdR proliferation assay. Each of the dose-dependent proliferation curves was normalised by dividing by the highest cpm value (=1). Half maximal effective concentration (EC_{50}) values were calculated from the sigmoidal curves using a nonlinear regression curve as illustrated in supplementary Fig. 1. The epitope specificity of individual Ag85A-specific CD4+ T cell lines was assessed using six pools of eleven synthetic peptides covering Ag85A: fifteen amino acid (aa) long and overlapping by ten residues (Mimotopes Pty Ltd., Australia). Individual peptides were used at a concentration of 5 µg/ml (for concentration of peptides in moles, please see supplementary Table 1) with CD14+ APC in a ³H-TdR proliferation assay (same cell concentrations as above). Each stimulation index (S.I.) was calculated by dividing cpm from peptide pool treated cells by the cpm of the negative control medium treated cells (S.I.

threshold $\geqslant 2$). Pokeweed mitogen (PWM) at 5 µg/ml (Sigma-Aldrich) was used as a positive proliferation control.

2.7. Statistics

All data analyses were performed using GraphPad Prism version 5.04 (GraphPad Software, La Jolla, CA, USA). Ag85A-specific CD4$^+$ T cell frequency data did not pass normality testing and was subjected to the Wilcoxon matched-pairs signed rank test. Total pathology score data did not pass normality testing therefore the correlation between CD4$^+$ T-cell frequency and pathology score was determined using a nonparametric Spearman correlation coefficient.

3. Results

3.1. Boosting BCG with Ad5-85A increases the frequency of Ag85A-specific CD4$^+$ T cells which correlate with protection against M. bovis pathology

To evaluate the effect of boosting BCG with Ad5-85A on the frequency of Ag85A-specific CD4$^+$ T cells, CD4$^+$ T cell libraries were generated from pre-boost (wk 8) and post-boost (wk 11) PBMC samples and compared to responses from animals vaccinated with BCG alone. Wk 11 was selected as peripheral blood responses at this time point had returned to background levels after the boosting response increasing the chances of detecting memory responses [19]; wk 11 was also the last sampling point after vaccination prior to challenge. The frequencies of Ag85A-specific T cells in the Ad5-85A-boosted wk 11 (post-boost) libraries were found to be significantly higher than the frequencies of Ag85A-specific CD4$^+$ T cells in the wk8 (pre-boost) libraries (Fig. 1A) – this trend was not found in the BCG-vaccinated control animals. This indicates that boosting BCG-vaccinated cattle with Ad5-85A increased the frequencies of Ag85A-specific CD4$^+$ T cells.

To determine whether increased Ag85A-specific CD4$^+$ T cell frequencies correlate with protection against *M. bovis*, wk 11 and wk 8 Ag85A-specific CD4$^+$ T cell frequencies were plotted against the total pathology scores that describe the extent and spread of tuberculous lesions within infected cattle (Fig. 1B). A significant negative correlation was found between the frequencies of post-boost (wk 11) Ag85A-specific CD4$^+$ T cells from Ad5-85A boosted animals

and their respective pathology score ($n = 9$: $r = -0.6331$, $P = 0.0317$) which was not observed when using frequency data from pre-boost (wk 8) Ag85A-specific CD4$^+$ T cells ($n = 9$: $r = -0.1743$, $P = 0.4696$). This suggests the increase in Ag85A-specific CD4$^+$ T cell frequencies, observed 3 wk after boosting with Ad5-85A, is associated with enhanced protection.

3.2. Assessment of T cell avidity pre- and post-boost

To further evaluate the functionality of Ag85A-specific CD4$^+$ T cell lines, an Ag85A-dose dependent proliferation assay was carried out and EC$_{50}$ values were calculated from the sigmoidal curves using a nonlinear regression curve (supplementary Fig. 1). Fig. 2 illustrates comparisons of Ag85A-specific CD4$^+$ T cell EC$_{50}$ values pre-boost and post-boost. We were able to make comparisons between the EC$_{50}$ values for 2 BCG animals (Fig. 2A) and for 3 BCG primed Ad5-85A boosted animals (Fig. 2B). Fig. 2C illustrates Ag85A-specific CD4$^+$ T cell EC$_{50}$ values for all wk 8 Ag85A-specific CD4$^+$ T cell lines at pre-boost (all BCG and to be-boosted animals – $n = 9$) and all Ad5-85A-boosted animals at wk 11 (post-boost, $n = 5$). Taken together, these data suggest that boosting does not increase T cell avidity.

3.3. Boosting BCG with Ad5-85A leads to minimal epitope spreading

To map the regions of Ag85A recognised after BCG priming and compare to the repertoire recognised post-Ad5-85A boost, the epitope specificities of the Ag85A-specific CD4$^+$ T cell lines were analysed using six pools of eleven peptides each (Fig. 3). There is marked variation in epitope specificities between individual animals where: only #6642 reacted to pool one; #6509/#6639 reacted to pool two; after boosting all three animals reacted to pool three which may contain the most dominant epitope; only #6509 reacted to pool four; and only #6642 reacted to pool six. Ag85A-specific CD4$^+$ T cell lines from these animals exhibited varying levels of reactivity to the Ag85A protein (Fig. 3G) but they all had the capacity to react to the PWM control (Fig. 3H). Only one out of the three animals tested (#6509) exhibited a noticeable spread in epitope specificity. This was indicated by positive reactions to pool three from five cell lines derived from this animal out of the eight post-boost Ag85A-specific CD4$^+$ T cell lines; in comparison to no positive reactions in the six pre-boost cell lines derived from
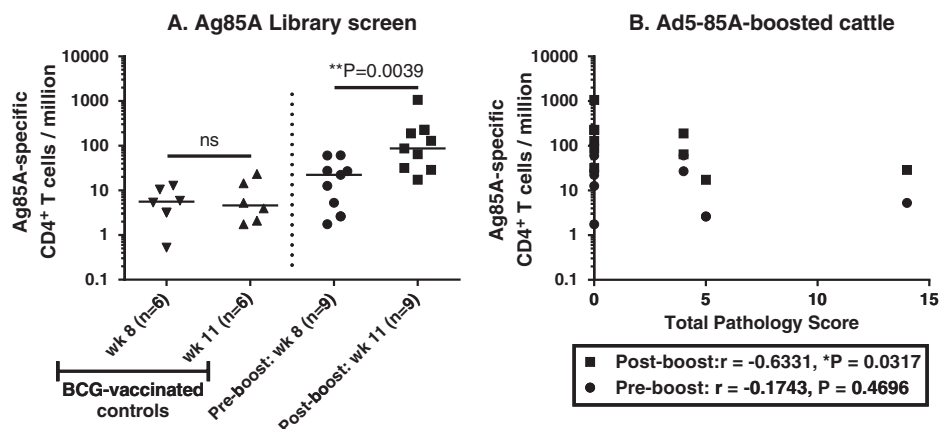

Fig. 1. Boosting BCG with Ad5-85A increases the frequencies of Ag85A-specific CD4$^+$ T cells which have a negative correlation with total pathology score. Polyclonal CD4$^+$ T cell libraries were generated from wk 8 and wk 11 PBMC samples collected from 9 BCG-primed Ad5-85A-boosted cattle and 6 BCG-vaccinated control cattle. Each library was screened for Ag85A-specific CD4$^+$ T cells using the ^{3}H-TdR proliferation assay as described. (A) Frequencies of Ag85A-specific CD4$^+$ T cells plotted in an aligned dot plot (lines = median) and compared using the Wilcoxon matched-pairs signed rank test (ns = no significance). (B) Pre-boost (wk 8) and post-boost (wk 11) Ag85A-specific CD4$^+$ T cell frequencies (Y-axis) from the BCG-primed Ad5-85A-boosted cattle were plotted against their respective Total Pathology Score (X-axis) and analysed using the Spearman Correlation Coefficient (data shown in the box). BCG-vaccinated control: wk 8 = up-side-down triangles; wk 11 = triangles. BCG-primed Ad5-85A-boosted: pre-boost (wk 8) = circles; post-boost (wk 11) = squares.
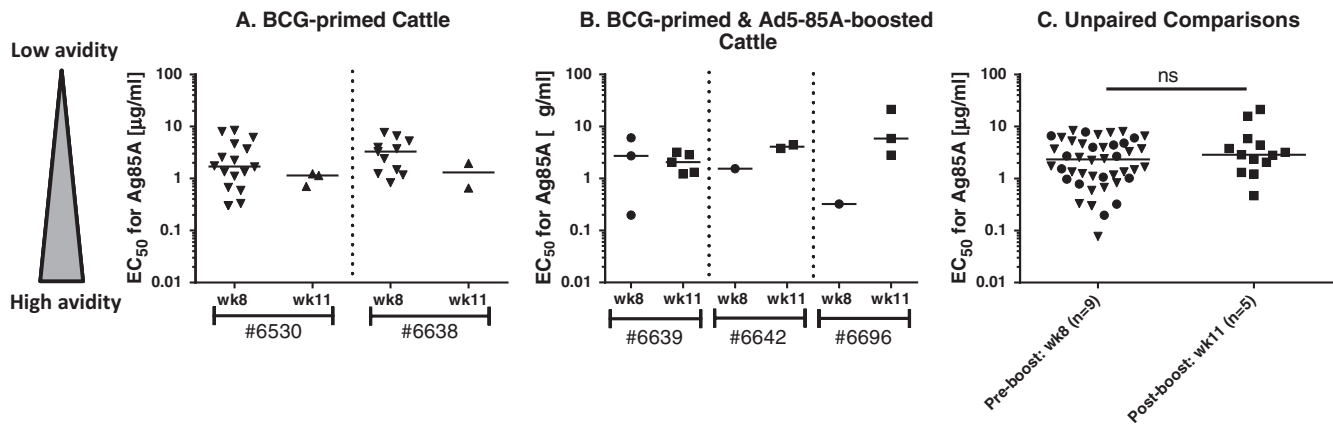
Fig. 2. Comparison of Ag85A-specific CD4^{+} T cell EC$_{50}$ values from BCG vaccinated cows and BCG-primed Ad5-85A-boosted cows. Ag85A-specific CD4^{+} T cell lines were evaluated in the Ag85A-dose dependent ^{3}H-TdR proliferation assay and EC$_{50}$ values were calculated from the sigmoidal curve using a nonlinear regression (Supplementary Fig. 1). Figures A and B illustrate EC$_{50}$ values (wk 8 vs wk 11) from 2 BCG-vaccinated control cows and 3 BCG-primed Ad5-85A-boosted cows, respectively. Figure C illustrates all the Ag85A-specific CD4^{+} T cell EC$_{50}$ values acquired from wk 8 (pre-boost) Ag85A-specific CD4^{+} T cell lines (from n = 9 animals) and Ad5-85A-boosted wk 11 (post-boost) Ag85A-specific CD4^{+} T cell lines (from n = 5 animals). BCG-vaccinated control: wk 8 = up-side-down triangles; wk 11 = triangles. BCG-primed Ad5-85A-boosted: pre-boost (wk 8) = circles; post-boost (wk 11) = squares. Horizontal lines represent the median. Statistical comparison in C was carried out using the Mann-Whitney test (ns = no significance: P = 0.3313).
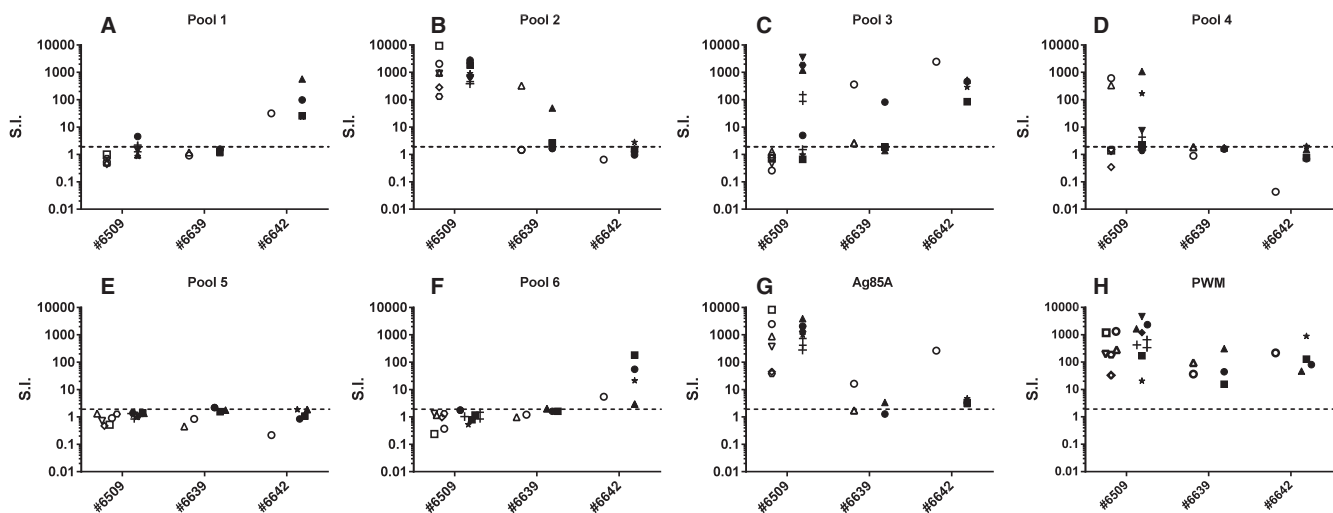

Fig. 3. Pre- and Post-boost epitope specificities within Ag85A-specific CD4^{+} T cell lines from three BCG-primed and Ad5-85A-boosted cows. Ag85A-specific CD4^{+} T lines were challenged with six pools of eleven overlapping Ag85A peptides in a ^{3}H-TdR proliferation assay. Stimulation indexes (S.I.) for each of 3 Ad5-85A-boosted animals (*X*-axis) were plotted in aligned dot-plots where open symbols represent pre-boost lines and closed symbols or crosses represent post-boost lines (left vs right, respectively, for each animal). The horizontal dotted black line represents the S.I. threshold (=2).

the same animal (Fig. 3C). No such widening of the epitope repertoire was observed when the peptide pool reactions were compared pre- and post-boost in the other two animals. These data indicate that boosting BCG-vaccinated cattle with Ad5-85A induces minimal epitope spreading.

4. Discussion

In this study, we sought to determine whether increased protection against *M. bovis* infection, induced by boosting BCG with Ad5-85A in cattle, was associated with changes in the: (i) frequency of CD4^{+} T cells recognising Ag85A; (ii) avidity for Ag85A; or (iii) changes in Ag85A epitope specificity. To this end, CD4^{+} T cell libraries were generated – from animals used in previously published vaccine efficacy experiments [19] – employing a method adapted from Geiger et al. [20]. Analysis of these libraries, showed that BCG vaccinated cattle boosted with Ad5-85A had increased

frequencies of Ag85A-specific CD4^{+} T cells and this correlated with enhanced protection against *M. bovis*-induced pathology. However, boosting with Ad5-85A did not enhance the avidity of the Ag85A-specific CD4^{+} T cell lines nor significantly alter their epitope specificity. Samples used to generate the post-boost libraries were from wk 11 – two weeks after the peak of the response following the boost [19] – when IFNγ whole blood responses had returned to background levels. This increased the probability of detecting memory responses rather than effector responses.

It has been shown that antigen (Ag) persistence – as well as dose – can lead to CD4^{+} T cell exhaustion (or terminal differentiation) [23–26]. However, as Lindenstrom et al. indicated, none of these studies examined differences in avidity or cytotoxic potential [25]. Results from the current study indicate that boosting BCG vaccinated cattle with Ad5-85A did not increase avidity; rather there may be a tendency to low avidity in two out of three post-boost libraries compared to the opposite trend in BCG-vaccinated control animals. A low Ag85A-specific avidity might be advanta-

geous during *M. bovis* infection because these cells may have enhanced functional persistence during recall, as demonstrated by Patke and Farber for influenza-specific CD4^{+} T cells [27]. Indeed, Caserta et al. found, in situations of Ag persistence (e.g. tumour-associated Ag), memory CD4^{+} T cells with low functional avidity are more effective immune cells than those with high functional avidity [28]. This may be due to: a delay in the expression of inhibitory receptors (e.g. TIM3) which have been linked with T cell exhaustion during bacterial recrudescence in chronic TB [29]; or a reduced risk of T cell receptor down-regulation which has been found to have a negative impact on the function (e.g. cytokine production, recall proliferation and mycobacterial inhibition) of T cells with high reactivity for ESAT6 in mice [30].

It is interesting to note one Ad5-85A boosted animal (#6639) with the lowest post-boost EC_{50} values (high avidity) had the highest total pathology score in the Ad5-85A boosted group. However, the frequencies of both pre- and post-boost Ag85A-specific CD4^{+} T cells in this animal (#6639) were at the lower end of the spectrum found in boosted cattle. This may suggest there was less efficient BCG priming in this animal which may have restricted the Ad5-85A boosting.

In this study, we found minimal evidence of epitope spreading – between the peptide pools – after boosting. In contrast, we found marked variations in epitope specificities between animals, as seen in the differences in Ag-specific reactivity to five out of the six Ag85A peptide pools. This is likely to be a consequence of variation in the Major Histocompatibility Complex (MHC) II alleles that presented antigen to CD4^{+} T cells. Indeed, molecular typing of animals used in this experiment for one of the MHCII loci, DRB3 [31], revealed diverse combinations of DRB3 alleles (data not shown) which will lead to differences in the epitopes presented by different animals. Diversity in MHC expression has been linked to variations in disease susceptibility [31] and it may have played a role in the outcome of this experiment, i.e. some animals in the boosted group were better protected than others.

Previous studies have reported different immunodominant CD4^{+} and CD8^{+} T cell epitopes in the highly conserved Ag85 complex (summarised by Huygen [32]). Interestingly, one of the three Ad5-85A boosted animals in the present study had Ag85A-specific CD4^{+} T cell responses to pool one (aa 1-55) (Fig. 3A) which includes a region reported as a signal peptide (aa 1-33 [33]); we are not aware of other studies that have reported epitopes in this region. We also found the number of Ag85A-specific CD4^{+} T cell responses to pool five (aa 221-285) was negligible (Fig. 3E) which is intriguing because at least one epitope with strong homology to this region has been frequently reported in mice, guinea-pigs and humans (reviewed by Huygen [32]). This may be an indication of species-specific differences in Ag85A epitopes.

There is evidence to suggest that strong T helper 1 (Th1) Ag-specific CD4^{+} T cell responses do not necessarily provide the best protection during pulmonary TB infection but may instead facilitate mycobacterial dissemination [34–39]. Indeed, Coscolla et al. found the majority of TB antigenic proteins are highly conserved (76% epitopes analysed were in conserved non-essential genes [34]) and hypothesise there may be a net evolutionary benefit from T cell recognition for transmission [34,36]. Thus, a more modulated immune response may be more effective at combating *M. bovis* infection as suggested previously by Woodworth et al. [36,38]. Therefore, it may be beneficial to select for CD4^{+} T cells with a low avidity for *M. bovis* Ag in future vaccine strategies.

In summary, although the number of animals used in this study is relatively small, the results from these experiments indicate that protection induced by boosting BCG with Ad5-85A is associated with increased frequencies of Ag85A-specific CD4^{+} T cells but not with increased avidity. This study also indicates that Ag-specific CD4^{+} T cell epitope spread does not necessarily correlate with

the level of protection conferred by the vaccination strategy. To our knowledge, this is the first study in cattle aiming to determine whether there is an association between vaccine-induced protection and Ag-specific CD4^{+} T cell avidity and repertoire.

Funding

Conflict of interest

None.

Author contribution

Hannah J. Metcalfe adapted the CD4^{+} T cell methods to the experiments, performed the experiments and wrote the paper; Sabine Steinbach optimised the bovine CD4^{+} T cell library/avidity methods and reviewed/edited the paper; Gareth J. Jones provided advice in the setting up of the system and reviewed/edited the paper; Tim Connelley was involved in design of the project and contributed to experimental planning; W. Ivan Morrison was involved in design of the project, contributed to experimental planning and reviewed/edited the paper; Bernardo Villarreal-Ramos and Martin Vordermeier were both involved in design of the project, contributed to experimental planning, provided advice in the setting up of the system and reviewed/edited the paper. All authors approved the final version of the submitted manuscript.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2016.07.055.

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