

## A quantitative assessment of primary and secondary immune responses in cattle using a B cell ELISPOT assay

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(Received 20 March 2008; accepted 29 September 2008)

**Abstract** – The aim of the study was to build a comprehensive picture of the appearance in the blood stream of Ag-specific plasma cells and memory B cells in the bovine model. For this purpose, we have developed a method allowing the detection and quantification of both cell types within individual calves immunised with ovalbumin. During the primary response, we detected a burst of ovalbumin-specific plasma cells at days 6 and 7 post-immunisation, which was followed by the production of specific Ab, whereas a gradual increase of memory B cells was only detected from day 15. As expected, a boost immunisation performed 7 weeks later induced a quicker and stronger secondary response. Indeed, a burst of plasma cells was detected in the blood at days 3 and 4, which was followed by a strong increase in Ab titres. Furthermore, a burst of memory B cells, and not a gradual increase, was detected at days 5 and 6 post-boost immunisation. Importantly, we showed a strong correlation between the anti-ovalbumin-specific IgG titres detected 5 months after secondary immunisation and the plasma cell numbers detected in the blood at the peak response after secondary immunisation. The detection and quantification of plasma cells following an immunisation/vaccination strategy could constitute a very effective means for predicting the magnitude and longevity of an Ab response.

**bovine immunology / memory B cells / plasma cells / humoral response / vaccines**

### 1. INTRODUCTION

The traditional method for assessing a B cell response generated after immunisation, vaccination or infection consists of quantifying specific Ab titres in the serum. Indeed, following a primary challenge with a T-dependent Ag, there is an initial phase when no specific Ab can be detected as it takes 1 to 2 weeks to observe a logarithmic rise of Ab titres, which then reach a plateau before declining again. In the primary response, the appearance of IgG is preceded by IgM. In comparison, the Ab titres following a secondary antigenic challenge appear more quickly, reach a higher level and can persist for very long periods of time. Moreover, the

secondary humoral response is characterised by the predominance of Abs of the IgG subclass with a greater affinity for the immunising Ag. This rapid and specific secondary response is dependent on the recruitment and activation of memory B cells (generated during the primary response), as these cells exhibit an intrinsic advantage over naïve B cells in both the time to initiate a response and in the division-based rate of effector cell development [17].

Even in the absence of re-immunisation, Ab levels in the serum can still be maintained for many years and even decades [12]. It is believed these Ab levels are maintained by the presence of long-lived plasma cells in the bone marrow that secrete Ab for long periods of time and/or the continuous differentiation of memory B cells into plasma cells in an Ag-independent manner [2, 11, 15]. Therefore, it is

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possible the enumeration of plasma cells and memory B cells following a vaccination, rather than only quantifying Ab titres, could provide a means to predict earlier and more efficiently the duration of immunity.

It is possible to detect plasma cells and memory B cells in the blood by the ELISPOT technique; the detection of the latter necessitates stimulating these quiescent memory B cells in order to induce their differentiation into Ab-secreting cells (ASC). Various methods have previously been described for inducing the differentiation of human memory B cells. These methods involve the culture of peripheral blood mononuclear cells (PBMC) or purified B cells for 5 to 6 days in the presence of polyclonal stimuli and cytokines, such as *Staphylococcus aureus* Cowan I (SAC) and interleukin (IL)-2 [7], pokeweed mitogen (PWM), unmethylated CpG oligodeoxynucleotides (CpG ODN) and SAC [5], CD40L-transfected CDw32L mouse fibroblasts plus IL-2 and IL-10 [20] or CpG and IL-15 [2].

The detection of plasma cells and memory B cells could also be useful for determining the efficacy of new vaccine formulations and schedules in cattle. However, most of the reagents used for stimulating human B cells are not suitable when using bovine B cells. Here, we describe a method allowing, in the bovine model, the detection and quantification in the blood of both Ag-specific plasma cells and memory B cells, using ovalbumin as a model T-dependent Ag. Using this model, we were able to sample sufficient amounts of blood at numerous time points that allowed us to build a comprehensive picture of the appearance of both these cell types within individual calves and to establish a correlation between the number of these cells and Ab titres detected following a boost immunisation.

## 2. MATERIALS AND METHODS

### 2.1. Calves and immunisation protocol

Calves (*Bos taurus*) were British Holstein Friesians conventionally reared at the Institute for Animal Health (Compton, UK). These animals were selected with ages ranging from 4 to 8 months

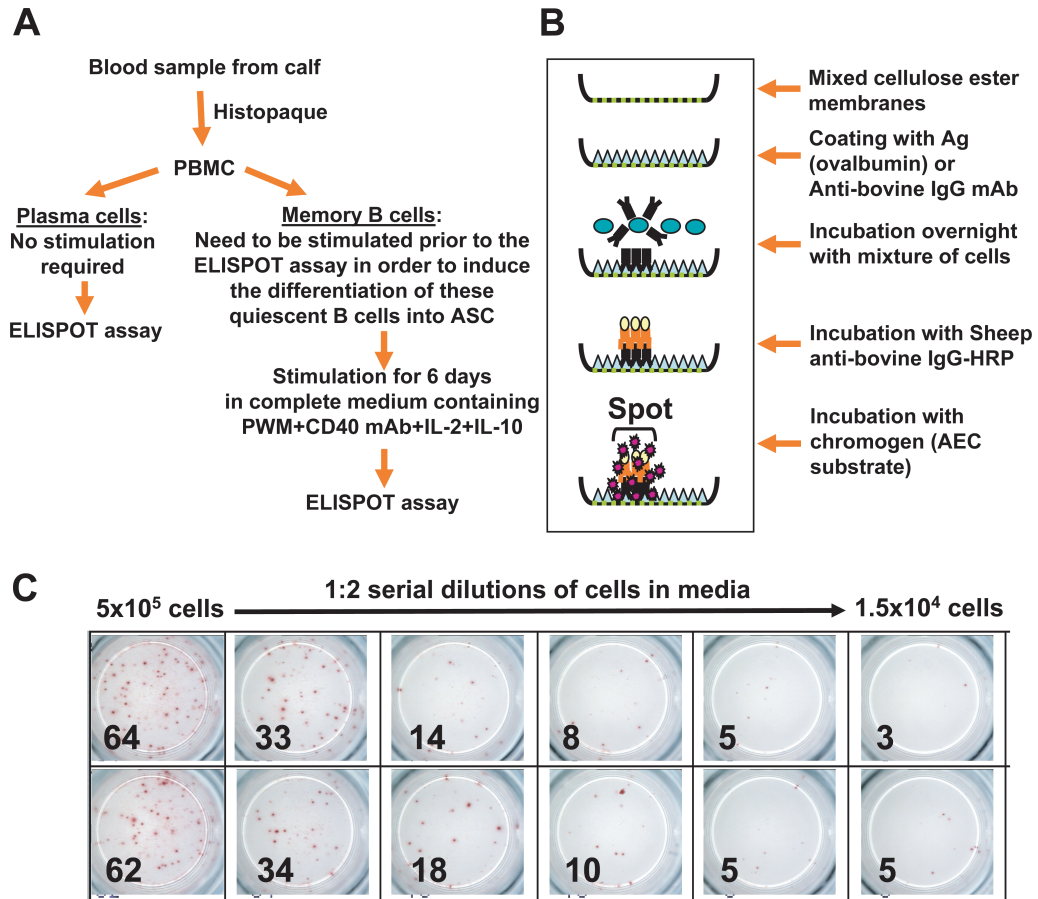
and weighing 100–160 kg. In the first (kinetic) experiment, 4 calves were sub-cutaneously injected with 40 mg ovalbumin (Sigma-Aldrich, Poole, UK) per animal in incomplete Freund's adjuvant (primary response), then boosted 49 days later with 10 mg ovalbumin per animal in incomplete Freund's adjuvant (secondary response). In the second experiment, 8 calves in total were sub-cutaneously injected with various doses of ovalbumin (40, 10, 1 or 0.1 mg per animal) in incomplete Freund's adjuvant, then boosted 51 days later with 10 mg ovalbumin per animal in incomplete Freund's adjuvant. Blood samples were taken at various time-points following these injections. All experiments were approved by the Institute's ethical review process and were in accordance with national guidelines on animal use.

### 2.2. Culture media

Iscove's Modified Dulbecco's Medium (IMDM) with L-Glutamine and 25 mM Hepes (Gibco, Paisley, UK) containing penicillin (100 units/mL), streptomycin (100 µg/mL), 1 × non-essential amino acids, 1 mM sodium pyruvate and 15% heat-inactivated horse serum (Sigma-Aldrich), subsequently termed complete medium.

### 2.3. Isolation and stimulation of bovine peripheral blood mononuclear cells (PBMC)

Ten milliliters of blood per calf was collected in heparinised tubes and bovine PBMC were obtained by density gradient centrifugation (1200 × g for 30 min over Histopaque<sup>®</sup> 1.083 g/mL, Sigma-Aldrich). Fresh PBMC were stored on ice until being used for the detection of spontaneous ASC and/or were stimulated as described below. Briefly, 20 × 10<sup>6</sup> PBMC were cultured for 6 days in 20 mL complete medium containing 5 µg/mL pokeweed mitogen (PWM, Sigma-Aldrich), 2 µg/mL anti-bovine CD40 mAb (ILA158, kindly donated by ILRI, Kenya), 20 ng/mL recombinant Human IL-2 (ImmunoKontakt, AMS Biotechnology, Abingdon, UK), 7.3 units/mL recombinant bovine IL-10 (kindly donated by G. Entrican, Moredun Research Institute, Penicuik, UK) or a combination of these. At the end of the culture period, cells were washed twice, resuspended in complete media and stored on ice until being used for the detection of induced ASC. Spontaneous and induced ASC were subsequently referred to as plasma cells and



**Figure 1.** Protocol and schematic representation of the B cell ELISPOT assay. (A) The B cell ELISPOT assay was performed with freshly isolated and 6 day-stimulated PBMC for the detection of plasma cells and memory B cells, respectively. (B) Schematic procedure of the detection of ASC by ELISPOT. (C) Ovalbumin-specific ASC enumeration: in this example, freshly isolated PBMC from a naïve calf immunised 7 days previously with ovalbumin were incubated overnight in pre-coated ELISPOT plates; the cells were seeded in duplicates (upper and lower wells) starting at  $5 \times 10^5$  cells per well and performing 1:2 serial dilutions down to  $1.5 \times 10^4$  cells per well; at the end of the assay, ASC were enumerated by counting the red-coloured immunospots (number indicated in bold in the lower left corner for each well) and results were expressed as ASC number per  $10^6$  PBMC (here,  $144 \pm 2$  ASC per  $10^6$  PBMC were detected).

memory B cells, respectively. A representation of this protocol is detailed in Figure 1A.

**2.4. Detection of anti-ovalbumin-specific IgG-secreting cells (plasma cells and memory B cells)**

A schematic representation of the detection procedure is shown in Figure 1B. Briefly, MultiScreen™-HA ELISPOT plates (Millipore,

Watford, UK) were coated for 2 h at 37 °C with 20 µg/mL ovalbumin in carbonate buffer, pH 9.6. Plates were washed 5 times with PBS and subsequently incubated for 2 h at 37 °C with 100 µL per well of blocking buffer (PBS containing 4% dried skimmed milk). Plates were then washed 5 times with PBS and stored at 4 °C until use.

Fresh or 6 day-stimulated PBMC were resuspended at  $5 \times 10^6$  cells/mL and 1:2 serial

dilutions of cells were performed in complete medium down to  $1.5 \times 10^5$  cells/mL. 100  $\mu$ L/well of each cell suspension (in duplicate) was added to coated ELISPOT plates and cultured overnight at 37 °C in a 5% CO<sub>2</sub> incubator. Cells were then washed off the plates using PBS containing 0.05% Tween20 and 100  $\mu$ L per well sheep anti-bovine IgG conjugated to horse radish peroxidase (1/1000, Serotec, Oxford, UK) was added to the plates for 3 h at room temperature. Plates were washed 5 times with PBS containing 0.05% Tween20 and 100  $\mu$ L per well 3-amino-9-ethylcarbazole (AEC) substrate (Merck Chemicals, Nottingham, UK) was added for 1 h at room temperature. Plates were rinsed with tap water and allowed to dry overnight at room temperature before counting the red-coloured immunospots using the AID ELISPOT reader (Fig. 1C). Results were expressed as ASC number per 10<sup>6</sup> PBMC for plasma cells and induced-ASC number per 10<sup>6</sup> cultured PBMC for memory B cells (mean of duplicates  $\pm$  SD).

## 2.5. Detection of total IgG-secreting cells (plasma cells and memory B cells)

The procedure was similar to that used for the detection of anti-ovalbumin-specific IgG secreting cells with minor modifications as described thereafter. For coating, ovalbumin was substituted with 4.5  $\mu$ g/mL mouse anti-bovine IgG mAb (clone BG-18, Sigma-Aldrich). Fresh and 6 day-stimulated PBMC were resuspended at  $5 \times 10^6$  and  $5 \times 10^5$  cells/mL, respectively, before performing 1:2 serial dilutions in complete medium and addition of 100  $\mu$ L/well of each cell suspension (in duplicate) in coated ELISPOT plates. Results were expressed as ASC number per 10<sup>6</sup> PBMC for plasma cells and induced-ASC number per 10<sup>6</sup> cultured PBMC for memory B cells (mean of duplicates  $\pm$  SD).

## 2.6. Flow cytometry

PBMC stimulated for 6 days were analyzed by indirect immunofluorescence using the following reagents: mouse anti-bovine CD4 (clone cc8), CD8 (clone cc63) or  $\gamma\delta$  T cells (clone cc15) IgG2a mAbs [13] (all were culture supernatants diluted at 1/10 from IAH, Compton, UK) followed by incubation with FITC-conjugated goat anti-mouse IgG2a Ab (1/200, Southern Biotechnology Associates, Birmingham, AL, USA). TRT6 was

used as an IgG2a isotype control mAb [4]. 5 000 viable cells per sample were analyzed using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA).

## 2.7. Cell staining by May Grünwald Giemsa and immunohistochemistry of cytopins

Cytopins (10<sup>5</sup> cells/slide) were prepared from 6 day-stimulated PBMC using a cytocentrifuge (Cytospin II, Shandon Scientific, Cheshire, UK) according to the manufacturer's instructions. Some cytopins were fixed in ethanol for 5 min followed by May-Grünwald-Giemsa staining. Alternatively, acetone-fixed cytopins were stained by immunohistochemistry using mouse anti-bovine light chain mAb (ILA58, culture supernatant diluted at 1/10 from IAH, Compton, UK) [21] and DakoCytomation EnVision+<sup>®</sup> System (DakoCytomation, Carpinteria, CA, USA) followed by May-Grünwald-Giemsa staining. The numbers of plasma cells expressing intracellular Ig and total cells were counted in five random  $\times$  40 microscopic fields (number of total cells per field ranging between 114 and 204) and results were expressed as the percentage of plasma cells  $\pm$  SD.

## 2.8. Detection of anti-ovalbumin Abs in serum by ELISA

Anti-ovalbumin IgG concentrations in sera were determined using a specific ELISA, as described below. 96 well plates were coated for 1 h at room temperature with 100  $\mu$ L per well ovalbumin (20  $\mu$ g/mL in carbonate buffer, pH 9.6). Plates were washed 5 times with PBS containing 1% Tween 20 (Sigma-Aldrich) and 100  $\mu$ L per well of PBS containing 1% Tween 20 and 4% skimmed milk (blocking buffer) was added for 1 h at room temperature in order to block any non-specific binding. After 5 washes, 100  $\mu$ L per well blocking buffer or 1:3 serial dilutions of sera were added and incubated for 1 h at room temperature. Plates were subsequently washed and 100  $\mu$ L of sheep anti-bovine IgG coupled to horse radish peroxidase (Serotec) diluted at 1/100 000 was added for 1 h at room temperature. After washing, 100  $\mu$ L 3,3',5,5-tetra-methyl benzidine (TMB) substrate (ICN biomedical, Irvine, CA, USA) was added and the reaction was stopped after 10 min by the addition of 50  $\mu$ L of 1.2 M H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) values at both 450 and 690 nm were

read for each well on a SpectraMAX 250 plate reader (Molecular devices, Sunnyvale, CA, USA) at dual wavelength. Anti-ovalbumin IgG titres (mean of duplicates  $\pm$  SD) were calculated as follows: the  $\log_{10}$  OD against  $\log_{10}$  sample dilution was plotted and a regression analysis of the linear part of this curve allowed calculation of the endpoint titre with an OD of twice the background.

### 2.9. Statistical analysis

The Pearson's correlation coefficient ( $r$ ) was derived from experimental data in Excel spreadsheets to examine the association between the anti-ovalbumin IgG titres and the plasma cell or memory B cell numbers detected at the selected time points.

## 3. RESULTS

### 3.1. Polyclonal stimulation of memory B cells in PBMC is necessary for their subsequent detection by the ELISPOT assay

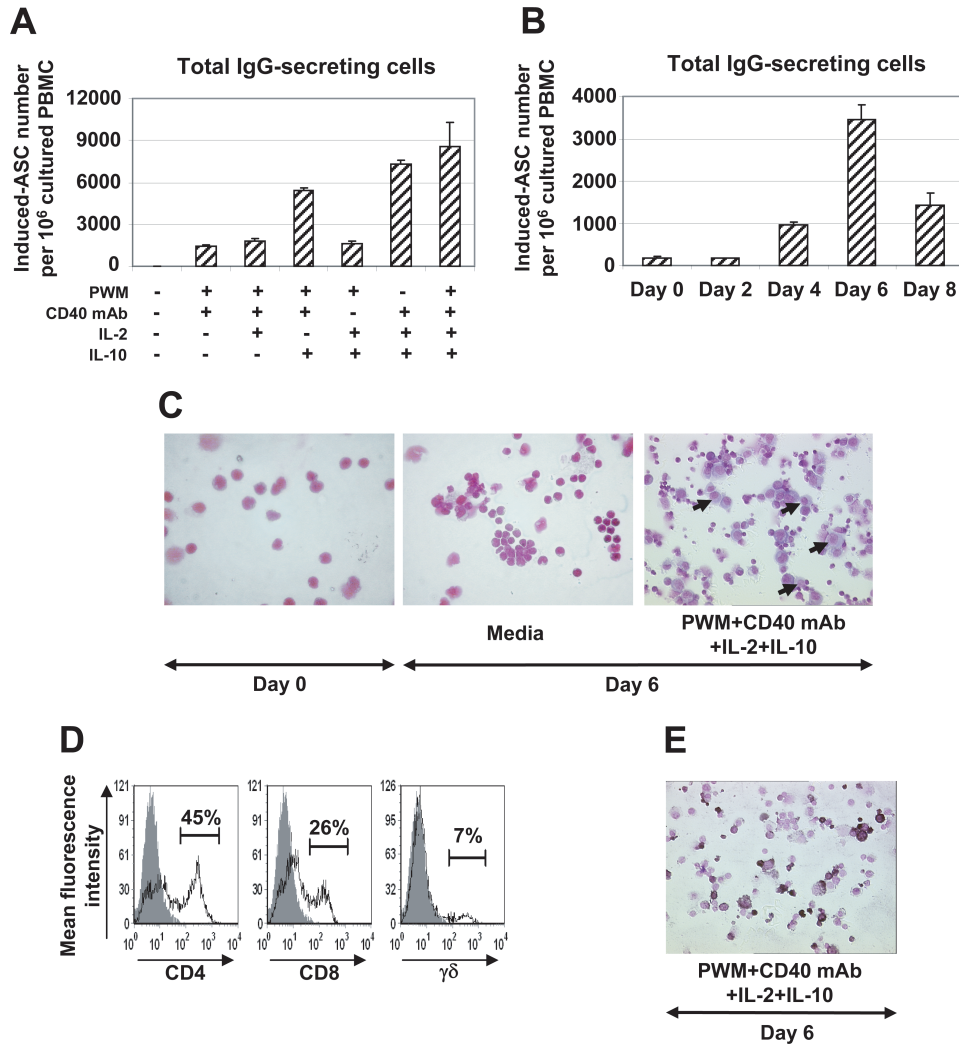
The B cell ELISPOT assay can only be used to detect and quantify cells actively secreting Abs. As plasma cells spontaneously secrete Ab, these cells can be directly detected following their culture overnight in pre-coated ELISPOT plates. In contrast to plasma cells, memory B cells are quiescent cells and it is therefore necessary to induce their differentiation into ASC prior to the ELISPOT assay. To determine the most potent differentiation stimuli, we incubated bovine PBMC with various combinations of stimuli for 6 days. The B cell ELISPOT assay was then used to determine the number of total IgG-secreting cells generated from  $10^6$  cultured PBMC in each culture condition. As expected, PBMC cultured for 6 days in media alone did not generate any ASC. Amongst the combinations of stimuli tested, PWM + CD40 mAb + IL-2 + IL-10 was the most potent stimulus to induce the differentiation of bovine PBMC, generating  $8\,556 \pm 1\,725$  total IgG-secreting cells per  $10^6$  cultured PBMC (Fig. 2A). Using this stimulus, we activated bovine PBMC for various periods of time before performing the ELISPOT assay and showed the optimal

duration of PBMC activation required for inducing their differentiation into ASC was 6 days (Fig. 2B). This was consistent with the findings of Crotty et al. [5]. As shown in Figure 2C, the stimulation of PBMC with PWM + CD40 mAb + IL-2 + IL-10 for 6 days resulted in a large proportion of blasts, which were not present when PBMC were cultured in media alone. Using FACS analysis, we showed these blasts were mostly T cells ( $38 \pm 6\%$  CD4<sup>+</sup>,  $16 \pm 8\%$  CD8<sup>+</sup> and  $21 \pm 11\%$   $\gamma\delta^+$  cells,  $n = 3$ , Fig. 2D). Parallel analysis of cytopspin preparations showed  $23 \pm 3\%$  of these stimulated cells were plasma cells, expressing intracellular Ig, as shown by their positive staining with an anti-bovine light chain mAb (Fig. 2E). This polyclonal stimulation, i.e. activation of PBMC for 6 days with PWM + CD40 mAb + IL-2 + IL-10, was subsequently used in all experiments aiming at the detection of Ag-specific memory B cells in the blood of immunised calves.

### 3.2. Ab titre, plasma cell and memory B cell kinetics during the primary and secondary immune responses

Using the B cell ELISPOT assay, we sought to determine the kinetics of ovalbumin-specific plasma cells and memory cells appearance in the blood during the primary and secondary immune responses and relate these to the kinetics of Ab titres. For this purpose, four calves were immunised with 40 mg ovalbumin, boost-immunised at day 49 with 10 mg ovalbumin and blood samples were collected at various time points for the quantification of ovalbumin-specific Ab titres, plasma cells and memory B cells.

The quantification of Ab titres constitutes the traditional method for assessing a B cell response generated after an immunisation, vaccination or infection and the kinetics of Ab production has been described in numerous reports. In agreement with these reports, we showed that, during the primary response, ovalbumin-specific IgG titres started to increase between days 6 and 15 post-immunisation and then reached a plateau (values ranging from 26 021 to 91 187 on



**Figure 2.** Phenotype of PBMC stimulated for 6 days with PWM, CD40 mAb, IL-2 and IL-10. **(A)**  $20 \times 10^6$  bovine PBMC were cultured for 6 days in 20 mL complete medium alone (media) or in the presence of various combinations of PWM, CD40 mAb, IL-2 and IL-10. The number of total IgG-secreting cells generated from  $10^6$  cultured PBMC (mean of duplicate determinations  $\pm$  SD) was determined for each culture condition. Representative data from 2 independent experiments are shown. **(B)**  $20 \times 10^6$  PBMC were cultured for various periods of time with PWM, CD40 mAb, IL-2 and IL-10. The number of total IgG-secreting cells generated from  $10^6$  cultured PBMC (mean of duplicate determinations  $\pm$  SD) was determined for each time point. Representative data from 2 independent experiments are shown. **(C)** Cytospin slides from fresh (day 0) and cultured (day 6) PBMC were stained with May-Grunwald-Giemsa (arrows indicate cells with plasma cell morphology). **(D-E)** PBMC were stimulated for 6 days with PWM, CD40 mAb, IL-2 and IL-10. At the end of the culture period, the phenotype of the cells in the resulting population was analysed: flow cytometry was used to determine the percentage of CD4<sup>+</sup>, CD8<sup>+</sup> and  $\gamma\delta$ <sup>+</sup> T cells **(D)** and immunohistochemistry to detect intracellular Ig (in cytopsin slides) using an anti-bovine light chain mAb and counter-staining with May-Grunwald-Giemsa **(E)**. Representative data from 3 independent experiments are shown. Original magnification:  $\times 40$ . ASC: Ab-secreting cells.

day 49 post-immunisation,  $n = 4$ ). During the secondary response, the Ab titres increased from an earlier time point (between days 3 to 4) and reached much higher values (ranging from 626 241 to 1 748 100 on day 9 post-boost immunisation,  $n = 4$ , Fig. 3A).

The kinetics of plasma cells detected in the blood was very different from the kinetics of Ab titres (Fig. 3B). Indeed, a sharp peak of plasma cells was detected at days 6 and 7 (range: 27 to 220 ovalbumin-specific ASC per  $10^6$  PBMC,  $n = 4$ ) during the primary response instead of the gradual increase previously observed for Ab titres. Similarly, we also detected a sharp peak of plasma cells during the secondary response but, consistent with the recognised feature of a secondary response, this peak occurred earlier (at days 3 and 4) and to a higher magnitude (range: 546 to 1 781 ovalbumin-specific ASC per  $10^6$  PBMC,  $n = 4$ ).

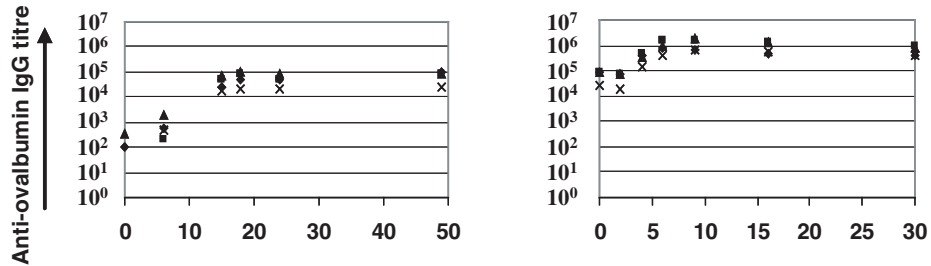
The kinetics of memory B cells detected in the blood (following the *in vitro* stimulation of PBMC for 6 days prior to performing the B cell ELISPOT assay) are shown in Figure 3C. During the primary response, the number of memory B cells started to gradually increase between days 15 and 20 and reached values ranging from 407 to 554 ovalbumin-specific ASC per  $10^6$  PBMC,  $n = 4$ , at day 49 post-immunisation (corresponding to day 0 of the boost immunisation with 10 mg ovalbumin). Following the boost immunisation, we initially observed a reduction in the memory B cell number detected in the blood (ranging from 16 to 58 ovalbumin-specific ASC per  $10^6$  PBMC at days 1 and 2 post-boost immunisation,  $n = 4$ ). This reduction probably reflected the recruitment of memory B cells from the blood to the lymphoid organs. Subsequently, the number of memory B cells peaked at days 5 and 6 post-boost immunisation. Interestingly, the increase in memory B cells detected during the secondary response started earlier and was stronger (ranging from 2 096 to 17 920 ovalbumin-specific ASC per  $10^6$  PBMC at days 5 and 6 post-boost immunisation,  $n = 4$ ) than during the primary response.

### 3.3. The Ab titres correlate with the plasma cell numbers detected at the peak of the secondary immune response

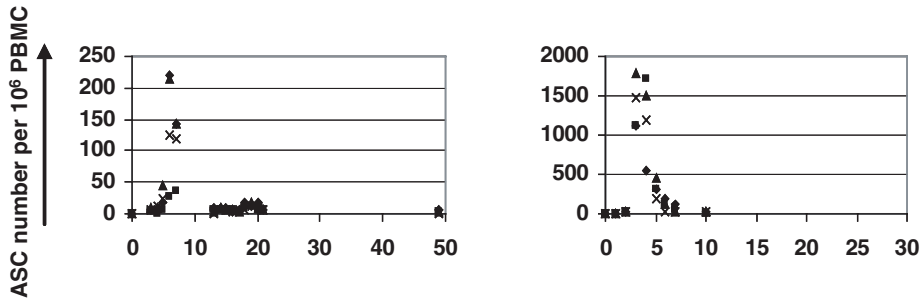
In order to try and establish a correlation between the Ab titres and the peak number of plasma cells or memory B cells generated, we designed a new immunisation protocol where calves were inoculated with various doses of ovalbumin. This time, we used 8 calves in total: 2 were immunised with 40 mg, 2 with 10 mg, 2 with 1 mg and 2 with 0.1 mg ovalbumin per animal and, at day 51 post-immunisation, each of these calves was boosted with 10 mg ovalbumin. Blood samples were then collected at the optimal time-points determined during the previous kinetic experiment and ovalbumin-specific Ab titres, plasma cells and memory cells were quantified.

During the primary response, despite reasonable ovalbumin-specific IgG titres, the memory B cells (assessed at days 21 and 51 post-immunisation) and plasma cells (assessed at days 6 and 7 post-immunisation) were detectable but their number too low to establish a correlation between B cell frequency and Ab response (Tab. I). Following the boost immunisation, the number of memory and plasma cells detected in the blood reached much higher values at the optimal time-points previously determined for each of these parameters: days 3 and 4 post-boost immunisation for plasma cells and days 5 and 6 post-boost immunisation for memory B cells (Tab. I). Our results showed there was no clear correlation between the doses of ovalbumin used in the primary immunisation regime and the Ab titres or memory B cell or plasma cell numbers detected at the peak response during the secondary immune response. Similarly, no correlation was found between the anti-ovalbumin IgG titres assessed 17 days post-boost immunisation and the memory B cell numbers detected at the peak response (6 days post-boost immunisation) during the secondary immune response ( $r$  value =  $-0.05$ ,  $n = 8$ , Fig. 4A). However, we found

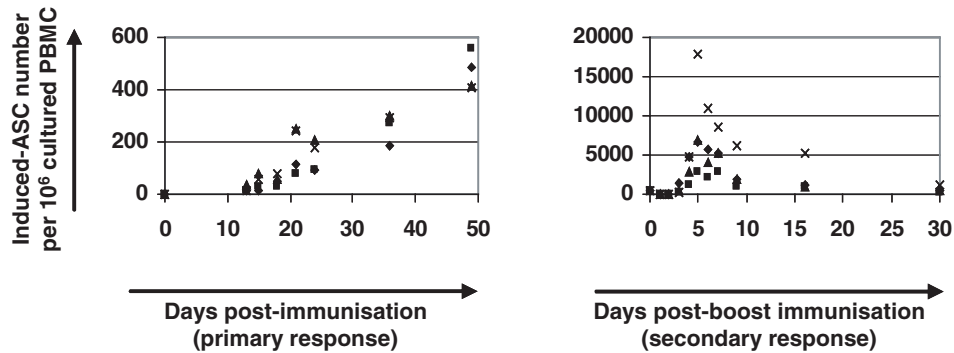
### A: Ab titres



### B: Plasma cells



### C: Memory B cells



**Figure 3.** Kinetics of Ab titres, plasma cell and memory B cell numbers after immunisation and boost-immunisation of calves with ovalbumin. Four calves were sub-cutaneously injected with 40 mg ovalbumin per animal in incomplete Freund's adjuvant (primary response, left panels), then boosted 49 days later with 10 mg ovalbumin per animal in incomplete Freund's adjuvant (secondary response, right panels). Blood samples were taken at various time-points following these injections and ovalbumin-specific IgG titres (Ab titre, **A**), plasma cell (**B**) and memory B cell (**C**) numbers were determined by ELISA and B cell ELISPOT. Results were expressed as ASC per  $10^6$  PBMC for plasma cells and induced-ASC per  $10^6$  cultured PBMC for memory B cells (mean of duplicate determinations).



**Table I.** Ab titres, plasma cell and memory B cell numbers following various immunisation regimes with ovalbumin.

Calf number	Immunisation regime Primary/Secondary <sup>a</sup>	Primary response		
		Ab titre (log10) (day 21 pi <sup>b</sup> )	Peak plasma cell number (day 7 pi <sup>b</sup> )	Memory B cell number before boost (day 51 pi <sup>b</sup> )
# 1	40 mg/10 mg	4.46 ± 0.04	138 ± 15	44 ± 7
# 2	40 mg/10 mg	4.62 ± 0.2	146 ± 30	221 ± 32
# 3	10 mg/10 mg	4.65 ± 0.01	40 ± 7	26 ± 6
# 4	10 mg/10 mg	4.62 ± 0.11	69 ± 22	6 ± 0
# 5	1 mg/10 mg	4.1 ± 0.11	15 ± 0	17 ± 4
# 6	1 mg/10 mg	3.91 ± 0.08	53 ± 10	72 ± 32
# 7	0.1 mg/10 mg	4.49 ± 0.01	16 ± 6	124 ± 1
# 8	0.1 mg/10 mg	4.4 ± 0.07	13 ± 1	6 ± 0

Calf number	Immunisation regime Primary/Secondary <sup>a</sup>	Secondary response			
		Ab titre (log10) (day 17 pb <sup>c</sup> )	Ab titre (log10) (5 months pb <sup>c</sup> )	Peak plasma cell number (day 4 pb <sup>c</sup> )	Peak memory B cell number (day 6 pb <sup>c</sup> )
# 1	40 mg/10 mg	5.28 ± 0.04	4.11 ± 0.01	70 ± 19	2 021 ± 0
# 2	40 mg/10 mg	5.86 ± 0.02	4.76 ± 0.03	581 ± 44	2 222 ± 226
# 3	10 mg/10 mg	5.96 ± 0.01	4.86 ± 0.03	728 ± 79	382 ± 25
# 4	10 mg/10 mg	5.19 ± 0.01	4.1 ± 0	187 ± 7	142 ± 14
# 5	1 mg/10 mg	5.44 ± 0.05	4.09 ± 0.01	229 ± 3	352 ± 28
# 6	1 mg/10 mg	5.87 ± 0.04	4.36 ± 0.01	618 ± 33	1 553 ± 21
# 7	0.1 mg/10 mg	6.13 ± 0.02	4.81 ± 0.01	1 601 ± 226	485 ± 23
# 8	0.1 mg/10 mg	6.25 ± 0.04	5.02 ± 0	2 879 ± 433	166 ± 11

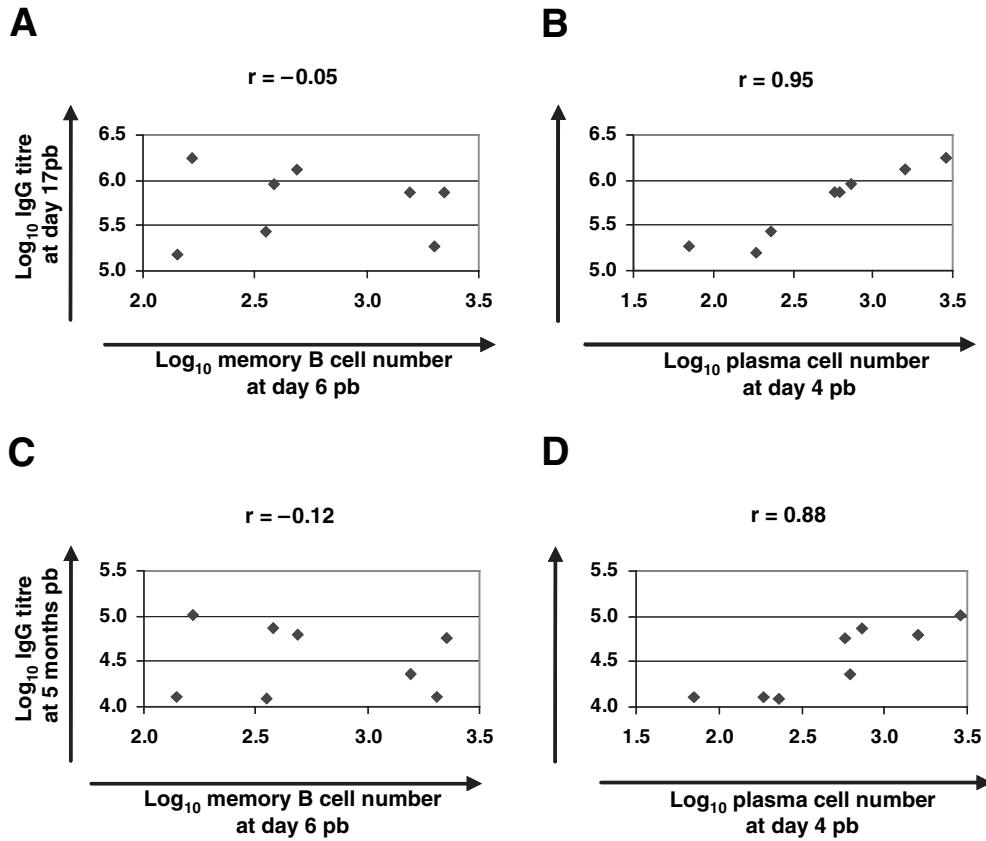
<sup>a</sup>Eight calves were sub-cutaneously injected with 40, 10, 1 or 0.1 mg ovalbumin per animal in incomplete Freund's adjuvant, and then boosted 51 days later with 10 mg ovalbumin per animal in incomplete Freund's adjuvant. Blood samples were taken at various time-points corresponding to the peak response for ovalbumin-specific plasma cells (day 7 pi and day 4 pb) and memory B cells (day 51 pi and day 6 pb) as well as at day 21 pi, day 17 pb and 5 months pb for ovalbumin-specific IgG titres (Ab titre). Plasma cell and memory B cell numbers were expressed as ASC number per 10<sup>6</sup> PBMC and induced-ASC number per 10<sup>6</sup> cultured PBMC, respectively (mean of duplicates ± SD).

<sup>b</sup>pi: post-immunisation;

<sup>c</sup>pb: post-boost-immunisation.

a very strong correlation between the anti-ovalbumin IgG titres and plasma cell numbers detected in the blood at the peak response (4 days post-boost immunisation) during the secondary response ( $r$  value = 0.95,  $n$  = 8, Fig. 4B). Interestingly, although the anti-ovalbumin IgG titres determined at a later time point, five months post-boost immunisation,

did not correlate with the memory B cell numbers detected in the blood at day 6 post-boost immunisation ( $r$  value = -0.12,  $n$  = 8, Fig. 4C), the five months post-boost Ab titres did strongly correlate with the plasma cell numbers detected in the blood at day 4 post-boost immunisation ( $r$  value = 0.88,  $n$  = 8, Fig. 4D).



**Figure 4.** Ovalbumin-specific plasma cell numbers detected at the peak of the secondary response strongly correlates with ovalbumin-specific IgG titres detected up to 5 months post-boost immunisation. Pearson's correlation coefficient ( $r$ ) was determined to examine the association between the  $\text{Log}_{10}$  anti-ovalbumin IgG titres and the  $\text{Log}_{10}$  memory B cell (A) or plasma cell (B) numbers detected at the peak response following the boost-injection of ovalbumin (day 17 pb, day 6 pb and day 4 pb, respectively). A similar analysis was performed to examine the association between the  $\text{Log}_{10}$  anti-ovalbumin IgG titres detected 5 months later and the  $\text{Log}_{10}$  memory B cell (C) or plasma cell (D) numbers detected at the peak response (day 6 pb and day 4 pb, respectively); pb: post-boost.

#### 4. DISCUSSION

Here, we describe a B cell ELISPOT assay allowing the detection and quantification of circulating bovine plasma cells and memory B cells generated, subsequent to an immunisation and a boost immunisation with ovalbumin. This assay enables us to detect and quantify bovine plasma cells circulating in the blood without any prior stimulation. Indeed, these cells secrete large amounts of Ab when cultured overnight in vitro, which constitutes the

first step of the B cell ELISPOT assay. In contrast, it is necessary to induce the differentiation of the quiescent memory B cells into ASC prior to the ELISPOT assay. Human memory B cells can be terminally differentiated using a combination of SAC, PWM and/or CpG ODN in the presence of recombinant human cytokines such as IL-2, IL-10 and/or IL-15 [2, 5, 7, 20]. Here, we showed the combination of PWM, anti-bovine CD40 mAb, recombinant human IL-2 and recombinant bovine IL-10 is a potent stimulus for the terminal

differentiation of bovine memory B cells into Ab-secreting cells. Indeed, it is likely the cells detected after the polyclonal stimulation for 6 days were derived from memory B cells for the following reasons. Firstly, these cells cannot be derived from naïve B cells as we showed no ovalbumin-specific ASC were detected following the 6-day stimulation of PBMC isolated on day 0 during the primary response in naïve calves. If naïve B cells specific for ovalbumin can be differentiated into ASC following the polyclonal stimulation for 6 days, it is possible that their initial scarcity results in a very low, undetectable number of ASC in our ELISPOT assay. Alternatively, naïve B cells may not be activated and/or terminally differentiated in our cultures as these conditions do not provide any triggering of the B cell receptor. This hypothesis is in agreement with a previous report published by Bernasconi et al. who showed the stimulation with anti-Ig Ab was absolutely required to induce the proliferation and terminal differentiation of purified naïve B cells in the presence of bystander T cell help [2]. Secondly, the ASC detected after the 6-day polyclonal stimulation cannot represent plasma cells that were present in the initial blood sample. Indeed, it is known plasma cells die quickly if appropriate signals, such as IL-5, IL-6, stromal-cell-derived factor-1 $\alpha$ , tumor necrosis factor (TNF) $\alpha$  and ligands for CD44 are not provided [3]. Accordingly, during the secondary response, we detected only low numbers of ovalbumin-specific memory B cells in the blood at day 3 post-boost immunisation whereas large numbers of ovalbumin-specific plasma cells were already detected at this time-point ( $764 \pm 708$  and  $1376 \pm 319$  ASC per  $10^6$  PBMC for memory B cells and plasma cells, respectively,  $n = 4$ ). It is however important to mention that the number of induced ASC per  $10^6$  cultured PBMC (referred to as “memory B cells” in the present manuscript) detected by the B cell ELISPOT assay is not equal to, but rather indirectly reflects, the number of memory B cells initially present in the blood. Indeed, previous reports have shown that B cells can divide up to 8 times following their culture for several days in the presence of

various polyclonal stimuli, therefore amplifying the final number of ASC detected [2,6,17].

Upon the encounter of a naïve B cell with its specific Ag and T cells, a primary humoral response is initiated within secondary lymphoid organs. Some naïve B cells proliferate and differentiate into plasma cells secreting low affinity Abs in extrafollicular areas, such as the medullary cords in lymph nodes (LN) or the red pulp in the spleen. These foci of ASC develop during the first week of the response and subsequently involute during the second week [16]. In parallel, a proportion of the Ag-activated B cells migrate to the B cell areas of secondary lymphoid organs, i.e. primary follicles, where the process of affinity maturation takes place [10]. This process involves the generation of secondary follicles from primary follicles, which can be observed from day 4 to day 21 post-immunisation in the absence of antigenic restimulation, and ultimately leads to (i) the improved affinity of serum antibodies during the course of a humoral response and (ii) the generation of plasma cells and memory B cells specific for the immunizing T-dependent Ag [1, 8, 9, 18, 19]. Some plasma cells will exit the lymphoid organ and migrate via the blood towards specific niches, such as the bone marrow, where they can reside as long-lived plasma cells and secrete Abs for long periods of time. On the other hand, memory B cells do not spontaneously secrete Abs but constantly recirculate between the blood and tissues in a quiescent state.

Using the B cell ELISPOT assay, we detected in the blood a burst of ovalbumin-specific plasma cells at days 6 and 7 post-immunisation during the primary response, which was followed by the production of specific Ab. The generation of memory B cells was only detected at a later time-point (starting between days 15 and 21) and gradually increased. Interestingly, the timing of the appearance of the memory B cells in the blood is consistent with the recognised timing required for a secondary follicle to form and be fully mature within the draining lymphoid organs [9].

In accordance with numerous reports, we showed the boost immunisation induced a

quicker and stronger secondary response. Indeed, a burst of plasma cells was detected in the blood at days 3 and 4, which was followed by a strong increase in Ab titres. Moreover, a burst of memory B cells, and not a gradual increase, was detected at days 5 and 6 post-boost immunisation.

Therefore, using the B cell ELISPOT assay, we were able to determine the kinetics of the appearance in the blood of bovine plasma cells and memory B cells generated after an immunisation with ovalbumin. We then showed a strong correlation between the anti-ovalbumin-specific IgG titres and plasma cell numbers detected in the blood at the peak response after secondary immunisation. Interestingly, a weak correlation (Pearson correlation coefficient,  $r = 0.58$ ) has previously been observed in humans between the increase in Ab level and the peak plasma cell response following an immunisation with a polysaccharide conjugate vaccine [7]. It is not clear why a much stronger correlation between those parameters was obtained in our model but it is possible that this reflects the immunisation regime chosen (using a strong T-dependent Ag, such as ovalbumin). Nevertheless, our results suggest it is possible to predict the intensity of the Ab response that will be generated following an immunisation by measuring the number of plasma cells detected in the blood at early time points (day 3 and 4 following a boost immunisation). Moreover, Rohner et al. also showed a correlation between the level of memory B cells detected in the blood following the primary immunisation of children with a protein-polysaccharide conjugate vaccine and the maintenance of protective Ab levels for several months afterwards [14]. Similarly, another report showed a strong correlation in patients between serum Ab levels and frequency of memory B cells under steady state conditions several years after antigenic stimulation [2]. Therefore, based on our own results and those of others, it appears the detection and quantification of plasma cells and/or memory B cells following an immunisation/vaccination strategy could constitute a very effective means to predict, at an early

time point, the magnitude and maintenance of the Ab response that will be generated afterwards.

*Acknowledgements.* The authors would like to thank the International Livestock Research Institute (ILRI, Kenya) for providing the ILA158 mAb and Dr Gary Entrican (Moredun Research Institute, UK) for the recombinant bovine IL-10. We also thank Brenda Jones and Gillian Hill for producing the hybridoma supernatants. Financial support for this research was given by the Biotechnology and Biological Sciences Research Council (BBSRC, grant number BBS/B/00549) and the Institute for Animal Health.

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