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Research paper

Natural and inducible regulatory B cells are widely distributed in ovine lymphoid tissues

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ABSTRACT

Regulatory B cells that produce IL-10 are now recognized as an important component of the immune system. We previously confirmed that IL-10 secreting $CD21^+$ regulatory B cells (B_{reg} cells) were present in ovine jejunal Peyer's patches (JPP) and this IL-10 production suppressed IL-12 and IFN-γ secretion. It is not known, however, whether ovine B_{reg} cells are restricted to JPP or are present in other lymphoid tissues. Therefore, $CD21^+$ B cells were purified from sheep JPP and from a variety of mucosal and systemic lymphoid tissues using magnetic cell sorting. Purified $CD21^+$ B cells were stimulated with a TLR9-agonist, CpG oligodeoxynucleotide (CpG ODN), and the frequency of spontaneous and inducible (i) IL-10-secreting B cells was evaluated by ELISPOT. Spontaneous IL-10 secreting $CD21^+$ B cells were present in mucosal (jejunal PP, parabronchial lymph nodes (LN), mesesnteric LN, and palatine tonsils) and systemic (spleen and blood) lymphoid tissues. Mucosal lymphoid tissues (parabronchial and mesenteric LNs and JPP) had the highest frequency of cells spontaneously secreting IL-10 while tonsils had the lowest. The frequency of B cells spontaneously secreting IL-10 was lowest in blood and spleen. There was large inter-animal variation in the frequency of $CD21^+$ B cells spontaneously secreting IL-10 and no significant difference was detected following CpG ODN stimulation. When comparing within individual animals there was, however, a consistent increase in the frequency of $CD21^+$ cells secreting IL-10 following CpG ODN stimulation *versus* stimulation with GpC control ODN. The presence of inducible (i)B_{reg} cells in ovine mucosal tissues supports previous evidence from mice indicating that B cells have the capacity to modulate inflammatory responses. The presence of iB_{reg} cells in ruminants may also provide a novel therapeutic target for both immunomodulatory drugs and vaccines designed to control antigen-specific mucosal inflammation.

1. Introduction

The immune system can be divided into the peripheral immune system, composed of the bone marrow, thymus, spleen and the lymph nodes, and the mucosal immune system, consisting of organized and diffuse mucosal associated lymphoid tissues (Beverley et al., 2014). Mucosal surfaces form the interface between the body and the external environment and play a central role in immune surveillance and protection against infection. The surface areas that comprise the mucosa are defined by the presence of a semipermeable epithelial barrier that is maintained by a variety of innate and adaptive immune mechanisms. Therefore, a protective immune activity must coexist with efficient regulatory mechanisms to maintain the health and function of mucosal tissues.

The hallmark of an innate immune response is inflammation. Early during infection, the inflammatory response is critical for containing and clearing pathogens and activating proteins that control wound healing (Medzhitov, 2008). If unresolved, this inflammatory response causes injury to host tissues, which can lead to the development of a wide variety of immune-mediated pathologies (Medzhitov, 2008). In the healthy individual, inflammation is self-limiting, and resolution is controlled by the release of anti-inflammatory mediators and cytokines, such as interleukin-10 (IL-10), produced by cells that have been termed "suppressive" or "regulatory" (Nathan and Ding, 2010). The idea that B cells could regulate immune responses originated in 1974, when the ability of B cells to suppress delayed-type hypersensitivity responses in guinea pigs was first described (Katz et al., 1974; Neta and Salvin, 1974). Almost two decades later,(Wolf, 1996) and colleagues were the first to provide direct evidence for a B cell regulatory role in a model of experimentally induced autoimmune encephalomyelitis (EAE) in mice

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deficient in B cells (μMT) (Alsalami and Filippich, 1999; Wolf et al., 1996). Mizoguchi and colleagues later coined the term regulatory B cells (B_{reg} cells) to designate B cells with regulatory properties independent of secreted immunoglobulins (Mizoguchi et al., 2002) (Mizoguchi and Bhan, 2006). Regulatory B cells, which are specifically induced under inflammatory conditions (iB_{reg} cells) and which are capable of suppressing inflammation, enhancing recovery, or inducing tolerance have also been implicated in the development of nasal tolerance to aeroallergens. B_{reg} cells exert their regulatory activities through IL-10 production and regulatory B cells have thus become a focus of increased investigations in recent years. These studies were conducted in mice and it is not known if B_{reg} cells are present in other species and play a similar role in regulating inflammatory responses.

Ovine $sIgM^+$ $CD21^+$ B cells are representative of naive B cells, are localized in the B cell follicles of spleen, lymph nodes (LNs) and Peyer's patches (PPs), and re-circulate in blood (Gupta et al., 1998; Young et al., 1999). We recently reported that ovine $CD21^+$ B cells isolated from jejunal PPs spontaneously secreted IL-10, had a regulatory function, and these B_{reg} cells were present in the fetus prior to antigen exposure (Booth et al., 2009; Jimbo et al., 2014). However, it is not known whether ovine B_{reg} cells are present in other lymphoid tissues and whether they continue to function following a cognate interaction with T cells that induce isotype switching. It is also not known whether the TLR9 agonist, CpG ODN, can induce B cells to produce IL-10. Therefore we investigated the distribution of B_{reg} cells in ovine mucosal and systemic lymphoid tissues and used an IL-10 ELISPOT assay to enumerate the frequency of IL-10 secreting B cells following CpG ODN stimulation.

2. Materials and methods

2.1. Animals

Suffolk sheep of either sex, between 3–4 months old were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). Experiments were conducted in accordance with the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. All experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

2.2. CpG oligodeoxynucleotides

Oligodeoxynucleotides of different classes have been shown to be biologically active in sheep both *in vitro* and *in vivo*. We used B-class CpG ODN 2007 to stimulate cells in our experiments. The CpG ODN 2007 and CpG ODN 2007 GC were obtained from Merial limited (Lyon, France). They have the following sequences and backbone structures; 2007 B tcgtcgttgtcgttttgtcgtt and 2007GC B tgctgcttgtgcttttgtgctt. The ODN doses were previously optimized and used at a dose of 5 μg/mL (Booth et al., 2009, 2007).

2.3. Isolation of leukocytes from jejunal Peyer's patches (JPP), parabronchial lymph nodes (BLN), mesenteric lymph nodes (MLN), palatine tonsils, spleen and blood

Blood was collected in EDTA and peripheral blood mononuclear cells (PBMC) were isolated as previously described (Booth et al., 2009). Lambs were then euthanized and JPP, BLN, MLN, tonsil and spleen tissues were immediately removed and placed in ice-cold DMEM (GibcoBRL) containing antibiotics, penicillin (100 U/mL), streptomycin sulfate (100 μg/mL), Amphotericin B (Sigma-Aldrich) (0.25 μg/mL). Single cell suspensions were prepared from JPP, BLN, MLN, tonsils and spleen as described previously (Griebel, 1996). The number of viable cells isolated from all tissues was determined by trypan blue dye exclusion and viable cells were counted with a hemocytometer under a light microscope. Cells were re-suspended at the appropriate concentrations in AIM V medium (supplemented with 2% FBS, 100 U penicillin /mL, 100 μg streptomycin sulfate /mL, 0.25 μg amphotericin B /mL, 2 Mm L-glutamine, 50 μM 2-mercaptoethanol and 10 μg polymyxin B sulfate (Sigma- Aldrich) /mL and plated in 100 μl medium/well in round bottomed 96-well plates (Nunc, Naperville, IL, USA).

2.4. Magnetic activated cell sorting (MACS)

JPP, BLN, MLN, tonsil and spleen cell suspensions and PBMC were stained with mouse anti-bovine CD21 antibody (IgG1 isotype; AbD Serotec, UK) for 15 min at 4 °C. The JPP, BLN, MLN, tonsil, spleen and PBMCs were then washed twice with MACS buffer (PBSA, 0.5 M EDTA, 10% BSA). Cells were pelleted by centrifugation for 8 min at 440 × *g*. The JPP, BLN, MLN, tonsil and spleen cell suspensions and PBMCs were then stained with goat anti-mouse IgG1 phycoerythrin (PE) conjugate (ABD Serotec, UK) for 15 min at 4 °C before washing as above. The JPP, BLN, MLN, tonsils, spleen and PBMCs were then incubated with anti-PE magnetic beads for 15 min at 4 °C and passed through the LC MACS column (Miltenyi Biotec, Bergish Gladbach, Germany) following the manufacturer's instructions. The $CD21^+$ B cell fraction was eluted, washed in PBSA and re-suspended in AIM V medium. The purity of eluted $CD21^+$ was confirmed to be > 95% (Data not shown).

2.5. Enzyme-linked immunosorbent SPOT assay (ELISPOT) for IL-10

Single cell suspensions were prepared from JPPs, BLN, MLN, tonsil and spleen tissues and PBMCs were isolated from blood. $CD21^+$ and $CD21^-$ cell populations were then fractionated from each cell population using magnetic cell sorting. The frequency of spontaneous IL-10-producing cells within unfractionated, $CD21^+$ and $CD21^-$ fractions of JPP, BLN, MLN, tonsil and spleen cells and PBMCs was determined by ELISPOT. Flat-bottom, polyvinylidene difluoride-coated, 96-well plates (Millipore, Bedford, MA, USA) were pre-wet with 100 μL 70% ethanol/well (Sigma Chemical Co.), washed with PBS, and coated overnight at 4 °C or for 1 h at 37 °C with mouse anti-recombinant bovine IL-10 antibody (Clone CC318; Serotec, MCA 2110) diluted to 1.0 μg/mL in coating buffer (carbonate/bicarbonate buffer pH 9.6). Plates were then washed 4 times with Tris buffered saline containing 0.05% Tween 20 (Sigma-Aldrich) (TBST) before adding 5×10^5 cells in 100 μl AIMV medium per well in triplicate wells for each cell fraction from each tissue. Plates were incubated at 37 °C, 5% CO_2, for 24 h, washed 4 times with PBS, and incubated for 2 h with 0.125 μg biotinylated mouse anti-bovine IL-10 antibody/well (Serotec, MCA 2111B). Plates were washed, and 100 μL of streptavidin-alkaline phosphatase (Mabtech, 1:1000 dilution) was added for 1 h. After washing, 100 μL freshly prepared NBT chloride (3 mg/mL)/5-bromo-4-chloro-3-indolyl phosphate-p-toluidine (1.5 mg/mL) substrate (BioRad, Hercules, CA, USA) in Tris buffer (pH 9.5) was added to each well for 20 min. Plates were washed with distilled water to stop the reaction and air-dried overnight before spot enumeration. The number of IL-10-producing cells per million cells from each tissue was determined by multiplying the number of spots/well by 2 and then averaging values from triplicate wells. The concentration of IL-10 in U/ml (units per ml) was defined previously by Kwong et al. (2002), as the biological activity of IL-10, with one unit being the reciprocal of the IL-10 dilution that inhibited 50% of the IFN-γ secretion by Cos-7 cells.

2.6. Tissue culture conditions and stimulation with TLR agonists

Isolated $CD21^+$ cells were re-suspended in AIM V medium. Aliquots of 5×10^5 unfractionated JPP, BLN, MLN, tonsil and spleen cells and PBMCs, 5×10^5 $CD21^+$ cells and 3×10^5 cells $CD21^-$ cells were cultured in triplicate wells in a final volume of 200 μL. Cells were stimulated with B-class CpG 2007 or GpC 2007 at 5 μg/mL and then incubated at 37 °C for 48 h in a 5% CO_2 atmosphere and 95% humidity.

For optimal detection of cytokines, cells were stimulated for 48 h as previously described (Griebel, 1996). Culture supernatants were stored at −20 °C until assayed for secreted cytokines.

2.7. Statistical analysis

Data analysis was performed with the package, Graph Prism 8 (Graphpad software). Individual group differences were examined by performing one way analysis of variance (ANOVA). Values of $P < 0.05$ and $P < 0.01$, were considered significant and very significant, respectively. Kruskal–Wallis tests and Dunn's tests were used to perform the post-hoc analyses.

3. Results

We had previously shown that IL-10 secreting $CD21^+$ B_{reg} cells were present in jejunal PPs of lambs of different ages (Jimbo et al., 2014). However, it was not determined whether these B_{reg} cells were also present in other lymphoid tissues. Therefore, we performed experiments to determine the frequency of B_{reg} cells among ovine spleen, MLN, tonsil, and BLN cells and PBMCs and compared these results to those for the JPP.

3.1. Frequency of IL-10 secreting cells in JPP

The frequency of cells spontaneously secreting IL-10 in the JPP of 3–4 month old lambs was determined with an ELISpot essay. There were on average 207 (range = 90–372) IL-10 secreting cells; (n = 5) per million cells in the unfractionated JPP population. To determine if a TLR9 agonist, CpG ODN specifically, induced IL-10 secretion we then calculated the net frequency of IL-10 secreting cells following stimulation with CpG ODN by subtracting the number of cells spontaneously secreting IL-10 from the number detected following GpC ODN stimulation. There was, however, no significant change in the frequency of IL-10 producing B cells following stimulation with CpG compared to the GpC ODN treated control (Fig. 1a). Enriched JPP $CD21^+$ B cells had a similar frequency of IL-10 secreting cells (124–500 cells per million). Moreover, the number of $CD21^+$ B cells secreting IL-10 did not change significantly following stimulation with CpG ODN when compared relative to the GpC control ODN (Fig. 1a). There were no or few (0– < 100 cells per million) IL-10 secreting cells in the unstimulated $CD21^-$ cell population and this also did not change with CpG stimulation. These results are consistent with our previous observations on PP cells isolated from this age group of lambs (Booth et al., 2009; Jimbo et al., 2014).

3.2. Frequency of IL-10 secreting cells in bronchial lymph nodes (BLN)

There were on average 80 (6–142 cells per million) IL-10 secreting cells per million unfractionated BLN cells cultured in medium. This frequency did not change following stimulation with CpG or GpC ODN (Fig. 1b). Similarly, unstimulated BLN $CD21^+$ B cells contained IL-10 secreting cells with an average frequency of 70 IL-10 secreting cells per million cells and this frequency did not change significantly following CpG or non-CpG ODN stimulation (Fig. 1b). There were fewer (8– < 38 cells per million) IL-10 secreting cells detected in the unstimulated $CD21^-$ cell population, and this frequency did not change significantly with CpG or non-CpG ODN stimulation. These results confirm that $CD21^+$ B cells spontaneously secreting IL-10 were present in BLN.

3.3. Frequency of IL-10 secreting cells in mesenteric lymph nodes (MLN)

There were on average 37 (10–80 cells per million) IL-10 secreting cells per million unfractionated and unstimulated MLN cells. The frequency of cells spontaneously secreting IL-10 in the MLN cell population did not increase significantly following stimulation with CpG compared to the GpC ODN treated controls (Fig. 1c). $CD21^+$ B cells enriched from MLN contained cells spontaneously secreting IL-10, with on average 32 IL-10 secreting cells per million cells, and this frequency did not change significantly following CpG ODN stimulation when compared to the GpC ODN treated controls (Fig. 1c). There were few IL-10 secreting cells in the $CD21^-$ cell population following CpG or GpC ODN stimulation. The average frequency of IL-10 secreting cells in the $CD21^-$ MLN cell population was 19 cells per million.

3.4. Frequency of IL10 secreting cells in the tonsils

IL-10 ELISpot analysis of cells isolated from tonsils revealed an average of 16 cells spontaneously secreting per million cells (8–22 cells per million). The frequency of IL-10 secreting cells did not increase significantly following stimulation with CpG when compared to the GpC ODN treated controls (Fig. 1d). Similarly, enriched $CD21^+$ B cells had an average frequency of 17 cells spontaneously secreting IL-10 per million cells and this frequency was not significantly changed following CpG ODN stimulation (Fig. 1d). No IL-10 secreting cells were detected in the $CD21^-$ MLN population, either before or after CpG or GpC ODN stimulation (Fig. 1d).

3.5. Frequency of IL-10 secreting cells in blood and spleen

The frequency of cells spontaneously secreting IL-10 in unfractionated PBMCs was low, with an average of 30 cells per million leukocytes (6–52 cells per million) and this frequency did not change significantly following stimulation with CpG ODN (Fig. 2a). Enriched $CD21^+$ PBMCs had a similar low frequency of IL-10 secreting cells (mean = 50 IL-10 secreting cells per million cells) and the frequency of IL-10 secreting cells was not significantly increased following CpG ODN stimulation (Fig. 2a). The $CD21^-$ PBMC fraction had few IL-10 secreting cells (0–62 cells per million) and this frequency (mean = 15 cells per million) did not change significantly following CpG ODN stimulation.

There was an average of 51 cells spontaneously secreting IL-10 (0–180 cells per million) in unfractionated splenocytes and this frequency did not change significantly following stimulation with CpG o ODN (Fig. 2b). A similar proportion of enriched $CD21^+$ splenocytes spontaneously secreted IL-10, with an average of 130 IL-10 secreting cells per million (26–280) and their frequency was not significantly changed following CpG ODN stimulation (Fig. 2b). There were, however, very few IL-10 secreting $CD21^-$ cells (0–11 cells per million), either with or without CpG or GpC ODN stimulation.

3.6. CpG ODN increases the frequency of IL-10 secreting $CD21^+$ B cells in both mucosal and systemic lymphoid tissues

Naturally occurring regulatory B cells have been shown to exist in ovine PP (Booth et al., 2009; Jimbo et al., 2014). However it is not known whether inducible (i) B_{reg} cells also exist in sheep. We previously confirmed that sheep B cells express TLR9 but do not proliferate or significantly increase IL-10 secretion following CpG stimulation (Booth et al., 2009; Jimbo et al., 2014). High inter-animal variation in spontaneous IL-10 secretion by B_{reg} cells (Fig. 1) may, however, limit our ability to detect iB_{reg} cell responses following CpG ODN stimulation. Therefore, the possible presence of iB_{reg} cells was investigated by comparing within each animal for the frequency of IL-10 secreting CD21+ B cells detected following GpC ODN stimulation *versus* CpG ODN stimulation. In this regard, CD21+ B cells were stimulated *in vitro* with either CpG ODN 2007 or the control GpC ODN and the number of IL-10 secreting $CD21^+$ B cells quantified. The number of iB_{reg} cells was calculated by subtracting the number of IL-10-secreting B cells present following GpC ODN stimulation from the number of IL-10 secreting B cells present following stimulation with CpG ODN (Fig. 3). This analysis revealed a consistent CpG ODN specific increase in the frequency of IL-

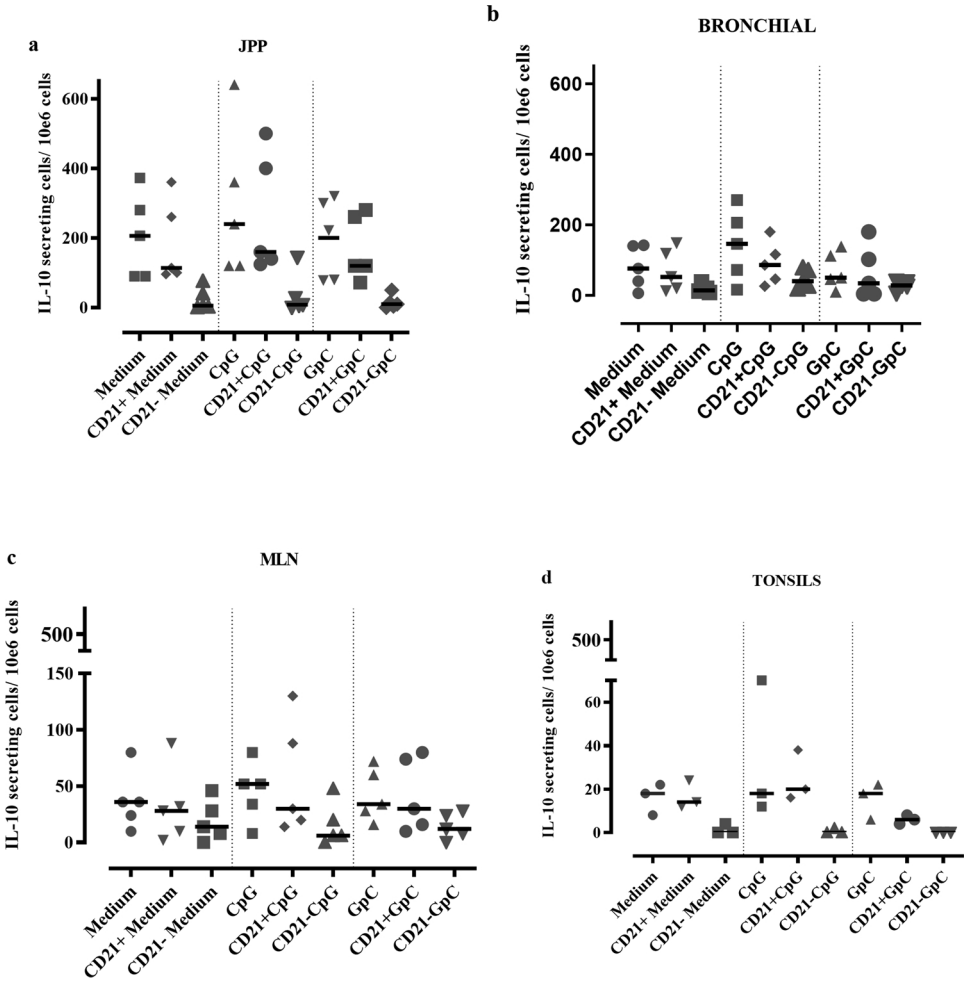

Fig. 1. IL-10 secreting cells in JPP (a), BLN (b), MLN (c) and tonsils (d). Unfractionated, $CD21^+$ B cells and $CD21^-$ JPP, BLN, MLN (jejunal Peyer's patches, mesenteric lymph nodes) and tonsil cells were cultured for 48 h in either medium alone or in the presence of 5 μg/ml CpG or GpC ODN. The frequency of IL-10 secreting cells was enumerated using ELISpot. Data for individual animals are presented with the mean value for each group (n = 5) indicated by a horizontal bar. ANOVA revealed no significant difference in the frequency of IL-10 secreting cells among mucosal lymphoid tissues or when comparing CpG *versus* GpC ODN or media.

10 secreting cells present in ovine JPPs, but the magnitude of this response varied markedly between individual animals. These $CD21^+$ iB_{reg} cells were also present in a variety of ovine mucosal and systemic tissues (Fig. 3) and again the frequency varied widely between individual animals, with no apparent response in some animals. There was no significant difference in the frequency of iB_{reg} cells between different lymphoid tissues.

4. Discussion

There is increasing evidence that B_{reg} cells play a significant role in regulating immune responses in both mucosal and systemic lymphoid tissues, but B_{reg} cell distribution has not been investigated in ruminants. In the current study we observed that IL-10 secreting $CD21^+$ B cells were present in both mucosal and systemic lymphoid tissues of sheep and confirmed that CpG ODN stimulation induced increased B_{reg} cell activity in a variety of ovine lymphoid tissues. This is the first evidence that iB_{reg} cells are present in sheep and their frequency varies substantially in individual animals and in different lymphoid tissues.

Our first aim in this study was to survey the distribution of $CD21^+$ B_{reg} cells in the various tissues of sheep. Two subpopulations of B cells have been described in sheep. These two B cell sub-populations have

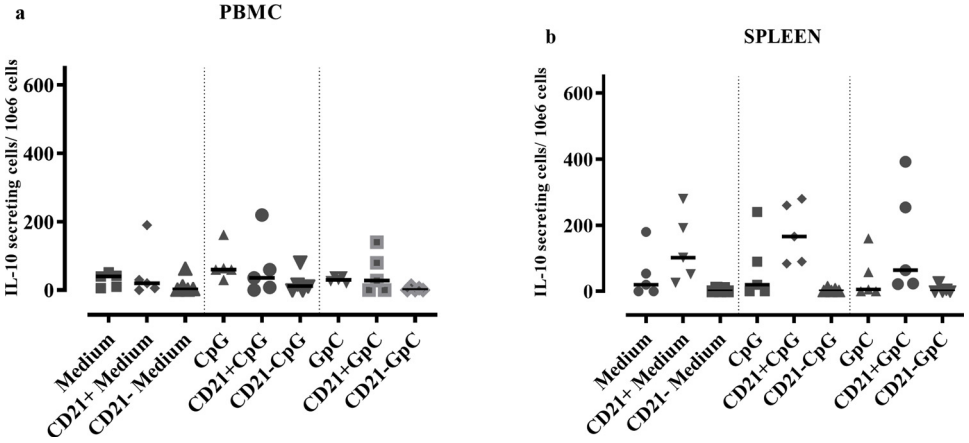

Fig. 2. IL-10 secreting cells in PBMC (peripheral blood mononuclear cells) (a) and spleen (b). Unfractionated, $CD21^+$ and $CD21^-$ PBMCs and splenocytes were cultured for 48 h in either medium alone or in the presence of 5 μg/ml CpG or GpC ODN. The frequency of IL-10 secreting cells was enumerated using ELISPOT. Data for individual animals are presented with the mean value for each group (n = 5) indicated by a horizontal bar. ANOVA revealed no significant difference in the frequency of IL-10 secreting cells when comparing CpG *versus* GpC ODN or media.

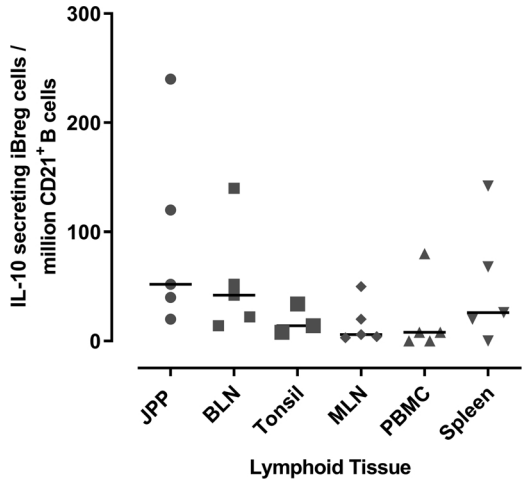

Fig. 3. CpG ODN induction of IL-10 secretion by CD21$^+$ B cells isolated from ovine mucosal and systemic lymphoid tissues. CD21$^+$ B cells were isolated from mucosal (JPP: jejunal Peyer's patches; BLN: bronchial lymph nodes; TONSIL: palatine tonsil; MLN: mesenteric lymph node) and systemic (PBMCs: peripheral blood mononuclear cells; Spleen) lymphoid tissues. CD21$^+$ B cells were stimulated *in vitro* with either CpG ODN 2007 or the control GpC ODN and the number of IL-10 secreting CD21$^+$ B cells quantified with an ELISPOT assay. The number of inducible (i) B_{regs} was calculated by subtracting the number of IL-10 secreting B cells present following GpC ODN stimulation from the number of IL-10 secreting B cells present following stimulation with CpG ODN. ANOVA revealed no significant difference in the frequency of iB_{regs} among lymphoid tissues.

distinct recirculation characteristics and tissue distributions (Gupta et al., 1998). Phenotypically, the two subpopulations are distinguished by their surface expression of the complement receptors, CD21 (CR2) and CD11b/ CD18 (CR3) (Gupta et al., 1998). The CD11b$^+$ B cells are non-circulating B cells. Unlike these cells, the CD21$^+$ B cells express low levels of sIgM and co-express L-selectin. CD21$^+$ B cells populate the splenic and PP follicles, are absent in the splenic marginal zones and are the only B cells found in afferent and efferent lymph and are present in all lymph nodes (Gupta et al., 1998). We now show that the frequency of IL-10 secreting CD21$^+$ B cells varies significantly in different lymphoid tissues and these CD21$^+$ B cells are similar to those described by Gupta et al, since they are also distributed in varying concentrations in different lymphoid tissues (Gupta et al., 1998).

The reason for the broad variation in B_{reg} cells frequency is presently unknown but there may be several possible explanations. One reason may be due to the need for higher B_{reg} cells presence in some tissues in order to maintain proper immune function. For example, mucosal tissues such as the gut mucosal tissues may require a higher frequency of B_{reg} cells due to constant exposure to microbial products. Thus, B_{reg} cells may be required to control the level of immune activation and avoid chronic inflammation (O'Byrne and Dalgleish, 2001). Secondly, some IL-10 secreting cells were also present among the CD21$^-$ population isolated from different tissues. This may be due to IL-10 secreting CD21$^+$ B cell contamination of the CD21$^-$ cell fraction, as MACs fractionation of JPP cells into CD21$^+$ and CD21$^-$ subpopulations is not completely effective.

B cells are characterized by their specific ability to produce antibodies (Adachi et al., 2000; Mizoguchi and Bhan, 2006). It is also evident that B cells can produce a wide spectrum of cytokines, both spontaneously and under inflammatory conditions. We have identified an inducible B_{reg} subset that develops in response to TLR stimulation and may control ongoing inflammation through the production of IL-10. These findings support the conclusion that effector and regulatory B cells may co-exist in the same immune compartment. The CD21$^+$ B_{reg} cells identified in the current study shared phenotypic and functional properties with ovine PP B_{reg} cells that we previously showed spontaneously secreted IL-10 (Booth et al., 2009; Jimbo et al., 2014). Based on these previous reports from our laboratory, we hypothesized that these unique intestinal B cells represent a precursor of iB_{reg} cells, which differentiate from immature/naive B cells and are present in the jejunal Peyer's patches, where their immune regulatory function is fully elicited. This observation may be particularly relevant when infections induce intestinal inflammation, leading to an increased need for IL-10 producing cells.

In conclusion, we confirmed that IL-10 secreting CD21$^+$ B cells were present in both mucosal and systemic lymphoid tissues of sheep. These findings provide insight into the possible role that B cells may play in regulating inflammatory responses and the identification of iB_{reg} cells indicates that this activity may be modulated by exposure to microbial-associated molecular pattern molecules. We predict that in the future, iB_{reg} cells may be of benefit for treatment of auto-inflammatory disease.

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