Veterinary Immunology and Immunopathology 174 (2016) 26–34

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Evidence for the existence of regulatory and effector B cell populations in Peyer's patches of sheep

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ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form 31 March 2016
Accepted 7 April 2016

Keywords:
Immune regulation
Regulatory cells
B_{regs}
Effector B cells
Peyer's patches
TLR9
CpG ODN

ABSTRACT

IL-10 secreting $CD21^+$ B cells exist in sheep Peyer's patches (PP). It's not known however, whether all PP B cells are regulatory or whether an effector population also exists in this tissue. To further characterize the subpopulations of B cells in PP's, highly purified B cells were negatively sorted from jejunal PP and fractionated according to co-expression of $CD72^+CD21^+$or $CD72^+CD21^-$ molecules and then stimulated with the TLR9-agonist, CpG ODN. IL-10, IL-12, IFN-γ, and IgM production were then assayed. We observed that only highly purified $CD72^+CD21^+$ B cells spontaneously secreted high levels of IL-10, but they did not produce any IL-12, IFN-γ or IgM suggesting that this cell population contains regulatory B cells. In contrast, $CD72^+CD21^-$ B cells did not secrete IL-10, but secreted IL-12, IFN-γ, and IgM, suggesting they include effector cells. In addition, B cells expressing surface IgA, IgM and IgG1 all secreted similar levels of IL-10. We further confirmed that only B cells produce IL-10, while other cells in the PP including DCs and T cells do not. Our investigations may provide evidence for the existence of two sub-populations in sheep PP; IL-10 secreting regulatory ($CD72^+CD21^+$) cells, and IL-12/IFN-γ/IgM-secreting effector ($CD72^+CD21^-$) cells.

1. Introduction

B cells are functionally characterised by the ability to produce antibodies (Mizoguchi and Bhan, 2006). They also have the capacity to produce cytokines and function as antigen presenting cells (APC) (Mizoguchi and Bhan, 2006). Based on their function, B cells can be divided into two subgroups; effector and regulatory B cells.

Effector B cells are regulators of immunity and based on their cytokine secretion patterns have been subdivided into B-effector-1 cells (Be1) and B-effector-2 (Be2) (Airoldi et al., 2000; Medzhitov, 2001). The Be1 cells secrete IL-12 and IFN-γ, whereas Be2 secrete IL-4, IL-5, IL-6, IL-10 and IL-13 (Medzhitov, 2001). In addition, effector B cells function as antibody producing cells, present antigens to T cells and modulate T cell-mediated responses (Graham et al., 2007). Effector B cells eventually differentiate into plasma cells, able to produce different isotype antibodies (Shapiro-Shelef and Calame, 2005).

The regulatory role of B cells was first reported by Janeway and colleagues in a murine model of experimental autoimmune encephalomyelitis (EAE) (Wolf et al., 1996). Using a B cell deficient model, they reported that B cells were not required for the induction of EAE but they contributed to immune regulation resulting in complete recovery from acute EAE. Further evidence for the existence of B_{regs}, was obtained from mouse models of inflammatory bowel disease (IBD) (Dalwadi et al., 2003; Mizoguchi et al., 2002), EAE (Fillatreau et al., 2002), arthritis (Mauri et al., 2003), and lupus (Brummel and Lenert, 2005; Lenert et al., 2005). These B_{regs} inhibited the progression of inflammation and/or hastened the recovery from the experimental inflammatory conditions. Notably, B_{regs} cells produce IL-10, the immuno-regulatory cytokine that can suppress harmful immune responses by regulating Th1/Th2 balance and directly dampening innate cell-mediated inflammatory responses (Fiorentino et al., 1991; Grünig et al., 1997; O'Farrell et al., 1998).

Recent investigations in our laboratory revealed a novel B cell population in sheep jejunal Peyer's patches with regulatory function (B_{regs}) that spontaneously produce IL-10 and suppresses TLR9-induced IFN-γ and IL-12 cytokine responses (Booth et al., 2009). We have subsequently obtained evidence that these regulatory B cells develop in utero prior to antigen exposure (Jimbo et al., 2014). In these previous studies, $CD21^+$ B cells were isolated using magnetic cells sorting, raising questions as to whether other

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http://dx.doi.org/10.1016/j.vetimm.2016.04.006
0165-2427/

contaminating cell types may be present. In the present investigation, we sought to determine whether $CD21^+$ B cells are the only source of IL-10 in Peyer's patches. We also investigated whether an effector B cell population co-existed with regulatory B cells in the PPs. Furthermore, we also investigated whether IL-10 secretion was limited to naïve $CD21^+IgM^+$ B cells, or B cells that had undergone isotype switching.

2. Materials and methods

2.1. Animals

Suffolk sheep of either sex were obtained from the Department of Animal and Poultry Science (University of Saskatchewan, Saskatoon, SK, Canada). 2–4 months old lambs were used in these studies. Experiments were conducted according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. All the experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

2.2. 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 GpC ODN 2007 GC were obtained from Merial Limited (Lyon, France). They have the following sequences and backbone structures; 2007 tcgtcgttgtcgttttgtcgtt and 2007GC tgctgcttgtgcttttgtgctt. The ODN doses were previously optimized and used at a dose of 5 ug/ml (Booth et al., 2009, 2007).

2.3. Isolation of jejunal Peyer's patch (JPP) cells for culture

Lambs were euthanized and jejunal Peyer's patch tissues immediately removed and placed in ice-cold DMEM (GibcoBRL) containing antibiotics (100 U/mL), Penicillin, 100 μg/mL Streptomycin sulfate, 0.25 μg/mL Amphotericin B (Sigma-Aldrich). Cells were isolated from JPP 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, 100U/mL penicillin, 100 μg/mL streptomycin sulfate, 0.25 μg/mL amphotericin B, 2 Mm L-glutamine, 50 μM 2-mercaptoethanol and 10 μg/mL polymyxin B sulfate (Sigma- Aldrich)) and added to round bottomed 96-well plates (Nunc, Naperville, IL, USA).

2.4. JPP Labelling for high speed Cell Sorting of negatively selected B cells

For negative sorting of B cells, 3×10^8 ovine JPP cells, at a final concentration of 1×10^8 cells/mL PBS, were labelled with 18 μg anti-ovine CD25, 18 μg anti-ovine CD11c, 9 μg anti-ovine CD14, 18 μg anti-ovine CD2, 18 μg anti-ovine CD4, 18 μg anti-ovine WC1-N2, 18 μg anti-ovine CD8, 18 μg anti-ovine SWC3 and 15 μg anti-ovine CD16. JPP cells were incubated with mAbs for 20 min at 4 °C, with gentle mixing every 5 min. Cells were then pelleted by centrifuging at $311 \times g$ for 8 min at 4 °C and washed twice with ice-cold PBS. The cell pellet was re-suspended in 3 ml PBS and the following fluorochrome-conjugated secondary antibodies were added: 20 μg goat anti-mouse IgG2a-PE; 20 μg of goat anti-mouse IgG1-PE; and 9 μg goat anti-mouse IgM- allophycocyanin. Cells were incubated in the dark at 4 °C for 15 min and then washed twice with ice-cold PBS.

2.5. High-speed cell sorting

Labelled JPP cells were adjusted to a final concentration of 1×10^8 cells/mL in ice-cold PBS, filtered through 35 μm cell strainer capped 12 × 75 mm polystyrene round bottom tubes (Cat. No. 352235) (BD Falcon ON, CA) to remove cell clumps. High-speed sorting was performed with a MoFlo XDP (Beckman Coulter Inc. CA, USA) equipped with a 488 argon and 633HeNe laser. FITC, PE and Allophycocyanin fluorescence was collected through 529/25, 575/25 and 670/30 band-pass filters, respectively. Dot scatter plots of forward light scatter (FSC) and 90° light scatter (SSC) were displayed on a linear scale and used to define the first sort region (R1) which excluded dead cells and debris (Fig. 1). Dot plots displaying FSC-Height/FSC-Width were used to define the second sort region (R2) to exclude doublets or larger cell clumps. To negatively sort for B cells, a sort region was set to include all PP B cells and exclude the rest of the cells (R3) (Fig. 1). R3 was set at different positions on the CD11c log scale and samples analyzed on cytospins to identify the gate yielding the highest purity of B cells (data not shown). Cells in the region R3 were restained with either with antibodies against CD21 or IgM, IgA and IgG1 and used when sorting B cells based on CD21 and immunoglobulin expression and CD11c and T cells based on the absence of CD11c, MHCII, and CD335. Sort conditions included a sheath pressure of 60 psi, a 70 μm nozzle, 0.3 psi differential pressure, and a sort rate of 18–22,000 events/s. Sorted cells were collected in sterile 12 × 75 mm polypropylene round bottom tubes (VWR international, Mississauga, ON, CA) pre-coated overnight with 4% bovine serum albumen (BSA, Sigma, Ontario CA). Collection tubes were kept on ice throughout the sort and cells were transferred every 30 min to ice-cold AIMV medium (Invitrogen, Burlington, ON) supplemented with plus 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON).

2.6. Tissue culture conditions and stimulation with TLR agonists

High-speed sorted cells were re-suspended in AIM V media. Aliquots of 5×10^5 cells for $CD21^+$ and 3×10^5 for $CD21^-$ cells were cultured in triplicate wells in a final volume of 200 μl. Cells were stimulated with TLR9 agonists B-class CpG 2007 or GpC 2007 as a control at 5 μg/mL and then incubated at 37 °C 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 cytokines assays were performed.

2.7. Enzyme-linked immunosorbent assay (ELISA) for IgM, IL-10, IL-12 and IFN-γ

ELISAs for quantifying IgM and cytokines in culture supernatants were performed according to previously described procedures as follows; IgM (Booth et al., 2010), IFN-γ (Mutwiri et al., 2000), IL-12 (Hope et al., 2002) and IL-10 (Kwong et al., 2002), with minor modifications. U/ml (units per ml) for IL-10 was defined previously by Kwong et al. (Kwong et al., 2002), as the biological activity of IL-10 with one unit being the reciprocal of the IL-10 dilution that inhibited IFN-γ secretion by 50% of Cos-7 cells.

2.8. Flow cytometry

JPP cells were re-suspended at a final concentration of 20×10^6 cells/ml in PBSA containing 0.03% sodium azide (EMD Chemicals). 50 μl aliquot of cells was added to each well of a U-bottom 96-well plate (Corning Inc. Life Sciences, Lowell, MA) and 5 μl aliquot of primary monoclonal antibody (mAb) for CD21, CD72, IgM, IgA, IgG1 (Table 1) were each added at a final concentration between 1–5 μg/mL. For each mAb the concentration was titrated to provide