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Co-stimulation with TLR7/8 and TLR9 agonists induce down-regulation of innate immune responses in sheep blood mononuclear and B cells

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ABSTRACT

Toll-like receptors (TLRs) play an important role in the activation of innate and adaptive immune responses. Stimulation with multiple TLR agonists may result in synergistic, complimentary or inhibitory effects on innate immune responses. In this study, we investigated the effects of co-stimulation of sheep peripheral blood mononuclear cells (PBMC) and B cells with agonists for TLR3, 4, 7/8 and 9. Sheep PBMC stimulated with either CpG (TLR9 agonist) or RNA oligoribonucleotides ([ORNs], TLR7/8 agonist) exhibited significant IL-12 production, but only CpG induced IFNα, IgM and proliferative responses. In contrast, poly(I:C) (TLR3 agonist) and LPS (TLR4 agonist) did not induce any of these responses. Interestingly, we observed that co-stimulation of PBMC with CpG + ORN or CpG + imiquimod (another TLR7/8 agonist) resulted in significant reduction in CpG-induced IFNα production, B cell proliferation and IgM responses. Pre-incubation of cells with CpG prior to exposure of the cells to imiquimod resulted in similar inhibitory responses indicating that the down-regulatory mechanisms are not associated with competition for cellular uptake or for receptors of the two agonists. Sheep B cells constitutively expressed TLR7, TLR8 and TLR9 mRNA transcripts, suggesting a possible role of TLR cross-talk in the down-regulatory mechanisms. Down-regulation of responses by co-stimulation with closely related TLRs may be a regulatory mechanism by which the host prevents overstimulation of innate immune responses.

1. Introduction

The innate immune system uses a set of germline-encoded pattern recognition receptors (PRR) to detect pathogen associated molecular patterns (PAMPs) present in microbial pathogens [1]. PRR are strategically located in various cells and have been grouped into different families that include Toll-like receptors (TLRs), card helicases, nucleotide oligomerization domains (NODs), C-type lectins, complement, complement receptors and others [2]. Toll-like receptors (TLRs), the main PRR family, are transmembrane signaling molecules currently comprised of at least 13 members in mammals named TLR1–TLR13 [1–4].

TLR3 recognizes viral double stranded (ds) RNA and poly(I:C) whereas TLR4 detects lipopolysaccharide (LPS). Endosomal TLR7 and TLR8 recognize viral single stranded (ss) RNA and the small antiviral compounds, imidazoquinolines [5,6], while TLR9 is the receptor for CpG-rich viral and bacterial DNA [7]. Agonists for TLR7/8 and TLR9 are potent activators of innate and adaptive immunities, and have attracted a great deal of attention due to their potential as vaccine adjuvants and immunotherapeutic agents. Activation of TLR7/8 predominantly induces Th1 cytokines and chemokines including IFNα, IP-10, IL-12, IL-6 and TNF-α [5,8–11], increased expression of costimulatory molecules and upregulation of the early activation marker CD69 [5,12–14]. Similarly, activation of TLR9 induces predominantly Th1 cytokines, chemokines and increased expression of costimulatory molecules, but also activates B cells to produce the cytokines IL-6 and IL-12, and to proliferate and secrete IgM [15].

Most studies assessing the immune effects of TLRs have focused on activation of a single TLR. However, experience with TLR9 agonist, CpG oligodeoxynucleotide (ODN) suggests that individual TLR agonists in ruminants are not as potent immune activators as reported in rodent models [16]. Given that viruses and bacteria express several TLR agonists and most likely pathogens stimulate multiple TLRs simultaneously, the consequences of activating multiple TLRs has generated a lot of interest. Evidence is accumulating to support the notion that activation of multiple TLRs can result in complimentary, synergistic or antagonistic effects. In this regard, simultaneous activation of dendritic cells (DCs) with TLR9 and TLR4 agonists, CpG ODN and LPS resulted in additive effects on IL-12 production [17]. Similarly, co-stimulation of TLR3 and TLR8, or TLR4 and TLR8 resulted in a synergistic IL-12

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response, but co-stimulation of TLR3 and TLR4 had no effect on the IL-12 response [18]. In contrast, simultaneous activation with agonists for TLR7/8 and TLR9 resulted in inhibition of IFNα responses [19,20]. While, complimentary or synergistic responses would result in better protection against infection, inhibition may be a safety mechanism to prevent overstimulation of innate immune responses which would otherwise lead to pathology. A profound understanding of the cellular events triggered by combinations of TLRs will be valuable in the rational design of more successful TLR-based immunotherapies and vaccination strategies. TLR agonists can be grouped into two main categories based on their dependence or independence on the adaptor molecule MyD88. It has been proposed that co-stimulation with agonists sharing common pathway (MyD88-dependent (D) or -independent (I) agonists) do not induce synergistic, but rather may lead to inhibitory responses. In contrast, co-stimulation with a D agonist and I agonist lead to synergistic responses [21].

It is thought that most of the beneficial effects of TLR7/8 and TLR9 activation are mediated primarily through IFNα produced by DC [22,23]. Consequently, studies exploring co-stimulation of these TLRs have focused primarily on IFNα responses in DCs, and relatively less is known about the effects of co-stimulation of other cells. B cells are one of the major cell populations which can be significantly influenced through TLR activation. Many B cells express TLR7/8/9 and, activation of TLR9 signal in B cells can synergize with the antigen-specific BCR signal resulting in enhanced antibody production [23]. More recently, it was suggested that in addition to $CD4^+$ T-cell help, generation of T-dependent antigen-specific antibody responses requires activation of TLRs in B cells [24]. Thus, TLR activation can have dramatic effects on the behavior of B cells, but not much is known about multiple TLR stimulation in these cells. One recent report suggested that simultaneous activation with TLR7 and TLR9 agonists had no effect on human B-cell responses [20].

It has been proposed that the use of combinations of TLR (and non-TLR) agonists may be necessary to induce strong immune responses in large animal [16]. However, the consequences of multiple TLR stimulation have not been explored in ruminants. Given that TLR responses can differ dramatically from one species to another, we investigated effects of co-stimulation of TLR3, TLR4, TLR7/8 and TLR9 in sheep. The objectives of the present studies were (i) to determine whether TLR agonists and their combinations can induce innate immune responses in ovine immune cells, and (ii) to characterize innate immune responses in ovine blood mononuclear and B cells following co-stimulation with TLR7/8 and TLR9 agonists.

We report that co-stimulation of ovine blood mononuclear and B cells with TLR7/8 and TLR9 agonists results in significant reduction in ensuing innate immune responses.

2. Materials and methods

2.1. TLR agonists and animals

The TLR7/8 agonists used included synthetic ssRNA oligoribonucleotide (ORN) 1075 obtained from Coley Pharmaceutical Group (Ottawa, ON, Canada) and a synthetic imidazoquinoline compound imiquimod purchased from Invivogen (San Diego, CA, USA). CpG ODN C-class ODN 2429; sequence; tcgtcgttttcggcggccgccg was provided by Coley Pharmaceutical Group, while B-class CpG ODN 2007; sequence tcgtcgttgtcgttttgtcgtt were obtained from Merial Limited (Lyon, France). All the TLR agonists had no detectable endotoxin levels as determined by the Limulus Amebocyte Lysate Assay (Biowhittaker, Wlakersville, MD, USA).

Suffolk sheep of either sex (1–4 months of age) were obtained from the Department of Poultry and Animal Science (University of Saskatchewan, Saskatoon, SK, Canada). The animals were housed at the Vaccine and Infectious Disease Organization (VIDO) animal facility and fed ad libitum on a ration of rolled barley and alfalfa hay. All experiments were carried out according to the Guide to the Care and Use of Experimental Animals, provided by the Canadian Council on Animal Care. Experimental protocols were approved by the University of Saskatchewan Animal Care Committee.

2.2. Isolation of mononuclear cells from peripheral blood

Jugular blood was collected from sheep in ethylene diamine tetra-acetic acid (EDTA)-treated VACUTAINER® tubes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and PBMC were isolated using 54% isotonic Percoll® (Pharmacia Biotech AB, Uppsala, Sweden) as previously described [25]. Cells were counted using a cell counter (Dual Diluter III, Coulter Electronics Ltd., Luton, England) and re-suspended in AIM V® medium (Invitrogen Corporation) containing 2% fetal bovine serum (FBS; GibcoBRL).

2.3. Magnetic activated cell sorting (MACS)

The $CD21^+$ B-cell fraction of PBMC was isolated as previously described [26] with minor modifications. Briefly PBMC were incubated with mouse anti-bovine CD21 antibody (IgG1, AbD Serotec, UK) for 15 min at 4 °C. The cells were then washed twice with magnetic activated cell sorting (MACS) buffer (PBSA, EDTA and 10% BSA) by spinning for 8 min at 440 × *g* and incubated with goat anti-mouse IgG1 phycoerythrin (PE) conjugate (Southern Biotech, AL, USA) for 15 min at 4 °C. The cells were then labeled with anti-PE magnetic beads for 15 min at 4 °C and eluted through the LC MACS® column (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to manufacturer's instructions. The $CD21^+$ fraction was flushed out, washed in PBS and re-suspended in AIM V® medium containing 2% FBS.

2.4. Tissue culture conditions and stimulation with TLR agonists

Cells were re-suspended in AIM V® medium supplemented with 2% FBS, 100 IU/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 96-well, round bottom plates (Nunc, Naperville, IL, USA). For each treatment, 5×10^5 cells were cultured in triplicate wells in 200 μl total volume. Cells were incubated at 37 °C in a 5% CO_2 atmosphere and 95% humidity. Optimal cell stimulation concentrations for TLR7/8 or TLR9 agonists were established previously [27,28]. Cells were stimulated with TLR7/8 agonists; ORN 1075 at 2.5 μg/ml or imiquimod 5 μg/ml and TLR9 agonists; C-class CpG 2429 and B-class CpG 2007 at 5 μg/ml. ORN was used with DOTAP at ORN:DOTAP ratio of 1:2. To further investigate the effect of immiquimod on CpG-induced responses, we co-stimulated cells with CpG (5 μg/ml) with varying concentrations of imiquimod ranging from 25 μg/ml to 0.25 μg/ml. For optimal detection of cytokines, cells were stimulated for 48 h as previously described [28]. Culture supernatants were stored at −20 °C until assayed for cytokines.

2.5. Enzyme-linked immunosorbent assay (ELISA) for IFNα, IFNγ, IL-10, IL-12 and IgM

ELISA for quantifying cytokines in cell culture supernatants were performed according to previously described procedures as follows; IFNα [29], IFNγ [30], IL-12 [31] and IL-10 [32], with minor modifications.

For IgM ELISA, polystyrene microtiter plates (Immulon® 2, Dynex Technology Inc., Chantilly, VA) were coated overnight at