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Effect of synthetic agonists of toll-like receptor 9 on canine lymphocyte proliferation and cytokine production in vitro

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Abstract

Synthetic agonists of TLR9 containing novel DNA structures and R′pG (wherein R = 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine) motifs, referred to as immune modulatory oligonucleotides (IMOs), have been shown to stimulate T_H-1-type-immune responses and potently reverse allergen-induced T_H-2 responses to T_H-1 responses in vitro and in vivo in mice. In order to investigate the immunomodulatory potential of IMOs in dogs, canine peripheral blood mononuclear cells (PBMC) from healthy dogs were stimulated with three different IMOs and a control IMO, alone or in combination with concanavalin A (ConA). Lipopolysaccharide (LPS) was used as a positive control for B lymphocyte activation. Carboxyfluorescein diacetate succinimidyl ester and phenotype staining was used to tag proliferating T and B lymphocytes ($CD5^+$ and $CD21^+$) by flow cytometry. Real-time PCR and ELISA were processed to assay cytokine production of IFN-γ, IL-10, TGF-β, IL-6 and IL-10. Like LPS, IMOs alone induced neither proliferation of $CD5^+$ T cells nor $CD21^+$ B cells, but both LPS and IMO had the capacity to co-stimulate ConA and induced proliferation of B cells. In combination with ConA, one of the IMOs (IMO1) also induced proliferation of T cells. IMO1 also significantly enhanced the expression of IFN-γ on the mRNA and protein level in canine PBMC, whereas expression of IL-10, TGF-β and IL-4 mRNAs was not induced by any of the IMOs. These results indicate that in canine PBMC from healthy dogs, IMO1 was able to induce a T_H-1 immune response including T- and B-cell proliferation.

Keywords: TLR9 agonists; Immunomodulatory oligonucleotides; Flow cytometry; Canine lymphocyte proliferation; Cytokines; Toll-like receptor 9

1. Introduction

The immune system of vertebrates recognizes unmethylated cytidine–phosphate–guanosine (CpG) dinucleotides, which are present in bacterial DNA, as a danger signal (Krieg, 2001). CpG dinucleotides trigger a protective immune response that improves the ability of the host to eliminate the pathogen (Wagner, 1999). This

Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; CpG, cytidine-phosphate-guanosine; IMO, immunomodulatory oligonucleotide; ODN, oligodeoxynucleotide; PE, phycoerythrin; RT, room temperature; TLR9, toll-like receptor 9; Treg, T regulatory cells.

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recognition is mediated by a molecular pattern recognition receptor called toll-like receptor 9 (TLR9) (Hemmi et al., 2000; Latz et al., 2004). As TLR9 agonists, CpG oligonucleotides initiate signaling pathways that lead to activation of several transcription factors including nuclear factor-κB and activator protein-1 (Stacey et al., 1996; Yi and Krieg, 1998). The ensuing biological effect includes activation of B cells, T cells, professional antigen-presenting cells (APCs), increased expression of MHC class II antigens, increased synthesis of RNA, DNA, cytokines (IL-6, IL-12, IFN-γ) and chemokines (Klinman et al., 1996; Pasare and Medzhitov, 2003a; Klinman, 2004; Li et al., 2005; Krieg, 2006). Due to this immunological activation, CpG oligodeoxynucleotides (CpG ODN) have a broad therapeutic potential in cancer treatment, infectious disease and allergy (Walker and Zuany-Amorim, 2001; Zuany-Amorim et al., 2002; Klinman, 2004; Krieg, 2006; Romagne, 2007). CpG ODN were tested for their use as allergen-adjuvants in specific immunotherapy and were shown to suppress established IgE-titers in mice (Johansen et al., 2005). They were also shown to prevent inflammatory disease manifestations in mice with established allergic disease, due to a potent T helper 1 (T_H-1) immune response (Kline et al., 1998; Jain et al., 2002).

Recent studies have described synthetic agonists of TLR9 containing novel DNA structure and synthetic immune stimulatory motifs (Kandimalla et al., 2001, 2003a,b, 2005), referred to as immune modulatory oligonucleotides (IMOs). A novel immune stimulatory motif R′pG (wherein R′ = 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine) has been shown to induce immune responses in a wide range of mammalian species. The R′pG motifs potently reverse allergen-induced T_H-2 responses to T_H-1 responses by inhibiting IL-5 secretion and augmenting secretion of IL-12 and IFN-γ in mice, and act as a T_H-1-type adjuvant with antigens and vaccines in mice (Kandimalla et al., 2003a,b; Li et al., 2005). Therefore, synthetic TLR9 agonists containing R′pG motifs could be useful to elicit T_H-1 type-immune responses as immunomodulatory therapy for allergic diseases, viral infections or neoplasia in dogs.

The aim of this study was to investigate the immunomodulatory potential of these synthetic agonists of TLR9 with R′pG motifs in canine cell-based studies. In particular, various synthetic agonists of TLR9 (IMO1–3 and control IMO4) were examined for their ability to induce proliferation of peripheral blood lymphocytes in peripheral blood mononuclear cells (PBMC) obtained from healthy dogs. We also investigated their ability to induce the expression of selected cytokines representing different T cell responses: IFN-γ as a T_H-1 cytokine, IL-4 as a T_H-2 cytokine, IL-10 and TGF-β as T regulatory cell (Treg) cytokines.

2. Materials and methods

2.1. Synthetic agonists of TLR9

Synthetic agonists of TLR9 containing R′pG motifs were synthesized as described elsewhere (Kandimalla et al., 2003a,b) by Idera Pharmaceuticals (Cambridge, MA, USA). The three TLR9 agonists containing R′pG motifs and control IMO used in the study were referred to as IMO1 (5′-TCAGT**R′**GTTAG-X-GATTG**R′**TGA-CT-5′), IMO2 (5′-T**R′**GAA**R′**GTTCT-X-TCTTG-**R′**AAG**R′**T-5′) and IMO3 (5′-T**R′**GTA**R′**GTACT-X-TCATG**R′**ATG**R′**T-5′) and control IMO4 (5′-ACA-CACCAACT-X-TCAACCACACA-5′) (wherein **R′** and X stand for 1-(2′-deoxy-β-D-ribofuranosyl)-2-oxo-7-deaza-8-methyl-purine and glycerol, respectively) in the text. All four compounds contained phosphorthioate backbone and the purity was greater than 92% full-length product with the rest being 1, or 2 nucleotides shorter as determined by anion-exchange high performance liquid chromatography, capillary gel electrophoresis, and/or denaturing polyacryl amide gel electrophoresis (PAGE). All compounds were characterized by matrix-assisted laser desorption/ionization-time-of-flight mass for their sequence integrity. All four compounds contained < 0.05 EU of endotoxin as determined by limulus amebocyte lysate assay.

2.2. Animals

Six healthy beagle dogs, with no previous history of disease from Novartis Centre de Recherche Santé Animale, St. Aubin, Switzerland were used as blood donors. Four males (4–7 years old) and two females (6 and 7 years old) were kept in their usual housing conditions. All procedures applied for this study were approved by the local animal welfare authorities.

2.3. Blood sampling

Fifty milliliters of blood was collected from each dog by veinpuncture of the jugular vein using tubes containing EDTA.

2.4. Isolation of PBMC

PBMC were prepared by density gradient centrifugation of EDTA–blood samples obtained from the