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Oligodeoxynucleotide inhibition of Toll-like receptors 3, 7, 8 and 9 suppresses cytokine production in a human rheumatoid arthritis model

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Abbreviations: oligodeoxynucleotide (ODN), ectodomain (ECD), phosphorothioate (PTO), phosphodiester (PD)

Abstract

Toll-like receptors (TLRs) are innate immune receptors that respond to both exogenous and endogenous stimuli and are suggested to contribute to the perpetuation of chronic inflammation associated with rheumatoid arthritis (RA). In particular, the endosomal TLRs 3, 7, 8 and 9 have more recently been postulated to be of importance in RA pathogenesis. In this study, pan inhibition of the endosomal TLRs by a phosphorothioate-modified inhibitory oligodeoxynucleotide (ODN) is demonstrated in primary human B cells, macrophages and RA fibroblasts. Inhibition of TLR8 was of particular interest as TLR8 has been associated with RA pathogenesis in both human and murine arthritis models. ODN1411 competitively inhibited TLR8 signaling and was observed to directly bind to a purified TLR8 ectodomain, suggesting inhibition was through a direct interaction with the receptor. Addition of ODN1411 to human RA synovial membrane cultures significantly inhibited spontaneous cytokine production from these cultures, suggesting a potential role for one or more of the endosomal TLRs in inflammatory cytokine production in RA and the potential for inhibitory ODNs as novel therapies.

Introduction

Toll like receptors (TLRs) are a family of innate immune receptors that recognize exogenous ligands generated by viral and bacterial pathogens. In addition, they can respond to endogenous host molecules that can be produced as a consequence of tissue damage or inflammation [1]. Ten human TLRs have been identified, the majority of which are expressed at the cell surface. The exceptions are TLRs 3, 7, 8 and 9 that recognize nucleic acid sequences that are predominantly localized in the endosome [2]. Stimulation of TLRs activates nuclear factor (NF)- κ B and interferon regulatory factors (IRFs) leading to the production of cytokines and other inflammatory mediators. In addition to their role in innate immunity, TLRs have been suggested to contributing to the pathogenesis of many autoimmune diseases, including systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [3, 4].

RA is a debilitating disease affecting approximately 1 in 100 people worldwide. It is characterized by an early and progressive destruction of the joints, associated with chronic inflammation which is sustained by infiltrating immune cells [5]. The factors responsible for perpetuating this chronic cycle of inflammation are still unclear. However, data from arthritis models have demonstrated that many of the TLRs can either induce and/or maintain joint inflammation in these models. In a human ex vivo model using RA synovial membrane cultures, we previously demonstrated a role for the TLR adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and MyD88-adaptor-like (Mal) in the spontaneous production of cytokines [6]. However, which TLRs were important remained unclear, as most TLRs are upregulated in synovial tissue and peripheral blood cells from RA patients [6-14]. In subsequent experiments, TLR8 was identified as a significant contributor of TNF production in human RA synovial tissue cultures. Furthermore, combined inhibition of TLRs 3, 7, 8 and 9, resulted in greater inhibition of TNF than blocking TLR8 alone, suggesting possible additional roles for other endosomal TLRs [7].

Similar data suggesting a role for the endosomal TLRs has been produced in rodent arthritis models. In the rat pristane-induced arthritis (PIA) model, decreased expression of TLR3 via siRNA knockdown reduced disease severity, whereas a TLR9 antagonist delayed disease onset and decreased severity of disease [15, 16]. Systemic knockout or intra-articular knockdown of TLR7 in rodent collagen induced arthritis (CIA) models has been reported to decrease established disease progression and radiological measures of disease activity respectively [17, 18]. Whereas, human TLR8 transgenic mouse chimeras develop spontaneous arthritis and show an enhanced susceptibility to CIA [19]. Together these experimental models along with the human tissue studies suggest that inhibition of TLR8 and possibly other endosomal TLRs may provide a novel way to control inflammation in RA joint tissue.

Several approaches are currently being developed to inhibit endosomal TLRs as future therapeutics. One promising class of inhibitors that have been under investigation for the treatment of SLE for many years, are inhibitory oligodeoxynucleotides (ODN) containing Phosphorothioate (PTO) modifications of the backbone. They are short single stranded DNA sequences that were initially described as inhibitors of TLR9 activation, but it was later discovered that some sequences could inhibit TLR3, TLR7 or both TLR7 and 9 [20-22]. There have also been reports that inhibitory ODNs can suppress disease severity in a murine CIA model and an inflammatory arthritis model induced by intra-articular injection of CpG DNA [23, 24]. The aims of this current study were to investigate the effect of an in-house ODN (ODN1411) on TLR activation in primary human cells and spontaneous cytokine production from human RA synovial membrane cultures.

Results

ODN1411 inhibits cytokine production induced by TLR3, 7, 8 and 9 from human cells

The effect of ODN1411 on TLR function was investigated in primary human cells. ODN1411 inhibited IL-6 induced by imiquimod (TLR7 ligand) and by ODN2006 (TLR9 ligand) in primary human B cells in a dose dependent manner (Figure 1A), with no effect on cell viability (Figure 1B). At the highest dose it significantly inhibited TLR7 and TLR9 induced IL-6 by $87.59\% \pm 5.041$ ($p=0.0033$) and $84.92\% \pm 3.043$ ($p=0.0013$) respectively (Figure 1C). To examine the function of the other TLRs, primary human RASFs and M-CSF-derived macrophages were used. Activation of TLR3 stimulates different pathways in these cell types. In RASF poly IC (TLR3 ligand) activates the NF- κ B pathway where IL-6 production was measured by ELISA. In these cells, TLR3 induced IL-6 production was inhibited in a dose dependent manner by ODN1411 (Figure 1D), with no effect on cell viability (Figure 1E). In pooled data there was a $95.56\% \pm 1.803$ ($p=0.004$) inhibition with the top concentration of ODN1411, but TLR4 induced IL-6 was unaffected (Figure 1F).

Poly IC does not activate NF- κ B in primary human macrophages but does stimulate the production of Type I interferon [25]. To examine TLR3 activation in macrophages IP-10 an interferon inducible chemokine, produced on activation of TLR3 or TLR4 was measured by ELISA. ODN1411 inhibited TLR3 induced IP-10 in a dose dependent manner (Figure 2A) with no effect on cell viability (Figure 2B). At the highest concentration ODN1411 inhibited TLR3 induced IP-10 by $79.25\% \pm 14.69$ ($p=0.0327$) but did not inhibit LPS activation of TLR4 (Figure 2C). However, macrophages can also respond to a wide variety of other TLR ligands to activate NF- κ B and generate TNF. In macrophages, ODN1411 also dose dependently inhibited TNF production produced by a TLR7/8 ligand R-848 (Figure 2D), with no effect

observed on cell viability (Figure 2E). At the highest concentration ODN1411 inhibited R-848 induced TNF by 93.94% \pm 2.698 ($p=0.0008$), but not by ligands that activate TLR1/2 (Pam3), TLR4 (LPS) or TLR5 (Flagellin) (Figure 2F). TLR7 ligands do not induce cytokine production from macrophages so it is assumed that R-848 activates TLR8 in these cells [7].

ODN1411 inhibition of TLR8-induced TNF in human M-CSF-derived macrophages is not sequence-specific

To assess the importance of the CCT and GGG motifs present in ODN1411, four other ODN sequences were tested in M-CSF derived macrophages. ODN1411-rev is the reverse sequence of ODN1411, although the sequence order has been changed this ODN still contains CCT and GGG motifs. ODN1412 also contains a CCT and a GGG motif where the rest of the sequence is random, ODN1413 contains two CCT motifs where the rest of the sequence is random and the fourth sequence ODN1612 does not contain either motif. ODN1612 was used as a control ODN in a previously published collagen induced arthritis study [23]. All of the ODNs inhibited TLR8 induced TNF production to an equivalent extent to ODN1411 (Figure 3A-B), with no loss in cell viability (Figure 3C). When data was pooled from 3 independent experiments from separate donors there was a significant inhibition of TNF production for all of the ODNs tested (Figure 3D).

ODN1411 inhibits TLR8 by inhibiting phosphorylation of signaling molecules and activation of NF- κ B

Several mechanisms have been proposed for the inhibitory action of ODNs, varying from inhibition of signaling pathways, interactions with accessory molecules to a direct interaction with the TLRs [20, 26-29]. To assess the level at which the inhibition occurred, the activation of p54 Jun N-terminal Kinase (JNK), p38 mitogen-activated

protein kinases (MAPK), p42/44 extracellular signal-regulated kinase (ERK) and NF- κ B were examined; these events are required for TNF and IL-6 production [30]. LPS was used as a control in these experiments as this also results in a powerful activation of these signaling pathways in macrophages and RASFs. In addition, LPS induced cytokine production was unaffected by ODN1411 in the previous experiments. Macrophages were stimulated for 15 minutes with LPS or 30 minutes with R-848; time points previously determined to give maximal phosphorylation of these proteins in macrophages [7]. ODN1411 inhibited phosphorylation of p54 JNK, P42/44 ERK and P38 MAPK in the R-848 treated cells but no inhibition was observed in the cells treated with LPS (Figure 4A). RASFs were stimulated for 30 minutes with LPS or 2 hours with poly IC. ODN1411 inhibited phosphorylation of P42/44 ERK and P38 MAPK in the poly IC treated cells but no inhibition was observed in the cells treated with LPS (Figure 4B). In addition, downstream activation of NF- κ B was measured. R-848 but not LPS activation of NF- κ B was suppressed in macrophages treated with ODN1411 by 53.3 % \pm 14.3 (p= 0.0078) (Figure 4C). In RASFs poly IC but not LPS activation of NF- κ B was suppressed by 100 % (p= 0.0053) (Figure 4D).

ODN1411 associates directly with TLR8 in a plate binding assay

The signaling data indicated that the inhibitory effect of ODN1411 on TLR8 was an early event. It seemed unlikely to be inhibition of an early signaling molecule as the cell surface TLRs use the same signaling pathways but were not inhibited by ODN1411. Inhibitory ODNs have been reported to directly bind to TLR3 and TLR9 [20, 26]. Therefore, to investigate the possibility of a direct interaction with TLR8, increasing concentrations of R-848 were added to macrophages in combination with a fixed dose of 10.5 μ M ODN1411. The results revealed that the inhibitory effect on TNF production was significantly decreased with increasing concentrations of R-848, demonstrating a competitive inhibition by ODN1411 (Figure 5A). The competitive

nature of the inhibition suggested that this could be a direct effect of ODN1411 on TLR8.

To examine the possibility that ODN1411 could directly interact with TLR8, a chimera of the ectodomain (ECD) of TLR8 fused to murine Fc was generated. This fusion protein could be coated onto a plate to act as a binding partner for a 3' fluorescein conjugated version of ODN1411 (ODN1411-Flu). A commercially available TLR1-Fc and the appropriate human and murine immunoglobulins (Ig) were used as controls. A TLR1-Fc was chosen as we had previously observed no inhibition of TLR1/2 activation by ODN1411. The ability ODN1411-Flu to bind each protein was measured by a fluorescence plate reader (Figure 5B). ODN1411-Flu bound to the TLR8-Fc but did not bind to TLR1-Fc or the Ig controls (Figure 5B).

ODN1411 inhibits the spontaneous production of cytokines from RA membrane cultures

The ability of ODN1411 to inhibit endosomal TLR activation in human cells and our previous study indicating a role for TLR8 and possibly other endosomal TLRs in RA, made ODN1411 a good candidate to test in the human ex vivo RA model [6, 7]. This model was used for the initial studies that identified the importance of TNF in RA and is an accepted model of human RA [31]. The model uses cells derived from RA synovial membranes removed from patients during elective surgery. These cultures comprise of T cells, B cells, macrophages and synovial fibroblasts that spontaneously release cytokines without the requirement of exogenous stimulation [6]. ODN1411 was found to dose dependently inhibit spontaneous production of TNF from RA synovial membrane cultures (Figure 6A) without any effect on cell viability (Figure 6B). The top concentration of ODN1411, led to a significant inhibition of spontaneous TNF ($44.1\% \pm 17.3$, $P=0.007$), IL-1 ($65.4\% \pm 7.6$, $P=0.004$) and IP-10 ($61.9\% \pm 16.4$, $P=0.0277$) production in data pooled from 8-14 unrelated donor RA synovial membrane cultures (Figure 6C).

Discussion

This study initially set out to investigate the effect of ODN1411 on TLR signaling in primary human cells. ODN1411 inhibited the endosomal TLRs 3, 7, 8 and 9 in primary human cells. The ability of an ODN to inhibit TLRs 3, 7 or 9 was consistent with earlier studies using other inhibitory ODNs [20-22]. In addition, we discovered that ODN1411 also inhibited R-848 induction of TNF in macrophages. Although R-848 is a TLR7/8 agonist, TLR7 ligands do not activate TNF production in macrophages, suggesting that R-848 acts solely through TLR8 in these cells [7]. This is the first study to our knowledge to report inhibition of TLR8 by an inhibitory ODN and consequently of an ODN that can inhibit all of the endosomal TLRs.

A diverse range of inhibitory ODNs have been reported to suppress TLR activation, with both the sequence and PTO modification suggested as important factors for this activity. PTO modification makes the DNA backbone more resistant to degradation by DNase than a phosphodiester linkage. In addition, the DNA sugar backbone with this modification has been shown to be inhibitory against TLR9 activation even in the absence of the nitrogenous bases [32]. The presence of the triplets CCT and GGG have been demonstrated to be important for inhibition of TLR9 whereas TLR7 inhibition is considered to be sequence independent [33]. ODN1411 contained both of these triplets which may explain why it was an effective inhibitor of both TLR7 and TLR9 in B cells. However, neither of these motifs appeared to be essential for the inhibition of TLR8 induced TNF in M-CSF derived macrophages. Moreover, reversing the sequence of ODN1411 also had no effect on the ability to inhibit TLR8 demonstrating that the order of the nucleotide sequence was also not essential for the inhibitory action. Alternatively, it may be the modified DNA sugar backbone that is sufficient for this inhibition, as was reported for TLR9 [32].

The mechanism by which inhibitory ODNs block TLR function has been suggested to be through direct binding to the TLR ectodomain, disruption of downstream signaling

molecules or a direct interaction with high mobility group box (HMGB) proteins that bind nucleic acids and thereby assist in TLR signaling [20, 26-29]. An investigation into the mechanism revealed that ODN1411 inhibited R-848 induced phosphorylation of p38 MAPK, P42/44 ERK and P54 JNK in macrophages, suggesting that inhibition was an early event, possibly at the level of receptor activation. Inhibition of early signaling molecules seemed less probable given that the signaling pathways leading to MAPK activation are shared by most of the TLRs, yet ODN1411 did not suppress activation of TLRs 2, 4 or 5. It also seemed unlikely that the inhibitory actions of ODN1411 were mediated by a HMGB protein, as ODN1411 was able to inhibit the small molecular weight TLR agonists R-848 and imiquimod that would not be expected to interact with HMGB proteins. Indeed, HMGB1 deficient plasmacytoid dendritic cells have previously been shown to be unaffected compared to wild type cells upon imiquimod activation [29]. However, experimental data would be required to exclude a role for HMGB proteins.

It was also unlikely that the inhibition of endosomal TLR signaling by ODN1411 was due to ODN1411 out-competing the uptake of the TLR ligands. Activation of the small molecular weight ligand R-848 that can diffuse across the membrane was competitively inhibited by ODN1411. In addition, confocal images of M-CSF derived macrophages in the absence or presence of 42 μ M ODN1411 after a 30 minute incubation with 20 μ g/ml fluorescence labelled Poly I:C did not show any difference in the uptake of Poly IC (data not shown).

It therefore appeared most likely that the inhibitory effect was mediated via a direct interaction with the endosomal TLRs. Evidence to support this hypothesis came from two studies demonstrating direct binding of inhibitory sequences to TLR3 and TLR9 [20, 26]. In the current study, inhibition of TLR8 was competitive *in vitro* and it was observed that ODN1411 may directly bind to the ECD of TLR8. The fact that

ODN1411 did not bind to TLR1-Fc was consistent with the observation that ODN1411 did not inhibit TLR1/2 induced TNF production in macrophages. This suggested that the mechanism of TLR8 inhibition may be mediated through a direct interaction with the receptor. However, further investigation of a direct binding event using other techniques such as surface plasmon resonance assay would be required for definitive confirmation of such an interaction. Given the previous report of ODN interactions with TLR3 and TLR9 and the fact that all of the endosomal TLRs recognise nucleic acid structures as their ligands, it is conceivable that ODN1411 may also inhibit TLR3, 7 and 9 through a similar mechanism [20, 26].

A potential application for inhibitory ODNs has been suggested for the treatment of autoimmune diseases and in particular SLE [4]. Early studies using inhibitory ODNs in murine models of arthritis have demonstrated suppression of disease activity in both a collagen induced arthritis (CIA) model and an inflammatory arthritis model [23, 24]. However, in both studies the ODNs were given prophylactically prior to disease induction by intra-articular injection of CpG DNA or complete Freund's adjuvant containing mycobacterium DNA, both activators of TLR9. As a result, it is difficult to determine if disease suppression by the ODN in these studies was due to an effect on preventing disease induction or was actually having an effect on disease pathology. Instead, in this study a human culture model of RA which spontaneously releases cytokines was used. ODN1411 significantly inhibited the spontaneous production of TNF, IL-1 and IP-10. Both TNF and pro-IL-1 are induced via the MyD88 TLR signaling pathway, with pro-IL-1 later being matured by caspase 1 of the NLRP3 inflammasome, whereas IP-10 is an interferon inducible gene that lies downstream of the TRIF TLR signaling pathway as a consequence of type I IFN production [34, 35]. The inhibition of TNF was consistent with our previous study suggesting that TLR8 was a significant contributor to TNF production from these cultures. In this study, TLR8 ligands but not those that activate TLR7 or 9 were able to induce TNF from RA

synovial membrane cultures [7]. However, TLR8 does not induce IFN leading to IP-10 in human macrophages, thus the inhibition of IP-10 in the RA synovial membrane cultures may suggest the involvement of another TLR such as TLR3 which is inhibited by ODN1411 and utilizes TRIF as its adaptor protein.

In summary, the data presented here demonstrate that ODN1411 specifically inhibits endosomal TLR function in primary human cells. Inhibition of TLR8 appeared to be mediated by a direct interaction with the ectodomain and occurred in a competitive manner. Furthermore, ODN1411 suppressed the production of cytokines from a human model of RA, supporting existing data demonstrating the role of the endosomal TLRs in the chronic inflammation observed in RA joint tissue. In addition to the prospect of developing inhibitory ODNs as a therapy for SLE, this study illustrates the potential benefit of therapeutically inhibiting the endosomal TLRs with ODNs in RA.

Materials and Methods

Reagents

Cell culture reagents used were Penicillin-Streptomycin, RPMI 1640 and DMEM obtained from Cambrex (Belgium), Indomethacin from Sigma (USA) and foetal bovine serum (FBS) from PAA (Austria). The TLR ligands used were chloroform extracted *Escherichia coli* (*E.coli*) LPS, resiquimod (R-848), CpG (ODN2006) and imiquimod from Invivogen (USA). Flagellin (purified) and Pam₃cys-ser(lys)₄.3HCl (Pam3) were from Alexis (UK). All reagents were tested for LPS using the limulus amoebocyte lysate (LAL) assay from Cambrex (USA) and found to have no detectable levels of LPS [36].

The antibodies used for western blotting were anti-tubulin antibody from Sigma (USA), antibodies recognizing phosphorylated forms of JNK (p46/54), p38, and ERK (p42/44) were from Cell Signaling Technology Inc (USA). ODNs were purchased from Eurofins MWG Operon (Germany), PTO modified bases are shown as lower case; ODN1411 5'-tgCTCCCTGGACGGGTgt-3', and ODN1411-Flu 5'-tgCTCCCTGGACGGGTgt-3'- fluorescein, ODN1411-rev 5'-tgtTGGGCAGGTCCCTCgt-3', ODN1612 5'-gcTAGATGTTAGcgt-3', ODN1412 5'-tccTTAAGTTCCTGCAggt-3' and ODN1413 5'-tccTGTAAGGGTTGTgt-3'. ODN1411 shares a 90% homology with IRS 954 [22].

SF9 insect cell line, insect cell expression vector pBi-Ex, Insect GeneJuice Transfection Reagent and Ultramobius 1000 Plasmid kit were all purchased from Merck. Plasmid pC DNA3.1-Fc encoding murine IgG2a Fc was a kind gift from Dr. Douglas Golenbock (USA). Goat anti-murine IgM/G/A (H+L) was purchased from Chemicon. Protease inhibitor cocktail was purchased from Sigma (USA). Murine Ig

was purchased from Becton Dickinson (UK) and human IgG was purchased from Sigma (USA).

Cell culture

RA synovial membrane cells were isolated from patients undergoing joint replacement surgery as previously described [31, 37]. Immediately after isolation cells were cultured at 1×10^5 cells/well in 96-well tissue culture plates (Falcon, UK) in RPMI 1640 containing 10% (v/v) FBS and 100 U/ml penicillin/streptomycin. All patients gave written informed consent and the study was approved by the Riverside Research Ethics Committee REC number: 07/H0706/81. Primary human RA synovial fibroblasts (RASFs) and peripheral blood monocytes were isolated and cultured as previously described [38-40]. Macrophages were derived from monocytes after differentiation for 4 days with 100ng/ml M-CSF and incubated at 1×10^5 cells/well in 96-well tissue culture plates for experiments. Cell viability was determined by the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma, USA) [41].

Western blotting

Human M-CSF macrophages and RASFs were pre-incubated with ODN1411 for 30min prior to stimulation with TLR ligands. Cell extracts were prepared in 100 μ l of lysis buffer (1% NP-40, 150mM NaCl, 20mM Tris, pH 7.5) containing 10mM EDTA, 10mM EGTA, 1mM Na_3VO_4 , 5mM NaF and a protease inhibitor cocktail (Sigma, USA). Extracts were separated on 10% SDS-PAGE gels and proteins transferred to PVDF membrane. Membranes were blocked in 2% BSA in Tris-buffered saline containing 0.1% tween 20 (TBST) and sequentially probed with antibodies recognizing phosphorylated p38 MAPK, JNK (p54), ERK (p42/44) and tubulin. Blots

were stripped of antibody between analysis using Re-blot (Chemicon, Temecula, CA) and blocked again in 2% BSA-TBST.

Luciferase Assay.

Macrophages were infected with a recombinant adenovirus containing a NF- κ B Luciferase reporter gene (kindly provided by Dr B Davidson, University of Iowa, U.S.A.) at a MOI of 50:1. After 24 hours, cells were stimulated for 6h. The cells were washed once in PBS and lysed with 100 μ l of CAT lysis buffer (0.65%(v/v) of Nonidet P-40, 10mM Tris-HCl pH 8, 0.1mM EDTA pH 8, 150mM NaCl). Fifty μ l of cell lysate were mixed with 120 μ l luciferase assay buffer (25mM Tris-phosphate pH 7.8, 8mM MgCl₂, 1mM EDTA, 1%(v/v) Triton X-100, 1%(v/v) glycerol, 1mM DTT, 0.5mM ATP) in the well of a luminometer cuvette strip. Luciferase activity was measured with a Luminometer (Thermo Labsystems, U.K.) by adding 30 μ l of luciferin (Bright-Glo luciferase assay system; Promega, Madison, WI) per assay point.

Cloning and expression of TLR8-Fc in SF9 cells

The ectodomain of human TLR8 (aa 1-827) was amplified by PCR (primers 5'GGTACCTAAGCTTCCACCATGGAAAACATGTTC3' and 5'ATTCCAGCGGC CGCAGTGACATCTGAAACA3') and cloned into pBi-Ex using NcoI and NotI restriction sites. A sequence encoding the murine IgG2a (Fc) was excised from pCDNA3.1-Fc vector using NotI and XhoI sites and cloned at the 3' of TLR8 to create pBiEx-TLR8-Fc and verified by sequencing. The plasmid was transfected into SF9 cells using the insect GeneJuice transfection reagent according to the manufacturer's instructions. At 48 h after transfection, cells were lysed with lysis buffer (1% Nonidet P-40, 150mM NaCl, 50mM Tris-HCL, pH 7.5 and 1x protease inhibitor cocktail). After removing cell debris by centrifugation, lysates were collected.

Binding assay for TLR1-Fc, TLR8-Fc and ODN1411-Flu.

A 96 well ELISA plate was either uncoated or coated with 25µg/ml goat anti-murine Ig antibody (Millipore, UK) or 25µg/ml anti-human Ig antibody (Millipore, UK) overnight at 4°C. The plate was then blocked with 2% BSA in PBS at room temperature for 1h. TLR8-Fc, TLR1-Fc (R&D systems, USA), murine Ig (as a control for the TLR8-Fc) or human Ig (as a control for the TLR1-Fc) were added at 1µg/ml to the plate in the appropriate wells and incubated at 4°C for 2h. The plate was then washed with 0.01% PBS-Tween before adding 10.5µM ODN1411-Flu for 1h at 4 °C in the dark. After a further wash, the fluorescence intensity was measured at emission λ 485 on a FLUOstar Omega plate reader (BMG Labtech).

ELISA (enzyme-linked immunosorbent assay)

Sandwich ELISAs were employed to measure IL-1 β (R&D systems, USA), TNF, IL-6, IL-10 and IP-10 (Pharmingen, UK). Absorbance was read on a spectrophotometric ELISA plate reader (Labsystems Multiscan Biochromic) and analyzed using Ascent software V2.6 (Thermo Labsystems, Cambridge, United Kingdom).

Statistical methods

Mean, standard deviation (SD), Standard error of the mean (SEM) and statistical significance were calculated using GraphPad version 3 (GraphPad Software Inc., USA). For statistical analysis, a two tailed Student's t-test of paired data was used with a 95% confidence interval. SEM was used for pooled experimental data whilst SD was used in graphs showing representative experiments.

**** = p <0.0001, *** = p <0.001, ** = p <0.01 and * = p <0.05.

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Figure legends

Figure 1. ODN1411 inhibits TLR3, -7 and -9-induced IL-6 production from primary human cells. (A-C) Primary human B cells were pre-incubated with varying concentrations of ODN1411 for 30 minutes prior to stimulation with imiquimod or ODN2006 for 24 hours, after which (A) IL-6 production was measured and (B) an MTT cell viability assay was performed. A dose response is shown as mean \pm SD of triplicates from one experiment representative of three performed with unrelated donors. (C) Pooled IL-6 data are shown as mean \pm SEM from 3 independent experiments in unrelated donors as the percentage of the maximal ligand response which is represented as a dotted line. (D, E) Primary RASFs were pre-incubated with varying concentrations of ODN1411 for 30 minutes prior to stimulation with 20 μ g/ml Poly IC for 18 hours after which (D) IL-6 production was measured and (E) an MTT cell viability assay was performed. A dose response is shown as mean \pm SD of triplicates from one experiment representative of three performed with unrelated donors. (F) Pooled percentage inhibition data are shown for RASFs pre-incubated with 42 μ M ODN1411 for 30 minutes prior to stimulation with 20 μ g/ml Poly IC or 10 ng/ml LPS for 18 hours. Data are the mean \pm SEM from 3 independent experiments in unrelated donors shown as the percentage of the maximal ligand response which is represented as a dotted line. All conditions were repeated in triplicate within each experiment. US, unstimulated ** $p < 0.01$, *** $p < 0.001$, two tailed Student's t-test.

Figure 2. ODN1411 inhibits TLR3- and TLR8-induced cytokine production from primary human macrophages. Primary M-CSF macrophages were pre-incubated with varying concentrations of ODN1411 for 30 minutes prior to stimulation with TLR ligands. (A, B) Cells were stimulated with 20 μ g/ml Poly IC for 24 hours after which (A) IP-10 production was measured and (B) an MTT cell viability assay was performed. A dose response is shown as mean \pm SD of triplicates from one

experiment representative of three performed with unrelated donors. (C) Pooled percentage inhibition data are shown for cells pre-incubated with 42 μ M ODN1411 for 30 minutes prior to stimulation with 20 μ g/ml Poly IC or 10 ng/ml LPS for 24 hours. Data are the mean \pm SEM from 3 independent experiments in unrelated donors shown as the percentage of the maximal ligand response which is represented as a dotted line. (D) Cells were stimulated with 1 μ g/ml R-848 for 6 hours after which (D) TNF production was measured and (E) an MTT cell viability assay was performed. A dose response is shown as mean \pm SD of triplicates from one experiment representative of three performed with unrelated donors. (F) Cells were stimulated with 100 ng/ml PAM3, 10 ng/ml LPS, 100 ng/ml Flagellin or 1 μ g/ml R-848 for 6 hours. Pooled percentage inhibition data are shown as mean \pm SEM from 3 independent experiments in unrelated donors as the percentage of the maximal ligand response which is represented as a dotted line. All conditions were repeated in triplicate within each experiment. US, unstimulated, * $p < 0.05$, *** $p < 0.001$, two tailed Student's t-test.

Figure 3. Inhibition of TLR8-induced TNF by ODN1411 is not sequence-specific in human M-CSF derived macrophages. Macrophages were either left untreated or incubated with (A) 42 μ M ODN1411, 42 μ M of two random sequences ODN1412 and ODN1413 or 42 μ M ODN1612 for 30 min, or (B) a titration of ODN1411 or ODN1411-rev, before stimulation with 1 μ g/ml R-848 for 6 hours. (C) Cell viability was measured by a MTT assay in experiments A-B. (A-C) Data shown as mean \pm SD of triplicates from one experiment representative of three performed with unrelated donors. (D) Pooled percentage inhibition data are shown as the percentage of the maximal ligand response which is represented as a dotted line. These experiments were performed in the presence of 42 μ M each of ODN1411, ODN1411-rev,

ODN1412, ODN1413 or ODN1612. Data are pooled from 3 independent experiments performed in triplicate in unrelated donors and are shown as mean \pm SEM. US, unstimulated, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two tailed Student's t-test.

Figure 4. ODN1411 inhibits activation of TLR8 signaling in primary human M-CSF macrophages and RA synovial fibroblasts. (A) Macrophages were either left untreated or incubated with varying concentrations of ODN1411 for 30 min before stimulation with R-848 for 30 min or LPS for 15 min. (B) RASFs were either left untreated or incubated with varying concentrations of ODN1411 for 30 min before stimulation with Poly IC for 2 hours or LPS for 30 min. (A, B) Lysates were examined for phosphorylation of p54 JNK, p38 MAPK and p42/44 ERK with tubulin as a loading control where indicated. All data are representative of three separate experiments from unrelated donors. (B) Images are from the same experiment but have had irrelevant lanes removed from the middle of each gel. (C) Macrophages and (D) RA synovial fibroblasts, were infected with a virus containing a NF- κ B luciferase reporter gene at a MOI of 50:1. After 24 h the cells were either left untreated or incubated with ODN1411 for 30 min before stimulation with 10ng/ml LPS, 1 μ g/ml R-848 or 20 μ g/ml Poly IC for 6 hours prior to measurement of luciferase activity. Pooled percentage inhibition data are shown as mean \pm SEM from five macrophage donors and three rheumatoid synovial fibroblast donors as the percentage of the maximal ligand response which is represented as a dotted line. ** $p < 0.01$, *** $p < 0.001$, two tailed Student's t-test.

Figure 5. ODN1411 competitively inhibits R-848-induced TNF production and interacts directly with the TLR8 ectodomain. (A) Primary human M-CSF macrophages were incubated with media containing R-848, with media alone or in

the presence of ODN1411 for 6 hours before measuring TNF production. Pooled percentage inhibition of the TNF production in the absence of ODN1411 are shown as mean \pm SEM from 3 independent experiments in unrelated donors, the maximal ligand response is represented as a dotted line. (B) TLR8-Fc, TLR1-Fc, murine Ig or human Ig were coated on wells of an ELISA plate at 1 μ g/ml before addition of 10.5 μ M ODN1411-Flu. Bound ODN1411-Flu was measured at λ 485. Data are pooled from 3 independent experiments. * p <0.05, a two tailed Student's t-test.

Figure 6. ODN1411 inhibits spontaneous production of TNF, IP-10 and IL-1 from RA synovial membrane cultures. Human RA synovial membrane cultures were incubated for 24 hours in the presence of media alone or media containing ODN1411. (A) Dose response of ODN1411-induced inhibition of spontaneous TNF was measured by ELISA, with (B) matched cell viability data from the same sample. Data shown as mean \pm SD of triplicates from one experiment representative of four performed with unrelated donors. (C) Production of TNF, IL-1 and IP-10 was measured by ELISA and data are shown for 8-14 independent experiments from independent RA donors. All experiments were performed in triplicate with the average plotted from each donor. * p <0.05, two tailed Student's t-test.