Competition by inhibitory oligonucleotides prevents binding of CpG to C-terminal TLR9

The MIT Faculty has made this article openly available. Please share how this access benefits you. Your story matters.

<table>
<thead>
<tr>
<th>Citation</th>
<th>Avalos, Ana M., and Hidde L. Ploegh. “Competition by inhibitory oligonucleotides prevents binding of CpG to C-terminal TLR9.” European Journal of Immunology 41, no. 10 (October 6, 2011): 2820-2827.</th>
</tr>
</thead>
<tbody>
<tr>
<td>As Published</td>
<td><a href="http://dx.doi.org/10.1002/eji.201141563">http://dx.doi.org/10.1002/eji.201141563</a></td>
</tr>
<tr>
<td>Publisher</td>
<td>Wiley Blackwell</td>
</tr>
<tr>
<td>Version</td>
<td>Author's final manuscript</td>
</tr>
<tr>
<td>Accessed</td>
<td>Fri Mar 08 19:45:59 EST 2019</td>
</tr>
<tr>
<td>Citable Link</td>
<td><a href="http://hdl.handle.net/1721.1/84991">http://hdl.handle.net/1721.1/84991</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>Creative Commons Attribution-Noncommercial-Share Alike</td>
</tr>
<tr>
<td>Detailed Terms</td>
<td><a href="http://creativecommons.org/licenses/by-nc-sa/4.0/">http://creativecommons.org/licenses/by-nc-sa/4.0/</a></td>
</tr>
</tbody>
</table>
Competition by Inhibitory Oligonucleotides Prevents Binding of CpG to C-terminal TLR9

Ana M. Avalos and Hidde L. Ploegh
Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA

Summary

TLR9 recognizes unmethylated CpG-containing DNA commonly found in bacteria. Synthetic oligonucleotides containing CpG-motifs (CpG ODNs) recapitulate the activation of TLR9 by microbial DNA, whereas inversion of the CG dinucleotide within the CpG motif to GC (GpC ODN) renders such ODNs inactive. This difference cannot be attributed to binding of ODNs to the full-length TLR9 ectodomain, as both CpG and GpC ODNs bind comparably. Activation of murine TLR9 requires cleavage into an active C-terminal fragment, which binds CpG robustly. We therefore compared the ability of CpG and GpC ODNs to bind to full-length and C-terminal TLR9, and their impact on cleavage of TLR9. We found that CpG binds better to C-terminal TLR9 when compared to GpC, despite comparable and low binding of both ODNs to full-length TLR9. Neither CpG nor GpC ODN affected TLR9 cleavage in murine RAW 264.7 cells stably expressing TLR9-Myc. Inhibitory ODNs (IN-ODNs) block TLR9 signaling, but how they do so remains unclear. We show that IN-ODNs do not impede TLR9 cleavage but bind to C-terminal TLR9 preferentially, and thereby compete for CpG ODN binding both in RAW cells and TLR9-deficient cells transduced with TLR9-Myc. Ligand binding to C-terminal fragment thus determines the outcome of activation through TLR9.

Keywords

TLR9; CpG ODN; inhibitory ODN; C-terminal TLR9

Introduction

Toll-Like Receptor 9 (TLR9) is an intracellular DNA sensor specific for unmethylated CpG motifs, defined as purine-purine-C-G-pyrimidine-pyrimidine [1]. TLR9 recognition of such sequences, abundantly present in bacterial DNA, triggers a signaling cascade that activates innate and adaptive immune responses [2]. Synthetic oligonucleotides with CpG motifs (CpG ODNs) constructed on a phosphorothioate backbone evoke TLR9-dependent responses and have been instrumental for our current understanding of TLR9 activation and function in mammalian cells [3]. Stimulatory CpG ODNs have their counterpart in oligonucleotides that are identical in composition but with an inversion of the CpG sequence, to GpC. CpG and GpC ODNs with phosphorothioate backbone have similar binding affinities for full-length TLR9 at physiologically relevant concentrations (0.01-100 nM) [4, 5], therefore the discrimination of...
such minor sequence variations is difficult to reconcile with intact receptor-ligand interactions. In antigen presenting cells of the myeloid lineage, TLR9 resides in the ER and moves to endolysosomal compartments upon addition of CpG ODN [4, 6]. TLR9 undergoes proteolytic conversion upon arrival in endolysosomes, a step required for activation [7, 8]. CpG ODNs bind better to cleaved TLR9 than to full length TLR9 [7, 8], suggesting that processed TLR9 is the physiologically relevant form responsible for sequence recognition and activation. Expression of the C-terminal fragment of TLR9 is sufficient to endow TLR9−/− BMDCs with the ability to respond to CpG [8, 9]. Whether C-terminal TLR9 can distinguish between CpG and GpC sequences is not known.

In addition to stimulatory and non-stimulatory ODNs, sequences that block responses to CpG DNA were originally discovered in adenoviruses [10]. Many such inhibitory ODNs (IN-ODNs), diverse in sequence, inhibit CpG and/or TLR9 activation with varying degrees of specificity and potency (reviewed in [11]). IN-ODNs were grouped into four classes [11] based on sequence requirements. Class I IN-ODNs contain the most potent and specific TLR9 inhibitory ODNs, which block CpG responses in vitro and in vivo [12-16]. These IN-ODNs include a previously identified inhibitory motif characterized by CC(T)(XXX)3-5GGG [17, 18]. How IN-ODNs decrease TLR9 responses is not known. Neither a decrease in surface binding nor uptake of CpG [15, 16, 19] can account for inhibition by IN-ODN: both CpG and IN-ODNs are taken up equally well [13, 15]. IN-ODN and CpG bind to full-length TLR9 with comparable affinities [20] and thus a competitive mechanism of inhibition, supported by functional data [12, 20] was proposed. Alternatively, binding of IN-ODNs to intact TLR9 could prevent access of proteases to the proteolytic site in the TLR9 molecule, inhibit cleavage and so compromise its activation.

The different outcomes for application of stimulatory, non-stimulatory and inhibitory ODNs cannot be fully explained by their comparable binding to full-length TLR9 [20]. Since TLR9 requires proteolytic conversion for ligand binding and activation, we hypothesized that ODN binding to C-terminal TLR9 may be a better correlate for the outcome of the response. Thus, we evaluated the ability of stimulatory, non-stimulatory and inhibitory ODNs to modulate TLR9 cleavage, to interact with C-terminal TLR9, and correlated ODN binding with functional outcomes of TLR9 activation.

Results

GpC ODN fails to efficiently bind C-terminal TLR9

Upon proteolytic conversion of TLR9, synthetic oligonucleotides containing CpG motifs (CpG ODNs) bind significantly better to the C-terminal TLR9 fragment when compared to full length TLR9 [7, 8]. We evaluated the ability of non-stimulatory ODNs containing an inversion in the CpG motif (GpC ODNs) to bind both intact and C-terminal TLR9, and compared it to CpG binding. We incubated RAW 264.7 macrophages that stably express Myc-tagged TLR9 [8] with increasing concentrations of biotinylated CpG (1826 ODN) and its control GpC ODN. We recovered bound materials on streptavidin (SA)-coated beads and carried out a digestion with PNGase F to remove complex type N-linked glycans and so improve resolution by SDS-PAGE. Binding of ODN to full-length or C-terminal TLR9 was visualized by immunoblot using an anti-Myc antibody. Both bio-CpG and bio-GpC bound to full-length TLR9 comparably, albeit weakly [4, 5, 20]. However, bio-GpC ODN bound less avidly to C-terminal TLR9 than did bio-CpG ODN (Figure 1A left panel). Decreased binding of bio-GpC was not due to less-efficient uptake of this ODN when compared to bio-CpG, as both ODNs could be recovered equally with SA beads when analyzed in TBE-urea acrylamide gels (Supplemental Figure 1). We confirmed that CpG ODN promoted TNFα production, while GpC ODN failed to do so [15] (Figure 1A right panel). Preferential CpG ODN binding (and activation) was also observed when ODNs bearing the same sequences as
CpG and GpC but containing phosphodiester bonds were delivered to RAW-TLR9 Myc cells using the transfection reagent DOTAP (Supplemental Figure 2). C-terminal TLR9 can thus distinguish the CG to GC inversion in the sequence of these ODNs whether constructed on a phosphodiester or a phosphorothioate backbone.

In APCs of myeloid origin, TLR9 resides in the ER, and trafficks to endolysosomal compartments upon addition of CpG [4, 6]. In these compartments, TLR9 cleavage occurs [7, 8]. In unstimulated Myc-tagged TLR9-expressing RAW macrophages, a sizable fraction of TLR9 appears to traffic to endolysosomes, since the C-terminal fragment of TLR9 is readily detected in pulse-chase experiments also in the absence of CpG [8]. We hypothesized that addition of CpG to RAW macrophages would enhance cleavage of TLR9 by stimulating the movement of full length TLR9 to lysosomal compartments, whereas addition of GpC would not affect cleavage. We therefore performed pulse-chase experiments where RAW cells expressing Myc-tagged TLR9 were pulsed for 2 h and chased for 0, 2 or 5 hours, in the absence or presence of CpG or GpC. TLR9 was recovered by immunoprecipitation with anti-Myc antibodies, and digested with PNGase F. We observed no differences in cleavage of TLR9 in cells treated with CpG compared to untreated controls (Figure 1B). At rest, a significant pool of C-terminal TLR9 must already be available for activation by ODNs, and addition of CpG ODN does not enhance trafficking of TLR9 in these cells.

**Inhibitory ODNs do not affect TLR9 cleavage**

DNA sequences with CpG inhibitory activity (IN-ODNs) were originally identified in adenoviral serotypes as ODNs capable of suppressing responses to E.coli DNA [10]. The mechanism by which IN-ODNs block TLR9 responses has not been elucidated. Since TLR9 requires proteolytic cleavage for activation, we asked whether inhibitory ODNs prevented TLR9 activation by affecting its proteolytic conversion. To test this possibility, we used the Class I inhibitory sequences 2088 and G-ODN. ODN 2088 blocks CpG-dependent IL-6 production, apoptosis and proliferation in B cells [16, 21], is protective in vivo in a mouse model of diabetes [22] and counteracts the acute inflammatory response triggered by adenoviral infection [23]. The IN-ODN G-ODN blocks CpG responses in murine RAW macrophages and BMDC in vitro, and in human pDCs. G-ODN is also protective in vivo in a mouse model of cytokine-mediated lethal shock [15]. To test whether 2088 and G-ODN blocked TLR9 cleavage, we performed pulse-chase experiments on Myc-TLR9 expressing-Raw macrophages in the presence of these IN-ODN, or in the presence of the cysteine protease inhibitor z-FA-FMK [24]. Treatment with IN-ODN did not change the cleavage profile of untreated cells, unlike treatment with z-FA-FMK, which blocks TLR9 cleavage (Figure 2). The apparent molecular weight of the C-terminal fragment was indistinguishable for all conditions examined and thus the site of cleavage is unlikely to be affected by inclusion of the different ODNs. IN-ODN 2088 on occasion appeared to promote cleavage at a slightly higher rate than seen for G-ODN or in untreated cells, but this was not observed consistently (Figure 2B). We conclude that inhibitory ODNs affect neither the rate of TLR9 cleavage nor the identity of the cleavage products generated.

**IN-ODNs bind C-terminal TLR9 preferentially**

Inhibitory ODN (IN-ODN) may block CpG responses through a competitive mechanism [12, 20]. We hypothesized that IN-ODNs may bind to C-terminal TLR9 without leading to activation, and thus not only prevent signal transduction but also binding of stimulatory CpG ODNs. Since IN-ODNs interact with intact TLR9, we compared binding of increasing concentrations of biotinylated versions of the IN-ODNs 2088 and G-ODN to both full-
length and C-terminal TLR9. Both ODNs showed significant binding to C-terminal TLR9 (Figure 3A). To compare binding of CpG, GpC and IN-ODNs to C-terminal TLR9, we incubated RAW macrophages with 1 μM each of biotinylated CpG, GpC and IN-ODNs. Inhibitory ODNs bind to C-terminal TLR9 better than CpG ODN (Figure 3B). A biotinylated version of the TLR4 ligand LPS failed to bind TLR9 in either full-length or its cleaved form (Figure 3B). Comparable amounts of biotinylated CpG, GpC and 2088 ODNs were found inside cells by SA-precipitations and detection in TBE-Urea Acrylamide gels. However G-ODN was found in higher concentrations inside cells (Supplemental Figure 1). Binding of IN-ODN to C-terminal TLR9 may therefore block activation simply through competitive binding to cleaved TLR9.

IN-ODNs block binding of CpG to C-terminal TLR9

To test whether IN-ODN may block TLR9 activation through competitive binding, we incubated TLR9-Myc-expressing RAW macrophages with increasing concentrations of unlabelled 2088 or G-ODN for 15 min, and then added 1 μM biotinylated CpG ODN for 2 h. Binding to C-terminal TLR9 was detected by recovery on streptavidin beads followed by immunoblotting using an anti-Myc antibody. Addition of G-ODN or 2088 inhibited binding of biotinylated CpG to C-terminal TLR9 (Figure 4A top two panels) in a manner that correlated with their ability to block TLR9-dependent TNFα production in response to CpG ODN (Figure 4B, top two panels). As expected, based on the low level of binding of GpC ODN to C-terminal TLR9, addition of GpC ODN did not affect binding of biotinylated CpG ODN to C-terminal TLR9 (Figure 4A bottom panel), but modestly blocked CpG ODN responses at high concentrations (Figure 4B bottom panel and [15]).

To eliminate the potential confounding effect of endogenous TLR9 in binding of BIO-CpG to TLR9, we transduced bone marrow-derived macrophages (BMDM) from TLR9−/− mice with retrovirus expressing Myc-tagged TLR9. TLR9-deficient cells transduced with TLR9-Myc responded to CpG ODN, and this activation was blocked by addition of IN-ODN 2088 (Figure 5A). As expected, addition of non-stimulatory GpC ODN did not affect the cytokine response of TLR9−/− BMDM transduced with TLR9-Myc. BIO-CpG bound preferentially to C-terminal TLR9, and this binding was prevented by addition of unlabelled 2088 but not by addition of GpC ODN (Figure 5B). We conclude that IN-ODN block activation of TLR9 responses through competitive binding to C-terminal TLR9.

Discussion

Activation of TLR9 requires two steps: ligand binding to the receptor and initiation of signaling by recruitment of MyD88 to the TIR domains present in the cytoplasmic region of TLR9 [25]. The presence of CpG motifs or CG repeats in both synthetic and natural DNA sequences is required for TLR9 signaling [1, 26]. So far it has been not possible to correlate TLR9 function with ODN binding to the intact TLR9 ectodomain. We propose that binding to C-terminal TLR9 and not to full-length TLR9 is the first step toward activation of TLR9. CpG ODN fails to activate TLR9 because it is unable to bind cleaved TLR9. In contrast, IN-ODNs bind C-terminal TLR9 but are unable to trigger responses due to the lack of CpG motifs. CpG ODNs both bind and include immunostimulatory CpG sequences and therefore induce activation. It is noteworthy that C-terminal TLR9 discriminates between the orientation of the CG repeat in the CpG motif, considering the overall similar properties of CpG and GpC ODNs. This result underscores the importance of cleavage for fine-tuning of sequence-specific recognition of ODNs by TLR9.

Perhaps unexpectedly, CpG ODN did not enhance cleavage of TLR9 in RAW cells even though translocation of TLR9 to lysosomes in human pDCs and mouse macrophages has been shown to be CpG dependent [4]. The pool of cleaved TLR9 present in the
endolysosomes in resting macrophages appears to be sufficient for CpG to promote activation, as this ODN reaches these compartments within 30 min of stimulation [27]. In this regard, RAW macrophages behave very much like primary murine immune cells of myeloid and lymphoid origin, where a significant amount of cleaved TLR9 is already found at rest (Brinkmann, Avalos, Ploegh et al, manuscript in preparation).

GpC ODNs bind only weakly to C-terminal TLR9, which accounts for their inability to fully block CpG binding to C-terminal TLR9, or to significantly decrease activation by CpG ODN at suboptimal and physiologically relevant concentrations (0.1-1 μM), in spite of the reported potential inhibitory effects exerted by these GpC at high concentrations [4, 20].

Binding of IN-ODNs to full-length TLR9 in all cases was very weak or undetectable, (Figure 3, and results not shown), even though the lysates used for immunoprecipitation contained comparable amounts of full-length and C-terminal TLR9 (Figure 1A and 3, lower panels). As shown for CpG ODNs, IN-ODNs may rapidly traffic to endolysosomes and bind preferentially to the existing pool of C-terminal TLR9, leaving little available ODN to bind the remaining intact TLR9 in these compartments. Consistent with this idea and with the results shown above, Class I IN-ODNs cause a decrease in colocalization of TLR9 and CpG in vesicles of possible endolysosomal origin [13].

Binding alone to C-terminal TLR9 is not sufficient for activation. In the case of IN-ODN 2088 and G-ODN, binding to C-terminal TLR9 did not translate into signaling and activation, possibly due to the absence of a canonical CpG motif, or a significant number of CG repeats in these ODNs. The identification of the minimal sequence required for binding to C-terminal TLR9 was beyond the scope of this manuscript, but preferential binding of IN-ODNs to C-terminal TLR9 may be facilitated by the formation of G-tetrads between ODN molecules [28], leading to larger structures able to obstruct CpG binding due to steric hindrance. Thus, the presence of the inhibitory motif CC(T)(XXX)3-5GGG may allow IN-ODN binding, and an increased number of G residues may favor interaction and/or uptake. The direct binding results shown here eliminate possible non-specific inhibitory effects due to interactions with other receptors for G-rich ODNs, such as scavenger receptors, as has been shown for other G-rich inhibitory ODNs [18]. Thus, the direct correlation between competition for binding and inhibition of cytokine production shown here, as well as the direct binding data, further clarify the action of at least 2088 and G-ODN on TLR9. The specificity of action of 2088 in spite of the possible formation of G-tetrads is corroborated by the finding that a deaza modification of a single G in ODN 2088, which should prevent G-tetrad formation, did not affect its inhibitory activity [16].

The deoxyribose backbone is claimed to be responsible for activation and binding of ODN to intact TLR9 irrespective of sequence [29]. However, sequence-dependent TLR9 activation of B cells by natural DNA sequences has also been recorded [26], and differences in concentration and uptake mechanisms may account for these discrepancies. Macrophages are less efficient at taking up phosphodiester backbone ODNs (PO-ODNs) when compared to their phosphorothioate counterparts [30] and therefore this system is not typically amenable for binding studies with PO-ODNs. To overcome this handicap, we delivered CpG and GpC ODNs containing phosphodiester bond using the transfection reagent DOTAP, a reagent used before to deliver TLR9 ligands to the endolysosome [29]. We and found that still C-terminal TLR9 could discriminate between CpG and GpC motifs independently of the nature of the backbone, clearly demonstrating that C-terminal TLR9 can distinguish these subtle sequence differences present in these ODNs.

Interestingly, mutations in N-terminal domain of TLR9 rendered the receptor inactive, suggesting that the N-terminal domain may exert some regulatory role on the activity of
TLR9 [31], perhaps by controlling the folding pathway of the nascent TLR9 polypeptide. The definitive answer as to how synthetic oligonucleotides and natural DNA sequences bind and induce conformational changes that lead to TLR9 signaling can be obtained only upon resolution of the structure of the TLR9 fragments resulting from ectodomain cleavage in the absence or presence of its ligands. The results shown here underscore the importance of studying the physical properties of C-terminal TLR9 as well as intact TLR9 and their agonists and antagonists to better understand the function of this receptor.

Materials and Methods

Cells and Reagents

RAW 264.7 macrophages stably expressing TLR9 with a Myc tag have been previously described [8]. Phosphothioate and phosphodiester backbone oligonucleotides 1826 (CpG) (5′-TCCATGACGTTCCTGACGTT-3′) and 1826 control (GpC) (5′-TCCATGAGCTTCCTGAGCTT-3′), and phosphothioate ODNs 2088 (5′-TCCTGGCGGGGAAGT-3′), G-ODN (5′-CTCCTATTGGGGTTTCCTAT-3′) and their biotinylated counterparts were from Integrated DNA Technologies (IDT). z-FA-FMK and LPS were from Sigma. Biotinylated LPS was from Invivogen.

Pulse chase analysis

RAW macrophages were starved in DME medium lacking cysteine and methionine and without serum for 1h. Cells were then incubated with DME lacking cysteine and methionine in the presence [35S] methionine and [35S] cysteine (Perkin Elmer) at 0.2 mCi/ml and dialyzed serum for 2 hours (“pulse”). Cells were washed three times with 10% FBS normal DME medium and incubated (“chased”) for the indicated times. During all three periods (starvation, pulse and chase) cells were incubated with ODNs or z-FA-FMK as indicated in the text. Cells were then lysed in 1% digitonin, radioactive labeling quantified using a scintillation counter, and Myc-TLR9 was immunoprecipitated and reimmunoprecipitated (IP and reIP) using anti-Myc antibody and protein G beads in lysates bearing equal cpm. Eluates from Protein G beads were digested using PGNase F and protein separated by SDS-PAGE and visualized in autoradiogram. Densitometry was performed by exposing gels to phosphorimager plates and analyzing the intensity of bands using the BAS-2500 Phosphoimager and Multi Gauge software (Fujifilm).

Binding of biotinylated ODNs to TLR9

TLR9-Myc expressing RAW macrophages or BMDM from TLR9−/− mice transduced with TLR9-Myc were stimulated for 2 h with biotinylated oligonucleotides and washed once with PBS. For competition studies, TLR9-Myc expressing RAW macrophages or BMDM from TLR9−/− mice transduced with TLR9-Myc were stimulated with unlabelled 2088, G-ODN or GpC for 15 min, then biotinylated CpG was added and stimulated for another 2 h. Plates were washed once with PBS before lysis. Cells were lysed using 1% digitonin buffer containing protease inhibitors. Biotin-ODN/protein conjugates were precipitated from lysates containing equal protein concentrations using streptavidin (SA) beads and digested with PGNase F. Immunoprecipitates and total lysates were run in SDS-PAGE, immunoblotted onto PVDF membranes and developed using anti-Myc antibody.

Retroviral transduction

HEK293T cells were transfected with plasmids encoding TLR9-Myc, VSV-G and Gag-Pol proteins. After 48 hours, supernatants containing virus were collected and filtered through a 0.45 μm filter. Virus-containing supernatants were added to bone marrow cells from TLR9−/− mice one day after they were harvested from the bone and incubated for 24 h in
the presence of 5% M-CSF. On the next day, cells were given fresh media in 5% M-CSF and cultured for 5 days. On day 6 cells were assayed for TLR9 function and ODN binding. The viral transduction efficiency was ~15%.

**TNFα production assays**

For detection of secreted TNFα, 0.5×10⁶ cells were seeded in 96-well plates, stimulated for 2 h and supernatants harvested. Titers of TNFα were determined using the OptEIA TNFα ELISA kit (Becton Dickinson) according to manufacturers specifications. For detection of intracellular TNFα, BMDM from TLR9−/− mice transduced with TLR9-Myc retrovirus were stimulated for 2 h in the presence of Brefeldin A. Cells were then trypsinized, permeabilized with 0.5% saponin in 5% FBS/PBS buffer, incubated with Alexa647-labelled anti-TNFα antibody (BD Biosciences) for 30 min and analyzed in LSRII flow cytometer (Becton Dickinson). MFI were calculated using the FlowJo software (Tree Star).

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

The authors wish to thank Boyoun Park for scientific discussion and technical support. This work was supported with grants from NIH to H.L.P. A.M.A. is a recipient of the Merck-MIT Postdoctoral Fellowship.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ODN</td>
<td>Oligonucleotide</td>
</tr>
<tr>
<td>TLR9</td>
<td>Toll-Like Receptor 9</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation Primary Response Gene 88</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 Receptor</td>
</tr>
<tr>
<td>z-FA-FMK</td>
<td>Benzyloxycarbonyl-Phenyl-Alanyl-Fluoromethylketone</td>
</tr>
</tbody>
</table>

**References**


Eur J Immunol. Author manuscript; available in PMC 2013 August 19.


Figure 1. CpG ODN bind better to C-terminal TLR9 when compared to GpC ODN

(A) (Left) Top panel: RAW macrophages expressing TLR9-Myc were incubated with increasing concentrations of biotinylated CpG or GpC or left untreated (−) for 2 h. CpG-bound materials were recovered using streptavidin beads and analyzed by immunoblotting with anti-Myc antibody. Bottom panel: TLR9-Myc levels were detected in total lysates by immunoblotting with anti-Myc antibody. (Right) RAW macrophages expressing TLR9-Myc were stimulated with increasing concentrations of CpG and GpC for 2 h, supernatants harvested and TNFα production measured by ELISA. (B) TLR9-Myc expressing RAW macrophages incubated with 1 μM CpG or GpC or left untreated (no addition) were pulsed for 2 h with [35S] cysteine/methionine and chased for 0, 2 and 5 h. Proteins were immunoprecipitated and reimmunoprecipitated using anti-Myc antibody, and analyzed by SDS-PAGE and autoradiography. Individual band intensity was analyzed by phosphorimaging, and shown are the intensity ratios of C-terminal (C-term)/intact TLR9. In A and B, noted are the locations of full length (intact) and C-terminal (C-term) TLR9. Results in (A) are representative of six and two independent experiments for left and right panels respectively. In (B), results shown are representative (left) and the mean±SD (right) of two independent experiments.
Figure 2. IN-ODN do not affect TLR9 cleavage

(A) RAW macrophages expressing TLR9-Myc were incubated with 1 μM of the IN-ODN 2088 and G-ODN, 20 μM of the cysteine protease inhibitor z-FA-FMK, or left untreated (no addition) during 1 h starvation, 2 h pulse with [35S] cysteine/methionine and 0, 2 and 5 h chase. Proteins were immunoprecipitated and reimmunoprecipitated using anti-Myc antibody, and analyzed by SDS-PAGE and autoradiography. Noted are the locations of full length (intact) and C-terminal (C-term) TLR9. (B) Individual band intensity was analyzed by phosphorimaging, and shown are the intensity ratios of C-terminal (C-term)/intact TLR9. Results shown are representative (A) and the mean±SEM (B) of three independent experiments.
Figure 3. IN-ODN bind to C-terminal TLR9

(A) RAW macrophages expressing TLR9-Myc were incubated with increasing concentrations of biotinylated G-ODN (left, 0.1, 0.25 and 0.5 μM) or 2088 (right, 0.1, 0.25 and 1 μM) for 2 h or left untreated (−). Biotin ODN/protein conjugates were recovered with streptavidin beads and analyzed by immunoblotting using anti-Myc. (B) RAW macrophages expressing TLR9-Myc were incubated with 1 μM biotinylated CpG, GpC, 2088, G-ODN (G-O), 100 ng/ml of biotinylated LPS or left untreated (−). Precipitation was performed using SA beads and bound materials analyzed by immunoblotting using anti-Myc. In A and B, bottom panels show TLR9-Myc levels in total lysates. Noted are the locations of full length (intact) and C-terminal (C-term) TLR9. Shown are representative results of three independent experiments.
**Figure 4. IN-ODNs block binding of CpG ODN to C-terminal TLR9**

(A) RAW macrophages expressing TLR9-Myc were incubated for 15 min with increasing concentrations of unlabeled 2088 (top), G-ODN (middle) and GpC (bottom) ODN before addition of 1 μM biotinylated CpG (A, *CpG) or media (−) and incubated for 2h. CpG-bound materials were recovered using streptavidin beads and analyzed by immunoblotting using anti-Myc antibody. (B) Cells pretreated under the same conditions as in (A) were incubated with 0.1 μM CpG or 20 ng/ml LPS for 2h. Cell supernatants were analyzed for production of TNFα by ELISA. In (A), noted are the locations of full length (intact) and C-terminal (C-term) TLR9. Shown are representative experiments of five (2088), four (G-ODN) and two (GpC) in (A), and two (2088), four (G-ODN) and two (GpC) in (B).
Figure 5. IN-ODNs prevent binding and activation of TLR9 in BMDM from TLR9−/− transduced with TLR9-Myc

Bone marrow cells from TLR9−/− mice were transduced with retrovirus expressing TLR9-Myc. At day 5 post-transduction, in (A) TLR9−/− BMDM transduced with TLR9-Myc (white bars) or WT BMDM (black bars) were unstimulated or preincubated with 1 μM 2088 or GpC for 15 min, and then stimulated with 1 μM of CpG or with 0.1 μM R848 for 4 h in the presence of 10 μg/mL Brefeldin A. Intracellular TNFα was measured by flow cytometry and median fluorescence intensity (MFI) calculated. Data shown is the mean±SD MFI of two independent experiments after subtracting MFI obtained in the unstimulated condition (222 for TLR9−/− and 262 for WT). In (B), TLR9−/− BMDM were either not transduced (−) or transduced with TLR9-Myc. Transduced cells were unstimulated (−) or preincubated with media (−), 0.5 μM 2088 or GpC for 15 min before addition of 0.5 μM BIO-CpG and incubated for 2 h. Cells were lysed and precipitation with SA-beads was performed. SA-recovered materials were analyzed by immunoblot using anti-Myc antibody. Shown is a representative result from two independent experiments.