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Synthetic Oligodeoxynucleotides Containing Suppressive TTAGGG Motifs Inhibit AIM2 Inflammasome Activation

John J. Kaminski,* Stefan A. Schattgen,* Te-Chen Tzeng,* Christian Bode,† Dennis M. Klinman,‡ and Katherine A. Fitzgerald*

Synthetic oligodeoxynucleotides (ODNs) comprised of the immunosuppressive motif TTAGGG block TLR9 signaling, prevent STAT1 and STAT4 phosphorylation and attenuate a variety of inflammatory responses in vivo. In this study, we demonstrate that such suppressive ODN abrogate activation of cytosolic nucleic acid-sensing pathways. Pretreatment of dendritic cells and macrophages with the suppressive ODN-A151 abrogated type I IFN, TNF-α, and ISG induction in response to cytosolic dsDNA. In addition, A151 abrogated caspase-1-dependent IL-1β and IL-18 maturation in dendritic cells stimulated with dsDNA and murine CMV. Inhibition was dependent on A151’s phosphorothioate backbone, whereas substitution of the guanosine residues for adenosine negatively affected potency. A151 mediates these effects by binding to AIM2 in a manner that is competitive with immune-stimulatory DNA and as a consequence prevents AIM2 inflammasome complex formation. Collectively, these findings reveal a new route by which suppressive ODNs modulate the immune system and unveil novel applications for suppressive ODNs in the treatment of infectious and autoimmune diseases. The Journal of Immunology, 2013, 191: 3876–3883.

The innate immune system provides an essential first line of defense against infection. Innate immune cells detect pathogens through distinct classes of pattern-recognition receptors including the TLRs, the C-type lectin receptors, the RIG-like helicases, the nucleotide-binding oligomerization domain-like receptors, and the PYHIN receptors. These pattern-recognition receptors respond to conserved pathogen- and danger-associated molecular patterns, allowing rapid recognition and response to infectious agents. Activated receptors initiate signaling cascades that lead to the production of cytokines, chemokines, and type I IFNs, all of which are vital for controlling pathogen loads directly and coordinating adaptive immune responses. Unrestricted or improper activation of the innate immune system can have dire consequences. Uncontrolled inflammation can cause extensive tissue damage, exacerbate septic shock, and contribute to the development of autoimmunity. Therefore, it is essential to establish balance between activation and suppression to ensure an appropriate and effective innate response.

Detection of DNA by the innate immune system is an important mechanism by which pathogens are recognized to turn on protective immunity. Recognition of DNA is complex and can be influenced by a variety of factors including sequence, secondary structure, subcellular localization, and covalent modification. Hypomethylated CpG motifs found in bacteria and certain viruses are detected by TLR9 (2, 3). In contrast, cytosolic DNA can be detected by a number of DNA sensors including IFN-γ-inducible protein-16 (IFI16) and AIM2, two members of the PYHIN protein family; DDX41, a member of the DEXDc helicase family; cytoplasmic GMP-AMP synthase, a recently identified nucleotidyltransferase; DNA-dependent activator of IFN-regulatory factors, as well as RIG-I via an RNA polymerase III–transcribed intermediate (4–9).

IFI16 was first identified as a potential intracellular DNA sensor in a screen using a 70-bp DNA motif derived from the vaccinia virus genome to affinity purity binding partners. Unterholzner et al. (10) found that IFN-β induction by this vaccinia virus 70-mer was independent of TLR, DNA-dependent activator of IFN-regulatory factors, and Pol III signaling but was attenuated following IFI16 knockdown. Further analysis revealed IFI16 also mediated IFN-β induction following transfection with a 60-bp motif derived from the HSV genome as well as by HSV-1 infection. Similarly, targeting of the IFI16 murine ortholog p204 attenuated IFN-β and TNF-α production in response to these dsDNA motifs and HSV-1, suggesting a role in both IFN regulatory factor (IRF) 3- and NF-κB–dependent inflammatory pathways. IFI16 mediated this response by engaging the crucial signaling component stimulator of IFN gene, leading to the activation of TNFR-associated factor family member–associated NF-κB activator–binding kinase 1 and nuclear translocation of IRF3 and p65 (10). Both IFI16 and p204 contain a DNA-binding HIN200A and HIN200B domain as well as a pyrin domain (11, 12). In contrast to IFI16, another member of the PYHIN family, AIM2, signals via assembly of an inflammasome.

The inflammasome is a large complex that provides a platform for the activation of caspase-1, an enzyme that cleaves the immature ILs pro–IL-1β and pro–IL-18 into their active forms. There

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Abbreviations used in this article: ASC, adaptor molecule apoptotic speck protein containing caspase activation and recruitment domain; BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; HA, hemagglutinin; HMGB1, high-mobility group box 1; IFI16, IFN-γ-inducible protein-16; IRF, IFN regulatory factor; MCMV, murine CMV; MHC, major histocompatibility complex; NF-κB, nuclear factor-κB; NP-40, Nonidet P-40; ODN, oligodeoxynucleotide; PD, phosphodiester; PO, phosphothioate; poly(dA:dT), poly(deoxyadenylic–deoxythymidylic) acid; qPCR, quantitative PCR; SeV, Sendai virus; sup ODN, suppressive oligodeoxynucleotide.
are distinct types of inflammasomes, differentiated by their protein constituents, activators, and effectors. In many cases, an inflammasome contains a nucleotide binding and oligomerization leucine-rich repeat protein. In addition, our laboratory and others (5, 6, 13, 14) have recently reported that the AIM2 protein directly binds to cytosolic bacterial and viral dsDNA, leading to the formation of an AIM2 inflammasome complex. The AIM2 inflammasome is activated in response to infection by bacteria such as Listeria monocytogenes as well as the viral pathogen murine CMV (MCMV), in which it plays an essential role in controlling early viral replication (5). AIM2 is composed of a DNA-binding HIN200C domain and a pyrin domain, which recruits caspase-1 via the adaptor molecule apoptotic speck protein with caspase activation and recruitment domain (ASC) (4, 6, 13–15).

Importantly, certain DNA sequences such as the TTAGGG repeat commonly found in mammalian telomeric DNA can serve to suppress innate immune activation. The therapeutic potential of these suppressive oligodeoxynucleotides (sup ODNs) has been demonstrated in murine models of inflammatory arthritis, toxic shock, systemic lupus erythematosus, atherosclerosis, and silica-induced pulmonary inflammation (16–20). Given the known roles of type I IFNs and the proinflammatory cytokines IL-1β and IL-18 in the development of many of these diseases, we set out to examine the effect of sup ODNs on cytosolic innate immune sensors, particularly those leading to inflammasome signaling (21). Synthetic sup ODNs were first recognized for their ability to prevent TLR9 activation by binding to unmethylated CpG DNA (22). Interestingly, the potency of these sup ODNs was found to be strongly affected by sequence, a phenomenon not explained by their relative avidity to the TLR9 ectodomain (23). In addition Shirota et al. (24) have shown that sup ODNs prevent Th1 differentiation in wild-type and TLR9-deficient CD4+ cells alike, suggesting that their biological activity is independent of their interaction with TLR9 and instead involves as yet undefined receptor(s). In this study, we demonstrate that treatment with the sup ODN A151, an ssDNA species composed of four repeats of the hexanucleotide TTAGGG motif, blocks cytosolic DNA-driven IFN and inflammatory cytokine production by binding to IFI16 and AIM2, respectively. A151-mediated inhibition of cytosolic DNA sensing was specific to dsDNA signaling and had no effect on NLRP3-mediated inflammasome activation, RIG-I, or NPS signaling. The inhibitory effect of A151 was dependent on a phosphorothioate backbone unless otherwise specified by IDT Technologies (Coralville, IA) (25–27). A 3′-biotin tag was added to the sup ODN sequence for pull-downs. MCMV (Smith strain) was a gift from R. Welsh (University of Massachusetts Medical School), L. monocytogenes (clinical isolate 10403s) was from V. Boyartchuk (University of Massachusetts Medical School), HSV-1 (7134) was a gift from D. Knipe (Harvard Medical School). Sendai virus (SeV; Cantrell strain) was purchased from Charles River Laboratories (Wilmington, MA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Genejuice was from Novagen (Madison, WI). ZVAD-FMK was from Calbiochem (San Diego, CA). Full-length human AIM2 was obtained by PCR from cDNA and fused into pEFBOS-C-terminal-FLAG/HIS as described (5, 6). Murine pro-IL-1β was obtained by PCR from cDNA and fused into pEFBOS-C-terminal-GLu/FLAG as described (5). Expression plasmids (pcET) encoding human ASC and caspase-1 were from Millenium Pharmaceuticals (Cambridge, MA). The expression plasmid containing the AIM2 HIN200 domain only (pCMV) was from T. Xiao (National Institutes of Health/National Institute of Allergy and Infectious Diseases).

**Mice**

C57BL/6 mice were from The Jackson Laboratory (Bar Harbor, ME). All experiments were conducted with mice maintained under specific pathogen-free conditions in the animal facilities at the University of Massachusetts Medical School and were carried out in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee.

**Cell culture, stimulation, and ELISA**

For reconstitution of the AIM2 inflammasome, HEK293T cells (5 × 10⁴ cells/ml) in 96-well plates were cotransfected in triplicate with Genejuice (4 μg/ml) with plasmids encoding pro-IL-1β and the expression plasmids listed previously (total DNA 200 ng) as described by Hornung et al. (6). Cultures were incubated for 2 h, then exposed to sup ODN (3 μM) or left untreated; 24 h later, supernatants and lysates were collected. Bone marrow–derived macrophages (BMDM) and bone marrow–derived dendritic cells (BMDC) were generated as described (6, 28). For experiments measuring IFI16/p204 activation, sup ODN was added 1 h before stimulation. For experiments measuring AIM2/NLRP3 activation, cells were primed with LPS (200 ng/ml) for 2 h prior to the addition sup ODN or CpG-ODN then incubated for an additional hour before secondary stimulation. ATP (5 mM) or nigericin (10 μM) was added 1 h before harvesting supernatants and lysates. Poly(dA:dT) was transfected using Lipofectamine 2000 (Invitrogen) at a concentration of 0.5 μg/ml, 6 h before harvesting. Cells were infected with MCMV and HSV-1 at a multiplicity of infection (MOI) of 10. Cells were exposed to SeV at 200 IU/ml. Cells were challenged with L. monocytogenes at an MOI of 5 for 1 h. Cells were then washed twice, and media containing gentamicin (100 μg/ml) was added. All infections were incubated for 16 h before harvest. Supernatants from cell-culture experiments were assayed for IL-1β (BD Biosciences, Franklin Lakes, NJ) and IL-18 (R&D Systems, Piscataway, NJ) by sandwich ELISA.

**Nanostring and quantitative RT-PCR experiments**

Cells were treated as described above, and RNA was purified using an RNeasy Mini Kit (Qiagen). Total RNA was hybridized to a custom gene expression CodeSet and analyzed on an nCounter Digital Analyzer (Nanostring Technologies). Counts were normalized to internal spike-in and endogenous controls per Nanostring Technologies’ specifications. A pseudocount was added to all values such that the smallest value in the dataset was equal to 1. Values were log-transformed and displayed via heat map (Euclidean clustering) generated using the ggplot package within the open source R software environment. cDNA was synthesized from total RNA, and quantitative RT-PCR analysis was performed as previously described (29). Gene expression is shown as a ratio of gene copy number per 100 copies of β-actin + SD.

**Immunoblotting**

Supernatants were harvested by precipitation with methanol chloroform extraction. Cells were washed twice with PBS and lysed using 1% Nonidet P-40 (NP-40) buffer. Immunoblotting was performed as described (5). Anti-Flag (M2) and anti-hemagglutinin (HA; HA-7) was from Sigma-Aldrich, anti-murine caspase-1 p10 (sc-514) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-murine caspase-1 p20 (5B10) from eBioscience, anti-murine IL-1β from R&D Systems (Minneapolis, MN), and anti-mouse high-mobility group box 1 (HMG1; 3E8) was from BioLegend (San Diego, CA).

**ASC oligomerization assay**

ASC oligomerization assay was performed as described with minor modifications (30). In brief, BMDM (1 × 10⁶ cells/condition) were primed with LPS (200 ng/ml) for 2 h prior to the addition of A151 or C151 (3 μM). After 30 min, 25 μM ZVAD-FMK was added, followed 30 min later by poly(dA:dT) transfection (0.5 μg/ml) using Lipofectamine 2000. Cells were washed and lysed with 1% NP-40 lysis buffer 3 h after poly(dA:dT) challenge. Lysates were cleared by centrifugation at 3000 g. Macromolecular structures were then pelleted by centrifugation at 45000 g, resuspended...
in 50 μl CHAPS buffer, and cross-linked with disuccinimidyl carbonate 2 μM (Pierce Thermo Scientific, Rockford, IL). Supernatant from this step was saved and run as lysate in ASC blots. The pellet was washed, resuspended in Laemmli buffer, incubated overnight with shaking at 4°C, then boiled, and run on a 12% SDS-acrylamide gel as the cross-linked fraction. Blots were probed with anti-ASC Ab (N-15-R; Santa Cruz Biotechnology).

Confocal microscopy

Confocal microscopy was performed using a Leica SP2 AOBBS confocal laser scanning microscope (Leica Microsystems). Immortalized murine macrophages stably expressing AIM2– or ASC–citrine constructs were plated at 2 × 10⁶ cells/ml on glass-bottom 35-mm culture dishes (MatTek, Ashland, MA) and allowed to adhere. A151 or C151 was added 1 h prior to transfection with poly(dA:dT) or exposure to nigericin. Two hours after poly(dA:dT) challenge or 30 min after nigericin exposure, cultures were photographed. The total number of fluorescent cells was recorded in >20 independent fields representing >1000 cells and divided into those displaying diffuse cytoplasmic staining and those exhibiting speck formation. Graphs quantifying speck formation were calculated by combining data from three independent experiments.

Pulldown assay

For pulldown of endogenous AIM2 and IFI16, immortalized murine macrophages (5 × 10⁶ cells/condition) were lysed in an ice-cold high-salt lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris HCl [pH 7.9], 100 mM EDTA, 10% glycerol, 10 mM NaF, DTT, and protease inhibitor mixture as described previously) (31). Cell debris was removed by centrifugation, and total lysate was incubated with 6 μg 3′-biotinylated A151 and prewashed streptavidin-agarose beads (50% w/v) for 2 h at 4°C. For competition assays, an increasing amount of poly(dA:dT) was mixed with biotinylated A151 before addition to the lysate. Bead pellets were washed, boiled in Laemmli buffer, and run on a 12% SDS-polyacrylamide gel. Blots were probed with polyclonal anti-mouse AIM2 Ab from Genentech (4G9; San Francisco, CA), monoclonal anti-mouse IFI16 (IFI-230; Abcam), and anti-mouse β-actin (AC-74; Sigma-Aldrich). For pulldown of the AIM2 HIN200 domain only, HEK293T (3 × 10⁶ cells/condition) were transfected with the AIM2 HIN200 domain containing pCMV vector using GeneJuice (4 μl/ml) and incubated 24 h before being lysed and processed as described above. Blots probed with anti-HA (HA-7) were from Sigma-Aldrich.

Statistical analysis

ELISA experiments are presented as the mean ± SD from three independent biological replicates and are representative of three experiments. Supplemental Fig. 1 represents combined data from three independent experiments and was analyzed using an unpaired Student t test with Welch correction via Prism 4 Software (GraphPad, San Diego, CA). The p values < 0.05 were considered significant.

Results

A151 has broad anti-inflammatory activities against cytosolic DNA-sensing pathways

To explore the immunosuppressive potential of sup ODN A151, BMDC were pretreated with A151 and then transfected with the synthetic dsDNA poly(dA:dT). Total RNA was isolated and subjected to multiplex gene expression analysis using nCounter (Nanostring). Poly(dA:dT) treatment of dendritic cells increased mRNA levels of a panel of inflammatory cytokines, type I IFNs and IFN-stimulated genes as well as other immune mediators and regulators. With very few exceptions, pretreatment with sup ODN A151 abrogated these responses, resulting in an expression profile that closely resembled media controls (Fig. 1A). In contrast, A151 had no inhibitory effect on induction of inflammatory genes following LPS treatment. To further define the specificity of the A151-mediated suppression, BMDC and BMDM were exposed to HSV-1 or MCMV, two herpesviruses, and IFN-βmediated suppression, BMDC and BMDM were exposed to HSV-1 (MOI 10), HSV-1 (MOI 10), and SenV (200 IU/ml). IFN-β and β-actin mRNA were measured by qPCR. IFN-β induction is represented relative to untreated controls. BMDC were stimulated with LPS (200 ng/ml), 300 ng of poly(dA:dT) [p(dA:dT)] complexed with Lipofectamine 2000, MCMV (MOI 10), and SenV (200 IU/ml). TNF-α and β-actin mRNA was measured by qPCR. TNF-α induction is represented relative to untreated controls.

FIGURE 1. Gene expression analysis reveals A151-mediated suppression of inflammatory gene expression in mouse cells. (A) RNA from mouse BMDC treated as described was subjected to nCounter Nanostring analysis. Gene expression profiles are displayed as a heat map (log10 transformed) with hierarchical clustering indicated by dendrogram. BMDC (B) and BMDM (C) were stimulated with LPS (200 ng/ml), 300 ng of poly(dA:dT) complexed with Lipofectamine 2000, MCMV (MOI 10), HSV-1, and SenV (200 IU/ml). IFN-β and β-actin mRNA were measured by qPCR. IFN-β induction is represented relative to untreated controls. BMDC were stimulated with LPS (200 ng/ml), 300 ng of poly(dA:dT) complexed with Lipofectamine 2000, and SenV (200 IU/ml). TNF-α and β-actin mRNA was measured by qPCR. TNF-α induction is represented relative to untreated controls.

A151 blocks AIM2 inflammasome activation in response to cytosolic dsDNA

We next explored the inhibitory potential of the sup ODN A151 on activation of the inflammasome. BMDC pretreated with A151 were exposed to a panel of inflammasome ligands and IL-1β secretion measured by ELISA. Pretreatment with A151 had no effect on IL-1β production in response to the NLRP3 ligands silica, nigericin, or ATP, whereas the response to the AIM2 ligand poly(dA:dT) was reduced (Fig. 2A). Combined data from three independent experiments using BMDC demonstrate that this inhibitory effect is significant (Supplemental Fig. 1). The pattern of AIM2-specific inhibition was also observed in BMDM and the human monocytic cell line THP-1 (Fig. 2B, 2C). A151 also suppressed IL-18 secretion
in response to poly(dA:dT) but not nigericin in BMDC (Fig. 2D). Western blot analyses confirmed the reduction of cleaved IL-1β in the supernatants of A151-treated BMDC challenged with poly(dA:dT) (Fig. 2E). Furthermore, these blots revealed a decrease in caspase-1 activity, as evidenced by the reduced levels of the active caspase-1 p10 and p20 subunits following A151 treatment. Importantly, exposure to A151 did not diminish levels of pro–IL-1β and pro–caspase-1 in cellular lysates, suggesting A151 treatment blocked the activity of the AIM2 inflammasome rather than modulating expression of the caspase-1 substrate. Secretion of the alarmin HMGB1 also requires caspase-1 activation, and much like IL-1β and IL-18, HMGB1 release into the supernatant was also suppressed by A151 (Fig. 2E) (32, 33). In addition to cytokine processing, AIM2 activation leads to a caspase-1–dependent, inflammatory form of programmed cell death known as pyroptosis (5, 34). Treatment with A151 prevented cell death following poly(dA:dT) exposure, indicating that A151 blocks AIM2-mediated pyroptosis in addition to cytokine maturation (Supplemental Fig. 2A, Fig. 2B).

Previous studies have shown that the deoxyguanosine residues found within A151’s TTAGGG motif play a role in suppression of CpG-induced TLR9 signaling as well as STAT1 and STAT4 phosphorylation. To determine if A151’s inhibitory effects were sequence dependent, we also monitored IL-1β and IL-18 production as well as cell death in all of these conditions using C151, a construct in which the guanosine triplet had been replaced with an adenosine sequence. In contrast to the inhibitory effect of A151 on DNA-induced cytokine secretion, C151 had no significant inhibitory effect (Fig. 2A–E). At a concentration of 3 μM, C151 was able to reduce pyroptosis, though, unlike A151, it was not able to completely block cell death (Supplemental Fig. 2A). The human HEK293T cell line has proven a useful tool for studying inflammasome activation. Transient transfection of plasmids encoding Aim2, Asc, and pro–IL-1β along with caspase-1 leads to the formation of a functional AIM2 inflammasome complex and IL-1β cleavage (5). Moreover, HEK293T are devoid of endogenous TLR expression, allowing us to examine the effects of sup ODN A151 in a system free of TLR signaling (35). Exposure of AIM2-reconstituted HEK293T cells to A151 drastically reduced IL-1β cleavage (Fig. 2F). Suppression was not observed when IL-1β cleavage was driven by caspase-1 overexpression alone, indicating that A151 inhibits inflammasome activation at a step prior to caspase-1 activation (data not shown). No inhibitory effect was observed in the C151-treated control.

Our laboratory has previously shown that AIM2 is essential for inflammasome activation in response to the viral pathogen MCMV (5). Secretion of IL-1β by BMDC challenged with MCMV was also markedly reduced by A151 pretreatment (Fig. 2G). A number of inflammasome receptors including NLRP3, NLRC4, and AIM2 have been implicated in the IL-1β response to L. monocytogenes (5, 36–39). A151 treatment reduced IL-1β production in BMDC treated as described, and IL-18 was measured by ELISA. (E) Immunoblotting of IL-1β, caspase-1, and HMGB1 in the supernatants and lysates from BMDC. (F) HEK293T cells were transfected with empty vector (pEFBOS) or 50 ng pro–IL-1β-FLAG (pEFBOS) together with 1 ng pro–Caspase-1 (pC1) (★ received 50 ng), 1 ng ASC (pC1), and 1 ng AIM2 (pEFBOS) as shown. A151/C151 (3 μM) was added 2 h posttransfection, and 24 h later, lysates were collected and immunoblotted with anti-Flag Ab. BMDC were primed with LPS (200 ng/ml) and challenged with MCMV or silica (G) and listeria or nigericin (H). IL-1β secretion into the supernatant was analyzed by ELISA. Data are presented as mean ± SD from three biological replicates representative of three experiments.
responding to L. monocytogenes, a reduction proportional to that observed in AIM2-deficient cells (Fig. 2H) (5). Collectively, these findings suggest that A151 blocks AIM2-mediated inflammasome signaling in response to the true dsDNA ligand poly(dA:dT) as well as pathogens such as MCMV and L. monocytogenes.

A phosphorothioate backbone is required for A151-mediated AIM2 inhibition, whereas sequence affects ODN potency

Although C151 had no significant inhibitory effect on DNA-induced inflammasome signaling at the doses used in the experiments above, we did find that at higher concentrations C151 could attenuate DNA-induced IL-1β and IL-18 secretion (Fig. 3A, 3B). However, A151 with an EC50 of 0.360 μM was 20 times more potent than C151 (EC50 = 6.16 μM). In contrast to this suppressive effect, CpG-ODN 2336, an A-class CpG oligonucleotide with a backbone consisting of approximately two-thirds phosphodiester (PD) linkages, had no such inhibitory effect (Supplementary Fig. 2B). To determine whether the phosphothioate (PO) backbone affected A151-mediated inhibition, this sup ODN was synthesized with a phosphodiester backbone (A151-PD) and tested in BMDC and BMDM (Fig. 3C, 3D). Unlike A151, pretreatment with A151-PD had no effect on IL-1β release or cell death following poly(dA:dT) challenge. Thus, a PO backbone is essential for the inhibition of A151-mediated IL-1β processing and pyroptosis, whereas the deoxyguanosine content positively affected potency.

A151 prevents ASC dimerization in vitro

We next wanted to understand the molecular basis for the suppressive effect of A151 on AIM2 inflammasome activation. We first examined the ability of A151 to modulate AIM2-ASC inflammasome complex assembly. To do so, BMDM were challenged with poly(dA:dT) in the presence or absence of A151, and whole-cell lysates were cross-linked and fractionated by sequential centrifugation. Following exposure to poly(dA:dT), we observed an increase in the presence of ASC dimers in the macromolecular pellet, a finding consistent with inflammasome activation (Fig. 4A) (30). Pretreatment with A151 reduced ASC dimer formation to levels observed in media controls, whereas C151 led to a modest decrease, although A151-PD had no effect. Consistent with these observations, A151-treated cells maintained ASC in its soluble, monomeric form, suggesting that A151 blocks recruitment of ASC to AIM2, preventing inflammasome assembly.

A151 blocks inflammasome assembly in an AIM2–citrine reporter cell line

A defining feature of inflammasome signaling is the formation of a large, multiprotein complex in the cytosol. This complex can be as large as 2 μm in size and offers a unique opportunity to analyze signaling by tracking the localization of inflammasome components in living cells (34). To visualize the formation of the AIM2 inflammasome, we employed an immortalized murine macrophage cell line stably expressing AIM2–citrine and monitored inflammasome activation in live cells. In resting macrophages, the AIM2–citrine fusion protein was diffusely cytoplasmic; however, stimulation with poly(dA:dT) caused ~50% of these cells to form fluorescent punctate structures or specks, indicative of inflammasome assembly (Fig. 4B). Pretreatment with A151 strongly inhibited the formation of AIM2–citrine specks in our poly(dA:dT)-treated reporter line; instead, the AIM2–citrine protein remained dispersed throughout the cytoplasm. A similar pattern of inhibition was observed using macrophages expressing an ASC–citrine construct (Fig. 4C). These results are consistent with our in vitro data and suggest that sup ODN A151 blocks the ability of AIM2 to engage downstream signaling components necessary for aggregation.

A151 binds to AIM2

A151 has been shown to exert its suppressive effects through direct association with stimulatory CpG DNA as well as by disrupting STAT signaling pathways (17, 22). AIM2 binds DNA via its C-terminal HIN200 domain, thus releasing it from a resting, autoinhibited conformation and allowing inflammasome formation (40). To determine whether A151 can interact with endogenous AIM2, immortalized murine macrophages were lysed, incubated with biotinylated A151, and exposed to streptavidin beads. These pull-down studies revealed that A151 was capable of interacting with AIM2 even in the absence of an activating stimulus (Fig. 4D, Supplemental Fig. 3A). Moreover, inclusion of an increasing amount of poly(dA:dT) in the binding step resulted in a proportional decrease in AIM2 recovery, suggesting A151 competes with poly(dA:dT) for AIM2 binding. To determine if the AIM2 HIN200 domain was sufficient for binding to A151, HEK293T cells were transfected with a pCMV vector containing the HIN200 domain. As expected, the HIN200 domain alone was able to pull down A151 (Supplemental Fig. 3B). Unterholzner et al. (10) have previously demonstrated that IFN-β induction and NF-κB activation in response to cytosolic DNA or HSV-1 infection is dependent on IFI16. Therefore, we also examined if A151 could bind IFI16. Similar to what we had seen above, we were also able to pull down IFI16 from THP1 cells using biotinylated A151, but not with biotinylated C151 or biotinylated A151 (PD) (Fig. 4E).

Discussion

This work is the first, to our knowledge, to identify a DNA species capable of preventing activation of cytosolic DNA-sensing pathways. Moreover, it establishes a novel mechanism through which the sup ODN A151 mediates suppression of innate immune responses via interaction with members of the IFI20X/IFI16 (PYHN) family. Our data show that A151 added to the media of primary
dendritic cells and macrophages prevents DNA induced IRF3- and NF-κB–dependent gene induction in response to cytosolic dsDNA as well as infection with HSV-1 and MCMV. As described previously, A151 did not inhibit LPS-driven cytokine production nor did it affect IFN-β induction by SeV via the RIG-I pathway (17). Notably, our Nanostring analysis revealed A151 treatment had little effect on the expression profile of resting cells, suggesting that A151 does not induce an anti-inflammatory state but rather blocks DNA sensing. A151 also prevented AIM2-mediated caspase-1 activation in response to dsDNA challenge, thereby reducing IL-1β/IL-18 processing, HMGB1 release, and pyroptotic cell death. Mechanistically, A151 appeared to prevent ASC dimerization in macrophages and decreased the formation of cytoplasmic inflammasome specks in both AIM2– and ASC–citrine reporter lines. Further insight came from experiments showing biotinylated A151 was able to pull down AIM2, suggesting that this sup ODN interacts with AIM2 to block inflammasome assembly. Consistent with these findings, A151 also inhibited AIM2-dependent IL-1β cleavage in BMDC challenged with the viral pathogen MCMV, which has been shown to be entirely dependent on AIM2 and also had a partial role in the response to the bacterial pathogen L. monocytogenes. Previously, Sato et al. (20) uncovered a role for A151 in the inhibition of silica-induced inflammation. Unfortunately, this paper did not examine processing of IL-1β or IL-18, two cytokines directly activated by the NLRP3 inflammasome following silica exposure. As the authors demonstrated, silica treatment leads to significant host cell death in vitro, and we theorize that the inhibitory effects observed were due to A151’s effect on cytosolic sensing of host dsDNA released from dead and dying cells rather than a direct effect on NLRP3 activation.

In keeping with A151’s effects on TLR9 activation, conversion to a PD backbone completely abolished A151-mediated inhibition of IFI16 and AIM2 at the concentrations tested (41). Sequence also proved to be important; substitution of A151’s guanosine triplet with adenosine residues reduced construct potency by 94%. The immunomodulatory affects of G-rich ODNs generated with a phosphorothioate backbone was first reported nearly a decade ago (42). Since then, the structural requirements for maximal inhibition have been defined in a number of in vitro models (24, 43). Interestingly, Ashman et al. (24) have recently reported that sequence-specific differences in sup ODN activity cannot be accounted for by their relative affinity to TLR9. We theorize that A151’s interaction with members of the PYHIN family may explain many of the in vitro effects of sup ODNs that cannot be explained by interaction with TLRs (24) as well as the robust and global changes often observed in disease models.

Recently, a crystal structure of the AIM2 HIN200C domain complexed with dsDNA was reported (40). This study indicated that DNA recognition was accomplished largely through electrostatic interaction patterns and are accompanied by graphs quantifying speck formation as a percent of the total fluorescent cells. Graphs are the combined data from three independent experiments. (D) Immortalized macrophage lysates were subjected to pulldown analysis using A151 (6 μg) with (lane 1) or without (lane 4) 3′-biotinylation. An increasing amount of poly(dA:dT) was included in lanes 2 (6 μg) and 3 (18 μg), and whole lysate was run in lane 5. Western blots were probed for the presence of AIM2. (E) Live THP-1 cells were pretreated with A151 or 3′-biotinylated A151, C151 or A151 (PD) (3 μM) and challenged with 0.5 μg/ml of poly(dA:dT) complexed with Lipofectamine 2000 for 2 h. Lysates were divided in half, with one half subjected to pulldown analysis using streptavidin-agarose beads and the other run as whole lysate. Western blots were probed for the presence of IFI16.
actions between the HIN domain and the DNA’s sugar–phosphate backbone and was therefore independent of sequence. Our pull-down studies indicated that the PO backbone is required to interact with IFI16 and AIM2. We also found that biotinylated phosphorothioate A151 pulled down more AIM2 and IFI16 from macrophores than did the equivalent C151 construct, in keeping with these constructs’ relative potencies. Moreover, the inclusion of increasing amounts of poly(dA:dT) during the binding step led to a proportional decrease in AIM2 recovery indicating an affinity-driven competition with poly(dA:dT). Results were similar whether biotinylated sup ODN was added to the media or directly to lysates, suggesting sequence affects AIM2 binding rather than sup ODN uptake. One explanation for this apparent sequence dependence is that A151 as an ssDNA species may theoretically allow HIN200 residues greater access to its nucleotide bases than a double-stranded construct, although we cannot rule out the potential influence of sequence-specific self-aggregation. Finally, we show that the AIM2 HIN200 domain alone is sufficient to mediate the interaction with A151. Thus, we propose a mechanism in which A151 competes with dsDNA for binding to IFI16 and AIM2 but does not itself promote AIM2 or IFI16 aggregation and activation of downstream signaling events.

Administration of A151 has been used in murine models of shock, lupus, inflammatory arthritis, and atherosclerosis. A growing body of evidence suggests cytoplasmic DNA sensing contributes to the pathogenesis of many of these same syndromes. The identification of A151 as an inhibitor of the AIM2 and IFI16 signaling of other which A151 may mediate many of its beneficial effects and invites further investigation into A151’s role in the signaling of other PYHIN family members.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: BMDCs were primed with LPS (200ng/ml; LPS prime alone is control) and challenged with nigericin (10μM) or 300ng of poly(dA:dT) complexed with lipofectamine 2000 (untreated). Cells were pretreated with A151 or C151 (3μM) as indicated and IL-1β secretion into the supernatant was measured by ELISA. Data are presented as mean ± SD from three independent experiments. * p<0.0001

Supplemental Figure 2: A, BMDMs were left unstimulated (media) or challenged with 300ng of poly(dA:dT) complexed with lipofectamine 2000 (untreated) for sixteen hours. Cells challenged with poly(dA:dT) were pretreated with A151, C151 or A151 (PD) and viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay. B, BMDC were primed and challenged as above and the effects of pretreatment with either A151, C151, 2336 (3μM) are shown.

Supplemental Figure 3: A, Live immortalized macrophages were pretreated with biotinylated A151 or C151 (3μM) and challenged with 0.5 ug/ml of poly(dA:dT) complexed with lipofectamine 2000 (untreated) for two hours. Lysates were divided in half, one half subjected to pull-down analysis using streptavidin-agarose beads and the other was run as whole lysate. Western blots were probed for the presence of AIM2. B, HEK293T cells were transfected with HA-tagged human AIM2 (HIN200 domain only; pCMV). Lysates were subjected to pull-down analysis using streptavidin-agarose beads and biotinylated A151. Western blots were probed for HA.
Supplemental Figure 1

BMDC

IL-1β ng/ml

Control  p(dA:dT)  Nigericin

untreated  A151  C151

*
Supplementary Figure 2

A

BMDM

Cell Viability

media untreated A151 C151 A151 (PD)

poly(dA:dT)

B

BMDC

IL-1β ng/ml

untreated A151 C151 Cop 2268 poly(dA:dT)

poly(dA:dT)
Supplementary Figure 3

A

- IP: DNA
- IB: AIM2
- IB: AIM2

Pull Down
Whole Lysate

B

- IP: DNA
- IB: HA
- IB: HA

Pull Down
Whole Lysate