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m6A methylation potentiates cytosolic dsDNA recognition in a sequence-specific manner

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Nucleic acid sensing through pattern recognition receptors is critical for immune recognition of microbial infections. Microbial DNA is frequently methylated at the N^6 position of adenines (m6A), a modification that is rare in mammalian host DNA. We show here how that m6A methylation of 5′-GATC-3′ motifs augments the immunogenicity of synthetic double-stranded (ds)DNA in murine macrophages and dendritic cells. Transfection with m6A-methylated DNA increased the expression of the activation markers CD69 and CD86, and of *Ifnβ*, *iNos* and *Cxcl10* mRNA. Similar to unmethylated cytosolic dsDNA, recognition of m6A DNA occurs independently of TLR and RIG-I signalling, but requires the two key mediators of cytosolic DNA sensing, STING and cGAS. Intriguingly, the response to m6A DNA is sequence-specific. m6A is immunostimulatory in some motifs, but immunosuppressive in others, a feature that is conserved between mouse and human macrophages. In conclusion, epigenetic alterations of DNA depend on the context of the sequence and are differentially perceived by innate cells, a feature that could potentially be used for the design of immune-modulating therapeutics.

1. Introduction

Innate immune cells can recognize invading pathogens through pattern recognition receptors (PRRs) [1]. This feature allows for rapid recognition of invading pathogens and for a swift onset of immune responses. De-regulation of PRR sensing signalling is associated with pathogenic and autoimmune conditions [2,3].

A wide range of PRRs localize in the endosomes and in the cytosol, where they detect bacterial and viral nucleic acids [3–5]. In the endosome, Toll-like receptors (TLRs) sense single-stranded (ss) and double-stranded (ds)RNA (TLR7 and TLR3, respectively), as well as conserved pathogen-derived ssDNA structures (TLR9) [3,6]. Engaging these TLRs leads to the induction of proinflammatory cytokines like Interleukin (IL)-6, Tumour necrosis factor (TNF)-α and type I Interferons (IFNs) in an NF-kB- and MYD88/TRIF-dependent manner [6–9]. In the cytosol, viral dsRNA is recognized by the RIG-I-like family of receptors (RLRs) and MDA5 [5]. Through the adaptor

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protein IPS1/MAVS, proinflammatory cytokines and type I IFNs are produced [5,10]. dsDNA present in the cytosol is primarily recognized by cGAS and AIM2, which promote the production of type I IFNs and IL-1β through STING and ASC, respectively [11,12]. Other DNA sensors include RNA polymerase III, IFI16 and DAI [4,5].

Recognition of pathogenic cytosolic DNA is influenced by sequence length, secondary structures and nucleotide overhangs [3,5]. For instance, the right-handed (B) form of DNA is well recognized by cytosolic DNA sensors [11,13,14]. Furthermore, guanosine overhangs in conserved Y-form DNA of retroviruses such as the human immunodeficiency virus type 1 (HIV-1) potentiate type I IFN production in human macrophages [15].

Eukaryotic and microbial DNA also differ in their epigenetic landscape, in particular methylation of adenines and cytosines. These modifications are catalyzed by DNA methyltransferases (MTases). Adenine and cytosine methylations are found in DNA of most prokaryotes [16] and are involved in bacterial defence, virulence, chromosomal replication and gene regulation [16,17]. The best-studied prokaryotic MTase is DNA adenine methyltransferase (Dam). Dam was originally described in *Escherichia coli* and methylates adenine in position N^6 (m6A) in 5′-GATC-3′ DNA motifs, generating a $G^{m6}ATC$ DNA motif [18]. Other sequence motifs in a variety of prokaryotes can also carry m6A [16].

Differences in the methylation status are used by the innate immune system to discriminate pathogen-derived DNA from host DNA. For example, CpG motifs are mostly unmethylated in microbial genomes [16], but frequently methylated in DNA across a variety of human and mouse tissues [19,20]. This difference is recognized by the PRR TLR9 [16,17], leading to the production of inflammatory cytokines. Thus, recognition of CpG motifs forms a prime example for immune cells to discriminate host DNA from the microbial genome. Much less is known about a putative immunogenic role of ubiquitous m6A modification in DNA, which is therefore the topic of this study.

M6A modification is present in human and mouse DNA, but it appears to be extremely rare (in the range of 0.0005–0.05% of all adenines) [21,22] compared to the pervasive presence in prokaryotic DNA [16]. This could thus be another basis for discrimination of host and pathogen DNA. Indeed, a previous study showed that systemic injection of DNA containing one $G^{m6}ATC$ motif resulted in increased blood levels on the proinflammatory cytokines TNF-α, IL-6 and IL-12 in mice [23]. However, which cells respond to m6A-methylated DNA and through which innate immune sensors is not well understood [24]. Furthermore, it is not known whether m6A recognition is restricted to $G^{m6}ATC$ motifs or whether it is also observed in another sequence context.

Here, we interrogated whether the cytosolic delivery of $G^{m6}ATC$ DNA provokes immune cell response in innate immune cells, and if so, through which mechanism. We found that synthetic dsDNA containing $G^{m6}ATC$ motifs potentiates the response of murine macrophages and dendritic cells. Irrespective of the motif, recognition of dsDNA requires stimulator of interferon gene (STING)- and cyclic GMP-AMP synthase (cGAS). Importantly, m6A methylation does not boost immune responses *per se*, but depends on the nucleotide sequence context, a feature that is conserved in mouse and in human macrophages.

2. Material and methods

2.1. Mice

C57BL/6 J mice (bred at the animal department of the Netherlands Cancer Institute, Amsterdam, The Netherlands), or mice deficient for MYD88xTRIF [8,25] (hereafter *Myd88*$^{-/-}$*Trif*$^{-/-}$), for IPS-1 [25] (*Ips*$^{-/-}$), for STING [26] (*Sting*$^{-/-}$) or for cGAS [27] (*cGas*$^{-/-}$) were used.

2.2. Generation of murine bone-marrow-derived macrophages and dendritic cells

Bone marrow (BM) cells were obtained from mouse tibias and femurs. Briefly, after BM was flushed from the bones, red blood cells were lysed with red blood cell lysis buffer containing 0.168 M NH_4Cl, and washed once with PBS [28]. Bone-marrow-derived macrophages (BMMs) were generated by seeding 2×10^6 BM cells in a 100 mm non-tissue culture treated dish in RPMI 1640 (Lonza) supplemented with 10% FCS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and β-mercaptoethanol together with 15% L-929 conditioned medium containing recombinant M-CSF for 8 days at 37°C and 5% CO_2. The medium was refreshed after 4 days.

Bone marrow-derived dendritic cells were generated with recombinant Flt3 L (Flt3 L-DCs) as previously described [28]. Briefly, BM cells were cultured at 1.5×10^6 cells ml^{-1} for 9–10 days at 37°C and 5% CO_2 in complete DC medium (RPMI 1640 supplemented with 5% FCS, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and β-mercaptoethanol) supplemented with 30% conditioned medium from CHO cells producing murine recombinant Flt3 L [29]. BMMs and Flt3 L-DC cultures were 95–99% F4/80$^+$ or CD11c$^+$, respectively.

2.3. Generation of human monocyte-derived macrophages

Peripheral mononuclear blood cells were isolated from peripheral blood or buffy coats of healthy individuals collected by Sanquin Blood Supply (Amsterdam, The Netherlands). The study was performed according to the Declaration of Helsinki (seventh revision, 2013). Written informed consent was obtained (Sanquin, Amsterdam, The Netherlands). Monocyte isolation was performed by gradient centrifugation on Percoll (Pharmacia, Uppsala, Sweden) following by magnetic-activated cell separation sorting using human CD14 Microbeads (Miltenyi Biotec). Freshly isolated CD14$^+$ monocytes were cultured for 7–8 days to differentiate into macrophages in IMDM medium supplemented with 10% FCS, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 2 mM L-glutamine and 20 ng ml^{-1} human macrophage colony-stimulating factor (M-CSF) (eBioscience).

2.4. Generation of double-stranded GATC and $G^{m6}ATC$ sequences

HPLC-grade DNA oligos (Sigma-Aldrich) were dissolved in sterile endotoxin-free water, aliquoted and stored at −20°C. To generate dsDNA, equimolar amounts of m6A-methylated or unmethylated complementary oligos were linearized at 95°C, annealed at 75°C for 5 min, and slowly cooled down

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Table 1. Oligos and melting temperature (T_m) of corresponding dsDNA used in this study. Also depicted are the motifs recognized by prokaryotic methyltransferases (MTses), and examples of bacterial strains expressing the MTses.

DNA sequence	T_m (°C)	recognition motif	MTses	bacterial strains	references
AAGGATCTCAAGAAGATCCTTTGATCTTTTCTAC AAGG^{m6}ATCTCAAGAAG^{m6}ATCCTTTG^{m6}ATCTTTTCTAC	68.7 63.4	GATC	numerous DNA adenine MTses	*Escherichia coli* *Klebsiella* sp. *Salmonella enterica* *Mycoplasma mycoides* *Legionella pneumophila* *Yersinia pseudotuberculosis* *Vibrio cholerae*	16,18,35
AAGCATGTCAAGAACATGCTTTCATGTTTTCTAC AAGC^{m6}ATGTCAAGAAC^{m6}ATGCTTTC^{m6}ATGTTTTCTAC	69.0 65.4	CATG	M. TvoI M. ThaIV	*Thermoplasmata*	16
AAGGTACTCAAGAAGTACCTTTGTACTTTTCTAC AAGGT^{m6}ACTCAAGAAGT^{m6}ACCTTTGT^{m6}ACTTTTCTAC	63.4	GTAC	M. HpyAXII	*Helicobacter pylori*	36

to room temperature. Double-stranded sequences were aliquoted and stored at −20°C. dsDNA of GATC DNA was generated from multiple batches. For T_m analysis of each batch, 1 µg dsDNA was incubated with Sybr Green mix (Applied Biosystems) for 5 min at room temperature. The melting curve was determined on the Step-OnePlus Real-Time PCR System (Applied Biosystems) with the standard temperature gradient from 40 to 95°C.

2.5. Stimulation and nucleic acid transfection

After generation, murine BMMs and Flt3 L-DCs, and human monocyte-derived macrophages were seeded for 1 h at 37°C and 5% CO_2 in 24- or 48-well non-tissue culture treated plates (BD) at a density of 1–2 × 10^5 cells ml^{-1}, before being cultured for indicated time points in FCS-free medium containing 1 µg ml^{-1} LPS (Invivogen), 1 µg ml^{-1} synthetic (B) form DNA analog poly(deoxyadenylic-deoxythymidylic) acid (poly(dA:dT)) (Invivogen) or 400 nM dsDNA containing GATC or G^{m6}ATC sequences, or variants thereof. Cells were transfected with poly(dA:dT), m6A methylated or unmethylated dsDNA with 0.1% Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells in medium alone (untransfected, ctrl) or in medium containing Lipofectamine 2000 (mock) served as controls for DNA stimulation and DNA transfection, respectively. After indicated time points, cells were harvested by scraping from culture plates for analysis.

2.6. Antibodies and flow cytometry

BMMs and Flt3 L-DCs were stained with antibodies directed against murine F4/80-APC (clone BM8), CD69-FITC (clone H1.2F3), CD11c-APC (clone N418) and CD86-FITC (clone GL1) (eBioscience). Stainings were performed in the presence of anti-CD16/CD32 block (2.4G2; kind gift from Louis Boon, Bioceros). Flow cytometry was performed with LSRII (BD Biosciences), and data were analysed with FlowJo software v.7.6.5 and v.10 (Tree Star, Inc).

2.7. Quantitative reverse transcriptase-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). cDNA was generated with SuperScript III reverse transcriptase (Invitrogen), dNTPs (Fermentas) and Random Primer (Promega) according to the manufacturer's protocol. Quantitative reverse transcriptase-PCR (RT-qPCR) was performed using SYBR Green mix on the Step-OnePlus System (Applied Biosystems). Primers used for gene expression analysis (electronic supplementary material, table S1) were validated by serial dilutions. Gene expression was normalized to *L32* (mouse genes) or *18s* (human genes).

2.8. Statistical analysis

Data were analysed for statistical significance with two-tailed unpaired or paired Student's *t*-test, as indicated (Prism v.5, GraphPad Software). Results are expressed as mean ± standard deviation (s.d.) and were considered statistically significant with p-values < 0.05.

3. Results

3.1. Cytosolic delivery of m6A-methylated dsDNA enhances macrophage and DC activation

We first examined whether N^6-methyl-adenine (m6A) modifications in GATC motifs alters the immunogenicity of dsDNA for macrophages and dendritic cells. To specifically study the role of m6A methylation and to prevent the engagement of any other pathways of the intricate microbial sensing machinery of mammalian cells, we made use of synthetic dsDNA. The sequence we selected for analysis is present in the genome of several bacterial strains, such as *E. coli*, *Salmonella enterica* and *Klebsiella pneumoniae*. The 34 bp long sequence contains a cluster of three GATC motifs but lacks CpG motifs (table 1). To exclude other immune stimulants in the preparations, we used HPLC-purified oligos that were dissolved in endotoxin-free H_2O. m6A modifications are abundant in bacteria on

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both DNA strands, which prompted us to study the response to double-stranded DNA (dsDNA). We determined the integrity of the generated dsDNA by measuring the melting temperature (T_m) of the m6A-methylated (GATC DNA) or unmethylated (G^{m6}ATC DNA) dsDNA. As expected, m6A modifications reduced the T_m of the dsDNA by approximately 5°C, as a consequence of altering the structure and by destabilizing double-stranded bonds (table 1).

Recognition of dsDNA by PRRs occurs primarily in the cytosol [3,4]. Therefore, to determine whether m6A modifications alter the immunogenicity of dsDNA, we delivered the dsDNA to BMMs from C57Bl/6 J mice through transfection with Lipofectamine 2000. As a control, we transfected poly(dA:dT), a well-studied (B) form dsDNA that elicits potent type I IFN response in both mouse and human cells [4]. Within 6 h of stimulation BMMs transfected with poly(dA:dT) showed increased expression of CD69 (figure 1*a*), an early macrophage activation marker [8,30]. Transfection with the 34 bp synthetic DNA sequences also resulted in increased CD69 expression (figure 1*a*). CD69 protein expression was even higher when cells were transfected G^{m6}ATC DNA compared to unmethylated DNA (figure 1*a*). CD69 expression was also increased at later time points, i.e. 24 h after transfection with G^{m6}ATC DNA (figure 1*b*). The induction of CD69 expression depended on intracellular delivery of the dsDNA, because the delivery of GATC or G^{m6}ATC DNA without Lipofectamine 2000 did not induce expression of CD69 (figure 1*b*).

Macrophage activation with dsDNA leads to rapid transcription of inflammatory molecules [31]. To determine whether m6A methylation alters the inflammatory gene expression profile of macrophages, we measured the mRNA levels of *Il6*, *Il10*, *Tnfα*, *Ifnβ* and *iNos*. *Il6*, *Il10* and *Tnfα* mRNA levels were increased upon transfection with both DNA variants, and it occurred irrespective of the methylation status of the dsDNA (figure 1*c*). We also observed increased mRNA levels of the early inflammatory genes *Ifnβ* and *iNos*, and both transcripts were more potently induced upon transfection with G^{m6}ATC DNA (figure 1*c*; $p = 0.005$ and $p < 0.0001$, respectively). Similarly, bone-marrow-derived DCs generated with Flt3 L showed increased levels of the costimulatory molecule CD86 upon transfection with G^{m6}ATC DNA when compared to transfection with GATC DNA (figure 1*d*). Thus, m6A modification in GATC motifs promotes the gene expression of several key inflammatory molecules.

3.2. STING and cGAS drive immune activation for both m6A-modified and unmodified DNA

We next interrogated which PRR mediates the recognition of the m6A-methylated dsDNA. TLR3, TLR7/8 and TLR9 which detect nucleic acids [32] signal through MYD88 and TRIF, the key adaptor molecules downstream of TLR signalling [8,9]. To determine whether TLRs can sense methylated dsDNA, we generated BMMs from *Myd88*$^{-/-}$*Trif*$^{-/-}$ mice. As expected, *Myd88*$^{-/-}$*Trif*$^{-/-}$ BMMs failed to respond to the TLR4 ligand LPS after 6 h of stimulation, but maintained their ability to respond to poly(dA:dT), which is sensed in an TLR-independent manner [13] (figure 2*a*,*b*). Transfection with GATC and G^{m6}ATC DNA resulted in identical effects in *Myd88*$^{-/-}$*Trif*$^{-/-}$ and *wt* BMMs, with higher CD69 expression upon transfection with G^{m6}ATC DNA (figure 2*a*,*b*). This finding indicated that TLRs are dispensable for dsDNA recognition. The adaptor protein IPS-1 that acts downstream of the dsRNA recognizing RIG-I-like receptors [25,33] was also not required for either GATC, or G^{m6}ATC DNA recognition (figure 2*c*).

STING was identified as a key adaptor molecule of cytosolic DNA sensing [26]. In line with this, we did not detect any upregulation of CD69 protein expression in *Sting*$^{-/-}$ BMMs upon transfection with poly(dA:dT), or with synthetic dsDNA (figure 2*d*). Intriguingly, the lack of recognition occurred independently of the m6A modification (figure 2*d*). We then questioned how cGAS, the sensor for cytosolic DNA upstream of STING [3,14,34] responded to cytosolic GATC, or G^{m6}ATC DNA. BMMs generated from mice that constitutively lack the cytosolic DNA sensor cGAS [27] failed to induce CD69 upon transfection with GATC, or with G^{m6}ATC (electronic supplementary material, figure S1). Thus, the cGAS–STING axis is required to recognize cytosolic synthetic dsDNA, and this recognition is permissive to epigenetic modifications within the DNA.

3.3. Enhanced BMM-activation by m6A-methylated DNA is sequence-specific

We then interrogated whether the increased immunogenicity of G^{m6}ATC DNA was a general feature of m6A-methylated DNA. In fact, in addition to the GATC sequence-specific Dam methyltransferase (MTse), a number of other m6A DNA MTses have been described [16,18,35]. For instance, *Thermoplasmata* express a m6A MTse that recognizes CATG sequences [16]. Another m6A MTse found in *Helicobacter pylori* recognizes adenine within GTAC motifs [36]. To determine whether m6A methylations within these motifs also increased the immunogenicity of DNA, we generated dsDNA with the identical 34 bp core sequence, but with the GATC motifs exchanged to m6A-methylated or unmethylated CATG and GTAC motifs (table 1). Similar to the GATC containing DNA, C^{m6}ATG and GT^{m6}AC DNA displayed a reduced T_m compared to the respective unmethylated dsDNA (table 1), indicating that m6A methylation also affects the strength of dsDNA bonds in these sequences.

Comparable to G^{m6}ATC DNA, transfecting BMMs with DNA containing GT^{m6}AC also induced higher CD69 expression levels than its unmethylated counterpart (figure 3*a*). However, this was not the case for C^{m6}ATG DNA. Transfecting BMMs with DNA containing C^{m6}ATG resulted in lower CD69 expression than transfection with the unmethylated DNA (figure 3*a*). Furthermore, whereas G^{m6}ATC and GT^{m6}AC were also superior in increasing *Ifnβ*, *iNos* and *Cxcl10* transcript levels compared to the respective unmethylated DNA, C^{m6}ATG-containing DNA rather hampered the induction of these key inflammatory genes (figure 3*b*–*d*). Thus, the observed enhanced immunogenicity of m6A methylation in DNA sequences is sequence-specific.

3.4. Sequence-specific recognition of m6A-methylated DNA is conserved in human macrophages

To determine whether the observed differences in sequence-specific immunogenicity were also found in humans, we generated M-CSF derived macrophages from peripheral blood-derived monocytes and compared the gene expression levels of effector molecules upon DNA transfection. Comparable to murine macrophages, transfecting human

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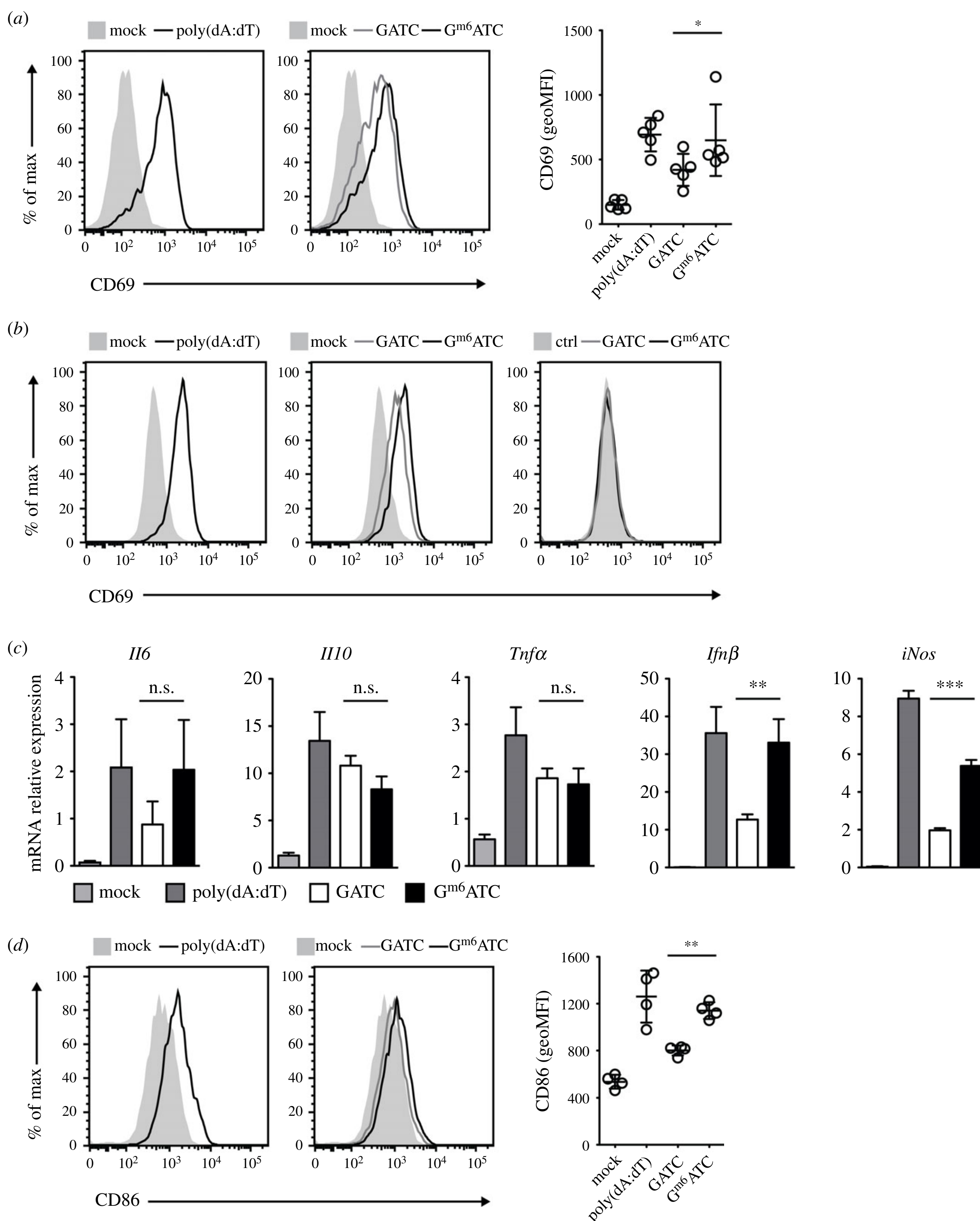

Figure 1. Cytosolic recognition of m6A-methylated dsDNA potentiates macrophage and dendritic cell activation. (*a*) Representative histogram of CD69 expression of bone-marrow-derived macrophages (BMMs) 6 h after transfection with 0.1% Lipofectemine 2000 and 1 µg ml^{-1} poly(dA:dT) (left panel), 400 nM unmethylated (GATC) or 400 nM methylated ($G^{m6}ATC$) DNA (middle panel). Transfection with 0.1% Lipofectamine 2000 alone served as control (mock). Right panel: CD69 expression levels (Geometric mean fluorescence intensity, geoMFI) compiled from five independently performed experiments. (*b*) CD69 expression of BMMs stimulated for 24 h with 1 µg ml^{-1} poly(dA:dT), or with GATC or $G^{m6}ATC$ DNA in the presence (middle panel) or absence (right panel) of Lipofectemine. Lipofectamine mock treated or untreated BMMs (ctrl) served as controls. (*c*) *Il6*, *Il10*, *Tnfα*, *Ifnβ* and *iNos* mRNA levels of BMMs activated for 6 h with indicated reagents. (*b*,*c*) are representative of two independently performed experiments. (*d*) Representative histograms (left) of CD86 expression and compiled data from 2 independently performed experiments (right) of BM-derived dendritic cells (Flt3 L-DCs) that were mock transfected or transfected overnight with poly(dA:dT), GATC or $G^{m6}ATC$ DNA. Paired (*a–e*) or unpaired (*c*) Student's *t*-test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

macrophages with $G^{m6}ATC$-containing DNA resulted in higher induction of *CXCL10* mRNA compared to unmethylated DNA (figure 4*a*). The increased immunogenicity of DNA was also conserved for $GT^{m6}AC$ DNA (figure 4*a*). By contrast, transfecting macrophages with $C^{m6}ATG$ DNA again lowered the induction of *CXCL10* mRNA (figure 4*a*).

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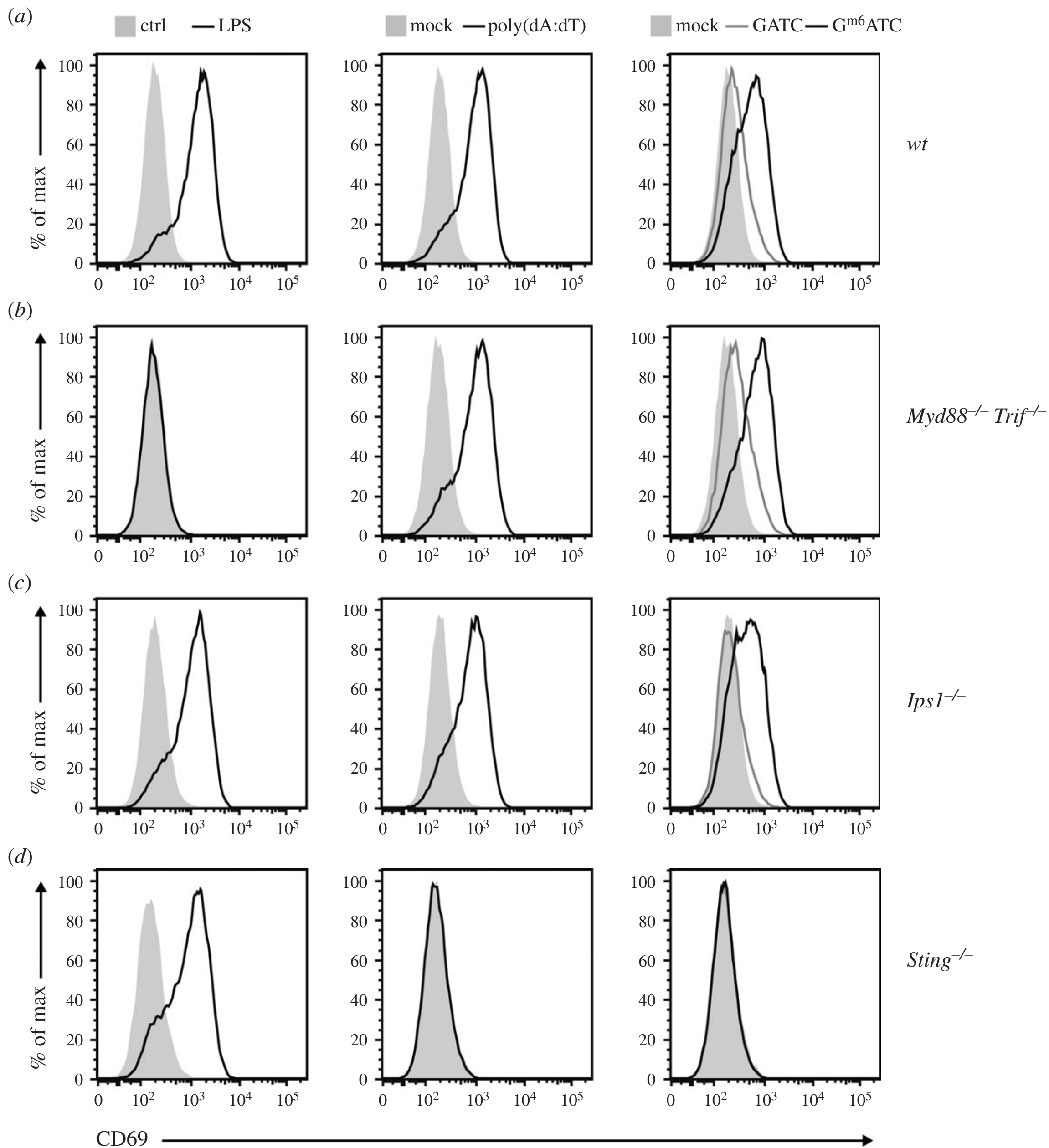

Figure 2. STING is required for macrophage activation by dsDNA irrespective of methylation status. CD69 expression levels determined by flow cytometry of BMMs from (*a*) *wt*, (*b*) *Myd88*$^{-/-}$*Trif*$^{-/-}$, (*c*) *Ips1*$^{-/-}$ or (*d*) *Sting*$^{-/-}$ mice activated for 6 h with 1 µg ml^{-1} LPS, or left untreated (Ctrl; left panels). Alternatively, BMMs were transfected with poly(dA:dT) or mock transfected (middle panels), or were transfected with GATC and G^{m6}ATC DNA, respectively (right panels). Data are representative of two independently performed experiments.

Because the C^{m6}ATG sequence in transfected DNA blocked the induction of proinflammatory molecules in macrophages, we investigated whether this sequence instead induced the expression of a prototypic anti-inflammatory cytokine, IL-10. However, we did not detect increased *IL10* mRNA levels with any of the m6A-methylated DNA sequences when compared to mock-transfected cells (figure 4*b*). In conclusion, the sequence-specific immunogenicity by m6A-methylated DNA motifs is conserved between mouse and human.

4. Discussion

Recognition of intracellular dsDNA is an important process that can occur during microbial infection and after cell damage [3]. Whereas length and structure was shown to modulate the immunogenicity of DNA [5], we show here that m6A methylation also alters the immunogenicity of cytosolic DNA. The response to m6A-methylated DNA is identical to unmethylated DNA: it is independent of MyD88/TRIF and IPS-1 signalling but requires the cGAS–STING axis. How m6A methylation influences the immunogenicity of cytosolic DNA is yet to be determined. dsDNA binds to cGAS by interacting with its two DNA-binding sites and zinc ribbon domain [37–39], and this interaction is mediated via the sugar-phosphate backbone of the DNA [37,39]. DNA binding leads to dimerization of cGAS and conformational changes, which spark the enzymatic activity of cGAS for the synthesis of the intermediate messenger cGAMP(2′-5′) [37–41]. m6A methylation affects the secondary structure of DNA, as observed by different T_m of methylated and unmethylated DNA. This may alter the local flexibility of DNA structures, and affect the DNA geometry and stiffness, as was recently reported for CpG motifs [42]. Whether and how these alterations in dsDNA structure and stiffness influence the binding affinity or avidity to cGAS, or its dimerization, is yet to be determined.

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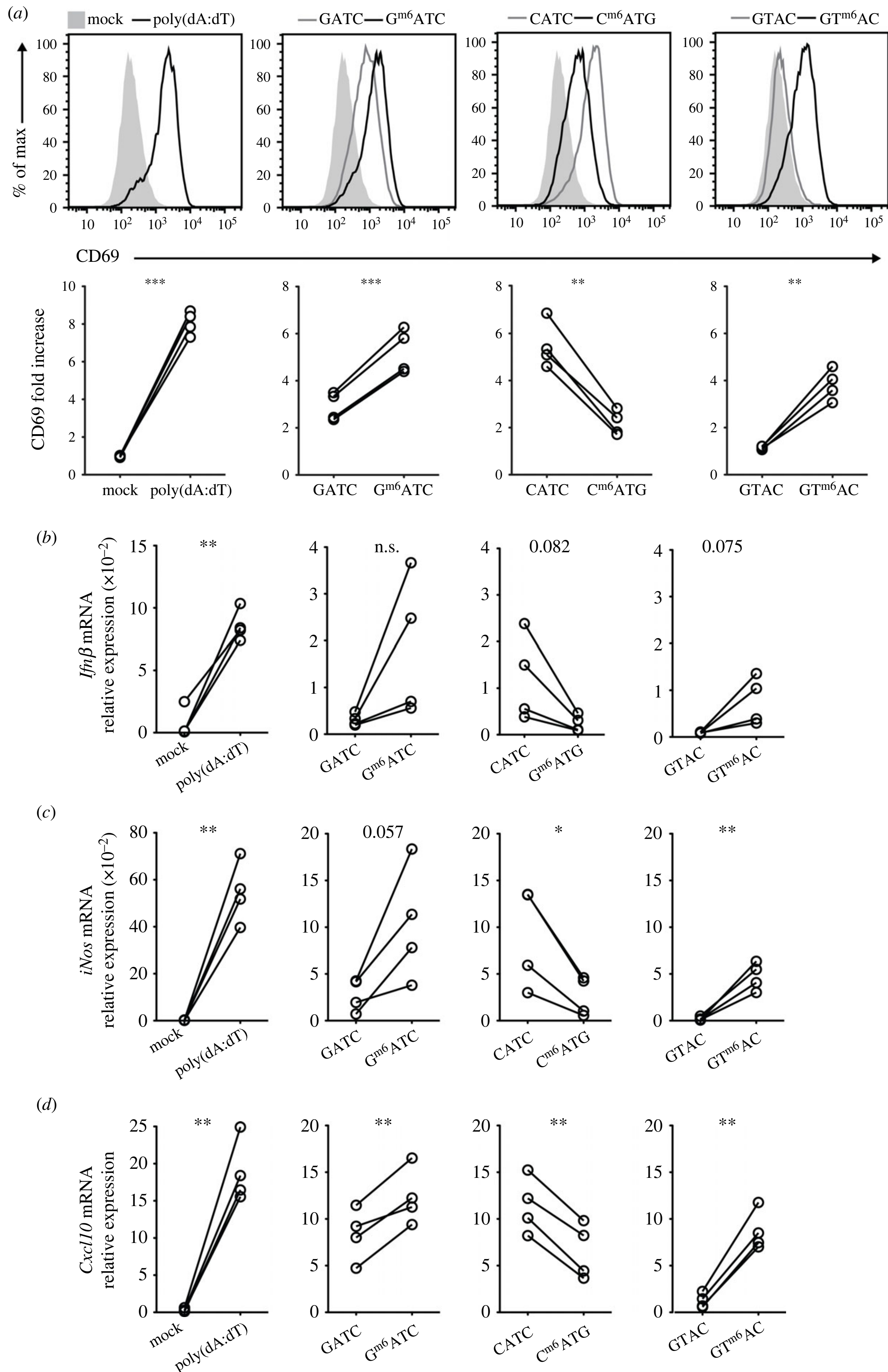

Figure 3. BMMs recognize m6A-methylated dsDNA in a sequence-dependent manner. (*a*) BMMs were mock transfected or transfected for 6 h with poly(dA:dT), (left panel), with GATC or G^{m6}ATC DNA (second panel), CATG or C^{m6}ATG (third panel), or GTAC or GT^{m6}AC DNA (right panel). For sequences see table 1. Top row: Representative histograms of CD69 expression measured by flow cytometry. Bottom row: Compiled data from BMM cultures of four mice from two independently performed experiments. (*b–d*) mRNA levels of *Ifnβ* (*b*) *iNos* (*c*) and *Cxcl10* (*d*) in BMMs after 6 h stimulation with indicated reagents, normalized to the expression of *L32*. Paired Student's *t*-test. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. n.s. = not significant).

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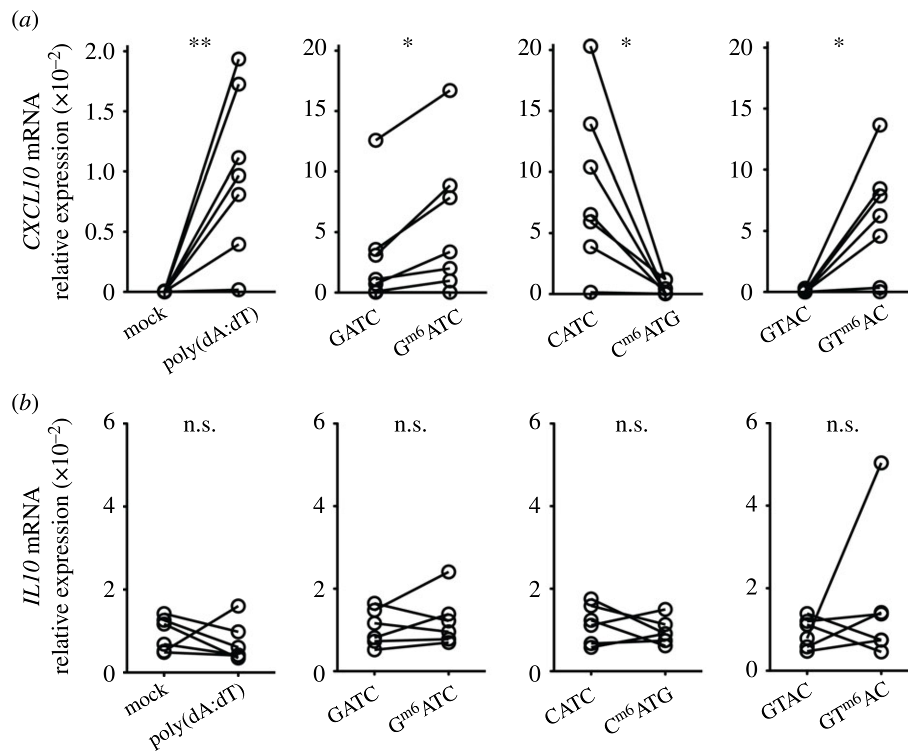

Figure 4. Sequence-specific recognition of m6A-methylated dsDNA is conserved in human macrophages. (*a*,*b*) M-CSF induced macrophages from human peripheral blood-derived monocytes were transfected with poly(dA:dT) (left panel), GATC or G^{m6}ATC DNA (second panel), CATG or C^{m6}ATG (third panel), or GTAC or GT ^{m6}AC DNA (right panel). mRNA levels of *CXCL10* (*a*) and *IL10* (*b*) were measured and normalized to the expression of *18S*. $n = 7$ independent donors, measured in four independently performed experiments. Paired Student's *t*-test. (*$p < 0.05$, **$p < 0.01$. n.s. = not significant).

Interestingly, m6A methylation in conserved GATC motifs in *E. coli* origin of replication enhances DNA-intrinsic and protein-dependent bending, and—as a consequence—binding to the DNA-binding protein IHF and other pre-replication complex proteins [43,44]. It is, therefore, tempting to speculate that such increased structural bending by m6A methylation could also influence the binding affinity of dsDNA to cGAS, promote cGAS dimerization or its enzymatic activity. Intriguingly, during *Listeria monocytogenes* infection, also bystander cells can be activated via the cGAS–STING pathway. In fact, bacterial DNA can be transferred to neighbouring cells through extracellular vesicles [45]. As *L. monocytogenes* contains ubiquitous m6A methylation [46], m6A methylation may not only be involved in effective recognition of bacterial DNA within infected cells, but also in engaging bystander cells.

However, m6A methylation of dsDNA does not increase its recognition *per se*, but rather depends on the sequence context. The nucleotides flanking the m6A methylation could possibly alter the DNA bending, as was previously suggested [43]. It is therefore conceivable that the poorly recognized C^{m6}ATG motif provokes structural changes in DNA that reduces its bending and therefore its immunogenicity. This sequence specificity of cGAS may also be a safeguard for recognizing self-DNA, as low levels of m6A methylation has been observed in mammalians, albeit in different motifs [21,22].

Lastly, it would be interesting to assess whether m6A can modulate innate immune responses to dsDNA. In fact, synthetic oligonucleotides derived from telomeric DNA can compete with endogenous DNA for cGAS activation, by binding to cGAS without eliciting conformational changes [47]. Similar effects could arise by pretreating BMMs with C^{m6}ATG sequences. Such approaches could thus help the design and development of novel therapeutic DNA-based inhibitors of cGAS-mediated signalling.

In conclusion, our study identifies a new role for m6A-DNA methylation in regulating innate immune responses to cytosolic DNA. Whether the observed sequence-specific recognition of m6A-methylated DNA is a specific feature of synthetic DNA or stems from different immune responses to various bacterial strains is yet to be determined. Our findings may help to increase the immunogenicity of DNA vaccines while preventing unwanted cytosolic DNA-mediated responses, and could potentially pave the way to unravel novel mechanisms of pathogen recognition and evasion in innate immune cells.

Ethics. All animal experiments were performed in accordance with institutional and national guidelines and approved by the Experimental Animal Committee of the Netherlands Cancer Institute, and of the Cincinnati Children's Hospital.

Data accessibility. Data supporting the findings of the study are available from the corresponding author upon request.

Author's contribution. M.B. and M.C.W. designed research, performed experiments, analysed data and wrote the manuscript; S.E. and A.J.d.J. performed experiments and analysed data; K.F., T.K.v.d.B., M.F.G., A.A. and E.M.J. provided material; B.v.S. designed research and provided intellectual input; S.E., A.J.d.J., K.F., T.K.v.d.B., E.M.J. and B.v.S. critically revised the manuscript.

Competing interests. We declare we have no competing interests.

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