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 N6 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-κB- 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...
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 N⁶ (m⁶A) in 5'GATC-3' DNA motifs, generating a G⁶mATC 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 G⁶mATC 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 G⁶mATC motifs or whether it is also observed in another sequence context.

Here, we interrogated whether the cytosolic delivery of G⁶mATC DNA provokes immune cell response in innate immune cells, and if so, through which mechanism. We found that synthetic dsDNA containing G⁶mATC 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

CS7BL/6J mice (bred at the animal department of the Netherlands Cancer Institute, Amsterdam, The Netherlands), or mice deficient for MYD88/Trif [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 NH₄Cl, and washed once with PBS [28]. Bone-marrow-derived macrophages (BMMs) were generated by seeding 2 × 10⁶ 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 b-mercaptoethanol together with 15% L-929 conditioned medium containing recombinant M-CSF for 8 days at 37°C and 5% CO₂. 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 × 10⁶ cells ml⁻¹ for 9–10 days at 37°C and 5% CO₂ 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 b-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 G⁶mATC 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...
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.

### 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 |
|--------------|----------------|------------------|-------|-------------------|------------|
| AAGGACTCAAGAAGATCCTGTTGATCTTTTAC | 68.7 | GATC | numerous | Escherichia coli | 16,18,35 |
| AAGGMCTCAAGAAGATCCTGTTGATCTTTTAC | 63.4 | Gm6A | DNA adenine | Salmonella enterica | |
| AAGCTCAAGAAGATCCTGTTGATCTTTTAC | 65.4 | ThaIV | MTses | Mycoplasma mycoides | |
| AAGCTCAAGAAGATCCTGTTGATCTTTTAC | 63.4 | GTm6A | Legionella pneumophila | Yersinia pseudotuberculosis | Vibrio cholerae |

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(deoxyadenylc-deoxithymidylic) acid (poly(dA:dT)) (Invivogen) or 400 nM dsDNA containing GATC or Gm6A TCT 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, Bioceres). 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 quantitative reverse transcriptase-PCR (RT-qPCR) using SYBR Green mix on the Step-OnePlus System (Applied Biosystems) with the standard temperature gradient from 40 to 95°C.

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\(_2\)O. m6A modifications are abundant in bacteria on the genome.
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 \((G^{\text{m6A}}\text{ATC DNA})\) 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 1a), an early macrophage activation marker [8,30]. Transfection with the 34 bp synthetic DNA sequences also resulted in increased CD69 expression (figure 1a). CD69 protein expression was even higher when cells were transfected with Gm6ATC DNA compared to unmethylated DNA (figure 1a). CD69 expression was also increased at later time points, i.e. 24 h after transfection with Gm6ATC DNA (figure 1b). The induction of CD69 expression depended on intracellular delivery of the dsDNA, because the delivery of GATC or Gm6ATC DNA without Lipofectamine 2000 did not induce expression of CD69 (figure 1b).

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, Tfha, Ifng and iNos. Il6, Il10 and Tfha mRNA levels were increased upon transfection with both DNA variants, and it occurred irrespective of the methylation status of the dsDNA (figure 1c). We also observed increased mRNA levels of the early inflammatory genes Ifng and iNos, and both transcripts were more potently induced upon transfection with Gm6ATC DNA (figure 1c; \(p = 0.005\) 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 Gm6ATC DNA when compared to transfection with GATC DNA (figure 1d). 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 2a,b). Transfection with GATC and Gm6ATC DNA resulted in reduced effects in Myd88\(^{-/-}\)/Trif\(^{-/-}\) and wt BMMs, with higher CD69 expression upon transfection with Gm6ATC DNA (figure 2a,b). This finding indicated that TLRs are dispensable for dsDNA recognition. The adaptor protein IFS-1 that acts downstream of the dsRNA recognizing RIG-I-like receptors [25,33] was also not required for either GATC, or Gm6ATC DNA recognition (figure 2c).

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 2d). Intriguingly, the lack of recognition occurred independently of the m6A modification (figure 2d). We then questioned how cGAS, the sensor for cytosolic DNA upstream of STING [3,14,34] responded to cytosolic GATC, or Gm6ATC DNA. BMMs generated from mice that constitutively lack the cytosolic DNA sensor cGAS [27] failed to induce CD69 upon transfection with GATC, or with Gm6ATC (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 Gm6ATC 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, Thermoplasma 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, Cm6ATG and GTm6AC 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 Gm6ATC DNA, transfecting BMMs with DNA containing GTm6AC also induced higher CD69 expression levels than its unmethylated counterpart (figure 3a). However, this was not the case for Cm6ATG DNA. Transfecting BMMs with DNA containing Cm6ATG resulted in lower CD69 expression than transfection with the unmethylated DNA (figure 3a). Furthermore, whereas Gm6ATC and GTm6AC were also superior in increasing Ifng, iNos and Cxcl10 transcript levels compared to the respective unmethylated DNA, Cm6ATG-containing DNA rather hampered the induction of these key inflammatory genes (figure 3b–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
macrophages with Gm6A TC-containing DNA resulted in higher induction of CXCL10 mRNA compared to unmethylated DNA (figure 4a). The increased immunogenicity of DNA was also conserved for GTm6AC DNA (figure 4a). By contrast, transfecting macrophages with Cm6A TG DNA again lowered the induction of CXCL10 mRNA (figure 4a).

**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% Lipofectamine 2000 and 1 µg ml⁻¹ poly(dA:dT) (left panel), 400 nM unmethylated (GATC) or 400 nM methylated (Gm6ATC) 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 Gm6ATC DNA in the presence (middle panel) or absence (right panel) of Lipofectamine. 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 Gm6ATC DNA. Paired (a–e) or unpaired (c) Student’s t-test. (*p < 0.05, **p < 0.01, ***p < 0.001).
Because the C<sup>6A</sup> 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 com-
pared to mock-transfected cells (figure 4b). 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 iden-
tical 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 inter-
acting with its two DNA-binding sites and zinc ribbon domain
[37–39], and this interaction is mediated via the sugar-phos-
phate 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 methyla-
tion affects the secondary structure of DNA, as observed by
different T<sub>m</sub> 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.
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 Gm6ATC DNA (second panel), CATC or Cm6ATG (third panel), or GTAC or GTm6AC 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 < 0.01, ***p < 0.001. 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<sup>n</sup>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 mammals, 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<sup>n</sup>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.

**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<sup>m</sup>ATC DNA (second panel), CATG or C<sup>m</sup>ATG (third panel), or GTAC or C<sup>mm</sup>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).

### Table 1. Relative mRNA expression of CXCL10 and IL10 in response to dsDNA transfection

| Condition | CXCL10 mRNA | IL10 mRNA |
|-----------|-------------|-----------|
| mock      | 1.0         | 1.0       |
| poly(dA:dT) | 2.0         | 1.5       |
| GATC      | 1.5         | 1.0       |
| G<sup>m</sup>ATC | 1.0         | 0.5       |
| CATG      | 2.0         | 1.5       |
| C<sup>m</sup>ATG | 1.5         | 1.0       |
| GTAC      | 1.0         | 0.5       |
| GT<sup>m</sup>AC | 0.5         | 0.5       |

**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., E.M.J. and B.v.S. provided material; M.F.G., A.A. and E.M.J. critically revised the manuscript.

**Competing interests.** We declare we have no competing interests.

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