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The RNA modification N^6-methyladenosine as a novel regulator of the immune system

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Protection from harmful pathogens depends on activation of the immune system, which relies on tight regulation of gene expression. Recently, the RNA modification N^6-methyladenosine (m^6A) has been found to play an essential role in such regulation. Here, we summarize newly discovered functions of m^6A in controlling various aspects of immunity, including immune recognition, activation of innate and adaptive immune responses, and cell fate decisions. We then discuss some of the current challenges in the field and describe future directions for uncovering the immunological functions of m^6A and its mechanisms of action.

RNA modifications are post-transcriptional changes to the chemical composition of RNA molecules that have the potential to alter RNA function. While over 100 different modified bases have been identified in RNA, the majority of these are restricted to non-coding RNAs, especially transfer RNA and ribosomal RNA. However, several distinct modifications have been reported to occur in mammalian messenger RNAs[1]. Of these, the most prevalent internally modified base is N^6-methyladenosine (m^6A). m^6A was first identified on cellular mRNAs in the 1970s[2,3]. However, the absence of methods to map the precise location of m^6A on transcripts, and the lack of information about the cellular factors that produce and recognize m^6A residues, imposed strong limitations on the field. Although the enzyme that adds the m^6A modification to mRNAs was identified in the late 1990s[4], the immense scientific interest in RNA modifications began with the development of genome-wide m^6A-mapping methods, which revealed the prevalence of m^6A throughout the transcriptome[5,6]. Since then, the role of m^6A in regulating mRNA fate and its functional importance in various cell types have been widely studied, and m^6A is emerging as a widespread regulatory mechanism that controls gene expression in diverse physiological processes.

In this Review, we summarize recent studies describing the role of the m^6A modification in fine-tuning the immune response. We discuss the functions of m^6A modification in the context of innate sensing, in regulation of the innate and adaptive immune response, and in immune system development. The studies presented here portray the importance of m^6A in shaping a balanced immune response and also emphasize the potential of using well-characterized immune regulation pathways and the corresponding in vivo model systems to shed new light on m^6A regulatory mechanisms.

m^6A writers

m^6A is added to mRNA by a multisubunit 'writer complex' composed of the METTL3–METTL14 heterodimer and numerous additional adaptor proteins. METTL3 is the enzymatic component of the complex, while METTL14 is an allosteric activator that also binds to the target RNA[7–10]. The writer complex also includes the WTAP, RBM15 or RBM15B, ZC3H13 and VIRMA (also known as KIAA1429) subunits. WTAP is essential for m^6A deposition and localizes METTL3–METTL14 heterodimers to transcription sites[11], ZC3H13 maintains the nuclear localization of the complex[12] and RBM15/15B and VIRMA are thought to provide additional specificity[13–15]. The writer complex methylates adenosine cotranscriptionally at the m^6A consensus sequence, DRACH (where D = A, G, or U; R = G or A; and H = A, C or U). While this sequence is promiscuous and every transcript is predicted to have many potential methylation sites, the deposition of m^6A is more restricted, as only certain mRNAs contain m^6A and only a small fraction of the consensus sites in these transcripts are methylated. Moreover, the stoichiometry of m^6A (that is, the ratio between modified and unmodified adenosines) at distinct sites can vary[16–18]. A recent analysis revealed that up to 45% of the variability in methylation levels may be explained by features in the flanking sequences[17], but many aspects of deposition specificity remain poorly understood.

As the writer complex acts cotranscriptionally[10,19], m^6A formation is thought to be primarily nuclear. Surprisingly, however, cytoplasmic RNA viruses—whose RNA is transcribed in the cytoplasm—were shown to be m^6A modified as well[20–22], suggesting that some form of the writer complex might also be present outside the nucleus.

In addition to the canonical writer complex, several other enzymes have been shown to act as m^6A methyltransferases: METTL16 modifies U6 small nuclear RNAs, the *MAT2A* transcript and possibly additional mRNAs[23]; ZCCHC4 and METTL5 add m^6A to18S and 28S ribosomal RNAs[24,25]; and PCIF1 catalyzes m^6A methylation on 2-*O*-methylated adenine—present at the transcription start site of m^7G-capped mRNAs—generating N^6, 2′-*O*-dimethyladenosine (m^6Am)[26–28]. The m^6A modified positions generated by these enzymes have not been associated with immunity; therefore, this Review focuses on the canonical m^6A mRNA modification.

m^6A readers

The RNA-binding proteins that bind to m^6A are referred to as m^6A readers. These proteins mediate the regulatory functions of m^6A on modified RNAs. YTH domain–containing proteins bind RNA in an m^6A-dependent manner[29–32]. There are five YTH domain–containing proteins in the mammalian genome. These proteins can be divided into three classes: YTHDC1, which is broadly expressed and is predominantly localized to the nucleus[33,34]; YTHDC2, whose

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expression is tissue restricted and can be both nuclear and cytosolic[35,36]; and the family of cytosolic YTHDF proteins, comprising three highly similar paralogs—YTHDF1, YTHDF2 and YTHDF3. YTHDF proteins share high similarity in their amino acid sequences, and there is conflicting evidence regarding whether they induce similar outcomes or whether each has a specialized effect on m^{6}A-modified mRNAs (reviewed in ref. [37]). Several studies have reported that YTHDF1 enhances translation of m^{6}A-modified mRNAs, YTHDF2 promotes their degradation and YTHDF3 enhances both functions[38–41]. However, other studies showed that YTHDF1, 2 and 3 had similar roles in mRNA degradation[41]. Furthermore, all three YTHDF proteins were recently demonstrated to mediate phase separation, which facilitates the targeting of m^{6}A-modified mRNA to P-bodies, stress granules and other RNA–protein assemblies[42], suggesting a common mechanism of action for the three YTHDF proteins.

YTHDC1 is a nuclear m^{6}A reader and has been linked to mRNA splicing[34,43], epigenetic silencing[15] and nuclear export of mRNA[44]. YTHDC2 is enriched in the testes and was suggested to be involved in translation and mRNA degradation[45]. YTHDC2 knockout mice show defects mainly in spermatogenesis, supporting the notion that it has a testis-specific function[36,46,47]. Nonetheless, on the basis of genomic database information, immune cells express moderate levels of YTHDC2, so it remains a possibility that this reader has a role in immune cells as well.

Besides the YTH domain–containing m^{6}A readers, several additional RNA-binding proteins have been reported to preferentially bind to m^{6}A-containing RNA. These include eIF3D, FMR1, IGF2BP1-3, HNRNPC, HNRNPG and HNRNPA2B1[48–54]. However, the regulatory functions of these RNA-binding proteins, and whether they bind m^{6}A directly rather than indirectly through canonical m^{6}A binders, remain unclear. Furthermore, since the presence of m^{6}A reduces the ability of RNA to form secondary structures[50,55], m^{6}A RNA modifications can provide greater access to RNA-binding proteins. This concept was demonstrated for HNRNPC, which preferentially binds m^{6}A-modified sites[50]. The propensity of m^{6}A to form unstructured regions in RNA makes it difficult to determine whether an RNA-binding protein binds directly to m^{6}A or whether it preferentially binds to m^{6}A-modified RNA due to an increase in accessibility[56].

m^{6}A erasers

Two enzymes, FTO and ALKBH5, have been suggested to remove m^{6}A from mRNA[57–59]. FTO likely demethylates both m^{6}A and terminal m^{6}Am[59], while ALKBH5 specifically demethylates m^{6}A[58,60]. Although m^{6}A erasers provide the means to regulate m^{6}A levels in a dynamic and signal-dependent manner, metabolic labeling studies in HeLa cells show that m^{6}A levels are largely stable throughout the mRNA life cycle[19], suggesting dynamic m^{6}A erasure may be limited to specific conditions or tissues[61,62]. The potential effects of RNA demethylation in additional physiological contexts need to be further evaluated.

m^{6}A-mediated molecular mechanisms

Studies using specific deletion of m^{6}A machinery components have revealed that m^{6}A regulates many aspects of the mRNA lifecycle. The route of m^{6}A-modified mRNA starts during transcription, when m^{6}A writing and erasing is initiated. Subsequently, the nuclear m^{6}A readers can affect mRNA splicing and export, and potentially other nuclear processes. Upon export to the cytoplasm, m^{6}A can affect the stability, translation and localization of mRNAs, and these effects are mainly mediated by the cytosolic m^{6}A readers (Fig. 1).

The most established function of m^{6}A is destabilization of mRNAs. This was indicated by metabolic radioisotope labeling studies in the 1970s[63]. Since then, probing METTL3 depletion in diverse cell types and organisms has shown that m^{6}A levels are globally associated with shorter half-lives for modified mRNAs[38,64–66]. Nonetheless, recognition of m^{6}A by the RNA-binding protein IGF2BP was also suggested to enhance mRNA stability[52,67–69]. Since m^{6}A deposition occurs in the nucleus, it is likely that m^{6}A serves as a mark that dictates the mRNA half-life once these mRNAs enter the cytosol compartment.

The ability of m^{6}A to mediate changes in translation is complex and probably more context dependent than its effects on RNA stability. Numerous studies have linked m^{6}A to an increased translation rate, but several distinct mechanisms have been suggested. One mechanism involves the reader YTHDF1, which recruits the eukaryotic translation initiation factor complex eIF3. This complex, in turn, recruits the small ribosomal subunit, leading to translation enhancement of m^{6}A-modified mRNAs[39]. Another mechanism of translation regulation involves direct binding of eIF3 to m^{6}A in the 5′ UTR (untranslated region)[53]. In both cases, the presence of m^{6}A bypasses the normal requirement for eIF4E (the main cytosolic cap-binding protein), allowing a subset of the m^{6}A-containing mRNAs to be translated under stress[53,70,71]. The third mechanism of translational enhancement involves direct activation by METTL3. In this case, METTL3 is suggested to remain bound to m^{6}A-modified transcripts and to directly recruit eIF3 in the cytosol[72,73].

Strong evidence that m^{6}A is involved in splicing regulation comes from studies in *Drosophila melanogaster*, where m^{6}A modification affects the splicing of a gene central to sex determination[74]. In mammals, the general link between m^{6}A and mRNA splicing is less clear. The overall number of METTL3-dependent splicing events is suggested to be small[64,66], indicating that if m^{6}A is involved in splicing regulation, it is probably for a limited number of genes or in specific conditions and should be further evaluated. Additionally, m^{6}A may enhance mRNA export from the nucleus[44,75]. However, it is still unclear whether there are global m^{6}A-dependent variations in mRNA export rates.

Methods for m^{6}A detection

The ability to understand the phenotypes caused by deletion of m^{6}A machinery proteins and how m^{6}A-containing transcripts are affected at the molecular level requires the ability to detect m^{6}A-modified transcripts and to quantify m^{6}A levels (that is, the stoichiometry of modified versus unmodified adenosine). m^{6}A-seq[5,6] involves transcriptome-wide sequencing of m^{6}A-antibody enriched regions as compared to input, and it provided the first global view of m^{6}A-modified transcripts. This genome-wide method, which is still widely used, facilitates the identification of modified transcripts and provides a proxy for m^{6}A-modified positions within those RNAs. However, the method lacks single-base resolution, is not quantitative in its simplest implementation, and is prone to false-positive results due to nonspecific binding of the antibody. Subsequently, new m^{6}A-antibody-based technologies that utilize ultraviolet cross-linking of the m^{6}A antibody were developed: namely, m^{6}A-CLIP and miCLIP[76–78]. These methods provide single-base resolution, but they are laborious and require large amounts of input, and the stoichiometric information is still lost.

Recently, several antibody-independent m^{6}A-identification methods were described. MAZTER-seq utilizes the ability of the MazF RNase to cleave RNA at unmethylated ACA motifs but not at their methylated counterparts. This method was the first to provide quantitative profiling of m^{6}A at single-nucleotide resolution, but due to the specificity of MazF, it detects only around 16% of mammalian m^{6}A-modified sites[17,18]. DART-seq, in which the cytidine deaminase APOBEC1 is fused to the m^{6}A-binding YTH domain, C-to-U deamination occurs at sites adjacent to m^{6}A residues, which are subsequently identified and quantified by RNA-seq. This method requires ectopic expression of the fusion protein in target cells[79]. Finally, direct RNA sequencing using nanopore technology allows detection of m^{6}A in native RNAs[80,81]. This detection is based

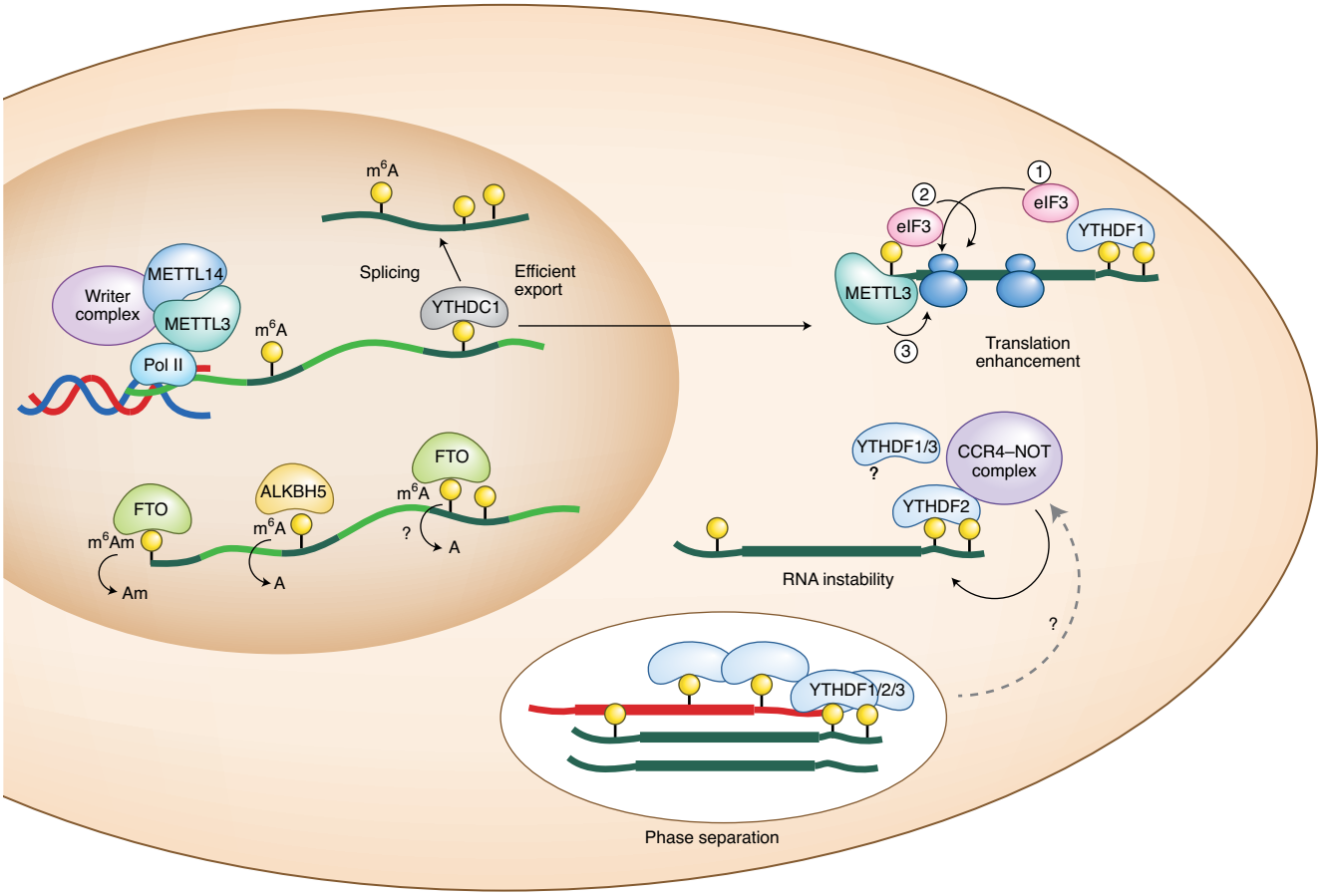

Fig. 1 | The cellular m⁶A machinery and its potential molecular functions. m⁶A is added cotranscriptionally by the writer complex, which consists of METTL3, METTL14 and a set of accessory proteins. m⁶A can be removed in the nucleus by two potential m⁶A demethylases, ALKBH5 and FTO. FTO can also remove m⁶Am methylation at the first base of the transcript. While in the nucleus, m⁶A can be recognized by nuclear reader proteins, mainly YTHDC1, which may regulate splicing and mRNA export. Upon mRNA export to the cytoplasm, m⁶A is recognized by cytoplasmic reader proteins, mainly YTHDF1, YTHDF2 and YTHDF3, which mediate diverse post-transcriptional processes, including mRNA translation, stability and localization. m⁶A is suggested to enhance mRNA translation through three distinct mechanisms: YTHDF1 binding, which then recruits the eukaryotic translation initiation factor eIF3; direct recognition of m⁶A in the mRNA 5′ UTR by eIF3, probably under stress conditions; and direct recruitment of the ribosome by METTL3. YTHDF2, and potentially also YTHDF1 and YTHDF3, accelerate the degradation of m⁶A-containing mRNAs. YTHDF1, 2 and 3 proteins lead to liquid–liquid phase separation in the presence of mRNAs containing multiple m⁶A sites. This phase separation may mediate the recruitment of m⁶A-containing mRNAs to membraneless compartments, such as stress granules and P-bodies.

on capturing errors and decreased base-calling accuracy that occur at m⁶A positions. Although the approach is still noisy and currently not quantitative, it may allow direct assessment of the relationship between m⁶A sites and different RNA isoforms.

m⁶A in innate immunity

Humans are exposed to diverse potential pathogens daily. During the first hours of exposure, the innate immune system provides protection by sensing and resisting the threat. m⁶A modification and m⁶A machinery proteins have been shown to play major roles in regulating the innate immune response to viral infection. m⁶A is involved both in the sensing of foreign RNAs and in the regulation of transcripts involved in innate immune signaling.

Invading pathogens are sensed as non-self entities by a growing collection of pattern-recognition receptors. In the context of viral infection, the non-self molecules that are recognized are predominantly nucleic acids. The detection of foreign RNA is dependent on a number of pattern-recognition receptors, including the RIG-I-like receptors RIG-I and MDA-5 and Toll-like receptors (TLRs) 3, 7 and 8[82]. These receptors can recognize foreign RNAs through specific features—some of which are well characterized, such as extended double-stranded regions or a 5′ triphosphate[83]. Once recognition occurs, a signaling program is activated, leading to the induction of cytokines—especially type I interferons (IFNs)—that lead to an efficient antiviral response. RNA modifications were suggested to play a role in this recognition step, as modified nucleotides, including m⁶A, that are added to in vitro–transcribed RNAs suppress the activation of TLR3 and RIG-I[84,85]. This discovery raised the possibility that viruses use m⁶A-modified viral RNAs as a strategy to evade innate immune recognition by the host. Furthermore, since m⁶A is added cotranscriptionally in the nucleus[10,19], this may serve as an efficient strategy to discriminate between RNAs that were synthesized in the nucleus and non-self RNAs that were generated in the cytosol.

Another subclass of RNAs in which m⁶A is suggested to regulate immune recognition is circular RNAs (circRNAs). CircRNAs are non-coding RNAs that are generated by back splicing, which produces covalently linked circles that are highly stable[86]. Due to their stability, circRNAs have great potential to be used as effective delivery agents, and several studies have examined their immunogenicity[87–89]. Synthetically derived circRNAs are immunogenic in a RIG-I-dependent manner[88]. Conversely, host circRNAs evade

immune detection, and this evasion was suggested to depend on m^{6}A modifications[89]. However, another study showed that a synthetic circRNA, generated by additional purification steps that ensured the complete removal of non-circular RNAs, is not immunostimulatory[87]. Aside from technical differences that may have contributed to the different circRNA immunogenicity observed in these studies, these experiments indicate that circRNA immunogenicity depends on diverse features and that m^{6}A modifications likely contribute to this process. It was also shown that, when adenines are replaced with m^{6}A in short interfering RNAs (siRNA), the immune response against these modified siRNAs is reduced, without any detectable effect on RNAi activity[90].

It was shown that the genomic RNAs of human metapneumovirus contain m^{6}A modifications and that the methylation is important for evading immune recognition. Specifically mutating viral m^{6}A sites with synonymous mutations or removing m^{6}A from viral RNA by demethylation resulted in m^{6}A-deficient human metapneumovirus RNA that induced significantly higher IFN production in a RIG-I-dependent manner[22]. With a resemblance to m^{6}A, internal 2′-*O*-methylation of human immunodeficiency virus (HIV)-1 RNA by the cellular methyltransferase FTSJ3 can also prevent cellular immune recognition. HIV-1 viruses that were produced in cells in which FTSJ3 was perturbed triggered the induction of IFNs through the RNA sensor MDA5[91].

Together, these studies illustrate the potential for m^{6}A and additional RNA modifications to serve as marks for self-recognition. However, it is not yet known in these systems whether m^{6}A is sensed directly by any of the innate sensors or whether modulation of recognition is due to the ability of m^{6}A to reduce RNA secondary structure[55].

While the involvement of m^{6}A and additional RNA modifications in immune recognition of endogenous transcripts still needs to be fully deciphered, the role of 2′-*O*-methylation of the mRNA cap is well established. Among the most highly upregulated interferon-stimulated genes (ISGs) are the IFN-induced proteins containing tetratricopeptide repeats (IFITs). One of these proteins, IFIT1, restricts the translation of viral RNAs that lack 2′-*O*-methylation on the mRNA cap by preventing the recruitment of translation initiation factors[92,93]. Indeed, several families of viruses encode their own 2′-*O*-methyltransferase activity, and viral mutants lacking these enzymes are severely impaired through an IFIT1-dependent mechanism[94]. Interestingly, most mammals encode several IFIT proteins, and these paralogs exhibit diverse affinities for distinct RNA ligands[93]. Therefore, it is possible that the absence of other cap-associated modifications, including m^{6}Am (methylation of 2-*O*-methylated adenine present at the transcription start site) could be detected. These discoveries illustrate the importance of identifying the specific RNA species sensed by each pattern-recognition receptor and the role RNA modifications play in this process. The utility of using RNA modifications to distinguish self versus non-self is reminiscent of the restriction-modification DNA recognition systems that are widely used in bacteria.

m^{6}A regulation of the antiviral response

Immunity to pathogens is characterized by the production of potent cytokines, such as type I IFNs, that induce autocrine and paracrine antiviral resistance states. The IFN response is initiated by recognition of pathogen-associated molecular patterns (PAMPs) by cellular sensors. These sensors trigger signaling cascades resulting in transcription and secretion of type I IFNs, namely IFNα and IFNβ. Subsequently, type I IFNs bind to the interferon receptor (IFNAR) and activate the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, leading to the transcription of hundreds of ISGs that mediate the antiviral response[95]. The type I IFN response is fine-tuned by opposing activating and suppressing signals; these signals are responsible for inducing a rapid and effective antiviral response, while restraining the magnitude and duration of the response to avoid attendant toxicity. Therefore, the gene expression program that is activated by innate immune recognition must be tightly controlled. Indeed, m^{6}A and its machinery play a pivotal role in the regulation of several central mRNAs that participate in the antiviral immune response (Fig. 2).

The Cao group connected m^{6}A to the cellular antiviral response by showing that m^{6}A promotes antiviral immunity. Their first study suggested that viral infection enhances the binding of the RNA helicase DDX46 to transcripts encoding the antiviral proteins MAVS, TRAF3 and TRAF6, leading to the recruitment of ALKBH5, which demethylates these transcripts[96]. This demethylation in turn leads to retention of these transcripts in the nucleus, decreasing their protein levels and consequently inhibiting the production of type I IFNs. In a second study, hnRNPA2B1 was identified as a novel nuclear receptor for viral DNA that is critical for initiating host responses to herpesvirus infection[62]. In this work, a dynamic interaction between hnRNPA2B1 and the m^{6}A eraser FTO was defined. Upon infection, hnRNPA2B1 prevented the recruitment of FTO to *CGAS*, *IFI16* and *STING* mRNAs, thereby blocking m^{6}A removal from these transcripts and enabling their efficient export[62]. Thus, in both studies, m^{6}A played a crucial role in amplifying the antiviral responses through enhancing the export of different components in the type I IFN pathway. However, in the first study, infection led to an increase in demethylation by enhancing the recruitment of ALKBH5[96], whereas the second study showed that infection impeded demethylation by blocking FTO recruitment[62].

In contrast to these studies, other groups demonstrated that m^{6}A modification inhibits antiviral responses. We, along with the Mohr group, showed that the mRNAs of *IFNB* and *IFNA*, genes encoding the main cytokines that drive the type I interferon response, are m^{6}A-modified and are stabilized following depletion of METTL3 or METTL14, leading to increases in type I IFN expression and ISG induction[97,98]. Consequently, propagation of different viruses in cells that lack METTL3 or METTL14 was suppressed in an interferon signaling–dependent manner[97,98]. Furthermore, ALKBH5 depletion resulted in reduced levels of *IFNB*[98] and an increase in viral propagation[97,98]. These results therefore indicate that, by dictating the fast turnover of interferon mRNAs, m^{6}A acts as a negative regulator of the antiviral response. Another study illustrated that the m^{6}A reader YTHDF3 also restrains the type I IFN response and ISG expression, but the mechanism was suggested to be m^{6}A-independent and to involve enhancement of FOXO3 translation, which acts as an IFN transcriptional repressor[99].

Finally, a recent study by the Cao group contradicts their initial findings about ALKBH5, as it shows the innate immune response is impaired, rather than bolstered, by *Alkbh5* deficiency[100]. While the authors still argue that ALKBH5 is proviral, they say that this is because ALKBH5 demethylates the α-ketoglutarate dehydrogenase (OGDH) transcript, which reduces its stability and protein expression, leading to a more virus-friendly metabolic state[100].

In addition to the substantial roles m^{6}A modification plays in regulating the innate immune response to viral infection, m^{6}A also acts directly on viral RNA. m^{6}A decoration of viral RNA was described over 40 years ago, but its functional role remained unclear. Recent work uncovered a wide spectrum of viruses that contain m^{6}A in their genome and transcripts, including positive-sense and negative-sense RNA viruses, retroviruses and DNA viruses. Elucidating m^{6}A regulatory roles in viral RNA is now an active field of research, and diverse functions have been revealed (recently reviewed in ref. [101]). Since studies to date examining the innate immune response to infection or viral gene expression use cells or mouse models that lack one of the m^{6}A machinery enzymes, any effects on viral propagation, which is often used as the functional readout, could be attributed to either the regulation of antiviral cellular genes or to

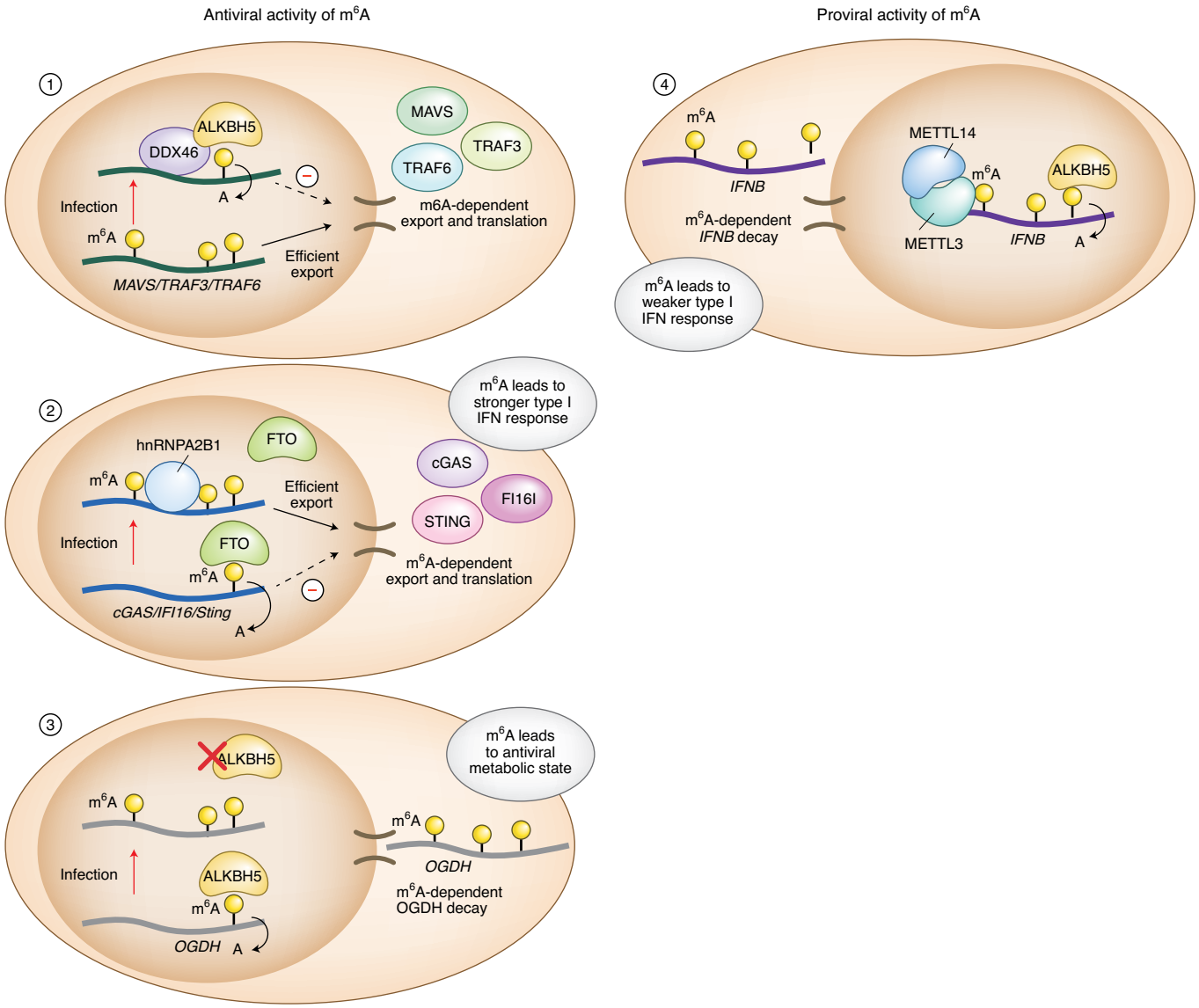

Fig. 2 | m⁶A regulates the innate immune response to infection through diverse mechanisms. Several mechanisms have been suggested for m⁶A involvement in the antiviral response. (1) During infection with vesicular stomatitis virus, the RNA helicase DDX46 recruits ALKBH5 to specifically erase m⁶A from *MAVS*, *TRAF3* and *TRAF6* transcripts. These m⁶A-deficient transcripts are retained in the nucleus, resulting in severe attenuation of the antiviral type I IFN response. (2) During infection with herpes simplex virus-1, hnRNPAB1 blocks FTO access to *cGAS*, *IFI16* and *STING* transcripts, thereby increasing their methylation levels and nuclear export, which produces a stronger antiviral response. (3) During infection with vesicular stomatitis virus or herpes simplex virus-1, ALKBH5 enzymatic activity is impaired, through demethylation of the protein. This results in elevated m⁶A levels, leading to reduced stability of the α-ketoglutarate dehydrogenase (*OGDH*) transcript and to a shift toward an antiviral metabolic state. (4) *IFNB* transcripts are regulated by m⁶A-mediated mRNA degradation. In the absence of m⁶A, the amounts of *IFNB* mRNA and protein increase, leading to a stronger antiviral response.

effects on viral RNA expression or processing. Inactivated viruses or artificial immune stimulators, such as poly(I:C) and synthetic double-stranded DNA, can allow the effects of interference with m⁶A machinery on the innate immune response to infection to be examined without the presence of viral transcripts[97,98]. On the other hand, JAK/STAT inhibitors that efficiently block signaling downstream of type-I IFNs can facilitate separation of the direct effects on viral mRNA from the indirect priming of the interferon pathway[97,98,102]. Extending such analysis to in vivo settings is more complex. One possible strategy is to use double-knockout mice in which both the IFN-I receptor and the m⁶A-machinery proteins are ablated (as was recently done by the Cao group[100]). This can enable dissection of whether in vivo changes in viral replication are independent of IFN-I signaling. However, these experiments should be interpreted cautiously, given the importance of the m⁶A machinery for immune system development and proper activation.

Ultimately, dissecting the direct effects of m⁶A modification on each of the identified transcripts (viral or cellular) via point mutations and by measuring their individual contribution to the observed phenotypes in diverse cell types and models will be an important focus for future studies.

m⁶A in adaptive immune responses

The immune system produces cells that circulate throughout the body and provide protection from invading microbes. These cells undergo progressive developmental stages in the process of acquiring effector functions. The adaptive immune response is an arm of the immune system that specializes in the clearance of specific

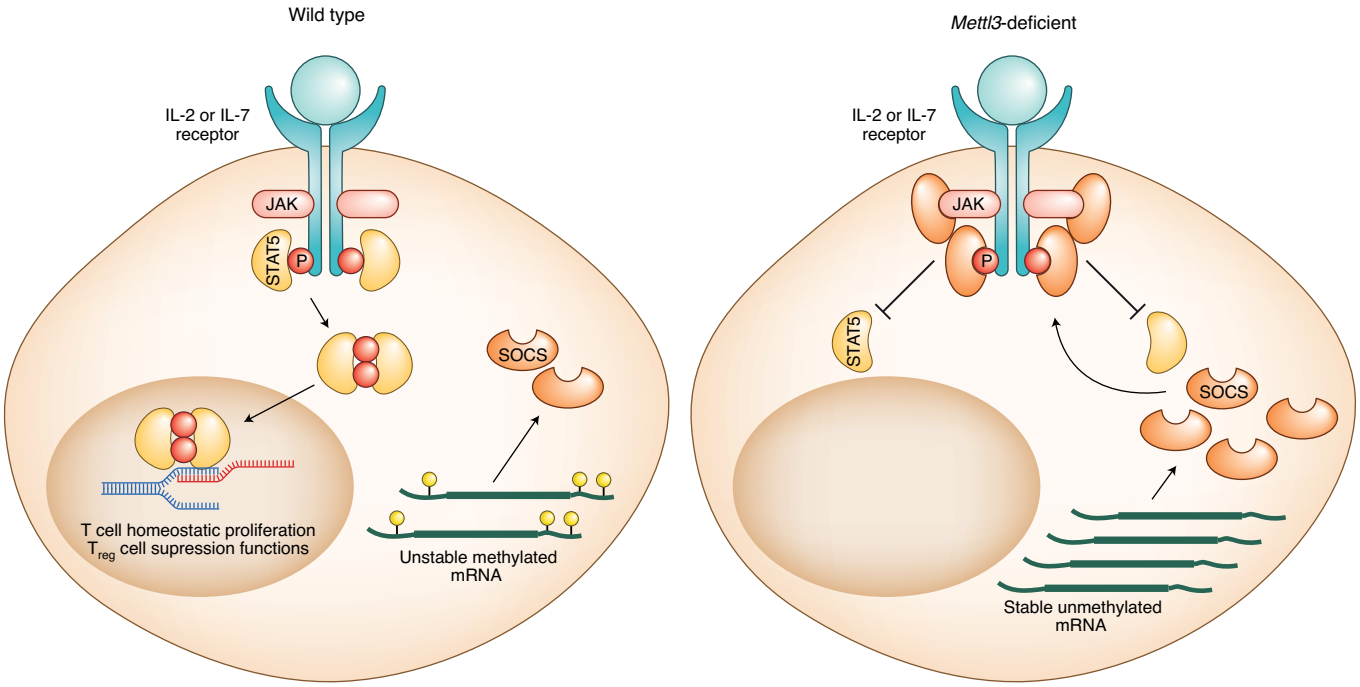

Fig. 3 | m⁶A modification regulates cytokine receptor signaling. *SOCS* genes are immediate early genes that impede cytokine receptor signaling and attenuate gene expression by interfering with STAT5 functions. The levels of *SOCS* transcripts are regulated by m⁶A-mediated mRNA degradation. In the absence of METTL3, the amounts of *SOCS* mRNA and protein increase. SOCS interferes with cytokine signal transduction by reducing STAT5 activation, leading to attenuation of homeostatic proliferation of CD4⁺ T cells and defects in T_{reg} cell suppression functions.

pathogens and the establishment of long-lasting immunological memory, involving intricate communication between multiple cell types. The mechanisms by which m⁶A regulates adaptive immunity is an emerging field of investigation. METTL3 deletion is embryonic lethal[64]; therefore, to study the role of m⁶A in immune cells in vivo, conditional knockout systems are being used. Vav1 is expressed in all immune cells, and expression of Cre recombinase under the Vav1 promoter is widely used in analyzing the immune response. However, deletion of METTL3 in the Vav1-Cre mouse strain results in non-viable progeny[103] (Z. Shulman and N. Stern-Ginossar, unpublished observation). Therefore, researchers have used transgenic mice that express Cre recombinase under cell-specific promoters such as CD4, CD11C and Foxp3. It is important to note that, in these mouse models, considerable levels of m⁶A were still detectable[65,104], probably reflecting incomplete METTL3 deletion.

The first study that investigated the function of m⁶A in immune cells focused on its role in CD4⁺ T helper cells. Deletion of METTL3 specifically in CD4⁺ T cells did not have a major effect on their generation and maturation in the thymus, a process that is highly dependent on T cell receptor (TCR) stimulation, as well as multiple costimulatory signals[65]. The role of m⁶A machineries in the thymic development of CD8⁺ T cells remains unknown and requires additional investigation. Furthermore, METTL3-deficient CD4⁺ T cells were able to respond to TCR stimulation in vitro, suggesting that the basic TCR machinery and downstream signal transduction do not depend on m⁶A modification in these settings. Nonetheless, a role for m⁶A machineries in TCR signaling in response to cognate antigen recognition in vitro, or following exposure to a pathogen in vivo, cannot be excluded. In particular, the role of mRNA methylation in TCR signaling in CD8⁺ T cells is yet to be determined.

In CD4-Cre *loxP*-flanked-METTL3 ($METTL3^{fl/fl}$) mice, the proportion of circulating naive cells was higher than in wild-type mice. Consistently, a lower number of activated CD4⁺ T cells were observed in a spontaneous colitis model, wherein CD4-Cre $METTL3^{fl/fl}$ T cells were adoptively transferred into RAG2-deficient mice, indicating that m⁶A plays a role in retaining naive cells in a quiescent state[65]. In this setting, METTL3-deficient T helper cells failed to proliferate under the lymphopenic conditions and were unable to differentiate into pathogenic effector T cells that induce colitis[65]. Consistently, T cells lacking METTL14, an additional component of the methylation complex, showed identical phenotypes[65]. IL-7 is essential for T cell homeostatic proliferation, and the phenotype of METTL3-deficient T cells shows striking similarity to that of CD4⁺ T cells that were adoptively transferred into IL-7-deficient mice[105]. Members of the *SOCS* gene family act as adaptors that bind cytokine receptors, including the IL-7 receptor, and prevent STAT5 activation and downstream signaling[106]. In the absence of *SOCS* genes, T cells are highly responsive to IL-7 signaling, whereas overexpression of these genes suppresses IL-7-dependent T cell survival[107]. Furthermore, *SOCS* genes belong to the immediate early gene group, which includes genes that are rapidly transcribed in response to acute signals[108]. These genes are characterized by a short mRNA half-life[109], and many of the transcripts within this group are methylated, suggesting that the degradation of immediate early genes might be regulated by m⁶A-mediated mechanisms[65]. Consistent with these observations, the mRNAs of the *SOCS* genes *Socs1*, *Socs3* and *Cish* are marked by m⁶A and exhibit slower mRNA decay and higher protein expression in METTL3-deficient T helper cells. Moreover, the higher SOCS expression likely impedes signal transduction through the IL-7 receptor (Fig. 3)[65]. Overall, this study reveals the critical role m⁶A plays in lymphocyte functions and suggests that this effect is mediated by regulation of SOCS mRNA stability[65]. Whether TCR signaling in vivo is also affected by m⁶A-mechanisms requires further investigation[110].

CD4⁺ regulatory T (T_{reg}) cells are a subset of differentiated CD4⁺ T cells that mediate the negative regulation of immune cell

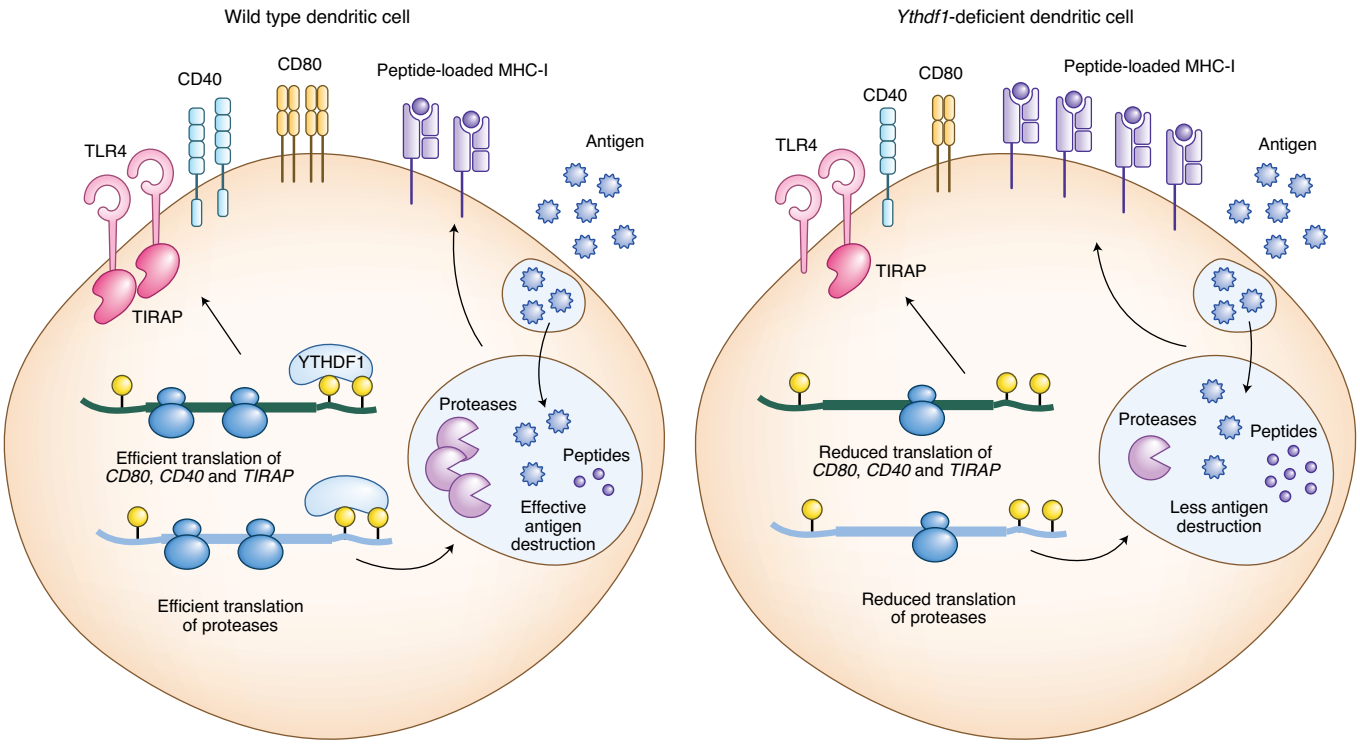

Fig. 4 | Dendritic cell functions are modulated by m^{6}A modification. In the absence of the m^{6}A reader YTHDF1, translation of genes encoding proteases is less efficient and, as a result, fewer antigen-degrading enzymes are found in the phagosome. Accordingly, more peptides are available for antigen cross-presentation on surface MHC class I molecules. Lack of m^{6}A in LPS-stimulated DCs also leads to inadequate translation of costimulatory molecules, such as CD80 and CD40 and the TLR4 adaptor TIRAP (also known as Mal).

functions and prevent the emergence of deleterious autoimmune diseases. The hallmark of this cell lineage is the transcription factor Foxp3, which is essential for its development and function. T_{reg} cells express high amounts of the IL-2 receptor α-chain, which activates STAT5, and this pathway is essential for their suppressive functions[111–113]. Mice that lack METTL3 specifically in T_{reg} cells (the Foxp3-Cre mouse strain) develop severe autoimmune disease and die only a few weeks after birth[65,114]. These mice develop normal numbers of Foxp3$^+$ T_{reg} cells, but the cells are unable to mediate their suppressive functions. Like depletion in total CD4$^+$ T cells, depletion of METTL3 specifically in T_{reg} cells leads to an increase in the mRNAs of the SOCS gene family[114]. Thus, in T_{reg} cells, the m^{6}A machinery enables efficient IL-2 receptor signaling through *SOCS* mRNA decay, thereby promoting T_{reg} suppressive functions (Fig. 3).

An interesting question that stems from these studies is why there is a need to use accelerated mRNA degradation as a mechanism to control SOCS gene expression. SOCS family members negatively regulate inflammatory cytokine signaling and, thereby, prevent unnecessary activation of the immune system[106]. Thus, it is possible that, to rapidly respond to cytokine stimulation under inflammatory conditions, SOCS are rapidly degraded. Yet IL-7 is not a classical inflammatory cytokine, so it is most likely that, under homeostatic conditions, IL-7 signaling must overcome the SOCS-dependent regulatory checkpoint to support steady-state proliferation.

An additional mechanism that regulates the fitness of T_{reg} cells involves miR-155, a microRNA that also limits SOCS1 mRNA levels[115]. Although both m^{6}A and miR-155 target SOCS1 mRNA, it seems that they are not redundant, and it will be interesting to characterize how these pathways interact to achieve optimal SOCS1 repression.

m^{6}A machinery regulation of dendritic cells

Lymph nodes and spleen are the primary sites where naive T cells interact with antigen-presenting cells (APCs), which display cognate peptides on their surface. The main APCs that activate T cells are dendritic cells (DCs), which are innate immune cells with the capacity to effectively take up, process and present antigen on the cell surface. Naive T cells are highly motile cells that constantly circulate through the blood and migrate to the lymphoid organs in search of DCs presenting cognate antigens[116]. Upon antigen encounter, T cells arrest their movement and form immunological synapses with DCs[117]. In addition to antigen presentation, interactions between costimulatory molecules on DCs and their ligands on T cells also contribute to proper T cell activation[118,119].

The m^{6}A writer complex, including METTL3, is expressed at higher levels in mature DCs as compared to their immature counterparts[104]. Similarly, METTL3 expression is enhanced in lipopolysaccharide (LPS)-treated cells derived from dental pulp, and these most likely contain many antigen-presenting DCs, among other immune cell types[120]. Depletion of *Mettl3* in DCs results in their impaired maturation in response to LPS, with decreased expression of the costimulatory molecules CD40 and CD80 and inflammatory cytokines, and a reduced ability to induce T cell responses. Knockdown of *YTHDF1* by shRNA also reduced the expression of these costimulatory molecules; however, these defects were not observed in DCs derived from YTHDF1-deficient mice that were stimulated with the FLT3 ligand[121]. Therefore, optimal expression of the costimulatory molecules CD40 and CD80 depends on m^{6}A, but the effect may be context dependent[104,121]. Furthermore, LPS-mediated DC activation also depends on the expression of the TLR4 adaptor TIRAP (also known as Mal), regulated by METTL3 functions[104]. Thus, the m^{6}A modification is required for DC maturation in response to LPS and for effective translation of T cell–priming

molecules (Fig. 4). However, other DC activation signals or DCs located in different microenvironments in vivo may not depend on m^{6}A machineries for their proper maturation and for the expression of costimulatory molecules.

These findings suggest METTL3-dependent mechanisms may enhance DC maturation and priming efficiency during an immune response against bacteria. In addition, higher expression of CD40 has the potential to affect the balance between induction of tolerance and activation of antigen-specific T cells[118,122]. It also remains to be determined whether this m^{6}A-regulated mechanism is specific for TLR4, or whether other pattern-recognition receptors that induce APC maturation, such as TLR2[123], show a similar dependence.

Studies in mice and clinical trials demonstrated that endogenous T cells have the ability to recognize tumor-derived antigens and facilitate the clearance of malignant cells. Tumor-specific T cells are primed by tumor-associated DCs that take up tumor antigens and present them to CD8$^+$ T cells[124]. The chain of events in which APCs take up, process and present extracellular antigens on major histocompatibility complex (MHC) class I is called antigen cross-presentation[121,125].

Antigen cross-presentation by tumor-infiltrating DCs in melanoma or colon tumors is more effective in the absence of YTHDF1, and mature *Ythdf1*-deficient DCs are more effective in T cell activation than wild-type cells, both in vitro and in vivo[121]. *Ythdf1* depletion in DCs was found to attenuate the translation of genes that are associated with the phagosome and lysosome pathways, including enzymes that are members of the cathepsin family[121]. These enzymes degrade proteins in the phagosome and thereby limit antigen cross-presentation through the destruction of antigens after uptake by DCs[125–127]. This low expression of cathepsin proteins in *Ythdf1*-deficient DCs results in slower antigen degradation and more efficient cross-presentation to CD8$^+$ T cells (Fig. 4). Furthermore, treatment of mice with an anti-PD-L1 antibody further potentiates the capacity of CD8$^+$ T cells that were primed by *Ythdf1*-deficient DCs to eliminate tumor cells[121]. Overall, m^{6}A plays a major role as DCs transition from immature cells into effective T cell activators that express costimulatory molecules and promote the initiation of the adaptive immune response through antigen cross-presentation.

m^{6}A and immune system development

Hematopoietic stem cells (HSCs) generate all blood cell types throughout the life of vertebrate organisms while maintaining self-renewal capacity[128]. These cells arise from a subset of endothelial cell progenitors known as hemogenic endothelial cells, which are found in the ventral wall of the dorsal aorta[129–131]. Hemogenic endothelial cells express high levels of METTL3, which is required for their differentiation into HSCs, both in zebrafish and in mice[132,133]. Notch signaling is central to this process, as it represses HSC reprogramming and maintains endothelial cell identity[134–136]. It was demonstrated that m^{6}A, through its reader YTHDF2, is critical for the transition of endothelial cells into HSCs by reducing the levels of Notch1a mRNA, thereby downregulating Notch1 signaling[132,133]. Deletion of METTL3 after HSCs are formed in an Mx1-Cre mouse model results in their massive accumulation in the bone marrow and spleen, and effective hematopoiesis progression is severely blocked[67,137]. An additional study that examined hematopoiesis capacity in METTL3-deficient HSCs by single-cell RNA sequencing (RNA-seq) revealed an additional cell population that showed an intermediate differentiation state and resembled multipotent progenitors[68]. As opposed to the initial findings, this study suggests that METTL3-deficient HSCs are depleted, whereas the cell population that accumulates in these mice are HSC multipotent progenitor–like cells with reduced self-renewal capacity[68]. The presence of this new cell population may have been missed in the initial studies due to analyses that employed bulk RNA-seq methods and different cell-specific markers. Nonetheless, regardless of the exact identity of the accumulating progenitor cell population, all three studies show that METTL3 is essential for hematopoiesis and the generation of immune cell lineages[67,68,137]. The hematopoiesis block caused by deletion of METTL3 is specific for HSC differentiation, as the deletion of METTL3 in mature differentiated immune cells, such as in myeloid cells and in CD4$^+$ T cells, had no substantial effect on their generation and maintenance[65,67]. Myc transcripts are enriched with m^{6}A modifications, and high Myc levels promote HSC differentiation[138,139]. Although Myc transcript levels were not significantly altered in METTL3-deficient HSCs, Myc target gene signatures were decreased, suggesting that m^{6}A may regulate Myc mRNA translation in HSCs[67]. In support of this model, forced expression of Myc rescued differentiation defects in METTL3-deficient HSCs[67]. Consistently, Myc transcripts are destabilized by m^{6}A, and the reduction in Myc expression affects multipotent progenitor self-renewal by promoting asymmetric cell division[68]. In summary, the ability to maintain sufficient levels of Myc and the generation of intact hematopoiesis depend on METTL3 activity in HSCs or HSC-like progenitors.

The finding that METTL3 is critical for HSC differentiation contradicts earlier reports that deletion of METTL3 or METTL14 in human CD34$^+$ hematopoietic progenitors in vitro promotes HSC differentiation[69,140]. It is most likely that these differences stem from the source of the cells or the knockdown approaches that were used in these studies. Alternatively, the physiological context in mice might lead to different outcomes in HSC differentiation.

One of the best-characterized consequences of m^{6}A modification is accelerated mRNA degradation (Fig. 1). Interestingly, YTHDF2-deficient mice also exhibited an increased number of HSCs[141,142]; however, in contrast to METTL3- and METTL14-deficient HSCs, YTHDF2-deficient HSCs demonstrated improved differentiation capacity and an increase in Myc transcripts[69,142]. These results may indicate that any deviation in Myc levels interferes with HSC differentiation capacity and that additional mechanisms involving other m^{6}A readers might contribute to enhancing HSC accumulation in mice.

Collectively, these studies demonstrate that m^{6}A modification is required for proper HSC differentiation, and that this process also likely involves m^{6}A-dependent regulation of Myc mRNA levels. Directly measuring m^{6}A stoichiometry during hematopoietic lineage differentiation and identifying the role of m^{6}A in the different developmental stages and branches will uncover how m^{6}A orchestrates immune cell differentiation.

Future directions

Recent advances in mapping m^{6}A in mRNAs, coupled with the ability to manipulate the enzymes involved in m^{6}A deposition and recognition, have begun to expose the different molecular consequences associated with RNA methylation and the central role it plays in diverse biological processes, including regulating different facets of immunity. In the cellular response to infection and in the few immune cell types that have been analyzed, depletion of m^{6}A writers, readers or erasers had significant phenotypic consequences. These findings portray RNA modifications as a novel critical layer of post-transcriptional gene regulation that controls immune cell functions and cell fate decisions in the hematopoietic lineage.

Although the field is advancing rapidly, major knowledge gaps remain to be filled. So far, most of the conclusions in the field were based on the deletion of one of the components of the m^{6}A machinery; therefore, bridging the gaps between phenotype, specific methylated mRNAs and molecular mechanism remains a major challenge. One straightforward strategy that could advance our understanding of how m^{6}A-modified transcripts lead to a certain phenotype is to conduct a deeper analysis of the phenotypic effects of different m^{6}A-readers. So far, the majority of immunological

phenotypes that depend on m^{6}A were studied using cells in which one of the essential components of the m^{6}A writer complex was deleted. Identifying which of the m^{6}A readers drives the observed phenotypes, and whether deletion of individual or multiple m^{6}A readers could recapitulate the effects seen when the modification is deleted, will provide a clearer view of the underlying molecular mechanism. Since some of the characterized readers seem to have opposing effects on protein production, these experiments would provide opportunities to decipher how these signals integrate, especially if they are extended to animal models. YTHDC1 and YTHDF1 were reported to enhance the expression of m^{6}A-containing transcripts by accelerating their export and translation, respectively. In contrast, YTHDF2 has so far been associated with increased decay of m^{6}A-containing transcripts, thereby leading to a reduction in their expression. Identifying whether there are corresponding opposing effects of these readers in vivo will advance our understanding of this complex regulation. This phenotypic dissection can then be extended to molecular analysis to understand the transcripts and mechanisms through which each of the m^{6}A readers act and how these are embedded and regulated within the cellular circuitry. Obtaining a better understanding of the factors involved in decoding the m^{6}A modification may provide the tools to determine whether the distinct molecular effects (for example, impact on stability, translation or localization) are truly independent of each other or whether they reflect the coupling of different steps in the mRNA cycle.

Conceptually, it is still unclear why and under which conditions cells rely on RNA modifications to control gene expression. The direct effects of m^{6}A modification on modified transcript expression are mostly subtle, so it is therefore likely that m^{6}A is used to fine tune dosage-sensitive genes, for which small fluctuations in protein expression may contribute to a substantial functional output. Alternatively, in analogy to models that have been proposed for miRNA regulation[143], RNA methylation can regulate groups of transcripts that are part of the same response or process, thus contributing to the robustness of specific functional responses or transcriptional programs that dictate cell fate decisions. However, in contrast to miRNAs, in which each miRNA molecule can target a different subset of genes, m^{6}A is a single modification that presumably affects all modified transcripts in a similar way. Thus, although it is possible that the effect of m^{6}A is dependent on its location in the transcripts or on its stoichiometry, the ability of m^{6}A to specifically regulate a confined group of genes is more limited and currently poorly characterized.

The ultimate challenge is to link specific methylation sites to phenotypes. This is a daunting task, as there are tens of thousands of methylated sites in the mammalian transcriptome, and deciphering which of them—or which combination of them—is causally linked to a phenotype is an immense challenge. This endeavor resembles the challenge of understanding which miRNA binding sites underlie phenotypes of miRNA deletions, which has lagged behind the description of these phenotypes by almost a decade[144]. In the context of immune regulation, several m^{6}A-modified transcripts are products of dosage-sensitive genes that were suggested to drive phenotypic changes in the immune system. Testing whether point mutations that eliminate methylation sites in these genes recapitulate some of the observed phenotypes generated through deletion of the m^{6}A writer complex will be a major step forward.

In the context of pro- and antiviral phenotypes attributed to the m^{6}A pathway, the studies to date indicate three potentially interconnected layers of m^{6}A regulation; sensing of foreign RNA, direct regulation of viral transcripts and regulation of transcripts involved in the cellular response to infection. While it is clear that m^{6}A modification regulates viral RNA and key transcripts in the antiviral type I IFN response, it will be important to substantiate the role of m^{6}A in sensing viral nucleic acids and to decipher how this process integrates to produce the observed phenotypes. Performing measurements of both viral and cellular RNA at high temporal resolution of infected cells that lack or express the m^{6}A machinery could be very informative for determining the order of events and the underlying mechanisms.

Finally, in the studies to date, complete loss-of-function mutants of m^{6}A machinery proteins were used. Studies analyzing how m^{6}A readers, writers and erasers are modulated in cells during immune responses may improve our understanding of the role the m^{6}A machinery plays in regulating immunity and will also help to reveal to what extent and when the m^{6}A modification is dynamically regulated in a physiological context.

Though vast advances have been made in recent years, our understanding of how the m^{6}A modification contributes to immunological phenotypes is still in its infancy. Deciphering how dysregulated methylation or dysregulated recognition of methylated positions lead to diverse immunological phenotypes will require additional research. While this review has focused on m^{6}A, it is becoming apparent that the collection of modifications on mRNAs might be wider. Subsequent analysis of RNA modifications in physiological settings in animal models will advance our knowledge concerning how these RNA modifications act and how they regulate immunity.

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Additional information

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