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Cutting Edge: Novel *Tmem173* Allele Reveals Importance of STING N Terminus in Trafficking and Type I IFN Production

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Cutting Edge: Novel *Tmem173* Allele Reveals Importance of STING N Terminus in Trafficking and Type I IFN Production

Guy Surpris,^{*} Jennie Chan,[†] Mikayla Thompson,[†] Vladimir Ilyukha,[‡] Beiyun C. Liu,^{*} Maninjay Atianand,[†] Shruti Sharma,[†] Tatyana Volkova,[‡] Irina Smirnova,[§] Katherine A. Fitzgerald,[†] and Alexander Poltorak^{*,‡,§,¶}

With the stimulator of IFN genes (STING) C terminus being extensively studied, the role of the N-terminal domain (NTD) of STING remains an important subject of investigation. In this article, we identify novel mutations in NTD of *Sting* of the MOLF strain in response to HSV and *Listeria monocytogenes* both in vitro and in vivo. These mutations are responsible for low levels of IFN- β caused by failure of MOLF STING to translocate from the endoplasmic reticulum. These data provide evidence that the NTD of STING affects DNA responses via control of trafficking. They also show that the genetic diversity of wild-derived mice resembles the diversity observed in humans. Several human alleles of *STING* confer attenuated IFN-I production similar to what we observe with the MOLF *Sting* allele, a crucial functional difference not apparent in classical inbred mice. Thus, understanding the functional significance of polymorphisms in MOLF STING can provide basic mechanistic insights relevant to humans. *The Journal of Immunology*, 2016, 196: 547–552.

The main pathway of cytosolic DNA-stimulated cytokine production, including type I IFNs, is mediated by stimulator of IFN genes (STING) (1, 2). Upon activation, STING moves from the endoplasmic reticulum (ER) and aggregates via oligomerization of the STING cytosolic C-terminal domain (CTD) (3). These aggregates provide a platform for binding and autophosphorylation of TBK1 (tank binding kinase 1), which then phosphorylates IRF3 (4). STING is activated by dsDNA via upstream DNA sensors such as IFI16 (5), or cGAS (6), or via direct binding

of cyclic dinucleotides as well as DMXAA (7–9), a tumor-vascular disrupting agent. In contrast with CTD (3), the role of the N-terminal domain (NTD) in activation of STING remains unclear. It is believed that the NTD anchors STING in the ER (10). Also, targeting of Atg9a (autophagy related protein) leads to aberrant activation of DNA response, suggesting importance of autophagosomes for STING function (11). Thus, STING's intracellular trafficking is important for its function, but the mechanism of trafficking is largely unknown.

In humans, *STING* locus is linked to susceptibility to esophageal cell carcinoma (12, 13) and responses to vaccines (14, 15). *HAQ* allele (R71H-G230A-R293Q) is linked to the loss of IFN- β production in response to cyclic dinucleotides (CDNs), whereas gain-of-function *A154S*, *V155M*, *V147L* alleles are associated with vasculopathy and elevated type I IFN (16). Additional SNPs in human STING have been identified that affect responses to CDNs (17). Together with the high frequency of human potentially nonfunctional alleles of *STING* (18), these data support clinical relevance of studies of STING-mediated responses to DNA. Using wild-derived MOLF mice, we positionally cloned a novel allele of *Sting* that fails to activate *Ifnb1* because of novel mutations L47V, A48G, and S53L in its NTD, which abrogate translocation of STING from the ER upon stimulation. Further genome-wide inquiry revealed that MOLF *Sting* is a hypomorphic allele that confers normal levels of IL-6, and several other cytokines, thus implying important biological functions of the NTD of STING.

Materials and Methods

Mice

C57BL6/J and MOLF/EiJ mice were from The Jackson Laboratory (Bar Harbor, ME); STING-deficient mice were from Glen Barber (University of Miami). B6.MOLF-*Tmem173*^{molf} (STING^{MOLF/MOLF}) congenic mice were

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The RNA-sequencing data presented in this article have been submitted to the National Center for Biotechnology Information's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50682>) under accession number GSE50682.

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Abbreviations used in this article: c-di-AMP, cyclic diadenylate monophosphate; c-di-GMP, cyclic diguanylate monophosphate; CDN, cyclic dinucleotide; CTD, C-terminal domain; dAdT, poly(deoxyadenylic-deoxythymidylic acid sodium salt); ER, endoplasmic reticulum; ISG, IFN stimulated gene; NTD, N-terminal domain; poly(I:C), polyinosinic-polycytidylic acid; STING, stimulator of IFN gene; TBK1, tank binding kinase 1.

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established via genotyping of the backcross mice at *Tmem173* locus. A panel of F2 intercross mice was generated first by producing F1 (C57BL6/J × MOLF) mice, followed by brother-sister intercrossing. Mice were phenotyped at the age of 6–8 wk. For phenotyping, peritoneal macrophages were plated at a density of 10^5 cells/well of the 96-well plate and transfected (Lipofectamine 2000) next day with cyclic diadenylate monophosphate (c-di-AMP) or cyclic diguanylate monophosphate (c-di-GMP) (2 μ g/ml) for 16 h. Genome-wide genotyping of the F2 panel of mice was performed with three polymorphic microsatellite markers per chromosome using MIT primer sequences obtained from The Jackson Laboratory Mouse Genome Informatics Web site: <http://www.informatics.jax.org/marker>. PCR fragments were amplified using Jumpstart REDtaq (Sigma) and resolved on 3% gels. J/qtl v.1.3.x and R.2.0 software was used for linkage analysis. For generation of the B6.MOLF-*Tmem173*^{molf} (STING^{MOLF/MOLF}) congenic mice, F1 (B6 × MOLF) hybrids were backcrossed for nine generations to C57BL6/J. Phenotyping assays with congenic mice were done using 8- to 12-wk-old females.

RNA-sequencing

Peritoneal macrophages were stimulated for 4 h with 2'3' cGAMP (4 μ g), dAdT (4 μ g), or DMXAA (10 μ g) as previously indicated. Total RNA was isolated using TRIzol and used to make a directional cDNA library using TrueSeq kit. Seventy-five bp pair-end reads from cDNA libraries were generated on MiSeq (Illumina) and aligned using TopHat2 and Cufflinks software. Log-transformed values were displayed by heat map. The data are available at the National Center for Biotechnology Information Gene Expression Omnibus: <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50682>.

Confocal imaging of MEFs

STING^{-/-} mouse embryonic fibroblasts were infected with lentiviral particles generated from pLEX-STING lentiviral constructs, pMD2.G and psPAX2 plasmids, followed by selection with puromycin (2 μ g/ml) for 2 d followed by recovery for at least 3 more days before use. The cells were grown, stimulated, and imaged on glass coverslips using a spinning-disc confocal microscope with a modified live-cell imaging system (UltraVIEW; Perkin-Elmer) consisting of a five-line laser launch (442/488/514/568/647 nm; Prairie Technologies), a spinning-disc confocal head (CSU-10; Yokogawa Corporation of America), an Axiovert 200M stand (Carl Zeiss), a 40× Plan-Neofluar oil immersion objective lens (NA 1.3; Carl Zeiss), and an intensified CCD camera (XR MEGA-10; Stanford Photonics). ImageJ analysis was done using ImageJ software (National Institutes of Health). Colocalization analysis was done using Coloc2 plugin.

Reporter assay

293T cells at density 5×10^4 cells/well were transfected (Lipofectamine 2000) with 12.5 ng Luciferase reporter plasmids carrying full IFN- β promoter, or PRDII (NF- κ B), or PRDIII (IRF3) promoter driving Luc gene, 12.5 ng Renilla Luciferase-pRL-TK, and 30 ng STING pEF-BOS plasmid encoding

STING. Sixteen hours after transfection, luciferase activity was determined with Dual-Glo Luciferase Assay System on Spectramax M5.

Reagents

Polyinosinic-polycytidylic acid [poly(I:C)] and poly(deoxyadenylic-deoxythymidylic acid sodium salt) (dAdT) were purchased from Sigma Aldrich. c-di-GMP, c-di-AMP, 2'3' cGAMP, 3'3' cGAMP, and DMXAA were purchased from Invivogen. Lipofectamine 2000 (Life Technologies) was used to transfect c-di-GMP, c-di-AMP, 2'3' cGAMP, 3'3' cGAMP, and dAdT. Xtremegene 9 to transfect poly(I:C). For stimulation experiments, macrophages were plated at a density of 10^6 cells/ml. C-di-nucleotide stimulations were done as indicated at 2 or 4 μ g/ml. Abs used were as follows: (Cell Signaling) STING 3337; p65 8242; Phospho-p65 3033; ERK 4695; Phospho-ERK 4370; p38 9212; Phospho-p38 4511; TBK1 3013; Phospho-TBK1 5483; Phospho-IRF3 4947; JNK 9258; Phospho-JNK 9251; HA-Tag 3724; (ENZO) Grp94, ADI-SPA-850.

Results and Discussion

MOLF macrophages have attenuated type I IFN responses to cytosolic DNA

In a genetic screen for DNA responses, we found impairments in the IFN response of MOLF macrophages to both HSV-1 and *Listeria monocytogenes*, whereas responses to Sendai virus, an RNA virus, were normal. In addition, MOLF macrophages produced significantly less IFN in response to dAdT, which requires cGAS (19) and 2'3' cGAMP (Fig. 1A) compared with B6 macrophages. Surprisingly, IL-6 production in response to all CDNs, but not DMXAA, was significantly higher in MOLF than in B6 macrophages (Fig. 1C) with very low levels of IFN for all the agonists. In extension of these findings, infection of MOLF mice in vivo led to low IFN-I but high IL-6 production in MOLF mice (Fig. 1B). Transcriptional analysis of IFN-stimulated genes (ISGs) in response to infection in MOLF further confirmed the defect in their IFN responses, although several NF- κ B-regulated genes, for example, *Il1a*, *Il1b*, *Tnf*, *Tlr2*, and *Il6*, were strongly upregulated (Supplemental Fig. 2D).

In support of signaling defects upstream of IFN, we found TBK1 and IRF3, a downstream target of TBK1 and the main transcriptional activator of type I IFNs, to be poorly activated in CDN-activated MOLF macrophages (Fig. 1D). Thus, low levels of IFN- β in MOLF macrophages in response to DNA can be explained by decreased activity of TBK1 and IRF3.

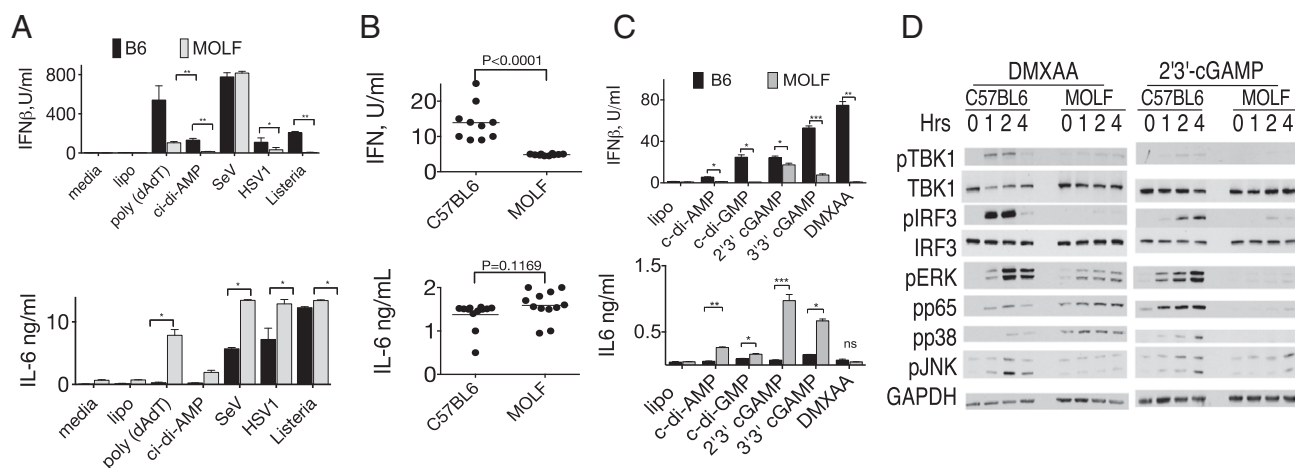


FIGURE 1. MOLF mice are defective in type I IFN production in response to cytosolic DNA: (A) IFN- β or IL-6 production in peritoneal macrophages after 16 h of treatment with dAdT, c-di-AMP, HSV, *Listeria*, or Sendai virus (SeV) (ELISA); (B) groups of B6 and MOLF mice were infected in vivo with *L. monocytogenes* or HSV-1 for 24 h followed by analysis of IL-6 and IFN- β ; (C) production of IFN- β and IL-6 in MOLF and B6 in response to synthetic DNA analogs; and (D) phosphorylation of TBK1 and IRF3 is attenuated in 2'3' cGAMP (4 μ g/ml) or DMXAA (10 μ g/ml) activated MOLF macrophages. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The defect in IFN production in response to DNA maps to *Sting* in MOLF

To identify the gene(s) that confer the IFN defect, we generated a panel of F2 intercross ($B6 \times MOLF$) mice, assayed their IFN responses to CDNs, HSV, and *L. monocytogenes*, and looked for association (linkage) between their phenotype and genotype (Supplemental Fig. 1A). All traits (except Sendai virus responses) were linked to *Tmem173*, the gene that encodes STING, a well-characterized mediator of DNA responses. High-resolution mapping confirmed STING as the gene candidate. Assortment of the F2 progeny by their haplotype at the *Tmem173* locus revealed that $B6/MOLF$ mice were phenotypically indistinguishable from $MOLF/MOLF$ individuals (Fig. 2A), suggesting dominance of the MOLF allele.

We identified multiple polymorphisms in MOLF STING—L47V, A48G, S53L, S103F, I114M, Y115C, Y126S, and 6-aa deletion, 116–121, all in the NTD, with one change, N210D, in the CTD of STING (Fig. 2B, Supplemental Fig. 1B). Structural analysis using the Smart Motifs (<http://smart.embl-heidelberg.de/>) and ELM (elm.eu.org) databases software predicted these polymorphisms would alter secondary structure and would disrupt the transmembrane regions of the

STING protein (Fig. 2B). Phylogenetic analysis of STING in other mouse subspecies suggested evolutionary significance of the MOLF allele of *Sting* (Supplemental Fig. 1C).

Alignment of MOLF STING against STING from other species revealed preservation of the L47, S53, and S103 aa through all the mammals (Supplemental Fig. 1D) and were predicted to disrupt, respectively, the BRCA1 and 14-3-3 interacting region, both being highly conserved motifs. To directly assess the ability of MOLF STING to activate downstream components, we examined the effect of MOLF STING on activation of luciferase reporter driven by the *Irfb1* promoter regulatory elements. We observed no activation of the reporter for MOLF STING compared with B6 STING (Fig. 2C). Thus, identified polymorphisms are mutations that disrupt function of STING.

It is established that STING oligomerization and departure from the ER is integral to its function. To determine the ability of MOLF STING's translocation, we expressed fluorescently tagged STING in MEFs deficient for STING. In resting cells, both B6 and MOLF STING colocalized with the ER marker. Upon activation with 2'3'cGAMP, B6 STING moved out of the ER and displayed a punctate nuclear localization (Fig. 2E,

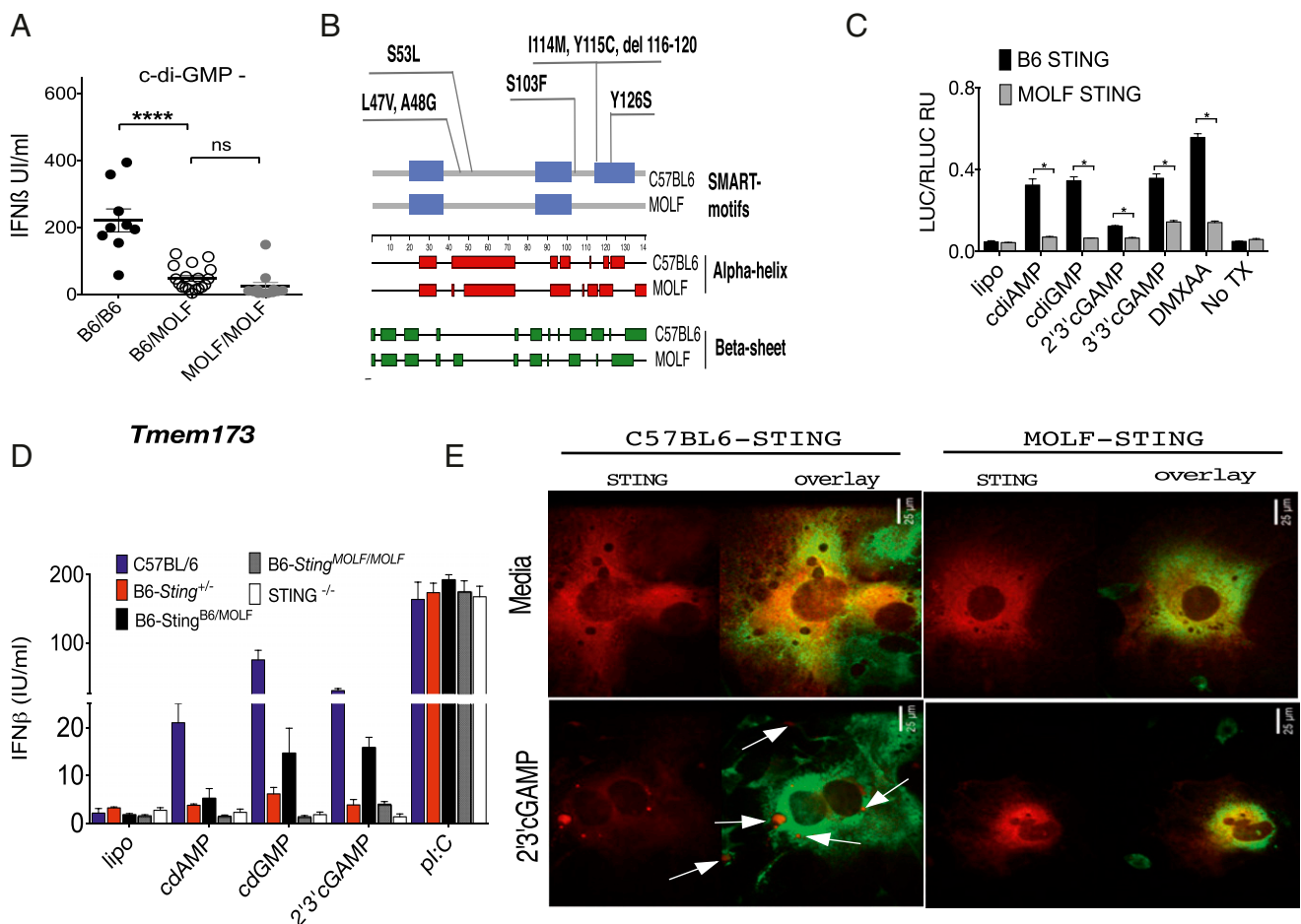


FIGURE 2. Defect in IFN production in MOLF is conferred by a dominantly inherited mutant allele of *Sting*. (A) Haplotype assortment of IFN- β by F2 mice at marker D18Mit202 in the vicinity of *Tmem173*. (B) Identified polymorphisms in MOLF STING change predicted motifs and structure of the protein. (C) 293T luciferase reporter assay with B6 or MOLF STING cDNA in pEF-*Bos* activating *Irfb1* promoter in response to different STING agonists. (D) Macrophages were transfected (Lipofectamine) with STING agonists or poly(I:C) for 16 h, and IFN- β was determined by ELISA. (E) STING KO MEFs stably reconstituted with B6 (left panels) or MOLF (right panels) mCherry-STING were stimulated for 2 h with Lipofectamine alone (media) or 2'3' cGAMP (4 μ g/ml). Cells were fixed and permeabilized with 4% PFA and MeOH, costained with ER marker, Grp94 (Green). * $p < 0.05$, **** $p < 0.0001$.

arrows). This was markedly different from the localization of MOLF STING, which displayed a more dispersed pattern (Fig. 2E, *bottom right panel*), thus suggesting that inability of STING to move out of the ER is the reason for the IFN defect in response DNA in MOLF.

Ability of B6 but not MOLF STING to translocate from the ER suggests that, in F1 (B6 × MOLF) mice, oligomerization of B6 allelic product should not be affected by MOLF allelic product, thus challenging interference with oligomerization as the main reason for the dominant effect of MOLF STING. Inheritance of a single MOLF mutant allele of *Sting* incurred a negative IFN-β phenotype (Fig. 2A), which could be explained by either dominant-negative effect of MOLF or by haploinsufficiency. To distinguish between these two possibilities, we crossed wild type and STING-deficient mice and compared their responses with responses in heterozygous, STING-knockout and wild type animals (Fig. 2D). Quite surprisingly, IFN production in the hemizygous macrophages was significantly lower than in the wild type cells and comparable with IFN levels in the heterozygous STING-congenic mice (Fig. 2D). These data support haploinsufficiency effect, which will be investigated further. One possibility is that certain critical amount of STING has to be translocated from the ER to facilitate activation of downstream components.

MOLF STING is a hypomorphic mutant

The relatively robust production of IL-6 (Fig. 1) suggested that the MOLF allele is partially responsive to stimulation with cytosolic DNA. To determine the role of STING in these differences, we generated *Sting*^{MOLF/MOLF} congenic mice (STING-congenic), which were similar to B6 parents in response to several TLR-agonists except DNA responses (Supplemental Fig. 2A). Specifically, macrophages from B6-*Sting*^{MOLF} congenic mice showed a profound IFN defect at the protein (Fig. 3A) or mRNA (Supplemental Fig. 2G) level across all STING agonists. However, IL-6 responses in B6-*Sting*^{MOLF} mice also remained low (Supplemental Fig. 2B), suggesting that genes other than STING of the MOLF mouse genomic background are required for robust IL-6 production in response to CDNs.

Dominant-negative inheritance of MOLF allele was recapitulated in STING-congenic macrophages (Supplemental Fig. 2H). Next, TBK1 and IRF3 were essentially not activated in B6-*Sting*^{MOLF} (Fig. 3B). In contrast, activation with 2'3' cGAMP resulted in similar kinetics and levels of TBK1 and IRF3 phosphorylation in congenic and B6 macrophages (Supplemental Fig. 2E), which prompted us to examine responses in STING-congenic mice to several agonists at the genome-wide level. The RNA-sequencing analysis (Fig. 3C) revealed a significant downregulation of DNA responses in B6-*Sting*^{MOLF}, albeit higher than in the *STING*^{-/-} mice and in agreement with ELISA and Western blot data. In summary, MOLF *Sting* is a partial-loss-of-function or hypomorphic allele, which leads to severe attenuation IFN-β production.

MOLF polymorphisms confer loss of function and aberrant translocation of STING from the ER

To further investigate individual STING polymorphisms, we examined the effect of individual MOLF polymorphisms on activation of luciferase reporter driven by IRF3/7, or NF-κB DNA binding elements. Several mutants such as 47, 48, and

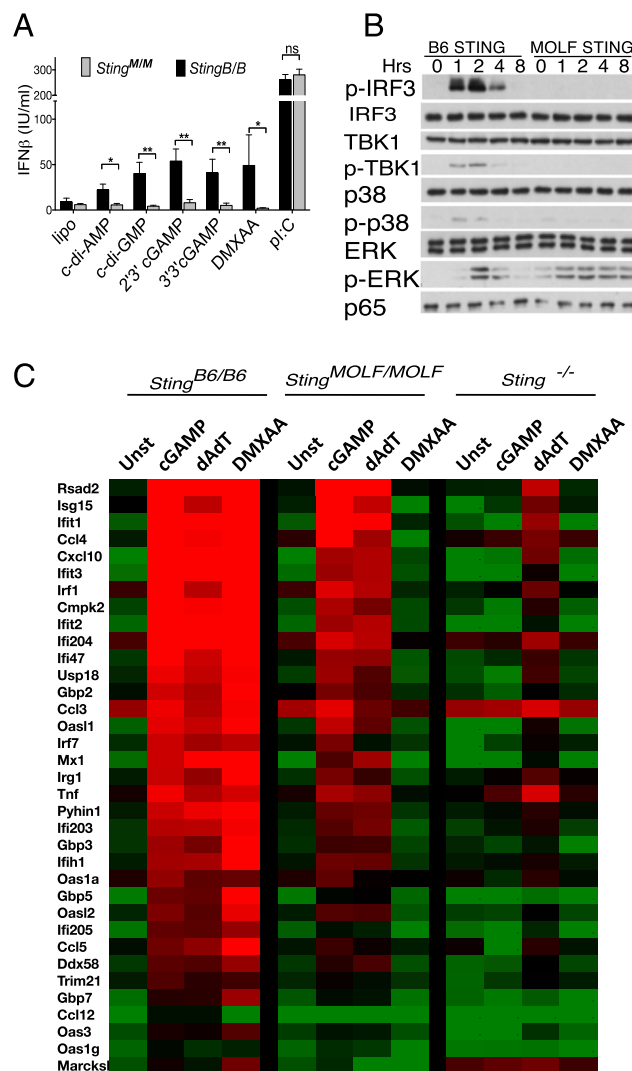


FIGURE 3. MOLF have hypomorphic allele of *Sting*. (A) Macrophages were activated with (4 μg/ml) CDNs for 16 h followed by ELISA of IFN-β. (B) Macrophages were stimulated with DMXAA for different time points followed by Western blot analysis of intracellular components of STING-mediated activation. (C) Macrophages were transfected with STING agonists for 4 h, and levels of ISGs mRNAs were compared via RNA-sequencing. **p* < 0.05, ***p* < 0.01.

53 failed to robustly activate any of the tested promoter elements in overexpression (Fig. 4A) or stimulus-dependent manner (Fig. 4B). The failure of 47, 48, and 53 mutants to activate IFN was further supported by Western blot analysis (Fig. 4D), revealing that TBK1 and IRF3 phosphorylation was concordant with the reporter activity of individual mutants. The remaining three polymorphisms (S103F, Y126S, N210D) individually exert incremental effects on STING (Fig. 4A, 4B) function but synergize to STING-dependent IFN responses when MOLF L47V-A48G-S53L mutations are reverted to MOLF V47L-G48A-L53S (Supplemental Fig. 2C). Thus, biochemical analysis of the MOLF STING polymorphisms revealed that L47V, A48G, and S53L abrogate STING-mediated signaling.

We also examined the effect of individual mutations on trafficking of STING and observed severely reduced formation of STING puncta and displacement from the ER of STING single-mutants (Fig. 4C). S53L STING, however, formed strange puncta even in unstimulated conditions (Fig. 4C,



provide evidence that NTD regulates trafficking of STING. Of additional significance is that the MOLF allele of *Sting* is a partial loss of function to DNA responses in MOLF/Ei mice, whereas defect in IFN- β presents with copious amounts of IL-6 protein (Fig. 1) that is lost in B6 STING^{MOLF/MOLF} congenics, suggesting that other differentially regulated genes might be involved in STING-dependent DNA responses in MOLF. Finally, although B6 macrophages are more responsive to DMXAA than other STING-agonists, MOLF cells are essentially nonresponsive to DMXAA but transcriptionally are moderately responsive to cGAMP and dAdT. Because CTD in both MOLF and B6 STING is identical, these data imply that NTD defines to some extent the specificity of STING-DNA interactions. Whether such bias is beneficial for the host will have to be further investigated with in vivo infection and DNA damage

response experiments using B6 *Sting*^{MOLF} mice. Another potential benefit from biased DNA responses is in auto-immune processes where chronic exposure to DNA leads to STING-mediated autoimmunity. Examining the role of MOLF STING in autoimmunity would be particularly interesting because several hyperresponsive and hyporesponsive to DNA human *STING* alleles have been described. Interestingly, both human and MOLF STING do not respond to Xanthone derivative, DMXAA, thus supporting further investigation of sensitivity to Xanthone derivatives found in higher plants and fungi on immunity using MOLF STING. Sensitivity of murine lymphocytes in the gut to xanthone derivatives in diet can affect mucosal immunity; thus, the MOLF STING variant may show a more human-like quality in these cases.

Finally, the clinical and biological implications of hypomorphic MOLF *Sting* alleles are also very important for anticipating the efficacy of STING-dependent cancer therapies that seek to break tolerance of tumors. The importance of induction of both IFN- β and ISGs downstream of STING for resolution of disease is not fully understood.

Altogether, our data highlight the importance of the STING NTD and its function, which to date has been largely ignored. Further studies of the NTD are necessary to fully understand its role in STING-mediated activation of innate responses.

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Disclosures

The authors have no financial conflicts of interest.

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