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The centrosomal hub unifies regulatory factors of NLRP3 inflammasome activation

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The NLRP3 inflammasome is central to host defense and sterile inflammation and forms condensates at the microtubule-organizing center (also known as the centrosome), although the mechanisms regulating this process remain unclear. Here we define a functional relationship among microtubule transport, the centrosomal kinase NEK7, priming, and NLRP3 abundance. We show that microtubule-dependent transport is required for NEK7-dependent NLRP3 activation and promotes NEK7 to the pericentriolar material (PCM). Microtubules, priming, and NEK7 synergistically converge on PCM abundance, thereby creating a permissive centrosomal environment for NLRP3 condensation and inflammasome assembly. Elevated NLRP3 expression compensates for limited PCM abundance, rendering K^+ efflux-induced activation independent of both NEK7 and priming in human and mouse macrophages. By contrast, NLRP3 overexpression only partially bypasses NEK7 dependence in response to the K^+ efflux-independent stimulus imiquimod, likely due to its activation of a non-trans-Golgi network pool of NLRP3 that is quantitatively limited. Together, these findings define a conserved spatial mechanism in which microtubule transport, NEK7 localization, priming, and NLRP3 abundance integrate at the PCM to establish the activation threshold and magnitude of NLRP3 inflammasome signaling across species and stimuli.

innate immunity | NLRP3 inflammasome | MTOC | NEK7 | pericentriolar material

Significance

The NLRP3 inflammasome is a master regulator of inflammation implicated in diverse pathologies, from metabolic disorders to neurodegeneration. However, the fundamental spatial principles governing its assembly remain elusive. Here we demonstrate that microtubules, NEK7, and cellular priming coordinate the expansion of centrosomes and pericentriolar material (PCM) to facilitate inflammasome organization. We further identify a compensatory relationship in which elevated NLRP3 expression can bypass the requirement for high PCM density. By defining these relationships between PCM architecture and protein abundance, our work provides a unified model for NLRP3 regulation and suggests avenues for therapeutic intervention in chronic inflammatory diseases.

The NLRP3 inflammasome is a critical component of innate immunity due to its sensing of a broad range of stimuli and its implicated role in the pathogenesis of numerous human diseases (1, 2). The NLRP3 inflammasome is a complex supramolecular assembly (3, 4), composed of the sensor protein NLRP3, the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and the effector caspase-1 as its core components. The active inflammasome generates mature caspase-1, which in turn cleaves interleukin 1β (IL-1β), interleukin 18 (IL-18), and the pore-forming protein gasdermin D (GSDMD), leading to cytokine maturation, cytokine release, and/or pyroptotic cell death. NLRP3 inflammasome activation is tightly regulated and typically involves two steps. First, a priming step mediated by Toll-like receptor ligands such as lipopolysaccharides (LPS) licenses the cells by upregulating the expression levels of NLRP3 and pro-IL-1β and by posttranslationally modifying key inflammasome components. Second, an activation step involving a wide range of stimuli, including bacterial toxins (e.g., nigericin), extracellular adenosine triphosphate (ATP, a damage-associated molecular pattern), and various pathogenic particulates (e.g., monosodium urate (MSU) crystals, cholesterol crystals, amyloids, and PM2.5 pollutants), triggers inflammasome assembly (5).

The NLRP3 inflammasome forms a single punctum or speck in each cell (also known as the ASC speck) containing all inflammasome proteins, which often localizes at the microtubule organizing center (MTOC) and acts as the caspase-1 holoenzyme (6–8). Most NLRP3 activation stimuli induce potassium ion (K^+) efflux from cells (9); by contrast, imiquimod and CL097 activate a K^+ efflux-independent NLRP3 pathway in a reactive oxygen species (ROS)-dependent manner (10). Some evidence suggests that K^+ efflux induces trans-Golgi network (TGN) dispersion, an early event in NLRP3 activation (11–13). In this model, priming increases NLRP3 recruitment to the TGN membrane through the interaction of the conserved, positively charged polybasic region of NLRP3 with phosphatidylinositol 4-phosphate (PI4P), and activating signals generate TGN-derived vesicles. Consistently, priming allows NEK7-independent activation of human NLRP3, at least partly by increasing NLRP3 recruitment to the TGN (11).

Our recent cryoelectron tomography (cryo-ET) study, which visualized the human NLRP3 inflammasome in its native cellular environment, revealed the trafficking of dispersed TGN vesicles to the MTOC and the subsequent NLRP3 condensate formation to support inflammasome activation (14). These studies are consistent with another study implicating signal-dependent NLRP3 condensation (15). Of note, the MTOC or the pericentriolar material (PCM) is also a condensate itself (16), suggesting that NLRP3 and

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the MTOC may cocondense within the same cellular compartment. Recent research further suggests that TGN dispersion may also be due to a disruption in endocytic trafficking by NLRP3 agonists, which prevents the recycling of TGN resident proteins and lipids, causing PI4P to accumulate on aberrant, dispersed endosomal vesicles to promote NLRP3 recruitment (17, 18).

Another important player in NLRP3 activation is the centrosomal kinase NEK7, which was first demonstrated by multiple laboratories to participate in the NLRP3 pathway in mouse macrophages as a scaffolding protein not requiring its kinase activity (19–21). By contrast, a recent study in human cells suggested that priming enables NLRP3 recruitment to PI4P-enriched TGN membranes and NLRP3 inflammasome activation independent of NEK7 (11). Along another thread, we and others showed previously in mouse macrophages that microtubule retrograde transport, which moves cargoes to the MTOC along microtubules, plays a critical role in mouse NLRP3 activation (6, 8). This transport machinery uses the dynein motor protein complex and the dynein adaptor histone deacetylase 6 (HDAC6) (8). However, the relationship between the requirements for NEK7 and microtubule transport have not been explored. Given its emergence as a potential therapeutic target with numerous ongoing clinical studies, defining the roles of NEK7 and microtubules in the human NLRP3 pathway is particularly important. Here, we examined the requirement for NEK7 and microtubule-dependent transport across both K^+ efflux-dependent and -independent conditions in human and mouse macrophages.

Results

NEK7 Increases the Kinetics of Unprimed NLRP3 Activation in Human Macrophages. The requirement of NEK7 in NLRP3 activation was well documented in mouse macrophages (19–21). To confirm this essentiality, we generated *Nek7* knockout (KO) mouse immortalized bone marrow derived macrophage (iBMDM) lines using the CRISPR-Cas9 system and two different guide RNAs (gRNAs), one of which, *Nek7* KO-1, was previously published (20) (*SI Appendix*, Fig. S1*A*). We randomly selected one monoclonal *Nek7* KO iBMDM line from each gRNA group (*SI Appendix*, Fig. S1*B*). Consistent with previous data, without NEK7, NLRP3 activity was markedly diminished in response to LPS priming (1 μg/mL for 4 h) and nigericin treatment (20 μM for 30 min) as shown by lactate dehydrogenase (LDH) release (*SI Appendix*, Fig. S1*C*). Nonetheless, low-level residual LDH release persisted in NEK7-deficient cells but was absent in NLRP3-deficient cells derived from *Nlrp3*$^{-/-}$ mice (22) (*SI Appendix*, Fig. S1*C*), suggesting that NEK7 plays a regulatory rather than a deterministic role in NLRP3 inflammasome activation.

Next, we turned to human macrophages by generating *NEK7* KO THP-1 monocytes, also using the CRISPR-Cas9 system (*SI Appendix*, Fig. S2*A*). Upon PMA-mediated monocyte differentiation into macrophages, the same priming and nigericin stimulation condition used for mouse iBMDMs resulted in similar NLRP3 activation in both wild-type (WT) and *NEK7* KO THP-1 cells, as revealed by LDH release, IL-1β secretion, and ASC speck formation (*SI Appendix*, Fig. S2 *B–E*). Notably, WT and *NEK7* KO THP-1 cells showed comparable IL-1β secretion at different time points after nigericin stimulation (*SI Appendix*, Fig. S3*A*). We also confirmed that the ASC-specks colocalized with FLAG-mScarlet (mSL)-fused NLRP3 in both WT and NEK7-deficient cells (*SI Appendix*, Fig. S2*F*). These results indicate that NEK7 has a strong regulatory role in NLRP3 activation in mouse macrophages, but not in human macrophages.

To rule out the possibility of incomplete KO in the *NEK7* KO pool (*SI Appendix*, Fig. S2*A*), we next isolated and conducted experiments using two monoclonal *NEK7* KO THP-1 lines (*SI Appendix*, Fig. S3 *B* and *C*). We also employed a gentler stimulation condition with a lower nigericin concentration of 10 μM and found that NEK7 is still not required for NLRP3 activation with LPS priming in PMA-differentiated human THP-1 macrophages, as revealed by membrane permeability to PI, LDH release, and ASC speck formation (Fig. 1 *A–C*). When we removed LPS priming, nigericin alone triggered robust NLRP3 activation only in WT, but not in *NEK7* KO human THP-1 macrophages (Fig. 1 *D–F*), suggesting that priming renders NEK7-independent NLRP3 activation in THP-1 macrophages.

As in mouse iBMDMs (*SI Appendix*, Fig. S1*C*), NEK7 deficiency also retained low level, residual NLRP3 activation without priming in human THP-1 macrophages (Fig. 1 *D* and *E*). We thus hypothesized that NEK7 may function as an accelerator rather than an essential component of NLRP3 inflammasome activation. To further investigate this, we extended the treatment duration to assess whether prolonged stimulation could compensate for the absence of NEK7 in THP-1 macrophages. The *NEK7* KO macrophages exhibited delayed NLRP3 activation upon nigericin stimulation when unprimed (Fig. 1*G*). However, prolonged stimulation still led to efficient inflammasome activation in *NEK7* KO macrophages, albeit with slower kinetics. Notably, when primed by LPS, NLRP3 activation levels were comparable between WT and *NEK7* KO macrophages throughout the time course (Fig. 1*H*). These data support the role of NEK7 as an inflammasome activation accelerator in unprimed human macrophages.

We further tested the NEK7-dependence of NLRP3 activation in undifferentiated THP-1 monocytes. As for THP-1 macrophages, NEK7 only enhanced NLRP3 activation without LPS priming, shown by both the pooled and monoclonal *NEK7* KO THP-1 monocytes (*SI Appendix*, Fig. S3 *D* and *E*). Thus, we conclude that priming renders NEK7-independent NLRP3 activation in human monocytes and macrophages, as also shown previously (11). Notably, nigericin alone cannot activate the NLRP3 pathway in mouse macrophages (*SI Appendix*, Fig. S3*F*).

LPS is a well-characterized pathogen-associated molecular pattern recognized by Toll-like receptor 4 (TLR4) (23) that licenses cells for NLRP3 pathway activation by, at least, upregulating the expression levels of NLRP3, pro-caspase-1, and pro-IL-1β (1). To test whether other priming regents could facilitate NEK7-independent NLRP3 activation in THP-1 macrophages, we assessed Pam3CSK4, a regularly used agonist for TLR1/2 (24). We observed that like LPS, Pam3CSK4 triggered NEK7-independent NLRP3 activation (*SI Appendix*, Fig. S3 *G* and *H*).

Microtubules and HDAC6 Facilitate NEK7-Dependent NLRP3 Activation. We previously showed that NLRP3 activation in mouse macrophages depends on HDAC6-dynein retrograde microtubule transport toward the MTOC (8). To investigate if this trafficking mechanism also functions in human macrophages, we used the microtubule disruptor colchicine and the HDAC6 inhibitor tubastatin A (TubA). We observed that colchicine and TubA had little effect on LPS-primed nigericin-stimulated NLRP3 activation in human THP-1 macrophages in comparison with the unprimed group, revealed by LDH release and ASC speck formation (*SI Appendix*, Fig. S4 *A–D*). We also utilized *HDAC6* KO THP-1 cells (*SI Appendix*, Fig. S3*I*) to examine HDAC6's role in NLRP3 activation by nigericin. Consistent with the effect of the HDAC6 inhibitor, *HDAC6* KO compromised NLRP3 activity in unprimed, but not in primed, THP-1 macrophages, shown by

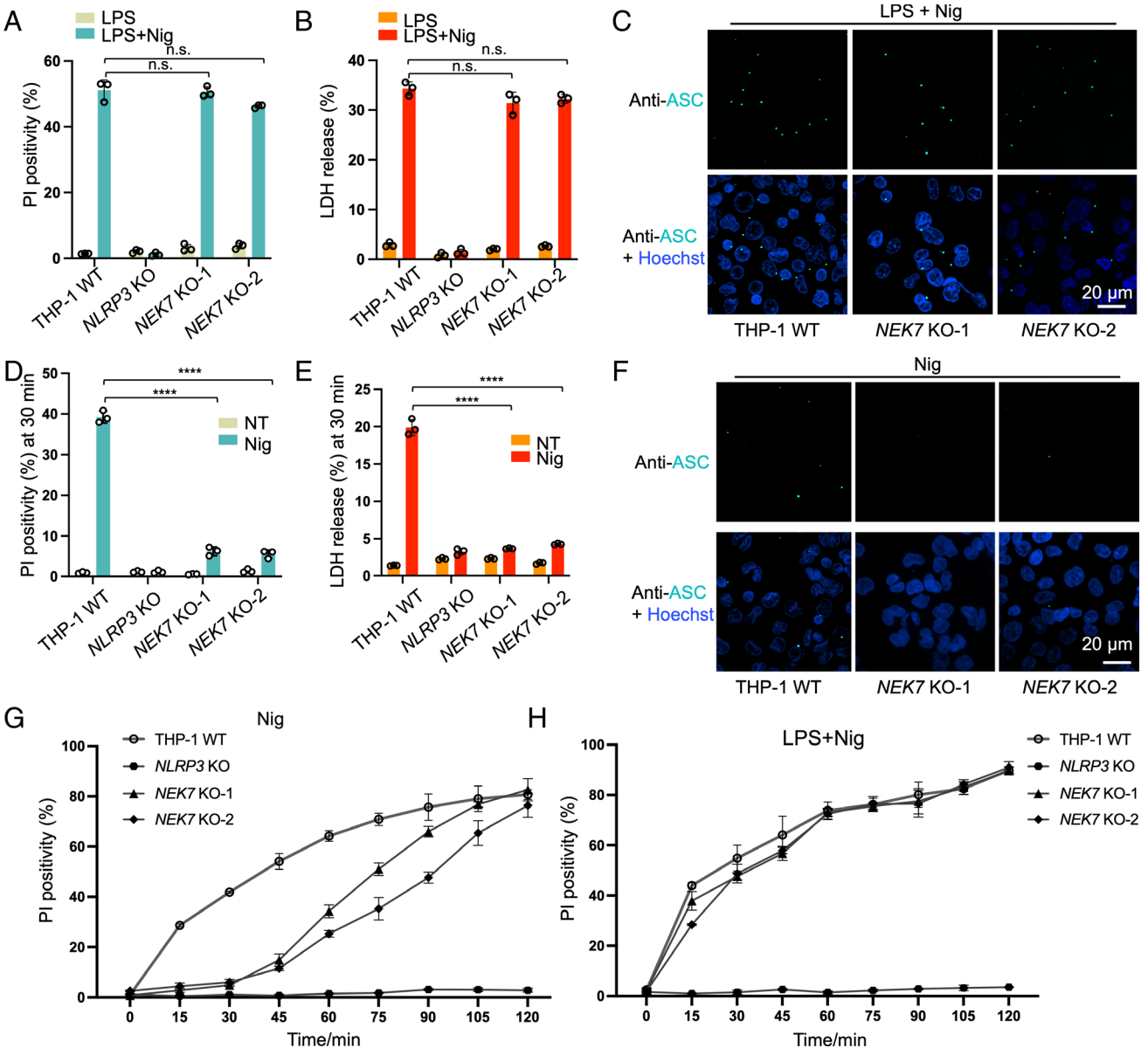

Fig. 1. Priming renders NEK7-independent NLRP3 activation in PMA-differentiated THP-1 macrophages. (*A–C*) Membrane permeability by propidium iodide (PI) staining (*A*) and percentage of LDH release in cell culture supernatants (*B*), and representative immunofluorescence images of ASC speck formation (*C*) from LPS-primed (1 μg/mL) THP-1 macrophages of the indicated genotypes treated with nigericin (10 μM for 30 min). *NEK7* KO-1 and *NEK7* KO-2: Two randomly selected *NEK7* KO THP-1 monoclonal lines. THP-1 macrophages were generated by PMA differentiation (50 nM, 48 h). (*D–F*) Membrane permeability by PI staining (*D*) and percentage of LDH release in cell culture supernatants (*E*), and representative immunofluorescence images of ASC speck formation (*F*) from THP-1 macrophages of the indicated genotypes treated with nigericin only. NT: nontreated. (*G* and *H*) Time course of membrane permeability by PI staining after nigericin treatment, without (*G*) or with LPS priming (*H*).

LDH release (Fig. 2 *A* and *B*), ASC speck formation (Fig. 2 *C* and *D*), and IL-1β secretion (Fig. 2 *E* and *F*).

In addition to nigericin, extracellular ATP and MSU also induce K^+ efflux-dependent NLRP3 activation (9). To determine if NEK7 or microtubule transport influences ATP and MSU-mediated NLRP3 activation, we used two clones each of *NEK7* KO and *HDAC6* KO THP-1 macrophages. Without priming, ATP and MSU induced a low level of NLRP3 activation shown by LDH release, which is dampened by either *NEK7* KO or *HDAC6* KO (Fig. 2*G*). By contrast, primed THP-1 macrophages showed equivalent NLRP3 activation by LDH release (Fig. 2*H*). For both unprimed and primed THP-1 macrophages, ATP and MSU-mediated activation was abolished by *NLRP3* KO (Fig. 2 *G* and *H*).

Given this apparent coupling between the roles of microtubules and NEK7 in NLRP3 activation, we used microtubule and HDAC6 inhibitors to infer the role of NEK7 in human primary immune cells, an important question due to the potential therapeutic utility of NLRP3 inhibition with many clinical studies ongoing. We obtained monocytes from human peripheral blood mononuclear cells (PBMCs) and stimulated them with nigericin alone or combined with LPS, with or without pretreatment with colchicine or TubA. As in THP-1 monocytes or macrophages, colchicine or TubA compromised NLRP3 activation in primary monocytes only in the absence of LPS priming (*SI Appendix*, Fig. S4 *E* and *F*), suggesting the NEK7 dependence of these monocytes under this condition.

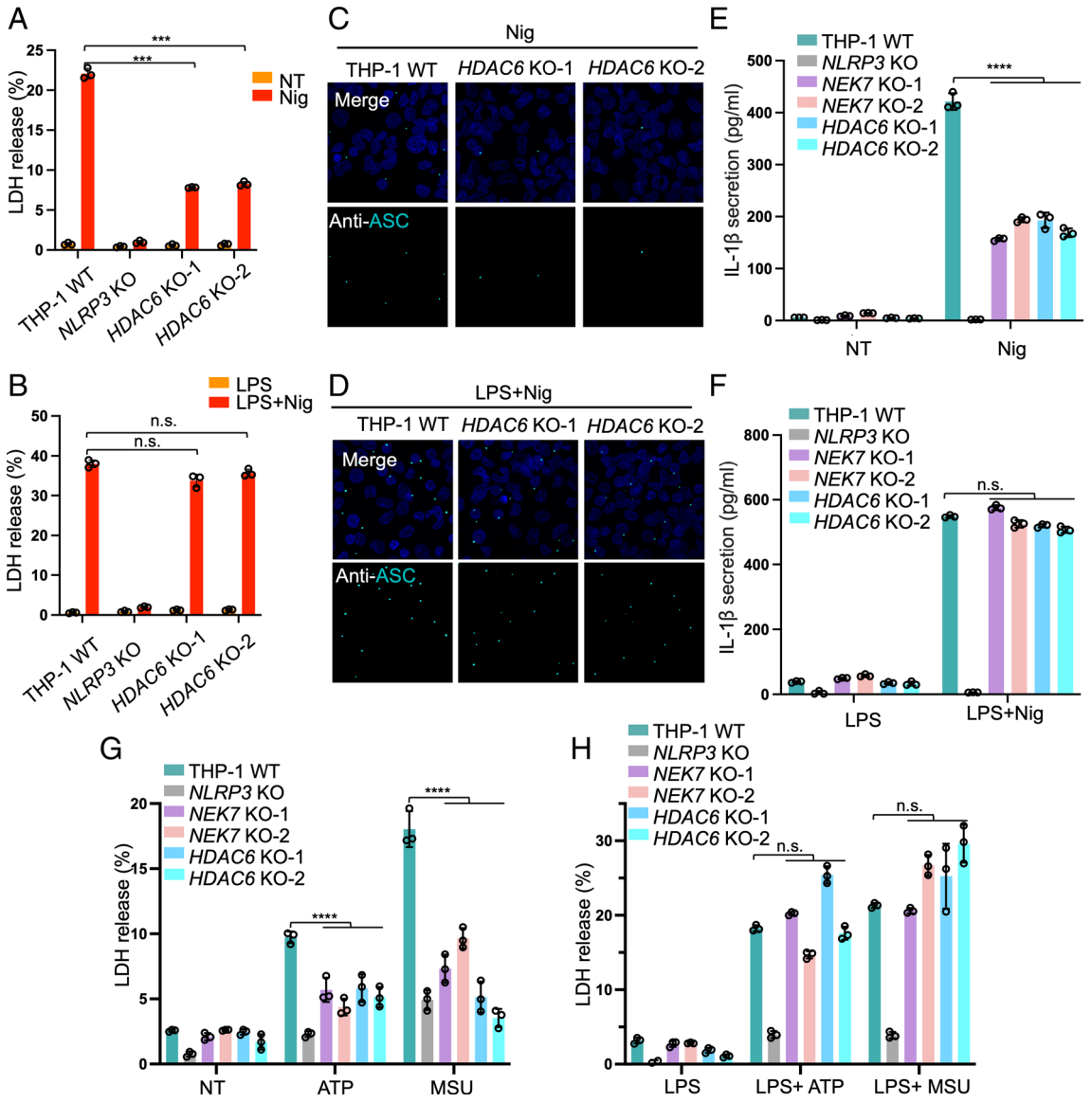

Fig. 2. HDAC6 is required for human NLRP3 activation without priming. (*A* and *B*) LDH release in cell culture supernatants from unprimed (*A*) and LPS-primed (*B*) THP-1 macrophages of the indicated genotypes treated with nigericin. (*C* and *D*) Representative immunofluorescence images of ASC speck formation from unprimed (*C*) and LPS-primed (*D*) THP-1 macrophages of the indicated genotypes treated with nigericin. (*E* and *F*) Amounts of IL-1β in cell culture supernatants analyzed by ELISA from unprimed (*E*) and LPS-primed (*F*) THP-1 macrophages of the indicated genotypes treated with nigericin. (*G* and *H*) LDH release in cell culture supernatants from unprimed (*G*) and LPS-primed (*H*) THP-1 macrophages of the indicated genotypes treated with ATP at 10 mM for 16 h (*G*) and 5 mM for 6 h (*H*), respectively, or MSU at 200 ng/mL for 6 h (*G* and *H*).

Exogenous High NLRP3 Expression Renders NEK7- and Priming-Independent Activation in Both Human and Mouse Macrophages. Because many mechanistic dissections of NLRP3 activation used overexpressed NLRP3 (7, 12, 25–27), we asked if and to what extent NLRP3 expression level influences its activation by different stimuli. To address this question, we first determined the copy numbers of NLRP3 in unprimed and LPS-primed THP-1 macrophages, respectively, as 280 and 870 per cell, by western blots using known amounts of recombinant human NLRP3 to create a standard curve (*SI Appendix*, Fig. S5*A*). We reconstituted mSL-NLRP3 in *NLRP3* KO or *NLRP3*/*NEK7* dKO THP-1 monocytes, and used sorting to obtain populations expressing low, medium, and high levels of FLAG-mSL-NLRP3, with the low expressors possessing a comparable NLRP3 level to WT THP-1 (*SI Appendix*, Fig. S5*B*). However, although PMA differentiation also elevated NLRP3 expression in WT THP-1 macrophages, the exogenous NLRP3 expression was enhanced more strongly such that even the reconstituted low expressing cells had more NLRP3 than WT macrophages (*SI Appendix*, Fig. S5 *C*–*E*). Nonetheless, priming by either LPS or Pam3CSK4 only boosted endogenous, but not exogenous, NLRP3 in THP-1 macrophages (*SI Appendix*, Fig. S5 *F* and *G*). Exogenously expressed mSL-NLRP3 at low, medium, or high levels in *NLRP3* KO THP-1 macrophages all induced robust LDH release with or without priming (Fig. 3 *A* and *B*), which is consistent with previous studies showing exogenous overexpression of NLRP3 resulted in priming-independent NLRP3 activation (28, 29). With NEK7 deficiency, however, the NLRP3 inflammasome was only strongly activated with, but not without, priming in all NLRP3 expressing THP-1 macrophages, shown by LDH release (Fig. 3 *C* and *D*). ASC speck formation (Fig. 3 *E* and *F*) and IL-1β release (Fig. 3 *G* and *H*) also confirmed the same conclusion. These data suggest that higher NLRP3 expression can bypass the NEK7 or priming requirement to allow NEK7- and priming-independent NLRP3 activation in THP-1 macrophages.

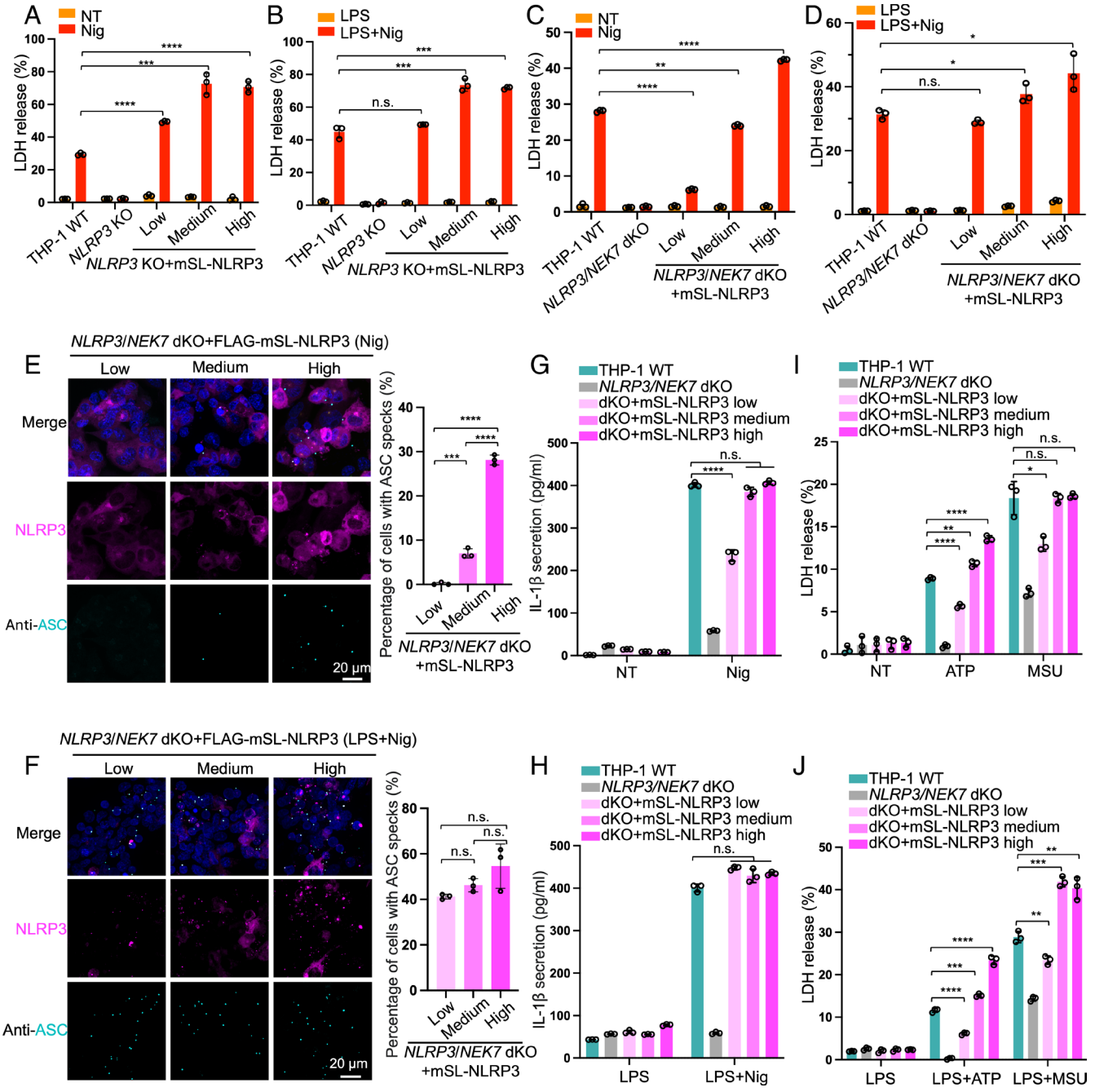

Fig. 3. NLRP3 expression level and priming render NEK7 independence. (*A* and *B*) LDH release in cell culture supernatants from unprimed (*A*) and LPS-primed (*B*) *NLRP3* KO THP-1 macrophages reconstituted with different levels of FLAG-mSL-NLRP3 and treated with nigericin. (*C* and *D*) LDH release in cell culture supernatants from unprimed (*C*) and LPS-primed (*D*) *NLRP3/NEK7* dKO THP-1 macrophages reconstituted with different levels of FLAG-mSL-NLRP3 and treated with nigericin. (*E* and *F*) *Left*: Representative immunofluorescence images of ASC speck formation from unprimed (*E*) and LPS-primed (*F*) endogenous *NLRP3/NEK7* dKO THP-1 macrophages reconstituted with different levels of FLAG-mSL-NLRP3 and treated with nigericin. *Right*: Quantification of the percentage of ASC speck-positive cells from the left images. (*G* and *H*) Amounts of IL-1β in cell culture supernatants analyzed by ELISA from unprimed (*G*) and LPS-primed (*H*) *NLRP3/NEK7* dKO THP-1 macrophages reconstituted with different levels of FLAG-mSL-NLRP3 and treated with nigericin. (*I* and *J*) LDH release in cell culture supernatants from unprimed (*G*) and LPS-primed (*H*) *NLRP3/NEK7* dKO THP-1 macrophages reconstituted with different levels of FLAG-mSL-NLRP3 and treated with ATP at 10 mM for 16 h and 5 mM for 6 h, respectively, or MSU at 200 ng/mL for 6 h (*I* and *J*).

To determine if this expression level-enabled bypass of NEK7 or priming requirement is true for other K^+ efflux-dependent stimuli, we tested ATP and MSU, two weaker NLRP3 agonists, for inducing NLRP3 activation. Without priming or with LPS priming, only medium and high NLRP3 expression levels, but not low NLRP3 expression level, compensated for the NEK7 loss. In fact, the high NLRP3 expression levels overcompensated for the NEK7 loss so that their LDH release exceeded those of WT under both no priming and priming conditions (Fig. 3 *I* and *J*). These data suggest that for ATP or MSU-induced NLRP3 activation, higher NLRP3 expression can also allow NEK7- and priming-independent NLRP3 activation in THP-1 macrophages.

We next asked whether reconstituting higher levels of NLRP3 in mouse macrophages could also render its inflammasome activation independent of NEK7 or priming. Using recombinant mouse NLRP3 to generate a standard curve, we determined that unprimed

and LPS-primed iBMDMs contain 603 and 3,086 NLRP3 molecules per cell, respectively (*SI Appendix*, Fig. S6*A*). We reconstituted three different levels of mouse NLRP3-FLAG in *Nlrp3* KO or *Nlrp3/Nek7* dKO mouse iBMDMs, with the lowest-level higher than unprimed, but comparable to LPS-primed, endogenous NLRP3 (*SI Appendix*, Fig. S6*B*). With all exogenous expression levels, NLRP3-FLAG-reconstituted *Nlrp3* KO iBMDMs were activated by nigericin with or without priming, which differs from lack of activation without priming for WT iBMDMs (*SI Appendix*, Fig. S6 *C* and *D*). We hypothesize that the higher expression even in low NLRP3 expressing cells rendered the NLRP3 activation independent of priming. In the absence of NEK7, unprimed reconstituted iBMDMs exhibited graded levels of activation depending on the exogenous NLRP3 expression levels, while endogenous NLRP3 in unprimed WT iBMDMs was not activated (*SI Appendix*, Fig. S6*E*). Like for WT iBMDMs, LPS priming enabled NEK7-independent NLRP3 activation in all reconstituted iBMDMs (*SI Appendix*, Fig. S6*F*). Thus, higher expression of NLRP3 in mouse macrophages also enables NLRP3 activation independent of priming and at least less dependent on NEK7.

Priming Enhances PCM Recruitment to the MTOC. We recently showed by cryo-ET that TGN-derived and NLRP3-bound vesicles appeared to traffic to the MTOC to form an inflammasome condensate (14). Since the MTOC is also a condensate itself (16), we wondered if the MTOC could be altered by priming or NEK7's presence to affect condensate formation by NLRP3. Thus, we evaluated centrosomal protein levels at the MTOC. While priming by LPS or Pam3CSK4 did not increase the global level of the centrosomal proteins pericentrin (PCNT) or γ-Tubulin (GTU) (Fig. 4*A*), it increased centrosomal levels of PCNT and GTU shown by immunofluorescence (Fig. 4 *B–D*), consistent with the PCM expansion and interphase centrosome maturation from a previous study (30).

In addition, centrosomal levels of GTU and PCNT were decreased in unprimed *NEK7* KO THP-1 cells, and this reduction could be reversed by LPS priming (Fig. 4 *E* and *F* and *SI Appendix*, Fig. S7*A*). This scenario suggests a correlation between PCM size and NLRP3 activation in the context of NEK7 and priming. Since the roles of NEK7 and microtubules are coupled in different NLRP3 activation contexts, we also examined centrosomal GTU

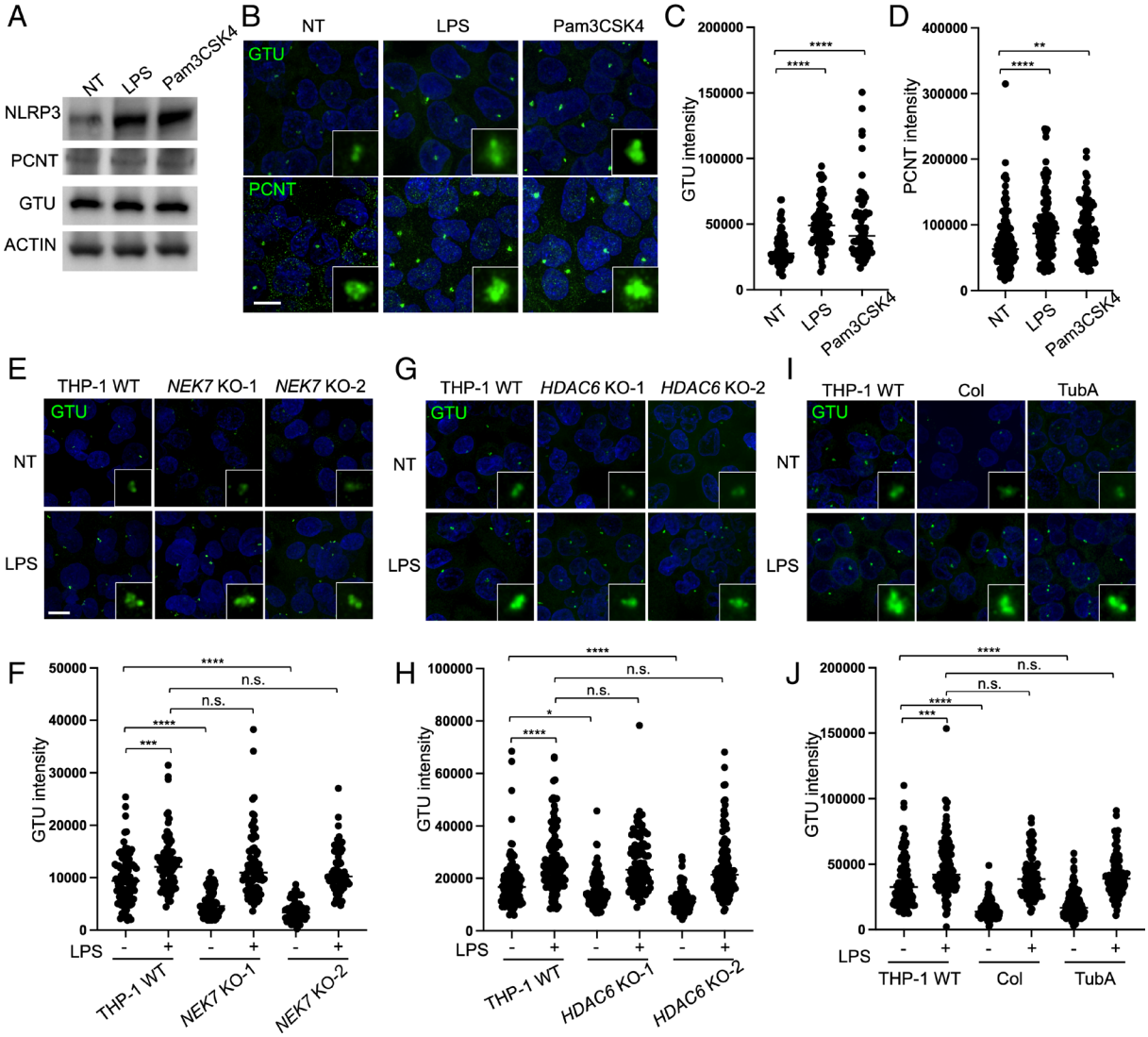

Fig. 4. Priming contributes to PCM protein recruitment to the MTOC. (*A*) Western blots of NLRP3, GTU, PCNT from unprimed and LPS- or Pam3CSK4-primed THP-1 macrophages. (*B*) Representative immunofluorescence images of GTU and PCNT from unprimed, LPS-primed, or Pam3CSK4-primed THP-1 macrophages. (*C* and *D*) Quantitative analysis of GTU (*C*) and PCNT (*D*) intensities at the MTOC from *B*. (*E–H*) Images (*E* and *G*) and quantitative analysis (*F* and *H*) of GTU at the MTOC from unprimed or LPS-primed THP-1 macrophages of the indicated genotypes. (*I* and *J*) Images (*I*) and quantitative analysis (*J*) of GTU at the MTOC from unprimed or LPS-primed THP-1 macrophages treated with colchicine or TubA.

and PCNT levels using immunofluorescence in *HDAC6* KO or microtubule-inhibited conditions in THP-1 macrophages. Strikingly, we observed consistent reduction of centrosomal PCM proteins, be it by HDAC6 KO, or treatment of colchicine or TubA in unprimed THP-1 cells, and again this reduction was reversed by LPS priming (Fig. 4 *G–J* and *SI Appendix*, Fig. S7 *B* and *C*). Collectively, these studies suggest that priming, NEK7, or microtubule transport may converge to PCM protein accumulation at the MTOC to promote NLRP3 pathway activation.

NEK7's Localization at the MTOC Is Dependent on Microtubule Transport. Because NEK7 is a centrosomal protein (31), we asked if its localization at the MTOC is dependent on microtubules or HDAC6. We initially performed immunofluorescence with several commercial NEK7 antibodies that worked well for western blotting but failed to produce a distinct centrosomal signal. We then reconstituted NEK7-mNG in *NEK7* KO THP-1 monocytes and differentiated them into macrophages for live cell imaging. Indeed, we observed a concentrated NEK7 intensity overlapping with the centrosomal maker, SiR-Tubulin, among the more diffuse NEK7 signals in the cytosol and the nucleus in unprimed or LPS-primed THP-1 macrophages (Fig. 5 *A* and *B*). Using cytosolic and nuclear fraction of WT THP-1 cells, we also confirmed that endogenous NEK7 is cytosolic, and the reason for a nuclear NEK7-mNG distribution could be due to overexpression (*SI Appendix*, Fig. S7*D*).

When treated by colchicine to disrupt the microtubule or TubA to inhibit HDAC6, unprimed NEK7-mNG THP-1 macrophages exhibited visibly reduced centrosomal localization of NEK7 (Fig. 5*A*). These effects were validated by quantification of the degree of colocalization within a 5 µm-diameter area surrounding the MTOC using two correlation coefficients, the PCC and the MOC (Fig. 5 *C* and *D*). While the PCC measured the linear correlation between the two channels (0 to 1), MOC calculated colocalization by the proportion of fluorescent signal from NEK7-mNG that overlaps with that of SiR-Tubulin. Both calculations demonstrated the prominent NEK7 localization at the MTOC and significantly reduced centrosomal localization when treated by colchicine or TubA (Fig. 5 *C* and *D*). The reduction in the NEK7 centrosomal signal in unprimed THP-1 macrophages was highly significant for both colchicine and TubA. The effect size was more pronounced for the microtubule disrupter colchicine than for the HDAC6 inhibitor TubA, likely due to the existence of other dynein adaptors other than HDAC6 responsible for NEK7 transport to the MTOC. In comparison with unprimed cells, LPS priming resulted in a modest increase of NEK7 accumulation surrounding MTOC in WT THP-1 macrophages, and this increase was visibly stronger in colchicine- or TubA-treated cells (Fig. 5*B*), as confirmed by both methods of quantification (Fig. 5 *C*and *D*). We thus conclude that microtubule transportation helps NEK7's MTOC localization and priming could further facilitate this process.

The K^+ Efflux-Independent Stimulus Imiquimod Requires NEK7 and HDAC6, and This Requirement Is Reduced, but Not Bypassed, by Priming or NLRP3 Overexpression. In addition to K^+ efflux-dependent NLRP3 agonists, we used a slow, and K^+ efflux-independent activator, imiquimod, which is also a TLR7 agonist (32), and was previously shown to require NEK7 for NLRP3 activation (10). Indeed, NEK7 is indispensable for imiquimod-mediated NLRP3 activity in both unprimed and primed human macrophages, shown by membrane permeability,

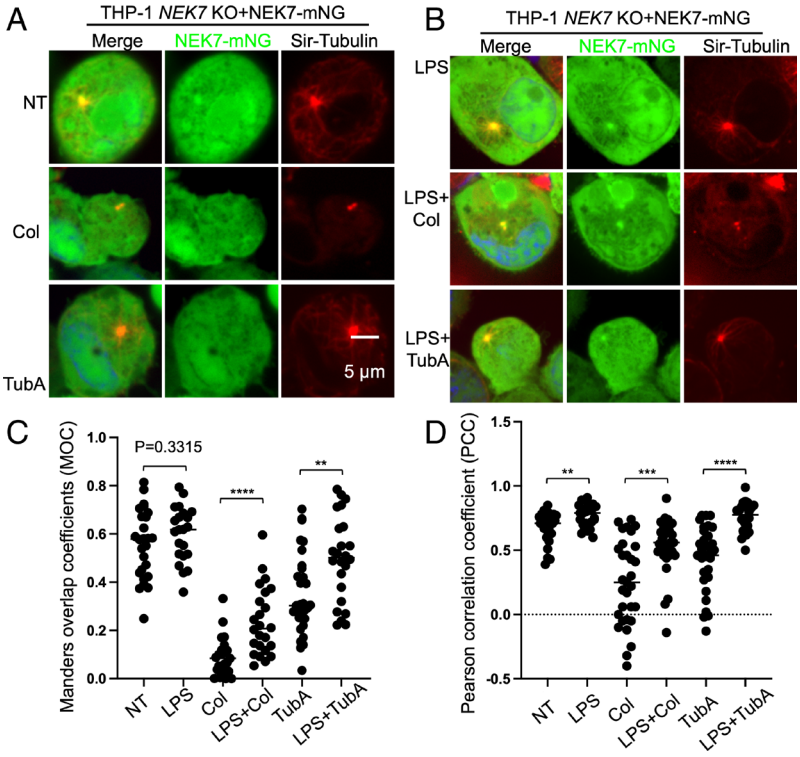

Fig. 5. Microtubule transport is essential for NEK7's localization at the MTOC. (*A* and *B*) Representative live cell images of NEK7-mNG and SiR-Tubulin from *NEK7* KO THP-1 cells reconstituted with NEK7-mNG and treated with colchicine or TubA, unprimed (*A*) and LPS-primed (*B*). (*C* and *D*) Quantitative analysis of the colocalization of NEK7-mNG and SiR-Tubulin from the images by Manders overlap coefficients (MOC) (*C*) and Pearson correlation coefficient (PCC) (*D*). Over 30 cells were analyzed in each group.

LDH release, and ASC speck formation (Fig. 6 *A*–*E*). This NEK7 dependence mirrored HDAC6 dependence (Fig. 6 *A*–*E*), like for K^+ efflux-dependent NLRP3 agonists. Together, these findings show that for both K^+ efflux-dependent and -independent stimuli, whenever NEK7 is required, microtubules and HDAC6 are also required to activate the NLRP3 inflammasome across several contexts. They suggest that the mechanism of microtubules and HDAC6 in controlling NLRP3 inflammasome activation is likely coupled to that of NEK7. In our *NLRP3/NEK7* double KO THP-1 macrophages reconstituted with different levels of MLRP3, priming enhanced imiquimod-induced NLRP3 activation in both WT and reconstituted cells; however, even the highest level of exogenous NLRP3 did not completely counteract the absence of NEK7 with or without LPS priming (Fig. 6 *F* and *G*).

Because the potential mechanism for imiquimod-induced NLRP3 activation has been linked to its role in disrupting mitochondrial function to heighten the production of ROS (10), we wondered if its activation of NLRP3 is still related to TGN. Immunofluorescence images of TGN38 and ASC from LPS-primed THP-1 macrophage treated with nigericin or imiquimod revealed that unlike TGN dispersion upon nigericin treatment, the TGN remained intact in imiquimod-treated THP-1 macrophages (Fig. 6*H*). These data suggest that while K^+ efflux-dependent stimuli mobilize TGN-recruited NLRP3, K^+ efflux-independent stimuli may utilize the cytosolic, not the TGN, pool of NLRP3. We hypothesize that the engagement of non-TGN NLRP3 may be a reason why priming, which increases recruitment of NLRP3 to the TGN upon stimulation by K^+ efflux-inducing agonists (11), does not render the NLRP3 activation independent of NEK7. In addition, the non-TGN pool is quantitatively limited relative to the TGN pool (*SI Appendix*, Fig. S2*F*), which may explain why NLRP3 overexpression did not completely compensate for lack of NEK7 even with priming (Fig. 6*G*).

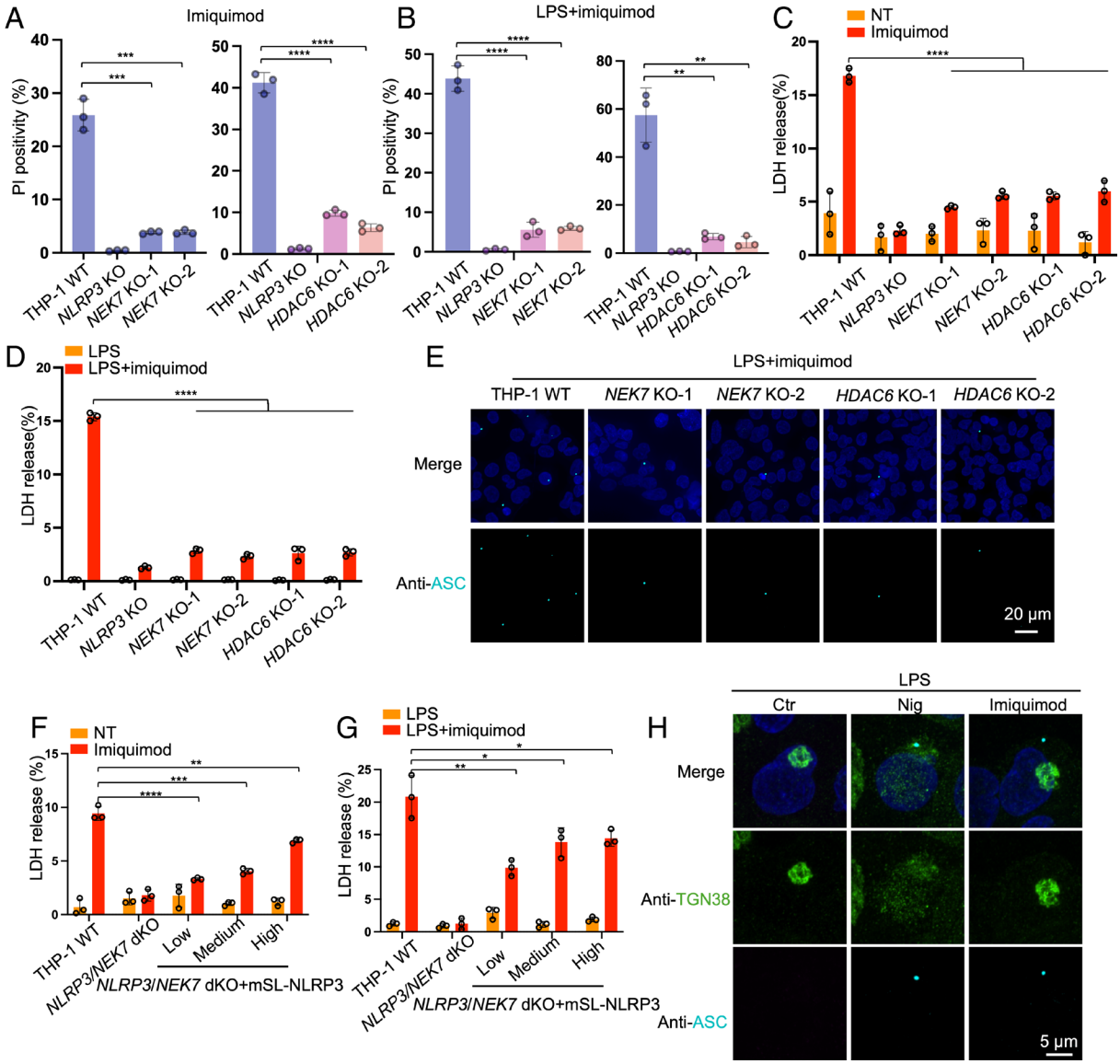

Fig. 6. K^+ efflux-independent NLRP3 activation requires NEK7 and HDAC6 in human macrophages. (*A* and *B*) Membrane permeability of THP-1 macrophages of the indicated genotypes treated with imiquimod (100 μM for 5 h) (*A*) or LPS (1 μg/mL for 4 h) + imiquimod (100 μM for 2.5 h) (*B*) by PI staining. (*C* and *D*) LDH release in cell culture supernatants form unprimed (*C*) and LPS-primed (*D*) THP-1 macrophages of the indicated genotypes treated with imiquimod. (*E*) Representative immunofluorescence images of ASC speck formation from LPS-primed THP-1 macrophage of the indicated genotypes treated with imiquimod. (*F* and *G*) LDH release in cell culture supernatants from unprimed (*F*) and LPS-primed (*G*) *NLRP3/NEK7* dKO THP-1 macrophages reconstituted with different levels of FLAG-mSL-NLRP3 and treated with imiquimod. (*H*) Representative immunofluorescence images of TGN38 and ASC from LPS-primed THP-1 macrophage treated with nigericin (10 μM, 30 min) or imiquimod (100 μM for 2.5 h).

GSDMD Mediates MT Network Dismantlement During NLRP3 Inflammasome Activation. Since the NLRP3 inflammasome has been shown to localize at the MTOC (7, 8, 14), we imaged *NLRP3* KO THP-1 macrophages reconstituted with mSL-NLRP3 in live cells, together with the microtubule dye SiR-Tubulin to mark the MTOC. Indeed, mSL-NLRP3 formed single puncta upon LPS priming and nigericin stimulation immediately adjacent to the MTOC (Fig. 7*A* and *SI Appendix*, Movie S1). Surprisingly, we noticed that the microtubule network appeared disintegrated by ~20 to 30 min after nigericin stimulation (Fig. 7*A*). In some cells, we also saw that the ASC speck moved away from the MTOC at later time points after nigericin stimulation (Fig. 7*B* and *SI Appendix*, Movie S2), which may explain some published observations that the ASC speck does not localize at the MTOC (33, 34). To further support this, we performed immunofluorescence on ASC and the MTOC marker protein PCNT at different time points after nigericin stimulation and examined the distance between ASC and PCNT (*SI Appendix*, Fig. S8 *A* and *B*). We classified the distances less than 0.5 μm as adjacent to the MTOC area. The statistical data showed ASC speck could originally form next to MTOC and move away as the microtubule network breaks down. In the context of imiquimod stimulation, we also observed the ASC speck formation in the MTOC-area (*SI Appendix*, Fig. S8*C*). This is in line with the necessary role of NEK7 and HDAC6 in transporting non-TGN localized NLRP3 to the MTOC region.

Immunofluorescence in fixed THP-1 macrophages also revealed microtubule disintegration during NLRP3 inflammasome activation (Fig. 7*C*). We next asked which component of the NLRP3 inflammasome is the key factor for microtubule network disintegration by examining the network in *NEK7* KO, *CASP1* KO, *GSDMD* KO, or *IL-1β* KO THP-1 macrophages. We found that microtubule network stayed intact in *CASP1* KO and *GSDMD* KO THP-1 cells (Fig. 7*C*). The use of glycine to inhibit NINJ1 activation and membrane rupture (35) did not rescue the phenotype of microtubule network breakage in WT THP-1 cells (Fig. 7*D*). Thus, these results let us speculate that membrane pore formation by GSDMD could be the reason for MT network disassembly.

Discussion

Mouse and Human NLRP3 Exhibit a Similar Activation Mechanism Modulated by TGN Recruitment, NLRP3 Levels, and the PCM. While it is clear that both human and mouse NEK7 interact with NLRP3 (19–21, 36), and that an active human NLRP3 inflammasome can be reconstituted with NEK7 in vitro (37), the role of NEK7 in human cells can be bypassed by priming (11), unlike the NEK7 dependence observed in mouse iBMDMs even after priming. Using comprehensive analyses of NLRP3 activation while perturbing NEK7, microtubules, HDAC6, or NLRP3 levels

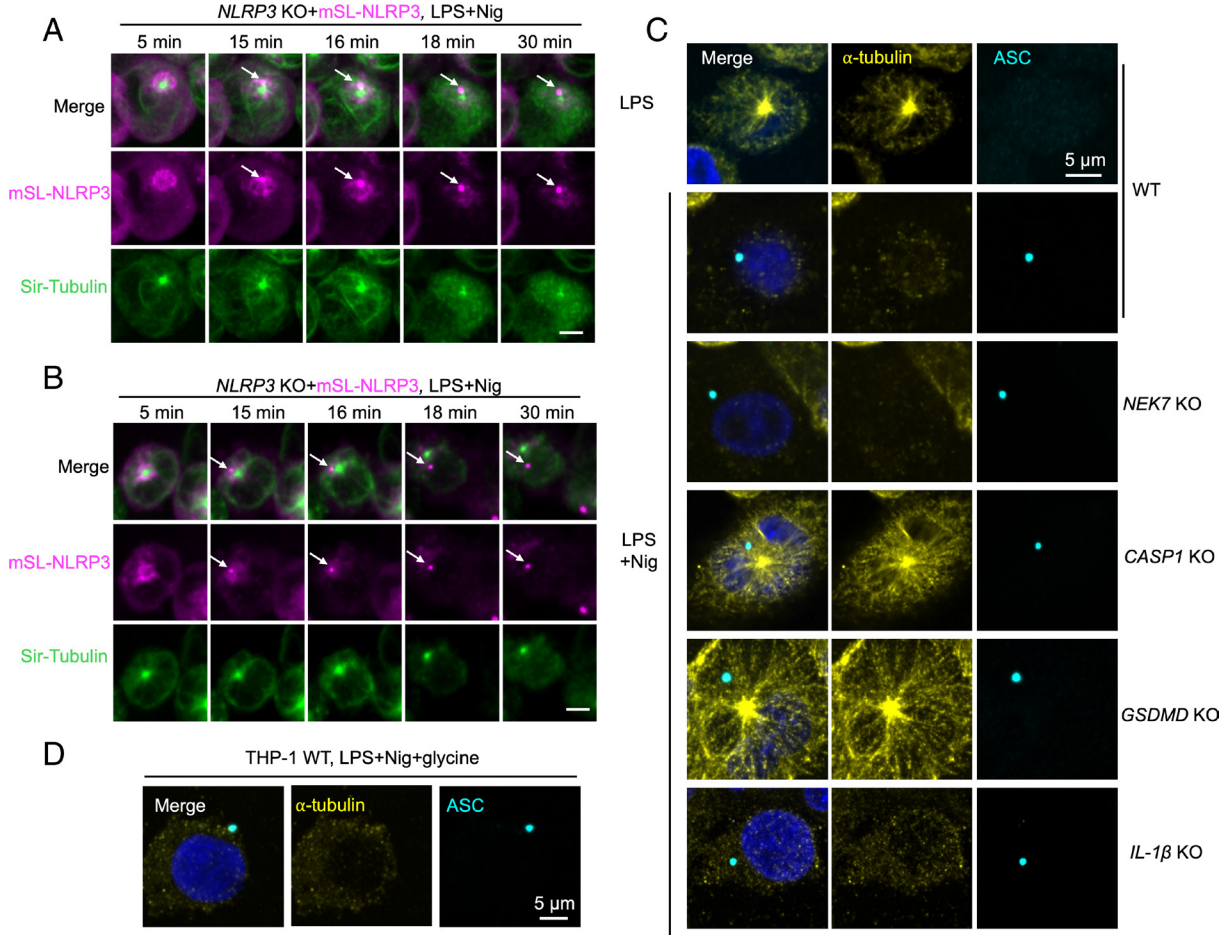

Fig. 7. GSDMD mediates microtubule network disassembly in NLRP3 inflammasome activation. (*A* and *B*) Live cell images tracking NLRP3 speck formation at different time points from *NLRP3* KO THP-1 cells reconstituted with mSL-NLRP3 and treated with LPS+nigericin, in which the NLRP3 speck remained close to (*A*) or moved away from (*B*) the MTOC. (*C*) Representative immunofluorescence images of ASC speck and α-Tubulin from LPS-primed THP-1 macrophages of the indicated genotypes treated with nigericin. (*D*) Representative immunofluorescence images of ASC speck and α-Tubulin from LPS-primed THP-1 macrophages treated with glycine and nigericin.

in human and mouse cells, we found that these factors set the threshold for NLRP3 activation, often by accelerating its kinetics. Our data, in conjunction with a previous study on priming's role in NLRP3 recruitment to the TGN and TGN vesicles (11), prompted us to propose a common mechanism of coupling among these factors, leading to an overarching model for the regulation of NLRP3 inflammasome activation. The model hinges on the abundance of NLRP3 on the TGN and the size and composition of the PCM, within both human and mouse cells, as well as across different modes of stimulation (*SI Appendix,* Fig. S9).

Specifically, for mouse NLRP3 stimulated by K^+ efflux triggers, endogenous NLRP3 can only be activated when priming, NEK7 and microtubule transport are all present to create an optimal PCM environment (scenario 1). For human NLRP3 stimulated by K^+ efflux triggers, endogenous NLRP3 can be activated even when NEK7 or microtubule transport is lacking (scenario 2), or without priming (scenario 3). This suggests a lower threshold for human NLRP3 activation, likely due to a less stringent need for an optimal PCM environment (see also next section). This dependence on priming, NEK7 or microtubule transport can be overcome by increasing the size of the NLRP3 pool through overexpression, where the abundance of NLRP3 compensates for a suboptimal PCM environment (scenario 4). Unlike K^+ efflux-associated NLRP3 stimuli, imiquimod requires NEK7 and microtubule transport to efficiently activate NLRP3 with or without priming (scenario 1 and 3); neither priming nor overexpression changes this requirement, likely due to the use of non-TGN NLRP3 in this mode of activation. We do not know if the non-TGN NLRP3 here is also associated with vesicles, e.g., endosomal vesicles (17, 18). Regardless, this model provides a conceptual framework for an integrated understanding of NLRP3 activation across species and stimuli.

Speculation on Why Mouse NLRP3 Exhibits an Apparent Higher Threshold for Activation. Intriguingly, human THP-1 macrophages have, on average, lower copy numbers of NLRP3 in unprimed or primed conditions than mouse iBMDMs; yet human cells are more easily activated. Sequence comparisons reveal that human and mouse NLRP3 share 83% sequence identity, a conserved domain architecture of PYD, NACHT and LRR, and an activation mechanism responsive to a broad and similar array of danger signals (38, 39). Structurally, both human and mouse NLRP3 can form cage-like oligomeric structures in the inactive state through their LRR domains and PYDs (12, 40, 41). Activation of NLRP3 requires a large conformational rearrangement, in which the N-terminal NACHT subdomains (NBD + HD1) rotate by ~90° relative to the C-terminal subdomains (WHD + HD2), thereby converting NLRP3 from an inactive to an active conformation (37). Thus, the NACHT domain lies at the core of NLRP3 activation; it also harbors the majority of disease-associated mutations and mediates the binding of NLRP3 inhibitors (42).

To undergo this activating conformational change, the inactive cage structure must be disrupted. Is it possible that the mouse NLRP3 cage is more stable than the human NLRP3 cage? In our previous experiments, we obtained mouse NLRP3 cages, but not human NLRP3 cages, when overexpressing NLRP3 in a mammalian cell line (12). By contrast, human NLRP3 cages can be purified from insect cells, which may not possess the appropriate posttranslational modifications for NLRP3 activation, thus stabilizing the cage structures. Interestingly, NEK7 also interacts with NLRP3 at its LRR domain (36), an interaction that may compete with the LRR–LRR interactions in the cage to facilitate its disruption. As such, the higher threshold for independence from NEK7, microtubule transport, or priming in mouse cells could be due to more stable cages that rely more heavily on NEK7 for disruption, a higher NLRP3 concentration to shift the equilibrium, or a larger PCM to support the condensate environment for mouse NLRP3 inflammasome assembly.

The MTOC Localization of Active NLRP3 May Change After Microtubule Disruption. The role of the PCM in NLRP3 activation rationalizes the MTOC localization of the NLRP3-ASC speck observed in this and previous studies (8). However, while ASC specks initially form at or near the MTOC, they often move away as the microtubule network breaks down at later time points following stimulation. This observation helps to explain why ASC specks are sometimes observed away from the MTOC (33, 34). We showed that GSDMD activation is required for this microtubule network dismantlement; however, we do not know whether this results from a direct effect of GSDMD pore formation or, more likely, is a secondary consequence of the rapid change in the intracellular ionic environment and the physical osmotic stress associated with cell swelling during pyroptosis. Additionally, NLRP3 agonists such as nigericin may induce ionic fluxes and cellular stress independently of the NLRP3 pathway, which may also cause organelle dispersion and microtubule disassembly.

Limitations. We acknowledge the limitations of biasing our conclusions mainly on immortalized cell lines. Future studies using primary human and mouse monocytes and macrophages will be essential to further validate our findings. Mechanistically, the interpretation of our data and the resulting overarching model may be affected by the complexity of both NLRP3 stimuli and their downstream effects. We have not tested our model in the context of mitochondrial and other organelle damage or ROS production (9, 43), whether occurring independent of NLRP3 activation or downstream of caspase-1 activation. Future studies should address these important questions to broaden our mechanistic insights and facilitate NLRP3-targeted therapeutic translation.

Materials and Methods

Human THP-1 cells, mouse iBMDMs, and primary human monocytes isolated from PBMCs were used for inflammasome assays. Cells were primed or not with LPS followed by stimulation with nigericin, ATP, MSU, or imiquimod to trigger NLRP3 inflammasome activation. Inflammasome activity was quantified by measuring cell death, ASC speck formation, and/or IL-1β release. Detailed experimental procedures, including gRNA sequences, cloning strategies, reagents, cellular fractionation protocols, and comprehensive imaging parameters, are provided in *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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