Tubular lysosomes harbor active ion gradients and poise macrophages for phagocytosis

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Abstract

The multiple functions of lysosomes have been linked to differences in their size, shape, location, abundance, and composition. Although lysosomes are generally vesicular, they can form tubules several microns long. New roles are emerging for such tubular lysosomes (TLs) (1–6). They facilitate antigen presentation, promote the efflux of bacterial peptides to the lysosomal lumen, and enhance antigen presentation. TLs harbor active ion gradients. Such gradients had been predicted but never previously observed. We identify a role for tubular lysosomes in promoting phagocytosis and activating MMP9. The ability to tubulate lysosomes without starving or activating immune cells may help reveal new roles for tubular lysosomes.

Introduction

Lysosomes adopt dynamic, tubular states that regulate antigen presentation, phagosome resolution, and autophagy. Tubular lysosomes are studied either by inducing autophagy or by activating immune cells, both of which lead to cell states where lysosomal gene expression differs from the resting state. Therefore, it has been challenging to pinpoint the biochemical properties lysosomes acquire upon tubulation that could drive their functionality. Here we describe a DNA-based assembly that tubulates lysosomes in macrophages without activating them. Proteolytic activity maps to single-lysosome resolution revealed that tubular lysosomes were less degradative and showed proximal to distal luminal pH and Ca2+ gradients. Such gradients had been predicted but never previously observed. We identify a role for tubular lysosomes in promoting phagocytosis and activating MMP9. The ability to tubulate lysosomes without starving or activating immune cells may help reveal new roles for tubular lysosomes.

Methods

A DNA nanodevice, Tudor, tubulates lysosomes. We describe the design, endocytic uptake pathway and activity of a DNA nanodevice that triggers lysosomes tubulation in macrophages without activating them. This DNA nanodevice, Tudor, is designed so that complementary DNA strands A1 and A2 display an Alexa 647 fluorophore, to form a 24-base pair duplex (double-stranded DNA (dsDNA)) (21). The dye on Tudor enables one to evaluate tubulation, its uptake efficiency, and its activity.

Results and Discussion

A DNA Nanodevice, Tudor, Tubulates Lysosomes. We describe the design, endocytic uptake pathway and activity of a DNA nanodevice that triggers lysosomes tubulation in macrophages without activating them. This DNA nanodevice, Tudor, displays an Alexa 647 fluorophore, to form a 24-base pair duplex (double-stranded DNA (dsDNA)) (21). The dye on Tudor enables one to evaluate tubulation, its uptake efficiency, and its activity. It activates MMP9 and triggers lysosome tubulation. Tubulation in turn promotes extracellular MMP9 activity, and the resultant positive feedback leads to sustained MMP9 secretion. MMP9 secretion by macrophages drives pathophysiological occurrences such as cancer, atherosclerosis, and renal fibrosis. Our studies reveal TLs aid the early stages of phagocytosis and promote extracellular MMP9 activity.

Significance

Lysosomes are organelles that also act as cell-signaling hubs. They regulate functions ranging from antigen presentation to autophagy. Spherical lysosomes can spontaneously elongate into tubules in starving or inflamed immune cells. We describe a DNA-based reagent, denoted Tudor, that tubulates lysosomes in macrophages without triggering either an immune response or autophagy. Chemical imaging revealed that tubular lysosomes differ from vesicular ones in terms of their pH, calcium, and proteolytic activity. Tudor revealed a role for tubular lysosomes in that they enhance MMP9 secretion and phagocytosis in resting macrophages. The ability to tubulate lysosomes in resting immune cells without starving or inflaming them may help reveal new insights into how tubular lysosomes function.

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subcellular distribution (Fig. 1A). The formation, integrity, and purity of Tudor were confirmed by gel electrophoresis (SI Appendix, Fig. S1).

Tudor is internalized by receptor-mediated endocytosis in RAW 264.7 cells. Tudor uptake is not via scavenger receptors (22) but rather via Ku70/80. Unlike dsDNA, Tudor uptake could not be competed out by excess maleylated bovine serum albumin (mBSA), a ligand for scavenger receptors (23–25) (Fig. 1B and C). However, depleting Ku70 or addition of excess, unlabeled SA43 completely abolished the uptake, revealing that Tudor internalization was mediated by engaging Ku70/80 at the cell surface (Fig. 1B and C and SI Appendix, Fig. S2). In addition to the nucleus, the Ku70/80 heterodimer is also seen on the surface of many cell types including macrophages (26). Immunostaining without permeabilizing the plasma membrane revealed cell-surface Ku70 in diverse macrophages such as RAW 264.7, J774A.1, and SIM-A9 (SI Appendix, Fig. S3) as well as primary macrophages such as naïve murine bone marrow-derived macrophages (BMDMs), peritoneal macrophages (Pmacs), and their polarized states including the M1 phenotype (LPS/interferon γ, proinflammatory) or the M2 phenotype (interleukin 4, antiinflammatory) (Fig. 1D and E and SI Appendix, Fig. S3).

Upon treating macrophages with 100 nM Tudor for 4 h, we found that it labeled vesicular as well as tubular organelles. When lysosomes in macrophages were prelabeled with TMR dextran they colocalized with internalized Tudor, revealing that the vesicular and tubular Tudor-containing compartments were lysosomes (Fig. 1F and SI Appendix, Fig. S4). TLs formed by Tudor treatment phenocopied those induced by LPS treatment (Fig. 1F and G). LPS tubulates lysosomes by a well-defined pathway but it also activates macrophages via TLR4 (3, 27). Tubulation was not observed when RAW 264.7 cells were either treated with dsDNA or an aptamer against a different cell surface protein, MUC-1 (28) (Fig. 1F and G and SI Appendix, Table S2). Treating RAW 264.7 cells with CpG DNA, which activates macrophages via TLR9 (3, 27), also failed to induce tubulation, suggesting that Tudor-induced tubulation was specifically triggered by SA43 and was not due to generic immunogenicity of DNA (SI Appendix, Fig. S2). We found that Tudor-induced TL formation was not linked to DNA damage. DNA damage induced by etoposide failed to tubulate lysosomes even after 8 h (SI Appendix, Fig. S6) (29). Tudor tubulated lysosomes in macrophages such as J774A.1 and SIM-A9 as well as primary macrophages such as BMDMs, Pmacs, and adipose tissue macrophages (Fig. 1G and SI Appendix, Figs. S7 and S8).

**Tudor Tubulates Lysosomes in Macrophages without Immune Activation.** We then tested whether Tudor treatment activated macrophages by quantifying the number of TLs per cell (Methods, Fig. 2A–C, and SI Appendix, Figs. S9 and S10). The extent and kinetics of Tudor-induced tubulation phenocopied those induced by LPS, suggesting that both agents could potentially act via a similar pathway (Fig. 2D). Messenger RNA (mRNA) expression data for Tudor-treated resting BMDMs or Pmacs (M0) revealed no significant up-regulation of M1 or M2 markers, indicating that Tudor did not polarize them toward either state (Fig. 2E and SI Appendix, Fig. S11). Additionally, Tudor treatment did not activate autophagy, ruling out the autophagy-induced lysosome tubulation cascade (SI Appendix, Fig. S12).
To identify the players responsible, we quantified TLs in RAW 264.7 cells treated with different pharmacological inhibitors (SI Appendix, Table S3). Unlike LPS, Tudor-induced tubulation was independent of TLR4 as well as MYD88 (Fig. 2F and SI Appendix, Fig. S134). Pharmacological inhibition of TLR5, TLR3, TLR2/TLR6, and TLR1/TLR2 prior to Tudor treatment revealed that these TLRs were not on the pathway (SI Appendix, Figs. S13 B and D). Treatment with Dynasore and Tudor still led to efficient tubulation, suggesting that Tudor acts primarily at the plasma membrane (SI Appendix, Fig. S14) (26). Ku80 in the Ku heterodimer activates MMP9 at the plasma membrane by interacting with its hemopexin domain (30). Indeed, we found that Tudor treatment activated MMP9 (SI Appendix, Fig. S13E) and treating cells with MMP9 inhibitor-I (31) abolished both Tudor and LPS-induced lysosome tubulation (Fig. 2F and G and SI Appendix, Fig. S134). These findings were recapitulated in primary BMDMs (Fig. 2G and SI Appendix, Fig. S13E).

To test whether LPS and Tudor worked via common players late in the tubulation pathway, we depleted mTOR, Akt, and PI3K by small interfering RNA (siRNA) as well as pharmacologically inhibited them with Torin-1 (32), Akt-I (3), or ZSTK474 (33), respectively (SI Appendix, Figs. S15 and S16). Indeed, both LPS and Tudor-mediated tubulation activated the PI3K–Akt–mTOR cascade, which was also confirmed by the increased phosphorylation levels of these proteins (Fig. 2F and SI Appendix, Fig. S17).

PI3K can be activated via cSrc, c-Cbl, Rac1, or Jak1/2 (26, 34, 35). Treatment with inhibitors for cSrc (Dasatinib), Rac1 (Rac1i), and Jak1/2 (barcitinib) revealed that neither Tudor nor LPS acted via these proinflammatory signaling pathways (SI Appendix, Fig. S13 C and D). We therefore targeted antiinflammatory pathways involving PI3K and Akt (36). In our hands, AMPK was significantly phosphorylated in both LPS- and Tudor-treated RAW 264.7 macrophages (SI Appendix, Fig. S17) and inhibiting AMPK with dorsomorphin (compound C) (37) impeded tubulation, despite prior work showing that AMPK activation prevents LPS-mediated tubulation (Fig. 2F) (3). SirNA knockdown of AMPK reaffirmed its involvement in Tudor-induced lysosomal tubulation (SI Appendix, Fig. S15). AMPK activity is regulated by LKB1 or TGF-β activating kinase 1 (TAK1) (38). We found that Tudor-induced tubulation required LKB1 activity but not TAK1 (Fig. 2F and SI Appendix, Figs. S13 A, C, and D, S15B, and S17E). Our studies reveal two additional players, LKB1 and AMPK, that negatively regulate mTOR, in the tubulation cascade.

**TLs Are Less Degradative.** TLs facilitate functions ranging from antigen presentation to autophagy, yet it is still unclear whether tubulation changes the luminal biochemistry. LPS stimulation induces TL formation but also up-regulates many lysosomal enzymes (39). We therefore quantitatively mapped enzymatic activity at the resolution of single lysosomes in live RAW 264.7 macrophages treated with either LPS or Tudor. We allowed cells with Alexa 488-dextran–labeled lysosomes to endocytose DQ BSA and compared the proteolytic activity of TLs and VLs (Fig. 3A and B and SI Appendix, Fig. S18). Regardless of how they were tubulated, increase in proteolysis was confined to VLs while that in TLs was unaffected (Fig. 3B).

DNA-based enzyme activity reporters can selectively address the contribution of specific lysosomal proteases (40). Cathepsin C (CTC) plays key roles in inflammation, interleukin 1β production, tumor necrosis factor α production, and macrophage reprogramming (41, 42). We therefore used a previously published ratiometric DNA-based CTC reporter to probe CTC activity in VLs and TLs in RAW 264.7 cells and BMDMs (Fig. 3D–H and SI Appendix, Figs. S19 and S20) (40). The CTC activity
also mapped Ca\textsuperscript{2+}, since TLs undergo active fission and fusion (in dsDNA, red), and cleaved module (Rhodamine 110, green). (lysosomes). (below. (Fig. 3).

Luminal pH and Ca\textsuperscript{2+} Maps Reveal Two Major Kinds of TLs. The differential proteolytic activity despite their comparable cathepsin content led us to map the luminal pH of TLs and VLs (43). We also mapped Ca\textsuperscript{2+}, since TLs undergo active fission and fusion that implicate the lysosomal Ca\textsuperscript{2+} channels P2X4, TCP2, and TRPML1 (44–46). Moreover, these Ca\textsuperscript{2+} channels strongly depend on mTOR activity, which is part of the tubulation cascade (47). We used a DNA-based, pH-correctable Ca\textsuperscript{2+} sensor, CalipHluor\textsuperscript{2.0} (SI Appendix, Fig. S23A), which carries 1) a pH-sensing dye, DCF (SI Appendix, Fig. S24), 2) a Ca\textsuperscript{2+}-sensing dye, Rhod-5F, and 3) a reference dye, Atto647N, for quantitative ratiometry (SI Appendix, Tables S1 and S4). We measured the stability of CalipHluor\textsuperscript{2.0} and found that it was ∼95% intact at time t = 2 h within lysosomes of RAW 264.7 cells (SI Appendix, Fig. S25). We mapped luminal Ca\textsuperscript{2+} and pH by treatment with Tudor then labeling lysosomes with CalipHluor\textsuperscript{2.0} and imaging cells in three channels, G, O, and R (Fig. 4A and SI Appendix, Figs. S23 and S26 A–C). According to the lysosomal pH and Ca\textsuperscript{2+} maps, the overall luminal pH and Ca\textsuperscript{2+} levels in TLs were comparable to those of VLs (Fig. 4 C and D).

Interestingly, the pH and the Ca\textsuperscript{2+} maps of TLs revealed a striking gradient of each ion along the long axis of the tubule, unlike VLs where they were homogenous (Fig. 4A–H). Areas of high acidity in TLs corresponded to low Ca\textsuperscript{2+}, and vice versa. NH\textsubscript{4}Cl neutralizes lysosomal pH and releases luminal Ca\textsuperscript{2+} from lysosomes (48). While NH\textsubscript{4}Cl treatment greatly reduced the number of TLs, pH and Ca\textsuperscript{2+} gradients were dissipated in the few TLs that did form (Fig. 4E and SI Appendix, Fig. S26B), indicating that the gradients were not an imaging artifact. Moreover, the ion gradients dynamically changed as TLs underwent growth or deformation. However, regions of high Ca\textsuperscript{2+} always correlated with regions of low acidity (Fig. 4 G and H). Such Ca\textsuperscript{2+} gradients are reporter is a DNA duplex bearing a reference dye and a Rhodamine 110 dye caged by phenylglycyl (FG) groups that are cleaved by CTC activity in lysosomes (SI Appendix, Table S4). In Tudor- or LPS-treated cells, VLs show much higher proteolysis (CTC activity) compared to TLs in the same cells. In fact, the level of proteolysis in TLs upon LPS/Tudor treatment is comparable to the basal level of hydrolysis observed in VLs of dsDNA-treated (i.e., resting) cells (Fig. 3 E and F and SI Appendix, Fig. S19 A–C). These trends were recapitulated in BMDMs (Fig. 3 G and H and SI Appendix, Fig. S19 D–K).

Importantly, the differences in lysosomal activity between TLs and VLs was not due to the relative abundance of hydrolases (4), since immunofluorescence revealed that cathepsin B (CTB) content was similar across lysosomes regardless of whether cells were treated with dsDNA, LPS, or Tudor (Fig. 3C and SI Appendix, Fig. S21 A–C). We quantified the total numbers of VLs and TLs per cell, Feret length, percentage of TLs per cell, and average number of VLs and TLs per cell (SI Appendix, Fig. S22). We found no perceivable differences in number of VLs in Tudor-treated or dsDNA-treated cells. However, the fraction of TLs consistently increases upon treatment with either Tudor or LPS.

Luminal pH and Ca\textsuperscript{2+} Maps Reveal Two Major Kinds of TLs. The differential proteolytic activity despite their comparable cathepsin content led us to map the luminal pH of TLs and VLs (43). We also mapped Ca\textsuperscript{2+}, since TLs undergo active fission and fusion that implicate the lysosomal Ca\textsuperscript{2+} channels P2X4, TCP2, and TRPML1 (44–46). Moreover, these Ca\textsuperscript{2+} channels strongly depend on mTOR activity, which is part of the tubulation cascade (47). We used a DNA-based, pH-correctable Ca\textsuperscript{2+} sensor, CalipHluor\textsuperscript{2.0} (SI Appendix, Fig. S23A), which carries 1) a pH-sensing dye, DCF (SI Appendix, Fig. S24), 2) a Ca\textsuperscript{2+}-sensing dye, Rhod-5F, and 3) a reference dye, Atto647N, for quantitative ratiometry (SI Appendix, Tables S1 and S4). We measured the stability of CalipHluor\textsuperscript{2.0} and found that it was ∼95% intact at time t = 2 h within lysosomes of RAW 264.7 cells (SI Appendix, Fig. S25). We mapped luminal Ca\textsuperscript{2+} and pH by treatment with Tudor then labeling lysosomes with CalipHluor\textsuperscript{2.0} and imaging cells in three channels, G, O, and R (Fig. 4A and SI Appendix, Figs. S23 and S26 A–C). According to the lysosomal pH and Ca\textsuperscript{2+} maps, the overall luminal pH and Ca\textsuperscript{2+} levels in TLs were comparable to those of VLs (Fig. 4 C and D).

Interestingly, the pH and the Ca\textsuperscript{2+} maps of TLs revealed a striking gradient of each ion along the long axis of the tubule, unlike VLs where they were homogenous (Fig. 4A–H). Areas of high acidity in TLs corresponded to low Ca\textsuperscript{2+}, and vice versa. NH\textsubscript{4}Cl neutralizes lysosomal pH and releases luminal Ca\textsuperscript{2+} from lysosomes (48). While NH\textsubscript{4}Cl treatment greatly reduced the number of TLs, pH and Ca\textsuperscript{2+} gradients were dissipated in the few TLs that did form (Fig. 4E and SI Appendix, Fig. S26B), indicating that the gradients were not an imaging artifact. Moreover, the ion gradients dynamically changed as TLs underwent growth or deformation. However, regions of high Ca\textsuperscript{2+} always correlated with regions of low acidity (Fig. 4 G and H). Such Ca\textsuperscript{2+} gradients are...
consistent with the hypothesis that tubulation requires the stringent control of TRPML1 activity since either overexpressing or inhibiting TRPML1 disintegrates TLs (49). We posit that the tight regulation of TRPML1 observed by others could potentially function to sculpt the $Ca^{2+}$ gradient.

Further analysis revealed at least three kinds of TL populations. Since TLs are stretched along microtubules, $\sim$95% of TLs radiated from the nucleus to the plasma membrane. We therefore classified radially oriented TLs based on whether their high $Ca^{2+}$ termini were nearer the nucleus (population A) or the plasma membrane (population B) or those with no ion gradient (n.g., Fig. 4 I and J). Nearly 53% of TLs fell under population B, $\sim$29% had the reverse orientation (population A), and $\sim$13% showed no gradients (Fig. 4 K and L). Our findings were recapitulated with LPS-induced TLs (SI Appendix, Fig. S26 C–G).

These different orientations suggest either that there are different tubulating mechanisms or different kinds of TLs, or both. Tubulation requires opposing pulls generated by Arl8b-SKIP-kinesin along the plus end of microtubules and Rab7-RILP-dynein complexes at the minus end (27, 50). In fact, Arl8b-SKIP and Rab7-RILP complexes regulate the spatial positions of VLs in cells (51). Further, peripheral VLs regulate plasma membrane repair while perinuclear VLs fuse with autophagosomes for autophagy (49, 52). Thus, these two populations of TLs also could function differently.

**TLs Promote Phagocytosis and Phagosome–Lysosome Fusion.** Phagocytosis drives key functions including antigen presentation and pathogen killing in innate immune cells (53). When macrophages are treated with LPS, inflammation is initiated and phagocytosis is enhanced (3, 54, 55). However, since LPS also tubulates lysosomes, we do not know whether increased phagocytosis is due to inflammation or lysosome tubulation, or both. To test this, we treated RAW 264.7 cells and Pmacs with either dsDNA, LPS, or Tudor and quantified phagocytosis of pHrodo Red–conjugated zymosan (Fig. 5 A and SI Appendix, Figs. S27 and S28). We found that lysosome tubulation alone was sufficient to enhance phagocytosis (Fig. 5 A and E and SI Appendix, Figs. S27 and S28). We then probed the effect of lysosome tubulation on phagosome maturation by quantifying TL–phagosome contacts and contact mixing, where lysosomes and phagosomes were labeled with Alexa 488 dextran and pHrodo Red zymosan, respectively (SI Appendix, Materials and Methods and Fig. 5D). In Pmacs, $\sim$30% of TLs contacted phagosomes, while in RAW 264.7 cells it was 15 to 20% (Fig. 5 B–F, H, and I and SI Appendix, Fig. S29). More contacts also correlated with more content exchange characteristic of more productive fusion events (Fig. 5 C and G). Tudor treatment did not alter fluid-phase endocytosis (SI Appendix, Figs. S27D and S29 G–I). Overall, our results show that TLs promote phagocytosis and aid phagosome–lysosome fusion.

To explicitly test the role of TLs in aiding phagocytosis, we knocked down Arl8b, an adaptor that connects lysosomes to kinesin, which lies downstream of mTOR activation and upstream of lysosome tubulation. Knocking down Arl8b suppressed lysosome tubulation and phagocytosis in Tudor–treated cells, demonstrating that tubulation promotes phagocytosis (Fig. 5 E and SI Appendix, Figs. S30 and S31).

**MMP9 Activation Drives Tudor–Mediated Tubulation.** To examine how TL formation aided phagocytosis, we pharmacologically inhibited mTOR and PI3K in Tudor–treated cells and observed a drastic decrease in phagocytosis in both cases (SI Appendix, Fig. S31).
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PI3K promotes phagocytosis by converting PI(4,5)P₂ on the inner leaflet of the plasma membrane to PI(3,4,5)P₃, which facilitates phagosome cup formation (56). Tudor treatment increased PI(3,4,5)P₃ on the plasma membrane, consistent with PI3K activity. Importantly, inhibiting mTOR did not affect PI(3,4,5)P₃ levels yet prevented lysosomal tubulation and impeded phagocytosis (Fig. 6A and B and SI Appendix, Fig. S31).

PI3K and Akt activation enhances MMP9 secretion (57). To test whether Tudor activated any secreted MMP9 which could feed back and further tubulate lysosomes, we probed the kinetics of extracellular MMP9 activity in RAW 264.7 cells treated with dsDNA, LPS, or Tudor. Arrow shows zymosan addition (100 nM) (N = 30 cells). (C) Percentage TLs contacting phagosomes (% TL-P contact) (N = 50 cells). (D) Extent of phagosome–lysosome fusion (P-L fusion (G/R)) given by mean G/R value in RAW 264.7 cells (n = 60 cells). (E) Average number of phagosomes versus time in RAW 264.7 cells treated with dsDNA, Tudor, or LPS. Arrow shows zymosan addition (N = 30 cells). (F) Percentage TLs contacting phagosomes in M0 Pmacs (N = 30 cells). (G) Number of phagosomes in M0 Pmacs (N = 30 cells). (H) Schematic of fusion assay in Tudor-treated cells where lysosomes and phagosomes are labeled with Alexa 488 dextran (G) and pHrodo Red zymosan (R), respectively. (I) Lysosomes (G) and phagocytosed zymosan particles (R) with or without dsDNA or Tudor treatment in RAW 264.7 cells (J) Inset of TLs-P contacts indicated by #. ***P < 0.0005; *P < 0.05 (one-way ANOVA with Tukey post hoc test). n.s: nonsignificant; n = 100 phagosomes wherever mentioned.

A Model for How TLs Promote Phagocytosis. We propose a model where Tudor acts as an extracellular ligand for plasma membrane-resident Ku70/80 (Fig. 6G). When Tudor binds the Ku heterodimer, Ku80 activates MMP9. Activated MMP9 interacts with and activates diverse receptor tyrosine kinases (RTKs) at the plasma membrane. We posit that MMP9 could activate PI3K via an unidentified RTK, possibly PAR-1 (60). PI3K activation, in turn, promotes phagocytosis through multiple mechanisms. First, it enriches PI(3,4,5)P₃ abundance (61). Second, PI(3,4,5)P₃ activates Akt, secreting more MMP9. This sets up positive feedback whereby Tudor at the cell surface activates newly secreted MMP9. Third, PI3K activity leads to mTOR activation tubulating lysosomes (3, 60). Suppressing any of these steps impedes phagosome formation and phagosome–lysosome fusion.

Our results also revealed that, like lysosomal Ca²⁺ channels, mTOR activity is also stringently regulated and underpins TL formation. LKB1 and AMPK activity contributed to lysosome tubulation. AMPK negatively regulates mTOR by phosphorylation at S722 and S792 (62). This likely prevents hyperphosphorylation and its downstream effects on macrophage function. Accordingly, we tested the effect of Tudor on primary M1, M0, and M2 macrophages derived from MMP9 knockout mice. Indeed, in all three types of macrophages Tudor treatment failed to tubulate lysosomes (Fig. 6D and SI Appendix, Fig. S33), and phagocytosis (Fig. 6E and SI Appendix, Fig. S34) and phagosome–lysosome fusion were diminished (Fig. 6F and SI Appendix, Fig. S35).

Fig. 5. Tubulation promotes phagocytosis and phagosome–lysosome fusion in macrophages. (A) Average number of phagosomes versus time in RAW 264.7 cells treated with dsDNA, LPS, or Tudor. Arrow shows zymosan addition (N = 30 cells). (B) Percentage TLs contacting phagosomes (% TL-P contact) (N = 50 cells). (C) Phagosome–lysosome fusion (P-L fusion) given by mean G/R value in RAW 264.7 cells (n = 60 cells). (D) Schematic of fusion assay in Tudor-treated cells where lysosomes and phagosomes are labeled with Alexa 488 dextran (G) and pHrodo Red zymosan (R), respectively. (E) Number of phagosomes in Tudor-treated RAW 264.7 cells depleted of Arl8b (N = 100 cells). (F) Lysosomes (G) and phagocytosed zymosan particles (R) with or without dsDNA or Tudor treatment in RAW 264.7 cells (J) Inset of TLs-P contacts indicated by #. ***P < 0.0005; *P < 0.05 (one-way ANOVA with Tukey post hoc test). n.s: nonsignificant; n = 100 phagosomes wherever mentioned.
MMP9 plays a role in the early stages of phagosome formation whereby they poise resting macrophages for phagocytosis. Tudor activates PI3K, enriching PI(3,4,5)P3 on the cell membrane, promoting phagocytosis (56). Our studies reveal that MMP9 activation is a crucial player in the lysosome tubulation cascade. MMP9 activation proceeds via positive feedback, leading to the sustained tubulation needed to support the extensive cell membrane ruffling and remodeling needed for phagocytosis. The ruffled border in osteoclasts is actually formed by secretory lysosomes where SNX10 is implicated in transporting secretory lysosomes to the plasma membrane (70). In fact, SNX10 promotes MMP9 secretion in osteoclasts (71). We therefore propose a model where the movement of the much larger TLs on force-generating microtubules could similarly push against the fluid, PI(3,4,5)P3-rich cell membrane, thereby causing the large-scale remodeling needed to engulf phagocytic cargo.

Accordingly, our studies mechanistically connect lysosome tubulation with MMP9 activation in macrophages. MMP9 is a key player in macrophage biology as its secretion by macrophages plays an important role in numerous diseases. In cancer, MMP9 secretion by macrophages promotes the epithelial–mesenchymal transition in pancreatic, gastric, colon, and bone cancers (3, 19, 49, 72).
MMP9 secreted by macrophages in tumors activates the PI3K–Akt pathway via protease activated receptor-1 (PAR-1) (72). In atherosclerosis, MMP9 secretion by macrophages enhances elastin degradation and induces significant plaque disruption (18). In renal fibrosis, MMP9 contributes to pathogenesis through osteoclast activation (17). Given the different types of TL populations, it is possible their roles in cell function may be more widespread than previously anticipated. The ability to switch on or switch off lysosome tubulation using Tudor or MMP9 inhibition will help uncover new roles of TLs and potentially modulate macrophage function.

**Methods**

Competition assays, tubulation assays, inhibitor assays, TL quantification, immunofluorescence, gene expression analysis, MMP9 activity, character-

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**Data Availability**

All data related to the study are included in the article and supporting information. The raw data supporting Figs. 1–6, respectively, are available for public access at Figshare (80–85): https://figshare.com/articles/dataset/Figure_1/16571561/1, https://figshare.com/articles/dataset/Figure_2/16571570/2, https://figshare.com/articles/dataset/Figure_3/16571579/2, https://figshare.com/articles/dataset/Figure_4/16571582/2, https://figshare.com/articles/dataset/Figure_5/16571585/2, and https://figshare.com/articles/dataset/Figure_6/16574231/2.

**Supplementary material**

Tables S1–S7 and SI Appendix. Material and Methods SI Appendix also contains supplementary notes and figures with figure legends as well as the legend for Movie S1.
Tubular lysosomes harbor active ion gradients and poise macrophages for phagocytosis.