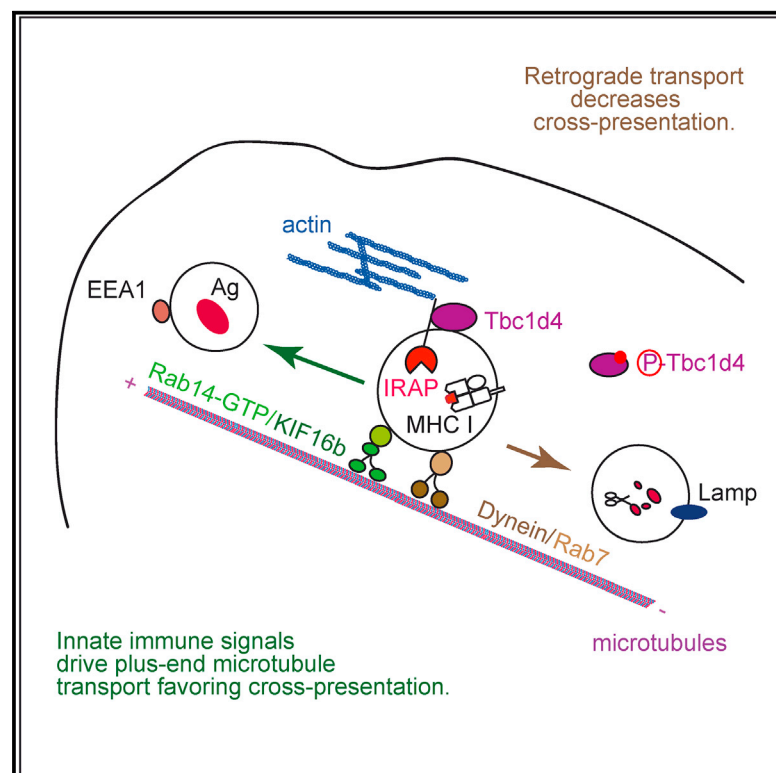


Innate Immune Signals Induce Anterograde Endosome Transport Promoting MHC Class I Cross-Presentation

Graphical Abstract



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In Brief

Weimershaus et al. identify a molecular complex that controls the intracellular trafficking along microtubules of antigens internalized by dendritic cells. They show that this trafficking is regulated by innate immune signals and regulates presentation of internalized antigens to T lymphocytes.

Highlights

- Innate immune signals induce formation of a complex of Rab14 with the kinesin KIF16B
- This complex promotes anterograde microtubule transport of endosomes and phagosomes
- Plus-end transport favors efficient cross-presentation of internalized antigens
- Disruption of the complex accelerates phagosome maturation and microbial killing



Innate Immune Signals Induce Anterograde Endosome Transport Promoting MHC Class I Cross-Presentation

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SUMMARY

Both cross-presentation of antigens by dendritic cells, a key pathway triggering T cell immunity and immune tolerance, and survival of several pathogens residing in intracellular vacuoles are intimately linked to delayed maturation of vesicles containing internalized antigens and microbes. However, how early endosome or phagosome identity is maintained is incompletely understood. We show that Toll-like receptor 4 (TLR4) and Fc receptor ligation induces interaction of the GTPase Rab14 with the kinesin KIF16b mediating plus-end-directed microtubule transport of endosomes. As a result, Rab14 recruitment to phagosomes delays their maturation and killing of an internalized pathogen. Enhancing anterograde transport by overexpressing Rab14, promoting the GTP-bound Rab14 state, or inhibiting retrograde transport upregulates cross-presentation. Conversely, reducing Rab14 expression, destabilizing Rab14 endosomes, and inhibiting anterograde microtubule transport by *Kif16b* knockdown compromise cross-presentation. Therefore, regulation of early endosome trafficking by innate immune signals is a critical parameter in cross-presentation by dendritic cells.

INTRODUCTION

Endocytosis, the active uptake of extracellular molecules, is used by all cells to sense, exploit, and interact with their environment. In myeloid cells of the immune system, endocytosis fulfills the additional functions of pathogen sensing and

destruction, and of extraction of antigenic peptides from internalized material for presentation by major histocompatibility complex (MHC) molecules. These functions are regulated by a process referred to as maturation of the vesicles containing internalized material (Huotari and Helenius, 2011). Vesicle maturation is the result of a series of “kiss-and-run” fusion and fission events between incoming and various intracellular vesicles. These events change the protein composition and pH of the former, eventually creating an acidic vesicle equipped for efficient and rapid degradation of all internalized biomolecules.

Cross-presentation, defined as the presentation by MHC class I molecules of peptides derived from internalized material, is one of the molecular events regulated by maturation (Cruz et al., 2017). Efficient cross-presentation depends on a delayed and/or attenuated maturation of vesicles containing antigenic material, thought to inhibit rapid destruction of internalized proteins and preserve antigenic information (Alloatti et al., 2016). Consistent with this, the speed of vesicle maturation is inversely correlated with the efficacy of cross-presentation, with a gradient from conventional dendritic cells (cDCs), cross-presenting with the highest efficacy, to macrophages and neutrophils (Amigorena and Savina, 2010).

Given that vesicle maturation transforms “early” to “late” vesicles, the prevalent concept is that an early endosome or phagosome environment is favorable for cross-presentation. In support of this, the efficacy of cross-presentation is inversely correlated with the expression of transcription factor EB (TFEB) in dendritic cells (DCs), a transcription factor enhancing lysosome biogenesis and activation (Samie and Cresswell, 2015). Moreover, inhibiting endosome maturation with chloroquine ameliorates cross-presentation (Accapezzato et al., 2005). Further support is provided by the observation that cell surface receptors retained longer in early endocytic compartments may be more suitable for targeting antigens for efficient cross-presentation than others rapidly trafficking to late compartments



(Chatterjee et al., 2012; Cohn et al., 2013). This distinction may also explain the relatively efficient cross-presentation of antigens targeted to the mannose receptor (MR) traveling to early endosomes (Burgdorf et al., 2007).

Given the importance of DC-specific maturation kinetics for cross-presentation, the mechanisms underlying have been subject to significant scrutiny. These studies have uncovered a number of features inhibiting the function or the recruitment to maturing vesicles of late endosomes and lysosomes. These include incomplete assembly of the proton pump V-ATPase (Delamarre et al., 2005), recruitment of the alkalinizing NADPH oxidase 2 (Savina et al., 2006), and lipopolysaccharide (LPS)-induced, Rab34-dependent perinuclear clustering of lysosomes. However, mechanisms regulating trafficking of early endosomes and phagosomes have not been reported so far.

Understanding of physiological cell biological pathways has in many instances benefitted from studying how pathogens subvert such pathways. Because phagosome maturation results in destruction of internalized pathogens, various microbes surviving and proliferating in intracellular vacuoles have developed mechanisms to block maturation of early to late phagosomes. One of these implicates Rab14, a small GTPase associated with early endosomes, that is recruited by mycobacteria, salmonella, legionella, and chlamydia to subvert phagosome maturation through an unknown mechanism (Capmany and Damiani, 2010; Hoffmann et al., 2014; Kuijl et al., 2007; Kyei et al., 2006). We investigated how Rab14 physiologically controls endosome and phagosome maturation. Here we describe a complete mechanism by which active Rab14 triggered by innate immune stimuli recruits the kinesin KIF16b to endosomes, thereby retaining incoming material in the cellular periphery, delaying fusion with acidic degradative vesicles and ultimately regulating cross-presentation.

RESULTS

Rab14 Highlights a Subpopulation of Early Endosomes in DCs

Rab14 is expressed in most cell types including macrophages and DCs. However, it has been studied most thoroughly in cells expressing the Glut4 glucose transporter such as adipocytes and muscle cells. In these cells, Glut4 localizes to a subpopulation of endosomes known as Glut4 storage vesicles (GSVs) (Rowland et al., 2011; Sadacca et al., 2013). Signaling by the insulin receptor results in a rapid increase in Glut4 density at the cell surface (Chen and Lippincott-Schwartz, 2013). Among several Rab proteins regulating trafficking to and from GSVs, Rab14 is thought to direct Glut4 from recycling endosomes and/or the *trans*-Golgi network (TGN) to GSVs (Sadacca et al., 2013). Thus, in muscle cells, Rab14 mediates retention of cell-specific proteins in an endosomal storage compartment (Jaldin-Fincati et al., 2017; Leto and Saltiel, 2012).

We wondered whether Rab14 might localize to a similar endosome subpopulation in DCs. In steady-state bone marrow-derived DCs (BM-DCs), Rab14 co-localized strongly with insulin-regulated aminopeptidase (IRAP), a hallmark constituent of GSVs in Glut4-expressing cells (Figure 1A) (Keller, 2003). Rab14 also co-localized strongly in peripheral endosomes, but

not in the TGN with syntaxin 6 (Stx6), a soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) involved in trafficking between the TGN and endosomes, the latter including GSVs (Du et al., 2014; Watson et al., 2008). Therefore, BM-DCs harbor endosomes with a protein profile resembling GSVs.

To further examine whether Rab14 localizes to general endosomes or to a GSV-like specific population, we knocked down *Rab14* and examined the effect on general and GSV-associated endosome markers. Efficient knockdown of *Rab14* (>70% reduction; Figure 1B) strongly reduced staining for IRAP and staining in the cellular periphery for Stx6 but had no notable effect on staining for early endosome antigen (EEA-1) or the MR, or on Stx6 staining in the TGN (Figure 1C). Moreover, co-localization between EEA-1 and the MR was not affected, whereas EEA-1 co-localization with Stx6 and IRAP, as well as MR co-localization with IRAP, were almost abolished (Figure 1D). This is consistent with the notion that Rab14 marks a specific subset of endosomes. We concluded that, in DCs, Rab14 not only marks a subtype of endosomes bearing GSV-associated markers but also seems to be required for formation or stability of these vesicles.

Effect of Rab14 on Phagosome Maturation and Cross-Presentation

Given that Rab14 is recruited to various microbe-containing vacuoles, we reasoned that Rab14 recruitment might be a regular event in DC phagosome maturation. To monitor phagosome maturation, we developed a protocol in which recruitment of EEA-1, Lamp1, and Rab14 to phagosomes of 1,000 BM-DCs per time point is evaluated quantitatively by imaging flow cytometry (Johansson et al., 2015) (Figures S1A–S1C). Using this method, we observed transient recruitment of Rab14 to BM-DC phagosomes at 20 min after the end of phagosome formation (Figure 1E).

Next, we examined the effect of Rab14 knockdown on phagosome maturation. Uptake of yeast was not affected by Rab14 knockdown (Figure S1D). In control cells, phagosomes initially stained for EEA-1 and acquired Lamp1 only at 35 min (Figure 2A). In contrast, Rab14 knockdown accelerated EEA-1 loss and Lamp1 acquisition. Quantitative evaluation by imaging flow cytometry confirmed EEA-1 loss starting at 30 min and enhanced Lamp1 acquisition starting at very early time points (Figure 2B). We concluded that low abundance of Rab14 results in accelerated phagosome maturation (Figure S1E). Consistent with this, killing of phagocytized *P. aeruginosa* was enhanced (Figure 2C).

To study the impact of Rab14 on cross-presentation capacity, we first used the model of HEK293 cells transfected with an Fc receptor and the murine H-2K^b MHC class I molecule (Giodini et al., 2009). These cells can cross-present immune complexes formed between ovalbumin (OVA) and anti-OVA antibodies that are internalized via the Fc receptor and degraded to the SIINFEKL (S8L) OVA peptide presented by H-2K^b. Peptide presentation can be read out using OT-I T cell receptor transgenic T cells (Hogquist et al., 1994) or the B3Z hybridoma. *Rab14* knockdown by lentivirus transduction did not affect H-2K^b expression or presentation of synthetic S8L by HEL cells or

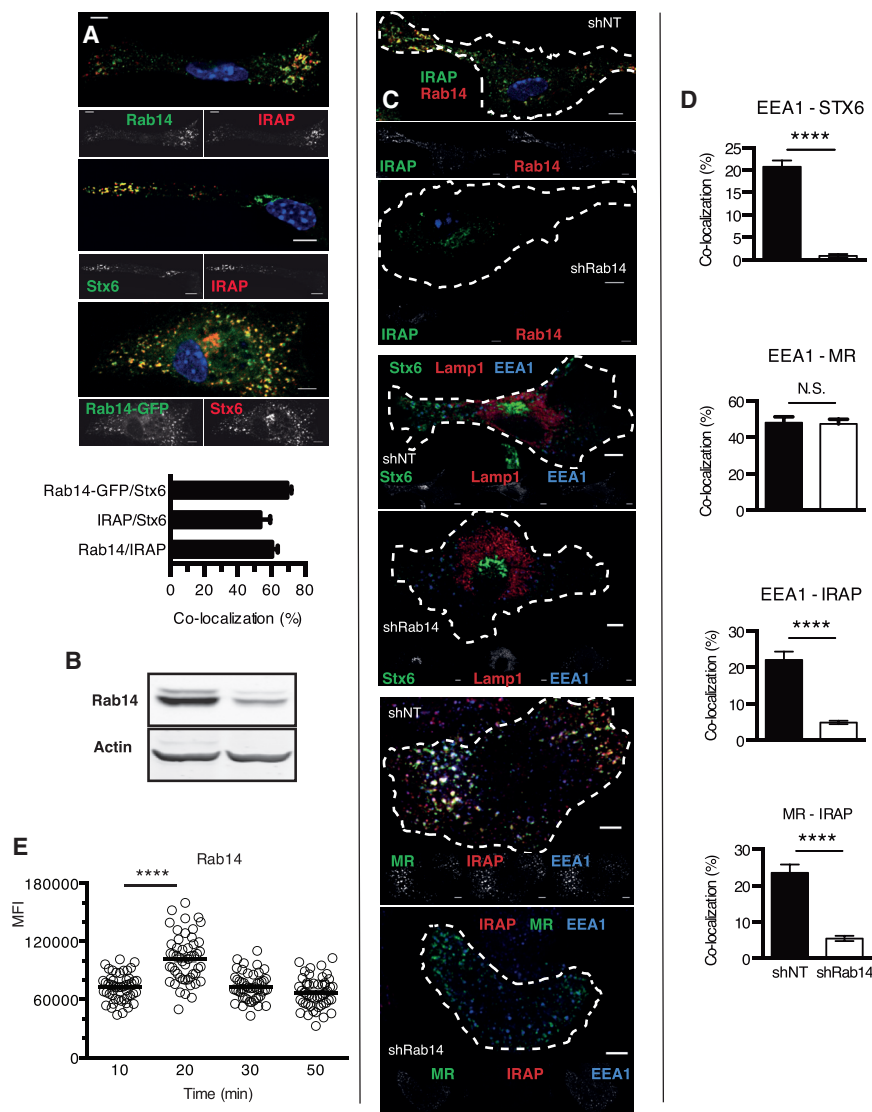


Figure 1. Rab14 Highlights a Subpopulation of Early Endosomes in DCs

(A) Expression and co-localization of Rab14 (endogenous or transfected as GFP fusion protein), Stx6, and IRAP in murine BM-DCs.

(B) Efficiency of *Rab14* knockdown in BM-DCs as judged by immunoblot.

(C) Co-localization of the markers indicated, in BM-DCs transduced with control non-targeting (shNT) or *Rab14*-targeting (shRab14) lentivirus.

(D) Quantitative analysis of the experiments shown in (C) ($n > 15$ cells).

(E) Imaging flow cytometry analysis of Rab14 recruitment by phagosomes for shNT-transduced BM-DCs.

All images and plots are representative of 3 experiments. Scale bars, 5 μ m. **** $p < 0.0001$. Graphics show means \pm SEM.

cells, and that the impact of Rab14 in cross-presentation extends to particulate and soluble receptor-targeted antigens.

Identification of the GTPase-Activating Protein Regulating Rab14

Rab GTPases are active when bound to GTP and inactivated by GTPase-activating proteins (GAPs) (Stenmark, 2009). Given the rarity of Stx6⁺IRAP⁺ endosomes observed upon *Rab14* knockdown, it was possible that the effect of Rab14 on phagosome maturation was due to a structural role rather than to recruitment of an effector by its active form. To differentiate between these two modes of action, we set out to identify a Rab14-specific GAP expressed in DCs and to test its impact. If Rab14 had a mere structural role, it was less likely to

BM-DCs (Figure S2A–S2C) but compromised cross-presentation of immune complexes by HEK293 cells (Figure 2D). Transfecting transduced cells with a *Rab14* cDNA not containing the target sequence of the short hairpin RNA (shRNA) more than fully restored cross-presentation, ruling out an off-target effect (Figure 2E). To further test the relationship between Rab14 expression and cross-presentation, we increased *Rab14* expression by transfecting a Rab14-mCherry fusion protein. Transfected cells formed enlarged early endosomes staining for EEA1, IRAP, and Stx6, thus resembling GSV-like DC vesicles (Figures S3A and S3B). Rab14 overexpression increased cross-presentation of immune complexes (Figure 2F). Conversely, knockdown of *Rab14* in BM-DCs compromised cross-presentation of both an OVA fusion protein targeted to the MR (Kratzer et al., 2010) and yeast cells bearing OVA at the surface (Saveanu and van Endert, 2013) (Figures 2G and 2H). We concluded that the capacity to cross-present correlates with Rab14 expression levels both in DCs and in non-professional antigen-presenting

be dependent on an intact nucleotide exchange cycle. In adipocytes and skeletal muscle, Tbc1d1 and Tbc1d4/AS160 act as GAPs for several Rab proteins including Rab14 (Sakamoto and Holman, 2008). Published microarray data suggest that DCs and macrophages express more Tbc1d4 than Tbc1d1 (<https://www.immgen.org>). We analyzed endosomes in BM-DCs lacking Tbc1d1, Tbc1d4, or both GAPs (Chadt et al., 2015) (Figures 3A–3D). In wild-type (WT) but also in *Tbc1d1* knockout (KO) cells, Rab14⁺Stx6⁺ vesicles belonged to early endosomes as documented by partial staining for EEA-1 and absence of Lamp1 staining. In contrast, absence of Tbc1d4 resulted in formation of enlarged Rab14⁺Stx6⁺ endosomes displaying reduced co-localization with EEA-1 and partial co-localization with Lamp1. Tbc1d4 could also be co-precipitated with IRAP, consistent with its recruitment to Rab14⁺ endosomes and with observations made in adipocytes (Rowland et al., 2011) (Figure 3E). These observations indicate that Tbc1d4 is a GAP for Rab14 in BM-DCs. Moreover, they suggest that the balance between the

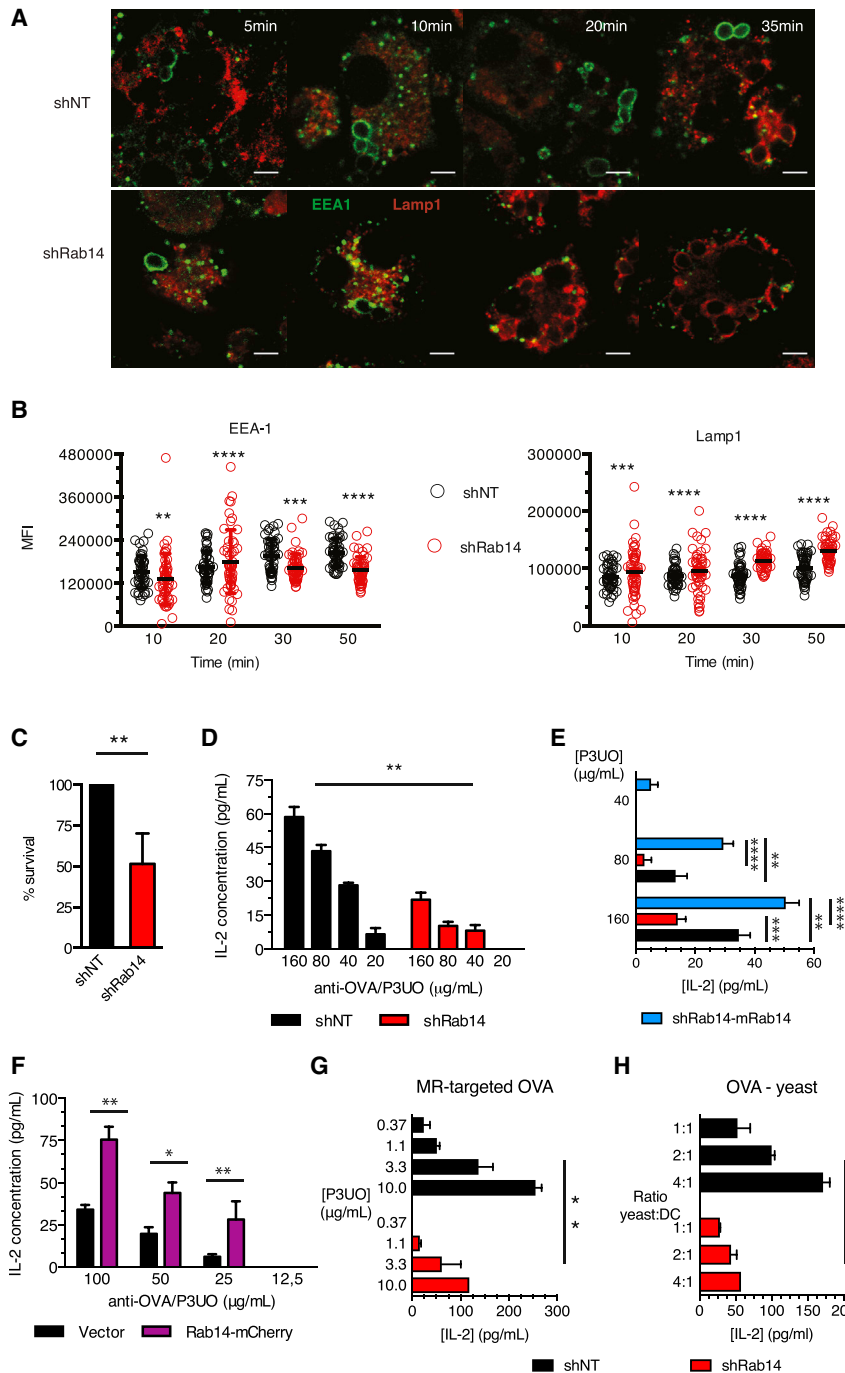


Figure 2. Rab14 Knockdown Accelerates Phagosome Maturation and Compromises Cross-Presentation

(A) BM-DCs transduced with control non-targeting (shNT) or shRab14 lentiviruses were pulsed with *S. cerevisiae* for 5 min and incubated at 37°C for the indicated periods of time before staining for EEA-1 and Lamp1.

(B) Imaging flow cytometry analysis of EEA-1 and Lamp1 recruitment for shNT and shRab14-transduced BM-DCs.

(C) Survival, as determined by colony formation assay, of *Pseudomonas aeruginosa* phagocytosed by shRNA-transduced BM-DCs.

(D) HEK293 cells transduced with lentiviruses were pulsed with OVA-anti-OVA immune complexes and used to stimulate B3Z hybridoma cells. Stimulation was read out as IL-2 secretion by ELISA.

(E) HEK293 cells were sequentially transduced with Rab14 shRNA and transfected with Rab14-mCherry before analyzing presentation of immune complexes.

(F) HEK293 cells were transfected with a plasmid expressing Rab14-mCherry or an empty vector-mCherry, and cross-presentation of OVA-anti-OVA complexes was analyzed as in (D).

(G and H) Lentivirus-transduced BM-DCs were pulsed with an OVA fusion protein targeted to the MR (left panel) (G) or with OVA-yeast cells (H) and then added to OT-I cells. Activation of OT-I CD8⁺ cells was read out as IL-2 secretion by ELISA. All images and plots are representative of 3 or 2 (B) experiments. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Graphs show mean \pm SEM.

a shift to the GTP-bound state of Rab14 promotes cross-presentation.

We also examined the effect of mutations locking Rab14 in the GTP- or GDP-bound state on endosome formation and cross-presentation. Transfection of Rab14 had no notable effect on BM-DC differentiation or activation, as documented by an unchanged proportion of CD11c⁺CD11b⁺ cells in cultures and identical CD80 expression (Figures S4A and S4B). Overexpressed WT Rab14 was found in enlarged endosomes and co-localized normally with Stx6 and IRAP, but not Lamp1 (Figures S4C and S4D). In contrast, the GTP-locked mutant

Q70L showed reduced co-localization with Stx6 and IRAP, and enhanced co-localization with Lamp1, thus resembling the distribution of endogenous Rab14 in *Tbc1d4* KO BM-DCs. The GDP-locked mutant S25N completely lacked co-localization with IRAP and co-localized only with Golgi-resident Stx6, consistent with retention in the Golgi and early secretory pathway, as reported previously (Junutula et al., 2004) (Figures S4C and S4D). In a cross-presentation assay, overexpression of WT Rab14 slightly enhanced cross-presentation, whereas not only

GTP- and GDP-bound states of Rab14 is important for the physiological trafficking of Rab14⁺Stx6⁺IRAP⁺ endosomes. However, when we tested cross-presentation of MR-targeted or yeast-decorating OVA by BM-DCs lacking either or both of the GAPs, absence of *Tbc1d4* enhanced cross-presentation of both antigen forms (Figure 3F). *Tbc1d4* KO also enhanced cross-presentation by inflammatory DCs (Cheong et al., 2010), the cells corresponding most closely to BM-DCs, produced *in vivo* by injection with LPS and soluble OVA, and tested *ex vivo* (Figure 3G). Therefore,

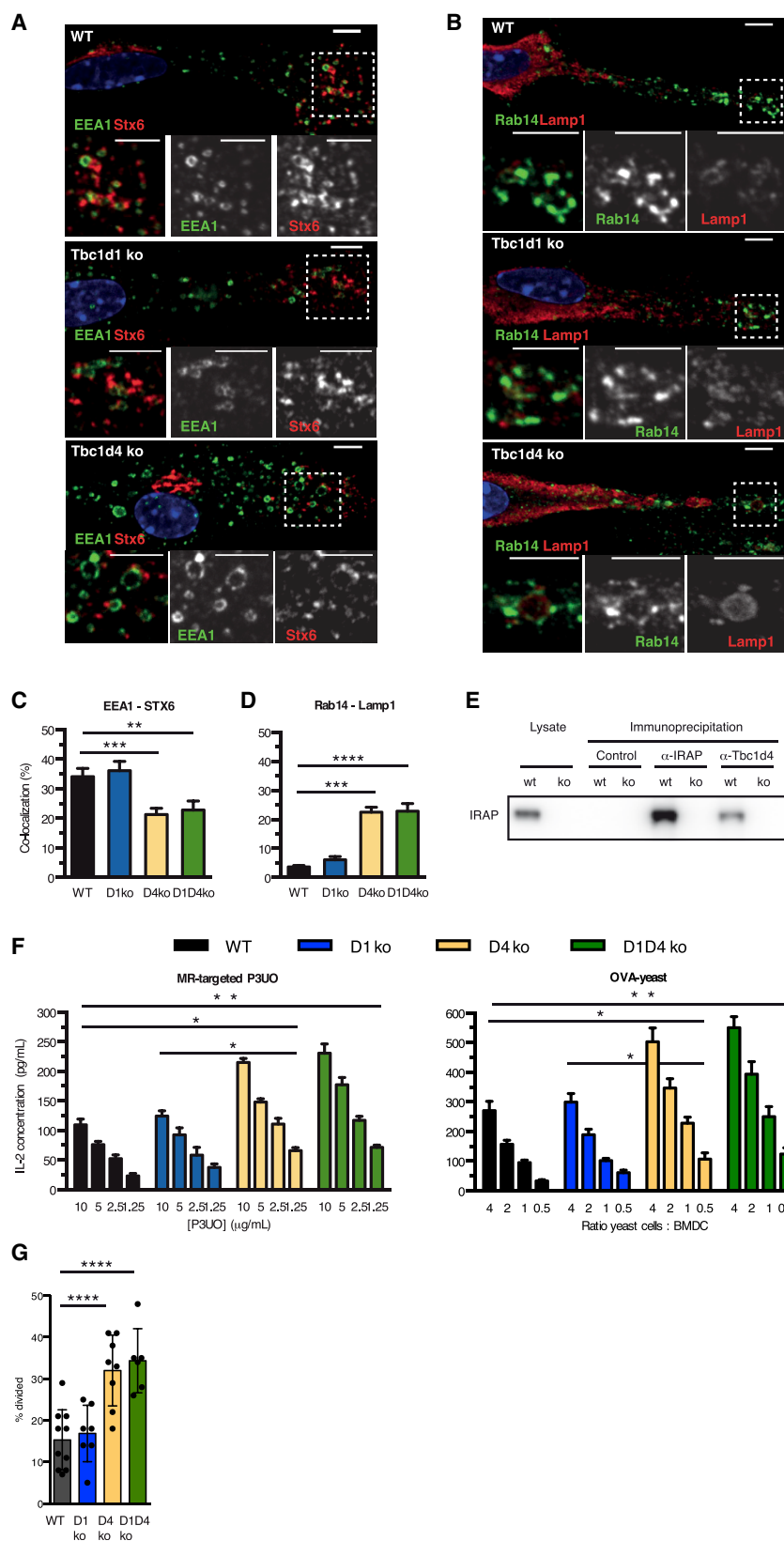


Figure 3. Tbc1d4 Is the GAP for Rab14 in DCs and Regulates Cross-Presentation

(A and B) Tbc1d1- or Tbc1d4-deficient BM-DCs were stained for Stx6 and EEA-1 (A), or for Rab14 and Lamp1 (B). The dotted boxes enclose the areas shown as split images below the larger images.

(C and D) Quantitative analysis of co-localization ($n = 10$ cells) of EEA1 with Stx6 (C) and of Rab14 with Lamp1 (D) in WT BM-DCs and DCs lacking Tbc1d1, Tbc1d4, or both (D1D4KO).

(E) Immunoprecipitation of Tbc1d4 and IRAP from WT or IRAP KO BM-DCs followed by immunoblot analysis of IRAP co-precipitation.

(F) Cross-presentation of MR-targeted OVA (left panel) and OVA-expressing yeast cells (right panel) to OT-I T cells by WT, Tbc1d1 KO, Tbc1d4 KO, and double-KO BM-DCs.

(G) WT and Tbc-deficient mice were first injected with LPS and then with OVA 2 hr before isolation of inflammatory DCs. DCs were added to dye-labeled OT-I at 1:3, and proliferation was measured after 3 days.

The experiments shown are representative of 3 (C) or 2 experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Scale bar, 5 μm . Graphs show means \pm SEM.

GDP-locked, as expected, but surprisingly also GTP-locked Rab14 compromised it in both BM-DCs and HEK293 cells (Figures S4E and S4F). Therefore, although shifting Rab14 to the GTP-bound state either through the Q70L mutation or through deletion of the Tbc1d4 GAP has very similar effects on Rab14 trafficking, the effects on cross-presentation differ markedly, presumably because of the inability of the mutant to hydrolyze GTP, which therefore is critical for cross-presentation.

Rab14 Interacts with a Kinesin

How does GTP-bound Rab14 promote cross-presentation? Rab GTPases act by recruiting effector proteins upon activation (Stenmark, 2009). Among these are motor proteins for microtubule transport. In most cells, kinesins mediate anterograde transport to the cell periphery and dynein retrograde centripetal transport to the microtubule organizing center (Hancock, 2014). Given that late endosomes and phagosomes accumulate at the cell center, we speculated that Rab14 interaction with a kinesin might delay endosome and phagosome contact with degradative vesicles, thus favoring cross-presentation. We tested our hypothesis first in Rab14-transfected embryonal fibroblasts strongly expressing Rab14⁺Stx6⁺IRAP⁺ vesicles (Figure S3C). We analyzed a potential interaction between Rab14 and kinesins using the proximity ligation assay (PLA) that detects proximity of proteins 40 nm or less distant from each other. PLA analysis using a pan-kinesin and a Rab14 antibody revealed proximity between Rab14 and one or several kinesins (Figures 4A and 4B). In Rab14/GFP-transfected HEK293 cells expressing an Fc receptor, Rab14-kinesin proximity was also present at the steady state and strongly enhanced upon immune complex binding to Fc γ receptors (Figures 4C and 4D). Control immunoglobulins combined with Rab14 antibodies did not give a PLA signal in transfected MEFs or HEK293 cells. To further test the specificity of the PLA signal, we performed an analysis using a pan-kinesin antibody combined with antibodies recognizing the three hallmark proteins of the GSV-like vesicles, Rab14, Stx6, and IRAP in BM-DCs. A PLA signal was obtained using only Rab14 antibodies, consistent with specific recruitment of a kinesin by the GTPase (Figure 4E). Incubation of BM-DCs with LPS for 1 hr increased this PLA signal (Figures 4F and 4G). In cells lacking the Rab14 GAP Tbc1d4, both the number of PLA dots and the endosome diameter were increased at steady state and upon LPS incubation, consistent with a role for GTP-bound Rab14 in kinesin interaction (Figures 4H–4J). We concluded that Rab14 is likely to recruit a kinesin as effector protein, and that this recruitment is stimulated by innate immune signaling through toll-like receptor 4 (TLR4) or Fc receptors.

Dynein-Mediated Retrograde Transport Compromises Cross-Presentation

Our finding of Rab14-kinesin proximity suggested that anterograde vesicle transport promoted cross-presentation. Reasoning that retrograde transport mediated by dynein might therefore reduce cross-presentation efficacy, we knocked down dynein heavy chain (Figure 5A) and examined the effect on Rab14⁺ endosomes and on cross-presentation. Knocking down dynein did not affect Rab14⁺Stx6⁺ endosomes in WT DCs (Figure 5B) or presentation of synthetic peptide S8L by them (Figure S2D). This was not

surprising given that WT Rab14 co-localizes very little with Lamp1 (Figure 3D), suggesting a physiological dominance of anterograde over retrograde transport of Rab14⁺ vesicles. We reasoned that the effect of retrograde transport on Rab14⁺ endosomes and cross-presentation might become evident in conditions destabilizing these vesicles.

We have recently described that IRAP stabilizes Stx6⁺ endosomes due to interaction with the actin-nucleation factor FHOD4, resulting in partial depletion of these vesicles in IRAP-deficient cells (Babdor et al., 2017). Like *Rab14* knockdown DCs, these cells display unperturbed co-localization of EEA-1 and MR in early endosomes but low co-localization of Stx6 with each of these markers; as a result of the depletion of IRAP⁺ endosomes, Rab14⁺EEA-1⁺ endosomes are virtually absent (Figure S5). In *IRAP* KO BM-DCs, Rab14 failed to be recruited to early phagosomes, and phagosomes displayed early loss of EEA-1 combined with accelerated acquisition of Lamp1 (Figure S6A). Moreover, Rab14 knockdown had no effect on the kinetics of maturation of phagosomes in *IRAP* KO cells (Figure S6B, left panel). Taken together, these findings suggest overlapping effects of Rab14 knockdown and *IRAP* KO on the GSV-like vesicles.

To assess the role of microtubule transport on Rab14⁺ vesicles, we first examined the effect of paclitaxel (Taxol) that inhibits microtubule disassembly on Rab14⁺ endosomes in WT and *IRAP* KO DCs. Microtubule stabilization had little effect on Rab14⁺ endosomes in WT DCs but restored these vesicles in *IRAP* KO cells (Figure 5C). Paclitaxel also restored endogenous Rab14 proximity with kinesin that was absent in untreated *IRAP* KO DCs, as measured by PLA (Figure 5E). In WT and *IRAP* KO fibroblasts transfected with Rab14-GFP, a pan-kinesin antibody co-precipitated Rab14, indicating that this PLA signal was due to physical interaction (Figure 5D). We concluded that depletion of Rab14⁺ endosomes and of Rab14-kinesin interaction in *IRAP* KO cells depended on microtubule transport.

Knockdown of dynein in *IRAP* KO BM-DCs indeed restored endosomes staining for Rab14 and Stx6, analogous to the effect of paclitaxel (Figure 5F). In *IRAP* KO cells depleted for dynein, Rab14 recruitment to phagosomes was restored, whereas Lamp1 recruitment and phagosome maturation were delayed (Figures 5G and S6B, center panel). Remarkably, dynein depletion fully normalized cross-presentation of both MR-targeted and yeast-decorating OVA by DCs, while increasing cross-presentation by WT cells only slightly (Figure 5H). Two additional shRNA sequences targeting dynein also restored cross-presentation, to an extent reflecting the magnitude of their effect on dynein expression (Figures S6C–S6E). Collectively, these data suggest that Rab14⁺Stx6⁺ endosomes are formed in *IRAP* KO cells and can promote cross-presentation but are destabilized by loss of the IRAP-formin anchor, resulting in dominant retrograde transport presumably followed by dissociation or degradation of Rab14.

Identification of the Kinesin Effector Protein Interacting with Rab14

Finally, we sought to identify the kinesin(s) interacting with Rab14. A literature search identified KIF16B, a member of the kinesin 3 family, as the single kinesin protein described as Rab14

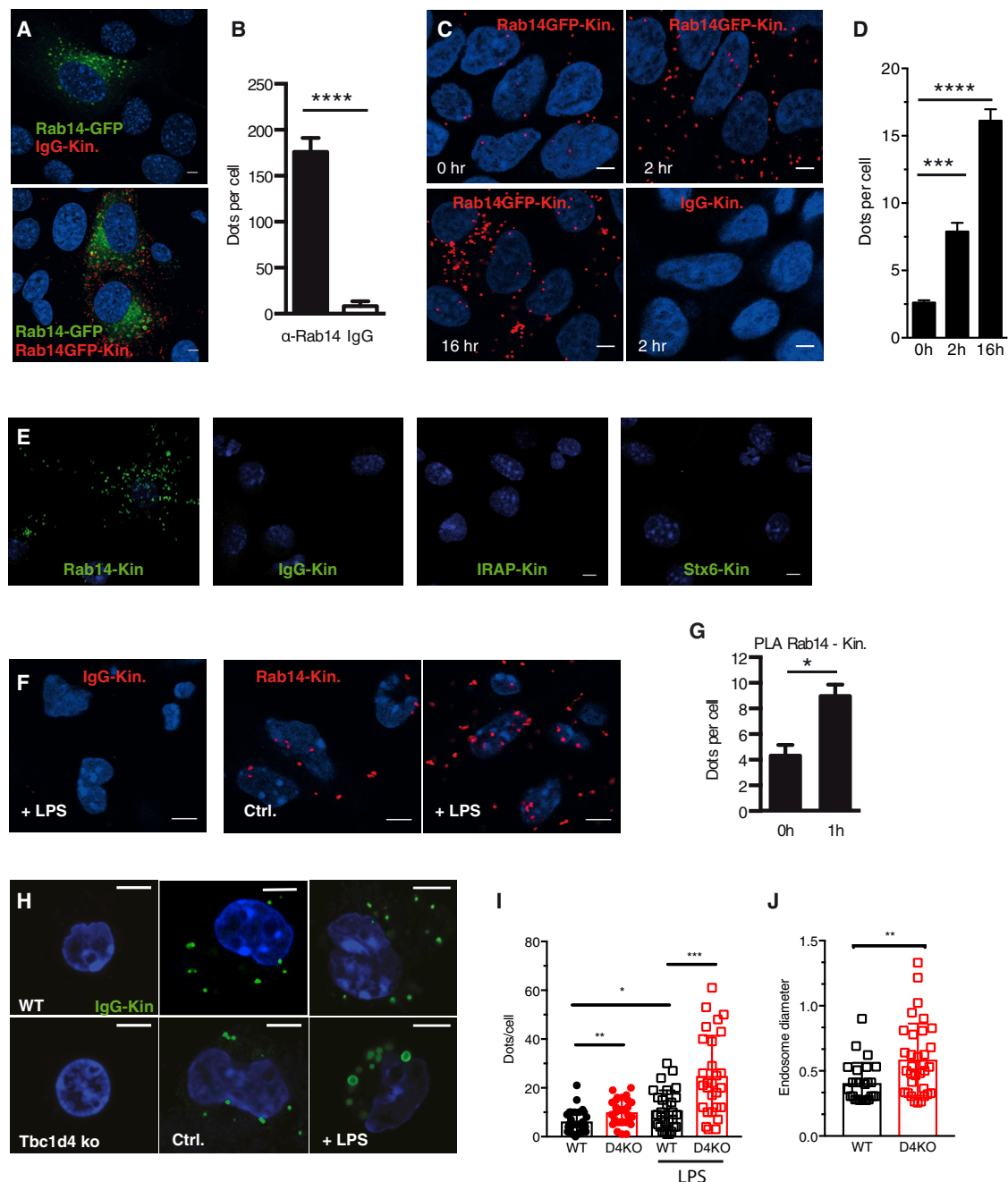


Figure 4. DC Rab14 Interacts with a Kinesin

(A) PLA on Rab14-GFP-transfected MEFs with Rab14 antibodies or control IgG together with pan-kinesin antibodies.
 (B) Quantification of the PLA signal for ≥ 30 cells per group obtained in the experiment shown in (A).
 (C) PLA analysis of Rab14/kinesin proximity in CD32a-expressing HEK293 cells stimulated with OVA-IgG immune complexes for the periods indicated.
 (D) Quantitative evaluation of (C), as in (B).
 (E) Analysis of Rab14-kinesin proximity to Rab14, Stx6, and IRAP in BM-DCs.
 (F) PLA analysis of BM-DCs stimulated or not for 1 hr with LPS.
 (G) Quantitative evaluation of the PLA analysis in (F), performed as in (B).
 (H) PLA analysis of Rab14-kinesin interaction in WT and Tbc1d4-deficient BM-DCs stimulated or not for 1 hr with LPS (n = 14 cells per group).
 (I) Quantitative evaluation of the PLA analysis in (H).
 (J) Quantification of PLA dot diameter (μ m) for LPS-treated WT and Tbc1d4-deficient BM-DCs.
 All experiments were performed at least twice. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Scale bars, 5 μ m. Graphs show means \pm SEM.

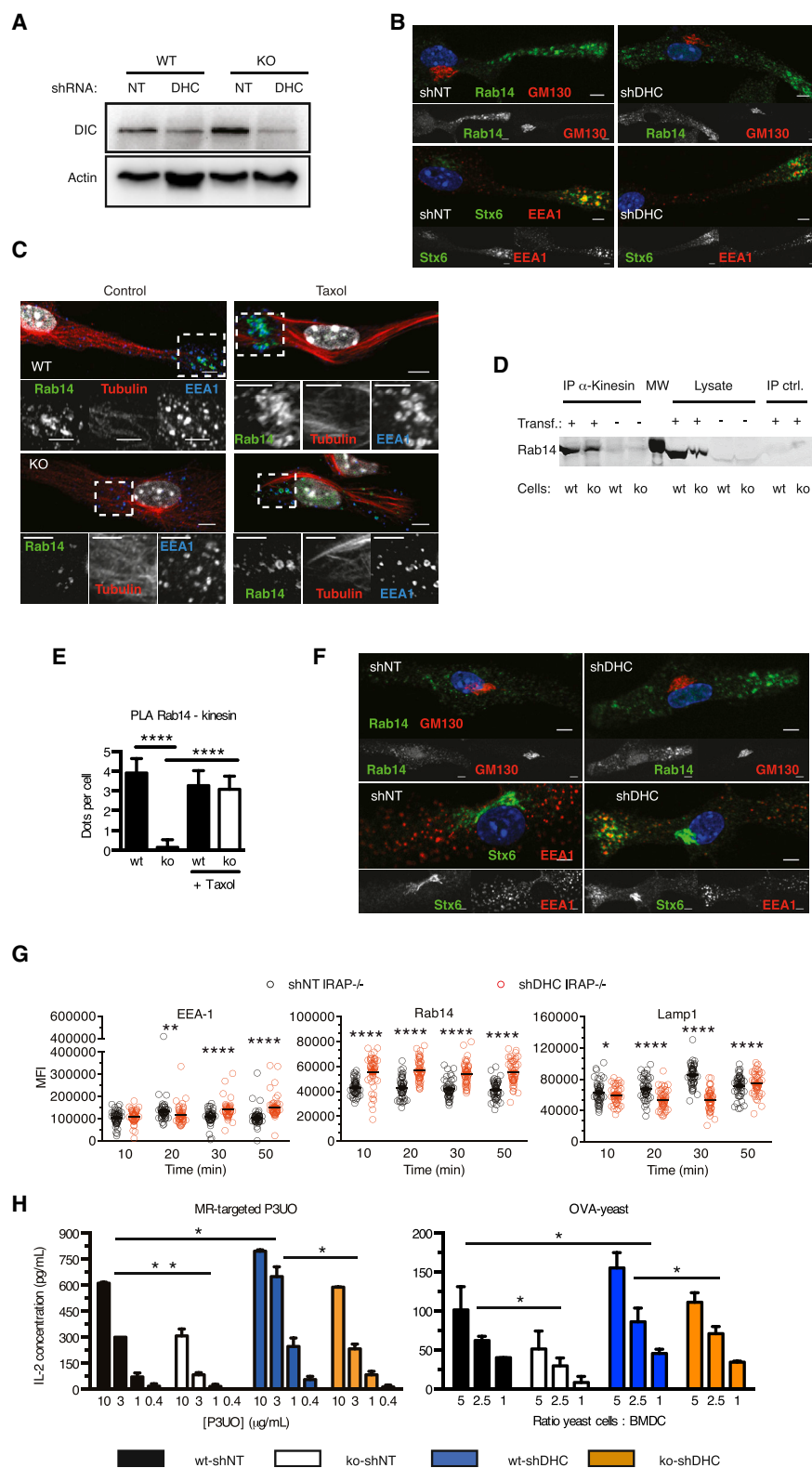


Figure 5. Rab14⁺IRAP⁺ Endosomes Inhibit Minus-End Microtubule Vesicle Transport Compromising Cross-Presentation

(A) The efficiency of lentivirus-mediated shDHC knockdown in WT and *IRAP* KO BM-DCs was analyzed by immunoblot for dynein intermediate chain (DIC), which is destabilized in the absence of dynein heavy chain (DHC).

(B) Effect of DHC knockdown on subcellular distribution of Rab14, GM130, Stx6, and EEA1 in WT BM-DCs.

(C) Effect of microtubule stabilization by paclitaxel on the subcellular distribution of Rab14, tubulin, and EEA-1 in WT and *IRAP* KO cells. The dotted boxes enclose the areas shown as split images below the larger images.

(D) Kinesin heavy chain was immunoprecipitated from MEFs transfected or not with Rab14-GFP and analyzed for co-precipitation of Rab14 by immunoblot.

(E) PLA analysis of Rab14/pan-kinesin proximity in BM-DCs treated with paclitaxel for 1 hr.

(F) Effect of DHC knockdown on the subcellular distribution of Rab14, GM130, Stx6, and EEA1 in *IRAP* KO BM-DCs.

(G) Mean fluorescence intensity for EEA-1, Lamp1, and Rab14 at the phagosome membrane for control non-targeting (shNT) or shDHC-transduced BMDCs, determined by imaging flow cytometry. For easier readability, each dot represents the mean of 50 cells; statistical evaluation was performed on 1,000 individual BMDCs. Black line represents mean value.

(H) Cross-presentation of MR-targeted OVA and of OVA-expressing yeast cells by BM-DCs transduced with control or DHC-targeting shRNA was assessed using OT-I T cells.

All experiments were performed at least twice, with analysis of 10 cells per condition for microscopy experiments. * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$. Scale bar, 5 μ m. Graphs show means \pm SEM.

effector. KIF16B mediates Golgi to endosome transport of fibroblast growth factor in mouse embryos (Ueno et al., 2011). It also relocates early endosomes to the periphery and inhibits their transport to the degradative pathway in HeLa cells (Hoepfner et al., 2005). Knockdown of *Kif16b* reduced its expression in HEK293 cells and BM-DCs by 75% (Figures 6A and 6B). Depletion of KIF16B in BM-DCs abolished peripheral clustering of Rab14⁺ and IRAP⁺ endosomes, instead scattering small endosomes across the entire cell, consistent with an interaction between Rab14 and KIF16B in BM-DCs (Figure 6C).

Lack of commercial antibodies suitable for immunoprecipitation of KIF16B or for a PLA analysis precluded obtaining direct evidence for this interaction. However, we reasoned that, if KIF16B was the principal kinesin interacting with Rab14, then knocking it down should strongly reduce the PLA signal obtained using pan-kinesin combined with Rab14 antibodies. Indeed, KIF16B depletion from HEK293 cells essentially abolished kinesin-Rab14 interaction at the steady state, as well as its increase triggered by immune complex binding in a PLA analysis (Figure 6D). Knockdown of KIF16B (like that of dynein) did not affect yeast uptake or presentation of synthetic peptide S8L by BM-DCs (Figures S2E, S7A, and S7B). Imaging flow cytometry demonstrated that KIF16B depletion abolished recruitment of Rab14 to phagosomes and accelerated their maturation, as reflected in accelerated loss of EEA-1 and acquisition of Lamp1 (Figures 6E and S7C). Indicative of an important role of the Rab14-KIF16B interaction in cross-presentation, *Kif16b* knockdown reduced cross-presentation of receptor-targeted and phagocytized antigen (Figure 6F) by BM-DCs, as well as cross-presentation of immune complexes by HEK293 cells (Figure 6G). In contrast, it did not further compromise cross-presentation by IRAP KO DCs (Figure 6F), a finding consistent with a model in which the impact of KIF16B depends entirely on its interaction with Rab14, which itself has no additive effect in IRAP KO BM-DCs (Figure S6B). Finally, consistent with the PLA analysis shown in Figures 4H–4J, an *in vitro* assay demonstrated exclusive KIF16B interaction with the GTP-locked form of Rab14 (Figure 6H).

DISCUSSION

This study reports three principal findings. First, we identify a critical role in cross-presentation of a small GTPase previously linked to GSV trafficking in adipocytes and muscle cells, and to establishment of microbe vacuoles in macrophages. Second, we determine the mechanism underlying this role, which implicates anterograde vesicle transport on microtubules. Finally, we identify KIF16B as the main kinesin mediating this transport. Collectively, our results suggest a scenario (Figure 7) in which BM-DC Rab14⁺Stx6⁺IRAP⁺ vesicles are recruited to internalized material early after receptor-mediated endocytosis or phagocytosis. Innate immune signaling downstream of Fc receptors and TLR4 phosphorylates and inactivates Tbc1d4, thus activating Rab14, which binds KIF16B upon GTP binding. KIF16B/Rab14 complexes then mediate dominant anterograde transport of endosomes and/or phagosomes, thereby delaying fusion with lysosomes and antigen degradation and promoting cross-presentation.

The favorable role of an “early endosome” environment in cross-presentation is an accepted concept in the field. However, in contrast to the common concept defining this environment as EEA-1⁺ vesicles, we show that such a favorable environment depends on a subtype of early endosomes defined by Rab14⁺IRAP⁺Stx6⁺. Compromising this subpopulation does not affect EEA-1⁺MR⁺ early endosomes but is sufficient to alter endosome maturation and cross-presentation. Our findings emphasize the dynamic nature of these vesicles that must be maintained by active microtubule transport, which in turn is regulated by innate immune signaling. The standard time span for early to late endosome maturation is estimated at 8–15 min (Huo-tari and Helenius, 2011), a duration likely insufficient for antigen degradation, transport, and loading on MHC class I molecules. Therefore, trafficking pathways delaying early endosome maturation and/or sorting early endosome cargo to slowly maturing vesicles are a requirement for efficient cross-presentation.

Knockdown of Rab14 resulted in strongly reduced abundance of Stx6⁺IRAP⁺ endosomes. This is likely due to rapid retrograde transport of these endosomes. The directional outcome of microtubule transport depends on the balance of recruitment of effectors mediating anterograde and retrograde transport; effectors mediating transport in the two directions may be recruited by distinct GTPases or by the same GTPase, as is the case for Rab7 (Wijdeven et al., 2015). Analysis of IRAP KO cells allowed us to directly demonstrate that “disappearance” of Stx6⁺ endosomes was due to dynein-mediated retrograde microtubule transport. Given that the effect of IRAP KO was indistinguishable from that of *Rab14* knockdown with respect to the abundance of Stx6⁺ endosomes and to phagosome maturation, and that *Rab14* knockdown had no effect on phagosome maturation in IRAP KO cells, it is reasonable to assume that the dearth of Stx6⁺ endosomes observed upon *Rab14* knockdown was due to the same mechanism. This notwithstanding, the available evidence indicates that the cell biological effects through which IRAP KO and *Rab14* knockdown confer unopposed dominance to the retrograde transport of Stx6⁺ endosomes are different: while IRAP KO removes static anchoring of the vesicles to the actin cytoskeleton through a formin, *Rab14* knockdown removes dynamic anterograde transport. As previously reported by us, IRAP also has an additional function as trimming peptidase (Saveanu et al., 2009). Our unpublished data suggest that this function is not dispensable, because reconstitution of IRAP KO BM-DCs with protease-dead IRAP reconstitutes Stx6⁺ endosomes but not cross-presentation fully.

Our observations led us to conclude that the effect of Rab14 on phagosome maturation and cross-presentation is due to its ability to recruit KIF16B. Studying how *Salmonella enterica* modulates vacuole maturation in a Rab14-dependent manner to promote its survival, Kuijl et al. (2013) identified its interaction with the dual effector Nischarin that directs maturation to Rab9⁺ as opposed to Rab7⁺ late endosomes. However, we did not observe any effect of Rab9 knockdown on cross-presentation (S. Montealegre and P.v.E., unpublished data), suggesting that the pathway proposed by these authors modulates pathogen survival, but not cross-presentation. We could obtain indirect but strong evidence for Rab14-KIF16B proximity at endosomes,

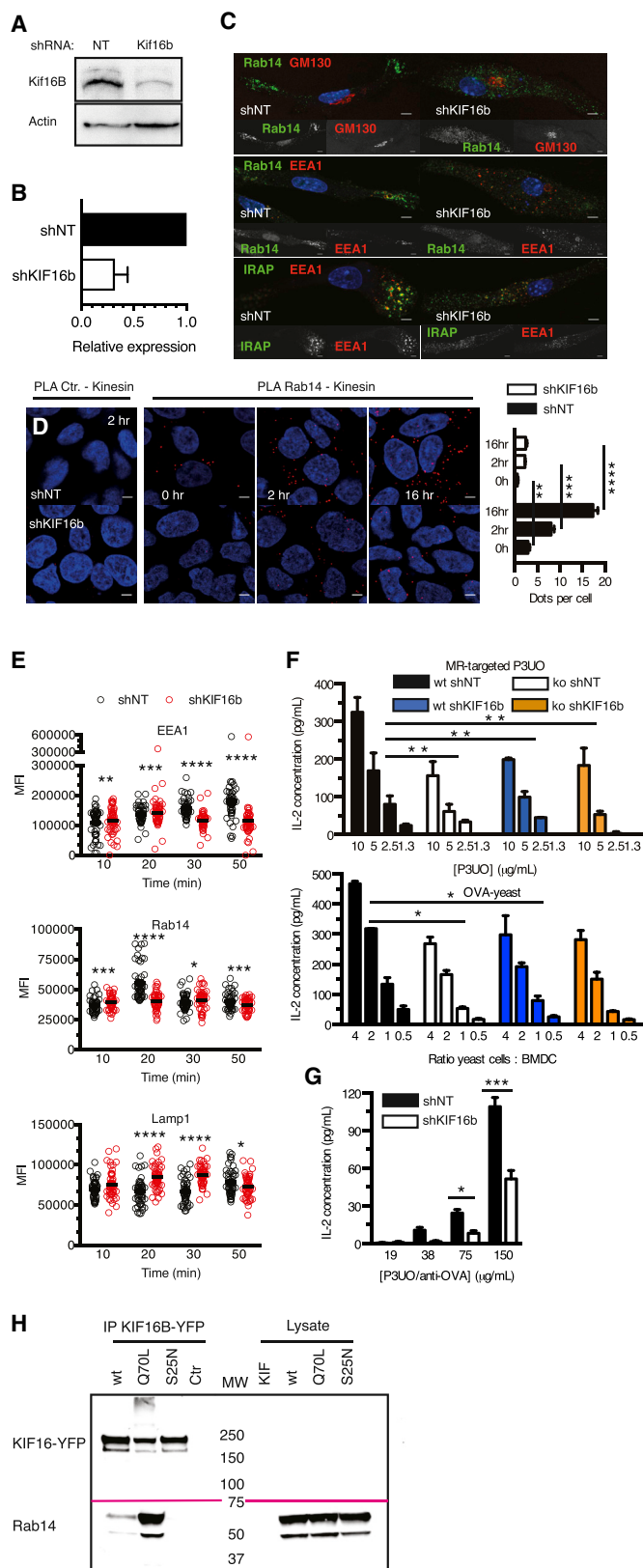


Figure 6. Rab14-KIF16B Interaction Delays Phagosome Maturation and Promotes Cross-Presentation

(A) Immunoblot showing expression of KIF16B by HEK293 cells expressing CD32a transduced with control or KIF16B-specific shRNA.

(B) KIF16B mRNA expression in BM-DCs transduced with specific shRNA, as percentage of expression in control non-targeting (shNT)-transduced cells.

(C) Expression and co-localization of indicated markers in BM-DCs transduced with non-targeting or KIF16B-specific shRNA.

(D) PLA analysis of Rab14/pan-kinesin proximity in CD32a-expressing HEK293 cells transduced with control or KIF16B-specific shRNA and stimulated with OVA-anti-OVA immune complexes for the indicated time periods. Quantitative evaluation of ≥ 30 cells per condition on the right.

(E) Imaging flow cytometry analysis of EEA-1, Rab14, and Lamp1 recruitment to phagosomes by shNT or shKIF16b-transduced BM-DCs. Representation as in Figure 3G.

(F) Cross-presentation of MR-targeted OVA and OVA-expressing yeast cells by BM-DCs transduced with control or KIF16b-targeting shRNA was assessed using OT-I T cells.

(G) Cross-presentation of OVA-anti-OVA immune complexes by HEK293 cells transduced with control or KIF16B-specific shRNA.

(H) *In vitro* interaction of KIF16B-YFP with WT, GTP-locked (Q70L), and GDP-locked (S25N) Rab14-mCherry. Rab14 proteins were immunoprecipitated by incubating lysates from single-transfected HEK cells (right-hand lanes) with KIF16B-YFP trapped on anti-GFP beads. The control (Ctr) represents the background of GTP-locked Rab14-mCherry binding to GFP-trap beads.

All experiments were performed at least twice. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Scale bar, 5 μ m. Graphs show means \pm SEM.

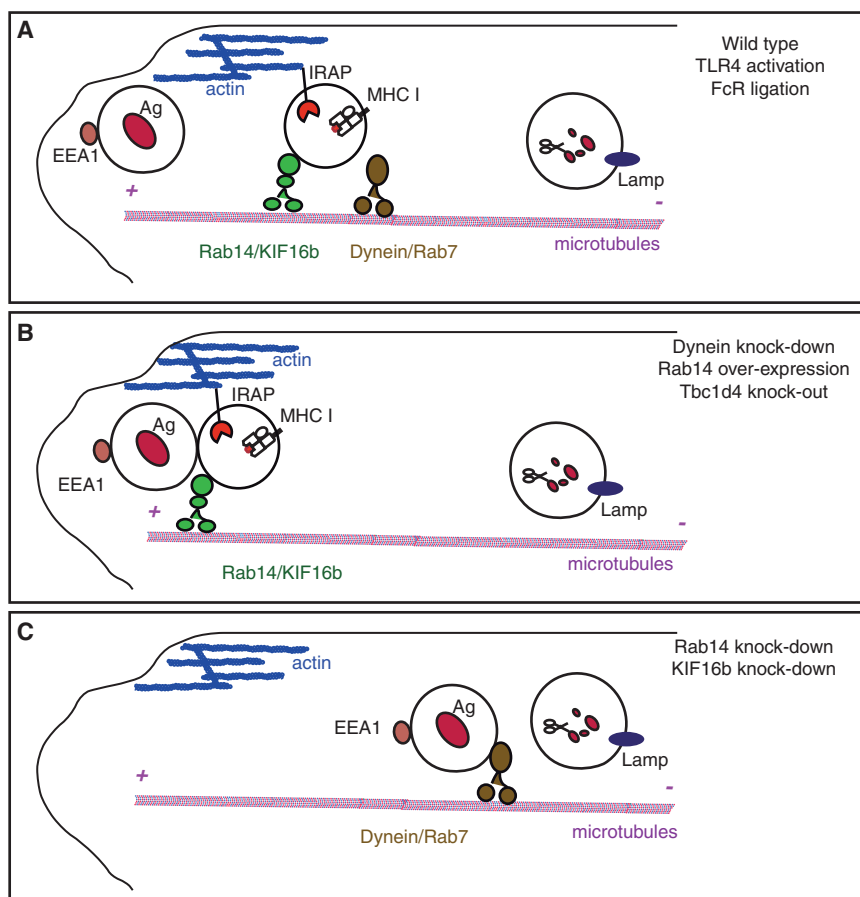


Figure 7. Role of Rab14 in Endosome Transport and Antigen Cross-Presentation

(A) In WT DCs, TLR4 or FcR activation triggers Rab14 interaction with KIF16B, thereby mediating the anterograde transport of endosomes along microtubules and their fusion with early phagosomes. Phagosomal recruitment of Rab14 delays phagosome maturation and pathogen killing. IRAP in Rab14 endosomes trims peptides to the correct length for MHC class I binding and also contributes to stabilization of early phagosomes by interaction with the actin remodeling protein, FHOD4.

(B) Overexpression of WT Rab14, inhibition of retrograde transport by dynein, knockdown, or shifting Rab14 to the GTP-bound form by deletion of Tbc1d4 enhance the anterograde transport of endosomes and upregulate cross-presentation.

(C) Conversely, the depletion of Rab14 or KIF16B shifts the equilibrium to retrograde transport of endosomes, accelerates the phagosome fusion with the late endosomes, and compromises antigen cross-presentation.

and direct evidence for a requirement of KIF16B for normal phagosome maturation and cross-presentation. We cannot rule out that KIF16B is required for early endosome positioning to the cellular periphery, facilitating Rab14 recruitment to phagosomes, which alone might promote cross-presentation. However, endosome and phagosome maturation are known to undergo similar events including microtubule transport (Flannagan et al., 2012). Therefore, the Rab14-KIF16B interaction is likely to be involved also in phagosome maturation.

Recruitment of KIF16B to endosomes was enhanced by signaling through Fc receptors and TLR4, both known to enhance cross-presentation (Joffe et al., 2012). Although studying the signaling pathways involved would have overstepped the context of this report, some reasonable assumptions can be made in view of the literature. Both TLR4 and Fc receptors can signal through the phosphoinositide 3-kinase (PI3K)/Akt pathway (Nair et al., 2011). In adipocytes and muscle cells, Tbc1d4, also known as Akt substrate of 160 kDa (AS160), is phosphorylated and thereby inactivated upon signaling by the insulin receptor through the PI3K/Akt pathway (Sadacca et al., 2013). Inactivation of the Tbc1d4 GAP in turn activates its substrates, among them Rab14. It is easily conceivable that signaling downstream of PI3K/Akt follows a similar pathway in BM-DCs. In support of this, Tbc1d4 KO not only enhanced cross-presentation suggestive of a positive effect on Rab14

function, but also resulted in a partial change of intracellular Rab14 localization identical to that characterizing the constitutively active Rab14 mutant Q70L, strongly suggesting that Tbc1d4 supports the Rab14 active cycle. We found enhanced cross-presentation *ex vivo* by Tbc1d4-deficient monocyte-derived DCs, the DC type resembling BM-DCs most. Public RNA-sequencing (RNA-seq) and microarray data (<https://www.immgen.org>) document limited expression of Tbc1d4 with high expression levels in cDC1 and cDC2, suggesting a broader impact of this GAP in cross-presentation *in vivo*.

The Q70L mutant and the GAP KO shared a partial endosomal co-localization with Lamp1 not observed for endogenous or overexpressed WT Rab14. This was at first sight surprising given that in phagosome maturation, Lamp1 acquisition was inversely correlated with cross-presentation efficacy. However, the presence of Lamp1 in a vesicle may not always indicate its nature as a late endosome or lysosome. Studying cells overexpressing Rab14Q70L by electron microscopy, Junutula et al. (2004) proposed that the mutant may localize to tubulo-vesicular transport intermediates between the Golgi and endosomes. Electron or super-resolution microscopy will have to reveal whether Rab14Q70L localizes to similar vesicles in DCs. Another interesting finding in this study is the discrepancy between the effects of the Q70L mutant and the *Tbc1d4* KO on cross-presentation that contrasts with their similar effects on steady-state endosomal distribution of Rab14. This observation must indicate that the cycle of GTP hydrolysis and re-binding by Rab14 is not relevant for endosomal Rab14 localization at steady state but essential for the role of Rab14 in cross-presentation.

In summary, we have demonstrated the important role of innate receptor-triggered anterograde endosome transport

mediated by the Rab14-KIF16B interaction in cross-presentation. This mechanism complements previously reported mechanisms, such as central lysosome clustering and phagosomal pH control, and ensures prolonged maintenance of a vesicular environment preventing premature antigen destruction. Whether the mechanism described here also promotes other key steps in cross-presentation such as antigen export to the cytosol, and how it is related to other parameters such as MHC class I trafficking and recycling, will be interesting questions for future studies.

EXPERIMENTAL PROCEDURES

Mice

Previously described IRAP KO (Keller et al., 2002) and RAG-deficient OT-I mice were bred in our animal facility. Mice lacking *Tbc1d1/d4* were also described previously (Chadt et al., 2015). The experiments were conducted with male or female mice, 8–12 weeks old, weighing 20–25 g, and housed under specific pathogen-free conditions in our animal facilities at 22°C ± 2°C with a 12-hr light-dark cycle, with access to food and water *ad libitum*. All animal care and experimental procedures were performed in accordance with the guidelines and regulations of the French Veterinary Department and approved by the Comité d'Éthique pour l'Expérimentation Animale Paris Descartes (n° P2.LS.156.10).

Cells

BM-DCs were obtained by culturing bone marrow cells in GM-CSF-containing supernatant from J558 cells (Weimershaus and van Endert, 2013). HEK293-FcR cells were a gift from P. Cresswell (Giodini et al., 2009), and the B3Z hybridoma a gift of N. Shastri.

Fluorescence Microscopy

For analysis of the steady state, the cells were plated on fibronectin-coated coverslips or on micro-pattern slides. For phagocytosis monitoring, cells seeded in IbiTreat channels (BioValley) were pulsed with latex beads (Polysciences) or yeast expressing OVA (Saveanu and van Endert, 2013). Images were acquired with Leica SP8 or Zeiss LSM800 microscopes, equipped with 40× and 63× oil immersion objectives, respectively.

Image Analysis

Marker co-localization and signal intensity were evaluated using the IMARIScoloc module for cells polarized on micro-patterns and with ImageJ for other images. For each image, a stack of at least 10 planes was acquired.

Cross-Presentation Assays

BM-DCs on day 7 were pulsed with preformed complexes of MR antibodies with P3UO fusion protein (Kratzer et al., 2010) or yeast OVA, before addition of OT-I cells for 20 hr. Where mentioned, cells were nucleofected 2 days before the assay with Rab14-GFP plasmids and sorted as CD11c⁺CD11b⁺GFP⁺ cells or transduced with lentiviruses. HEK293 cells were incubated with complexes of polyclonal mouse IgG and P3UO, optionally after transfection or lentiviral transduction 2 days prior to the assay, and fixed before addition of B3Z cells. T cell stimulation was read out by interleukin-2 (IL-2) sandwich ELISA. *Ex vivo* cross-presentation by monocyte-derived DCs primed with OVA and LPS *in vivo* was analyzed adopting a published protocol (Cheong et al., 2010).

Lentivirus Production and Transduction

Viruses were produced using standard protocols (Tiscornia et al., 2006) and pLKO.1 plasmids obtained from Open Biosystems, plasmids packaging pCMVDelta8.2, and envelope pMD2G and HEK293 cells. Infection of BM-DCs and HEK cells was performed 4 days before assays (day 3 for BM-DCs) with an MOI of 10, followed by puromycin selection.

Proximity Ligation Assays

Duolink (OLINK Biosciences) assays were performed following entirely the manufacturer's instructions.

Phagosome Maturation Assay

Phagosome maturation was evaluated by imaging flow cytometry adopting a published protocol (Johansson et al., 2015), with some modifications detailed in the Supplemental Information. Images were acquired on an ImageStreamX MkII machine (Amnis).

Co-precipitation and Immunoblots

Co-precipitation of IRAP and Tbc1d4, and of Rab14-GFP with kinesin, was performed using standard protocols and 1% Triton X-100. To analyze KIF16B interaction with Rab14 variants, HEK293 cells were separately transfected with KIF16B-YFP and Rab14-mCherry. Three days later, CHAPS lysates of KIF16B-YFP containing cells were incubated with GFP-trap beads (Chromotek), followed by addition of lysates containing Rab14-mCherry. Eluates in sample buffer were analyzed by immunoblots. All immunoblots were performed using transfer in 10 mM N-cyclohexyl-3-aminopropanesulfonic acid (CAPS) (pH 11) buffer to Immobilon membranes (Millipore). Proteins were visualized using an enhanced chemiluminescence (ECL) system or the Odyssey LI-COR imaging system and appropriate secondary antibodies.

Statistical Analysis

Values are expressed as mean ± SEM, unless otherwise specified. Statistical significance between two groups was analyzed using the unpaired Student's *t* test, or the Mann-Whitney U test in image analysis experiments, whereas the two-way ANOVA test with a Bonferroni post-test adjusted for multiple comparisons was used in *in vitro* and *ex vivo* experiments evaluating antigen presentation and phagosome maturation by imaging flow cytometry. *p* values are indicated as: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001. GraphPad Prism version 6.0 was used to perform the statistical analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.08.041>.

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AUTHOR CONTRIBUTIONS

L.S., M.W., and F.-X.M. designed, performed, and interpreted experiments. C.A., J.B., A.A., S.M., M.L., K.J.H., M.D., and I.E. performed experiments. P.V. and A.M.L.-D. provided micro-pattern slides. A.C. and H.A.-H. provided live Tbc1 KO mice and BM-DCs. M.Z. and T.B. helped with lentivirus production. P.v.E. designed and interpreted research and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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