**Immunology:**

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Tubulation of endosomal structures in human dendritic cells by Toll-like receptor ligation and lymphocyte contact accompanies antigen cross-presentation.

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Short title: TLR/T cell-mediated human DC endosomal remodeling

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Key words: Human, Dendritic cells, Intracellular trafficking, Recycling, Endosomes, T-cell receptor, Toll-like receptors (TLR), Tubulation, Cell-cell interaction, Live cell confocal microscopy.

Background: Mouse dendritic cells can transform their vesicular late-endosomes into long tubules, essential for MHC Class-II transport.

Results: In human dendritic cells, late-endosome tubules are induced by TLR-ligation, and for recycling-endosomes additional T-cell-mediated ICAM-1 and MHC Class-I ligation. Tubulation accompanies antigen cross-presentation. Tubulation accompanies antigen cross-presentation.

Conclusion: Human dendritic cells transform endosomal compartments upon distinct triggers.

Significance: Induced endosomal tubules regulate human dendritic-cell functioning.

ABSTRACT

Mouse dendritic cells (DCs) can rapidly extend their Class II MHC-positive late endosomal compartments into tubular structures, induced by Toll-like receptor (TLR) triggering. Within antigen-presenting DCs, tubular endosomes polarize towards antigen-specific CD4⁺ T cells, which are considered beneficial for their activation. We here describe that also in human DCs, TLR triggering induces tubular late endosomes, labeled by fluorescent LDL. TLR triggering was insufficient for induced tubulation of transferrin (Tfn)-positive endosomal recycling compartments (ERCs) in human monocyte-derived DCs. We studied endosomal remodeling in human DCs in co-cultures of DCs with CD8⁺ T cells. Tubulation of ERCs within human DCs requires antigen-specific CD8⁺ T cell interaction. Tubular remodeling of endosomes occurs within 30 minutes of T cell contact and involves ligation of HLA-A2 and ICAM-1 by T cell-expressed T cell receptor and LFA-1, respectively. Disintegration of microtubules or inhibition of endosomal recycling abolished tubular ERCs, which coincided with reduced antigen-dependent CD8⁺ T cell activation. Based on these data, we propose that remodeling of Tfn-positive ERCs in human DCs involves both innate and T-cell-derived signals.

Endocytosis and recycling of lipids and receptor-bound proteins in the plasma membrane is a highly dynamic and
organized process (1). Engulfed material enters the endosomal pathway, first localizing to early endosomes (EE). From here, most cargo is recycled back to the cell surface via two main recycling pathways that each consists of vesicular and tubular structures. The fast recycling route recycles cargo directly from EE to the plasma membrane, whereas slow recycling occurs via a juxtanuclear-positioned endocytic recycling compartment (ERC) (2). Non-recycled cargo in EEs transits into late endosomes (LE) that eventually fuse with lysosomes where remaining cargo is degraded (3).

Activation of CD4$^+$ and CD8$^+$ T cells requires presentation of peptide/MHC complexes of the appropriate specificity. DCs are the most adept antigen presenting cells. Presentation of extracellular antigens requires antigens to be internalized into endosomes, their processing into peptides, assembly of antigenic peptide/MHC complexes, and transport of these complexes to the cell surface. While loading of exogenous antigen-derived peptides onto Class II MHC molecules occurs in LE, the loading of Class I MHC molecules occurs in the endoplasmic reticulum or, as recent work supports, in endosomal compartments (4-6).

Encounter of antigen-specific CD4$^+$ T cells induces the formation of late endosomal Class II MHC-positive tubules in murine DCs. These intracellular tubules extend approximately to 5-15 µm in length (7-11). The LE tubules transform in a TLR-dependent manner, and rearrange transport of Class II MHC molecules between LEs and the DC surface, presumed to facilitate the ensuing CD4$^+$ T cell response (7:8;10;12). In early studies, next to LE tubules also elongated tubular recycling endosomes (REs) are observed. In HeLa cervical cancer cells, infection by S. typhimurium or overexpression of Eps15 homology domain (EHD) 1 induces formation of these long endosomal tubules (13;14). These RE tubules mediate efficient Class I MHC recycling towards the cell surface (14). For human DCs, morphology of the endosomal pathway and signals that induce rearrangement of endosomal structures during immune activation are not understood. We used live cell confocal microscopy to investigate endosomal remodeling in human DCs stimulated with TLR-ligands and upon cognate interaction with CD8$^+$ T cells. We demonstrate 3 modes of inducing endosomal tubulation, triggered by distinct signals for tubular remodeling of late and recycling endosomes. Live cell confocal microscopy experiments reveal an unexpected role for ICAM-1 and class I MHC molecules in remodeling of the endosomal recycling compartment.

**EXPERIMENTAL PROCEDURE**

*In vitro generation of human monocyte-derived DCs.* Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors after informed consent by centrifugation (2300 rpm, 20 min, RT) on ficoll-paque (GE Healthcare). Monocytes were isolated from PBMCs by centrifugation (2900 rpm, 45 min, RT) on 3 layer isotonic percoll density gradient (from top-to-bottom: 34%, 47.5% and 60%; Sigma Aldrich). Monocytes are stored in freeze medium (10% DMSO (Sigma Aldrich) in heat inactivated-FCS) at -80°C for maximally 8 weeks.

Monocytes were cultured in 8 wells Nunc® Lab-Tek® II chambered coverglass (Thermo Scientific). These were pre-coated with 1% w/v Alcian Blue 8GX (Klinipath) in PBS for 30 min at 37°C. Cells were maintained in a humidified incubator at 37°C with 5% CO$_2$ for 5 days in differentiation medium: RPMI 1640 with 1% v/v PenStrep (Invitrogen), and 1% v/v Gibco® GlutaMAX (Invitrogen), and 10% v/v human AB+ serum (Sanquin) + 500 U/ml GM-CSF and 100 U/ml IL4 (Immunotools).

*Monocyte-derived DC maturation.* MoDC maturation was induced by addition of 200 ng/ml Lipopolysaccharide Ultrapure from E. coli strain 0111:B4 (LPS-EB...
ultrapure, Invitrogen), 5 µg/ml pI:C (Sigma Aldrich) 4 hours prior to microscope analysis. This occurred in presence or absence of viral antigens; 3 µg/ml HCMV-derived pp65 antigen (Miltenyi Biotec) or dialysed recombinant EB2 protein.

Confocal microscopy and imaging analysis. Mature moDCs were washed with RPMI 1640 without phenol red, supplemented with 0.2% v/v Bovine Serum Albumin (BSA) (Roche) and 10 mM HEPES, and subsequently incubated for 30 min at 37°C with 20 µg/ml Dil-conjugated LDL (Biomedical Technologies) and 5 µg/ml Alexa Fluor 647-conjugated transferrin (Invitrogen Molecular Probes). Hereafter, cells were washed twice with RPMI 1640 without phenol red, supplemented with 0.2% BSA and 10 mM HEPES, and used for live cell imaging. Prior to any stimulation, at least ten positions are chosen and locked to be able to track the exact same cells over time. Scoring was done for presence of motile LDL or Tfn-positive tubular structures emanating from the center of moDCs, by two independent observers in a double-blinded manner. The percentage of LDL⁺ tubular moDCs is relative to LDL-loaded moDCs, whereas Tfn⁺ tubular moDCs is determined relative to LDL⁺ tubular moDCs. Live cell imaging was performed on a Zeiss LSM710 confocal microscope equipped with live-cell chamber device to maintain 37°C and 5% CO₂ condition during experiments. Images are obtained with 1.3x optical zoom using “Plan-Apochromat” 63x 1.40 oil DIC M27 objective (Zeiss) and processed using Zen 2009 software (Zeiss Enhanced Navigation).

T cell clone antibody-blocking experiments. HLA-A*0201–restricted, HCMV pp65–specific CD8⁺ T cell clones were prepared as published (15). HLA A2/NLVPMVATV-restricted CD8⁺ T cell clones were freshly thawed and incubated in ice cold PBS at 4°C for 1 hour in presence or absence of 1 µg/ml mouse-anti-human CD11a antibody (anti-LFA-1, HI111, BioLegend) or mouse-anti-human CD127 antibody (purified in house from R34-34 hybridoma).

T cells were washed with ice cold RPMI 1640 without phenol red, supplemented with 0.2% BSA (Roche) and 10 mM HEPES and kept on ice; 5 min prior to incubation with moDCs, T cells were warmed up to 37°C and used for live cell confocal microscopy.

Beads-antibody coating and bead binding assays. Dynabeads® M-450 Epoxy beads (Dynal) were coated with mouse-anti-human CD19-biotin antibody (HIB19, BD Pharmingen), or mouse-anti-human CD54-biotin (ICAM-1) antibody (HA58, eBioscience), or mouse anti-human HLA-A2 (Thermo Scientific Pierce), or combination of both anti-ICAM-1 and anti-HLA-A2 antibodies, according to the manufacturers instructions. For imaging experiments, beads were warmed up to 37°C prior to administration.

Pharmacological inhibition of endosomal remodeling. Human moDCs (day 5) are pulsed for 4 hours with 3 µg pp65 in presence of 200 ng/ml LPS (LPS-EB ultrapure, Invitrogen) and 5 µg/ml pI:C (Sigma Aldrich). Upon staining of ERCs (30 min, 37°C) with 5 µg/ml Alexa Fluor 647-conjugated transferrin (Invitrogen Molecular Probes), vesicle-to-tubule transformation is stimulated by co-culture of antigen-specific (NLV) CD8⁺ T cells for 1 hour. MoDC with tubular ERCs are imaged prior to any stimulation, and 20 to 40 minutes after administration of either 50 μM primaquine biphosphate (Sigma Aldrich), 10 μM nocodazole (Sigma Aldrich), PBS, or DMSO (Sigma Aldrich).

Pharmacological inhibition of antigen cross-presentation. Human moDCs (day 5) are loaded with 3 µg/ml CMV pp65 antigen (Miltenyi Biotec) or NLVPMVATV-peptide (Pepscan) overnight in presence of 200 ng/ml LPS (LPS-EB ultrapure, Invitrogen) and 5 µg/ml pI:C (Sigma Aldrich). Subsequently, moDCs are exposed for 30 minutes to
either primaquine biphosphate (50 µM), or nocodazole (10 µM), or carrier controls PBS, and DMSO, respectively (all Sigma Aldrich). Hereafter, DC cultures are thoroughly washed to remove inhibitors. HLA-A2/NLVPVMVATV-restricted CD8+ T cells are added, and DC/CD8+ T cells are co-cultured for a further 5 hours at 37°C. Activation of CD8+ T cells is measured by induced antigen-driven production of IFNγ and TNF, and surface-expressed LAMP1 by flow cytometry. DC viability was determined by Annexin V and 7-AAD staining (Biosciences). Surface expression was determined by staining DC with fluochrome conjugated anti-HLA-A2, CD80 (both Biosciences), HLA-DR and ICAM-1 (both Biolegend).

**Statistics** Flow cytometry data were collected on FacsCanto II and analyzed with BD FACSDiva v6.1.3 and Flowjo 7.6 software (Treestar). All data were statistically analyzed and plotted with GraphPad Prism® 5 software. P<0.05 was considered statistically significant.

**RESULTS**

*TLR stimulation of human dendritic cells triggers remodeling of late endosomes into tubular structures.* LPS stimulation of murine DCs induces elongated tubular structures emanating from LEs in a time- and dose-dependent manner (7-11). To examine the effect of TLR triggering in LE remodeling of human DCs, we cultured monocyte-derived DCs (moDCs, 5 day culture in presence of IL-4 and GM-CSF(15)), and performed live cell confocal microscopy. To allow visualization of LE, DCs were pulsed with fluorescently labeled DiL-Low Density Lipoprotein (LDL, 30 minutes, 37°C (7)), followed by washes to remove unbound LDL.

We visualized DiL-LDL-pulsed moDCs after addition of TLR4 ligand LPS, or PBS as control in a time-lapse manner (500,000 DCs/coverslip well, LPS 200ng/ml; 0, 20, 60 and 240 minutes, schematically depicted in Figure 1A. Scoring was done for presence of motile LDL-positive tubular structures emanating from the center of moDCs, by two independent observers in a double-blinded manner. LPS treatment induced a rapid and steady increase in long tubular endosomes in the majority of moDCs (55% of DCs at 1 hour; 80% of DCs at 4 hours, Figure 1B and C). PBS-treated DCs never showed tubular endosomes in more than 25% of DCs, a background level that may relate to spontaneous DC maturation (our unpublished data).

Polyinosinic:polycytidylic acid (pI:C)/LPS combined treatment induced a similar level of tubular endosomes as LPS treatment alone, measured after 4 hours. Of note, moDC-expressed tubular endosomes are stable, yet dynamic structures that last at least 6 hours, or the duration of experiments (Figure 1D, individual tubules time-lapse captures, and Figure S1, movie).

*Late endosome tubular remodeling in human dendritic cells occurs independently of cognate T cell interaction.* Cognate DC-T cell interaction induces T cell-polarized tubular endosomes in murine DCs, in a TLR-dependent manner (7;8). We now asked whether in human moDCs, cognate T cell interaction in itself causes tubulation of LE or that TLR triggering is required. Therefore we pulsed 5-day moDCs with human CMV protein pp65 that is cross-presented to HLA-A2/NLVPVMVATV-specific CD8+ T cell clones (15). We then added PBS or TLR ligands LPS and pI:C (200 ng/ml and 5µg/ml, respectively; 4 hours). Next, DCs were pulsed with DiI-LDL for 30 minutes (according to the scheme in Figure 1E), to allow for visualization of LE compartments. DCs were washed, T cells were added (1:1 DC-T cell ratio) and DCs were assayed for development of tubular endosomes by time-lapse confocal microscopy (0, 20, 60 minutes after T cell addition). Cognate DC-CD8+ T cell interaction in the absence of LPS/pI:C could not significantly induce...
LE tubular remodeling (Figure 1F), indicating the crucial role of TLR ligation in LE tubulation. In contrast, we observed tubular LDL\(^+\) compartments in 60-70% of antigen-laden LPS/pl:C-treated DCs. The fraction of antigen-laden DCs expressing tubular LDL\(^+\) endosomes did not significantly increase further upon addition of antigen-specific CD8\(^+\) T cells (Figure 1G). In conclusion, TLR-induced formation of tubular endosomes is conserved between murine and human DCs. We found no effect of additional antigen-specific CD8\(^+\) T cell contact on LE remodeling as in contrast to addition of antigen-specific CD4\(^+\) T cells in murine DCs (8).

Efficient tubular remodeling of recycling endosome in human dendritic cells requires cognate T cell interaction. Does antigen-specific T cell contact perhaps trigger tubular remodeling of other endosomal compartments in human DCs? To address this question, we visualized within moDCs the juxtanuclear located ERC, which is characterized by presence of Tfn receptors (16). Endosomal recycling can occur via tubular recycling endosomes extending intracellular, as shown in EHD 1 over-expressing system within HeLa cells. These elongated recycling endosomes facilitate efficient recycling of both Tfn and Class I MHC molecules (118).

We visualized ERC in moDCs by incorporation of fluorescent Tfn (according to scheme shown in Figure 1A). Prior to stimulation, most moDCs have a vesicular Tfn\(^+\) ERC (Figures 2A). In contrast to LE compartments, the addition of LPS or a combination of LPS and pl:C in absence (Figure 2B) or presence of viral antigens pp65 (Figure 2D) or EB2 (Figure 2E) to moDCs did not induce significant vesicular-to-tubular transformation of the ERC. When pp65-specific CD8\(^+\) T cells were added to pp65-laden LPS/pl:C-treated moDCs, tubular transformation of Tfn\(^+\) compartments ensued in 60% of LDL-tubular moDCs (Figure 2C and 2D). Similar results are obtained by using EB2-specific CD8\(^+\) T cell clone and EB2-laden LPS/pl:C treated moDCs (Figure 2E). Incubating EB2-laden LPS/pl:C treated moDCs with pp65-specific CD8\(^+\) T cells, avoiding cognate DC-T cell interaction, showed significantly reduced induction of tubular Tfn\(^+\) endosomes compared to Tfn\(^+\) tubulating pp65-laden DCs (Figure 2C and 2D, 60 minutes after addition of pp65-specific CD8\(^+\) T cells).

In conclusion, TLR signaling alone is not sufficient to drive remodeling of Tfn\(^+\) ERC. Instead, functional cognate DC-CD8\(^+\) T cell interaction induces remodeling into elongated Tfn\(^+\) tubular structures. This remodeling requires TLR stimulation, as tubular transformation did not occur when pp65 antigen-specific CD8\(^+\) T cells were cultured with pp65-laden moDCs in absence of TLR stimulation (Figure 2G). The TLR-dependent remodeling of Tfn\(^+\) compartments in moDCs upon cognate interaction of antigen-specific CD8\(^+\) T cells is rapid and dynamic (Figure 2F and Figure S2: single cell images and movie of Tfn\(^+\) compartments).

ICAM-1 clustering provokes tubulation of Tfn\(^+\) endosomal recycling compartments in human dendritic cells. Efficiency of tubular remodeling of Tfn\(^+\) ERC is higher upon cognate moDC/CD8\(^+\) T cell interaction compared to antigen independent DC-CD8\(^+\) T cell interaction. We hypothesized that interaction of DC-expressed intercellular adhesion molecule-1 (ICAM-1) with lymphocyte function-associated antigen 1 (LFA-1) on interacting T cells facilitates tubulation of Tfn\(^+\) ERC in human moDCs for the following reasons. First, TLR4 ligation stimulates surface expression of ICAM-1 within a few hours (our unpublished data). Second, when antigen-bearing DCs enter the lymph node and are scanned by T cells, the initial DC-T cell interaction is antigen-independent and involves association of LFA-1 with ICAM-1 (17). And third, upon recognition of peptide MHC complexes by antigen specific T cell receptors (TCRs),
TCR signaling drives LFA-1 in a state that binds with increased affinity to ICAM-1 (18). Of additional consideration was that ICAM-1 clustering facilitates antigen presentation by recruiting HLA-A2 to the T cell contact zone (19) and that blocking of LFA-1 on antigen-specific CD4+ T cells hampers tubular remodeling of LE in murine DCs (20).

To productively engage ICAM-1, we coated beads with stimulating antibodies (Ab) against ICAM-1.(19) As negative control we used beads coated with isotype-identical Ab specific for CD19, which is not expressed on moDCs. We performed the live-cell confocal imaging experiments using Ab-coated beads similar as earlier imaging experiments (Figure 3A). Addition of anti-ICAM-1 mAb coated beads to these moDCs induced tubular transformation of Tfns+ juxta-nuclear positioned endosomes within 30 minutes, reaching 60-70% of moDCs showing tubular recycling endosomes at 60 minutes. Addition of anti-CD19 mAb coated beads did not induce remodeling of Tfns+ compartments (Figure 3B and 3C). Similar data was obtained using anti-CD45 beads (our unpublished data).

To confirm whether absence of ICAM-1/LFA-1 interaction counteracts CD8+ T cell-induced tubular remodeling of ERCs, we pre-incubated CD8+ T cells with anti-LFA-1 (anti-CD11a) blocking Ab and added these to antigen-laden moDCs. CD127 (IL-7 receptor alpha) molecules are not involved with DC-T cell interaction, and were therefore blocked on CD8+ T cells as a control. We found that CD8+ T cell pre-treatment with anti-LFA1 mAb counteracted the induction of tubular Tfns+ ERCs in interacting moDCs, whereas pre-treatment with anti-CD127 did not (Figure 3D and 3E). Thus, ICAM-1/LFA-1 interaction between moDCs and interacting CD8+ T cells instigates ERC remodeling into elongated tubular structures in moDCs.

Class I MHC clustering, or simultaneous ICAM-1/Class I MHC clustering, provokes tubulation of Tfns+ endosomal recycling compartments in human dendritic cells. ICAM-1/LFA-1 binding induces moderate levels of tubular transformation of Tfns+ ERC in moDCs in comparison to cognate DC-CD8+ T cell. During cognate DC-CD8+ T cell interaction, both peptide/Class I MHC binding to the antigen-specific TCR and binding of ICAM-1 to LFA-1 occurs in parallel. We next asked whether ligation of MHC complexes, or co-ligation together with ICAM-1, is responsible for more tubular endosomal transformations in moDCs. To address this question, we exposed moDCs to anti-Class I MHC (anti-HLA-A2) mAb coated beads or beads coated with both anti-HLA-A2 and anti-ICAM-1 (Figure 4A). After 60 minutes of bead binding, up to 60-70% of LE remodeled human DCs showed tubular recycling endosomes (Fig 4B and C). Thus, both anti-ICAM-1 mAb-coated and double-coated (anti-A2/anti-ICAM-1 mAb) beads efficiently induced tubular remodeling of Tfns+ ERC.

In conclusion, sufficient cross-linking of HLA-A2 and/or ICAM-1 molecules on the DC surface by mAb-coated beads or antigen-specific T cells drives Tfns+ endosomal tubular remodeling.

Elongated recycling endosomal tubules require an intact microtubule cytoskeleton and unperturbed endosomal recycling in human dendritic cells. In murine DCs, tubulation of LE compartments requires the support of an intact microtubule-driven cytoskeleton (7). The cellular requirements for ERC remodeling are unknown. Therefore, we tested whether recycling from the endosomal pathway to the DC surface is necessary, and whether an intact microtubule cytoskeleton is required. We made use of the reversible inhibitors primaquine (50 µM) or nocodazole (10 µM) (Figure 5A-C) (11;15). We used these inhibitors as published in DCs (21;22). We induced tubulation of Tfns+ ERCs by 1-hour culture of antigen/LPS/pI:C-stimulated
moDCs with antigen-specific CD8+ T cells (Figure 5, A: schematic outline of the experiment; B: confocal image). Thirty minutes of primaquine or nocodazole treatment results in a significant reduction in moDCs with tubular Tfn+ endosomes to 8 and 17%, respectively (Figure 5B and C). Thus, tubular Tfn+ recycling endosomes require intact microtubules and continuous endosomal recycling.

Abolishment of tubular ERC compartments in human dendritic cells associates with reduced ability to activate CD8+ T cells. Does tubular transformation of ERCs have a functional consequence to antigen presentation by DCs? Molecules that selectively support tubular endosomes are not yet described, precluding a knockdown-based approach. We therefore used pharmacological reagents primaquine and nocodazole to address this question, as in figure 5A, B and C. We recently established a human DC-based cross-presentation model (15), which we adapted to compare antigen-specific CD8+ T cell activation by moDCs that are either able or temporarily unable to express tubular ERCs, by thirty minute-treatment of DCs with primaquine (50 µM) or nocodazole (10 µM) just prior to administration of T cells (schematically depicted in Figure 5D). Using this approach, advert effects of the drugs on moDC-mediated antigen uptake or processing, as well as direct effects on CD8+ T cells, were prevented (11;15). Antigen-specific CD8+ T cells were next added to untreated, primaquine or nocodazole-treated moDCs (5 hour culture, 37°C). Both reversible primaquine and nocodazole thirty minute-treatment significantly reduced antigen-specific CD8+ T cell activation, as measured by decreased IFNγ production (Figure 5F, 27% and 22% reduction, respectively). Concomitantly, TNF production and surface expressed LAMP1 on CD8+ T cells were reduced as well (Figure 5E and 5F). Both reversible primaquine and nocodazole thirty minute-treatment did not significantly affect presentation of pre-processed pp65-derived NLV-peptide (Figure 5G and 5H), DC surface expression of HLA-A2, ICAM-1, and CD80 (Figure 5I), and DC viability (Figure 5J). Because both inhibitors were added after antigen uptake and overnight antigen processing, possible effects on antigen uptake and processing are excluded. All together, this shows that primaquine and nocodazole affect endosomal tubulation, but does not interfere with other processes that are pivotal to antigen-dependent CD8+ T cell activation.

Taken together, abolishment of the tubular structure of ERC in human DCs associates with reduced ability of DCs to activate antigen-specific CD8+ T cells.

DISCUSSION

Various environmental cues, including TLR ligands, induce DC maturation. During maturation the DCs rapidly transform from endocytic cells that survey their immediate surroundings, into cells dedicated to antigen presentation. As the processing of antigen and assembly of peptide-loaded MHC complexes occurs at intracellular locations, transport of peptide/MHC complexes to the DC surface is critical for display to T cells. We here show that TLR triggering-induced maturation rapidly drives vesicle-to-tubule transformation of late endosomal compartments in human DCs. This corroborates earlier studies performed on murine DCs (8;10). TLR signaling does not suffice to drive Tfn+ recycling endosomal tubulation, which suggests that induction of endosomal tubulation does not necessarily direct DCs towards maturation. However, as antigen-specific CD8+ T cells only induced tubulation of recycling endosomes in presence of LPS and polyI:C, we believe that DC maturation is a prerequisite for endosomal tubulation. Whether there is selection of endosomal tubulation in response to distinct TLR stimuli, as was proposed for phagosome maturation, is yet unknown (23).
It is reported that in absence of TLR stimuli, T cells cannot stimulate late endosomal tubulation in both human and mice (8). In contrast to murine DCs, addition of T cells in presence of TLR ligand does not further stimulate late endosomal tubulation. Whether this is due to usage of human CD8\(^+\) T cells instead murine CD4\(^+\) T cells is not known.

We confirmed the necessity of ICAM-1/LFA-1 and HLA-A2/TCR interactions in remodeling of Early/Recycling endosomal compartments in DCs that bind T cells, by use of antibody-coated beads as surrogate T cells as well as with blocking experiments. Of note, in our bead experiments, we found equal efficiency at inducing tubular remodeling of Tfn\(^+\) endosomal compartments using HLA-A2 mAb-, ICAM-1 mAb-, or double mAb-coated beads (Figure 3C, 4B and 4C). Whether this finding is relevant to DCs that interact with T cells or only true to those that interact with mAb-coated beads, we could not fully address. However, since DC-T cell contact induces the rearrangement of HLA-A2 and ICAM-1 into immune synapse-like structures on the DC surface (24), we consider it unlikely that singular HLA-A2 clustering drives ERC tubular remodeling. In our bead assays, supra-physiological cross-linking of either HLA-A2 or ICAM-1 molecules may already facilitate immune synapse-like structures. Indeed, in live cells, HLA-A2 and ICAM-1 have increased association with each other upon cross-linking of either ICAM-1 or HLA-A2 (19).

We are not the first to relate Tfn\(^+\) compartments in DCs to Class I MHC-mediated stimulation of antigen-specific CD8\(^+\) T cells. It had been known that peptide-receptive Class I MHC molecules are present in endosomes (25). Moreover, Class I MHC molecules are present in primaquine sensitive or Tfn\(^+\) compartments (26;27). In murine DCs, soluble antigen derived peptide loading onto Class I MHC molecules occurs within Tfn\(^+\) endosomes in an LPS-dependent manner (5). Murine DCs that lack Class I MHC molecules in recycling endosomes due to an aberrant tyrosine-based internalization motif, were shown to be defective in cross-presentation (28). Finally, tubular recycling endosomes can mediate efficient Class I MHC recycling in HeLa cells, and HLA-A and ICAM-1 signaling is essential in viral antigen presentation to CD8\(^+\) T cells (14;19). All together, data by us and others provide experimental support that in human DCs tubular transformation of Tfn\(^+\) ERCs modulates the recycling peptide/Class I MHC complexes, and their display to antigen-specific CD8\(^+\) T cells.

Recently, it was shown that infection of HeLa or RAW cells by \textit{S. typhimurium} promote LE tubulation in these cells to increase cell-to-cell transfer of Salmonella (13). These data show that endosomal tubular transformation is not restricted to DCs. In addition, it raises the possibility that pathogens may exploit interference of endosomal tubulation to inhibit surface-directed transport of peptide/MHC complexes. Discovery of pathogen-derived molecules that selectively inhibit endosomal tubulation would be beneficial to determine molecular mechanisms involved in endosomal remodeling.

The stimulation and clonal expansion of CD8\(^+\) T cells by antigen presenting DCs requires the sequential interaction of an estimated 200 TCR molecules with antigen-specific peptide/Class I MHC complexes (29). As DC maturation does not drastically increase surface expression of Class I MHC molecules (30-32) selective recruitment of specific peptide/MHC complexes towards the DC-T cell contact zone must occur. We believe such recruitment is supported by endosomal tubules that polarize towards the cell surface. We show that the induced transformation of tubular ERC structures occurs efficient only when (a) sufficient clustering of HLA-A2 and/or ICAM-1 occurs at the DC surface and (b) TLR
stimulation is provided. The requirement for innate stimulation through for example TLRs restricts remodeling to “dangerous” antigens and not endogenous self-peptides. The requirement of TLR triggering prior to CD8\(^+\) T cell activation also ensures that DCs are optimally primed for antigen presentation. Our findings collectively support a two signal-model in which the DC through tubular endosome transformation facilitates selective clonal CD8\(^+\) T cell expansion. Only antigen-specific CD8\(^+\) T cells induce sufficient ICAM-1 and HLA-A2 clustering that allow for the tubular transformation of Tfn\(^+\) ERC in DCs. Accordingly, only a sufficiently high qualitative signal, triggering of the high affinity TCR would rally the quantitative response (peptide/MHC I complexes) that is required for full CD8\(^+\) T cell activation.

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FOOTNOTES

Contribution: E.B.C., T.W.H.F., and M.B. conceived the project and designed the experiments. E.B.C., T.W.H.F., and M.E.H. performed experiments. L.B. provided essential reagents. E.B.C, T.W.H.F., and M.B. wrote the manuscript. All authors commented on and
approved the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests.

FIGURE LEGENDS

FIGURE 1. TLR stimulation of human dendritic cells triggers remodeling of Late Endosomes into tubular structures.
(A) Schematic outline of live cell confocal microscopy experiment in B and C. Fluorescent cargo LDL-Dil and Transferrin-Alexa647 are staining after 30 minutes incubation the late and recycling endosomes, respectively. Just before or after stimulation with LPS or PBS. (B) Confocal image of moDCs with vesicular (left) or tubular (right) LDL⁺ endosomes (60 minutes 37°C, 200 ng/ml LPS). (C) Percentage of moDCs expressing tubular LDL⁺ endosomes. Time points indicate a 20-minute time window immediately before treatment and around indicated time points (20, 60 minutes); PBS (white bars), 200 ng/ml LPS (black bars), or mix of 200 ng/ml LPS and 5 µg/ml pI:C (grey bars). Data represent mean ± SEM of 4 independent experiments. (D) Time-lapse captures of tubular LDL⁺ endosomes in LPS-treated moDCs (time points indicate seconds). (E) Schematic outline of live cell confocal microscopy experiment in F and G. (F and G) Percentage of moDCs expressing tubular LDL⁺ endosomes after culture in the presence of pp65 (3 µg antigen, 4 hour time point) and pp65-specific CD8⁺ T cells (1:1 ratio) in absence (F) or presence of LPS and pI:C combined (G). Data represent mean ± SEM of at least 3 independent experiments. Two-tailed Mann-Whitney U test. *P<0.05, **P<0.01. bar 5µm.

FIGURE 2. Efficient Recycling Endosome tubular remodeling in human dendritic cells requires cognate T cell interaction.
(A) Representative images of moDCs with LDL⁺ late endosomes (red), and Tfn⁺ recycling endosomes (green) in absence or presence of LPS stimulation (60 minutes, 200 ng/ml). (B) Percentage of LDL⁺ tubular moDCs expressing tubular Tfn⁺ endosomes prior to stimulation (t=0) or around indicated time points. 200 ng/ml LPS (black bars), or mix of 200ng/ml LPS and 5 µg/ml pI:C (grey bars). Data represent mean ± SEM of at least 3 independent experiments. (C). Representative images of moDCs upon CD8⁺ T cell contact in antigen-independent (upper images) or antigen-dependent manner (lower images); LDL (red), Tfn (green), colocalization LDL and Tfn (yellow), pp65-specific CD8⁺ T cell (“T”). (D) Percentage of LDL⁺ tubular moDCs expressing tubular Tfn⁺ endosomes after 4 hour culture in the presence of 3 µg HCMV-derived pp65/LPS/pI:C (black circles) or EBV-derived EB2/LPS/pI:C (grey triangles), at indicated time points. Two-tailed, Mann-Whitney U test P<0.01. Data represent mean ± SEM of at least 4 independent experiments. (E) Percentage of LDL⁺ tubular moDCs expressing tubular Tfn⁺ endosomes after 4 hour culture in the presence of 3 µg EBV-derived EB2 with LPS and pI:C (black circles) at indicated time points. Data represent mean ± SEM of at least 3 independent experiments. (F) Time-lapse captures of Tfn⁺ tubular endosomes in moDCs (time points indicate seconds). bar 5 µm. (G) Percentage of LDL⁺ tubular moDCs expressing tubular Tfn⁺ endosomes upon culture in the presence of 3 µg HCMV antigen pp65 (4 hours) in absence (grey bars) or presence of LPS/pI:C (black bars). Data represent mean ± SEM of at least 3 independent experiments; *P<0.05.

FIGURE 3. ICAM-1 clustering provokes tubulation of Tfn⁺ endosomal recycling compartments in human dendritic cells.
(A) Schematic outline of live cell confocal microscopy experiment in B and D. (B) Representative images of moDCs upon anti-CD19 (upper images) or anti-ICAM-1 (lower images) mAb-coated beads contact. (C) Percentage of LDL⁺ tubular moDCs expressing
tubular Tfn\(^+\) endosomes after 4 hour culture in the presence of 3 \(\mu\)g HCMV-derived pp65/LPS/pI:C. Prior to stimulation (t=0) or around indicated time points upon addition of anti-CD19 (grey circles), anti-ICAM-1 (black triangles) mAb coated beads (1:4 DC/bead ratio). One-tailed, Mann-Whitney U test; *P<0.05. Data represent mean ± SEM of 4 independent experiments. (D) Representative images of moDCs upon CD11a (LFA-1, left 3 images) or CD127 (IL7R, right 3 images) blocked pp65-specific CD8\(^+\) T cell contact. (E) Percentage of LDL\(^+\) tubular moDCs expressing tubular Tfn\(^+\) endosomes after 4 hour culture in the presence of 3\(\mu\)g HCMV-derived pp65/LPS/pI:C. Prior to stimulation (t=0) or around indicated time points upon addition of CD11a (LFA-1, grey triangles) or CD127 (IL7R, black circles) blocked pp65-specific CD8\(^+\) T cells (1:1 DC/T cell ratio). One-tailed, Mann-Whitney U test; *P<0.05. Data represent mean ± SEM of 6 independent experiments. Boxes are zoomed part of images. LDL (red), Tfn (green), colocalization LDL and Tfn (yellow), CD11a or CD127-blocked pp65-specific CD8\(^+\) T cell (“T”), mAb coated bead (illustrated by “B”). bar 5 \(\mu\)m.

**FIGURE 4.** Class I MHC clustering, or simultaneous ICAM-1/Class I MHC clustering, provokes tubulation of Tfn\(^+\) endosomal recycling compartments in human dendritic cells.

(A) Schematic outline of live cell confocal microscopy experiment in B. (B) Representative images of moDCs upon anti-HLA-A2 mAb (left 6 images) or anti-ICAM-1/anti-HLA-A2 (right 6 images). LDL (red), Tfn (green), colocalization LDL and Tfn (yellow), mAb-coated beads (illustrated by “B”), Bar 5 \(\mu\)m. (C) Percentage of LDL\(^+\) tubular moDCs expressing tubular Tfn\(^+\) endosomes after 4 hour culture in the presence of 3 \(\mu\)g HCMV-derived pp65/LPS/pI:C. Prior to stimulation (t=0) or around indicated time points upon addition of anti-ICAM-1/anti-HLA-A2 (black triangles) or anti-HLA-A2 mAb (grey circles) coated beads (1:4 DC/bead ratio). One-tailed, Mann-Whitney U test; *P<0.05. Data represent mean ± SEM of 3 independent experiments.

**FIGURE 5.** Disintegration of tubular Tfn\(^+\) endosomal recycling compartments in human dendritic cells associates with reduced ability of dendritic cells to activate antigen-specific CD8\(^+\) T cells.

(A) Schematic outline of live cell confocal microscopy experiment in 5B and C. Representative images (B) and the percentage (C) of selected 3 \(\mu\)g HCMV-derived pp65/LPS/pI:C-laden moDCs after 1 hours of co-culture with pp65-specific CD8\(^+\) T cells and pre-treatment (left 3 images in B, white bars in C) or after 30 minutes of indicated drug treatment (right 3 images in B, black bars in C). “PQ” (primaquine, 50 \(\mu\)M), “noc” (nocodazole, 10 \(\mu\)M), Transferrin (green), Bar 5 \(\mu\)m. Data represents mean ± SEM of at least 4 independent experiments. (D) Schematic outline of experimental setup for 5E, F, G and H. (E) Human moDCs were loaded (O/N, 37\(^\circ\)C) with 0 or 3 \(\mu\)g HCMV-derived pp65 in presence of 200 ng/ml LPS and 5\(\mu\)g/ml pI:C. MoDCs were treated (30 min., 37\(^\circ\)C) with either 50 \(\mu\)M primaquine biphosphate, 1 \(\mu\)g/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing (3 washes). Next, pp65-specific CD8\(^+\) T cells were added for co-culture with the treated moDCs (1:1, 5 hours, 37\(^\circ\)C). DC mediated activation of antigen-specific CD8\(^+\) T cells was measured by analysis of induced production of IFN\(\gamma\), TNF, or surface expressed LAMP1 high (bar in dot plot is background, based on 1% cytokine positive CD8\(^+\) T cells upon culture with LPS and pI:C-treated moDCs in absence of antigen). (F) Percentage of antigen-specific activated CD8\(^+\) T cells, determined relative to matched PBS treated-moDCs. (G) Human moDCs were loaded (O/N, 37\(^\circ\)C) with pp65-derived NLV-peptide in presence of 200 ng/ml LPS and 5\(\mu\)g/ml pI:C. MoDCs were treated (30 min., 37\(^\circ\)C) with either 50 \(\mu\)M primaquine biphosphate, 1 \(\mu\)g/ml nocodazole, or carrier controls PBS, and DMSO, followed
by extensive washing (3 washes). Next, pp65-specific CD8$^+$ T cells were added for co-culture with the treated moDCs (1:1, 5 hours, 37°C) and antigen-specific CD8$^+$ T cell activation was determined. (H) Percentage of antigen-specific activated CD8$^+$ T cells, determined relative to matched PBS treated-moDCs. Human moDCs were loaded (O/N, 37°C) with 3 µg HCMV-derived pp65 in presence of 200 ng/ml LPS and 5µg/ml pI:C. MoDCs were treated (30 min., 37°C) with either 50 µM primaquine biphosphate, 1 µg/ml nocodazole, or carrier controls PBS, and DMSO, followed by extensive washing (3 washes). Hereafter, HLA-A2, ICAM-1, and CD80 expression on (I) or viability (J) of moDCs is determined by flow cytometry analysis. Data represents mean ± SEM of at least 3 independent experiments. Two-tailed, Mann-Whitney U test; *P<0.05, **P<0.01.

FIGURES