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CD40L Induces Functional Tunneling Nanotube Networks Exclusively in Dendritic Cells Programmed by Mediators of Type 1 Immunity

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The ability of dendritic cells (DC) to mediate CD4+ T cell help for cellular immunity is guided by instructive signals received during DC maturation, as well as the resulting pattern of DC responsiveness to the Th signal, CD40L. Furthermore, the professional transfer of antigenic information from migratory DC to lymph node–residing DC is critical for the effective induction of cellular immune responses. In this study we report that, in addition to their enhanced IL-12p70 producing capacity, human DC matured in the presence of inflammatory mediators of type 1 immunity are uniquely programmed to form networks of tunneling nanotube-like structures in response to CD40L-expressing Th cells or rCD40L. This immunologic process of DC reticulation facilitates intercellular trafficking of endosome-associated vesicles and Ag, but also pathogens such as HIV-1, and is regulated by the opposing roles of IFN-γ and IL-4. The initiation of DC reticulation represents a novel helper function of CD40L and a superior mechanism of intercellular communication possessed by type 1 polarized DC, as well as a target for exploitation by pathogens to enhance direct cell-to-cell spread. The Journal of Immunology, 2015, 194: 000–000.

Dendritic cells (DC) play a central role in the initiation and regulation of the immune response. They bridge the innate and adaptive branches of immunity by gathering pathogen- and tissue-derived environmental cues and translating this information into the development of appropriate adaptive immune responses following their migration to draining lymph nodes (1). The combination of exogenous and endogenous activation signals received in the affected tissue during their immature stage results in their differentiation into mature, preprogrammed DC capable of inducing differentially polarized, Ag-specific immune responses (2, 3).

The ability of DC to drive the appropriate type of adaptive immune response to effectively counter a particular pathogen assault is greatly influenced by their interaction with CD4+ T cells and their responsiveness to Th cell–associated CD40L, a critical factor in licensing or enabling DC to promote cellular immunity (4–6). Type 1 polarized DC (DC1) (2), or DC matured under proinflammatory conditions by immune mediators typically associated with acute viral infections, such as viral RNA (3), type 1 IFN (7), and activated NK cells (8), respond to CD40L by producing enhanced levels of IL-12p70, a key driving factor of Th1-biased cellular immunity (9). Conversely, standard or type 2 polarized DC (DC2) (2), such as those matured in the presence of histamines or PGE2 (3, 10), drive Th2-biased responses, display a diminished capacity to produce IL-12p70 upon CD40 ligation, and are less effective at driving cell-mediated immunity.

DC migration and transportation of Ag to draining lymph nodes are critical for the initiation of CTL responses (1). This process also involves immune communication with a subset of lymph node–resident DC that possess an enhanced ability to cross-present Ag to CD8+ T cells (11, 12). Transfer of antigenic information between migratory and lymph node–residing DC has been shown to be essential in models of immunity to viruses (12, 13), but the exact mechanisms involved in this Ag exchange are unclear. In situ imaging studies have revealed that migratory DC undergo dramatic morphological alterations upon entry into lymph nodes, including the formation of extended membrane processes, as they are integrated into a network of lymphoid-residing DC (14), thus supporting the concept of direct Ag transfer. One proposed mode of direct intercellular Ag exchange occurs through the facilitation of tunneling nanotubes (TNTs), or thin F-actin–based membrane protrusions that form direct cytoplasmic connections between proximal and remote cells (15, 16). TNTs can support the intercellular transfer of organelles, cytoplasmic and cell surface proteins, calcium fluxes, as well as some pathogens (16). Although TNTs and their function in the transmission of signaling fluxes have been described in immature DC (iDCs) (17), little information exists concerning the nature of their induction in mature DC, their function in DC-mediated communication, or their role in innate and adaptive immunity.

Abbreviations used in this article: cIMDM, IMDM supplemented with 10% FBS; DC, dendritic cell; DC1, type 1 polarized DC; DC2, type 2 polarized DC; DIC, differential interference contrast; EGFP, enhanced GFP; iDC, immature DC; rh, recombinant human; SEB, staphylococcal enterotoxin B; TNT, tunneling nanotube; TT, tetanus toxoid; VZV, varicella zoster virus; YG, yellow-green.

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In this study we describe a novel immunologic process by which networks of TNTs are induced as an exclusive trait of mature, high IL-12–producing DC1 in response to the Th cell activation signal, CD40L. We show that these CD40L–induced structures indeed support the direct intercellular transfer of cytoplasmic and cell surface–associated material between DC. Moreover, this novel process of DC “reticulation” dramatically increases cell surface area and spatial reach, thus enhancing the likelihood of their contact with Ag-specific T cells and other DC. Importantly, the ability of DC to reticulate in response to CD40L is impaired during maturation by exposure to type I inflammatory mediators, which are typically present during acute viral infection. Although the induction of reticulation represents a novel helper function of CD4+ T cells that serves to facilitate efficient DC1–mediated intercellular communication, this immune process can also be exploited by pathogens such as HIV-1 for direct cell-to-cell spread.

Materials and Methods
Isolation of human primary cells
Whole blood products (buffy coats) from healthy,anonymous donors were purchased from the Central Blood Bank of Pittsburgh. Autologous CD14–monocytes, CD3+ T cells, CD4+ T cells, CD8+ T cells, and myeloid blood–derived DC were isolated from PBMC by density gradient separation (18) followed by immunomagnetic negative selection of the respective cell types (EasySep: StemCell Technologies, Vancouver, BC, Canada).

Generation of DC
Monocytes were cultured for 5–7 d at 37°C in IMDM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (cIMDM) in the presence of GM-CSF and IL-4 (both 1000 IU/ml; R&D Systems, Minneapolis, MN). DC generated under serum-free conditions using either AIM-V (Life Technologies) or CellGenix base media were also tested and yielded similar results (data not shown). On day 5, iDC were differentially exposed to activation factors for 48 h. For mature DC1, the activation factors consisting of either polyinosinic-polycytidylic acid (20 μg/ml), IFN-α (3000 U/ml), TNF-α (50 ng/ml), IL-1β (25 ng/ml), and IFN-γ (1000 IU/ml) (7), LPS (250 ng/ml) and IFN-γ (1000 IU/ml), or R848 (2.5 μg/ml) (Enzo Life Sciences, Farmingdale, NY) and IFN-γ (1000 IU/ml). Alternatively, DC1 were generated by coculturing iDC with IL-18–primed NK cells in the presence of IL-15 (1 ng/ml) (8), or CD8+ T cells in the presence of staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, St. Louis, MO) (19). Sigma-Aldrich, St. Louis, MO). Mature, low IL-12p70–producing DC2 were generated using a modified version of a previously described mixture consisting of TNF-α (50 ng/ml), IL-1β (25 ng/ml), and PGE2 (10-6 mol/l) (7), a combination of LPS (250 ng/ml) and PGE2, (10-6 mol/l) (10), or R848 (2.5 μg/ml and PGE2, (10-6 mol/l) (10). Monocyte-derived DC0 were generated by exposure to TNF-α, LPS, or R848 alone for 24 h (10, 19). Similarly, freshly isolated blood-derived myeloid DC were treated for 24 h with TNF-α prior to secondary stimulation.

CD40L-induced activation of mature DC
Differentially matured DC were stimulated for 24 h with either recombinant human (rh)CD40L (0.5 μg/ml) (MegaCD40L; Enzo Life Sciences) or CD40L–expressing J558 (5558-CD40L) cells. (Dr. P. Lane, University of Birmingham, Birmingham, U.K.) (10), which were added to DC cultures at a 1:1 ratio. Where specified, IL-4 (5000 IU/ml), IL-10 (0.1 μg/ml), or IFN-γ (5000 IU/ml) was also included during CD40L stimulation.

IL-12p70 production and screening
DC were harvested, washed, and plated in 96-well flat-bottom plates (3 × 105 cells/well) and stimulated with either J558-CD40L cells or rhCD40L. IL-12p70 was measured in 24 h supernatants using the MSD electrochemiluminescence detection system (Meso Scale Discovery, Rockville, MD).

DC–T cell cocultures
Differentially matured DC (1.25 × 10^5 cells/ml) were cocultured with CD4+ or CD8+ T cells (3.75 × 10^5 cells/ml) in the presence of absence of SEB (1 μg/ml). When used, CD40L blocking mAb (Enzo Life Sciences) or control mAbs (BD Biosciences, San Jose, CA) were added to the cultures. Bright-field microscopic images (×400) were collected from 5 to 10 randomly selected fields in independent experiments from three healthy donors.

Flow cytometry
The following immunostaining Ab reagents were used for flow cytometry analysis: mouse-anti-human CD83-PE, CD86-PE, OX40L-PE, CD40L-PE, CD3-FITC, CD4-allophycocyanin, CD8-allophycocyanin, CD19-PE, CD3-FITC, HLA-DR-FITC (all from BD Biosciences), CD40L-PE, CD56-PE (Beckman Coulter, Indianapolis, IN), CCRR7-FITC (R&D Systems), CD14-PE, CD1c-PE (Miltenyi Biotec, San Diego, CA), and the respective matched isotype controls (BD Biosciences). Prior to analysis for expression of CD40L, isolated CD4+ and CD8+ T cells were stimulated for 24 h with anti-CD3/CD28 activating Dynabeads (Life Technologies). Purity was determined by the exclusive expression of either CD4 or CD8 on the CD3+ gated lymphocytes. Purity of blood-isolated DC was delineated using the following gating strategy: lineage (CD3, CD14, CD19, CD56)- and CD1c+HLA-DR+. Analysis was performed using the BD Biosciences LSRFortessa cell analyzer and FlowJo version 7.6 software.

Immunocytochemistry
DC1 were transferred to chambered borosilicate coverglass slides (Lab-Tek; Thermo Fisher Scientific, Rochester, NY) and treated with rhCD40L or media for 18–24 h prior to processing. Samples were fixed and permeabilized with 0.1% Triton X-100 and nonspecific mAb binding blocked with 2% BSA. Primary mouse anti-human early endosomal Ag 1 or isotype control mAb (1.25 μg/ml) (BD Transduction Laboratories, San Jose, CA) were added, followed by secondary goat anti-mouse Alexa Fluor 488–conjugated mAb and rhodamine-conjugated phallolidin (Molecular Probes/Invitrogen, Eugene, OR) to label primary mAb and filamentous F-actin, respectively. Nuclei were stained with Hoescht stain (Invitrogen Life Technologies).

Microscopy
Live cell differential interference contrast (DIC) and confocal images were collected using a Nikon Eclipse Ti and Photometrics Evolve camera system with a Nikon Apo TIRF ×60 OIL DIC N2 objective lens with a numerical aperture of 1.49, and NIS-Elements software was used to collect and analyze data. Additionally, images of fixed cells were collected on the Olympus Fluoview 1000 microscope system with Olympus ×60 objective (1.4 numerical aperture of 1.49) and an ×20 objective lens (numerical aperture of 0.5) using a Leica EC3 camera system, and images were analyzed using Leica Application Suite software. Live cell cultures were maintained in an imaging chamber at 37°C and 5% CO2 in media (cIMDM) during image acquisition, whereas fixed cells were maintained in PBS.

Morphological analysis of DC by high-resolution imaging
DC (1.25 × 10^5 cells/ml) were cultured in glass-bottom microwell imaging dishes (MatTek, Ashland, MA). Time-lapse live cell DIC imaging was captured at time points and durations specified, with illumination intervals ranging from 2 to 6 min. For confocal imaging experiments, DC were either maintained in media alone (resting) or exposed to rhCD40L for 18–20 h, followed by surface staining using anti-human HLA, ABC/Alexa Fluor 488 Ab (AbD Serotec, Raleigh, NC) as previously described (17, 20), and cell nuclei were stained with HCS NuclearMask red stain (Invitrogen Life Technologies). To quantitate total cell surface area and membrane morphologies, the cytoplasm was additionally stained with 0.39 μg/ml (0.4 μM) CFSE (Molecular Probes/Invitrogen), and Immars imaging software (Bitplane, South Windsor, CT) was used for data analysis.

Peptide and protein Ags
The following Ag sources were used at the concentrations listed in the described Ag transfer experiments: 0.2 μg/ml CMV Ag (a pool of 9–12 mer peptides that comprise the CMV portion of the common control CMV, EBV, and influenza A virus peptide pool) (21); 0.4 μg/ml varicella zoster virus (VZV) Ag (a peptide pool of 18 mers overlapping by 11 aa, spanning the entire glycoprotein E; Sigma-Aldrich); and 0.5 μg/ml tetanus toxoid (TT) Ag (whole protein; Astarte Biologics, Bellevue, WA).

Intercellular bead and Ag transfer experiments
Donor DC1 and DC2 were generated by pulsing iDC with 40-nm yellow-green (YG) latex nanobeads (Molecular Probes/Invitrogen) at 1 × 10^10 beads/ml and exposed them to the respective polarizing mixtures. For Ag transfer experiments, CMV, VZV, and TT Ags were added along with the beads and again after 24 h. Donor DC containing beads and recipient DC lacking beads were labeled with Cy5 or Cy3 dye (GE Healthcare, Piscataway, NJ), respectively, for 20 min at room temperature. Donor and
recipient DC1 or DC2 were harvested, washed five times, and cocultured for 20 h in the presence of absence of rhCD40L. Where stated, a 0.45-μm transwell system (Corning Costar, Tewksbury, MA) was used to separate cocultured DC. Bead transfer to recipient DC was assessed by flow cytometry, as well as by live cell confocal microscopy.

For Ag transfer experiments, donor DC1 were cocultured with Cy5-labeled recipient DC1 for 20 h in the presence of rhCD40L. Cy5-labeled recipient DC1 were then differentially sorted based on YG bead expression (as a marker for donor-to-recipient DC1 intercellular exchange) using a BD FACSAria IIu cell sorter. Sorted YG⁺ (bead-containing) and YG⁻ (bead-deficient) recipient DC1 were used as Ag-presenting stimulators of autologous T cell responses in a modified version of a previously described extended in vitro sensitization and IFN-γ ELISPOT assay (22). Briefly, the sorted DC1 and autologous CD3⁺ T cells were cocultured for 10 d at a DC1/T cell ratio of 1:10, with rIL-2 (100 IU/ml; Novartis, New York, NY) added on day 3. The cultured T cells (3 × 10⁶/well) were tested by ELISPOT for reactivity to the individual CMV, VZV, and TT Ags in the presence of autologous monocytes (5 × 10⁴/well). Spots were counted using an automated ELISPOT reader (AID) and are expressed as mean Ag-specific IFN-γ spot-forming units (SFU) per 10⁶ cells, subtracting values from Ag-negative control wells.

Detection of intercellular trafficking of pathogens

Bacteria transfer studies. DC1 were cultured in imaging dishes and stimulated with CD40L for 8-10 h prior to placement into the imaging chamber, which was maintained at 37°C and 5% CO₂. Live enhanced GFP (EGFP)-expressing bacteria (Escherichia coli strain BL21DE3) suspensions were prepared by picking a green isolated colony and suspending it in 200 μl cDMEM. Ten microliters E. coli mix was then injected into the media directly above cells. Cultures were incubated for 2 h to allow bacteria to settle in the proximity of the DC monolayer, and sequential imaging were generated using confocal resonance imaging methods.

Virus-like particle transfer studies. HIV-1–like particles were generated by transfecting 293T cells with pGag-EGFP, pRev, pGag/Pol, and pHXB2-J558 cells. When stimulated with the J558-CD40L cells, DC1 developed membrane protrusions similar to those induced by the CD4⁺ T cells (Supplemental Fig. 1C). However, this did not occur with exposure to the CD40L J558 control cells (Supplemental Fig. 1C), and once again DC2 failed to develop these formations (data not shown). We also tested the direct effect of adding rhCD40L to DC types (Fig. 1A), which induced a similar network of TNT-like membrane processes in DC1, but not DC2, in up to 25 donors tested. When CD40L-blocking mAb was added to 24 h DC1/CD4⁺ T cell cocultures, the formation of the membrane extensions was inhibited (Fig. 1B). Taken together, these data definitively show that the T cell factor CD40L is an inducer of the described morphological alterations occurring exclusively in DC1.

Live cell confocal fluorescence microscopy revealed that DC1 develop extensive networks of TNT-like processes in response to CD40 ligation (Fig. 1C), and we used Imaris three-dimensional imaging analysis software to quantitate the morphological changes observed in the DC types (Fig. 1D). This evaluation showed that the total cell surface area dramatically increased in CD40L-activated DC1 compared with resting DC1, and to a lesser degree in DC2 upon activation (Fig. 1E). Importantly, a significant difference in surface area was shown between DC1 and DC2 following rhCD40L treatment (Fig. 1E). In their resting state, we found that 27.2% of DC1 already displayed five or more long, ultrafine, nonbranching TNTs similar to those previously described in iDC (17), whereas only 2.4% of resting DC2 displayed multiple TNTs (Fig. 1F). CD40L-activated DC1 displayed a substantial increase in TNT-like extensions, which varied widely in length, diameter, and complexity, established multiple linkages between neighboring cells, and they were occasionally detected above the substratum (Supplemental Video 1). In contrast, the CD40L-activated DC2 were typically smaller and more rounded than DC1, and they displayed membrane ruffling as opposed to TNT-like extensions (Supplemental Video 2). For analysis of the more complex membrane formations, we characterized each filament (Fig. 1D, middle panel), defined as a group of connected dendrite- and TNT-like segments that branched from a single origin point at the edge of the cell body. The analysis showed that 100% of CD40L-activated DC1 displayed a reticulate membrane morphology (five or more filaments per cell), compared with 16.7% of CD40L-activated DC2 (Fig. 1G). Among the reticulation-positive cells, filaments expressed by DC2 tended to be lesser in number, shorter in length, and displayed less complexity of branching.
than did those of of DC1 (Supplemental Fig. 1D–G). Furthermore, CD40L-induced membrane extensions dramatically extended the spatial reach of DC1 compared with DC2 (Fig. 1H), as determined by measuring the shortest distance from the origin point to the distal terminal point of each filament (Fig. 1D, right panel).

We were curious whether the CD40L-induced reticulation process was related to the particular mixture(s) of activation factors used in our initial experiments, or whether this was in fact a general characteristic of DC1. Although a variety of pathogen- or host-derived signals can induce DC maturation, the presence of either IFN-γ or PGE2 during the maturation process is particularly important for driving differential DC polarization (2, 8, 10, 19, 23). To further explore the role of these key polarization factors in priming DC for reticulation, we tested the CD40L effect using alternative methods for generating DC0, DC1, and DC2 from iDC precursors utilizing different TLR agonists, such as LPS (TLR4) or R848 (TLR7/8) alone, or in combination with IFN-γ or PGE2, respectively (10, 24, 25). DC1 generated using these alternative methods formed extensive TNT networks in response to secondary CD40L activation, whereas DC2 failed to do so, and nonpolarized DC0 displayed an intermediate morphology (Fig. 2A, 2B). Additionally, we tested other previously published cellular methods to induce DC1 using either activated NK cells (8) or Ag-stimulated CD8+ T cells (19, 26). As was found with the other DC1 types, both NK cell– as well as CD8+ T cell–induced DC1 responded to secondary CD40L stimulation by forming networks of TNTs (Fig. 2C). Taken together, these data highlight that reticulation is indeed a characteristic trait of DC matured under type 1 inflammatory conditions, whereas PGE2-exposed DC2 are refractory to the reticulation process.
We speculated that IFN-γ can also induce high IL-12 production, similar to that of DC1. The Th1 cytokine IFN-γ has been established that concomitant exposure of TNF-α–matured, myeloid DC to the Th1 cytokine IFN-γ enhanced the formation of networks of ultrafine TNTs in response to CD40L (Supplemental Fig. 2). TNTs observed in peripheral blood DC tended to be thin and nonbranching, as opposed to the complex structures observed in monocyte-derived DC, but these structures established multiple intercellular connections and were frequently detected above the substratum (data not shown), similar to those observed in monocyte-derived DC1.

CD40L-induced reticulation supports the direct transfer of cellular contents between DC1

TNTs described in the present literature establish both membrane and cytoplasm continuity between connected cells, which in turn provides a pathway for direct intercellular communication (16). Indeed, TNTs can facilitate the direct exchange of organelles and both cytoplasmic and membrane-associated components (16, 28). We therefore speculated that the reticulation process could allow for efficient transfer of cellular contents between proximal and remote DC1. We first used high-resolution DIC imaging to capture the formation of the TNT-like networks over time and to search for visual evidence of the trafficking of cellular content between live DC via CD40L-induced TNTs. In doing so, we were able to clearly observe the dynamic immune process of reticulation, whereby DC actively formed numerous membrane extensions within hours of exposure to rhCD40L, ultimately establishing a network of interconnected processes between proximal and remote DC (Fig. 4A, Supplemental Video 3). Moreover, a number of endogenous vesicles could be seen traveling rapidly from one cell to another through these structures (Fig. 4B, Supplemental Video 4). We hypothesized that the membrane conduits were facilitating direct DC-to-DC transfer of endosomal vesicles, as has been shown in other cell types (15, 29). To address this, we fixed DC1–matured, myeloid DC and stained them with early endosomal Ag 1 mAb and rhodamine-conjugated phalloidin for labeling endosomes and F-actin–based TNTs, respectively. We were able to clearly detect early endosome localization within CD40L-induced TNTs (Fig. 4C) by confocal microscopy, despite the disruption of most fine membrane extensions by the fixation and staining process. These data indicate that DC1 can use the process of reticulation to exchange endosome-associated vesicles between interconnected cells.

To test whether exogenous material could also be acquired and subsequently exchanged between DC1 by the same mechanism, we pulsed iDC with YG-labeled nanobeads representing Ag, and then differentially matured them to achieve their DC1 or DC2 status. We labeled bead-containing donor DC and bead-deficient recipient DC with Cy5 and Cy3, respectively, and cocultured the DC for 20 h in the presence or absence of rhCD40L. Using time-lapse confocal imaging of live cells, beads were observed to localize to the TNTs of donor DC1 and rapidly traverse the length of these structures (Fig. 5A, Supplemental Video 5). Furthermore, the pathway of individual beads could be traced as they traveled through blue donor DC1 membrane extensions into the recipient cell, where they finally collected in the cell body (Fig. 5B).

Opposing roles of IFN-γ and IL-4 in regulating the CD40L-induced DC reticulation process

Preprogrammed DC1 can be generated from iDC by treatment with maturation factors in combination with IFN-γ, but concomitant activation of a mature nonpolarized DC0 with IFN-γ and CD40L can also induce high IL-12 production, similar to that of DC1 treated with CD40L alone (18, 19). We speculated that IFN-γ might also act in an analogous fashion, along with CD40L, as a costimulator of the reticulation process in DC0. Therefore, we tested the costimulatory effect of this Th1-associated cytokine, as well as the respective Th2- and regulatory T cell–associated cytokines IL-4 and IL-10 (3) on reticulation in TNF-α–matured DC0. We found that, in addition to augmented IL-12 production (data not shown), IFN-γ costimulation enhanced the ability of DC0 to reticulate in response to CD40L (Fig. 3A). Intriguingly, coexposure of DC0 to CD40L and IL-4 substantially reduced the percentage of reticulation-positive cells, whereas IL-10 had no significant impact on reticulation (Fig. 3A). We next investigated the effect of these differential Th cell–associated cytokines on CD40L–induced reticulation in preprogrammed DC1 and revealed an even more substantial inhibitory effect of IL-4 on this process (Fig. 3B). Again, the addition of IL-10 had no significant impact on the ability of DC1 to reticulate.

The method of generating DC from monocytes in vitro allows for sufficient cell numbers to be obtained for research studies and clinical applications (1), and it represents an established model for migratory DC that arise in vivo from the differentiation of monocyte precursors (13, 27). Nevertheless, we were interested to see whether DC isolated directly from human peripheral blood could be induced to form TNT networks by CD40L in an IFN-γ–dependent manner, analogous to the behavior of monocyte-derived DC. CD1c+HLA-DR+ myeloid DC were isolated from fresh PBMC by magnetic bead enrichment and matured with TNF-α for 24 h prior to treatment with media, rhCD40L, or IFN-γ plus rhCD40L, followed by live cell confocal imaging. Although low cell numbers limited the scope of these experiments, we established that concomitant exposure of TNF-α–matured, myeloid DC to the Th1 cytokine IFN-γ enhanced the formation of networks of ultrafine TNTs in response to CD40L (Supplemental Fig. 2).
In parallel to, and in support of, the live cell imaging studies, bead transfer was quantified by flow cytometry, as determined by detection of fluorescent beads in Cy3-labeled recipient cells in overnight resting or activated cocultures. Using this method, we demonstrated that the transfer of labeled beads from donor to recipient DC was enhanced in CD40L-activated DC1 compared with resting DC1, as well as resting and activated DC2 (Fig. 5C). Moreover, whereas a small fraction of DC2 recipients in the CD40L-activated conditions acquired beads, the mean fluorescence intensity of the bead-containing DC1 recipients was nearly 4-fold greater than that of the DC2 recipients (Fig. 5C), indicating a higher number of beads transferred to recipient DC1 on a per cell basis. We next evaluated the ability of recipient DC1 to functionally use and present Ags acquired from donor DC1 following reticulation. Briefly, we pulsed iDC with a combination of CMV, VZV, and TT Ags in addition to YG nanobeads during their exposure to type 1 polarizing maturation factors to generate Ag- and bead-loaded donor DC1. These donor DC1 were cocultured with Cy5-labeled recipient DC1 in the presence of rhCD40L for 20 h. Using the YG beads from donor DC1 as a marker for CD40L-induced intercellular exchange, two distinct populations of recipient DC1 (YG+ and YG−) were isolated by FACS sorting. The sorted YG+ and YG− recipient DC1 were assessed for their differential ability to drive Ag-specific recall responses in autologous T cell using an established 10-d in vitro sensitization assay followed by an IFN-γ ELISPOT readout. Sorted YG+ transfer recipient DC1 displayed an enhanced ability to drive Ag-specific T cell responses compared with YG− DC1 (Fig. 5D). Conversely, recipient DC1 separated from reticulating donor DC1 using a transwell system in parallel cocultures failed to generate substantial Ag-specific T cell responses (Supplemental Fig. 3), indicating that the functional transfer of Ag during the reticulation process was contact-dependent.

The DC reticulation process facilitates intercellular trafficking of microbial pathogens

Although the CD40L-mediated induction of TNT networks could play an important role in intercellular communication or Ag transfer between interconnected DC, the present literature suggests that bacterial (29) as well as viral pathogens such as HIV-1 (16, 30, 31) can exploit TNTs for direct cell-to-cell transmission. We hypothesized that CD40L-induced reticulation in DC1 could similarly provide an efficient pathway for intercellular trafficking of microbes.

To determine whether direct cell-to-cell bacterial transfer can occur via CD40L-induced TNT networks, we first injected a suspension of live EGFP-expressing E. coli into DC1 cultures following

![Image 228x488 to 539x732](http://www.jimmunol.org/)
their 10 h stimulation with rhCD40L. Time-lapse confocal resonance scanning was conducted 2 h later to capture the trafficking of live bacteria between DC1. Similar to what has been described in macrophages (29), this experimental strategy clearly revealed bacteria rapidly surfing along the outside of TNT-like membrane bridges from one cell to another (Fig. 6A, Supplemental Video 6). Notably, individual bacterium could simultaneously travel in opposite directions along the same tube, as well as change directions in midtransfer. CD40L-induced extensions could also be seen to capture bacteria and draw them toward the cell body for subsequent internalization (data not shown). These data reveal a mechanism by which CD40L-activated DC can use TNT-like extensions to probe for pathogens in tissues, or a pathway by which bacteria can spread from cell to cell over long distances, potentially facilitating their dissemination.

To confirm that DC reticulation could also support intercellular trafficking of viral pathogens, we pulsed Cy5-labeled DC1 with EGFP-expressing HIV-1–like particles and cocultured these donor DC with Cy3-labeled recipient DC1. The DC cocultures were then stimulated with rhCD40L for either 10 or 20 h prior to live cell confocal microscopy. Maximum intensity projection images revealed particle localization to TNTs of the reticulating donor cells that connected with recipient cell bodies at both 10 (Fig. 6A, Supplemental Video 6) and 20 h time points (Fig. 6C). Furthermore, HIV-1–like particles were frequently detected at the interface between donor cell TNTs and recipients, prior to their transfer to recipient cell bodies, up to 24 h after the donor DC first acquired the particles (Fig. 6C). Taken together, these findings demonstrate that the induction of DC reticulation by CD40L can facilitate rapid and direct spread of pathogens such as bacteria or HIV-1 between interconnected immune cells.

**Discussion**

In this study, we describe the induction of the immune process of reticulation, a novel aspect of CD40L-mediated CD4+ T cell help that results in the formation of dynamic networks of TNT-like membrane extensions between proximal and remote DC. We determined that the ability of human myeloid DC to reticulate is imprinted by specific combinations of pathogen- and host-derived inflammatory signals that they receive during maturation. Importantly, migratory, high IL-12p70–producing DC1 appear to be licensed to reticulate in response to CD40 ligation, whereas DC2 are refractory to this process.

Present research suggests a multifarious role for TNTs in intercellular communication, but their modes of induction and function in the context of DC-mediated immunity have not been fully elucidated. Studies using iDC have demonstrated that TNTs induced by mechanical stimulation or E. coli supernatants support cell-to-cell propagation of calcium fluxes, which are integral to the regulation of DC activation (17). A recent investigation showed that MHC class II+ DC in mouse corneas form TNTs in response to trauma or LPS activation, highlighting a potential link between inflammation and TNT formation for the first time in vivo (32). We showed that DC matured under type 1 inflammatory conditions display an enhanced number of unbranched ultrafine TNTs compared with DC2 in their resting state prior to in secondary activation. However, the distinctive responsiveness of preprogrammed
DC1 to secondary CD40L stimulation, or DC0 to concomitant CD40L and IFN-γ stimulation, results in the formation of a far more extensive and complex network of TNTs. Although CD40L-induced TNTs appear to be most similar to those described in macrophages due to their heterogeneous structure (29), they typically exhibit a unique branching pattern that results in a highly complex, interconnected web of DC.

Since the initial description of TNTs by Rustom et al. (15) in 2004, TNTs have been shown to facilitate the direct transfer of cytosolic and membrane components, such as endosome- and lysosome-associated vesicles and MHC molecules (16, 28). Previous in vivo studies have also shown that nonmigratory lymph node–residing DC specializing in cross-presentation acquire Ag and Ag-loaded MHC molecules from migratory DC for the efficient induction of CTL responses (12, 13). Although Ag acquisition by DC has been demonstrated through a number of alternate mechanisms (33–35), we demonstrate that the reticulation process can facilitate intercellular Ag exchange for the enhancement of Ag-specific T cell responses, and we propose that this represents an additional novel mechanism by which the acquisition occurs in vivo.

Recent in situ imaging advances have provided evidence of dynamic DC membrane extensions forming intercellular networks in lymph nodes or facilitating probing for microbial pathogens in nonlymphoid tissues. In lymph nodes, resident DC are positionally fixed in a distributed manner along a complex fibroblastic reticular network, which defines the T cell zones and guides T cell migration and scanning of DC (36). These DC are linked by the tips of extended membrane processes, which also facilitate rapid and pervasive probing (37). Migratory DC that travel to the T cell areas of regional lymph nodes undergo frequent changes in morphology, including the formation of dendrite-like extensions (14, 37), and they become sessile within 2 d as they are likely integrated into the resident DC network (37). These studies collectively provide in vivo evidence of a meshwork of DC fixed on the fibroblastic reticular network of lymph nodes. Our study demonstrates the unique ability of Th cells to regulate the formation of intercellular TNT networks in differentially polarized DC, yet the exact contribution of CD40L-induced reticulation to migratory and resident DC interactions in vivo remains to be determined. In addition to DC dynamics in nodes, this process may also assist pathogen acquisition by sentinel DC in mucosal tissues. Interestingly, intravital imaging of the lamina propria has revealed an increase in dynamic transepithelial DC extensions upon introduction of enteric bacterial pathogens (38). We have shown that, in addition to type 1 inflammatory mediators, DC exposed to signals from pathogens develop TNTs, and we speculate that contact with CD40L-expressing effector Th cells could further enhance the number of pathogen-probing extensions formed by DC in the mucosa.

Whereas DC reticulation is likely important for direct communication between immune cells, this process may conversely facilitate progression of chronic diseases such HIV-1 or cancer. The bidirectional transfer of vesicles, proteins, and mitochondria via TNTs has been recently observed between malignant human pleural mesothelioma cells and lung adenocarcinoma tumor specimens (39), suggesting a detrimental role for TNTs in cancer. Similarly, TNTs can be hijacked by intracellular pathogens to enhance rapid spread to distant cells while avoiding the inhospitable extracellular milieu. HIV-1 was shown to induce TNTs in infected macrophages and use them for high-speed transmission to neighboring uninfected macrophages (30, 31). Interestingly, HIV-1 infection does not induce these structures in infected CD4+ T cells, but their existing TNTs support the direct transfer of HIV-1 to uninfected T cells over longer distances (16). DC can act as a Trojan horse by sequestering intact HIV-1 virions and mediating transinfection of CD4+ T cells, but their existing TNTs support the direct transfer of HIV-1 to uninfected T cells over longer distances (16). DC can act as a Trojan horse by sequestering intact HIV-1 virions and mediating transinfection of CD4+ T cells, but their existing TNTs support the direct transfer of HIV-1 to uninfected T cells over longer distances (16). However, in our study we demonstrate that HIV-1–like particles can be directly transferred from DC to DC via CD40L-induced TNT networks. In addition to providing a direct conduit for HIV-1 dissemination from migratory DC to susceptible T cells, reticulation may facilitate viral spread from migratory to lymph node–resident DC, greatly amplifying the proportion of cells harboring and able to transmit the virus. Interestingly, we have recently shown that HIV-1 can selectively use CTL helper activity in the absence of killing as a means to induce and program DC1 capable of efficiently mediating HIV-1 transfection of CD4+ T cells (26). Although this study shows that such CD8+ T cell–matured DC1 reticulate in response to CD40L, questions...
remain regarding the role of reticulation in the scenario of transfection and the exact mechanism involved in HIV-1 transfer during natural infection.

DC reticulation may help or harm depending on the context in which it is induced, yet this phenomenon likely represents a fundamental aspect of DC effector function. The increased surface area and spatial reach afforded by this dynamic process can enhance not only the ability of IFN-γ-polarized DC1 to directly transfer activation signals or Ags to other DC subsets, but can also provide DC a greater opportunity to encounter rare cognate T cells for the efficient initiation of adaptive immune responses. Although elevated IL-12p70 production in part explains why DC1 are highly effective inducers of CD8+ T cell responses (7, 24), their ability to reticulate and maximize opportunities for critical contact with T cells as well as exchange Ags between interconnected DC are also likely contributing factors.

In addition to demonstrating that preprogrammed DC1 respond to CD40L by forming TNT networks, we show that CD40L and the cytokine IFN-γ can costimulate reticulation in mature, non-polarized DC0. These data collectively provide support for the existence of a positive feedback loop whereby successful DC1-driven IFN-γ responses from Th1, CTL, and/or NK cell immune effectors in turn promote continued DC1 polarization (41) and therefore reticulation. Conversely, exposure of DC to the chronic inflammatory mediator PGE2 during early stages of maturation generates DC2 that not only have a diminished capacity to produce IL-12p70 and promote type 1 immunity (7, 10, 42), but also fail to reticulate in response to CD40 ligation. Furthermore, the Th2 cell–associated cytokine IL-4 substantially inhibits this CD40L-induced process in both DC0 and DC1, suggesting a Th2-driven negative feedback mechanism for inhibiting the reticulation process. Although further study is required to fully understand the regulation and immunologic activity of DC reticulation in vivo, these findings advance our basic comprehension of the mechanisms by which DC bridge innate and adaptive immunity. These results also have important implications in the ongoing quest for therapies to combat cancer and viral pathogens such as HIV-1.

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Disclosures

The authors have no financial conflicts of interest.

References


**Supplemental Figure 1. Phenotypic characteristics of differentially polarized DC and their functional responsiveness to CD40L.** (A) DC1 and DC2 were harvested and analyzed by flow cytometry for the following cell surface markers: CD83, CD86, CCR7, and OX40L (left panel). IL-12p70 production of DC1 and DC2 in response to secondary rhCD40L stimulation was determined by MSD electrochemiluminescence (right panel). (B) Flow cytometric analysis showing the differential surface expression of CD40L on 24 h CD3/CD28-activated CD4+ and CD8+ T cells (left panel), and comparable surface expression of the receptor CD40 on DC1 and DC2 (right panel). (C) DC1 co-cultured with either a J558-CD40L cell line (left panel) or the control parental CD40L-deficient J558 cell line (right panel) for 20 h and analyzed by bright field microscopy (400X) for the presence of induced TNT-like protrusions (arrows). (A–C) Data are representative of 6 independent experiments using cells from 3 healthy donors. (D) Graphical depiction of the mean number of filaments per cell in reticulation positive DC1 compared to DC2. (E) Graph of the mean number of segments per filament in individual reticulating DC1 versus DC2. (F) Mean filament length per cell, defined as the sum length (μm) of its segments, graphically depicted in reticulating DC1 compared to DC2. (G) Graph of the mean full branch depth of filaments per cell, delineated as the maximum number of bifurcations from the origin point to a terminal point in an individual filament, in reticulation positive DC1 versus DC2. (D–G) Complex membrane filaments, displayed by 100% of individual CD40L-activated DC1 and 16.7% of DC2, were analyzed using IMARIS. Data are represented as mean ± SD from independent experiments of 3 healthy donors, and p-values <0.0001, <0.001, <0.01 and <0.05 are represented by ****, ***, **, and *, respectively.
Supplemental Figure 2. IFN-γ-dependent reticulation of peripheral blood-isolated DC in response to CD40L. Z plane reconstruction image (1000X) of TNF-α-treated, human peripheral blood-isolated myeloid DC (lineage-, HLA-DR+ and CD1c+) following 20 h activation with rhCD40L alone or in combination with IFN-γ. Confocal mages are representative of 3 healthy donors independently tested.
Supplemental Figure 3. Ag exchange between reticulating DC1 is contact dependent. IFN-γ ELISPOT assays measuring Ag-specific recall responses of cultured T cells following their in vitro sensitization with YG bead- and Ag-containing donor DC1 or recipient DC1 separated from reticulating donor DC1 by a trans-well system. Data are represented as mean ± SD of 2 replicate experiments and p-values <0.0001, <0.001, <0.01 and <0.05 are represented by ****, ***, **, and *, respectively.
Video Legends

Video 1. CD40L-treated DC1 express TNT-like protrusions that establish multiple connections between neighboring cells. DC1 were treated with rhCD40L for 20 h prior to staining for cell surface MHC class I (green) and nuclei (blue), followed by confocal imaging. The rotating volume view video displaying 12.5 frames per second (FPS) was generated from a representative Z plane reconstruction image, which highlights numerous TNT-like extensions that formed simultaneous intercellular connections on or above the substratum. Related to Figure 1.

Video 2. CD40L-treated DC2 typically display membrane ruffling, but fail to form TNTs. PGE$_2$-polarized DC2 were stained for cell surface MHC class I (green) and nuclei (blue), and confocal images collected 20 h post-addition of rhCD40L. A rotating volume view video (12.5 FPS) was generated from a representative Z plane reconstruction image, emphasizing the rounded cell morphology, typical membrane ruffling, and absence of TNT-like membrane extensions. Related to Figure 1.

Video 3. Live-cell imaging captures the dynamic CD40L-induced reticulation process in DC1. DC1 were hyper-stimulated with rhCD40L for 4 h prior to high resolution time-lapse DIC imaging for a duration of 3.6 hours to capture the reticulation process, whereby DC1 membrane extensions formed a dynamic and increasingly complex network between proximal and remote cells. The video is comprised of 36 total frames, which were collected every 6 min and displayed at 5.1 FPS. Related to Figure 4.

Video 4. TNTs support direct intercellular vesicle transfer between CD40L-activated DC1. Mature DC1 were hyper-stimulated with rhCD40L for a duration of 8 h, and subsequently analyzed using live-cell DIC microscopy. A representative 2.5 h time-lapse video reveals multiple endogenous vesicles exiting one cell body into a TNT-like membrane bridge formed between 2 adjacent cells, followed by vesicles trafficking through the tube, and finally entering the connected cell body. The video is composed of 75 total frames collected every 2 min and displayed at 7.5 FPS. Related to Figure 4.
**Video 5. Exogenous beads representing Ag rapidly traverse TNTs of donor DC1.** Donor DC1 (blue) containing fluorescently labeled 40 nm latex beads (green) were co-cultured with recipient DC1 (red) for 20 h in the presence of rhCD40L prior to live-cell confocal imaging. The video captures the interaction between donor and recipient DC membrane extensions and multiple beads rapidly traversing a dynamic TNT-like structure (blue) expressed by a donor DC1 over a duration of 28 min. Video is comprised of 46 total frames collected every 37 s and displayed at 11.5 FPS. Related to Figure 5.

**Video 6. Bacterial pathogens can traffic between DC1 along CD40L-induced TNTs.** Labeled DC1 monolayers (blue) stimulated with rhCD40L for 10 h were subsequently exposed to EGFP-expressing *Escherichia coli* for 2 h prior to microscopy. Time-lapse confocal resonant scanning methods were used to capture the bi-directional trafficking of individual bacterium between interconnected DC1 along a membrane bridge (arrows) for a duration of 34 min. Video is made up of 103 total frames collected every 20 s and shown at 14.7 FPS. Related to Figure 6.