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Migrating Lymph Dendritic Cells Contain Intracellular CD40 That Is Mobilized to the Immunological Synapse during Interactions with Antigen-Specific T Lymphocytes

Neil Foster,* Emma L. Turnbull,† and Gordon Macpherson‡

Steady state migrating rat lymph dendritic cells (LDC) are semimature, expressing high levels of surface MHC class II, but low levels of surface costimulatory molecules. In this study, we show that surface CD40 is not detectable, but LDC contain intracellular CD40. Multiple isoforms of CD40 were detected, including the type I isoform required for signal transduction. Culture of LDC with syngeneic T cells does not induce redistribution of cytoplasmic CD40. When LDC were cultured with naive allogeneic CD4 T lymphocytes, polarization of CD40 to the immune synapse occurred between 3 and 6 h postculture. By 24 h, although large numbers of T cells were engaged with LDC, CD40 could not be detected in LDC or at the synapses. We conclude that migrating LDC contain stores of CD40 that can be mobilized rapidly to the sites of interaction with Ag-specific T cells. The disappearance of CD40 by 24 h may help in the regulation of T cell activation. The Journal of Immunology, 2012, 189: 000–000.

Dendritic cells (DC) are unique among APCs in that they are the only cells that can activate naive T lymphocytes under physiological conditions (1, 2). Activation of T lymphocytes not only requires recognition of Ag and MHC by the cognate TCR, but also requires a second (costimulatory) signal (3). One such important costimulatory molecule is CD40, which is expressed by a variety of cells, including DC, macrophages, B lymphocytes, activated CD8+ T lymphocytes, and epithelial cells (4). When CD40 engages with CD40L (CD154/CD40L) on the surface of T lymphocytes, TNF-associated factor (TRAF) proteins are stimulated to interact with specific docking sites on the CD40 cytoplasmic tail (5), although TRAF-independent pathways are also known (6). The downstream effect of this CD40/ TRAF interaction includes increased B7.1 expression, IFN-γ production (7), and significant production of IL-12p70 (8). Other studies have shown that CD40−/− knockout mice have impaired T cell activation (9), although upstream calcium signaling (10) and recruitment of Wiskott-Aldrich syndrome protein are also known to be critical to CD40-dependent immune responses (11). Mobilization of CD40 to the immune synapse (IS) is therefore a very important event that occurs during cross-talk between Ds and T lymphocytes during the formation of the synapse. It is known that DC and T lymphocytes can interact in steady state (SS) conditions (12), but because this behavior does not result in T cell activation, it is possible that CD40/CD154 interaction does not occur or that the effect of CD40/CD154 ligation is inhibited under SS conditions. Recent studies have shown that in T lymphocytes CD154 becomes polarized to the IS during interaction with human monocyte-derived DC and polarization is required for CD154-dependent IL-12 secretion by DC (13). Such DC are not, however, representative of those DC that activate naive CD4 T cells in vivo, and little is known about the behavior of CD40 during interactions between T cells and physiologically relevant DC.

DC that are migrating from the rat small intestine in pseudo-afferent lymph (lymph DC [LDC]) can be collected (14). These LDC have left the intestine only minutes before their collection and are the DC that transport Ag to the mesenteric lymph nodes. They are also extremely potent activators of naive CD4 T cells (2) and B cells (15). LDC therefore represent one of the most physiologically relevant DC populations. We have shown that LDC are semimature, expressing high levels of surface MHC class II, but only low levels of CD80 and CD86 (16). LDC are, however, exceptionally potent activators of naive CD4 T cells in vitro (17), which implies that the interaction of an LDC with a naive, Ag-specific CD4 T cell induces upregulation of costimulatory molecules by the DC. The properties and behavior of LDC during such interactions have not, however, been studied, and it was the aim of this study to examine the localization and properties of CD40 during the interaction of LDC with allogeneic, naive CD4 T cells. Allogeneic T cells are used in this study because of the nonavailability of TCR transgenic T cells in the rat.

Our results show that CD40 is stored intracellularly in SS LDC and that multiple isoforms of CD40 exist in rat LDC. Following interaction with allogeneic, but not syngeneic CD4 T cells, CD40 redistributes to DC/T cell interface within 3–6 h, but by 24 h CD40 cannot be detected either in the DC cytoplasm or at the synapses. The presence of stored CD40 that can be mobilized rapidly to the site of interaction with an Ag-specific CD4 T cell may be important in facilitating rapid activation of the T cell. The lack of surface CD40 expression by LDC may be important in inducing tolerance in T cells specific for self or harmless foreign Ags. The focusing of CD40 to the immunological synapse may be important in preventing bystander activation of T cells, making non-Ag-specific interactions with a DC.
Materials and Methods

Isolation of rat pseudo-afferent lymph DC and CD4+ T lymphocytes

Pseudo-afferent lymph was collected via cannulation of the thoracic duct from mesenteric lymphadenectomized PVG (RT1c) rats, as previously described (18). In some experiments, Salmonella typhimurium LPS (50 μg; Sigma-Aldrich, Poole, U.K.) was administered as an i.v. bolus 24 h prior to isolating lymph for the collection of LPS-induced DC. Thoracic duct cells were collected over ice in PBS containing 10 mM EDTA and 20 U/ml heparin. Lymph cells were then passed through a 70-μm pore-size cell strainer (BD Biosciences, Oxford, U.K.) prior to removal of erythrocytes using ammonium chloride-potassium carbonate lysis buffer. CD11c+ LDC were then isolated using anti-CD11c MACS beads and AutoMACS (Miltenyi Biotec, Bicester, U.K.), according to the manufacturer’s protocols. A fraction of these LDC was then stained for MHCI and analyzed using a FACSCalibur 2, flow cytometer (BD Biosciences). CD4+ lymphocytes were isolated from the blood of conventional Lewis rats by cell sorting using a MoFlo (DakoCytomation, Ely, U.K.). A fraction of these was then stained for TCRβ and FACS analyzed, as stated above. The purity of the MoFlo-sorted cell populations associated with it were counted on five separate slides in each of three independent experiments.

LDC phenotype

FACS analysis (by methods stated earlier) was also used to determine the phenotype of LDC used throughout the study. These were as follows: CD11chigh, CD86high, MHCI/IIhigh (Fig. 1).

Analysis of CD40 localization in LDC by confocal microscopy

Cytopsins of SS rat pseudo-afferent lymph were prepared on glass microscope slides at 1500 rpm for 10 min using a Shandon II centrifuge (Thermo Scientific, Basingstoke, U.K.). Lymphatic leukocytes were permeabilized for 10 min by incubation with Triton X-100 (0.05%; Sigma-Aldrich) and washed three times in PBS before being fixed for 20 min in paraformaldehyde. After further washing, the cells were incubated with mouse anti-rat CD103 (α9 integrin, OX62) Ab (5 μg/ml; Serotec, Abingdon, U.K.) for 60 min on ice, washed three times with PBS prior to incubation for 30 min with 10% rat serum. Following further washing, the steps were incubated in the dark and on ice for 60 min with goat anti-mouse IgG1 conjugated to FITC (1:400; Sigma-Aldrich). In other preparations, anti-CD103 was replaced by goat anti-mouse CD40 (T-20 Ab; 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), followed by rabbit anti-goat IgG conjugated to FITC (1:400; Serotec). Lymph cells were then counterstained with 7-aminoactinomycin D (7-AAD; 25 μg/ml; Invitrogen, Paisley, U.K.). Anti-fade (DakoCytomation) was then applied to the cells prior to mounting under coverslips. All samples were viewed using TCS-NT confocal laser-scanning microscopes (Leica, Heidelberg, Germany), and images were analyzed using Leica software.

Following allogeneric DC/T lymphocyte culture, cells were treated prior to microscopy, as stated above, but without CD103 Ab and 7-AAD staining. The cells were then incubated with goat anti-mouse CD40 (T-20 Ab; 1:100; Santa Cruz Biotechnology), followed by rabbit anti-goat IgG conjugated to FITC (1:400; Serotec) and mouse anti-CD3 (1:100; Serotec), followed by goat anti-rat IgM conjugated to tetramethylrhodamine isothiocyanate (1:500; Serotec).

Analysis of CD40 distribution in rat LDC by flow cytometry

CD103+ LDC contained within density-enriched SS and LPS-induced pseudo-afferent leukocytes were positively selected using biotinylated CD103 mAb/streptavidin-MACS beads (Miltenyi Biotec) (purity >95% for both cell populations). LDC were double immunolabeled using anti-CD103 biotin/streptavidin-PE and L-17 (anti-CD40) polyclonal Ab (Santa Cruz Biotechnology) prior to Western blotting. Whole-cell lysates from CD103+ SS and LPS-induced LDC were prepared, and the protein equivalent of 5 × 106 cells was separated in a 10% SDS-PAGE gel/4% stacking gel prior to being electrophoretically transferred to a Hybond-C nitrocellulose membrane (Amersham, Bucks, U.K.). Blots were probed with L-17 (N terminus) or T-20 (C terminus) Ab (400 ng/ml; Santa Cruz Biotechnology), followed by anti-rabbit HRP-conjugated Ab prior to development using ECL (Fisher Scientific, Loughborough, U.K.). To verify the specificity of the T-20 Ab, cell lysates were also incubated with T-20 blocking peptide (4 μg/ml; Santa Cruz Biotechnology) prior to Western blotting.

Allogeneic DC/T lymphocyte culture

Density-enriched CD11c+ LDC (PVG RT1c) and CD4+ lymphocytes (Lewis) were cultured in RPMI 1640 media at a ratio of 50 DC to 500 lymphocytes (1:10) for a total of 24 h. At 3, 6, and 24 h, the mean numbers of T cells associated with LDC were assessed. CD40 localization was also assessed at these time points (by methods stated above). To assess the number of LDC/T cell interactions in SS lymph, cytospins of pseudo-afferent lymph were stained with anti-CD40 (T-20), and 7-AAD (as previously stated) was used. Each LDC and the number of T lymphocytes associated with it were counted on five separate slides in each of three independent experiments.

Results

CD40 in SS LDC is present in the cytoplasm and is not surface expressed

Fig. 1 shows the phenotype of LDC. As shown previously (14), these were uniformly CD103high, MHCIILhigh, MHCI/IIhigh, CD86high, and CD11chigh. In most cells that express CD40, the molecule is expressed on the plasma membrane (4). However, examination of SS LDC by immunocytochemistry on cytospins suggested that CD40 was distributed throughout the cytoplasm (Fig. 2F–H). To confirm this, LDC were examined by flow cytometry. No expression of CD40 could be detected on nonpermeabilized LDC (Fig. 3A). However, following permeabilization, strong expression of CD40 was detected in all LDC (Fig. 3B). Injection of LPS i.v. into rats results in a dramatic increase in the numbers of LDC migrating in pseudo-afferent lymph (16). In LDC collected under these conditions, CD40 remained confined to the cytoplasm with no evidence of surface expression (Fig. 3C, D).
To further characterize CD40 expression in LDC, we employed Western blotting using the L-17 (anti-N terminus) CD40 Ab. This revealed the presence of multiple isoforms of CD40 in LDC with molecular mass of between 35 and 50 kDa (Fig. 4A). We then analyzed the expression of the type 1 isoform of CD40, which is known to be required for signal transduction (19) using the type 1 isoform C terminus-specific T-20 Ab. The T-20 Ab detected a protein with a molecular mass of ∼40 kDa constitutively expressed by SS LDC and also by LPS-induced LDC (Fig. 4B). To confirm the specificity of the T-20 Ab, we probed transferred LDC protein with T-20 that had been preabsorbed using CD40 blocking peptide. Preabsorption with the blocking peptide resulted in complete abrogation of the CD40 (40-kDa) signal in both SS and LPS-induced LDC (Fig. 4C).

Interaction of LDC with syngeneic, SS T cells does not lead to CD40 polarization

LDC interactions with mixed syngeneic T cells under SS conditions were examined by immunocytochemistry on cytospins or coccular images. Arrows show points of contact between lymphocytes and DC. (F) CD40 distribution throughout the cell membrane of a pseudo-afferent DC in SS conditions. (G) CD40 distribution remains throughout the cell membrane during SS conditions even when DC interacts with lymphocyte. (H) The 90° reconstructed image of pseudo-afferent DC closely interacting with at least two lymphocytes during SS conditions. CD40 still remains throughout the cell membrane and is not polarized toward the lymphocytes. Scale bars at bottom left of images (A) and (F), 10 μm.

FIGURE 3. Freshly isolated SS and LPS-induced LDC show intracellular expression of CD40. FACS histograms showing density-enriched SS and LPS-induced LDC were double immunolabeled using anti-CD103 biotin/streptavidin-PE and L-17 (anti-CD40) polyclonal Ab. For surface labeling (A), cells remained unfixed and nonpermeabilized throughout, and for intracytoplasmic labeling (B), cells were fixed and permeabilized prior to CD40 labeling. CD40 expression by SS LDC and LPS-induced LDC is shown by the filled area, and isotype controls are shown by the dashed line. Cells were either unstimulated (steady state) (A, B) or stimulated by LPS (C, D). Each plot is representative of three independent experiments.
ture. This showed that DC were frequently in contact with T cells (Fig. 2A–E), although in general only small numbers of T cells were adherent. In all instances where syngeneic T cells were in contact with LDC, CD40 remained distributed throughout the LDC cytoplasm, with no evidence of polarization toward the sites of interaction (Fig. 2G, 2H).

**CD40 becomes polarized to the immunological synapse during interactions with allogeneic T cells**

Following coculture of LDC (CD103+/MHCII<sup>high</sup>) (Fig. 5A) with allogeneic CD4<sup>+</sup> (TCR<sup>high</sup>) T lymphocytes (Fig. 5B), we counted the number of T cells associated with individual DC and assessed whether polarization of CD40 to the IS had occurred during the 24-h culture period.

Initially (30–60 min), a mean of 48% of LDC was associated with T lymphocytes (Fig. 5C). At 3 h postculture, 66% of LDC interacted with between 1 and 7 T lymphocytes (Fig. 5D). By 6 h postculture, 86% of LDC were associated with between 1 and 9 T lymphocytes (Fig. 5E), and this was further increased at 24-h interaction with 100% of LDC interacting with 2–23 T lymphocytes (Fig. 5F).

Immunocytochemical examination of these LDC/T cell cultures showed that, after 3-h culture, CD40 was not polarized to the IS and remained distributed throughout LDC cytoplasm (Fig. 6A–D). However, by 6 h, CD40 was strongly polarized to the IS in all LDC that had engaged with a lymphocyte (Fig. 6E–H). Twenty percent more LDC had interacted with T lymphocytes at 6 h compared with 3 h, and this was reflected in larger lymphocyte clusters surrounding single LDC after 6 h (as shown in Fig. 5). The largest clustering of T lymphocytes with a single LDC was observed at the 24 h postculture point (Fig. 6I–L), and larger T lymphocytes (probably T lymphoblasts) were observed within these clusters (Fig. 6I). Importantly, we could not detect any CD40 expression in clusters at this time point, either in the cytoplasm of the LDC or at the synapse.

**FIGURE 4.** Freshly isolated SS and LPS-induced LDC express multiple protein isoforms of CD40. Western blots show CD40 protein isolated from density-enriched SS and LPS-induced LDC. Whole-cell lysates (protein equivalent of 5 × 10<sup>6</sup> LDC) were electrophoretically separated prior to transfer onto a nitrocellulose membrane. CD40 immunoreactivity was detected using anti-goat HRP-conjugated secondary Ab prior to peroxidase development using ECL with an exposure time of 5 min. (A) Blots probed with L-17 CD40 polyclonal Ab; (B) blots probed with type I CD40 isoform-specific Ab (T-20); (C) blots probed with T-20 Ab that had been preadsorbed with CD40 blocking peptide.

**FIGURE 5.** Allogeneic CD4<sup>+</sup> T cell interaction with pseudo-afferent rat DC. (A) CD11c<sup>+</sup> cell-sorted pseudo-afferent LDC expressing high levels of MHCII. (B) CD4<sup>+</sup> cell-sorted blood lymphocytes expressing high TCR αβ. (C–F) The mean number of T lymphocytes interacting with LDC in culture at time 0 (C) and in allogeneic reactions at time points ranging from 3 h postculture (D), 6 h postculture (E), and 24 h postculture (F). Each graph shows the number of lymphocytes associated with LDC. Each experiment was repeated five times on three separate occasions. Bars above each mean = SD from the mean.
FIGURE 6. Polarization of CD40 to the IS in rat pseudo-afferent LDC occurs only after at least 3-h culture with allogeneic CD4+ lymphocytes and is absent after 24 h. Confocal images of CD11c+ LDC and CD4+ T lymphocyte cultures shown in Fig. 5. (A, E, and I) CD3/tetramethylrhodamine isothiocyanate immunolabeling of LDC/T lymphocyte clusters; (B, F, and J) CD40/FITC immunolabeling of LDC/T lymphocyte clusters and (C, G, and K) overlay of previous images. (D, H, and L) Transmitted light images of the same LDC/T lymphocyte clusters. CD40 polarization to the IS occurs after 6-h culture (E–G), but is absent after 24 h (I–K). Images shown are representative of images obtained from five slides on three separate occasions. Scale bars at bottom left of images (A), (E), and (I), 10 μm.

Discussion

Naive T cells are first activated in the T cell areas of secondary lymphoid organs. DC acquire Ags in peripheral tissues and migrate via afferent lymph (nodes) or within the spleen or mucosal lymphoid tissues to T cell areas, where they are interrogated by recirculating naive T cells, forming short-lived associations. If the T cell recognizes its cognate peptide–MHC complex, it remains attached. If this is the only signal it receives (signal 1), it may become partially activated, but then apoptose or become anergic. If, however, it receives costimulatory signals (signal 2), it can become fully activated. Among these signals are the B7 family (CD80, 86), recognized by CD28 on the T cell, and CD40, recognized by CD40L on the T cell. Resting DC are generally low expressers of B7 and CD40, but can be stimulated to upregulate expression by signals from the innate immune system via TLR and other receptors.

The model we have developed enables us to study DC under near-physiological conditions (18). These DC have left the intestine seconds or minutes before they are collected. They migrate constitutively in the absence of any inflammatory stimuli (even in germ-free rats). These DC are semimature—they express high levels of surface MHC class II, but low levels of CD80 and CD86 (16). CD40 expression by these cells has not been examined previously. We were surprised to find that we could not detect expression of CD40 on the surface of LDC, but that both immunocytochemistry and intracellular flow cytometry showed abundant intracellular expression. That we were detecting CD40 was confirmed by Western blotting, which showed that several isoforms of CD40 were present, including the type 1 isoform required for signal transduction in mice, and that preincubation of the anti-CD40 Ab with a CD40 blocking peptide abolished the CD40 signal. Immunocytochemistry suggested that CD40 is present within a vesicular compartment in LDC, but the nature of this compartment has not been explored. A recent study (20) has reported increased cytoplasmic CD40 in murine LPS-stimulated peritoneal macrophages, but CD40 was not detected on the plasma membrane, whereas cytoplasmic localization of CD40 has also been reported in human airway epithelial cells (21). It has also been reported that murine Langerhans cells migrate to draining skin lymph nodes in an immature state, and, although CD40 expression was not studied, these migrating cells express low CD86 and cytoplasmic rather than membrane MHCII. The study also showed that when these cells were modeled in vitro using monocyte-derived DC, they did not mature in response to either LPS or heat-inactivated Escherichia coli (22). In accordance with these studies, we show that intracellular stores of CD40 are translocated to the IS when LDC are cocultured with allogeneic T lymphocytes, but not during SS syngeneic LDC/lymphocyte interaction, following migration into the pseudo-afferent lymph fluid or following culture of LDC with S. typhimurium LPS. Taken together, these studies may indicate that activation of migrating DC is a more tightly controlled event than was previously realized, and that the hypothesis that DC mature and become immunogenic as they migrate to draining lymph nodes may not be as robust as was previously thought. A recent study (23) may also provide evidence to suggest this because, in this latter study, it was shown that, although migrating lung DC upregulate both Cd40 gene and surface expression of CD40 protein, the cells also upregulate a number of different suppressive genes and proteins including the following: programmed cell death 1 ligand, protein inhibitor of STAT 3, and CD200.

The redistribution of CD40 in rat LDC, following culture with allogeneic CD4 T cells, took several hours to occur, which may reflect complex signaling events required between the LDC. That CD40 only redistributes to the immunological synapse may be crucial in restricting activation to Ag-specific T cells and preventing bystander activation. Surprisingly, by 24 h after the initiation of cultures between allogeneic CD4 T cells and LDC, no CD40 could be detected in the clusters. This suggests that the cytoplasmic stores of CD40 had been exhausted, and that synthesis of further CD40 did not occur. The physiological significance of the loss of CD40 is not clear, but may be important in restricting the ability of DC to activate CD4 T cells to a short time period after their arrival in the lymph node. We have not explored the signaling
events involved in the redistribution of CD40 in LDC. The primary event, however, must be the recognition of cognate peptide–MHC by the T cell, as this is the only difference between syngeneic and allogeneic interactions between the two cells. This recognition by the T cell must then result in signaling events occurring from the T cell to the LDC. The nature of these events is unknown, but cannot depend on upregulation of CD40L by the T cell, as the LDC do not express surface CD40. Given the very low expression of CD80 and CD86 by LDC, it is possible that other, unidentified molecules are involved in the signaling events.

In conclusion, we have shown that migrating SS rat LDC contain cytoplasmic stores of CD40, including the signaling isoform, and that this CD40 redistributes to the sites of interaction with allogeneic, but not syngeneic CD4 T cells. Following this redistribution, CD40 expression is lost from LDC. We suggest that these events are important in regulating the processes of naive CD4 T cell activation by DC in vivo.

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Disclosures
The authors have no financial conflicts of interest.

References
The authors have no financial conflicts of interest.

CD40 DYNAMICS IN RAT LYMPHATIC DC