T Cell Polarity at the Immunological Synapse Is Required for CD154-Dependent IL-12 Secretion by Dendritic Cells

Marie Tourret, Sarah Guégan, Karine Chemin, Stéphanie Dogniaux, Francesc Miro, Armelle Bohineust and Claire Hivroz

J Immunol published online 27 October 2010
http://www.jimmunol.org/content/early/2010/10/27/jimmunol.1001501

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/10/28/jimmunol.1001501.DC1

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
T Cell Polarity at the Immunological Synapse Is Required for CD154-Dependent IL-12 Secretion by Dendritic Cells

Marie Tourret,1 Sarah Guégan,1,2 Karine Chemin, Stéphanie Dogniaux, Francesc Miro,3 Armelle Bohineust, and Claire Hivroz

Ag-specific interaction between T lymphocytes and dendritic cells (DCs) leads to both T cell and DC activation. CD154 (CD40 ligand)/CD40 interactions have been shown to play a major, although not exclusive, role in this functional cross-talk. Interactions between T cells and DCs are structured by an immunological synapse (IS), characterized by polarization of the T cell microtubule cytoskeleton toward the interacting DCs. Yet the role T cell polarization may play in T cell-induced DC activation is mostly unknown. In this study, we address the role of T cell polarity in CD154-dependent activation of DCs in a human model, using two different tools to block T cell polarity (i.e., a microtubule depolymerizing drug and an inhibitor of atypical protein kinase C). We show that CD154 is recruited and concentrated at the IS formed between human primary T cells and autologous DCs and that this recruitment requires T cell polarity at the IS. Moreover, we show that T cell polarization at the IS controls T cell-dependent CD154–CD40 signaling in DCs as well as CD154-dependent IL-12 secretion by DCs. This study shows that T cell polarity at the IS plays a key role in CD154/CD40-dependent cross-talk between CD4+ T cells and DCs. The Journal of Immunology, 2010, 185: 000–000.

T he adaptive immune response is initiated through the recognition by Ag-specific T lymphocytes of peptides bound to MHC molecules presented at the surface of dendritic cells (DCs). This Ag-specific interaction leads not only to T cell activation but also to reciprocal DC activation, characterized by expression of costimulatory molecules and cytokine production by DCs. CD154 (CD40 ligand [CD40L])/CD40 interactions have been shown to play a major, although not exclusive, role in this functional cross-talk between T cells and DCs (1–3). This T cell-induced modification of DCs has been called “licensing” or “education” (4, 5). Contacts between T lymphocytes and APCs including DCs are structured by the formation of a specialized junction called the immunological synapse (IS) (6–9). One of the hallmarks of IS formation is the reorientation of the T cell microtubule organizing center (MTOC), along with the microtubule cytoskeleton toward the interacting APCs (10, 11) and reviewed in Ref. 12. This MTOC reorientation requires recognition of the Ag by the TCR and is controlled by TCR signaling (13–18). MTOC polarization at the IS induces a reorientation of the Golgi and secretory apparatus to the cell–cell interface, which controls the polarized secretion of lymphokines (19–21) and lytic granules (22, 23) toward the interacting APC or target cell. Polarized secretion does not only concern soluble proteins, such as cytokines, but also transmembrane proteins, such as Fas ligand (24) and CTLA-4 (25, 26). Although the role of MTOC polarization in T cells is not entirely unraveled, the polarized delivery of effectors may be required—to establish a high local concentration of effectors at the IS and/or—to ensure the Ag specificity of the immune responses by avoiding bystander effects.

In this study, we examine the role T cell polarity at the IS may play in CD154/CD40-dependent cross-talk between human CD4+ T lymphocytes and autologous DCs using two different tools to inhibit T cell polarity (i.e., colchicine [Colx], an alkaloid that depolymerizes the microtubules and an inhibitor of atypical protein kinase Cs (PKCs), which are known to control polarity of several cell types (27) including T cells (28, 29)). Our data show that CD154 concentration in the synaptic zone requires T cell polarity at the IS and is instrumental in IL-12 secretion.

Materials and Methods

Reagents and Ab

The media used are the following: RPMI 1640 Glutamax, 1% pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 10% FCS (Biowest, Miami, FL). Recombinant human IL-4 and GM-CSF were from Brucells (Brussels, Belgium). Recombinant bacterial superantigens were from Toxin Technology ( Sarasota, FL). Mouse mAbs against human CD4, CD69, CD40, CD80, CD83, CD86, CD154, and isotypic controls coupled to fluorochromes were from BD Biosciences (San Jose, CA). Rabbit polyclonal Abs against human Lamp1 and Lamp2 were from Abcam (Cambridge, MA). Mouse mAbs against human CD3ε UCHT1 and CD43 were a gift, respectively, from D. Cantrell (University of Dundee, Dundee, U.K.) and F. Sanchez-Madrid (Autonomous University of Madrid, Madrid, Spain). Rabbit Ab against human Rab 6 was from Santa Cruz Biotechnology (Santa Cruz, CA), rat anti–α-tubulin was from Sigma-Aldrich (St.

Received for publication May 6, 2010. Accepted for publication September 27, 2010.

This work was supported by Institut Curie, Institut National de la Sante et de la Recherche Medicale, Association pour la Recherche contre le Cancer, and Fondation pour la Recherche Medicale. M.T. and A.B. are recipients of a grant from the Ligue contre le Cancer, and K.C. is a recipient of a grant from Fondation pour la Recherche Medicale.

Address correspondence and reprint requests to Dr. Claire Hivroz, Institut Curie, Institut National de la Sante et de la Recherche Medicale, Association pour la Recherche contre le Cancer, and Fondation pour la Recherche Medicale. M.T. and A.B. are recipients of a grant from the Ligue contre le Cancer, and K.C. is a recipient of a grant from Fondation pour la Recherche Medicale.

The online version of this article contains supplemental material.

Abbreviations used in this paper: aPKC, atypical protein kinase C; aPKC inh., atypical protein kinase C inhibitor; CD40L, CD40 ligand; Colx, colchicine; DC, dendritic cell; HA, hemagglutinin; IS, immunological synapse; MTOC, microtubule organizing center; PKC, protein kinase C; sAg, superantigen; TSST-1, toxic shock syndrome toxin-1.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10/$16.00
Luis, MO). The myristoylated protein kinase Ca-C, pseudosubstrat was from Invitrogen. The agonist anti-human CD40 mAb was a gift from Y. Richard (Service of Immuno-Virology, Commissariat à l’Énergie Atomique, Unité Mixte de Recherche-E1, Université Paris-Sud, Orsay, France), and an IgG1 mouse anti-hemagglutinin (HA) mAb was used as a control. Secondary Abs used for microscopy (anti-rat, rabbit, and mouse Ig) coupled to Alexa Fluor 568 were from Invitrogen.

**Constructs and transfection of primary T cells**

Human CD154 cDNA followed by a short g1-gly-gly-ser-ser-gly-gly-gly linker was subcloned into the pEGFP-C1 vector (BD Clontech, Palo Alto, CA) between the Xhol and BamHI restriction site. Human fresh PBMCs were transfected with the Amaza Nucleofector technology (Amaza, Köln, Germany), according to the manufacturer’s instructions, and were used 18 h after transfection.

**Cells**

DCs were generated from human monocytes of healthy donors as described previously (30). CD4+ T cells were negatively selected from PBMCs, by depletion of CD14+ cells, using the T cell activation kit II from Miltenyi Biotec (Auburn, CA). Sorted CD4+ T cells were 97–99% CD4+/CD3+. PBMCs were obtained from a patient presenting with CD154 mutation, resulting in a complete defect in CD154 expression (31). This study was conducted according to the Helsinki Declaration, with informed consent obtained from the patient’s family, as requested by our Institutional Review Board.

**Colx pretreatment of T cells**

Purified CD4+ T cells were treated with 50 μg/ml Colx for 5 min at 37°C, extensively washed, and cultured for 1 h in complete medium to get rid of the excess of Colx. T cells were then washed again and used in cocultures.

**Cytokine detection**

Cytokine secretion was measured in the supernatants of DC/T cells cocultures performed in 96 round-bottom well plates by ELISA using matched paired Abs specific for IL-12p70 (DuoSet; R&D Systems, Minneapolis, MN). A cytometric bead array (BD Biosciences) was used to measure IL-8 and IL-6, produced in the cocultures.

**Flow cytometric analysis of T cell viability formation and T/DC conjugate**

For T cell viability, T cells were left untreated or treated with Colxs, or the myristoylated protein kinase Ca-C, pseudosubstrat DAPI was added at 0.5 μM just before acquisition on a MACSQuant analyzer (Miltenyi Biotec). For the analysis of T/DC conjugates, cells were cocultured in 96 round-bottom well plates at a 2:1 ratio, washed twice in PBS and BSA 0.5% without EDTA, labeled with anti-CD4 and anti-CD1a in mAbs at 4°C, and washed twice again in PBS and 0.5% BSA. Stained cells were then acquired on a FACScan (BD Biosciences) without prior vortexing. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

**Flow cytometric analysis of CD154 expression**

For the surface mobilization assay, CD4+ T cells incubated with a 1:10 dilution of PE-labeled dialyzed anti-CD154 (clone TRAP1; BD Biosciences) or control IgG1 were cocultured with DCs in the presence or absence of superantigens. After different coculture times, cells were stained with anti-CD4 and anti-CD1a mAbs for FACS analysis. In transfected cells, CD1a signal was stained, brefeldin A (5 μg/ml) was added during the last 3 h of cocultures. Cells were then labeled with anti-CD4 and anti-CD1a mAbs, fixed with 3% formaldehyde, and permeabilized with the Cytoperm/Wash kit from BD Biosciences before labeling with anti-CD154 mAb. Stained cells were acquired on a FACScan (BD Biosciences) and CD154 expression measured on CD4/CD1a cells. Data were analyzed with FlowJo software (Tree Star).

**Flow cytometric analysis of Stat3 phosphorylation**

Cells were labeled with anti-CD4 and CD1a mAbs, fixed with 3% formaldehyde, and permeabilized by adding ice-cold 100% methanol to prechilled cells for a final concentration of methanol of 90%. Cells were then stored at −20°C until labeling. Cells were washed with PBS and BSA 0.5% and incubated for 10 min with PBS and BSA 0.5% before labeling with anti-phospho-Stat3 (1:200) and then with secondary anti-rabbit Ig Abs coupled to Alexa 488. Stained cells were acquired on a FACScan (BD Biosciences), and Stat3 phosphorylation was measured on a “logical” gate CD1a and not CD4+ cells or CD4+ and not CD1a+ cells. Data were analyzed with FlowJo software (Tree Star).

**Microscopy**

In the experiments designed to follow the localization of CD154 at the IS, DCs and T cells were cocultured in the presence of superantigen mixture (staphylococcal enterotoxin E+ staphylococcal enterotoxin B + toxic shock syndrome toxin-1 [TSST-1]) on poly-(lysine)-coated coverslips. Six hours later, cells were fixed with 3% formaldehyde (Carlo Erba, Val de Reuil, France), incubated in PBS glycine (10 mM) to quench free aldehyde groups, and permeabilized with 0.05% saponin. Cells were then incubated with a first Ab against Lamp1, Lamp2, Rab6, CD43, CD3, or tubulin and subsequently with an Alexa Fluor 568-labeled secondary Ab. Then an anti-CD154 mouse mAb (clone TRAP1; BD Pharmingen) was used, which had been previously coupled with the DyLight 488 Protein Labeling Kit (Thermo Fisher Scientific, Waltham, MA). Coverslips were mounted onto glass slides using Fluoromount G (Southern Biotechnology Associates, Birmingham, AL). Images were acquired with a wide-field Eclipse 90i Upright Microscope (Nikon, Melville, NY) equipped for image deconvolution. Acquisition was performed using a ×100 Plan Apo VC 1.4 Oil objective and a highly sensitive cooled interline charge-coupled device camera (Roper CoolSnap HQ2). Z-positioning was accomplished by a piezoelectric motor (linear variable differential transformer; Physik Instrument, Karlsruhe, Germany), and a Z-series of images was taken every 0.2 μm. After deconvolution, images were segmented with the multidimensional image analysis interface nerve running under MetaMorph (Universal Imaging, Ypsilanti, MI) based on wavelet decomposition 17. Images were analyzed with Metamorph and ImageJ software. Pearson’s coefficient was quantified using the JACOP plugin from ImageJ (32).

Quantification of MTOC polarization and CD154 and CD3ε enrichment at the IS

MTOC polarization at the IS was scored blinded. Cells were analyzed by three-dimensional microscopy, with the same conditions used for control and drug-treated T cells. The proportion of conjugates with MTOC recruited at the immune synapse was calculated by random selection of >50 conjugates selected on transmission images from three independent experiments. Three-dimensional maximal projections of the centri or α-tubulin labeling were used to visualize the MTOC. They were generated to produce a z-stack projection of the region of cell–cell contact. The MTOC was scored as polarized when it was juxtaposed to the contact area with the DCs. CD154- or CD3-associated fluorences were quantified using the Metamorph software. Detectors were set to detect an optimal signal below the saturation limits. Image sets to be compared were acquired during the same session and using the same acquisition settings. Quantification of the recruitment of CD154 at the IS: average fluorescence intensity of CD154 in a fixed region of the synapse was divided by the average total fluorescence intensities inside T cells measured on three-dimensional projections of the CD154 deconvoluted images (Supplemental Fig. 1A, 1B).

Quantification of the recruitment of CD3ε at the IS: fluorescence intensity of CD3ε in a fixed region of the synapse was divided by the mean of the average intensities measured in three regions of the same size on the plasma membrane outside of the synapse. These values were calculated on two intermediate z-planes, averaged, and plotted (Supplemental Fig. 1C).

**Videomicroscopy**

For videomicroscopy experiments, we used the Leica DM IRBE microscope with the ×40 or ×100 1.4 NA oil immersion objective. Coverslips covered with T cells were placed into a chamber on the microscope at 37°C in a 5% CO2 atmosphere. At time 0, superantigen-loaded or untreated DCs were added, and at every 20 s, one phase contrast image and a stack of z-planes (step 0.3 mm) were collected with the green filter set. Movies consisting in the phase contrast image and the best focus plan of GFP–CD40L were accelerated ×90.

**Results**

CD154 is recruited at the IS formed between CD4+ human T cells and DCs

CD40L (CD154) expression by both murine and human CD4+ has been shown to play a crucial role in T cell-induced DC activation (1–3, 30, 33, 34). Moreover, others and we have shown that the IS formation plays a key role in T/DC cross-talk (30, 35–38). We thus examined the distribution of CD154 at the IS formed between human monocyte-derived DCs and autologous CD4+ T cells after 6 h of contact. No CD154 labeling was detected in T cells cocultured with DCs alone (data not shown), whereas in T cells for-
ming conjugates with superantigen-pulsed T cells, CD154 was observed at the IS “en face” of the DC in 74% of the CD4+ T cells forming conjugates (Fig. 1A–E and data not shown). CD154 did not colocalize with LAMP1 (Fig. 1A; Pearson coefficient $p$, quantification on 15 images $1F; p = 0.085$) or LAMP2 (Fig. 1B; quantification on 15 images $1F; p = 0.170$)-positive lysosomal compartments in T cells forming an IS but mainly colocalized with the Golgi, trans-Golgi marker Rab6 (Fig. 1C; quantification on 15 images $1F; p = 0.440$). Moreover, CD154 did not colocalize with the dSMAC marker CD43 (Fig. 1D; quantification on 15 images $1F; p = 0.060$), which is excluded from the IS but colocalized with CD3ε at the center of the synapse (Fig. 1E; quantification on 15 images $1F; p = 0.757$).

These results show that CD154 is recruited at the IS formed between human CD4+ T cells and DCs and cosegregates with CD3ε.

**CD154 recruitment at the IS formed between CD4+ T cells and DCs requires T cell polarity at the synapse**

T cell MTOC polarization at the IS induces a reorientation of secretion of lymphokines (19–21), lytic granules (22, 23), and transmembrane proteins (39, 40) at the synapse. We thus asked whether

**FIGURE 1.** CD154 localization in human CD4+ T cells forming synapse with autologous DCs. DCs and T cells were incubated in the presence of a superantigen mixture on poly (l-lysine)-coated coverslips during 6 h and observed by three-dimensional microscopy. A, Deconvoluted images are shown. White dotted lines indicate the position of T cells. Three-dimensional reconstruction of the interface (“en face view”) revealed that CD154 is clustered at the center of the synapse wherein CD43 is excluded and that CD3ε and CD40L cosegregate in this central zone. The Pearson’s coefficients ($p$) calculated for the shown merge images are encrusted in the image. Original magnification $\times 100$. Scale bars, 5 µm. F, Pearson’s coefficients ($p$) calculated for each pair of markers.
T cell polarity was required for CD154 recruitment. To answer this question, we pretreated human primary CD4+ T lymphocytes with Colx, which blocks polymerization of microtubule. Polarization of T cell MTOC toward superantigen-pulsed DCs was blocked by this pretreatment (Supplemental Fig. 2A) but did not significantly affect T cell viability (Supplemental Fig. 2B), T cell activation as measured by expression of CD154 (Fig. 2A and B). CD154 expression was not affected by Colx pretreatment of T cells. CD4+ human T cells were left untreated or pretreated for 5 min with 50 μg/ml Colx. A, Surface mobilization assay. Untreated or Colx-treated CD4+ T cells and autologous DCs pulsed with superantigen (sAg) were cocultured with an anti-CD154 fluorescent Ab (black histograms) or an isotype control (gray histogram) for 6 h. Labeling in the same conditions of CD4+ T cells from a CD154-deficient patient is shown as control (right bottom panel, CD154 def.). B, Intracellular labeling of CD154. Untreated or Colx CD4+ T cells and DCs pulsed with sAg were cocultured, and brefeldin A was added for the last 3 h. Cells were fixed, permeabilized, and labeled. Zebra plots of a representative experiment showing CD154 intracellular labeling in CD4+ T cells, percentage of cells, and geometric mean in the gate are shown. Labeling of CD4+ T cells from a CD154-deficient patient is shown as control (left panels, CD154 def.). C, Untreated or Colx-treated T cells were cultured on poly-(L-lysine)-coated coverslips with sAg-pulsed DCs for 6 h. Cells were fixed, permeabilized, and stained with α-tubulin (in red) and anti-CD154 or anti-CD3ε (in green). Coverslips were mounted for three-dimensional microscopy. Deconvoluted images. Original magnification ×100. Scale bars, 5 μm. D and E, Quantification of CD154 and CD3ε enrichment at the IS. D, Fluorescence intensity of CD154 in a fixed region of the synapse was divided by the total fluorescence intensity inside the T cell measured on three-dimensional projections of the CD154 deconvoluted images. E, Fluorescence intensity of CD3ε in a fixed region of the synapse was divided by the average intensities measured in three regions of the same size at the plasma membrane outside of the synapse. Each dot represents one T cell.
by CD69 expression (Supplemental Fig. 2C), or conjugate formation (Supplemental Fig. 2D). These results showed that interaction between Colo-treated T cells and DCs did occur and were productive in terms of T cell activation. We then controlled CD154 expression by T cells pretreated with Colo. Conventional methods for surface staining of CD154 at the surface are inefficient because of its high endocytosis and degradation rates (41); we thus adapted a method reported earlier (42, 43) to follow CD154 surface expression. Fluorescently conjugated Ab against CD154 or isotypic controls were added for the duration of the cocultures, allowing the accumulation of fluorescence in cells. Specificity of the labeling was controlled with T cells from a patient presenting with a complete defect in CD154 expression (Fig. 2A, lowest right panel). Untreated and Colo-treated CD4+ T cells cultured alone (data not shown) or with DCs showed a low, as reported by others (43, 44), but comparable surface expression of CD154.

In this test, however, addition of superantigen in the cocultures did not significantly increase CD154 surface expression. This was probably due to the instability of the CD154 Ab reported by others (42) that precluded accumulation of the complexes inside the cells. We thus measured the intracellular total amount of CD154 produced by T cells in the presence of brefeldin A, which blocks protein secretion thus allowing its accumulation. As shown in Fig. 2B, Colo neither modified the basal production of CD154 by T cells (Fig. 2B), nor the superantigen-induced CD154 expression confirming that T cell activation was not inhibited by Colo treatment (see Supplemental Fig. 2C for CD69 expression).

Knowing that CD154 was normally expressed by Colo-treated T cells, we then studied the effect of Colo on CD154 enrichment at the IS. Colo-treated T cells showed a scattered distribution of the CD154 labeling by fluorescence microscopy. Moreover, whereas the ratio between the synaptic CD154 fluorescence intensity and the total fluorescence intensity was $\geq 1$ in untreated CD4+ T cells, this ratio was $\leq 1$ in Colo-treated T cells revealing that Colo treatment inhibited CD154 recruitment at the IS (Fig. 2C and quantification in Fig. 2D). In contrast, an enrichment of CD3ε at the IS was still observed in Colo-treated T cells (Fig. 2C and quantification in Fig. 2D), showing, as demonstrated by us before (15), that CD3ε is still recruited at the IS in unpolarized T cells. This result is different from what has been reported by Das et al. (45), who showed a reduced accumulation of TCR in 30 min synapses formed between Colo-treated Jurkat T cells and superantigen-pulsed Raji B cells. Yet, this discrepancy may be due to the cells and kinetic used in this study (i.e., primary T cells and DCs and 6 h of contact).

We then studied the kinetic of recruitment of CD154 at the IS by introducing a GFP–CD154 chimeric molecule in primary CD4+ T cells. As shown in Fig. 3A and Supplemental Movie 1, CD154 was present both at the plasma membrane and in intracellular compartments of T cells. Concentration of CD154 at the IS was observed 3 min after contact and lasted for the whole length of the movie (20 min). In contrast, no recruitment of the GFP–CD154 chimeric molecule was observed in Colo-treated CD4+ T cells (Fig. 3B, Supplemental Movie 2).

Altogether, these results demonstrate that CD154 is rapidly polarized and recruited at the center of the IS formed between human CD4+ T cells and DCs and that this recruitment requires microtubule polymerization in T cells.

**T cell-induced IL-12 secretion by DC requires microtubule polymerization in T cell**

The role T cell MTOC polarization plays in T cell/APC cross-talk is mostly unknown. We addressed in this study the role it might play in T cell-induced DC activation. We have previously shown that CD4+ T cells induce increased expression of CD40, CD80, CD83, and CD86 in monocyte-derived DCs in the presence of superantigen (30). We thus analyzed the phenotype of DCs cocultured with untreated or Colo-treated T cells. Increased expression of CD40, CD80, CD83, and CD86 by DCs was equivalent when induced by polarized and unpolarized T cells (Fig. 4A). Moreover, Colo-treated T cells and untreated T cells also induced equivalent secretion of IL-6 and IL-8 (Fig. 4B, 4C) by DCs demonstrating that unpolarized T cells can induce DC activation. In contrast, Colo-
treated T cells induced significantly less IL-12p70 production by DCs than untreated CD4+ T cells (Fig. 4D), suggesting that polarization of T cells at the IS is required for T cell induced secretion of IL-12 by DCs.

CD154–CD40 signaling in DCs leading to IL-12 production requires T cell polarization at the IS

We have shown herein that microtubule polarization in T cells controls CD154 recruitment in the contact zone with DCs and IL-12 secretion by DCs. We thus hypothesized that the polarity-dependent CD154 recruitment at the IS is required for CD154/CD40 signaling in DCs and for T cell-induced IL-12 production by DCs, which has been shown by others and us to depend on CD154 (30, 33, 46). To test this hypothesis, we studied by FACS analysis the phosphorylation of Stat3, which has been shown to be induced by CD40 stimulation (47), in DCs activated by untreated or Colx-treated CD4+ T cells wherein CD154 is not recruited at the IS. Because Stat3 is also induced by cytokines including IL-6 (48), we measured phosphorylation of Stat3 at a time point (2 h) at which no cytokines were detectable in the supernatant. Moreover, we used a T/DC ratio (10:1) that ensured that most DCs were in contact with at least one superantigen-specific T cell. When cocultured with untreated autologous CD4+ T cells and superantigen, DCs presented with an increased phosphorylation of Stat3 (Fig. 5A, left panel). This required an antigenic stimulation because no increase of phospho-Stat3 was observed in DCs cultured with CD4+ T cells in the absence of superantigen compared with DCs (data not shown). No induction of Stat3 phosphorylation was observed in DCs cocultured with Colx-treated T cells and superantigens (Fig. 5A), suggesting that unpolarized T cells could not induce Stat3 signaling in DCs. Addition of an anti-CD40 agonist mAb to the cocultures containing Colx-treated CD4+ T cells completely restored Stat3 phosphorylation in DCs, whereas an anti-HA mAb of the same isotype did not (Fig. 5A, right panel). These results thus

FIGURE 4. T cell-dependent induction of IL-12p70 secretion by DCs requires microtubule polymerization in T cells. DCs (5 × 10^4) were cultured for 20 h with or without untreated or Colx-treated CD4+ T cells (10^5) and the superantigen (sAg) TSST-1 (100 ng/ml). A. Flow cytometric analysis of DC maturation markers. Histograms of a representative experiment. For each marker, expression was plotted as a ratio between the median fluorescence intensity obtained in the various conditions and the median fluorescence intensity measured on DCs alone (fold increase). White bar, DCs alone; black bar, DC+TSST-1+T; and gray bar, DC+TSST-1+Colx–treated T cells. IL-6 and IL-8 secretion (B, C) and IL-12p70 secretion (D) were measured by cytometric bead array and ELISA, respectively, in the supernatants from individual donors, and each circle represents an individual donor (black, untreated; gray, Colx). Significant differences between the groups were assessed by paired t test (IL-6 and IL-8, p = NS; IL-12p70, p = 0.003).

FIGURE 5. CD154–CD40 signaling in DCs leading to IL-12 production requires microtubule polymerization in T cells. A. DCs were incubated with untreated T cells or Colx-treated T cells in the presence of a superantigen (sAg) mixture. Anti-CD40 mAb or anti-HA at 3 μg/ml were added during the 2-h cocultures. Expression of phospho-Stat3 in DCs was measured by flow cytometry. B. DCs were cultured for 20 h with or without untreated (black histograms) or Colx-treated CD4+ T cells (gray histograms) and sAgs. A total of 3 μg/ml anti-HA or anti-CD40 mAb were added at the beginning of the coculture. IL-12p70 secretion was measured by ELISA. One representative experiment of three is shown in A and B.
suggest that unpolarized T cells, although expressing CD154 (Fig. 2B, 2C) are unable to induce CD154/CD40 signaling in DCs. We then studied whether the anti-CD40 agonist mAb, which restores CD154/CD40 signaling in DCs, could also restore IL-12 production by DCs activated by unpolarized T cells. Indeed, addition of the anti-CD40 mAb but not of the control anti-HA mAb restored the IL-12 secretion by DCs to levels that were induced by untreated CD4+ T cells (Fig. 5B).

Colx treatment does not only block MTOC polarity but also destroys the microtubule network in T cells. To demonstrate that MTOC polarity was indeed required for CD154-dependent signaling in DCs, we inhibited atypical PKCs, which have been shown to control the polarity of cells (27) including T cells (28, 29), and tested the effect of this inhibition on CD154-dependent IL-12 production by DCs. A cell-permeable myristoylated pseudosubstrate of atypical PKCs was added to the T cell/superantigen-pulsed DCs cocultures. This inhibitor used at 12.5 μM did not affect significantly T cell viability (Supplemental Fig. 3A), T cell activation as measured by CD69 expression (Supplemental Fig. 3B), or conjugate formation measured by flow cytometry (Supplemental Fig. 3C), showing that T/DC contacts were still productive. In contrast, polarization of T cell MTOC toward superantigen-pulsed DCs was inhibited (Fig. 6A and quantification in Fig. 6B), whereas T cell microtubule network (Fig. 6A4) was not affected. This absence of T cell MTOC polarity was accompanied by an inhibition of the recruitment of CD154 at the IS (Fig. 6A and quantification in Fig. 6C). The atypical PKC pseudosubstrate, when added to DC/superantigen/CD4+ T cell cocultures, inhibited IL-12 production by DC (Fig. 6D, 6E). Washing away the atypical PKC pseudosubstrate, which unlike Colx is a reversible inhibitor, partially restored IL-12 production by DC (Fig. 6E), showing that T cells after removal of the inhibitor were still able to induce IL-12 production by DCs. Moreover, as shown above for Colx treatment (Fig. 5B), addition of the anti-CD40 agonist mAb to cocultures containing the atypical PKC inhibitor restored the IL-12 secretion by DCs to levels that were induced by untreated CD4+ T cells, showing that atypical PKC pseudosubstrate that inhibits T cell MTOC polarity and CD154 recruitment at the IS inhibits CD154-dependent IL-12 production by DCs.

Altogether, our results demonstrate that unpolarized T cells, which show no CD154 recruitment at the synapse, do not induce IL-12 secretion by DCs because of defective CD154-dependent signaling in DCs.

**Discussion**

Polarization of T cells toward the interacting APCs has been described more than 20 y ago (11). Yet, the role this polarization may play in the immune response is not fully characterized. In CD8+ T cells, MTOC polarization controls the directional secretion of cytotoxic granules toward the target cell (reviewed in Ref. 49). In CD4+ T cells, MTOC polarization coincides with accumulation of some cytokines at the IS (19, 21). However, the role T cell polarity may play has not been envisaged on the APC’s point of view. We demonstrate in this paper that T cell polarity is required for CD154-dependent IL-12 secretion by DCs. Indeed, T cell polarity controls correct CD154 recruitment and concentration at the IS, which are themselves required for CD154/CD40 signaling in DCs.

We have investigated in this study the role of CD154 clustering at the IS in T/DC cross-talk. However, CD40–CD154 interactions do not only regulate cross-talk between T cells and DCs but also T-B...

---

**FIGURE 6.** An atypical PKC inhibitor blocks T cell polarity, CD154 enrichment at the IS, and CD154–CD40-dependent IL-12 production by DCs. A, DCs were incubated with T cells and superantigens (sAgs) in the absence or presence of 12.5 μM of an atypical PKC inhibitor on coverslips for 6 h. Cells were fixed permeabilized and stained with α-tubulin (in red) and anti-CD154 (in green). Deconvoluted images. Original magnification ×100. Scale bar, 5 μm. B, MTOC polarity at the IS was scored blindly in T cells in contact with DCs. Percentages of the T cells with a polarized MTOC are shown. C, Enrichment of CD154 in the IS was measured as a ratio of the CD154 fluorescence intensity in the synapse versus the total CD154 fluorescence in the cell. D and E, DCs, sAgs, and autologous CD4+ T cells were cocultured for 20 h with a mixture of sAgs in the absence or presence of 12.5 μM atypical PKC inhibitor (aPKC inh). D, A total of 3 μg/ml anti-CD40 mAb were added at the beginning of the coculture (gray histograms). IL-12p70 secretion was measured by ELISA. E, IL-12 secretion is expressed as a percentage of the maximum IL-12 secretion (black, no inhibitor; dashed, aPKC inh). In the right part of the histograms, cocultures were washed twice with medium and left in culture for an additional 12 h. One representative experiment of three (A–D) and two for the “washing” experiments.
cooperation. CD154 has major effects on B cell activation by regulating B cell proliferation, differentiation, and Ig production (reviewed in Ref. 50). This pivotal role of CD40–CD154 in the generation of efficient immune responses in humans is highlighted by the fact that patients presenting with mutations in CD154 develop defects of humoral immunity. Indeed, CD154 deficiency results in the X-linked hyper-IgM syndrome, a severe immunodeficiency characterized by low or absent IgG and IgA (reviewed in Ref. 51). CD154 enrichment has already been described at the IS formed in vitro between a mouse CD4+ T cell line and a B cell hybridoma presenting the right MHC-peptide (52) and in vivo in B/T cell IS formed after viral infection of primate brains (53).

CD154-expressing T cells have also been shown to be present in germinal centers of human tonsils (54). Thus, polarization-dependent CD154 enrichment at the IS might be involved in CD154-dependent B cell activation.

What could be the mechanisms for the polarization-dependent enrichment of CD154 at the IS? Specialized endocytic compartments such as secretory lysosomes are polarized toward the APC and fused at the synapse (reviewed in Ref. 55). This contributes to the polarized delivery of transmembrane receptors such as CTLA-4 (25, 26) and Fas ligand (24), which are present in secretory lysosomes. Fusion of the secretory lysosomes at the IS has been shown to require both microtubule polymerization to drive these compartments toward the MTOC (55) and MTOC recruitment to the IS to enable fusion of the compartments (49).

We have used in this study two different tools to block T cell polarity at the IS: inhibition of microtubule polymerization in T cells by treatment with Colx and inhibition of T cell MTOC polarity by treatment with a pseudosubstrate of atypical PKCs (αPKCs), which has been shown to inhibit T cell polarity (28, 29) but does not perturb microtubule network formation (Fig. 6). In both cases, CD154 was not enriched at the IS, yet when the microtubule network was absent (Colx treatment; Fig. 2C, Supplemental Fig. 1), CD154 endocytic compartments were scattered inside T cells, whereas when the microtubule network was present but not polarized (αPKC inhibitor; Fig. 6A) CD154 compartments were concentrated around the MTOC. These results suggest that recruitment of CD154 endocytic compartments and secretory lysosomes relies on similar mechanisms. Our results show that CD154 is rapidly recruited at the IS. The dynamic analysis of the GFP–CD154 recruitment at the IS suggests that two pools of CD154 are recruited: one that is at the plasma membrane and one that is intracellular. Because CD154 surface expression is low even in activated T cells (Fig. 2A), recruitment of the endocytic pool is probably crucial to provide a “bolus” of CD154 signaling. Yet, although CD154 was recently shown to be present in secretory lysosomes of mouse CD4+ and CD8+ T cells (43), we did not see any colocalization of CD154 with Lamp1 or Lamp2 in human CD4+ T lymphocytes. Alternatively, because CD154 is rapidly endocytosed (41), its enrichment at the IS may involve polarization of the recycling endosomal compartments toward the T cell–APC contact zone. Such a mechanism has been shown to contribute to the enrichment of TCRs, CD71, and the signaling molecule linker for activation of T cells at the IS (16, 45, 56). This polarized secretion of CD154 at the IS is probably crucial for CD154 signaling by nonactivated memory and naïve T cells that express low levels of CD154 (43, 44) and in this study (Fig. 2A, compare CD154 labeling of nonactivated T cells and T cells from CD154 deficient patient). In these cells polarized recruitment of the endocytic pools of CD154 may increase CD154/CD40 signaling in the interacting APC. Requirement for CD154 clustering at the IS of T cell polarization, which itself requires antigenic stimulation of T cells (13–15), could thus secure the antigenic specificity of the CD154-dependent signal.

Recruitment of CD154 at the IS might also account for its downregulation (41). Indeed, formation of the IS has been shown to facilitate TCR downregulation in the central zone of the synapse (57); the same may be true for CD154 that is also present in this central zone (Fig. 1D, 1E).

Several lines of evidence suggest that CD40 trimerization is required for initiating downstream signaling cascade (58–60). Clustering of CD154 at the IS may allow an efficient multimerization of CD40 leading to signaling in the interacting APC. We show in this paper that CD154 clustering in the synaptic zone controls Stat3 phosphorylation induced by CD154/CD40 signaling. The signaling pathways induced downstream of CD40 engagement on DCs activate multiple genes important for DC function. The signaling pathways activated downstream of CD40 depend on JAK/Stat signaling (47) as well as the recruitment of TNFR-associated factor family of proteins, which are recruited by the cytoplasmic tail of CD40 (reviewed in Ref. 61). These signaling pathways have been shown to induce IL-12 production as well as upregulation of costimulatory molecules such as CD80 and CD86 (62). It was thus somehow surprising to observe normal expression of activation markers in DCs activated by unpolarized T cells. Yet, as shown previously in our model (30), CD154 expression by T cells is mandatory for IL-12 production by DCs but not for expression of the CD40, CD80, CD83, and CD86 activation markers, which are probably induced by the TNF-α produced in the cocultures (63). Thus, unpolarized T cells, which are activated as shown by their expression of CD69, can still modulate DC function as revealed by expression by DCs of maturation markers (CD80, CD86, and CD40) and inflammatory cytokines (IL-8 and IL-6). These results imply that, in poor polarizing conditions [i.e., low number of TCR ligands or pathological conditions such as tumoral environment wherein defective T cell polarization has been reported (64)], T cells can induce some activation of DCs but no IL-12 production. Thus, DCs that have been activated by nonpolarized T cells are probably unable to induce Th1 responses because Th1 polarization has been shown to require IL-12–dependent signals (reviewed in Ref. 65). CD154–CD40 interactions have also been shown to provide antiapoptotic signal to DCs (66–68), to control cross-presentation of Ag by DCs (4), and to overcome peripheral T cell tolerance (69). The role T cell polarity and CD154 recruitment at the IS might play in these CD154-dependent functions should be investigated.

In conclusion, our results demonstrate that T cell polarity at the IS is required for CD154/CD40-dependent signaling in, and IL-12 secretion by, DCs, thus revealing a new role for CD4+ T cell polarity in functional T cell/DC cross-talk.

Note added in proof. While revising our manuscript, a study from Bertrand et al. (70) came out in The Journal of Immunology, which shows that PKCζ activation in T cells is required for the polarization of CD40L at the immunological synapse and the induction of IL-12 production by cognate DCs.

Acknowledgments We thank Prof. Alain Fischer (Unité d’Immuno-Hématologie Pédiatrique, Assistance Publique-Hôpitaux de Paris, Hôpital Necker-Enfants Malades, Paris, France) for providing access to PBMCs from a CD154-deficient patient, Lucie Sengmanivong and the Nikon Imaging Center at Institut Curie-Centre National de la Recherche Scientifique, Patricia Le Baccon and Vincent Fraizier (Imagerie Cellulaire et Tissulaire [Pict-IBI SA], Institut Curie, Paris, France) and Vassili Soumelis and Ana-Maria Lennon for discussion and critical reading of the manuscript.

Disclosures The authors have no financial conflicts of interest.
References


