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Article

The *Shigella flexneri* Type Three Secretion System Effector IpgD Inhibits T Cell Migration by Manipulating Host Phosphoinositide Metabolism

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SUMMARY

***Shigella*, the Gram-negative enteroinvasive bacterium that causes shigellosis, relies on its type III secretion system (TTSS) and injected effectors to modulate host cell functions. However, consequences of the interaction between *Shigella* and lymphocytes have not been investigated. We show that *Shigella* invades activated human $CD4^+$ T lymphocytes. Invasion requires a functional TTSS and results in inhibition of chemokine-induced T cell migration, an effect mediated by the TTSS effector IpgD, a phosphoinositide 4-phosphatase. Remarkably, IpgD injection into bystander T cells can occur in the absence of cell invasion. Upon IpgD-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), the pool of PIP_2 at the plasma membrane is reduced, leading to dephosphorylation of the ERM proteins and their inability to relocalize at one T cell pole upon chemokine stimulus, likely affecting the formation of the polarized edge required for cell migration. These results reveal a bacterial TTSS effector-mediated strategy to impair T cell function.**

INTRODUCTION

Pathogens have evolved strategies to avoid or resist host defense mechanisms. Although there is ample information on the mechanisms used by pathogenic bacteria to subvert the innate immune defense system, very little is known about how they affect the adaptive immune response (Hornef et al., 2002). This is also the case for *Shigella*, a facultative intracellular Gram-negative enteroinvasive bacterium. *Shigella* is the causative agent of shigellosis or bacillary dysentery and accounts for about one-third of the total annual deaths due to enteric infections (von Seidlein et al., 2006). *Shigella*'s virulence relies on the expression of a type III secretion system (TTSS) and the rapid injection of several effector proteins upon cell contact to modify host cell functions (Parsot, 2009). As recently exemplified for malaria (Weiss et al., 2010), protection occurs only after multiple infections and is of short duration, suggesting that *Shigella* has the capacity to dampen the host adaptive immune response.

In contrast, *Shigella* induces acute inflammation, a hallmark of the host innate response to infection, which requires TTSS effector secretion to be elicited. As a consequence of the proinflammatory process, massive dendritic cell (DC) and B and T lymphocyte cell death has been observed in rectal biopsies of *Shigella*-infected individuals (Raqib et al., 2002), suggesting that such an inflammatory response has an impact on the development of adaptive immunity. Furthermore, in a model of human intestinal xenograft, *Shigella*-infected intestinal epithelial cells (IECs) exhibit a dramatic decrease in the production of the chemokine CCL20, resulting in weak recruitment of DCs to the site of infection (Sperandio et al., 2008). However, during natural infection, *Shigella* crosses the intestinal barrier via M cells located in the lymphoid-associated epithelium where it is likely to directly encounter DCs and lymphocytes (Sansonetti and Phalipon, 1999). Moreover, after rupture of the integrity of the epithelial barrier, *Shigella* gets access to the lamina propria where DCs and mainly activated lymphocytes reside.

Whereas some data are available on the effect of *Shigella* infection on DCs (Edgeworth et al., 2002; Kim et al., 2008), the consequences of the interaction between *Shigella* and lymphocytes have not yet been investigated. Hence, we investigated the outcome of the interaction of *Shigella flexneri* with primary human $CD4^+$ T lymphocytes and Jurkat cells, a human T cell line largely used to dissect the molecular mechanisms underlying T cell function. We demonstrate that T lymphocytes are invaded by *Shigella* and that invasion impairs chemokine-induced migration, and identify IpgD as the TTSS effector responsible for the effect. In addition, we provide evidence that IpgD is injected into T cells in the absence of invasion, putting forth a model where immune cell functions can be affected with no need for the bacterium to be intracellular. These results

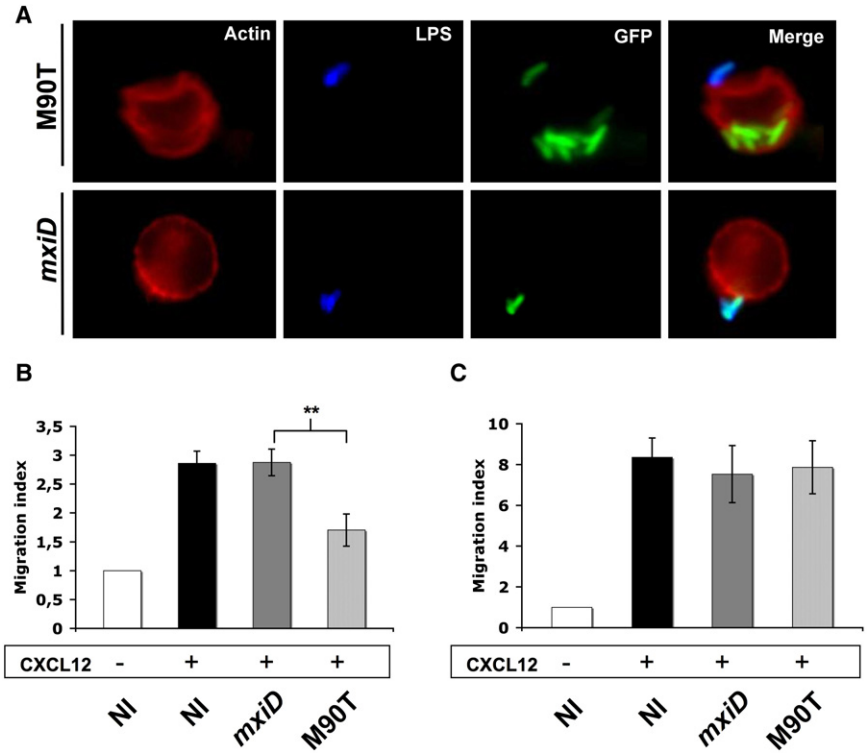

Figure 1. *Shigella* Invades Activated Primary Human T Cells and Impairs Their Migration

Activated primary human CD_4^+ T cells were infected with the *Shigella* wild-type strain M90T or the TTSS mutant *mxiD*. (A) Extra- and intracellular bacteria were visualized by immunofluorescence microscopy (IF) of cells infected for 30 min with GFP-expressing M90T (upper lane) and GFP-expressing *mxiD* (lower lane). Total and intracellular bacteria are seen in green (GFP), extracellular bacteria labeled prior to cell permeabilization with anti-LPS antibodies in blue, and actin labeled after cell permeabilization in red. Migration assays were performed with activated (B) and unactivated (C) primary human CD_4^+ T cells infected for 30 min with M90T or *mxiD* strains at a moi of 10. NI, not infected. Results represent the mean ± SEM calculated from a minimum of three independent experiments. $^{**}p < 0.01$.

start to unravel the possible mechanisms by which TTSS-expressing pathogens manipulate adaptive immunity.

RESULTS

Shigella Invades Activated Primary Human $CD4^+$ T Lymphocytes and Impairs Their Chemokine-Induced Migration

A possible crosstalk between *Shigella* and T lymphocytes was first assessed by analyzing the ability of *Shigella* to invade primary human T cells in an unactivated or activated state. $CD4^+$ T lymphocytes purified from PBMCs were incubated at moi of 10 or 100 bacteria per cell for 30 min with the invasive *Shigella* strain M90T or the noninvasive *Shigella* mutant *mxiD* that does not assemble the TTSS needle, and therefore does not secrete effectors (Allaoui et al., 1993b). In parallel experiments, $CD4^+$ T lymphocytes were activated with PMA prior to infection. To visualize intra- or extracellular bacteria, immunofluorescence analysis with anti-*Shigella* LPS antibodies on nonpermeabilized samples (staining only extracellular bacteria) and GFP-expressing bacteria (total and intracellular bacteria) was performed. Surprisingly, no intracellular bacteria were detected in unactivated $CD4^+$ T lymphocytes regardless of the moi (data not shown), while PMA activation rendered $CD4^+$ T cells susceptible to invasion by M90T (at both mois) but not by the mutant *mxiD* (Figure 1A). About 10% of activated primary $CD4^+$ T cells were invaded at an moi of 10 and 70% at an moi of 100.

Since lymphocyte migration is of utmost importance for T cells to exert their effector function (Kunkel and Butcher, 2002), we addressed whether *Shigella* infection of activated $CD4^+$ T lymphocytes had an effect on their ability to migrate in response to a chemoattractant stimulus. An moi of 10 was chosen for this essay because it resulted in less than 10% cell death for both noninfected and infected cells during the time course of the Transwell chamber migration assay (data not shown). We observed a 50% reduction in the ability of activated primary human $CD4^+$ T cells to migrate toward the chemoattractant CXCL12 when infected with M90T (Figure 1B). In contrast, upon infection with the *mxiD* mutant, the cell migration index was similar to that obtained with the noninfected cells (Figure 1B). Migration of unactivated cells was not affected regardless of the *Shigella* strain used for infection (Figure 1C). These results provide evidence that *Shigella* invasion of activated primary human $CD4^+$ T lymphocytes is dependent on a functional TTSS and that invasion has a significant impact on one of the critical functional features of T cells.

The *Shigella* Effector IpgD Impairs Chemokine-Induced Migration of Activated Primary Human $CD4^+$ T Lymphocytes

The T lymphocyte migration process induced in response to a chemoattactant is complex. At an early stage, the ERM (ezrin, radixin, and moesin) proteins, a family of membrane-cytoskeleton cross-linkers implicated in cell cortex organization, play a crucial role (Charrin and Alcover, 2006). ERM activation/inactivation state is dependent on the concentration of phosphatidylinositol 4,5-bisphosphate (PIP_2) at the cell membrane (Fehon et al., 2010). PIP_2 is also the substrate of the *Shigella* TTSS effector IpgD. IpgD is a phosphoinositide 4-phosphatase that generates phosphatidylinositol 5-monophosphate (PI5P) from PIP_2 (Niebuhr et al., 2000, 2002). Hence, we hypothesized that the T cell migration impairment observed upon *Shigella* infection was the result of the enzymatic activity of IpgD within the cells. The cell migration assay was thus performed with activated primary human $CD4^+$ T cells previously infected with the *ipgD* mutant and the

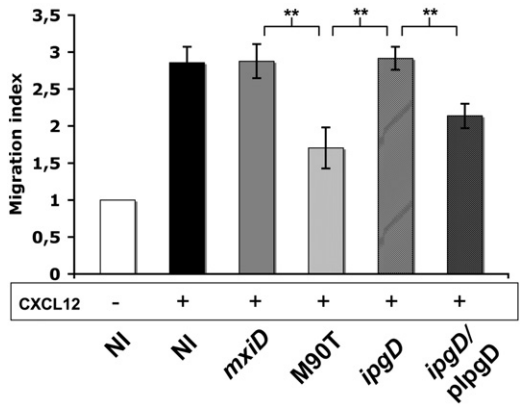

Figure 2. The *Shigella* Effector IpgD Accounts for the Reduction of Migration of Activated Human Primary T Cells

Migration assays were performed as described in Figure 1 with activated human CD_4^+ T cells infected with M90T, the mutant *mxiD*, the mutant *ipgD*, and its counterpart complemented strain *ipgD*/pIpgD. NI, not infected. Results represent the mean ± SEM calculated from a minimum of three independent experiments. **$p < 0.01$.

corresponding complemented strain *ipgD*/pIpgD. *ipgD* mutant and M90T infection gave rise to the same percentage of infected T cells (see Figure S1A available online). However, infection with the *ipgD* mutant resulted in a migration index comparable to that of T cells infected with the *mxiD* mutant or to the noninfected cells used as a positive control (Figure 2). Complementation of the *ipgD* mutation with *ipgD* resulted in a migration phenotype similar to that of M90T (Figure 2). These results demonstrate that the virulence effector IpgD is necessary and sufficient to impair the migration of activated primary $CD4^+$ T lymphocytes.

IpgD Inhibits the Migration of *Shigella*-Infected Jurkat Cells

We then used Jurkat cells (a human $CD4^+$ T cell line) to dissect the IpgD-mediated molecular mechanism involved in the impairment of T cell migration observed in the activated primary human $CD4^+$ T cells. We first assessed whether, upon *Shigella* infection, Jurkat cells displayed the same phenotype as primary human T cells. M90T and *mxiD* infection at an moi of 10 resulted in detection of intracellular bacteria only in Jurkat cells infected with M90T, and bacterial replication was shown to take place over time (Figure 3A). M90T infection resulted in ~10% invasion and a 30% reduction in CXCL12-induced migration when compared to noninfected cells or cells infected with the *mxiD* mutant. In addition, IpgD was shown to account for the impaired cell-migration phenotype (Figure 3B). These results are all consistent with the invasion and migration phenotypes observed upon *Shigella* infection of activated primary human $CD4^+$ T cells.

Interestingly, FACS analysis of M90T-GFP-infected Jurkat cells from the upper and lower compartments of the Transwell chamber revealed a population of GFP-high positive cells found only in the upper compartment (Figure 3C, red circle). IF analysis demonstrated that the GFP-high positive, nonmigrating Jurkat cell population was indeed cells heavily invaded by *Shigella* (Figure 3C, top panels). In contrast, cells that had migrated into the lower compartment were GFP-negative noninvaded cells (Figure 3C, blue circle, bottom panels). Thus, migration was inhibited in 100% of *Shigella*-invaded Jurkat cells, similarly to what was observed in human primary T cells (Figure S2). No difference in cell death was observed between the upper and lower wells, as monitored by PI staining (Figure 3C). In addition, no difference in Jurkat cell death occurred between M90T and *mxiD* under these experimental settings (data not shown).

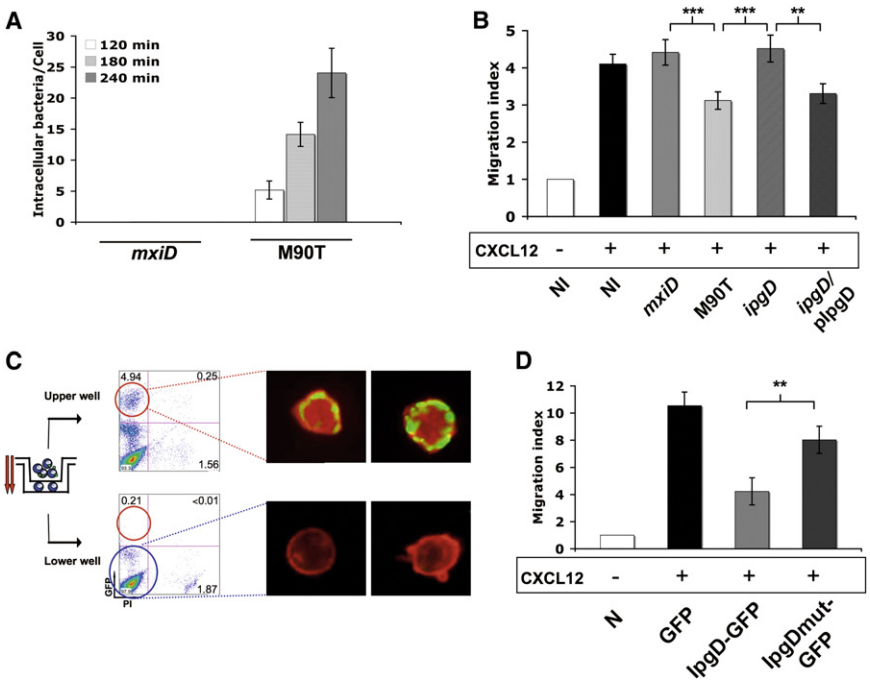

Figure 3. IpgD Is Responsible for the Inhibition of Jurkat Cell Migration

(A) Counting of intracellular bacteria in Jurkat cells infected with M90T or *mxiD* at an moi of 10 after gentamicin treatment. (B) Migration assay as previously described in Figures 1 and 2 with Jurkat cells infected with the strains M90T, *mxiD*, *ipgD*, *ipgD*/pIpgD, or not infected (NI). (C) FACS and IF analysis of Jurkat cells infected with GFP-expressing M90T present in the upper and lower wells after the cell migration assay. The GFP-high-positive cell population that is only found in the upper well (nonmigrating cells) corresponds to heavily invaded cells (red circle). In the lower well, the cells that have migrated are noninvaded cells (blue circle). IF pictures show actin in red and GFP-expressing M90T in green (D). Migration assay with Jurkat cells expressing IpgD-GFP, IpgDmut-GFP, GFP alone, or not transfected (N). Results represent the mean ± SEM calculated from a minimum of three independent experiments. **$p < 0.01$. ***$p < 0.001$.

Furthermore, migration analysis of Jurkat cells expressing IpgD-GFP or an inactive IpgD point mutant (IpgDmut-GFP) demonstrated that inhibition of T cell migration was dependent on the enzymatic activity of IpgD (Figure 3D). Therefore, both activated primary human $CD4^+$ T lymphocytes and Jurkat cells are susceptible to *Shigella* invasion and to the IpgD-mediated inhibition of their migration.

It is worth mentioning that a battery of *Shigella* mutants deleted for the expression of effectors reported to target different pathways involved in actin cytoskeleton rearrangement in EC (Tran Van Nhieu and Sansonetti, 1999) have been tested in the migration assay with Jurkat cells. For all of them, the migration index of the corresponding infected cells was similar to that of M90T-infected Jurkat cells (Figure S1B).

IpgD Is Injected into Jurkat Cells in the Absence of *Shigella* Invasion

It was intriguing that 10% invasion resulted in 50% and 30% reduction in T cell migration for both activated primary human $CD4^+$ T cells and Jurkat cells infected with M90T, respectively. This suggested that another phenomenon occurred in addition to invasion that contributed to the inhibition of T cell migration. Since injection of IpgD has indirectly been shown to occur in epithelial cells in the absence of invasion (Niebuhr et al., 2002), we hypothesized that this could also be the case for T cells. To test this, we adopted a FRET pair-based approach, previously reported to monitor effector translocation of Gram-negative bacteria into host cells (Mills et al., 2008). Briefly, to monitor translocation of IpgD into T cells, cells were preloaded with the cephalosporin-derived CCF4 probe and infected with *Shigella* M90T or *mxiD*, both expressing IpgD fused to β-lactamase (M90T-IpgD-bla and *mxiD*-IpgD-bla). Upon translocation of the effector fusion protein, the FRET pair CCF4 within the cell is cleaved by β-lactamase, and the fluorescence emission of the cells shifts from 535 nm (green) to 450 nm (blue). The threshold of detection for the FRET pair CCF4 cleavage depends on the amount of fusion protein delivered to the host cell and the kinetics of the enzymatic cleavage. To obtain a proper readout, we used an moi of 500 and a 2 hr incubation period of the bacteria with the cells after addition of gentamicin to kill the extracellular bacteria. To assess whether the translocation of IpgD from extracellular bacteria occurred in the absence of invasion, T cells were preincubated with cytochalasin D, an inhibitor of *Shigella* invasion (Mounier et al., 1997). In the presence of cytochalasin D, cleavage of the FRET pair occurred in about 30% of Jurkat cells infected with M90T-IpgD-bla, while no cleavage was detected in *mxiD*-IpgD-bla-infected cells (Figure 4), demonstrating that IpgD is injected into T cells in the absence of invasion.

IpgD Decreases the PIP_2 Pool at the Plasma Membrane

IpgD-mediated hydrolysis of PIP_2 has been shown to occur in epithelial cells (Niebuhr et al., 2002) and in Jurkat cells (Guittard et al., 2009). Since most of the PIP_2 pool is located at the plasma membrane, we expected that the IpgD-mediated PIP_2 hydrolysis in T cells would affect the plasma membrane PIP_2 pool. To visualize PIP_2 localization in Jurkat cells, the pleckstrin homology (PH) domain of PLCδ1 was used as a tool for confocal microscopy. The PH domain displays high affinity for PIP_2, and it is

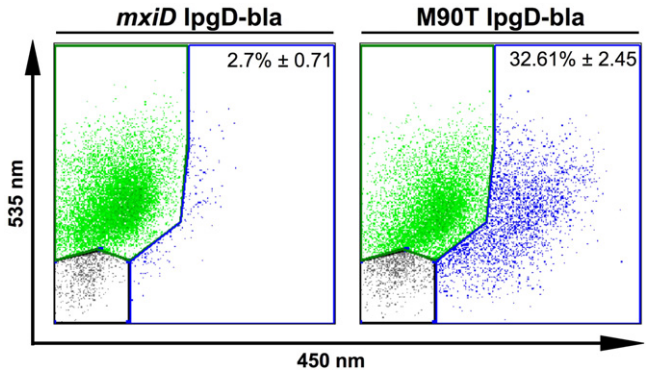

Figure 4. Injection of IpgD into Jurkat Cells in the Absence of Invasion

FACS analysis of cells loaded with CCF4 and infected with *mxiD* IpgD-bla (left panel) or M90T IpgD-bla (right panel) in the presence of Cytochalasin D. Translocation of the effector fusion protein leads to cleavage of CCF4 and to an emission switch from 535 nm to 450 nm. Results represent the mean ± SEM calculated from three independent experiments.

bound to the plasma membrane when PIP_2 is present but translocates to the cytosol when PIP_2 is hydrolyzed (Varnai et al., 2002). Jurkat cells were transfected to express the PH domain of PLCδ1 coupled to the red fluorescent protein (RFP) (PH-PLCδ1-RFP) together with either wild-type IpgD-GFP or its inactive form (IpgDmut-GFP). Localization of PH-PLCδ1-RFP at the plasma membrane was observed in 92% and 96% of cells transfected with PH-PLCδ1-RFP only and in cells cotransfected with IpgDmut-GFP, respectively. In contrast, the localization of PH-PLCδ1-RFP at the plasma membrane was lost in 81% of cells cotransfected with IpgD (Figure 5A). Quantitative analysis revealed a statistically significant decrease of the ratio fluorescence intensity at the plasma membrane versus cytosol for IpgD as compared to IpgDmut, which displayed a ratio similar to that of the control (Figure 5B). Moreover, the decrease of PIP_2 level at the plasma membrane occurred as soon as Jurkat cells were infected with M90T, but not with the *ipgD* mutant, and was sustained for at least 1 hr postinfection (Figure S3). These results indicate that PIP_2 is a target of IpgD in Jurkat cells and that the resulting IpgD-mediated PIP_2 cleavage modifies the PIP_2 pool at the plasma membrane.

IpgD Induces Dephosphorylation of Phospho-ERM Proteins, Affecting Their Polar Redistribution upon Chemokine Stimulus

As previously mentioned, the concentration of PIP_2 at the plasma membrane is crucial for the dynamics of the ERM activation/inactivation state which is important in the early steps of cell cortex organization during T cell polarization in response to a chemokine stimulus (Fehon et al., 2010). We thus investigated the ratio of nonphosphorylated (inactive) versus phosphorylated (active) ERMs (phospho-ERMs) in Jurkat cells upon *Shigella* infection. Whereas the pool of total ERMs remained unchanged, a dramatic sustained decrease in the pool of phospho-ERMs occurred upon infection with M90T, but not with the *ipgD* mutant, as compared to noninfected cells (Figure 6A). These results were confirmed in Jurkat cells expressing either IpgD-GFP or

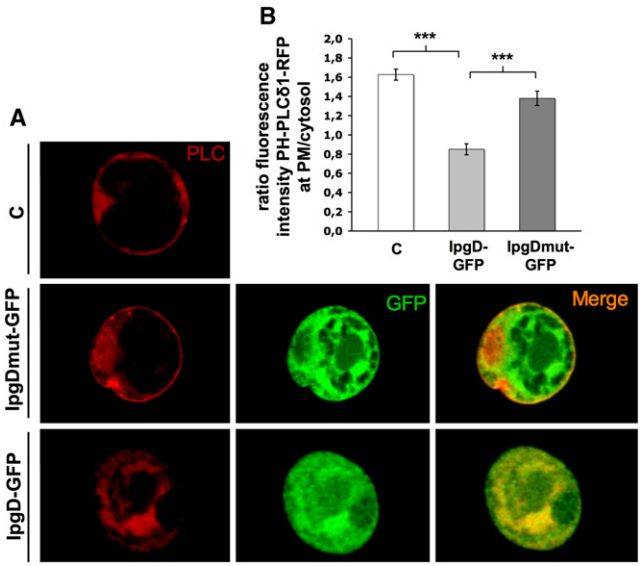

Figure 5. IpgD Reduces the PIP_2 Pool at the Plasma Membrane

(A) The pleckstrin homology (PH) domain of PLCδ1 was used to monitor PIP_2 localization in Jurkat cells expressing either PLCδ1PH-RFP (C for control, upper lane), PLCδ1PH-RFP and IpgDmut-GFP (middle lane), or PLCδ1PH-RFP and IpgD-GFP (lower lane).

(B) The ratio of the fluorescence intensity of PLCδ1PH-RFP at the PM (average fluorescence intensity of four defined boxes of 2 × 10 pixels of the plasma membrane per cell) to that of the cytosol (average fluorescence intensity of one box of a defined area of 11 × 11 pixels per cell) was determined by using Image J software. Bars represent mean ± the SEM of two independent experiments (25 cells scored for each construct). ***$p < 0.001$.

IpgDmut-GFP (Figure 6B) and in human primary T cells infected with M90T versus the *ipgD* mutant (Figure S4A).

We then used Jurkat cells expressing either IpgD-GFP or IpgDmut-GFP to assess phospho-ERMs redistribution upon chemokine stimulus. The transfected cells were incubated for 45 s with CXCL12 followed by anti-phospho-ERM antibodies to visualize phospho-ERMs localization. The number of Jurkat cells displaying recruitment of phospho-ERMs at one pole of the cell were counted by IF microscopy, and it was observed in cells expressing GFP as a control or IpgDmut-GFP. In contrast, phospho-ERMs localization at the cell pole was barely detected in Jurkat cells expressing IpgD-GFP, whose phenotype was similar to that of cells incubated in the absence of CXCL12 (Figures 6C and 6D). Those results were consistent with the observation that IpgD-mediated dephosphorylation of phospho-ERMs remained unchanged in the presence of CXCL12 both for *Shigella*-infected Jurkat cells (Figure S4B) and primary human T cells (Figure S4C). Our data collectively suggest that inhibition of T cell migration induced by IpgD is the consequence of the blockage of one of the early steps in the process of cell migration, i.e., cell polarization in response to a chemoattractant involving PIP_2 and ERMs.

DISCUSSION

Four main messages arise from this study. First, we show that *Shigella* invades activated human $CD4^+$ T cells in a TTSS-dependent manner. Second, injection of TTSS effectors takes place in T cells in the absence of invasion. Third, *Shigella* invasion and TTSS injection result in the inhibition of chemokine-induced T cell migration. Fourth, inhibition of T cell migration relies on the bacterial TTSS effector IpgD that hydrolyses PIP_2 through its phosphoinositol 4-phosphatase activity. Our results suggest that the subsequent reduction of the PIP_2 pool at the plasma membrane impairs the dynamics between the inactive (non-phosphorylated) and active (phosphorylated) forms of the ERM proteins. This presumably affects the polar cap formation (also called posteriorization of the plasma membrane), a critical step for the switch, upon chemokine stimulation, from unpolarized to polarized cell morphology that further allows T cell chemotaxis to occur (Charrin and Alcover, 2006).

Invasion of T cells by *Shigella* requires a functional TTSS, as previously reported for other nonphagocytic cells including IECs (Allaoui et al., 1993b). This suggests that the effectors and mechanisms triggering actin cytoskeleton rearrangements for *Shigella* entry into IECs (Tran Van Nhieu and Sansonetti, 1999) may be similar in T cells. Accordingly, we observed that the phenotype of the *ipaA* mutant when incubated with T cells was similar to the phenotype previously reported with IECs, i.e., a reduced invasion capacity as compared to wild-type *Shigella* (Figure S1A) (Tran Van Nhieu et al., 1997). IpaA is a TTSS effector that binds vinculin, a key component of focal adhesions, and stimulates actin depolymerization. By targeting β1-integrin, IpaA also stimulates the GTPase activity of RhoA, thereby inducing the loss of actin stress fibers (Demali et al., 2006). *Salmonella typhimurium* and *Yersinia entercolitica* are two Gram-negative enteroinvasive bacteria that express a TTSS. *S. typhimurium* has been reported to invade in vitro T cell lines, whereas *Y. enterocolitica* has not (Verjans et al., 1994).

Interestingly, activated but not unactivated primary human $CD4^+$ T cells are invaded by *Shigella*. We speculate that, as compared to unactivated T cells, activated cells upregulate the expression of surface molecules that would favor interactions with *Shigella* effectors involved in the entry process. For instance, the expression of CD44 and $\alpha_5\beta_1$ integrins is modulated upon lymphocyte activation (Kinashi, 2007; Ponta et al., 2003). IpaB, one of the effectors required for *Shigella* entry into EC, has been shown to interact with the CD44 receptor located within the lipid rafts (Skoudy et al., 2000), and cholesterol depletion impairs *Shigella* invasion (Lafont et al., 2002). IpaB also interacts with $\alpha_5\beta_1$ integrins like IpaC and IpaD, two other key players of the bacterial entry process into EC (Watarai et al., 1996). If $CD4^+$ T cells are invaded by *Shigella* in vivo, discrimination between activated and unactivated T cells would result in a preferential targeting of activated T cells in the lamina propria as opposed to the lymphoid follicules associated to the intestinal mucosa and their population of naive T cells.

We found that *Shigella* invasion and presumably injection of effectors in the absence of invasion into the T cell cytoplasm results in the inhibition of chemokine-induced migration of T lymphocytes. T cell trafficking is essential for efficient T cell functions. The ordered, directional migration of T lymphocytes is indeed a key process in their development, immune surveillance, and the immune response (Kunkel and Butcher, 2002). Inhibition of cell migration has been reported for other pathogens. For

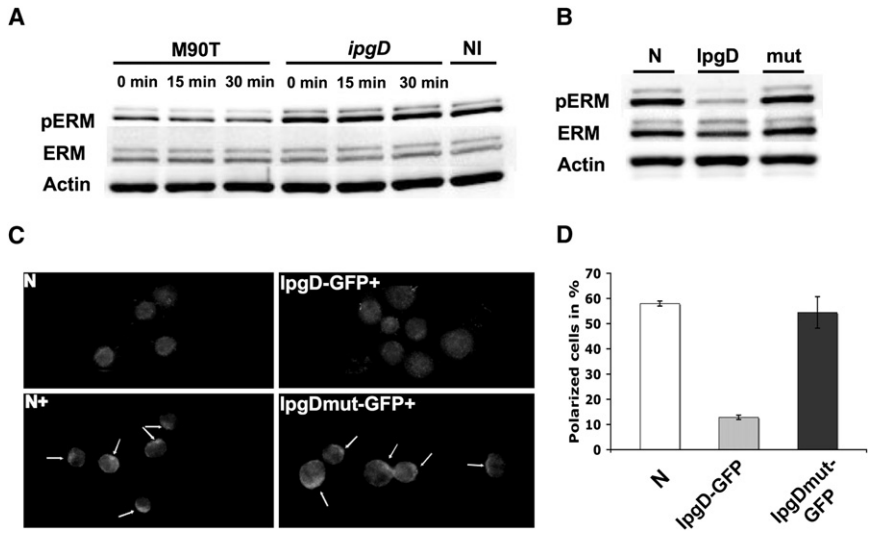

Figure 6. IpgD Induces ERM Dephosphorylation, Thus Blocking Chemokine-Induced Phospho-ERM Polarization in Jurkat Cells

Immunoblots using anti-ERM or anti-phosphoERM (pERM) antibodies of whole-cell lysates of Jurkat cells (A) infected with the strains M90T or *ipgD* at different time points postinfection or not infected (NI) or of Jurkat cells (B) expressing IpgD-GFP, IpgDmut-GFP, or not transfected (N). Jurkat cells were lysed 18 hr after transfection. Anti-ERM and anti-actin antibodies were used as loading controls. (C) pERM IF staining on Jurkat cells not stimulated and not transfected (N), CXCL12-stimulated and not transfected (N+), CXCL12-stimulated and expressing IpgD-GFP, CXCL12-stimulated and expressing IpgDmut-GFP. White arrows show localization of pERM at one pole of the cell. (D) Quantification of the cells displaying pERM polarization as observed in (C). For quantification, 150 cells were counted per condition and experiment. Results are representative of three independent experiments. Error bar represents the standard error of the mean (±SEM).

instance, the adenylate cyclase toxin CyaA released by *Bordetella pertussis* inhibits T cell chemotaxis by interfering with chemokine receptor signaling through its cyclic AMP (cAMP)-elevating activity (Paccani et al., 2008). The HIV-1 protein Nef inhibits T cell chemotaxis in response to the ligand CXCL12 (Choe et al., 2002) by downmodulating LFA-1 expression on T cells, therefore diminishing adhesion and polarization of T cells, resulting in decreased migration across the endothelium (Park and He, 2009). In addition, Nef strongly induces phosphorylation of cofilin, thus inactivating this evolutionarily conserved actin-depolymerizing factor that promotes cell motility when unphosphorylated (Stolp et al., 2009). The hepatitis C envelope 2 protein inhibits T cell motility by targeting protein kinase C signaling upon LFA-1 integrin ligation (Volkov et al., 2006). Our data reveal another type of virulence effector and mechanism involved in the impairment of T cell migration.

To date, no TTSS effector has been demonstrated to be involved in dampening T cell chemotaxis. We provide evidence that IpgD-mediated inhibition of T cell migration is dependent on its phosphoinositol 4-phosphatase activity, which leads to hydrolysis of PIP_2 with a resulting decrease of its pool at the plasma membrane. In epithelial cells, IpgD-mediated PIP_2 cleavage is responsible for dramatic morphological changes that lead to a decrease in membrane tether force associated with membrane blebbing and actin filament remodeling (Niebuhr et al., 2002). As shown here, IpgD is not required for T cell invasion (Figure S1A). This is fully consistent with the fact that, although involved in the formation of the fully structured entry foci, IpgD is not involved in invasion of IECs (Niebuhr et al., 2002). As previously illustrated with the *Yersinia* TTSS effectors (Trosky et al., 2008), our results emphasize the extraordinary power of such an injection device to deliver a given effector with a particular enzymatic activity into different cell types, thereby triggering a diversity of outcomes to modulate the host immune response.

As to the mechanism involved, it is known that pathogens target the phosphoinositides (PIs) network (Bakowski et al., 2010; Smith et al., 2010). PI metabolism plays a key role in the regulation of receptor-mediated signal transduction, actin remodeling, and membrane trafficking in eukaryotic cells (De Matteis and Godi, 2004). Thus, it is not surprising that several intracellular bacterial pathogens modulate and exploit PI levels, directly or indirectly, to ensure their survival and efficient intracellular replication. Like *Shigella*, *Salmonella enterica*, *Mycobacterium tuberculosis*, and some *Escherichia coli* secrete effectors mimicking mammalian phosphatases. For example, the *Salmonella* effector SopB shares similarity with mammalian PI4P and PI5P phosphatases (Marcus et al., 2001; Norris et al., 1998) mainly in the catalytic domain (Ungewickell et al., 2005). SopB was found to diminish specifically the cortical PIP_2 pool, thus destabilizing cytoskeleton-plasma membrane interactions (Terebiznik et al., 2002). Viruses also exploit PIs, as exemplified by the HIV Tat protein that binds with a high affinity to PIP_2, resulting in the perturbation of the PIP_2-mediated recruitment of cellular proteins to the plasma membrane (Rayne et al., 2010). However, *Shigella* is the first pathogen to be reported to provoke a massive and sustained dephosphorylation of phospho-ERMs by means of IpgD-mediated PIP_2 hydrolysis. Acute ERM protein inactivation plays a critical physiological role in lymphocytes. Lymphocyte recirculation from blood into tissues and then back into blood is crucial for efficient adaptive immune responses. While in blood, the cytoskeleton of the lymphocyte assures that it is spherical and relatively rigid, allowing it to survive the hemodynamic stress of circulation. Regulated binding to the vascular endothelium and migration into tissue are triggered by chemokines on the endothelial surface that activate G protein-coupled receptors (GPCRs) on the lymphocyte. One very rapid consequence is global reorganization of the cytoskeleton into a configuration appropriate for a flexible migration-capable cell. Because ERMs provide a conformationally regulated connection from the cortical actin cytoskeleton to the plasma membrane (Niggli and Rossy, 2008), rapid conversion of ERMs from their active to inactive conformations plays a key role in this process (Charrin and Alcover, 2006). The

importance of PIP_2 in ERMs function was just highlighted using a recently devised strategy for inducing rapid hydrolysis of PIP_2. The authors showed that PIP_2 hydrolysis in itself is sufficient to induce ERMs dephosphorylation, therefore proposing a key role of PIP_2 in ERM protein biology, namely hydrolysis-mediated ERM inactivation. In fact, our results show that *Shigella*, via IpgD, triggers this hydrolysis-mediated ERM inactivation. In addition, our data reveal that interfering with PI metabolism does not only help pathogens to improve their ability to enter or survive into host cells but also offers the opportunity, depending on the targeted host cell type, to modulate the host immune response by affecting cell migration.

Although we cannot formally exclude the possibility that additional mechanisms might be involved, our data suggest that alteration of ERMs dynamics is the key process. First, we did not detect any difference in the expression levels of the CXCL12 receptor, CXCR4, in Jurkat cells infected with wild-type *Shigella* or the mutant *ipgD*, suggesting that regulation of chemokine receptor expression is not involved (data not shown). Second, no difference in T cell migration was observed between the *ipgB1*, *ipaA*, *icsB*, *ipaC/ipaC351* mutants (Allaoui et al., 1992; Hachani et al., 2008; Menard et al., 1993; Mounier et al., 2009) when compared to wild-type *Shigella* (Figure S1B). Since IpaA, IpaC, IpgB1, and IcsB target different pathways involved in actin cytoskeleton rearrangement in EC (Tran Van Nhieu and Sansonetti, 1999), these data indicate that those pathways are not involved in the inhibition of T cell chemotaxis.

In conclusion, besides the indirect manipulation of the T cell-mediated immunity due to the proinflammatory environment induced by *Shigella*, leading to a predominant priming of *Shigella*-specific Th17 cells (Sellge et al., 2010), we are elucidating a strategy in which the microorganism has the capacity to directly interfere with T lymphocytes, and in particular with their dynamics. Our findings suggest that direct manipulation can occur via bacterial invasion of T cells or injection of effectors into T cells without invasion. As for the latter, one may easily imagine the efficiency of a strategy consisting of injecting effectors as soon as bacterium-cell contacts occur without the need of invading the cell, a surprising observation in view of the *Shigella* intracellular lifestyle. The importance of an "injection-only"-based pathway to "freeze" the host immune response has been underestimated so far and deserves further investigation. Whether *Shigella* via IpgD impairs T cell migration in vivo, and the consequence in terms of priming of *Shigella*-specific immunity, will be further analyzed. Interestingly enough, preliminary results indicate that upon crossing the intestinal barrier and reaching the mucosa-associated lymphoid follicles, *Shigella* can invade T cells in vivo (Figure S5). We provide insights into the understanding of the manipulation of T cell-mediated immunity by *Shigella* that leads to the poor priming of short-lasting protective immunity during natural infection in humans.

EXPERIMENTAL PROCEDURES

Bacterial Strains

The following *Shigella* strains were used: M90T, the *Shigella flexneri* 5a wild-type strain (Sansonetti et al., 1982); SF401, the *mxiD* mutant (M90T-Δ*mxiD*); SF701, the *IpgD* mutant (M90T-Δ*IpgD*); SF709 (M90T-Δ*ipgD*-pAB17) (Allaoui et al., 1993a, 1993b); GFP-expressing M90T, and GFP-expressing Δ*mxiD* (Jaumouille et al., 2008). Δ*mxiD* and M90T-expressing IpgD fused to β-lactamase were generated by transfecting the strains with a pBAD18 vector (Guzman et al., 1995), encoding for the chloramphenicol resistence gene, full-length IpgD, and TEM-1 (accession number AB282997, residues 24–286) introduced into the multiple cloning site using NheI and XbaI sites (for IpgD) and XbaI and HindIII (for TEM-1). Bacteria were grown at 37°C on trypticase soy (TCS) (Becton Dickinson) agar plates containing 0.01% Congo red (Serva). For cell invasion, a Congo red-positive colony was picked for an overnight (O/N) culture at 37°C in TCS medium, followed by a subculture in the same medium to grow the bacteria to logarithmic phase. Bacterial concentrations were calculated from the optical density of the culture at 600 nm.

Plasmid DNA Purification

E. coli DH5α strains (Invitrogen) containing the eukaryotic expression vectors were grown O/N in LB medium with corresponding antibiotic at 37°C. The EndoFree Plasmid Maxi Kit (QIAGEN) was used for the purification of plasmid DNA, according to the manufacturer's recommendations. The following plasmids were used: pKN16, GFP-tagged IpgD- Kan^R (Niebuhr et al., 2002); mut-GFP, GFP-tagged IpgD- Kan^R with a point mutation of Cys438 changed into Ser (Niebuhr et al., 2002); GFP, EGFP-Kan^R (Niebuhr et al., 2002); and mRFP-PLCδ1PH- Amp^R, mRFP-tagged PH domain of PLCδ1 (kind gift from Dr. T. Balla) (Varnai and Balla, 1998).

T Cell Lines and Primary T Cells

Jurkat, Clone E6-1 cells (ATCC TIB-152) were used as a human T cell line. Human, monocyte-depleted, peripheral blood mononuclear cells (PBMCs) were collected from blood samples. Informed consent was obtained from donors according to French ethical laws and agreement between Institut Pasteur and "Centre National de Transfusion Sanguine." Human CD_4^+ T cells were isolated with the CD_4^+ T cells isolation kit II (Milteny Biotec), according to the manufacturer's recommendations. Jurkat T cells, human PBMCs, and purified CD_4^+ T cells were cultured in RPMI medium (RPMI 1640 GIBCO, Invitrogen) supplemented with 10% decomplemented fetal bovine serum (FBS) (Biowest), 100 U/ml penicillin (Sigma-Aldrich), and 100 μg/ml streptomycin (Sigma-Aldrich), at 37°C with 5% CO_2. For activation, T cells were incubated for 3 days with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich). The cell concentration was determined by using a Malassez counting chamber.

T Cell Infection and Gentamicin Assay

For infection with *Shigella*, T cells were centrifuged for 10 min at 300 g, resuspended in prewarmed RPMI medium without FCS, and seeded in round-bottomed 96-well plates (TPP) at a concentration of 3×10^5 cells in 100 μl per well. Bacteria were centrifuged for 10 min at 4000 g and resuspended in RPMI medium without FCS. A bacterial concentration of 6×10^7 bacteria/ml was used for an moi of 10. T cell infection started by adding 50 μl of the bacterial solution to the cells, followed by a centrifugation of 5 min at 300 g, and incubated at 37°C in 5% CO_2. For the quantification of intracellular bacteria, extracellular bacteria were killed by gentamicin treatment (Sigma-Aldrich) at concentration of 50 μg/ml 1 hr postinfection. At indicated time points, cells were lysed with 0.5% sodium desoxycholate and dilutions plated. Enumeration of the bacteria was performed after O/N incubation at 37°C.

Cell Transfection

Jurkat cells were transfected by electroporation using the Amaxa Cell Line Nucleofector Kit V (Lonza). The cells were electroporated with 10 μg DNA encoding IpgD-GFP or IpgDmut-GFP and 5 μg DNA encoding PH-PLCδ1-RFP per 5×10^6 cells using the Nucleofector Device electroporator with the Nucleofector Program X-005 (Lonza). After electroporation, cells were incubated in RPMI containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% FBS for 1 day at 37°C.

FACS Analysis

For cell death quantification, cell suspensions were incubated for 1 min with propridium iodide (PI) (dilution 1:1000 of the stock solution) (Sigma-Aldrich) and FACS analysis performed to detect the PI-positive cell population.

Immunofluorescence Staining

T cells were grown and infected as described above, using GFP-expressing bacteria. After 1 hr of infection, T cells were fixed for 15 min with 4% paraformaldehyde (PFA) in PBS. Samples were transferred onto glass coverslips precoated with 10 μg/ml poly-L-lysine and centrifuged for 1 min at 300 g. After PBS washing, cells were incubated for 30 min with rabbit polyclonal serum specific for *Shigella*-LPS (collection of the laboratory) (dilution 1:100 in PBS). The cells were washed with PBS and incubated for 30 min with anti-rabbit-Alexa Fluor 350 antibodies diluted 1:100 in PBS (Invitrogen). Cell permeabilization for actin staining was done with 0,1% Triton-X100 for 5 min, followed by washes with PBS and incubated for 30 min with phalloidine-rhodamine. The coverslips were mounted using ProLong-mounting medium (Invitrogen). Immunofluorescence pictures were acquired either by inverted widefield (Carl Zeiss Inc.) or confocal microscopy (SP5, Leica) under oil immersion.

Migration Assay

T cell migration was performed as previously described (Ottoson et al., 2001) with the addition of gentamicin treatment to kill extracellular bacteria. For transfected Jurkat cell, no gentamicin was used. The migration index corresponds to the percentage of migrated cells at a particular condition normalized to the percentage of migrated cells in the noninfected control in the absence of CXCL12 (negative control). Hence, a migration index of 1 corresponds to that of the negative control. For the migration assay with transfected Jurkat cells, only GFP-positive cells were taken into account.

Western Blotting

Whole T cell lysates were obtained as previously described (Ottoson et al., 2001) from 10^6 cells. SDS-PAGE (8%) and immunoblotting were performed as previously described (Kufer et al., 2006) with anti-phospho-ERM and anti-ERM Abs (Cell Signal Technology), peroxydase-conjugated goat anti-rabbit Ab (Nordic Immunology) and the Bio-Rad Substrate ECL kit (Bio-Rad) followed by chemiluminescence, according to the manufacturer's instructions. To reuse the membrane after immunoblotting, Bio-Rad stripping buffer (Bio-Rad) was used according to the manufacturer's instructions.

Phospho-ERM Localization

Transfected Jurkat cells were starved for 1 hr in serum-free RPMI medium. Cells (5×10^5 in 500 μl) cells were then transferred into 12-well plates containing poly-L-lysin-precoated coverslips. Cells were allowed to settle down for 20 min at 37°C. CXCL12 (Preprotech) (200 ng/ml final concentration) was added for 45 s at 37°C, 5% CO_2. Cells were fixed by adding ice-cold 4% PFA for 15 min. After PBS washings, samples were blocked for 10 min with PBS-BSA (1 mg/ml) and then incubated for 1 hr with mouse anti-human-CD28 (Biolegend) (diluted 1:100 in PBS-BSA). After PBS washings, samples were incubated with anti-mouse-Cy3 (Jackson Medicorp Inc) Ab (diluted 1:100 in PBS-BSA). Cells were permeabilized for 5 min with 0.1% Triton X-100, blocked for 10 min with PBS-BSA, and rabbit anti-Phospho-ERM Ab (Cell Signal Technology) added for 1 hr (diluted 1:100 in PBS-BSA). Anti-rabbit-Alexa Fluor 647 (Invitrogen) diluted 1:100 in PBS-BSA was added for 1 hr. Coverslips were mounted on glass slides using ProLong mounting medium.

Injection Assay

Previous to the injection experiments, the bacterial strains M90T and *mxiD* IpgD-bla were tested for secretion of the fusion protein as previously described (Mounier et al., 1997) and the β-lactamase enzymatic activity tested by cleavage of Nitrocefin. For this, bacterial cytosolic extracts were obtained by shaking bacteria with glass beads (<106 μm from Sigma) for 10 min at 30 s^{-1} at 4°C. Samples were then centrifuged at 13,500 rpm for 5 min at 4°C. Twenty microliters of extracts were incubated with 100 μl Nitrocefin (0.1 mM) for 30 min at room temperature and the absorbance was measured at 486 nm. For the infection experiments, the optimal cytochalasin D concentration to fully inhibit invasion of T cells by *Shigella* was titrated using the gentamicin assay described above. Jurkat T cells were centrifuged for 10 min at 300 g, resuspended in EM buffer (120 mM NaCl, 7 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5 mM glucose, and 25 mM HEPES [pH 7.3]) containing Solution B (according to the manufacturer's recommendation, Invitrogen), 2 μM Probenecid, 5 μg/ml cytochalasin D, and 1 μM CCF4 (Invitrogen) and seeded in round-bottomed 96-well plates (TPP) at a concentration of 3×10^5 cells in 100 μl per well. Loading was carried out for 1 hr at RT in the dark. Subsequently, cells were centrifuged and resuspended in 100 μl RPMI containing 2 μM Probenecid and 5 μg/ml Cytochalasin D. Infection was performed as described above with the M90T IpgD-bla and *mxiD* IpgD-bla strains at an moi of 500, and 100 μg/ml gentamicin was added after 20 min. After an additional incubation of 2 hr, cells were washed with PBS and transferred to flow cytometry tubes for analysis. Data acquisition was carried out with a CyAn ADP flow cytometer (DakoCytomation). Live, single cells were gated, and the fluorescence intensities of cleaved and uncleaved CCF4 were detected with the 405 nm excitation laser and 450 nm and 535 nm emission filters.

Image Analysis

Analysis was performed using the public domain Image J64 program (version 1.42q NIH) as previously described (Kong et al., 2006). Briefly, the fluorescence intensity was measured in cells as the average pixel fluorescence intensity within an area of defined size drawn over four distinct areas of the plasma membrane (2 × 10 pixels) or the average of one box in the cytosol (11 × 11 pixels). Ratios of plasma membrane to cytosolic pixel fluorescence intensity were determined and subjected to statistical analysis.

Statistical Analysis

The t test was used to compare two groups, and p values < 0.05 were considered statistically significant. Significant statistically differences were indicated by asterisks: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The error bars represent the standard error of the mean (SEM).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article at doi:10.1016/j.chom.2011.03.010.

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