The adaptor molecules LAT and SLP-76 are specifically targeted by *Yersinia* to inhibit T cell activation

Christiane Gerke, Stanley Falkow, and Yueh-hsiu Chien

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305

T cell responses are critical to the survival of *Yersinia*–infected animals. *Yersinia* have the ability to directly suppress T lymphocyte activation through the virulence factor YopH, a tyrosine phosphatase. Using single cell video microscopy and FACS analysis, here we show that even an average of one *Yersinia* per T cell is sufficient to inhibit or alter T cell responses. This efficient inhibition is traced to specific targeting by YopH of the adaptor proteins, linker for activation of T cells (LAT) and SH2-domain–containing leukocyte protein of 76 kD (SLP-76), which are crucial for T cell antigen receptor (TCR) signaling. A catalytically inactive YopH translocated via the type III secretory pathway from the bacteria into T cells primarily binds to LAT and SLP-76. Furthermore, among the proteins of the TCR signaling pathway, the tyrosine phosphorylation levels of LAT and SLP-76 are the most affected in T cells exposed to low numbers of *Yersinia pseudotuberculosis*. This is the first example showing that a pathogen targets these adaptor proteins in the TCR signaling pathway, suggesting a novel mechanism by which pathogens may efficiently alter T cell–mediated immune responses.

CORRESPONDENCE
Yueh-hsiu Chien:
chien@stanford.edu

Abbreviations used: FAK, focal adhesion kinase; Gads, Grb2-like adaptor protein downstream of Shc; GST, glutathion *S*-transferase; HA, hemagglutinin; HRP, horseradish peroxidase; LAT, linker for activation of T cells; MCC, moth cytochrome *c*; MOI, multiplicity of infection; PH, pleckstrin homology; PI3K, phosphoinositide 3–kinase; SLAP-130, SLP-76–associated phosphoprotein of 130 kD; SLP-76, SH2-domain–containing leukocyte protein of 76 kD; YFP, yellow fluorescent protein.

The success of a pathogen in establishing an infection is dependent on its ability 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 (for review see reference 1). Yet, it is clear that the T cell response remains essential for the clearance of bacterial pathogens, including the pathogenic members of the genus *Yersinia* (2, 3). The genus *Yersinia* includes *Yersinia pestis*, the causative agent of plague, as well as *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. The latter two cause gastroenteritis and lymphadenitis and, in susceptible individuals, the infections have also been associated with the development of reactive arthritis (4).

Yersinia's virulence strategy is to modify host cell functions by injecting several effector proteins (the *Yersinia* outermembrane proteins, Yops) by a type III secretory pathway into the host cell upon contact (5). Studies of the in vitro effects of the Yops on macrophages and epithelial cell lines have been extensive (for reviews see references 5, 6). During the course of infection, *Yersinia* are also likely to encounter lymphocytes as the bacteria colonize and multiply extracellularly in the Peyer's patches, lymph nodes, and subsequently in the spleen and liver. T cells as well as macrophages are present in *Yersinia*-induced lesions in spleen and liver (2). We found that T cells transiently exposed to *Yersinia* are inhibited in their ability to be activated through the TCR and, thus, cannot mount subsequent effector functions (7). This is one of the first examples of a bacterial pathogen that directly inhibits T cell activation.

The virulence factor YopH, a potent phosphatase (8–10), was primarily responsible for this inhibitory effect (7). Surprisingly, YopE, YopT, and YpkA that are known to disrupt actin filaments leading to alterations in the cytoskeleton of both phagocytes and epithelial cells (5, 6) have no effects on T cells despite the fact that a redistribution and reorientation of the cytoskeleton occurs during lymphocyte activation. Furthermore, although the virulence factor YopJ was found to induce apoptosis in macrophages in vivo (11) and to suppress TNF-α and IL-8 production in macrophages in vitro (5, 6), it has no effect on T cells or TCR-mediated cytokine secretion. Thus, it

appears that T lymphocyte functions are specifically targeted by YopH.

YopH was shown in in vitro assays to block the oxidative burst of macrophages (12), to inhibit calcium signaling in neutrophils (13), to induce disassembly of focal adhesions in epithelial cells (14, 15), and to inhibit phagocytosis (16). These studies and our own previous investigation were all performed with a rather high multiplicity of infection (MOI) of >50. Yet, in vivo the bacteria to host cell ratio must be low, at least at the time of initial infection. Therefore, we set out to determine the efficacy and mechanism of YopH's inhibition of T cell activation.

Here, we report that YopH alters T cell function even at a level of just one bacterium per T cell. Consistent with such high efficiency, we show that the adaptor molecules linker for activation of T cells (LAT) and SH2-domain–containing leukocyte protein of 76 kD (SLP-76) that are phosphorylated in response to TCR stimulation and are essential for T cell activation are primary targets of YopH. We find that although YopH has a broad targeting capability, it is quite specific in an infection where the bacteria/target cell ratio is low.

RESULTS

Yersinia inhibit T cell responses efficiently

To determine the efficacy of YopH's inhibition of TCR-mediated activation, we analyzed the calcium flux of *Y. pseudotuberculosis*–exposed T cells as well as the recruitment of phosphoinositide 3–kinase (PI3K) to the immunological synapse, which both are important early events in T cell signaling (17–21).

T cell blasts prepared from 5C.C7 αβ TCR (specific for moth cytochrome *c* [MCC] peptide/I-E^k) transgenic mice (22) were exposed to wild-type *Y. pseudotuberculosis* at MOIs of 1, 2, 5, 10, 20, and 50 for 1 h and concurrently loaded with the calcium-sensitive dye indo-1 for 30 min. Their ability to flux calcium in response to CD3ε cross-linking was analyzed by flow cytometry. Ratiometric analysis showed that the calcium flux of a T cell population exposed to an MOI of 1 was substantially reduced (Fig. 1 A). Incubations with higher MOIs resulted in a delayed response and a further reduction of the intracellular calcium flux levels. Consistent with our previous finding that YopH is the primary virulence factor affecting T cell responses (7), we found no inhibition of calcium flux when T cells were exposed to a *Yersinia* mutant expressing a catalytically inactive YopH, even at an MOI of 50 (Fig. 1 A). The catalytically inactive YopH stably traps its targets and thereby likely impairs downstream events. However, because only a limited mount of YopH is translocated into the host cell even at an MOI of 50, a large number of phosphorylated signaling proteins is not bound to the substrate trap (unpublished data).

Analysis of calcium levels in individual cells at the peak of calcium flux (Fig. 1 A, asterisks) showed that both the number of cells fluxing calcium and the magnitude of the calcium flux are reduced in T cell populations exposed to *Y. pseudotuberculosis* (Fig. 1 B). The dose response curve fits the model of a Gaussian distribution of the number of bacteria encountering the T cells where one bacterium per T cell is sufficient to inhibit the T cell response. Similar results were obtained using the human Jurkat T cell line (unpublished data).

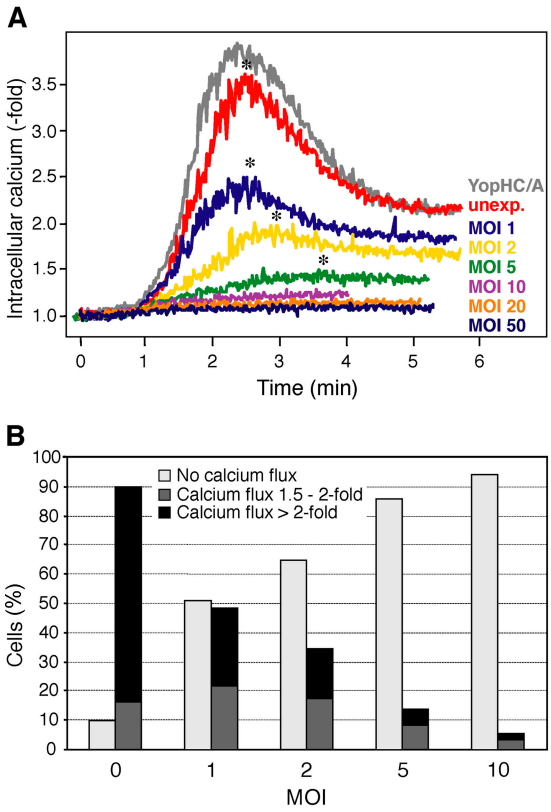

Figure 1. TCR-stimulated calcium flux in 5C.C7 T cell blasts exposed to different MOIs of wild-type *Y. pseudotuberculosis*. (A) Calcium flux of 5C.C7 T cell blasts either unexposed (unexp.) or exposed to wild-type *Y. pseudotuberculosis* at MOIs of 1, 2, 5, 10, 20, and 50 or to *yopHC403A-HA* (YopHC/A) at an MOI of 50, loaded with the calcium-sensitive dye indo-1 and activated through the TCR by the addition of anti-CD3ε at time 0, was measured by flow cytometry. The calcium flux kinetics are plotted as the median of the intracellular calcium levels in the population measured in three independent experiments versus time. (B) Relative number of cells at the peak of the population's calcium flux (as marked by asterisks in A) that show no significant calcium flux or intracellular calcium levels of 1.5-fold or 2-fold higher than the initial levels.

To evaluate the response of individual cells, the calcium flux of fura-2 (a calcium-sensitive dye) loaded 5C.C7 T cell blasts exposed to *Yersinia* at different MOIs and stimulated by murine CH27 B cells presenting the MCC peptide was analyzed using video fluorescence microscopy (Fig. 2). As seen before (Fig. 1), with increasing MOI, an increasing percentage of the cells could no longer flux calcium. Interestingly, a subset of exposed T cells (~10% at an MOI of 1 and 50% at an MOI of 10) exhibited a "sparking" phenotype where the initial increase of intracellular calcium was almost at the level of stimulated unexposed T cells, but was followed by a rapid decline to the basal level in ~5 min. In contrast, unexposed T cells (Fig. 2) or T cells incubated with a mutant strain of *Y. pseudotuberculosis* expressing inactive YopH (not depicted) maintained their elevated calcium concentrations for >10 min.

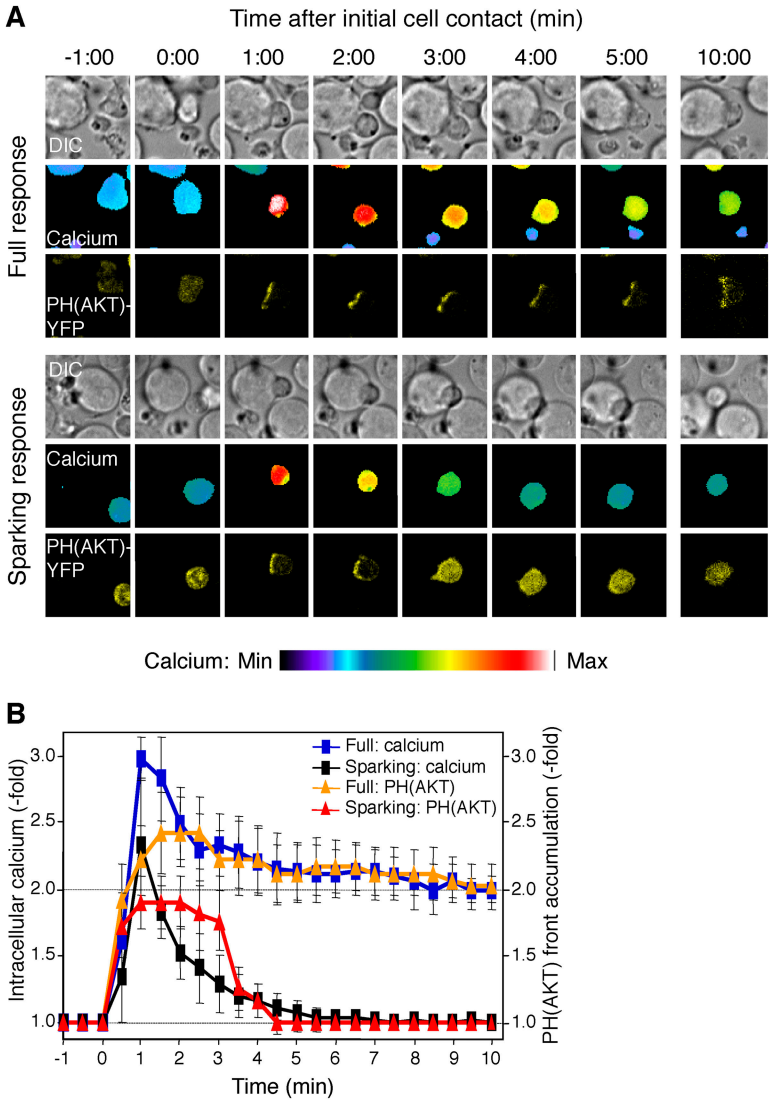

Figure 2. Antigen-stimulated calcium flux and PI3K accumulation in *Yersinia*-exposed T cells. (A) 5C.C7 T cell blasts expressing PH(AKT)-YFP were exposed to wild-type *Y. pseudotuberculosis* or left unexposed, loaded with the calcium sensitive dye fura-2, mixed with MCC peptide presenting CH27 cells, and analyzed by video microscopy. The differential interference contrast (DIC), the fura-2 ratio in ratio-dependent false color (calcium), and a midplane section of the YFP z-stack (PH[AKT]-YFP) are shown over a time frame of 6 min and at 10 min after contact. The full antigen-stimulated response in calcium flux and PI3K activity was observed in unexposed 5C.C7 T cell and the sparking response was observed in *Y. pseudotuberculosis*–exposed cells. Image quality of the PH(AKT)-YFP accumulation was improved by using a blind deconvolution algorithm to remove out-of-focus light from the z-stack. (B) Images of 15 cells of each phenotype were analyzed using the Metamorph program. Averages of intracellular calcium levels and PH(AKT)-YFP front-accumulation were plotted relatively to their corresponding base levels (-fold) versus time. Error bars represent standard deviation. Data were acquired at intervals of 30 s over a time frame of 10 min.

PI3K activity was assayed indirectly by expressing the pleckstrin homology (PH) domain of AKT (also known as protein kinase B) fused to the yellow fluorescent protein (YFP) (PH[AKT]-YFP) in T cells as described by Huppa et al. (21). The binding of 3′-phosphoinositides (the products of PI3K) by the PH domain results in an accumulation of PH(AKT)-YFP at sites of PI3K activity (20, 21). In unexposed 5C.C7 T cell blasts expressing PH(AKT)-YFP, antigen stimulation resulted in a rapid and sustained accumulation at the immunological synapse (front-accumulation) of PH(AKT)-YFP (Fig. 2). In *Y. pseudotuberculosis*–exposed T cell blasts, PH(AKT)-YFP accumulation was affected. T cells that did not respond to antigen-presenting cells by elevating their level of intracellular calcium also did not accumulate PH(AKT)-YFP. Observations that YopH inhibits PI3K-dependent T cell proliferation and IL-2 production (23) are consistent with this result. Exposed T cells exhibiting the sparking calcium flux also showed a transient front-accumulation of PH(AKT)-YFP. These results demonstrate that low numbers of *Y. pseudotuberculosis* can either abolish or alter T cell activation. Infection with *Yersinia* strains lacking a catalytically active YopH did not have any inhibitory effect (un-

published data). The simultaneous inhibition of calcium flux and PI3K activity suggests that YopH targets a common step in the signaling cascade upstream of PI3K and phospholipase C-γ1, which is essential for the calcium flux. Partial inhibition of this step may lead to the sparking phenotype.

Phosphorylated LAT and SLP-76 in activated T cells are primarily targeted by YopH

After T cell activation through the TCR, the TCR CD3ζ chains, antigen receptor-associated tyrosine kinases, such as Lck and ZAP-70, and adaptor/linker proteins, including LAT and SLP-76, are phosphorylated immediately. This precedes all downstream signaling events including calcium flux and the activation of PI3K. To understand how YopH inhibits TCR signaling, we followed up on our previous finding that, in activated Jurkat T cells exposed to *Y. pseudotuberculosis* at an MOI of 50, most of the TCR signaling–associated molecules were either not phosphorylated, or hypophosphorylated (7). To test whether YopH broadly targets most members of the TCR signaling complexes, or selectively few members of the complex whose lack of phosphorylation would lead to the hypophosphorylation of other members of the TCR signaling complex and the inability of inducing calcium flux and PI3K activity, we analyzed the tyrosine phosphorylation patterns of activated T cells exposed to wild-type *Y. pseudotuberculosis* at lower MOIs. Initial experiments were performed in Jurkat cells. T cell activation of primary murine 5C.C7 T cells and the human Jurkat T cells are similar in their sensitivity to *Y. pseudotuberculosis* infection (unpublished data). However, the biochemistry of antigen receptor signaling complexes is well characterized in Jurkat cells and mutant Jurkat lines defective in the TCR signaling pathway are available.

As shown in Fig. 3, even in activated Jurkat cells exposed to *Y. pseudotuberculosis* at an MOI of only 1.5, the overall tyrosine phosphorylation in response to TCR stimulation was reduced. The most substantial decrease was observed for phosphorylated LAT and SLP-76 (Fig. 3, A, lanes 2 and 4, and B). In contrast, Lck, Tyr394, CD3ζ, and ZAP-70, which are phosphorylated earlier in the signaling cascade, and SLP-76–associated phosphoprotein of 130 kD (SLAP-130; an adaptor protein binding to SLP-76, also known as Fyb, or ADAP) were not as affected. This trend continued in activated Jurkat cells exposed at an MOI of 5. After exposure at an MOI of 15, most TCR signaling proteins were uniformly hypophosphorylated, but Tyr394-phosphorylated Lck was still clearly detectable. The efficient dephosphorylation of TCR signaling proteins is consistent with the results that

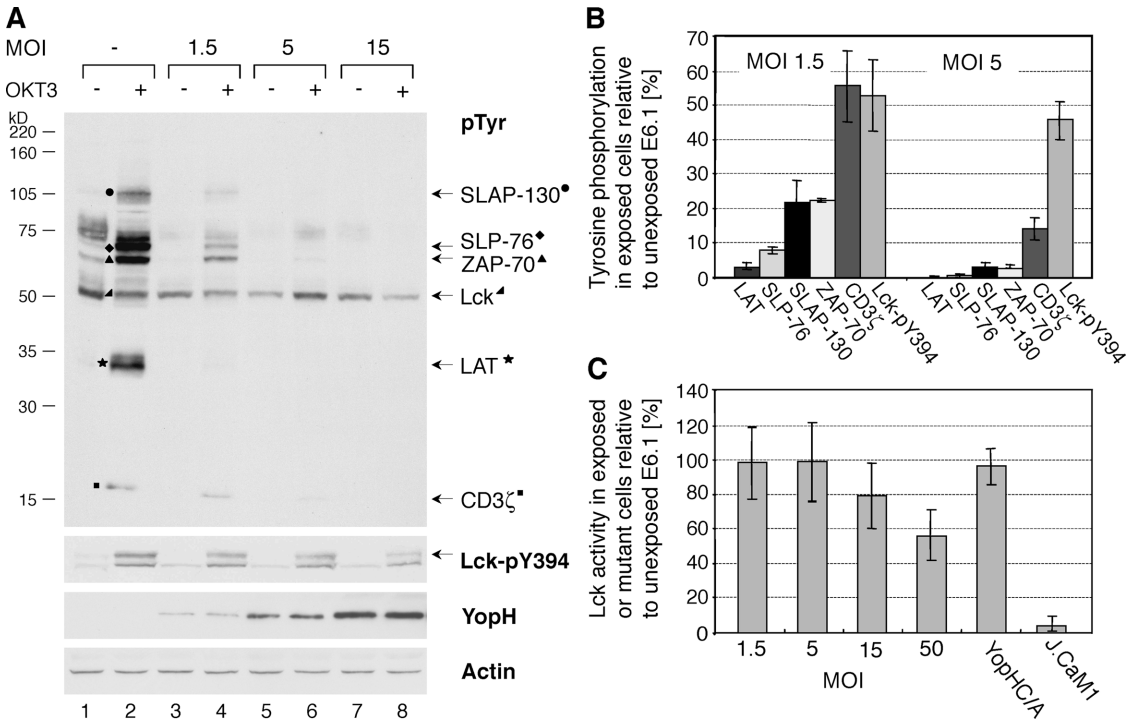

Figure 3. Inhibition of tyrosine phosphorylation in Jurkat T cells exposed to wild-type *Y. pseudotuberculosis*. Jurkat T cells were exposed to *Y. pseudotuberculosis* at MOIs of 1.5, 5, and 15 for 1 h or left unexposed (−). Total lysates of 4×10^5 resting (−) or OKT3-activated (+) T cells were analyzed by antiphosphotyrosine blotting (pTyr). Subsequently, blots were stripped and reprobed with antibodies reacting with phosphotyrosine-394-Lck (Lck-pY394), YopH, and actin. Labeled proteins were identified by reprobing with the corresponding antibodies. A representative result of three independent experiments is shown. (B) Amounts of tyrosine phosphorylated proteins were quantified in three independent experiments using the NIH Image. Error bars represent standard deviation. (C) Kinase activity of Lck in E6.1 Jurkat T cells unexposed or exposed to wild-type *Y. pseudotuberculosis* at MOIs of 1.5, 5, 15, and 50 or to *yopHC403A-HA* (YopHC/A) at an MOI of 50 was measured by ELISA in three independent experiments. Lck-deficient J.CaM1 Jurkat cells were used as negative control. Error bars represent standard deviation.

calcium flux and PI3K activity of T cells are efficiently altered by exposure to *Y. pseudotuberculosis* (Figs. 1 and 2). Recently, it was demonstrated that YopH inhibits the activity of recombinant Lck in vitro and that Tyr394-phosphorylated Lck is a target of YopH in in vitro immunoprecipitations with relatively high amounts of YopH (24). This has led to the hypothesis that YopH prevents T cell antigen receptor signaling by inhibiting Lck as the first step in the signaling cascade. Therefore, we analyzed Lck activity in *Yersinia*-exposed T cells. As shown in Fig. 3 C, no difference in the activity of Lck in T cells exposed to low MOIs of *Yersinia* (MOIs 1.5 and 5) or unexposed cells was observed, indicating that the hypophosphorylation of signaling molecules at these MOIs does not result from a reduction of Lck activity. Only at higher MOIs (15 and 50), we did find an inhibition of Lck activity. The results indicate that although YopH appears to have the ability to target multiple proteins in T cells at higher MOIs, LAT and SLP-76 or molecules responsible for their phosphorylation, but not Lck, are primary targets of YopH at a low bacteria to T cell ratio.

YopH dissociates from its substrates after dephosphorylation. Catalytically inactive forms of YopH that forms stable complexes with its substrates (8) have been used as a substrate trap to identify its catalytic targets (14, 15, 24–27). It is well documented that signaling complexes in mammalian cells are organized in modules. Preferential sequestration and localization of molecules in the modules confer the specificity and the selectivity of activation. Thus, to identify YopH's targets in infected cells, we constructed two *Y. pseudotuberculosis* strains (designated as *yopHC403A-HA* and *yopHD356A-HA*) that can deliver a hemagglutinin (HA)-epitope tagged substrate trap form of YopH (YopHC403A-HA or YopHD356A-HA) into host cells through their type III secrectory pathway. This was to ensure that the translocated substrate-trap YopH would be similarly localized in T cells as wild-type YopH. The YopHC403A substrate trap has previously been used extensively to identify YopH targets in macrophages and epithelial cells (14, 15, 25–27). For some phosphatases though, mutation of the conserved aspartate (D356 in YopH) has been found to result in a more efficient substrate trap (28). We performed identical experiments and found no difference in any of the results. For simplicity, only the results obtained using *yopHC403A-HA* are presented. A *Y. pseudotuberculosis* strain that delivers YopH-HA (designated as *yopH-HA*) was also generated as a control.

Jurkat cells were exposed to wild-type, YopH-deficient (ΔYopH), *yopH-HA*, and *yopHC403A-HA Y. pseudotuberculosis*, followed by activation with the CD3ε-specific antibody OKT3. As shown in Fig. 4, wild-type– as well as *yopH-HA*–exposed Jurkat cells showed no observable phosphorylation in response to TCR stimulation (lanes 8 and 10), indicating that the HA-tag does not affect the activity of YopH. In contrast, exposure to ΔYopH or *yopHC403A-HA Yersinia* did not inhibit TCR signaling even at an MOI of 50 (lanes 4 and 6). Comparable amounts of YopH, YopH-HA, and YopHC403A-HA were present in the exposed T cells (Fig. 4, YopH).

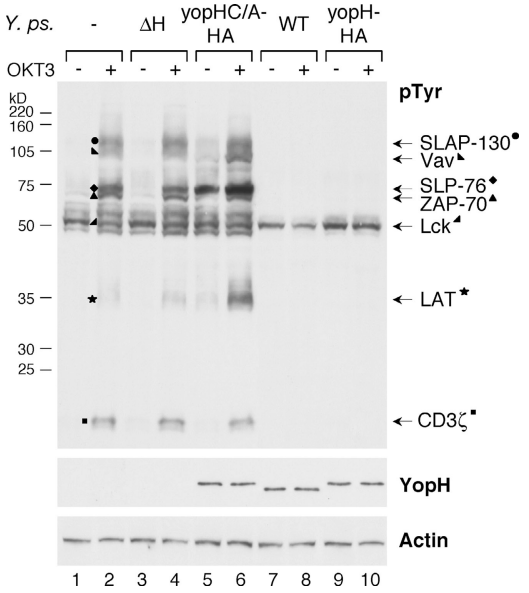

Figure 4. Analysis of the effects of YopH variants on TCR–stimulated tyrosine phosphorylation. Immunoblot analysis of resting (−) and OKT3-activated (+) Jurkat T cells that were either unexposed (−) or exposed for 1 h at an MOI of 50 of the following *Y. pseudotuberculosis* strains: WT, YopH-deficient (ΔH), *yopHC403A-HA* (yopHC/A-HA), and *yopH-HA* (yopH-HA). Total lysates of 4×10^5 T cells were first probed with antiphosphotyrosine antibodies (pTyr). Subsequently, the blot was stripped and reprobed with anti-YopH and anti-actin antibodies. Labeled proteins were identified by reprobing with the corresponding antibodies. A representative result of three independent experiments is shown.

The level of SLP-76 phosphorylation before TCR stimulation was much higher in Jurkat cells exposed to *yopHC403A-HA* at an MOI of 50 (lane 5) than in unexposed or ΔYopH-exposed cells (lanes 1 and 3). Also LAT, SLAP-130, and Vav, were also found to be slightly hyperphosphorylated before TCR stimulation and significantly hyperphosphorylated after TCR activation. It has been observed previously that binding of a phosphatase substrate-trap to its target protects it from endogenous phosphatases, resulting in a relative increase in the phosphorylated form of these molecules (28). These results suggest that phosphorylated LAT, SLP-76, SLAP-130, and Vav, rather than molecules responsible for their phosphorylation, are direct targets of YopHC403A-HA in T cells exposed at the MOI of 50.

Lck, CD3ζ, and ZAP-70 are phosphorylated earliest in the TCR-mediated signaling pathway. This leads to the phosphorylation of LAT and the recruitment of several proteins, including phospholipase C-γ1, PI3K, and Grb2-like adaptor protein downstream of Shc (Gads). SLP-76 is constitutively associated with Gads and, thus, is recruited to the TCR signaling complex and subsequently phosphorylated. Phosphorylated SLP-76 attracts more proteins, including SLAP-130 and Vav, to the protein assembly, referred to as the LAT/SLP-76 signalosome (19, 29, 30).

As shown in Fig. 5, a significant amount of phosphorylated SLP-76, and in addition phosphorylated LAT and SLAP-130, the unphosphorylated Gads and two unidentified

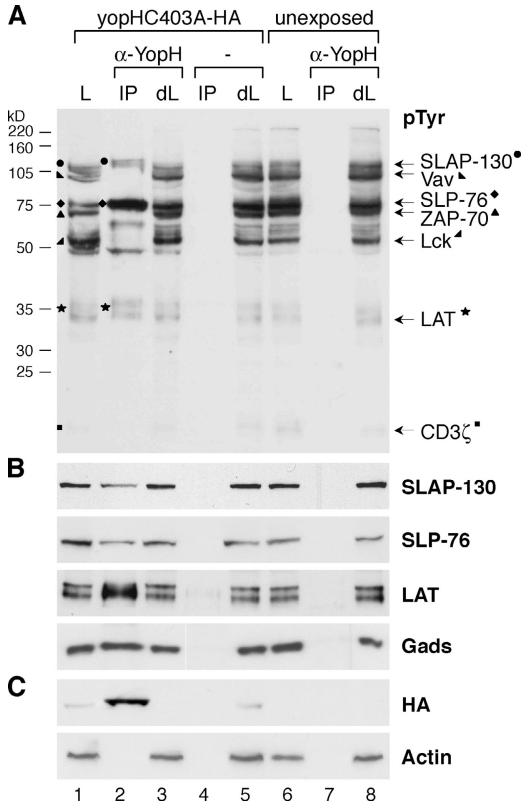

Figure 5. Substrate trap YopH associates with multiple components of the LAT/SLP-76 signalosome in activated Jurkat T cells. Detergent lysates of OKT3-activated Jurkat T cells exposed to *yopHC403A-HA Yersinia* at an MOI of 2 for 1 h, or left unexposed, were subjected to immunoprecipitation with affinity-purified anti-YopH antibodies (α-YopH) or protein G alone (−). (A) Antiphosphotyrosine blot of 4×10^5 T cell equivalents of the lysates before (L) and after (dL) immunoprecipitation and the immunoprecipitated complexes (IP) from 3×10^7 T cell equivalents. A representative result of at least three independent experiments is shown. (B) Blots were stripped and reprobed with a panel of antibodies to identify the precipitated proteins. Anti-SLAP-130, SLP-76, LAT, and Gads antibodies bound to proteins coprecipated with YopHC403A-HA (lane 2). The phosphorylated proteins of 50-kD and 65-kD size (A, lane 2) could not be identified. The white line indicates that intervening lanes have been spliced out. (C) Blots were reprobed with anti-HA-tag antibodies (to detect YopHC403A-HA) and actin antibodies.

phosphorylated proteins of ~50-kD and 65-kD size (lane 2) coprecipitated with YopHC403A-HA in lysates of activated Jurkat cells exposed to *yopHC403A-HA* at an MOI of 2. Phosphorylated Vav was present in the precipitate derived from T cells exposed at an MOI of 50 (unpublished data). Also a small amount of Lck was detected in YopHC403A precipitates from cells exposed to an MOI of 50. The precipitated 50- and 65-kD protein are likely to be part of the LAT/SLP-76 signalosome because phosphorylated proteins of 50- and 65-kD size also coprecipitated with LAT or SLP-76 from extracts of activated Jurkat cells (unpublished data).

Because the coupling of the integrin signaling pathway with the TCR activation pathway is known to be defective in Jurkat cells and *Yersinia* attachment was found to trigger the integrin signaling cascade in macrophages and epithelial cells, we assessed targets of YopHC403A-HA in 5C.C7 T cell blasts. We did not find focal adhesion kinase (FAK), paxillin, or $p130^{cas}$, which are primary targets of YopH in macrophages and epithelial cells (14, 15, 25, 26), nor did we find the closely related PYK2 (to FAK) and $p105^{CasL}$ (to $p130^{cas}$), but again found LAT, SLP-76, and SLAP-130 and two proteins of 50 and 65 kD to be targets (unpublished data).

To verify that binding of YopHC403A-HA to the coprecipitating proteins occurred in the T cells and was not a result of YopHC403A-HA binding to host proteins in the lysate, we incubated Jurkat T cells with a mutant *Yersinia* strain that is incapable of translocating YopH into eukaryotic cells, an *lcr* mutant (31) expressing YopH-HA. The infection did not affect TCR-stimulated tyrosine phosphorylation (Fig. 6 A), and we were not able to precipitate YopH-HA from lysates of the exposed T cells, even though the *lcr* mutant synthesized similar amounts of the epitope-tagged YopH compared with the wild-type strain (Fig. 6, B and C). These results showed that, under the experimental conditions we used for T cell lysis, there was no concomitant lysis of *Yersinia* and that the precipitated YopH protein was that which had been injected into T cells.

Although we did not detect CD3ζ or ZAP-70 in our coprecipitates when substrate trap YopH was injected into T cells by *Yersinia*, CD3ζ and ZAP-70 were found to coprecipitate with YopHC403A in in vitro immunoprecipitations when the protein was added to lysates of activated Jurkat cells in amounts similar to what is translocated by the bacteria at an MOI of 2 (unpublished data). Thus, YopHC403A appears to bind less specifically in vitro. Also when using WT YopH for in vitro immunoprecipitations, we found LAT, SLP-76, SLAP-130, ZAP-70, and CD3ζ, indicating

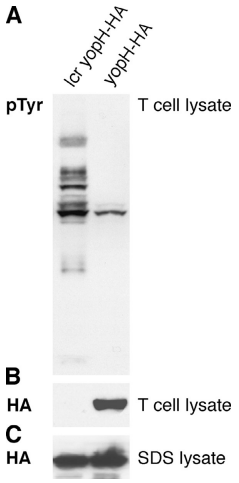

Figure 6. YopH precipitated from T cell lysates is YopH that had been injected. (A) Antiphosphotyrosine blot of total lysates of 4×10^5 OKT3-activated Jurkat T cells exposed to *yopH-HA* or a YopH-HA–expressing *lcr* mutant strain (*lcr yopH-HA*) at an MOI of 50 for 1 h. (B) Anti-HA blot of the anti-YopH immunoprecipitated proteins from lysates of 4×10^6 Jurkat cells was exposed and activated as described in A. (C) Anti-HA blot of lysates of 4×10^5 T cells was exposed as in A, but lysed by boiling in SDS sample buffer, which also lyses the bacteria.

that binding to these proteins is not limited to substrate trap YopH (unpublished data).

Because several phosphorylated proteins of the LAT/SLP-76 signalosome were found to precipitate with the substrate-trap YopH in exposed T cells, it was possible that the substrate trap YopH bound directly to every coprecipitated protein, or that it bound to one or a few of the proteins and precipitated the entire complex. To distinguish between these possibilities, we characterized YopH targets in Jurkat cells that were either SLP-76 deficient (J14; reference 32) or LAT deficient (J.CaM2; reference 33). In J14 cells, LAT and the 65-kD protein still coprecipitated with YopHC403A-HA (Fig. 7 A, lane 9). SLAP-130 and the 50-kD protein were barely detectable. This indicates that their association with YopHC403A-HA in wild-type T cells is mediated largely via SLP-76. In activated J.CaM2 cells, the lack of LAT expression resulted in a general reduction of phosphorylation level. Phosphorylated SLP-76 could not be detected in the T cell lysate. No phosphorylated proteins were found to coprecipitated with YopHC403A-HA (lane 7) after exposure to an MOI of 2. Because the signaling cascade from the T cell receptor is interrupted in these Jurkat mutants, we stimulated *yopHC403A-HA*–exposed Jurkat cells (MOI 2) with per-vanadate to increase the phosphorylation of signaling molecules (Fig. 7 B). The Lck-deficient Jurkat line J.CaM1 (34) was also included in these experiments. In activated J.CaM1 cells, the overall phosphorylation level was greatly reduced compared with wild-type Jurkat cells. However, SLP-76, LAT, the 65-kD protein, and SLAP-130 still coprecipitated with YopHC403A, indicating that the presence of Lck is not required for the binding of those proteins to YopHC403A. In the LAT-deficient Jurkat line, SLP-76, SLAP-130, and the 50- and 65-kD proteins were detected, indicating that the binding of YopHC403A to these proteins is not dependent on LAT. In addition, we detected ZAP-70 coprecipitating with YopHC403A in these experiments, suggesting that artificial hyperphosphorylation of signaling molecules allows YopH to bind to additional proteins.

Targeting of additional proteins by YopH could also be observed by in J.CaM2 cells exposed to *yopHC403A-HA* at an MOI of 50 and activated through the TCR. Under these conditions, phosphorylated SLAP-130 as well as phosphorylated CD3ζ and ZAP-70 coprecipitated with YopHC403A-HA, indicating that a loss of YopH specificity occurs when it is present at high concentration. This finding correlates with the observation that at high MOIs, CD3ζ and ZAP-70 were dephosphorylated by *Y. pseudotuberculosis* (Fig. 3), as were most of the TCR signaling proteins. These results indicate that, whereas YopH has a preference for LAT and SLP-76 in T cells, in their absence, or at higher concentrations of YopH, additional proteins will be targeted.

DISCUSSION

Suppression or evasion of host immune responses is necessary for pathogenic bacteria to establish infection. In previous work, we showed that *Yersinia* have the ability to directly suppress T cell reactivity and that this inhibition is dependent on the virulence factor YopH (7). In this paper, we showed that the inhibitory effect of YopH is very potent as an average of one bacterium per T cell is sufficient to inhibit or alter T

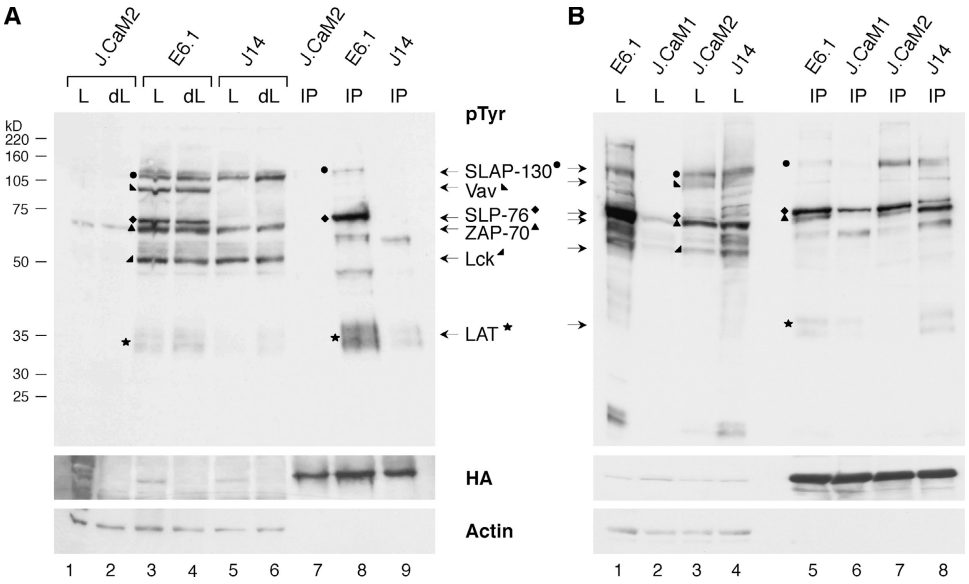

Figure 7. YopH substrate trap–associated tyrosine phosphorylated proteins in LAT- and SLP-76–deficient Jurkat cells. (A) Antiphosphotyrosine blot of anti-YopH immunoprecipitated proteins from total lysates of OKT3-activated Jurkat T cells (E6.1) and the E6.1-derived mutants J14 (SLP-76 deficient) and J.CaM2 (LAT deficient), exposed to *yopHC403A-HA Yersinia* at an MOI of 2 for 1 h. Lysates of 4×10^5 T cells before (L) and after (dL) immunoprecipitation and the precipitated immunocomplexes (IP) of 3×10^7 T cell equivalents were analyzed. A representative result of three independent experiments is shown. Blots were reprobed with actin antibodies and anti–HA-tag antibodies (to detect YopHC403A-HA). (B) Tyrosine-phosphorylated proteins associated with YopHC403A in per-vanadate–stimulated E6.1, J14, J.CaM2, and J.CaM1 (Lck deficient) Jurkat cells were analyzed as in A. A representative result of three independent experiments is shown.

cell responses. We traced the efficient inhibiting effect of YopH to its primary targeting of the adaptor molecules LAT and SLP-76 in the TCR signaling cascade. In addition, a 65-kD protein that coprecipitated with YopHC403A in wild-type and SLP-76–deficient Jurkat T cells also appears to be a primary target of YopH. This protein is likely to be part of the LAT/SLP-76 signalosome and it will be interesting to reveal its function. LAT and SLP-76 are essential for T cell activation and function (32, 33, 35) and targeting of LAT and SLP-76 alone by YopH can explain the YopH-dependent effects in T cell signaling we observed. This is the first time that LAT and SLP-76 are found to be the targets of a pathogen and underscores the importance of the adaptor proteins in the T cell response in an infection.

Our results are different from those reported by Alonso et al. (24), who found Lck, especially tyrosine 394 of Lck, to be the main target of YopH. The discrepancies are most likely due to differences in the amount of YopH used and the manner of its delivery into the cells. We constructed *Yersinia* strains that inject substrate trap YopH into cells to ensure that the mutant YopH is delivered by the same route and in similar quantity as translocated WT-YopH. Alonso et al. expressed YopH in transiently transfected T cells, treated T cells with membrane-permeable YopH, or added YopH to lysed cells. Furthermore, to identify targets of YopH, Alsonso et al. added ~150 times as much substrate trap YopH to T cell lysates as is translocated by *Yersinia* at an MOI of 2. Indeed, at an MOI of 50, we also see Lck being targeted by YopH.

The ability to alter T cell signaling as seen in the sparking phenotypes of calcium flux and PI3K activity is consistent with our identification of LAT and SLP-76 as major targets of YopH. The sparking pattern of calcium flux is similar to the ones observed in T cells with a deletion of Itk, a Tec family kinase that associates with phosphorylated SLP-76 (36), or a mutation in LAT. In the Itk-deficient T cells, TCR stimulation results in a substantial reduction in IP3 generation and defects in IL-2 production. Jurkat cells expressing LAT Tyr132Phe or T cells from "knockin" mice homozygous for LAT Tyr136Phe (Tyr136 in murine LAT is equivalent to Tyr132 in human LAT) also are deficient in their ability to respond to TCR stimulation (37, 38). Partial dephosphorylation of LAT and SLP-76 might be conferred by very limited quantities of injected YopH and, thus, could profoundly alter TCR-mediated signaling and the subsequent responses in *Yersinia* infection. This suggests that, during a *Yersinia* infection, a wide range of T cell–mediated responses may be affected. In addition, abnormal T cell signaling has been linked to the development of autoimmune diseases, particularly rheumatoid arthritis, in which the aberrant T cell signaling has been linked to severely impaired phosphorylation of LAT (39). One might speculate that the ability of *Y. pseudotuberculosis* to disrupt or alter T cell signaling could also play a role in triggering this disease.

When exposing T cells to *Yersinia* at low MOIs, wild-type as well as substrate trap YopH injected into T cells through the type III secretion system primarily targets LAT and SLP-76, but not Lck, CD3ζ, or ZAP-70, even though the latter three molecules are phosphorylated earlier during T cell activation. The selectivity of YopH may in part result from the amino acid sequences of the target proteins. In vitro analysis has shown that the optimum peptide sequence for YopH catalysis is D/E D/E pYxxP (40). The amino-terminus of YopH that exhibits a substrate-binding domain that binds substrates directly in a phosphotyrosine-dependent manner (25) contributes to this selectivity. Both, SLP-76 and LAT, but not CD3ζ, ZAP-70, and Lck, feature tyrosine phosphorylation sites comprising the optimal motif for YopH's phosphatase activity. However, when T cell lysates were incubated with an amount of YopHC403A similar to the amount injected by *Yersinia* into T cells at an MOI of 2, CD3ζ and ZAP-70 were found to bind to substrate trap YopH, indicating that there may be additional unidentified targeting mechanisms that contribute to YopH specificity. An additional targeting mechanism might be provided by the proline-rich region of YopH that shows the consensus motif for binding to Src homology 3 domains (25, 41) and that might confer binding in a phosphorylation-independent manner. Studies in macrophages did not find this region of YopH to be important for the targeting of YopH (25, 42). However, Src homology 3 domains are present (e.g., in the small adaptor Gads), but we did not find any evidence that catalytically active YopH has the ability to bind to Gads (unpublished data).

The difference in the specificity of YopH when delivered into live cells compared with in vitro experiments suggests that the accessibility of signaling complexes in live cells plays an important role. Many signaling complexes in mammalian cells are organized into modules and preferential sequestration and localization of these modules allows for specificity and selectivity of activation. Furthermore, cellular compartmentalization and localization is important for the in vivo substrate specificity of phosphatases that have a broad substrate spectrum in vitro (43). Thus, delivery of YopH through the type III secretory pathway likely determines the interactions of YopH with the host cell signaling modules. In addition, specificity of YopH is also influenced by its concentration. In T cells exposed to higher MOIs, YopH targets additional proteins, including Lck. This indicates that, although YopH shows broad targeting capabilities, it is very specific at low bacteria to host cell ratios, a likely situation in infections in vivo.

Despite the observation that the action of Yop effectors shows no cell specificity among various adherent cells lines (44), YopH is the only *Yersinia* effector protein affecting T cells (7). The data presented here suggest that *Yersinia* may have developed a sophisticated mechanism to target effector proteins to special cellular compartments so that the effect of the few virulence factors delivered by a few bacteria can be maximized. This strategy may not be limited to *Yersinia* because type III secretion systems have been found in other pathogenic microorganisms, such as *Salmonella*, *Shigella*, and *Chlamydia*, that are also associated with reactive arthritis in

humans (45). It is likely that these bacteria secrete molecules analogous to Yops that may alter host cell function and the immune response to the benefit of the microbe. For example, *Salmonella* translocates the effector protein, SptP, which has domains corresponding to those found in the *Yersinia* YopH and YopE (46).

In conclusion, we have identified a novel and efficient mechanism by which bacteria alter T cell responsiveness. These findings may have general applicability to the understanding of bacterial pathogenicity and the development of immune responses during infection.

MATERIALS AND METHODS

Cell lines, *Y. pseudotuberculosis* strains, and growth conditions. Wild-type Jurkat T cells E6.1 (47) and the mutant Jurkat T cell lines J.CaM1 (Lck-deficient; reference 34), J.CaM2 (LAT-deficient; reference 33), and J14 (SLP-76 deficient; reference 32) were maintained in IMDM medium (GIBCO BRL) containing 5% calf serum with iron and the standard supplements 5 mM L-glutamine, 50 μM β-mercaptoethanol, and 50 μg/ml gentamycin at 37°C in 5% CO_2. T cell blasts were derived from 5C.C7 TCR transgenic mice (specific for MCC/I-E^k; reference 22) by stimulating freshly isolated lymph node cells with MCC peptide (88–103) in supplemented RPMI 1640 medium containing 10% FBS. 25 U/ml IL-2 was added 24 h after stimulation. 5C.C7 T cell blasts expressing PH(AKT)-YFP were generated by retroviral transfection as described by Huppa et al. (21). T cell blasts were used on days 6 and 7 after initial stimulation. The B cell lymphoma line CH27 was used as antigen-presenting cells and maintained in complete RPMI 1640.

Wild-type *Y. pseudotuberculosis* YPIIIpIB1 (48), the YopH-deficient (ΔYopH) strain YPIIIp502 (49), and newly generated mutant strains were grown in 2YT medium at 26°C and the appropriate antibiotics: 100 μg/ml ampicillin, and 32 μg/ml chloramphenicol. For T cell infections, overnight cultures were diluted to an OD_{578} of 0.1 into 2YT supplemented with 20 mM sodium oxalate and 20 mM magnesium chloride, grown for 2 h at 26°C, and shifted to 37°C for 2 h. Bacterial numbers were determined by $OD_{578} = 10^9$ bacteria/ml.

Y. pseudotuberculosis strains that secrete HA-tagged YopH or HA-tagged substrate trap YopH (designated as *yopH-HA*, *yopHC403A-HA*, and *yopHD356A-HA*, respectively), were generated as follows. The genes encoding YopH or YopHC403A were amplified including the promoter regions from YPIIIpIB1 or the substrate trap YopHC403A-expressing strain YPIIIpIB1C403A (9) and were inserted into pACYC184 (50). The HA-tag was introduced at the 3′-end of *yopH* and *yopHC403A*. The resulting plasmids p*yopH-HA* and p*yopHC403A-HA* were introduced into YPIIIp502. In addition, p*yopH-HA* was also introduced into the *lcr* mutant YPIIIpIB71 (31), yielding the strain *lcr* (p*yopH-HA*). p*yopHD356A-HA* was generated by site-directed mutagenesis of p*yopH-HA* using Quikchange (Stratagene) and introduced into YPIIIp502.

His-tagged YopHC403A (His-YopHC403A) as well as a glutathion *S*-transferase (GST) fusion protein of YopHC403A (GST-YopHC403A) were expressed in *Escherichia coli*. *yopHC403A* was inserted into pQE30 (QIAGEN) to produce His-YopHC403A. GST-YopHC403A was encoded by a pGEX-4T-1 (Amersham Biosciences) derivative containing *yopHC403A*.

Antibodies. A polyclonal antiserum to YopH was generated at Josman, LLC by immunizing rabbits with purified His-YopHC403A. The crude antiserum was affinity purified (51) using GST-YopHC403A fusion protein bound to glutathione sepharose (Amersham Biosciences). Anti–human CD3ε (OKT3) was purified from hybridomas (American Type Culture Collection). In addition, antibodies against the following proteins and residues were used in this study: antiphosphotyrosine (4G10), LAT, Lck, Gads, ZAP-70, Vav, FAK (Upstate Biotechnology); CD3ζ, actin (Santa Cruz Biotechnology, Inc.); SLP-76, SLAP-130, paxillin, $p130^{cas}$, PYK2 (Transduction Laboratories); phospho-Lck Tyr394 (detected by anti–phospho-Src family [Tyr416] antibody; Cell Signaling), mouse CD3ε (2C11)-biotin (BD Biosciences); HA-tag (HA.11; Covance). Anti–mouse IgG horseradish peroxidase (HRP) linked whole antibody and anti–rabbit IgG HRP linked F(ab′)2 fragments were purchased from Amersham Biosciences.

T cell infection and activation. 10^6 cells/ml Jurkat T cells or 2×10^6 cells/ml 5C.C7 T cell blasts were exposed to specific *Y. pseudotuberculosis* at a specified MOI for 1 h in prewarmed supplemented IMDM or RPMI 1640 without gentamycin in tissue culture plates at 37°C and 5% CO_2. To facilitate contact between the bacteria and the T cells, the plates were spun for 3 min at 1,200 revolutions/min at the beginning of the infection. After the exposure, the T cells were collected in 1.5-ml tubes by centrifugation for 1.5 min (2 min for T cell blasts) at 1,600 revolutions/min (200 *g*) and washed four times with prewarmed PBS (37°C) to remove most of the unattached *Yersinia*.

Jurkat T cells were activated at 4×10^7 cells/ml in PBS at 37°C by adding OKT3 to a final concentration of 10 μg/ml. For the activation of T cell blasts, anti-CD3ε 2C11-biotin was preincubated with streptavidin and added at a final concentration of 10 μg/ml. For stimulation with per-vanadate (100 μM final concentration), ortho-vanadate was oxidized using H_2O_2, which was subsequently removed using catalase. After incubation for 2 min (vanadate 3 min), cells were spun down for 10 s at 4°C and lysed at 4×10^7 cells/ml (Jurkat) or 10^8 cells/ml (T cell blasts) in ice-cold TTS (25 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM β-glycerol-phosphate, 10 mM NaF, Complete protease inhibitors (Roche). Nuclei were removed by centrifugation for 10 min at 13,000 revolutions/min (16,000 *g*) at 4°C. Total lysates of 4×10^5 Jurkat cells or 2×10^6 T cell blasts were resolved by SDS-PAGE. Phosphotyrosine proteins were detected by immunoblot analysis using 4G10 antibody and the enhanced chemiluminescence system ECL (Amersham Biosciences). Blots were stripped according to the protocol provided by Amersham Biosciences and subjected to reprobing with appropriate antibodies. Results were quantified using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image).

Immunoprecipitation of YopH-associated proteins. To identify YopHC403A/YopHD356A targets in exposed T cells, 3×10^7 Jurkat T cells were exposed to *yopHC403A-HA* or *yopHD356A-HA* at an MOI of 2, activated, and lysed as described in the previous paragraph. Extracts were precleared using protein G agarose (Sigma-Aldrich) and subsequently incubated for 2 h at 4°C with affinity purified anti-YopH antibodies or anti-HA monoclonal antibodies (final concentration of 10 μg/ml) and protein G agarose. The agarose was collected by centrifugation for 3 min at 16,000 *g* at 4°C, washed three times with TTS buffer, and boiled with reducing SDS sample buffer. The proteins were separated by SDS-PAGE and analyzed by Western blot analysis. For in vitro immunoprecipitations, Jurkat cells were activated and lysed, and YopHC403A, YopHD356A, or YopH were added to the precleared lysate. YopH was preincubated in lysis buffer with 1 mM vanadate for 15 min to inactivate it before adding it to the lysate. To match the amount of purified YopH proteins added to the in vitro experiments with the amount translocated by *Yersinia*, we compared the amounts of YopH injected into Jurkat T cells with a titration curve of YopH. We found that ~20 ng of protein are translocated by *Yersinia* into 10^7 T cells exposed at an MOI of 2.

Lck kinase assay. Jurkat T cells were exposed to *Yersinia* as described before, stimulated with OKT3, and lysed in kinase assay buffer KAB (25 mM Hepes, pH 7.4, 150 mM KCl, 5 mM MnCl, 5 mM MgCl2, 1 mM DTT, 1% Triton X-100, 1 mM Na_3VO_4). Lysates were precleared and incubated with 10 μg/ml anti-Lck monoclonal antibody and protein G agarose. The agarose was washed three times in KAB. Kinase activity was measured by ELISA as follows. The cytoplasmic domain of CD3γ ([Biotin][AHX] GGQDGVRQSRASDKQTLLPNDQLYQPLKDREDDQYSHLQGNQL RRN) was used as a substrate for Lck and was bound on streptavidin-

coated Immulon 4 plates. Precipitated Lck was added for 30 min at 32°C in KAB containing 20 μM ATP. Phosphorylation of the CD3γ-peptides was quantified using antiphosphotyrosine antibody 4G10, anti–mouse IgG HRP conjugate and the HRP substrate OPD (Sigma-Aldrich) in comparison to a standard curve of phosphorylated CD3γ-peptide (0.05–12.5 ng/ml). Lck-deficient Jurkat cells (J.CaM1) were used as negative control.

Calcium flux and PI3K activity analysis. To analyze calcium flux by flow cytometry, 10^6 T cell blasts were exposed to specified *Y. pseudotuberculosis* strains and concurrently loaded with 2 μM indo-1, AM (Molecular Probes) for 30 min. T cells were collected by centrifugation at 1,600 revolutions/min (200 *g*) for 2 min in 1.5-ml tubes and washed twice with prewarmed antibiotic-free RPMI 1640 containing 25 mM Hepes, pH 7.4. Indo-1 loaded T cells were activated by the addition of 2C11-biotin antibody (preincubated with streptavidin) to a final concentration of 10 μg/ml. Calcium flux was analyzed at 37°C on a modified FACStar (FACS facility, Stanford University) by monitoring the ratio of 405/485 nm emission and time. As a positive control, 1 μM ionomycin (Sigma-Aldrich) was added to the T cells at the end of the measurement.

Calcium flux and the PI3K activity of T cells responding to antigenic stimulation were analyzed by single cell video microscopy using a Zeiss/Universal Imaging three-dimensional time-lapse system as described previously (21). 2×10^5 T cell blasts expressing the PH(AKT)-YFP were exposed to *Yersinia* and loaded with 1 μM fura-2, AM (Molecular Probes). After washing, the cells were resuspended in 200 μl prewarmed antibiotic-free RPMI 1640 supplemented with 25 mM Hepes, pH 7.4, and seeded into an eight-well glass-bottom chamber slide (Labtek/Nunc). 10^5 CH27 cells, preloaded with 1 μM MCC peptide for 1 h, were added to the T cells. Images were taken every 15 or 30 s for 15 min. At each time point, images of the two-dimensional differential interference contrast, the fura-2 fluorescence images (340/380 nm) and a z stack of YFP were recorded. Metamorph (Universal Imaging Corp.) was used for microscope control, data acquisition, and image analysis.

We thank J.B. Huppa for the phoenix cell lines producing PH(AKT)-YFP–expressing virus and introduction into live cell imaging, B.F. Lillemeier for the CD3γ peptides and advice on the Lck kinase assay, and J.B. Bliska for the *Yersinia* strain YPIIIpIB1C403A. We thank M.M. Davis, J.B. Huppa, Y. Konigshofer, Q-J. Li, B.F. Lillemeier, D.M. Monack, A. Müller, S. Shin, and L.C. Wu for advice and critical reading of the paper and M.R. Amieva, J.C. Boothroyd, J.-T. Chi, M.A. Purbhoo, R. Sciammas, and T. Yao for discussion.

This work was supported by funding from the National Institutes of Health and the Stanford Digestive Disease Center grant no. DK56339 (to Y.-h. Chien and S. Falkow).

The authors have no conflicting financial interests.

Submitted: 4 June 2004
Accepted: 22 November 2004

REFERENCES

1. Hornef, M.W., M.J. Wick, M. Rhen, and S. Normark. 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat. Immunol.* 3:1033–1040.
2. Autenrieth, I.B., U. Vogel, S. Preger, B. Heymer, and J. Heesemann. 1993. Experimental *Yersinia enterocolitica* infection in euthymic and T-cell-deficient athymic nude C57BL/6 mice: comparison of time course, histomorphology, and immune response. *Infect. Immun.* 61: 2585–2595.
3. Autenrieth, I.B., M. Beer, E. Bohn, S.H.E. Kaufmann, and J. Heesemann. 1994. Immune responses to *Yersinia enterocolitica* in susceptible BALB/c and resistant C57BL/6 mice: an essential role for gamma interferon. *Infect. Immun.* 62:2590–2599.
4. Toivanen, A., K. Granfors, R. Lahesmaa-Rantala, R. Leino, T. Stahlberg, and R. Vuento. 1985. Pathogenesis of *Yersinia*-triggered reactive arthritis: immunological, microbiological and clinical aspects. *Immunol. Rev.* 86:47–70.
5. Cornelis, G.R., A. Boland, A.P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M.-P. Sory, and I. Stainier. 1998. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol. Mol. Biol. Rev.* 62:1315–1352.
6. Juris, S.J., F. Shao, and J.E. Dixon. 2002. *Yersinia* effectors target mammalian signalling pathways. *Cell. Microbiol.* 4:201–211.
7. Yao, T., J. Mecsas, J.I. Healy, S. Falkow, and Y.-h. Chien. 1999. Suppression of T and B lymphocyte activation by a *Yersinia pseudotuberculosis* virulence factor, YopH. *J. Exp. Med.* 190:1343–1350.
8. Guan, K.L., and J.E. Dixon. 1990. Protein tyrosine phosphatase activity of an essential virulence determinant in *Yersinia*. *Science.* 249:553–556.
9. Bliska, J.B., K.L. Guan, J.E. Dixon, and S. Falkow. 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. *Proc. Natl. Acad. Sci. USA.* 88:1187–1191.
10. Zhang, Z.-Y., J.C. Clemens, H.L. Schubert, J.A. Stuckey, M.W. Fischer, D.M. Hume, M.A. Saper, and J.E. Dixon. 1992. Expression, purification, and physicochemical characterization of a recombinant *Yersinia* protein tyrosine phosphatase. *J. Biol. Chem.* 267:23759–23766.
11. Monack, D.M., J. Mecsas, D. Bouley, and S. Falkow. 1998. *Yersinia*-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. *J. Exp. Med.* 188:2127–2137.
12. Bliska, J.B., and D.S. Black. 1995. Inhibition of the Fc receptor-mediated oxidative burst in macrophages by the *Yersinia pseudotuberculosis* tyrosine phosphatase. *Infect. Immun.* 63:681–685.
13. Andersson, K., K.-E. Magnusson, M. Majeed, O. Stendahl, and M. Fällman. 1999. *Yersinia pseudotuberculosis*-induced calcium signaling in neutrophils is blocked by the virulence effector YopH. *Infect. Immun.* 67:2567–2574.
14. Black, D.S., and J.B. Bliska. 1997. Identification of $p130^{Cas}$ as a substrate of *Yersinia* YopH (Yop51), a bacterial protein tyrosine phosphatase that translocates into mammalian cells and targets focal adhesions. *EMBO J.* 16:2730–2744.
15. Persson, C., N. Carballeira, H. Wolf-Watz, and M. Fällman. 1997. The PTPase YopH inhibits uptake of *Yersinia*, tyrosine phosphorylation of $p130^{Cas}$ and FAK, and the associated accumulation of these proteins in peripheral focal adhesions. *EMBO J.* 16:2307–2318.
16. Rosqvist, R., I. Bölin, and H. Wolf-Watz. 1988. Inhibition of phagocytosis in *Yersinia pseudotuberculosis*: a virulence plasmid-encoded ability involving the Yop2b protein. *Infect. Immun.* 56:2139–2143.
17. Cantrell, D.A. 2002. T-cell antigen receptor signal transduction. *Immunology.* 105:369–374.
18. Kane, L.P., J. Lin, and A. Weiss. 2000. Signal transduction by the TCR for antigen. *Curr. Opin. Immunol.* 12:242–249.
19. Jordan, M.S., A.L. Singer, and G.A. Koretzky. 2003. Adaptors as central mediators of signal transduction in immune cells. *Nat. Immunol.* 4:110–116.
20. Costello, P.S., M. Gallagher, and D.A. Cantrell. 2002. Sustained and dynamic inositol lipid metabolism inside and outside the immunological synapse. *Nat. Immunol.* 3:1082–1089.
21. Huppa, J.B., M. Gleimer, C. Sumen, and M.M. Davis. 2003. Continuous T cell receptor signaling required for synapse maintenance and full effector potential. *Nat. Immunol.* 4:749–755.
22. Fazekas de St. Groth B., P.A. Patten, W.Y. Ho, E.P. Rock, and M.M. Davis. 1993. An analysis of T cell receptor-ligand interaction using a transgenic antigen model for T cell tolerance and T cell receptor mutagensis. *In* Molecular Mechamisms of Immunological Self-Recognition. F.W. Alt and H.J. Vogel, editors. Academic Press, San Diego, CA. 123–127.
23. Sauvonnet, N., I. Lambermont, P. van der Bruggen, and G.R. Cornelis. 2002. YopH prevents monocyte chemoattractant protein 1 expression in macrophages and T-cell proliferation through inactivation of the phosphatidylinositol 3-kinase pathway. *Mol. Microbiol.* 25:805–815.
24. Alonso, A., N. Bottini, S. Bruckner, S. Rahmouni, S. Williams, S.P. Schoenberger, and T. Mustelin. 2004. Lck dephosphorylation at Tyr-394 and inhibition of T cell antigen receptor signaling by *Yersinia* phosphatase YopH. *J. Biol. Chem.* 279:4922–4928.
25. Black, D.S., L.G. Montagna, S. Zitsmann, and J.B. Bliska. 1998. Identification of an amino-terminal substrate-binding domain in the *Yersinia* tyrosine phosphatase that is required for efficient recognition of focal

adhesion targets. *Mol. Microbiol.* 29:1263–1274.
26. Hamid, N., A. Gustavsson, K. Andersson, K. McGee, C. Persson, C.E. Rudd, and M. Fällman. 1999. YopH dephosphorylates Cas and Fyn-binding protein in macrophages. *Microb. Pathog.* 27:231–242.
27. Black, D.S., A. Marie-Cardine, B. Schraven, and J.B. Bliska. 2000. The *Yersinia* tyrosine phosphatase YopH targets a novel adhesion-regulated signalling complex in macrophages. *Cell. Microbiol.* 2:401–414.
28. Flint, A.J., T. Tiganis, D. Barford, and N.K. Tonks. 1997. Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. USA.* 94: 1680–1685.
29. Tomlinson, M.G., J. Lin, and A. Weiss. 2000. Lymphocytes with a complex: adapter proteins in antigen receptor signaling. *Immunol. Today.* 21:584–591.
30. Griffiths, E.K., and J.M. Penninger. 2002. ADAP-ting TCR signaling to integrins. *Sci. STKE.* 2002:RE3.
31. Bliska, J.B., M.C. Copass, and S. Falkow. 1993. The *Yersinia pseudotuberculosis* adhesin YadA mediates intimate bacterial attachment to and entry into HEp-2 cells. *Infect. Immun.* 61:3914–3921.
32. Yablonski, D., M.R. Kuhne, T. Kadlecek, and A. Weiss. 1998. Uncoupling of nonreceptor tyrosine kinases from PLC-γ1 in an SLP-76-deficient T cell. *Science.* 281:413–416.
33. Finco, T.S., T. Kadlecek, W. Zhang, L.E. Samelson, and A. Weiss. 1998. LAT is required for TCR-mediated activation of PLCγ1 and the Ras pathway. *Immunity.* 9:617–626.
34. Straus, D.B., and A. Weiss. 1992. Genetic evidence for the involvement of the lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell.* 70:585–593.
35. Zhang, W., B.J. Irvin, R.P. Trible, R.T. Abraham, and L.E. Samelson. 1999. Functional analysis of LAT in TCR-mediated signaling pathways using a LAT-deficient Jurkat cell line. *Int. Immunol.* 11:943–950.
36. Liu, K.-Q., S.C. Bunnell, C.B. Gurniak, and L.J. Berg. 1998. T cell receptor–initiated calcium release is uncoupled from capacitative calcium entry in Itk-deficient T cells. *J. Exp. Med.* 187:1721–1727.
37. Zhang, W., R.P. Trible, M. Zhu, S.K. Liu, C.J. McGlade, and L.E. Samelson. 2000. Association of Grb2, Gads, and phospholipase C-γ1 with phosphorylated LAT tyrosine residues. Effect of LAT tyrosine mutations on T cell antigen receptor-mediated signaling. *J. Biol. Chem.* 275:23355–23361.
38. Sommers, C.L., C.-S. Park, J. Lee, C. Feng, C.L. Fuller, A. Grinberg, J.A. Hildebrand, E. Lacaná, R.K. Menon, E.W. Shores, et al. 2002. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. *Science.* 296:2040–2043.
39. Gringhuis, S.I., A. Leow, E.A. Papendrecht-Van Der Voort, P.H. Remans, F.C. Breedveld, and C.L. Verweij. 2000. Displacement of linker for activation of T cells from the plasma membrane due to redox balance alterations results in hyporesponsiveness of synovial fluid T lymphocytes in rheumatoid arthritis. *J. Immunol.* 164:2170–2179.
40. Zhang, Z.Y., A.M. Thieme-Sefler, D. Maclean, D.J. McNamara, E.M. Dobrusin, T.K. Sawyer, and J.E. Dixon. 1993. Substrate specificity of the protein tyrosine phosphatases. *Proc. Natl. Acad. Sci. USA.* 90:4446–4450.
41. Bliska, J. 1996. How pathogens exploit interactions mediated by SH3 domains. *Chem. Biol.* 3:7–11.
42. Persson, C., R. Nordfelth, K. Andersson, Å. Forsberg, H. Wolf-Watz, and M. Fällman. 1999. Localization of the *Yersinia* PTPase to focal complexes is an important virulence mechanism. *Mol. Microbiol.* 33: 828–838.
43. Zhang, Z.-Y. 2002. Protein tyrosine phosphatases: structure and function, substrate specificity, and inhibitor development. *Annu. Rev. Pharmacol. Toxicol.* 42:209–234.
44. Boyd, A.P., N. Grosdent, S. Tötemeyer, C. Geuijen, S. Bleves, M. Iriarte, I. Lambermont, J.N. Octave, and G.R. Cornelis. 2000. *Yersinia enterocolitica* can deliver Yop proteins into a wide range of cell types: development of a delivery system for heterologous proteins. *Eur. J. Cell Biol.* 79:659–671.
45. Galán, J.E., and A. Collmer. 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. *Science.* 284:1322–1328.
46. Kaniga, K., J. Uralil, J.B. Bliska, and J.E. Galán. 1996. A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella typhimurium*. *Mol. Microbiol.* 21:633–641.
47. Weiss, A., R.L. Wiskocil, and J.D. Stobo. 1984. The role of T3 surface molecules in the activation of human T cells: a two-stimulus requirement for IL-2 production reflects events occurring at a pre-translational level. *J. Immunol.* 133:123–128.
48. Portnoy, D.A., H. Wolf-Watz, I. Bolin, A.B. Beeder, and S. Falkow. 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. *Infect. Immun.* 43:108–114.
49. Mecsas, J., B. Raupach, and S. Falkow. 1998. The *Yersinia* Yops inhibit invasion of *Listeria, Shigella* and *Edwardsiella* but not *Salmonella* into epithelial cells. *Mol. Microbiol.* 28:1269–1281.
50. Chang, A.C.Y., and S.N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134:1141–1156.
51. Harlow, E., and D. Lane. 1988. Antibodies: a Laboratory Manual. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY. 349 pp.