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Salmonella enterica serovar Typhimurium modulates P-glycoprotein in the intestinal epithelium

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Siccardi D, Mumy KL, Wall DM, Bien JD, McCormick BA. *Salmonella enterica* serovar Typhimurium modulates P-glycoprotein in the intestinal epithelium. *Am J Physiol Gastrointest Liver Physiol* 294: G1392–G1400, 2008. First published April 10, 2008; doi:10.1152/ajpgi.00599.2007.—Studies over the last decade have shown that *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) is able to preferentially locate to sites of tumor growth and modulate (shrink) the growth of many cancers. Given this unique association between *S. typhimurium* and cancer cells, the objective of this study was to investigate the capacity of this microorganism to modulate the plasma membrane multidrug resistance (MDR) protein P-glycoprotein (P-gp), an ATP-binding cassette transporter responsible for effluxing many cancer drugs. Using an in vitro model of *S. typhimurium* infection of polarized human cancer intestinal cell lines, we have found that this enteric pathogen functionally downregulates the efflux capabilities of P-gp. Specifically, we show that *S. typhimurium* infection of human intestinal cancer cells results in the enhanced intracellular accumulation of a number of P-gp substrates that corresponds to the posttranscriptional downregulation of P-gp expression. Furthermore, cells expressing small interfering RNAs against *MDR1*, the gene encoding P-gp, were significantly more susceptible to the cytotoxic effects of bacterial infection. This result is consistent with our observation that *S. typhimurium* was significantly less able to invade cells overexpressing *MDR1*. Taken together, these results reveal a novel role for P-gp in the maintenance of homeostasis in the gastrointestinal tract in regard to bacterial infection. Thus the regulation of P-gp by *S. typhimurium* has important implications not only for the development of new cancer therapeutics aimed at reversing drug resistance but also in the understanding of how microbes have evolved diverse strategies to interact with their host.

ATP-binding cassette transporter; inflammation; multidrug resistance 1

BACTERIA HAVE BEEN INVESTIGATED as therapeutic agents for tumors for over 150 years, when it was first observed by William B. Coley that a fraction of cancer patients who developed postoperative bacterial infections went into remission and were cured of their tumors (33). Although the mechanisms underlying this observation were unclear, it was known even then that bacteria exhibit immunostimulatory properties. Moreover, it has been understood for over half a century that anaerobic bacteria can selectively grow within tumors. In 1997, Pawelek et al. (27) reported that *Salmonella* could preferentially infect and accumulate within implanted tumors in mice, achieving tumor-to-normal-tissue ratios of ~1,000:1. It is clear that such microbes have the potential to sidestep many of the delivery barriers that hinder conventional chemotherapeutics. In addition, the conditions that permit anaerobic bacterial growth, such as impaired circulation and extensive necrosis, are found in many tumors, supporting the ideology that bacterial therapeutic conduits may serve as a unique portal to a wide variety of malignancies (21).

Collectively, these observations suggest that *Salmonella*, or aspects thereof, has the potential to be developed as clinically useful anticancer agents. In particular, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) produces self-limited enteritis in most healthy adults, can easily be manipulated to carry foreign genes, and infects both mice and humans, allowing testing of appropriate animal models. This species also exists as a facultative anaerobe, allowing for survival in both oxygenated and hypoxic conditions, and thus it would be expected that this organism could colonize both small metastatic lesions as well as larger tumors with an internal anaerobic environment. Recently, Low and co-workers (22) took advantage of these unique properties and developed a strain of *S. typhimurium* (VNP20009) that is nonpathogenic in mice, pigs, and humans and accumulates 2,000-fold more in tumors. Although this strain was able to retard tumor growth and prolong survival in mice, it was not successful at slowing tumor growth in humans (22).

Given that *S. typhimurium* can localize to tumors and modulate numerous biochemical pathways of intestinal epithelial cells, we reasoned that perhaps this enteric pathogen has evolved mechanisms that interact/interfere with multidrug resistance (MDR) transporters that complicate drug treatment. Consequently, we have investigated one of the membrane transport proteins that promote the expulsion of xenobiotics, the well-characterized P-glycoprotein (P-gp), a 170-kDa ATP-dependent multispecific drug transporter (16). Reports linking overexpression of the *MDR1* gene (leading to increased levels of P-gp) to adverse treatment outcomes in many cancers, including colon cancer, provided the evidence necessary to implicate this MDR phenotype as an important biologic target for pharmacologic modulation (15, 19). In this study, we examined where the two paths of targeting the MDR phenotype, specifically the inherent anti-tumor properties of P-gp and *Salmonella*, cross in regard to cancer therapeutics and host-tumor/pathogen interactions. We demonstrate that apical colonization of colon cancer cell lines by wild-type *S. typhimurium* leads to a profound functional decrease in the MDR protein transporter, P-gp. In a physiological context, this is consistent with our observation that the presence of P-gp adversely influences the ability of *S. typhimurium* to invade host cells. This is the first observation to link a microorganism

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that is targeted specifically to tumors with the regulation of MDR transporters.

MATERIAL AND METHODS

Chemicals. Anti-P-gp mouse mAb (C219) and DEVD-CHO were purchased from Calbiochem (La Jolla, CA). Rhodamine 123, cyclosporine A, verapamil, sodium orthovanadate, LPS (from *Salmonella typhimurium*), and ^{14}C-mannitol were purchased from Sigma Chemical (St. Louis, MO). DiOC2(3)(3,3′-diethyloxacarbocyanine iodide) was purchased from Anaspec (San Jose, CA). [^{3}H(G)]Digoxin was from Perkin Elmer (Boston, MA). Recombinant human tumor necrosis factor-α (TNF-α) was purchased from Pierce Biotechnology (Rockford, IL).

Cell culture. The human intestinal adenocarcinoma cell-lines (HCT8, ileocaecal and T84, colonic) were maintained in DMEM (Gibco, Carlsbad, CA) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at 50,000/cm^2 in tissue culture flasks and cultured until confluent at 37°C in 90% relative humidity and 5% CO_2. The media was changed every 48 h. The cells were detached from the flasks by washing with PBS, followed by 0.5% trypsin and 0.2% EDTA in PBS. T84 cells were used between *passages 40* and *58*. Human A549 cells, a lung adenocarcinoma cell line, were maintained in F-12 nutrient mixture (Gibco) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2. The A549 cells were passaged at 80–100% confluence using 0.5% trypsin and 0.2% EDTA in PBS. For permeability experiments T84 cells were seeded at 250,000/cm^2 on inverted polycarbonate membrane Transwell inserts (0.33-cm^2 diameter; Corning Costar, Corning, NY) and cultured same as above with the medium changed every 48 h. The transport studies were conducted using monolayers after 8 to 14 days of growth. For efflux experiments, HCT8 cells (*passages 30–40*) were seeded on inverted Transwells at a seeding density of 40,000/cm^2 and grown for 7–10 days. The formation of restrictive monolayers was monitored for all cell types by measurement of transepithelial electrical resistance (TEER) using an EVOM Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL). T84 and HCT8 monolayers were used with TEER ranging from 700 to 1,500, and 140–160 Ω/cm^2, respectively.

The Madin-Darby canine kidney II (MDCKII) cell line was previously modified to stably overexpress *MDR1* (MDCKII-MDR1), as described earlier (3, 12). Wild-type MDCKII and MDCKII-MDR1 cells were maintained in DMEM (Gibco) with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 90% relative humidity and 5% CO_2. Cells were seeded and maintained in the same manner as HCT8 and T84 cell lines described above, with the exception of MDCKII lines having been grown nonpolarized in 24- and six-well polystyrene Costar culture plates (Corning) for 3 and 7 days, respectively.

Bacterial strains, plasmids, and growth media. Luria-Bertani (LB) broth, LB agar, and MacConkey agar (Difco Laboratories, Detroit, Michigan) were prepared according to manufacturer's instructions. *S. typhimurium* strains were grown in LB broth overnight at 37°C under microaerophilic conditions to late-log phase (~16 h) (23). After overnight growth, all bacteria were resuspended in Hank's buffered salt solution, plus Ca^{2+} and Mg^{2+} (Sigma) with 10 mM HEPES, pH 7.4 (hereafter termed HBSS$^+$) and added to polarized monolayers (23) at a multiplicity of infection (MOI) of 100 bacteria/epithelial cell. This optimal dose was determined after evaluating varying doses (MOI of 1, 10, 100, and 1,000) of *S. typhimurium* at which experimental effects are observed.

Invasion assays. Infection of T84 and A549 monolayers was performed by the method described previously (23). Briefly, monolayers of T84 and A549 cells were prepared as described above. Following confluency the monolayers were drained of media and gently washed with HBSS$^+$. The Transwells were placed in a new 24-well tissue culture plate with 1.0 ml sterile HBSS$^+$ in the lower (basolateral) well and 50 μl HBSS$^+$ in the upper (apical) well. After a 30-min equilibration, 10 μl of bacteria (washed and resuspended in HBSS$^+$) was added apically to each monolayer (MOI of 20). *S. typhimurium* attachment to and entry into T84 intestinal epithelial and A549 lung epithelial cells was assessed after 1 h. Cell-associated *Salmonella*, representing populations of bacteria attached to and/or internalized into the monolayers, were released from the monolayer by incubation with 0.1 ml of 1% Triton X-100 (Sigma). Internalized bacteria were those collected following lysis of the epithelial cells with 1% Triton X-100 after the addition of gentamicin to kill extracellular *Salmonella* (23). Gentamicin dose-response studies defined the conditions required to achieve bacteriocidal effects on the strain used. LB broth (0.9 ml) was added after the Triton X-100 incubation step, and each sample was vigorously mixed and quantified by plating for colony-forming units (CFU) on MacConkey agar medium.

MDCKII wild-type and MDCKII-MDR1 cells were grown nonpolarized in 24-well culture plates and infected with 120 μl of bacteria (added to 50 μl HBSS$^+$). The remainder of the procedure was carried out the same as described above, with the exception of plating for CFU on LB agar with streptomycin (10 μg/ml).

Intracellular P-gp substrate accumulation studies. Uptake and retention of P-gp substrates rhodamine 123, DiOC2(3) and [^{3}H(G)] digoxin were measured to evaluate P-gp activity in T84 and HCT8 cells grown on 96-well plates or 24-well Transwell filters (Corning). HCT8 cells generating small interfering RNAs (siRNA) against *MDR1* were included as a negative control. After reaching confluence, cells were preincubated at 37°C for 30 min with serum-free DMEM. Next, bacteria were added directly to the top of monolayers at an MOI of 100. Infection was allowed to proceed at 37°C for the times indicated. Bacteria were removed by gentle washing of the monolayers with warm DMEM. Rhodamine 123 (5 μM), DiOC2(3) (1 μM), or [^{3}H(G)]digoxin (15 nM) were then added for 60 min. When Transwell filter supports were used, the probe was supplied to either the apical or basolateral compartment. After incubation, the cells were washed three times with ice-cold PBS and solubilized with 1% Triton X-100. Intracellular probe concentrations were determined from the fluorescence values by the construction of standard curves. Fluorescence was measured with Ex. 530, Em. 590 nm for rhodamine 123 and Ex. 488, Em. 525 nm for DiOC2(3), using an LS-5 spectrofluorometer (Perkin Elmer). Radioactivity was counted using a Beckman LS 6500 Beta Counter (Beckman Coulter, Fullerton, CA).

Generation of siRNA for suppression of MDR1 expression. Plasmids used to generate siRNAs were constructed using the pSUPER vector (Oligoengine, Seattle, WA) using the method described by Brummelkamp et al. (6). Two oligonucleotides were designed incorporating a 19-nt sequence from the target *MDR1* transcript shown in italics (GenBank accession no. NM_000927) or its reverse compliment separated by a short-spacer region and a portion of a *Bgl*II or *Hin*dIII restriction site: 5′-GATCCCC*GGATATTAGGACCATAAAT*TTCAAGAGA*ATTTATGGTCCTAATATCC*TTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAA*GGATATTAGGACCATAAAT*TCTCTTGAA*ATTTATGGTCCTAATATCC*GGG-3′, or random control sequences of 5′-GATCCCC*AGGATATTAGGACCATAAA*TTCAAGAGA*TTTATGGTCCTAATATCCT*TTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAAA*GGATATTAGGACCATAAA*TCTCTTGAA*TTTATGGTCCTAATATCCT*GGG-3′. Oligonucleotides were annealed, after which double-stranded DNAs had overhanging restriction sites and were ligated into digested pSUPER. Constructs were transformed into competent *Escherichia coli* (*E. coli*) DH5-α by standard methods, and transformants were plated on LB with ampicillin (50 μg/ml). Plasmids were extracted, purified (QIAprep Spin Mini-prep Kit; Qiagen, Valencia, CA), and sequenced for confirmation. Once confirmed, bulk plasmid was prepared for transfection using Qiagen Plasmid Midi Kit (Qiagen).

Transfection of HCT8 intestinal epithelial cells. HCT8 cells (kind gift of Cheleste Thorpe, Tufts University School of Medicine) are a

polarizing intestinal human transformed cell line and were used for this protocol because of its high transfection efficiency. HCT8 cells were transfected with the modified pSUPER with Lipofectamine 2000 (Invitrogen) per manufacturer's instructions. Briefly, 4 μg of plasmid was diluted into RPMI 1640 without serum (Invitrogen). Separately, Lipofectamine 2000 (Invitrogen) was diluted into RPMI 1640 with FBS and incubated at room temperature for 5 min. Following incubation, the two mixtures were combined and incubated at room temperature for 20 min. This cocktail was added to HCT8 cells with RPMI 1640, and the cells were incubated in RPMI with 8% vol/vol FBS without selection. Cells were passaged into fresh media with selection added the next day (neomycin-G418, 1 mg/ml, Sigma). Cells underwent two additional cycles of growth/passage in G418 before use.

Western blot analysis. T84 cell monolayers grown on 4.5-cm^2 Transwell filters (Corning Costar) were infected with 1 ml of bacterial suspension at an MOI of 200 per epithelial cell and incubated at 37°C; uninfected monolayers served as the negative control. Following infection, the cell monolayers were harvested on ice in 350 μl lysis buffer (1% Triton X-100, 100 mM NaCl, 10 mM HEPES, 2 mM EDTA, 4 mM Na_3VO_4, 40 mM NaF, 200 mM PMSF, and a protease inhibitor cocktail) (Complete Mini, Roche Molecular Biochemicals, Mannheim, Germany). Samples were centrifuged at 10,000 *g* for 30 min, and protein concentrations in the supernatant were determined using the Biorad protein assay kit (Biorad, Hercules, CA) and either frozen at −20°C or used immediately for immunoblotting. Total protein (equivalent to 30 μg for each sample) was loaded and resolved by 7.5% SDS-PAGE and then electroblotted to nitrocellulose (0.2-μm pore size) membrane. Rainbow prestained molecular weight markers (Pierce) were concurrently run. For signal generation, the membrane was incubated at room temperature first for 1 h with 5% nonfat dried milk in Tris-buffered saline with 0.1% Tween 20 (TBST, 0.1 M, pH 7.4) and then for 16 h at 4°C with the monoclonal P-gp C219 antibody (Calbiochem) diluted 1:200 in TBST. The membrane was then washed three times in TBST and further incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse IgG rabbit antibody (Sigma). MDCKII wild-type and MDCKII-MDR1 cells were grown nonpolarized in six-well culture plates and lysed, and the membrane proteins were collected for analysis of P-gp as described above.

Cytotoxicity. The cytotoxic effects of intestinal epithelial infection with *S. typhimurium* were measured using a lactate dehydrogenase (LDH) kit (Sigma), according to the manufacturer's instructions.

RESULTS

Intracellular accumulation of P-gp substrates during infection. To determine whether *S. typhimurium* could modulate P-gp transport activity during infection of the human intestinal cell line, T84, we assessed the intracellular accumulation of P-gp substrates. This assay measures the intracellular accumulation of fluorescent P-gp substrate probes, and we utilized rhodamine-123, a classic tool for measuring P-gp functionality (34, 35), in addition to DiOC2(3) and the radiolabeled probe digoxin. As shown in Fig. 1, we found that there was a marked increase in the accumulation of the P-gp substrate rhodamine 123 when T84 cells were infected with *S. typhimurium* (4 h postinfection). Treatment of T84 cell monolayers with verapamil, a potent and selective inhibitor of P-gp (28), substantially normalized the efflux of rhodamine 123 in the uninfected controls to that of *S. typhimurium*-infected monolayers. This result suggests that *S. typhimurium* is able to induce the retention of the P-gp substrate rhodamine 123 to the same extent as pharmacologic inhibition of the efflux pump. Each treatment condition in the infected group accumulated the probe to similar degrees ($P > 0.05$, Fig. 1). The experiment was repeated using a separate P-gp inhibitor, cyclosporine A, and provided similar results (data not shown). It should also be noted that the use of rhodamine 123 and inhibitors did not affect viability of the cells, as measured by the amount of LDH released (data not shown).

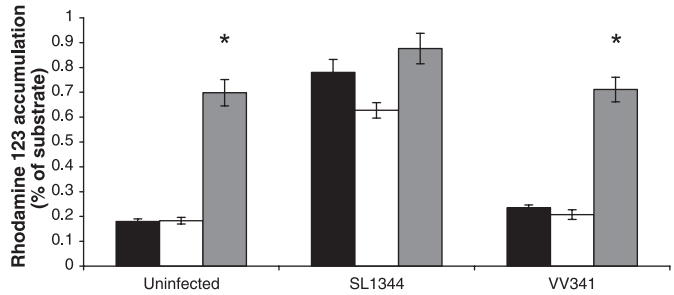

Fig. 1. Rhodamine 123 accumulation in T84 cells infected with *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) (4 h postinfection). Uninfected monolayers or monolayers infected with bacteria for 1 h were incubated in DMEM (black bars) or DMEM containing gentamicin (100 μg/ml, white bars) or verapamil (40 μM, gray bars) for 2 h at 37°C. Rhodamine 123 (5 μM) was then added and allowed to incorporate for 1 h at 37°C. Uninfected monolayers, monolayers infected with pathogenic *S. typhimurium* (SL1344), and monolayers infected with the noninvasive but adherent *hilA* mutant (VV341) are shown. The data are expressed as the means ± SD ($n = 8$) for all conditions tested and represent 1 of at least 3 independent experiments performed with similar results. Statistical analysis by Student's *t*-test. *Statistical difference ($P < 0.05$) compared with the respective control.

Since invasion into host cells is a critical step in the process of *S. typhimurium* pathogenesis, we next determined whether the ability of this pathogen to invade human colonic cancer cells was required to functionally modify the transport activities of P-gp. *S. typhimurium* has evolved into a sophisticated mechanism that allows this microorganism to enter nonphagocytic host cells. This process requires the expression of Salmonella pathogenicity island-1 (SPI-1), which encodes a specialized type III protein secretion system (TTSS). This TTSS delivers a set of effector proteins into the host cell that triggers a marked rearrangement of the host cytoskeleton, which drives bacterial entry. HilA is a key regulator of this process as it directly activates the expression of two SPI-1 operons that encode TTSS apparatus components. As shown in Fig. 1, we found that functional impairment of P-gp in cells exposed to *S. typhimurium* appears to be dependent upon a mechanism requiring SPI-1, since the noninvasive but still adherent *hilA*-mutant (vv341) was unable to cause P-gp inhibition. This finding suggests that a specific mechanism stimulated by *S. typhimurium* cell invasion, and SPI-1 specifically, is responsible for the intracellular accumulation of the probe. In addition, gentamicin treatment of *S. typhimurium*-infected monolayers (eliminating extracellular bacteria) before applying the probe did not substantially change the accumulation profile of rhodamine 123 with respect to the untreated control, implying that the adhesion of the bacteria to epithelial cells does not have an effect on the accumulation (Fig. 1). It should also be noted that rhodamine 123 accumulation following *S. typhimurium* infection was evaluated with varying doses of bacteria (MOI of 1, 10, 100, and 1000) and indicated that a threshold is met at an MOI of 100, where an MOI greater than 100 does not result in any additional substrate accumulation and less than 100 does not result in any significant accumulation (data not shown).

Therefore, an MOI of 100 was the optimal dose of *S. typhimurium* that was used for all probe accumulation experiments. The results of rhodamine 123 accumulation during infection with *S. typhimurium* were substantiated by the accumulation of other P-gp probe substrates, namely [^{3}H]digoxin and DiOC2(3). Similar to rhodamine 123, we found that accumulation of [^{3}H]digoxin was clearly enhanced by bacterial invasion. In particular, using T84 cells grown on 24-well plates, the accumulation of [^{3}H]digoxin was 2.65 ± 0.30% and 6.10 ± 0.52% in uninfected and *S. typhimurium*-infected monolayers (4 h postinfection), respectively. In addition, the presence of verapamil (a P-gp inhibitor) lead to a significant increase in the accumulation of [^{3}H]digoxin for both uninfected and *S. typhimurium*-infected monolayers (6.19 ± 0.95% and 8.28 ± 0.48%, respectively). This finding suggests that *S. typhimurium* increases the retention of the P-gp probe substrate perhaps by exploiting a mechanism different than substrate-binding inhibition. We also assessed DiOC2(3), a fluorescent probe known to be a substrate for P-gp, but not the MDR-associated protein (MRP) family members (25). As expected, the retention pattern of this probe in *S. typhimurium*-infected T84 cells was similar to that determined for rhodamine 123. In a representative experiment, the accumulation of applied DiOC2(3) in uninfected monolayers was 0.41 ± 0.04% (mean ± SD), whereas the addition of a potent P-gp inhibitor, cyclosporine A, significantly increased ($P < 0.05$) the accumulation of DiOC2(3) to 0.79 ± 0.11% (mean ± SD). However, infection of monolayers with *S. typhimurium* for 4 h led to a significant accumulation of the probe (1.27 ± 0.15%) in the absence of the inhibitor. A similar amount of P-gp substrate was also accumulated in infected cells in the presence of cyclosporine A (1.40 ± 0.58%), suggesting that a maximum threshold of inhibition was reached.

Specificity of intracellular accumulation of P-gp substrates during infection. As entry into epithelial cells appears to be a central virulence factor common to Salmonellae, we next determined the specificity of different *Salmonella* strains to functionally downregulate P-gp. Thus we evaluated two wild-type *S. typhimurium* strains with different genetic backgrounds (SL1344 and SR11) as well as a strain representing a different *Salmonella* serovar, *Salmonella enterica* serovar Dublin (*S. dublin*). *S. dublin* is a host-restricted strain and thus is adapted to cattle where it causes systemic and enteric disease. Infrequently, however, serovar Dublin does cause septicemia and enteric disease in humans (18). As shown in Fig. 2, the two different wild-type background strains of *S. typhimurium* (SL1344 and SR11), in addition to *S. dublin*, retained the ability to functionally inhibit P-gp. These strains were able to adhere to and enter into polarized monolayers of T84 cells at similar levels (data not shown). However, *E. coli* F-18, a normal flora isolate of the human large intestine (9), failed to modulate the transport activity of P-gp. *E. coli* F-18 was able to attach to T84 polarized monolayers at similar levels as *S. typhimurium*, although no significant internalization was observed (data not shown).

We next sought to determine whether *S. typhimurium* could inhibit P-gp functional activities in cells other than those of intestinal origin. Therefore, we employed A549 human pulmonary cells, which express little, if any, P-gp (7). In contrast to the T84 intestinal cell line, we found that rhodamine 123 failed to accumulate in A549 cells infected with either *S. typhimurium* or the opportunistic lung pathogen *Pseudomonas aeruginosa* (PAO1) (Fig. 3*A*). Interestingly, probe accumulation was significantly less ($P < 0.05$) in *S. typhimurium*-infected monolayers than it was in uninfected controls. Treatment with verapamil also significantly inhibited the accumulation of the probe in both uninfected and *S. typhimurium*-infected monolayers compared with respective controls. Thus we speculate that a transporter involved in rhodamine 123 uptake that is sensitive to verapamil may be present on the apical surface of A549, although we did not further investigate this issue. Notably, although the mechanism of PAO1 pathogenicity is not fully understood, we found that *S. typhimurium* was able to invade A549 cells with more efficiency than T84 cells (Fig. 3*B*). Therefore, the failure of *S. typhimurium* to increase accumulation of the P-gp probe substrate within infected A549 cells is an indication that the retention of the probe is a mechanism specific for some cell lines, rather than a nonspecific effect of invasion, such as membrane permeabilization or pinocytosis. This result may also imply that expression of *MDR1* lowers the invasion capability of *S. typhimurium*. To further examine this notion, we performed invasion assays in a cell line that stably overexpresses *MDR1* (MDCKII-MDR1; see MATERIALS AND METHODS for description). Compared with wild-type MDCKII cells, we confirmed through Western blotting that the MDCKII-MDR1 cells did indeed overexpress P-gp (Fig. 4*A*). Interestingly, when we examined the ability of *S. typhimurium* to invade this cell line, we found that this enteric pathogen was significantly less able to invade the cell line stably overexpressing *MDR1* as compared the wild-type cell line (Fig. 4*B*).

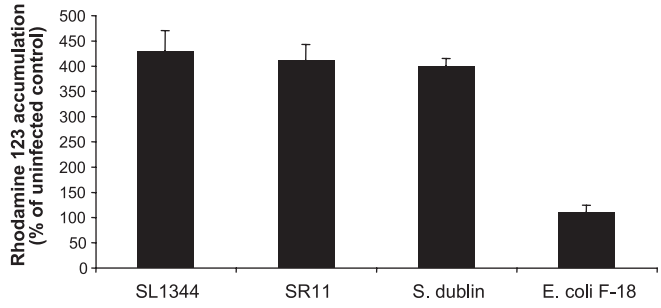

Fig. 2. Rhodamine 123 accumulation in T84 cells infected with different strains of *S. typhimurium* (SL1344 and SR11) and a normal flora *Escherichia coli* (*E. coli*) isolate (4 h postinfection). T84 cell monolayers were infected with bacteria for 1 h, washed, and further incubated in DMEM for 2 h at 37°C prior to the addition of rhodamine 123 (as described in MATERIALS AND METHODS). *E. coli* F-18 is a normal flora isolate of a healthy human intestine. The data are plotted as the percent (%) of the uninfected controls and are expressed as the means ± SD ($n = 8$) for all conditions tested. 1 representative experiment of at least 3 independent experiments performed with similar results.

Having established some insight regarding the specificity of intracellular accumulation of P-gp substrates during *S. typhimurium* infection, we next examined whether key inflammatory and apoptotic mediators, such as LPS and TNF-α, could also modulate P-gp functional activity under the same experimental conditions carried out for *S. typhimurium* infection. We found that cellular accumulation of rhodamine 123 in T84 cells was not affected by exposure to either LPS (0.1–100 μg/ml) applied apically or TNF-α (1–100 μg/ml) applied apically and basolaterally to monolayers grown on Transwell filters (data

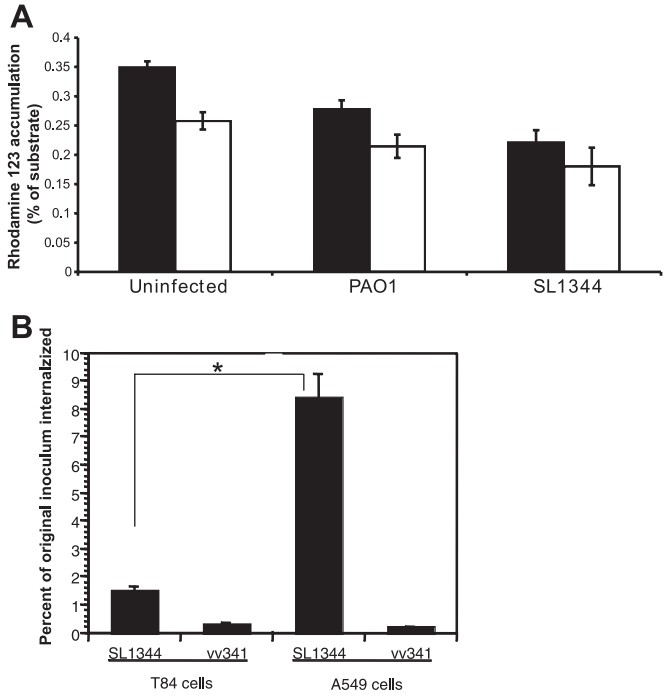

Fig. 3. Specificity of intracellular accumulation of P-glycoprotein (P-gp) substrates during bacterial infection. *A*: rhodamine 123 accumulation in A549 cells (4 h postinfection). Uninfected monolayers or monolayers infected with either *Pseudomonas aeruginosa* (PAO1) or wild-type *S. typhimurium* (SL1344) for 1 h were subsequently incubated in serum-free media (black bars) or serum-free media containing verapamil (40 μM, white bars) for 2 h at 37°C before the addition of rhodamine 123 (5 μM). The data are expressed as the means ± SD ($n = 8$) for all conditions tested and represent 1 of at least 3 independent experiments performed with similar results. *B*: invasion of T84 cells and A549 cells by *S. typhimurium*. A standard gentamicin protection assay was performed (see MATERIALS AND METHODS) to determine those bacteria that entered into epithelial cells. The number of internalized bacteria was calculated as the percent of the original inoculum. The data represent the means ± SD of triplicate samples. 1 representative experiment of 3 performed (all showing the same result) is presented. Statistical analysis by Student's *t*-test. *Statistical difference ($P < 0.01$) compared with the respective control.

not shown). Recently it has also been suggested that infection-induced hypoxia may play a role in the upregulation of P-gp expression (10). Although these observations were made at significantly later times than the time points used in this study (i.e., 18 h), we did evaluate the potential role of hypoxia. To accomplish this, we carried out identical infections and rhodamine 123 accumulation experiments in both a standard 37°C room incubator, as well as in a 37°C incubator at ambient CO_2 conditions (5%) and found there to be no significant difference in the amount of rhodamine 123 that accumulated (data not shown). Thus we conclude that changes in oxygen levels did not significantly impact our experiments at the times evaluated during this study. In addition, since BSA is a protein known to stimulate cellular uptake via caveolin structures (30), we used BSA (10–10,000 μg/ml) to assess any nonspecific mechanisms of probe uptake mediated by endocytosis. Although caveolin and P-gp often colocalize, and overexpression of caveolin-1 has been found to reverse the MDR phenotype in breast adenocarcinoma cells by inhibiting P-gp transport activity (37), BSA did not affect cellular accumulation of rhodamine 123 (data not shown).

Assessment of basolateral and apical uptake mechanisms. Since it has been reported that an active uptake mechanism facilitates the entry of rhodamine 123 into polarized monolayers of Caco-2 cells from the basolateral, but not the apical surface (31), we next sought to evaluate the intracellular concentration of probes internalized from the basolateral or apical compartments of T84 cell monolayer grown on Transwell filters. Consistent with this earlier study (31), we observed greater probe retention when rhodamine 123 was supplied from the basolateral side compared with the apical side (0.45 ± 0.003 nmol vs. 0.03 ± 0.003 nmol, respectively, Fig. 5). Moreover, the inhibitor cyclosporine A produced an ~50% increase in retention when the probe was administered from either the basolateral or the apical compartment (0.091 ± 0.008 nmol and 0.074 ± 0.005 nmol, respectively). As expected, *S. typhimurium* caused a significant increase of intracellular rhodamine 123 accumulation (0.15 ± 0.01 nmol, $P < 0.01$) compared with uninfected controls when probe substrate was supplied from the apical chamber; by contrast, a marked reduction of probe accumulation (0.21 ± 0.02 nmol) was seen when the probe was administered basolaterally. The concomitant use of cyclosporine A when administered from either the apical or basolateral chamber in *S. typhimurium*-infected monolayers did not significantly ($P > 0.05$) increase retention of the probe compared with the infected monolayers without the inhibitor. Thus the inability of cyclosporine A to increase accumulation when the probe is administered in the apical chamber suggests that the bacteria have reached a maximal level of inhibition. In the case of basolateral administration the fact that cells infected with *S. typhimurium* accumulated sig-

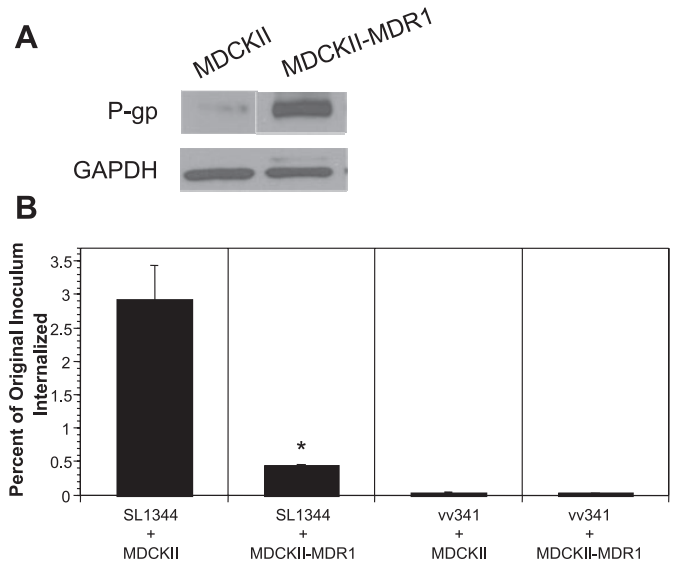

Fig. 4. Madin-Darby canine kidney II (MDCKII) cells overexpressing multidrug resistance (*MDR*)*1*. *A*: immunoblot of MDCKII and MDCKII-MDR1 cells showing different amounts of *MDR1* expression. Detection of GAPDH levels served as the internal control for protein loading conditions. The data represent a Western blot analysis from an individual experiment performed at least 3 times. *B*: monolayers of MDCKII or MDCKII-MDR1 cells were infected from the apical pole with wild-type *S. typhimurium* (SL1344) and an invasion-defective isogenic mutant, VV341, and bacterial internalization was quantified as described in MATERIALS AND METHODS. Results are the means ± SD of 3 independent filters. Data are representative of 3 experiments performed. Statistical analysis by Student's *t*-test. *Statistical difference ($P < 0.01$) compared with the respective control.

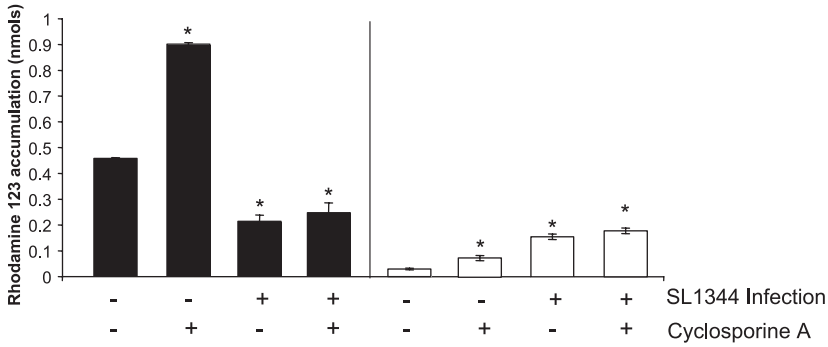

Fig. 5. Intracellular accumulation of rhodamine 123 into T84 polarized cell monolayers under conditions where the P-gp substrate is supplied from either the basolateral (solid bars) or apical (open bars) side of the Transwell filter support. Uninfected control cells or cells infected with *S. typhimurium* were treated in the absence and presence of 10 μM cyclosporine A, as described in MATERIALS AND METHODS. The data (4 h postinfection) are expressed as the means ± SD ($n = 6$) for all conditions tested and represent 1 of at least 3 independent experiments performed with similar results. Statistical analysis by Student's *t*-test; *$P < 0.05$ compared with control monolayers.

nificantly less probe than the controls (even in the presence of cyclosporine A) strongly suggest that an uptake transporter for rhodamine 123 is indeed present on the basolateral membrane of T84 cells and that the uptake ability of the same transporter has been inhibited by *S. typhimurium* (Fig. 5). A similar pattern was also observed when a different human intestinal epithelial cell line, HCT8, was infected with *S. typhimurium* in the absence and presence of the inhibitor verapamil (not shown).

The functional role of P-gp during S. typhimurium infection. Our results thus far suggest that upon *S. typhimurium* infection P-gp plays a determinant role in the mechanism of efflux, presumably through a functional downregulation of this transporter. Thus we next determined the extent to which changes in transporter expression contributed to the *Salmonella*-induced decrease in P-gp-mediated transport. We performed a biochemical analysis in which polarized T84 cell monolayers were apically infected with *S. typhimurium* for 3 h. As shown in Fig. 6, *S. typhimurium* interaction with the apical surface of T84 cell monolayers resulted in the ability of this enteric pathogen to substantially downregulate the expression of P-gp. Under similar conditions, we also measured *MDR1* mRNA levels by real-time PCR and determined that the levels of *MDR1* mRNA were the same regardless of the presence of *S. typhimurium* (data not shown). Time courses including earlier time points (1 and 2 h) following infection with *S. typhimurium* also showed no difference in the expression pattern of *MDR1* mRNA (data not shown). Consequently, these data suggest that the ability of *S. typhimurium* to downregulate the expression of P-gp is posttranscriptionally regulated.

To further assess the functional role of P-gp during *S. typhimurium* infection, polarized monolayers of intestinal epithelial cells were transfected with a plasmid generating siRNA, which bind and degrade *MDR1* mRNA, thereby decreasing P-gp expression (Fig. 7*A*). As expected, rhodamine 123 accumulation was nearly double in the cells exhibiting a decrease in P-gp with respect to parent cells expressing wild-type levels of P-gp. Wild-type HCT8 cells exposed to *S. typhimurium* accumulated significantly more rhodamine 123 than uninfected controls ($P < 0.05$). However, accumulation of rhodamine 123 was not significantly different ($P > 0.05$) when comparing the *S. typhimurium*-infected and uninfected monolayers of cells expressing *MDR1* siRNAs (Fig. 7*B*).

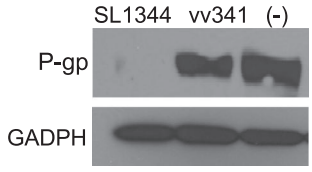

Fig. 6. P-gp (MDR1) protein expression pattern in T84 cells infected with *S. typhimurium* (3 h postinfection). Immunoblot showing levels of P-gp (MDR1) expression in cells infected with wild-type *S. typhimurium* SL1344 and the *HilA*-mutant VV341. Detection of GAPDH levels served as the internal control for protein loading conditions. The experiment was repeated 3 times, and the results are from 1 representative experiment.

The function and anatomic localization of P-gp in the gastrointestinal tract suggest that this transporter acts as a protective barrier to keep toxins out of the body by secreting them into the intestinal lumen. However, a clear physiological role for P-gp has not yet been clearly defined. Thus to further dissect the functional role of P-gp during infection with *S. typhimurium*, we examined epithelial cell cytoxicity using intestinal epithelial cells silenced for the expression of P-gp. Using a LDH cell viability assay, we found that cells exhibiting a decrease in P-gp functionality, with respect to parent cells expressing wild-type levels of P-gp, displayed a profound susceptibility to damage following infection with *S. typhimurium* (Fig. 7*C*), suggesting that P-gp is necessary for maintaining cell viability in the infected epithelium.

DISCUSSION

The enteric pathogen, *S. typhimurium*, initiates infection by invading enterocytes predominantly located within the distal ileum. This mode of entry is principally achieved though a process requiring the type III secretion machinery (SPI-1) (29), in which *S. typhimurium*-secreted effector proteins are translocated into the target cell cytoplasm (11). As a consequence, these effector proteins coopt host cell signal transduction pathways resulting in localized actin polymerization, membrane ruffling, and, ultimately, pathogen entry (14). Because of the close association of the cytoskeleton with the clinically relevant *MDR1* gene product, P-gp, a plasma membrane efflux transporter (2, 13, 16), we investigated the effects of *Salmonella* invasion of enterocytes with regard to the expression and functionality of this transporter. Herein, our studies are the first to demonstrate that the enteric pathogen, *S. typhimurium*, is able to decrease the abundance and function of P-gp.

MDR1 overexpression is one form of the MDR phenotype, which can be acquired by cancer patients that are initially responsive to chemotherapy. Thus much effort has been put forth to identify clinically effective inhibitors of P-gp (8). At least nine ABC (ATP-binding cassette) proteins related to P-gp have been characterized to date, and these have been shown to mediate efflux of small molecules from cells (15). In addition to P-gp, the MRP2 (or ABCC2) and breast cancer resistance

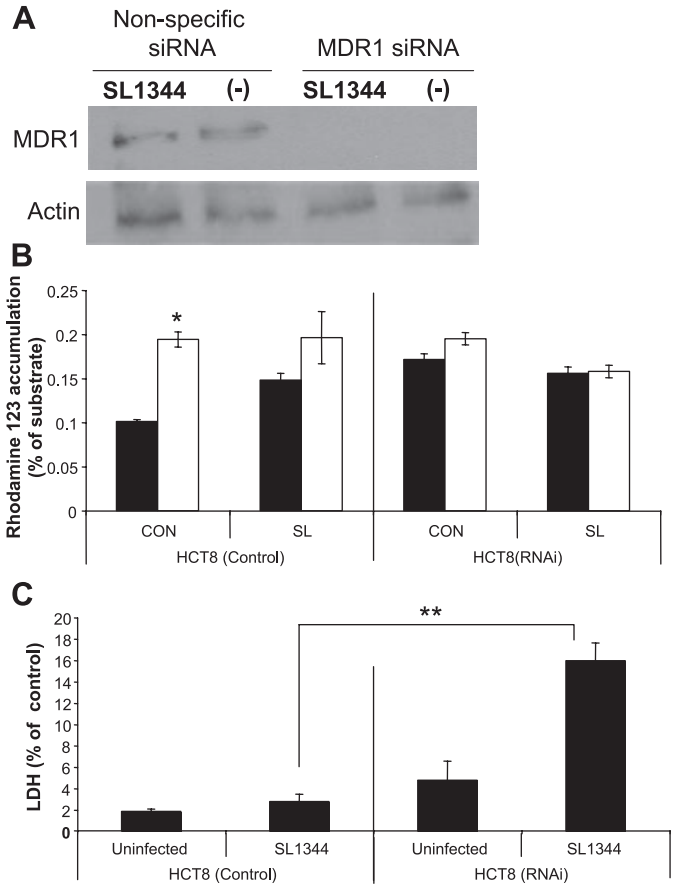

Fig. 7. Rhodamine 123 uptake and measurement of cytotoxicity in HCT8 cells silenced for the expression of P-gp (MDR1). *A*: Western blot analysis showing the level of P-gp in HCT8 cells transfected with a plasmid-generating siRNA targeted at decreasing the level of P-gp (MDR1 siRNA) or control siRNA (nonspecific siRNA). Transfected cells were exposed to buffer only (−) or infected with wild-type *S. typhimurium* SL1344. Detection of actin levels served as the internal control for protein-loading conditions. The experiment was repeated 3 times, and the results are from 1 representative experiment. *B*: rhodamine 123 accumulation in HCT8 cells, control or transfected with MDR1 RNA interfering vector HCT8 (RNAi) infected with *S. typhimurium* (4 h postinfection). Uninfected or monolayers infected with bacteria for 1 h were subsequently incubated in serum-free media for 2 h at 37°C. Rhodamine 123 (5 μM) was then added and allowed to incorporate for 1 h at 37°C in the absence (solid bars) or presence (open bars) of the P-gp inhibitor cyclosporine A (10 μM). Fluorescence was measured by spectrofluorometry. The experiment was repeated 3 times, and the results shown a representative experiment. Values represent the means ± SD ($n = 8$). *Statistically significant difference with respect to control ($P < 0.05$). *C*: evaluation of cell cytotoxicity during infection with *S. typhimurium*. Control HCT8 cells and HCT8 cells silenced for the expression of *MDR1* were infected with *S. typhimurium* for 4 h at 37°C. Release of LDH into the medium was measured using a colorimetric assay as described in the MATERIALS AND METHODS section. The data are expressed as the means ± SD ($n = 6$) for all conditions tested and are plotted as the percent (%) of the lysis control and 1 of at least 3 independent experiments performed with similar results. **Statistically significant difference with respect to control ($P < 0.01$).

protein (BCRP or ABCG2), also mediate MDR in tumor cells and, together with P-gp, are found on the apical membrane of intestinal epithelial cells.

In this study, we measured the activity of P-gp using three select probe substrates. DiOC2(3) is highly specific for P-gp and is not transported by the related MDR protein, MRP1 (25). Rhodamine 123 is effluxed by P-gp and, to a lesser extent, by MRP transporters and thus serves as a broader indicator of total cellular efflux activity. Another member of the ABC family, BCRP, weakly transports DiOC2(3) but does not transport rhodamine 123 (24). In addition to the hydrophilic, cationic probes such as rhodamine 123 and DiOC2(3), the lipophilic probe, [^{3}H]digoxin, was also assessed. Digoxin, in contrast to rhodamine 123 and DiOC2(3), is not known to accumulate inside intracellular stores, nor it is sensitive to cellular metabolic changes possibly arising from apoptotic or other toxic events.

The fundamental observation of this investigation was that infection of polarized intestinal cell monolayers with *S. typhimurium* led to a profound increase in the intracellular accumulation of rhodamine 123, DiOC2(3), and [^{3}H]digoxin. A mechanistic explanation for the enhanced retention of P-gp probes within bacterial-infected cells is largely based on the biochemical analysis, which revealed a profound decrease of P-gp in protein extracts of infected cells (Fig. 6). Since the levels of *MDR1* mRNA measured by real-time RT-PCR remained unchanged, we speculate that a process leading to the degradation of the protein expressed on the cell membrane may be activated by *S. typhimurium* invasion. Since other possibilities such as receptor internalization are also plausible, further dissection of this mechanism is currently under investigation.

Two lines of evidence indicate that such P-gp-specific substrate accumulation required bacterial invasion. First, the adherent but non-invasive *hilA* mutant (VV341) did not promote the accumulation of rhodamine 123 or DiOC2(3), demonstrating that bacterial adhesion in and of itself is not sufficient for drug accumulation. This interpretation was substantiated by the observation that gentamicin treatment of colonized monolayers (which eliminates extracellular adherent bacteria) did not substantially change the accumulation profile of rhodamine 123 with respect to untreated colonized controls (Fig. 1). This finding also indicates that bacterial-mediated endocytosis did not significantly contribute to nonspecific uptake of the P-gp-specific substrates. Of note, such accumulation of P-pg substrates was not the result of a loss in cell membrane integrity caused by necrosis in response to bacterial injury, as we routinely monitored LDH release into the culture medium following bacterial infection (D. Siccardi and B.A. McCormick, unpublished observations).

In contrast to the findings with intestinal cells, the lung adenocarcinoma cell line A549, which has been previously reported to be colonized by *S. typhimurium* (17), demonstrated a decrease in rhodamine 123 accumulation upon infection with *S. typhimurium*. This was not entirely unexpected since A549 cells express only modest levels of P-gp, if any (7, 36). Thus the intracellular accumulation pattern of rhodamine 123 in A549 cells substantiates that membrane permeability events driven by bacterial invasion (if responsible for the efflux of the probe from A549) are not involved in probe accumulation in the intestinal cell lines. However, the substantial invasion of bacteria into A549 cells, which we noted to be significantly higher compared with the intestinal cell line T84, suggested the possibility that P-gp may play a role in regulating bacterial entry into the cell (Fig. 3*B*). This was further substantiated by our results demonstrating that *S. typhimurium* was significantly less able to invade cells that overexpress *MDR1* (Fig. 4). Furthermore, similar to the P-gp-negative A549 cells, cells silenced for the expression of *MDR1* also showed that rhoda-

mine 123 did not accumulate in *S. typhimurium*-infected monolayers (Fig. 7*B*), suggesting that P-gp function is necessary for probe accumulation.

In the evaluation of the intracellular concentration of P-gp substrates taken up from either the basolateral or apical surface of polarized T84 cell monolayers, we found evidence of a second transporter system, one located on the basolateral side of T84 cells, which is involved in the active uptake of rhodamine 123. This finding is consistent with prior studies that identified rhodamine 123 uptake from the basolateral membrane of Caco-2 monolayers functions by an unidentified transporter (31). In our study, rhodamine 123 applied to the basolateral surface of polarized T84 cell monolayers accumulated intracellularly at a higher rate than when administered from the apical surface (Fig. 5), confirming the existence of such an uptake system in T84 cells. This basolateral transporter was inhibited by *S. typhimurium* but not cyclosporine A or verapamil. The inability of cyclosporine A (MRP1, MRP2, and BCRP inhibitor) or verapamil [MRP1, h organic cation transporter (OCTN)1, and OCTN inhibitor] to rescue the uptake of rhodamine 123 supplied to the basolateral chamber excludes the possibility that the activation of these efflux transporters subsequent to bacterial invasion may be involved. The exact nature of this uptake transporter has not been investigated in this study, but it is noteworthy that *S. typhimurium* infection can cause dramatic changes in the host cell such as the modulation of a protein carrier physically distant from the site of invasion.

P-gp is a plasma membrane protein that is involved in the active efflux of xenobiotics from cells. Although the role of P-gp in contributing to the MDR phenotype of many cancer cells resistant to antineoplastic agents is well known (8), a clear physiological role of this transporter has never been established. An intriguing finding in this study was the decreased resistance to *S. typhimurium* infection when P-gp expression was silenced by means of siRNA (Fig. 7, *B* and *C*). Moreover, there is our observation of the poor invasion potential of *S. typhimurium* in cells overexpressing *MDR1* (Fig. 4*B*). On the basis of these findings, it is tempting to speculate that P-gp may be important in maintaining homeostasis in the gut through modulation of intestinal responses to bacteria. In support of this contention, the role for P-gp in inflammation has been suggested given that LPS, IL-2 (31), and TNF-α (4) have been shown to reduce P-gp activity in the intestine, liver, and brain. Furthermore, using *MDR1* knockout mice, a relationship between the downregulation of P-gp and the development of inflammatory bowel disease has been demonstrated (26). Consistent with this finding, the reduced expression of *MDR1* gene has also been observed in intestinal biopsies obtained from patients with gastrointestinal disorders (1, 5, 16, 20). However, whether this is a direct consequence or a cause of the disease has never been established. Low levels of P-gp have also been suggested to aggravate intestinal inflammation. In keeping with these observations, our preliminary findings have further revealed that infection of mice with wild-type *S. typhimurium* results in the near complete loss of P-gp protein expression (D. Siccardi and B.A. McCormick, unpublished observations). Therefore, our findings are in agreement with the idea that P-gp plays an important role in maintaining gastrointestinal homeostasis and, for the first time, suggest a specific physiological role for P-gp in the intestinal epithelium. Furthermore, given that *S. typhimurium* localizes to tumors and in this report we have shown *S. typhimurium* to functionally downregulate P-gp, this provides the impetus for the development of an active MDR-reversing agent.

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