

## **A Role for Stem Cell Factor (SCF): c-kit Interaction(s) in the Intestinal Tract Response to *Salmonella typhimurium* Infection**

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### **Summary**

Cholera toxin (CT) has been shown to induce stem cell factor (SCF) production in mouse ligated intestinal loops. Further, SCF interaction(s) with its receptor (c-kit) was shown to be important for the intestinal tract secretory response after CT exposure. In this study, we have investigated whether SCF production is induced in the intestinal tract after exposure to *Salmonella typhimurium* and whether this production could be an important intestinal tract response to *Salmonella* infection. Using a mouse ligated intestinal loop model, increased levels of SCF mRNA were detected at 2–4 h post-*Salmonella* challenge. Intestinal fluid obtained from *Salmonella*-challenged loops contained high levels of SCF by ELISA. Human and murine intestinal epithelial cell lines were also shown to have increased levels of SCF mRNA after exposure to *Salmonella*. Inhibition of *Salmonella* invasion of epithelial cells was shown to be one potentially important role for SCF:c-kit interactions in host defense to *Salmonella* infection. Pretreatment of human or murine intestinal cell lines with SCF resulted in a cellular state that was resistant to *Salmonella* invasion. Finally, mice having mutations in the white spotting (W) locus, which encodes the SCF-receptor (c-kit), were significantly more susceptible to oral *Salmonella* challenge than their control littermates. Taken together, the above results suggest that an important intestinal tract response to *Salmonella* infection is an enhanced production of SCF and its subsequent interactions with c-kit.

**S**tem cell factor (SCF), sometimes referred to as mast cell growth factor or c-kit ligand (KL) and its receptor, c-kit, have been extensively studied and are the subject of many recent reviews (1, 2). SCF exists in both membrane-bound and soluble forms, with the soluble form representing a cleavage product of a surface bound protein (3, 4). The c-kit protein (CD117), the receptor for SCF, is a tyrosine kinase-containing receptor of the PDGF/CSF-IR receptor family (5, 6). SCF:c-kit interactions have been shown to play different roles in a wide range of physiological functions. SCF:c-kit interactions are important for mast development and function (1, 2), melanocyte development (7, 8), germ cell development (9, 10), and may play a role in the development and function of the nervous system (11, 12). Additionally, SCF:c-kit interactions have been shown to be important for the enteric nervous system (13–15) and for development of TCR $\gamma\delta^+$  intraepithelial lymphocytes (IEL) (16). Thus, SCF:c-kit interactions appear to be important for certain aspects of intestinal tract physiology. However, the importance of SCF:c-kit interactions in the

intestinal tract response to infection has not been fully explored. Using a mouse ligated intestinal loop model, we have recently shown that cholera toxin (CT) induces the production of elevated levels of SCF in the intestinal tract, and that SCF:c-kit interactions are necessary for CT-induced intestinal fluid secretion (17). In the present study, we have investigated whether SCF-production and/or SCF:c-kit interactions are important intestinal tract responses to *Salmonella* infection. In this paper, we show the following: (a) *Salmonella typhimurium* induces enhanced production of SCF in human and mouse intestinal epithelial cell lines and in mouse ligated intestinal loops, (b) pretreating human or mouse intestinal cell lines with SCF results in a cellular state that is resistant to *Salmonella* invasion, and (c) mice with mutations in the white spotting (W) locus, which encodes the SCF receptor (c-kit), are more susceptible to oral *Salmonella*-challenge than their control littermates. Our results suggest that SCF production and its subsequent interaction(s) with c-kit may play an important role in the intestinal tract response to *Salmonella* infection.

## Materials and Methods

**Mice.** All mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed under pathogen-free conditions. Female mice at 6–8 wk of age were used from the following mouse strains: WBB6F<sub>1</sub>/JW/W<sup>V</sup> and WBB6F<sub>1</sub>/J<sup>+</sup>. In some experiments, mice were challenged orally with varying numbers of *S. typhimurium*. LD<sub>50</sub> values were determined by the method of Reed and Muench (18).

**Reagents.** *Salmonella typhimurium* TML-R66 were used in all experiments. Cholera toxin (CT) was obtained from List Biological Laboratories, Inc. (Campbell, CA). Recombinant rat and human SCFs were purified as previously described (19).

**Cell Lines.** The human colonic epithelial cell lines HT-29 and T-84 were obtained from the American Type Culture Collection (Rockville, MD). The human intestinal myofibroblast cell line 18CO has been previously described (20) and was a generous gift from Dr. Don Powell (UTMB). The MODE-K cell line is a mouse intestinal epithelial cell line which has been previously characterized (21) and was obtained from Dr. Dominique Kaiserlian (Unite'd Immunologie et de Strategie Vaccinale Institute Pasteur de Lyon, Cedex, France). All cell lines were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 2 mmol/L L-glutamine and antibiotics. In some experiments, RNA was obtained from different cell lines at varying times post-exposure to *S. typhimurium*. In these experiments, cells were grown to confluency in 75-cm<sup>2</sup> flasks (5 × 10<sup>7</sup> cells). Cells were grown in antibiotic-free medium and exposed to *Salmonella* (MOI 10:1) for 90 min. Cells were then washed five times and cultured with medium containing 2× antibiotics and RNA obtained at varying times post-exposure.

**Northern Blot Analysis.** Human and murine SCF probes have been previously described (22, 23). A cDNA probe for the housekeeping gene glyceraldehyde-3 phosphate dehydrogenase (G3PDH) was obtained from Clontech Laboratories (Palo Alto, CA). The SCF-cDNA was nick-translated in preparation for RNA hybridization. Northern blot analysis was performed as we have previously described (21, 25). Total mucosal RNA or RNA from different cell lines was extracted using guanidine isothiocyanate and purified by cesium chloride gradient centrifugation. RNA samples were electrophoresed through a 1.4% agarose gel in the presence of formaldehyde then transferred to nylon membranes. Filters were UV crosslinked and baked at 80°C for 2 h and prehybridized for 18–24 h at 42°C in 6× SSPE, 5× Denhardt's solution, 0.5% SDS, 50% formamide, and 300 µg/ml denatured salmon sperm DNA. Hybridization was performed at 42°C for 18–24 h in 6× SSPE, 5× Denhardt's solution, 0.1% SDS, 50% formamide, 100 µg/ml denatured salmon sperm DNA and 1 × 10<sup>6</sup> CPM/ml [<sup>32</sup>P]-DNA. Initial washes were performed at room temperature with 5× SSPE/0.5% SDS for 15 min, followed by 15 min under the same conditions. Filters were then washed twice in SSPE/0.5% SDS at 42°C for a total of 15 min. Autoradiography was performed with intensifying screens at -70°C with an exposure of 1–3 d to XAR-5 film.

**Ligated intestinal loop assay:** The ligated loop surgery was performed on mice as previously described (24, 25). Mice were fasted for 24 h before surgery. A midline abdominal incision was made to expose the small intestine, and a single segment (~5 cm) was ligated with 00 silk suture. Loops were injected with one of the following: 100 µl of phosphate-buffered saline (PBS) only, PBS containing *S. typhimurium* (10<sup>7</sup> CFU), or PBS containing cholera toxin (1 µg). Incisions were closed with staples and mice were sacrificed at varying times post-surgery. At that time, loops

were removed, measured, fluid volume determined, fluid saved for SCF-ELISA, and total RNA isolated as described below.

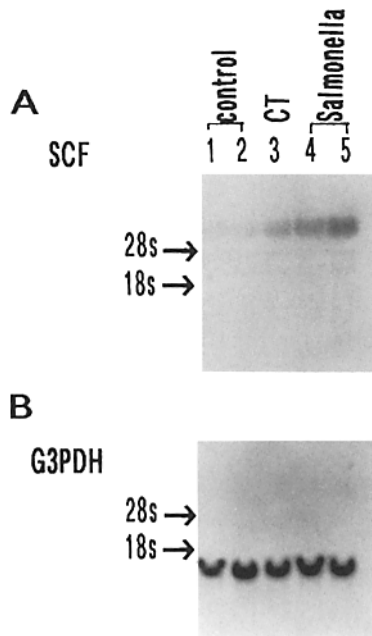
**Quantitation of Bacteria-infected Cells.** The agarose-agar overlay assay was used to quantitate the number of intestinal epithelial cells containing *Salmonella*. This assay has been previously described (26). Briefly, triplicate wells of MODE-K cells or 18CO cells were treated overnight with varying concentrations of rSCF or medium alone. Before exposure to *Salmonella*, cell monolayers were maintained in antibiotic-free medium and then exposed to *S. typhimurium* (MOI for MODE-K 1:4, MOI for 18CO 10:1) for 1 h. Noninternalized bacteria were then counterselected by exposing monolayers to medium containing antibiotics for 2 h at 37°C. Monolayers were then extensively washed with antibiotic-free medium and the number of bacteria-infected cells (CFU/well) determined.

**Mouse SCF ELISA.** An ELISA for quantitating mouse SCF was developed using the following reagents: (a) capture antibody was a rat monoclonal antibody specific for mouse SCF (JES9-28F4) and (b) detecting antibody was a purified and biotinylated rabbit IgG specific for mouse SCF (17). Immulon-2 plates (Dynatech Laboratories, Chantilly, VA) were first coated with 2 µg/ml of capture antibody for 24 h at 4°C. Wells were then washed twice with PBS/Tween-20 and blocked for 2 h with PBS containing 10% fetal calf serum. Wells were then washed twice with PBS/Tween-20 and ligated loop fluid and varying concentrations (10 ng/ml to 100 pg/ml) of rSCF added to wells for overnight incubation at 4°C. The ELISA was developed by sequential addition of detecting IgG (1 µg/ml) followed by avidin-peroxidase and substrate. Optical densities (405 nm) were next read after 30–45 min and SCF concentrations determined from a SCF standard curve. The SCF-ELISA was sensitive to a level of 400 pg/ml.

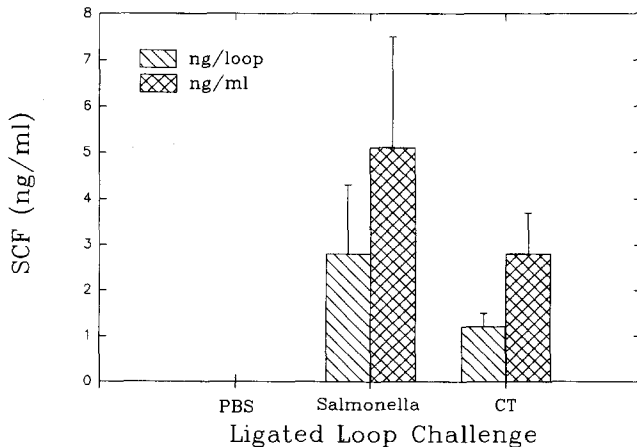
## Results and Discussion

*Salmonella* infection of the gastrointestinal tract results in fluid secretion and inflammation. Using a mouse ligated intestinal loop model, we have previously shown that ligated intestinal loops challenged with *S. typhimurium* have elevated levels of mRNA for IL1, IL6, TNFα and IFNγ and that these cytokines can be detected in the fluid obtained from such infected intestinal loops (24, 25). The cellular source for these cytokines is still not completely understood. Mast cells represent potentially important sources for several of these cytokines and could play an important role in the intestinal tract inflammatory response (27). Mast cells have been shown capable of producing a number of different cytokines including IL1, IL6, TNFα, and IFNγ (28). Additionally, mast cells are abundant in mucosal tissues and have been shown to phagocytize and kill bacteria (29, 30). SCF has been shown to be an important mediator of mast cell development, mast cell survival, mast cell chemotaxis, and mast cell production of different cytokines (1, 2). Besides these functions, SCF has also been shown to be important for homeostasis of the intestinal immune system. SCF:c-kit interactions were shown to be required for the maintenance and generation of γδ<sup>+</sup> IELs (16). Recently, we have shown that CT induces enhanced production of SCF by the intestinal tract and that SCF:c-kit interaction(s)

## Ligated Intestinal loop



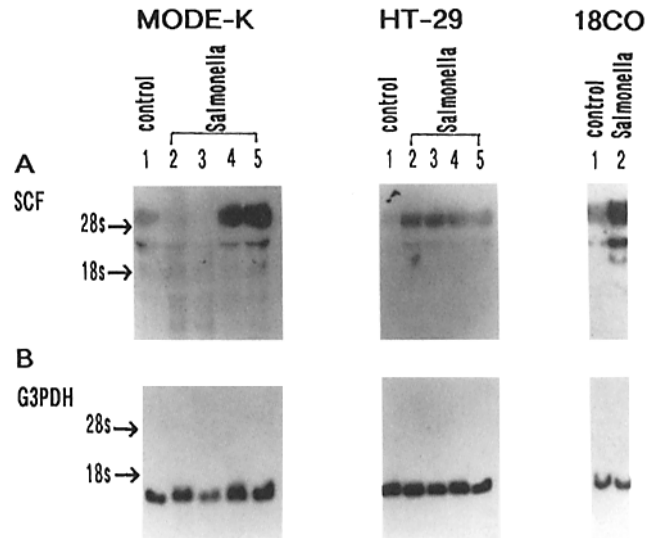
**Figure 1.** Ligated intestinal loops challenged with *S. typhimurium* have elevated levels of SCF mRNA. Ligated intestinal loops from C57BL/6 mice were challenged with one of the following: (a) PBS (lanes 1 and 2), (b) Cholera toxin (1  $\mu$ g, lane 3), or (c) *S. typhimurium* ( $10^7$  cfu, lanes 4 and 5). RNA was obtained at 2 h post-challenge. Each lane represents RNA obtained from an individual ligated intestinal loop. Northern blot analysis was carried out with a cDNA probe for SCF (A) or for G3PDH (B).



**Figure 2.** High levels of SCF are present in the fluid obtained from ligated intestinal loops challenged with *S. typhimurium*. Fluid was obtained at 10 h from ligated loops from C57BL/6 mice challenged with one of the following: (a) PBS, (b) CT (1  $\mu$ g), or (c) *S. typhimurium* ( $10^7$  CFU). The data presented was from fluid obtained from four mice per condition. Control loops (injected with PBS) were gavaged with a volume of PBS that was equivalent to the volumes of fluid contained in *Salmonella*-challenged loops. Ligated intestinal loops injected with CT or *Salmonella* yielded roughly equivalent levels of fluid: *Salmonella* =  $615 \pm 45$   $\mu$ l per loop; CT =  $585 \pm 70$   $\mu$ l per loop. SCF levels are expressed as either ng per ml of fluid or ng per loop.

is important for intestinal tract fluid secretion induced by CT (17). Thus, we initiated experiments to investigate whether SCF production is upregulated in the intestinal tract after *S. typhimurium* infection. As seen in Fig. 1, ligated intestinal loops challenged with *Salmonella* contained elevated levels of SCF mRNA. Of the three different sized mRNA transcripts for SCF (6.5–7 kb, 4.5–5 kb, and 3–4 kb) the 6.5-kb transcript appeared to be the most dramatically enhanced. CT also induced increased levels of SCF mRNA but at levels significantly lower than that present in *Salmonella*-challenged intestinal loops. Fluids obtained from intestinal loops challenged with *Salmonella* versus CT were also assessed for the presence of SCF protein by ELISA. Fluids obtained from either *Salmonella*- or CT-challenged intestinal loops contained significant levels of SCF protein (Fig. 2).

To explore what cell types might be involved in the enhanced production of SCF after *Salmonella* challenge, we investigated human and mouse intestinal epithelial cell lines for their ability to produce SCF after exposure to *Salmonella*. As seen in Fig. 3, mouse (MODE-K cells) and human (HT-29 and 18CO) intestinal cell lines contained elevated levels of SCF mRNA after exposure to *Salmonella*. In these cell lines, the 6.5- and 4.5-kb transcripts appeared to be the

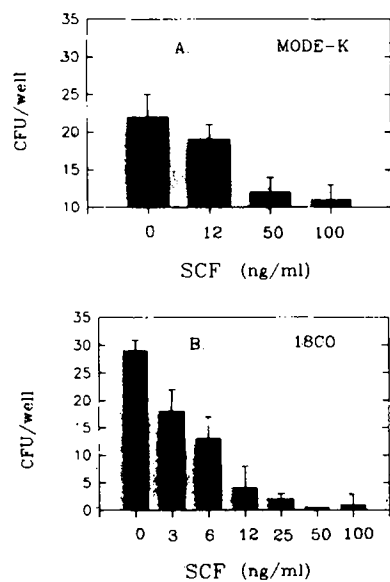


**Figure 3.** Human and murine intestinal epithelial cell lines exposed to *Salmonella* have increased levels of SCF mRNA. Levels of SCF mRNA were assessed by Northern blot analysis from intestinal cell lines from mouse (MODE-K) and human (HT-29 and 18CO) origin as described in Materials and Methods section. RNA was obtained from MODE-K cells exposed to medium only for 4 h (lane 1, control), or from MODE-K cells at the following times post-exposure to *S. typhimurium*: 30 min (lane 2), 1 h (lane 3), 2 h (lane 4) and 4 h (lane 5). RNA was obtained from HT-29 after the following treatments: medium only for 1 h (lane 1, control), *S. typhimurium* for 30 min (lane 2), 1 h (lane 3), 2 h (lane 4), and 4 h (lane 5). RNA was obtained from T84 cells after 4 h with medium only (lane 1, control) and after 4 h exposure to *S. typhimurium*. Northern blot analysis was carried out with a cDNA probe for either mouse or human SCF (A) or for G3PDH (B).

most dramatically enhanced. The above results suggest that exposure of the intestinal tract to *Salmonella* results in an elevated production of SCF and that one potential source for SCF production is the intestinal fibroblast and/or epithelial cell.

We (31) and others (32, 33) have previously shown that pretreatment of human or mouse epithelial cells or fibroblasts with different cytokines results in a cellular state that is resistant to subsequent invasion by *Salmonella* or other invasive bacteria. Since *Salmonella* invasion induced an enhanced production of SCF, we investigated whether SCF pretreatment of fibroblasts or epithelial cells could induce a cellular state that is resistant to *Salmonella* invasion. Cultures containing MODE-K or 18CO (a human intestinal fibroblast cell line) were pretreated with varying concentrations of rSCF and then assessed for their susceptibility to *Salmonella* invasion using a previously described quantitative invasion assay (26). As seen in Fig. 4, SCF pretreatment of MODE-K cells or 18CO cells resulted in a cellular state that was resistant to *Salmonella* invasion. This effect was dependent upon the concentration of SCF, and when high concentrations of SCF were used, a 80–95% reduction in *Salmonella* infected cells was achieved. These results suggest that SCF:c-kit interactions may play a role in host defense by inducing a cellular state that does not allow for efficient bacterial invasion of epithelial cells and/or fibroblasts.

To further explore the possible role of SCF:c-kit interactions in host defense, we investigated whether WW<sup>V</sup> mice have alterations in their resistance to oral *Salmonella* chal-

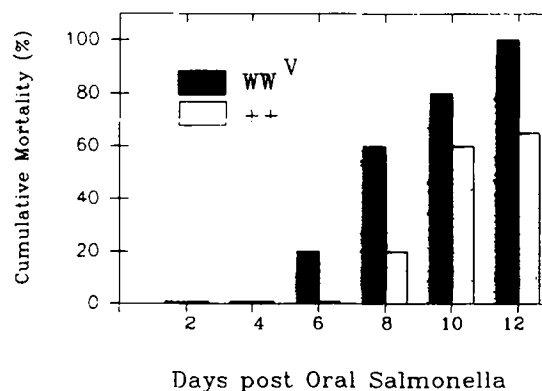


**Figure 4.** SCF inhibits *Salmonella* invasion of intestinal cell lines. Triplicate wells of MODE-K cells (A) or 18CO cells (human myofibroblast cell line, B) were treated overnight with varying concentrations of rSCF or medium alone. Before exposure to *Salmonella*, cell monolayers were washed in antibiotic-free medium and then exposed to *S. typhimurium* (MOI for MODE-K 1:4, MOI for 18CO 10:1) for 1 h. Noninternalized bacteria were then counterselected by exposing monolayers to medium containing antibiotics for 2 h at 37°C. Monolayers were then extensively washed with antibiotic-free medium and the number of bacteria-infected cells (CFU/well) determined using an agarose-agar overlay assay.

lenge. To investigate this possibility, we determined the LD<sub>50</sub> for WW<sup>V</sup> versus their littermate controls (++) for oral challenge with *S. typhimurium*. Groups of mice (6 per group) were given varying doses of *Salmonella* (10<sup>2</sup> to 10<sup>8</sup> cfu/mouse) orally and then assessed daily for 10 d. The LD<sub>50</sub> for WW<sup>V</sup> mice was 10<sup>5.7</sup>, while the LD<sub>50</sub> for their littermate controls was 10<sup>7.5</sup>. Fig. 5 presents results from a representative experiment where WW<sup>V</sup> and their littermate controls (++) (10 mice per group) were given (orally) 5 × 10<sup>6</sup> cfu of *Salmonella* and followed daily for survival. Results from these experiments showed clearly that WW<sup>V</sup> mice were more susceptible to oral challenge with *Salmonella*.

Puddington et al. (17) have shown that WW<sup>V</sup> and SI/SI<sup>d</sup> mice have a significantly decreased number of γδ<sup>+</sup> IEL and an increased number of αβ<sup>+</sup> IEL. This shift in γδ<sup>+</sup> versus αβ<sup>+</sup> IEL occurred in WW<sup>V</sup> mice from 6 to 14 wk of age. The mice used in our studies were 8–14 wk of age, and therefore probably lacked or had significantly reduced numbers of γδ<sup>+</sup> IEL. Thus, a major question concerning our in vivo results is whether the enhanced susceptibility of WW<sup>V</sup> mice to oral *Salmonella* challenge is due to altered numbers of γδ<sup>+</sup> versus αβ<sup>+</sup> IEL and/or to some other SCF:c-kit-dependent function.

Epithelial cells are now recognized as an important cell for regulating both natural and acquired immune functions at mucosal surfaces (34). An important component of this epithelial cell regulation could be the enhancement of SCF and/or c-kit expression after infection. Enhanced expression of SCF/c-kit by epithelial cells could result in mast cell chemotaxis, enhancement of mast cell cytokine production and survival, regulation of γδ IEL, inhibition of bacterial invasion of adjacent epithelial cells, and/or to some as yet characterized function(s) that is dependent upon SCF:c-kit interactions. In summary, results presented in this study show that an early response of the intestinal tract to *Salmonella* infection is the enhanced production of SCF and that a potential source of this SCF is the intestinal epithelial cell.



**Figure 5.** WW<sup>V</sup> mice have enhanced susceptibility to oral *Salmonella* challenge. WW<sup>V</sup> mice and their control littermates were given an oral dose of 5 × 10<sup>6</sup> CFU of *S. typhimurium*. 10 mice per group were followed for 12 d and assessed daily for survival. The difference in cumulative mortality between WW<sup>V</sup> versus ++ control littermate mice was statistically significant ( $P < 0.001$ ).

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