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L-Asparaginase II Produced by *Salmonella* Typhimurium Inhibits T Cell Responses and Mediates Virulence

Amy L. Kullas,[1,2] Michael McClelland,[5,6] Hee-Jeong Yang,[7] Jason W. Tam,[1,2] AnnMarie Torres,[1,3] Steffen Porwollik,[5] Patricio Mena,[1] Joseph B. McPhee,[1,8] Lydia Bogomolnaya,[7] Helene Andrews-Polymenis,[7] and Adrianus W.M. van der Velden[1,2,3,4,*]
[1]Center for Infectious Diseases
[2]Department of Molecular Genetics and Microbiology
[3]Program in Genetics
[4]Department of Pathology
Stony Brook University, Stony Brook, NY 11794-5120, USA
[5]The Vaccine Research Institute of San Diego, San Diego, CA 92121-1130, USA
[6]Department of Pathology and Laboratory Medicine, University of California, Irvine, CA 92697-4800, USA
[7]Department of Microbial and Molecular Pathogenesis, Texas A&M University System Health Science Center, Bryan, TX 77843-1114, USA
[8]Present address: Department of Biochemistry and Biomedical Sciences, McMaster University, Hamilton, ON L8N 3Z5, Canada
*Correspondence: vandervelden@notes.cc.sunysb.edu
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SUMMARY

***Salmonella enterica* serovar Typhimurium avoids clearance by the host immune system by suppressing T cell responses; however, the mechanisms that mediate this immunosuppression remain unknown. We show that *S.* Typhimurium inhibit T cell responses by producing L-Asparaginase II, which catalyzes the hydrolysis of L-asparagine to aspartic acid and ammonia. L-Asparaginase II is necessary and sufficient to suppress T cell blastogenesis, cytokine production, and proliferation and to downmodulate expression of the T cell receptor. Furthermore, *S.* Typhimurium-induced inhibition of T cells in vitro is prevented upon addition of L-asparagine. *S.* Typhimurium lacking the L-Asparaginase II gene (*STM3106*) are unable to inhibit T cell responses and exhibit attenuated virulence in vivo. L-Asparaginases are used to treat acute lymphoblastic leukemia through mechanisms that likely involve amino acid starvation of leukemic cells, and these findings indicate that pathogens similarly use L-asparagine deprivation to limit T cell responses.**

INTRODUCTION

Salmonellae are a leading cause of worldwide morbidity and mortality in humans (Pegues et al., 2005). Infections with *Salmonellae* range in severity from self-limiting gastroenteritis to typhoid fever. Nontyphoidal *Salmonellae* such as *S*. Typhimurium are a leading cause of inflammatory enterocolitis and death due to foodborne illness. Typhoidal *Salmonellae* such as *S*. Typhi cause systemic infections characterized by bacterial penetration of the intestinal barrier and extraintestinal dissemination to the liver and spleen, where the microorganisms replicate in professional phagocytes. Septic shock and death can occur if the infection is left untreated. *S*. Typhimurium cause a typhoid-like systemic illness in susceptible strains of mice, and experimental infection of mice with *S*. Typhimurium has served as a useful model for the human disease caused by *S*. Typhi (Tsolis et al., 2011).

Many bacterial pathogens induce acute immunosuppression to establish infection. A number of bacterial pathogens subvert pathways of the innate immune system, but how bacterial pathogens overcome pathways of the adaptive immune system is not well understood (Hornef et al., 2002). Recent studies demonstrating that *S*. Typhimurium, *Shigella flexneri, Yersinia spp.*, and *Helicobacter pylori* directly inhibit the response of T cells provided insight into the types of strategies used by bacterial pathogens to overcome pathways of the adaptive immune system (Algood and Cover, 2006; Fischer et al., 2009; Konradt et al., 2011; van der Velden et al., 2005, 2008; Viboud and Bliska, 2005). In humans and animal models, *S*. Typhimurium induce acute immunosuppression and delay the onset of a protective immune response (Dougan et al., 2011; Luu et al., 2006; Srinivasan et al., 2004; Tsolis et al., 2011). Immunity that eventually develops against *S*. Typhimurium requires humoral and cell-mediated immune responses (Dougan et al., 2011). T cells play a critical role in clearance of *S*. Typhimurium (Dougan et al., 2011), but the T cell response to these microorganisms is thwarted during infection (Bueno et al., 2007; Ertelt et al., 2011; Johanns et al., 2010; Sad et al., 2008; Srinivasan and McSorley, 2007).

We previously demonstrated that T cells fail to proliferate in response to antigen-pulsed dendritic cells when *S*. Typhimurium are present (van der Velden et al., 2005). Although *S*. Typhimurium can kill dendritic cells by a mechanism that depends on host caspase-1 and the *Salmonella* pathogenicity island 1 (SPI-1)-encoded type 3 secretion system (van der Velden et al., 2003), the killing of dendritic cells alone is not

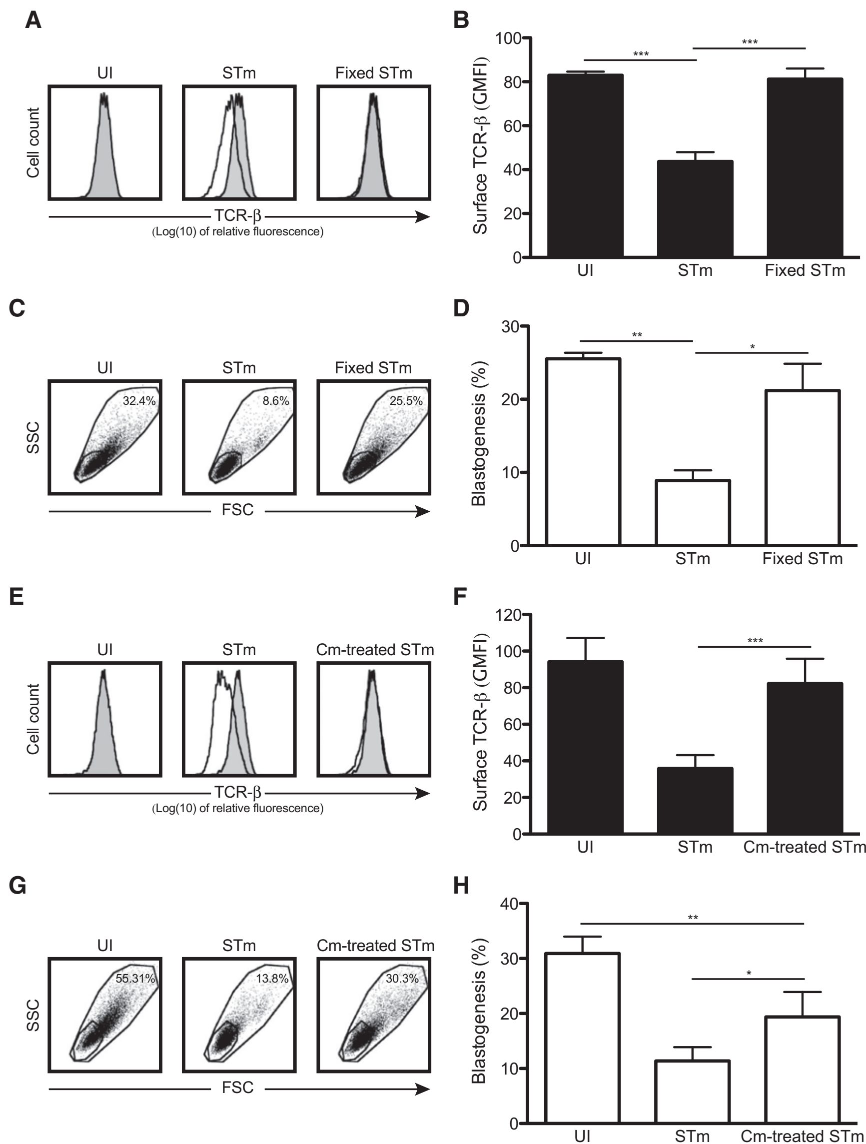


Figure 1. Only Viable *S.* Typhimurium and Those Capable of Synthesizing New Proteins Are Able to Downmodulate TCR Expression and Suppress T Cell Blastogenesis

(A and B) Expression of surface TCR-β by CD25+ T cells left uninfected (UI, gray in histograms) or cultured with viable *S*. Typhimurium (STm) or formaldehyde-fixed *S*. Typhimurium (Fixed STm); p = 0.0002.

(C and D) Blastogenesis of CD25+ T cells treated as in (A); p = 0.0054.

(E and F) Expression of surface TCR-β by CD25+ T cells left uninfected (UI, gray in histograms) or cultured with untreated *S*. Typhimurium (STm) or chloramphenicol-treated *S*. Typhimurium (Cm-treated STm); p < 0.0001.

(G and H) Blastogenesis of CD25+ T cells treated as in (E); p = 0.0004. Data are representative of (A), (C), (E), and (G), or show the mean with SEM (B, D, F, and H) from at least three independent experiments.

responsible for the lack of T cell proliferation (van der Velden et al., 2005). When T cells are stimulated in the absence of dendritic cells through ligation of the T cell receptor (TCR) complex, the robust proliferation of T cells that usually occurs fails to occur when *S*. Typhimurium are present (van der Velden et al., 2005). A proteinaceous factor present in medium harvested from T cells cultured with *S*. Typhimurium is responsible for this inhibition (van der Velden et al., 2005, 2008), but the identity of the factor, which may be produced or induced by *S*. Typhimurium, has remained elusive.

Here, we demonstrate that *S*. Typhimurium utilizes L-Asparaginase II to inhibit the response of T cells and that production of L-Asparaginase II by *S*. Typhimurium is both necessary and sufficient to cause inhibition of T cells. The identification of the factor responsible for *S*. Typhimurium-induced T cell inhibition provides an understanding of a mechanism by which these microorganisms can establish infection in the mammalian host and avoid clearance by the immune system.

RESULTS

Only Viable *S.* Typhimurium and Those Capable of Synthesizing New Proteins Are Able to Downmodulate TCR Expression and Suppress T Cell Blastogenesis

S. Typhimurium-induced inhibition of T cell proliferation correlates with downmodulation of TCR-β, a receptor required for antigen recognition and T cell function (van der Velden et al., 2005, 2008). To determine whether downmodulation of TCR-β by *S*. Typhimurium requires viable microorganisms, we cultured T cells with live or formaldehyde-fixed *S*. Typhimurium. After 20 hr of incubation, T cells cultured with viable *S*. Typhimurium expressed significantly less surface TCR-β than T cells left uninfected (Figures 1A and 1B). In contrast, T cells cultured with formaldehyde-fixed *S*. Typhimurium expressed levels of surface TCR-β that were comparable to the levels expressed by T cells left uninfected (Figures 1A and 1B). Additionally, blastogenesis of T cells cultured with viable *S*. Typhimurium was significantly reduced compared with that of T cells left uninfected or cultured with formaldehyde-fixed *S*. Typhimurium (Figures 1C and 1D).

To determine whether new protein synthesis by *S*. Typhimurium is required for downmodulation of TCR-β, we added chloramphenicol to T cells cultured with chloramphenicol-pretreated bacteria. After 20 hr of incubation, T cells cultured with chloramphenicol-treated *S*. Typhimurium expressed levels of surface TCR-β that were comparable to the levels expressed by T cells left uninfected (Figures 1E and 1F). Additionally, blastogenesis of T cells cultured with *S*. Typhimurium was significantly increased when chloramphenicol was present (Figures 1G and 1H). Thus, *S*. Typhimurium

must be viable and able to synthesize new proteins to downmodulate TCR-β expression and suppress T cell blastogenesis.

STM3106 Is Required for *S*. Typhimurium to Downmodulate TCR Expression, Suppress T Cell Blastogenesis, Block Cytokine Production, and Inhibit T Cell Proliferation

To identify the *S*. Typhimurium genes required for inhibition of T cells, we screened a library of 156 mutants. Each mutant in this library carries a targeted deletion of multiple linked, nonessential genes (~1,700 genes are deleted in the library). The mutants were screened individually to identify those that were unable to downmodulate TCR-β surface expression. One mutant, *ΔSTM3104-7*, was unable to downmodulate TCR-β (Figures 2A and 2B). To identify the gene(s) deleted in this mutant required for *S*. Typhimurium to downmodulate TCR-β, we generated strains with targeted deletions of *STM3104*, *STM3105*, *STM3106*, or *STM3107*. Only *ΔSTM3106 S*. Typhimurium were unable to downmodulate TCR-β (Figure 2C). The presence of *STM3106* in *trans* complemented the phenotype of *ΔSTM3106 S*. Typhimurium (Figures 2D, 2E, and S1A–S1F available online). Consistent with these results, *S*. Typhimurium lacking *STM3106* also were unable to suppress T cell blastogenesis (Figures 2F and 2G) and inhibit production of interferon (IFN)-γ (Figures 2H, 2I, S1G, and S1H) and interleukin (IL)-2 (Figures 2J, 2K, S1I, and S1J). *S*. Typhimurium did not efficiently invade T cells (Figures S1K and S1L), and *S*. Typhimurium lacking *STM3106* grew normally in bacterial culture medium (Figure S1M) and bone-marrow-derived macrophages (Figure S1N), indicating that *ΔSTM3106 S*. Typhimurium do not have a generalized growth defect.

To determine whether *STM3106* is required for *S.* Typhimurium to inhibit T cell proliferation, we labeled T cells with carboxyfluorescein succinimidyl ester (CFSE) and cultured them with wild-type (WT) or *ΔSTM3106 S*. Typhimurium. After 3 days of incubation, T cells cultured with WT *S*. Typhimurium had not proliferated in response to stimulus (Figures 2L and 2M). In contrast, T cells cultured with *ΔSTM3106 S*. Typhimurium, like the T cells that were left uninfected, had proliferated extensively (Figures 2L and 2M). Collectively, these results demonstrate that *STM3106* is required for *S*. Typhimurium to downmodulate TCR-β expression, suppress T cell blastogenesis, block cytokine production, and inhibit T cell proliferation.

L-Asparaginase II Encoded by *STM3106* Is Necessary and Sufficient to Cause Inhibition of T Cells

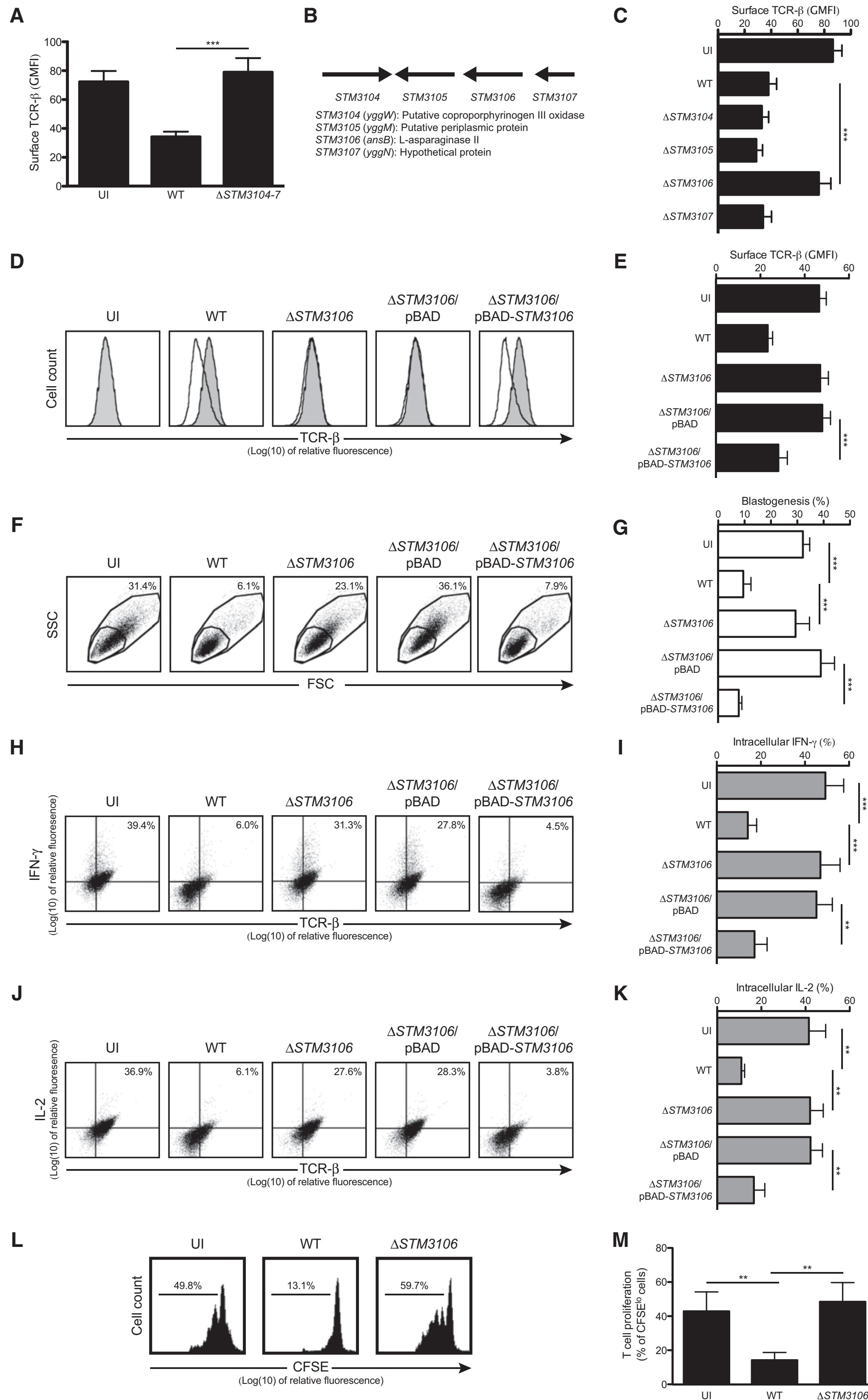
STM3106 (*ansB*) encodes L-Asparaginase II, an extracytoplasmic enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia (Figure 3A). We previously showed that a proteinaceous factor capable of downmodulating TCR-β was present in medium harvested from T cells cultured with *S*. Typhimurium (van der Velden et al., 2008). To determine whether this inhibitory factor could be L-Asparaginase II produced by *S*. Typhimurium, we subjected medium harvested from T cells cultured with *S*. Typhimurium to western blot analysis. The presence of L-Asparaginase II in medium could not be detected (Figure S2A), but the capacity of the medium to downmodulate TCR-β was lost when L-Asparaginase II-specific antibody was used in immunodepletion experiments (Figure 3B). In contrast, the capacity of the medium to downmodulate TCR-β was retained when control antibody or no antibody was used (Figure 3B). Thus, L-Asparaginase II is necessary for *S*. Typhimurium to cause downmodulation of TCR-β.

To determine whether L-Asparaginase II of *S*. Typhimurium is sufficient to cause downmodulation of TCR-β, we treated T cells with purified L-Asparaginase II. After 20 hr of incubation, T cells treated with purified L-Asparaginase II expressed significantly less surface TCR-β than did T cells left untreated (Figures 3C and S2A–S2C). Furthermore, T cells treated with purified L-Asparaginase II expressed levels of surface TCR-β that were comparable to the levels expressed by T cells cultured with WT *S*. Typhimurium (Figures 3C, S2B, and S2C). T cells treated with purified L-Asparaginase II also expressed significantly less intracellular IFN-γ and IL-2 than did T cells left untreated (Figures S2D and S2E). Thus, L-Asparaginase II of *S*. Typhimurium is sufficient to cause inhibition of T cells.

To determine whether depletion of L-Asparaginase II substrate is responsible for *S*. Typhimurium-induced inhibition of T cells, we added L-asparagine to T cells cultured with *S*. Typhimurium. Exogenous L-asparagine, but not L-aspartate or L-glutamine, prevented *S*. Typhimurium-induced downmodulation of TCR-β (Figure 3D). These results suggest that L-Asparaginase II-mediated depletion of L-asparagine contributes to *S*. Typhimurium-induced downmodulation of TCR-β.

An *S*. Typhimurium *ansB* Mutant Unable to Express L-Asparaginase II Is Attenuated for Virulence

To determine whether *STM3106* (*ansB*) is required for virulence of *S*. Typhimurium, we performed survival assays using 129X1/SvJ mice, which have served as a useful model for studying persistent salmonellosis. Significantly more mice survived infection with *ΔSTM3106 S*. Typhimurium than with WT *S*. Typhimurium (Figure 4A). A similar trend was observed for C57BL/6J mice, which are more susceptible to infection with *S*. Typhimurium (Figures S3A and S3B). Organ burden assays were performed on the surviving mice to determine bacterial loads. After 60 days of infection, a long-term time point used to assess bacterial persistence (Johanns et al., 2010; Lawley et al., 2006; Mittrücker et al., 2002), significantly fewer *ΔSTM3106 S*. Typhimurium than WT *S*. Typhimurium were recovered from livers, but not spleens and mesenteric lymph nodes (Figure 4B). Additional organ burden assays were performed to determine the kinetics of WT and *ΔSTM3106 S*. Typhimurium infection. Similar numbers of WT and *ΔSTM3106 S*. Typhimurium were recovered from spleens and livers during early stages of infection (Figures 4C and 4D). In contrast, significantly fewer *ΔSTM3106 S*. Typhimurium than WT *S*. Typhimurium were recovered from livers, but not spleens, during later stages of infection (Figures 4C and 4D). The presence of *STM3106* in *trans* complemented the colonization defect of *ΔSTM3106 S*. Typhimurium in liver (Figures 4E and S3C). Multiparametric flow cytometry was used to determine whether *S*. Typhimurium utilize L-Asparaginase II to inhibit the response of T cells in vivo. More T cells, including activated T cells and IFN-γ-producing T cells, were recovered from the livers and spleens of mice infected with *ΔSTM3106 S*. Typhimurium than from the livers and spleens of mice infected with WT


A
B
C
D
E
F
G
H
I
J
K
L
M
Surface TCR-β (GMFI)
UI
WT
ΔSTM3104-7
STM3104
STM3105
STM3106
STM3107
STM3104 (yggW): Putative coproporphyrinogen III oxidase
STM3105 (yggM): Putative periplasmic protein
STM3106 (ansB): L-asparaginase II
STM3107 (yggN): Hypothetical protein
ΔSTM3104
ΔSTM3105
ΔSTM3106
ΔSTM3107
ΔSTM3106/ pBAD
ΔSTM3106/ pBAD-STM3106
Cell count
TCR-β
(Log(10) of relative fluorescence)
SSC
FSC
31.4%
6.1%
23.1%
36.1%
7.9%
Blastogenesis (%)
IFN-γ
39.4%
6.0%
31.3%
27.8%
4.5%
Intracellular IFN-γ (%)
IL-2
36.9%
6.1%
27.6%
28.3%
3.8%
Intracellular IL-2 (%)
CFSE
49.8%
13.1%
59.7%
T cell proliferation (% of CFSE^{lo} cells)

S. Typhimurium (Figures S3D–S3I). However, these differences did not reach statistical significance. Collectively, these results demonstrate that *STM3106* (*ansB*), and therefore L-Asparaginase II, is required for virulence of S. Typhimurium in mouse models of infection, and that S. Typhimurium utilize L-Asparaginase II to colonize and persist in the liver. Furthermore, these results suggest that the attenuation in virulence of *ΔSTM3106* S. Typhimurium may be due to a reduced ability of these bacteria to inhibit the response of T cells in vivo.

DISCUSSION

We report that S. Typhimurium utilize L-Asparaginase II to inhibit the response of T cells, and that production of L-Asparaginase II by S. Typhimurium is both necessary and sufficient to cause inhibition of T cells. Our identification of the factor responsible for S. Typhimurium-induced T cell inhibition provides an understanding of a previously unrecognized mechanism by which these microorganisms can establish infection in the mammalian host and avoid clearance by the immune system. An immediate implication of our results is that production of L-Asparaginase II by S. Typhimurium causes depletion of exogenous L-asparagine, leading to downmodulation of TCR-β expression, suppression of T cell blastogenesis, blockade of cytokine production, and, ultimately, inhibition of T cell proliferation. Consistent with a role for L-Asparaginase II in S. Typhimurium-induced T cell immunosuppression, which was previously shown to also involve S. Typhimurium genes encoded by the virulence plasmid (Hoertt et al., 1989) and SPI-2 (Ertelt et al., 2011), in vivo phenotypes revealed themselves during later stages of infection, when the adaptive immune system is fully engaged. Another important and more general implication of our work is that a metabolic enzyme can function beyond its canonical role in catalyzing the hydrolysis of L-asparagine to aspartic acid and ammonia.

L-asparaginases are used clinically to treat acute lymphoblastic leukemia (ALL) by a mechanism that is thought to involve depletion of exogenous L-asparagine and, to a lesser extent, L-glutamine (Avramis and Tiwari, 2006; Pieters et al., 2011). In a significant number of patients with ALL, malignant cells exhibit a metabolic defect in L-asparagine synthesis and are dependent on an exogenous source of L-asparagine for survival (Avramis and Tiwari, 2006). Normal cells are able to synthesize L-asparagine and thus are less affected by the rapid depletion of this important amino acid following treatment with L-asparaginase (Avramis and Tiwari, 2006).

T cells are extremely sensitive to amino acid deprivation (Powell and Delgoffe, 2010) and, like malignant cells in ALL, may be dependent on an exogenous source of L-asparagine when undergoing the metabolically demanding process of clonal expansion. Thus, the mechanism by which S. Typhimurium uses L-Asparaginase II to inhibit the response of T cells may be similar to the mechanism by which clinically administered L-asparaginases help eliminate human cancers. The mechanistic details of how inhibition occurs are supported by recent studies demonstrating that purified L-Asparaginase II from S. *enteritidis* and *H. pylori* can inhibit protein synthesis, block cell-cycle progression, and suppress replication of cultured cell lines (Iwamaru et al., 2001; Scotti et al., 2010; Shibayama et al., 2011). Additional support comes from our own work indicating that S. Typhimurium expressing catalytic-site mutants of L-Asparaginase II are unable to inhibit the response of T cells (A.T. and A.W.M.v.d.V., unpublished data).

Although the canonical function of L-Asparaginase II is to catalyze the hydrolysis of L-asparagine to aspartic acid and ammonia, it is striking to consider the possibility that an enzyme that is used clinically to suppress T cell cancers also serves as a virulence determinant that allows S. Typhimurium to suppress T cells directed against it. Our work establishes that L-Asparaginase II has important functions in infection and immunity, leaving open the possibility that S. Typhimurium produces and secretes L-Asparaginase II to consume L-asparagine and thus compete with the host for nutrients. The L-Asparaginase II gene *ansB* is highly conserved in Gram-negative bacteria and has been shown to contribute to the virulence of several important human pathogens, including S. Typhimurium (this study), *H. pylori* (Shibayama et al., 2011), and *Campylobacter jejuni*, which also use L-Asparaginase II to colonize and persist in the liver (Hofreuter et al., 2008). Thus, L-Asparaginase II could prove to be a promising target for the development of innovative, broad-spectrum therapeutic approaches and preventive measures to overcome bacterial infections.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Strain Construction, and Culture Conditions

S. Typhimurium strain 14028 (American Type Culture Collection) and the isogenic, spontaneous, nalidixic acid-resistant derivative S. Typhimurium strain IR715 (Stojiljkovic et al., 1995) were used as WT strains. S. Typhimurium were grown aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar. WT S. Typhimurium, but not *ΔSTM3106* S. Typhimurium, expressed L-Asparaginase II under these conditions (Figure S2F). Where noted, S. Typhimurium were fixed with Fixation Buffer (BioLegend) for 30 min or treated with

Figure 2. *STM3106* Is Required for *S*. Typhimurium to Downmodulate TCR Expression, Suppress T Cell Blastogenesis, Block Cytokine Production, and Inhibit T Cell Proliferation

(A) Expression of surface TCR-β by CD25$^+$ T cells left uninfected (UI) or cultured with either WT or *ΔSTM3104-7* S. Typhimurium; $p < 0.0001$.

(B) Genetic organization of the chromosomal region deleted in *ΔSTM3104-7* S. Typhimurium.

(C) Expression of surface TCR-β by CD25$^+$ T cells left UI or cultured with WT, *ΔSTM3104*, *ΔSTM3105*, *ΔSTM3106*, or *ΔSTM3107* S. Typhimurium; $p < 0.0001$.

(D and E) Expression of surface TCR-β by CD25$^+$ T cells left UI (gray in histograms) or cultured with WT S. Typhimurium, *ΔSTM3106* S. Typhimurium, *ΔSTM3106* S. Typhimurium carrying plasmid pBAD18-Cm (*ΔSTM3106*/pBAD), or *ΔSTM3106* S. Typhimurium carrying a derivative of pBAD18-Cm encoding *STM3106* (*ΔSTM3106*/pBAD-*STM3106*); $p < 0.0001$.

(F and G) Blastogenesis of CD25$^+$ T cells treated as in (D); $p < 0.0001$.

(H and I) Expression of intracellular IFN-γ by CD25$^+$ T cells treated as in (D); $p < 0.0001$.

(J and K) Expression of intracellular IL-2 by CD25$^+$ T cells treated as in (D); $p = 0.0001$.

(L and M) Proliferation of CFSE-labeled T cells left UI or cultured with WT or *ΔSTM3106* S. Typhimurium; $p = 0.0021$. Data are representative of (D), (F), (H), (J), and (L), or show the mean with SEM (A, C, E, G, I, K, and M) from at least three independent experiments. See also Figure S1.

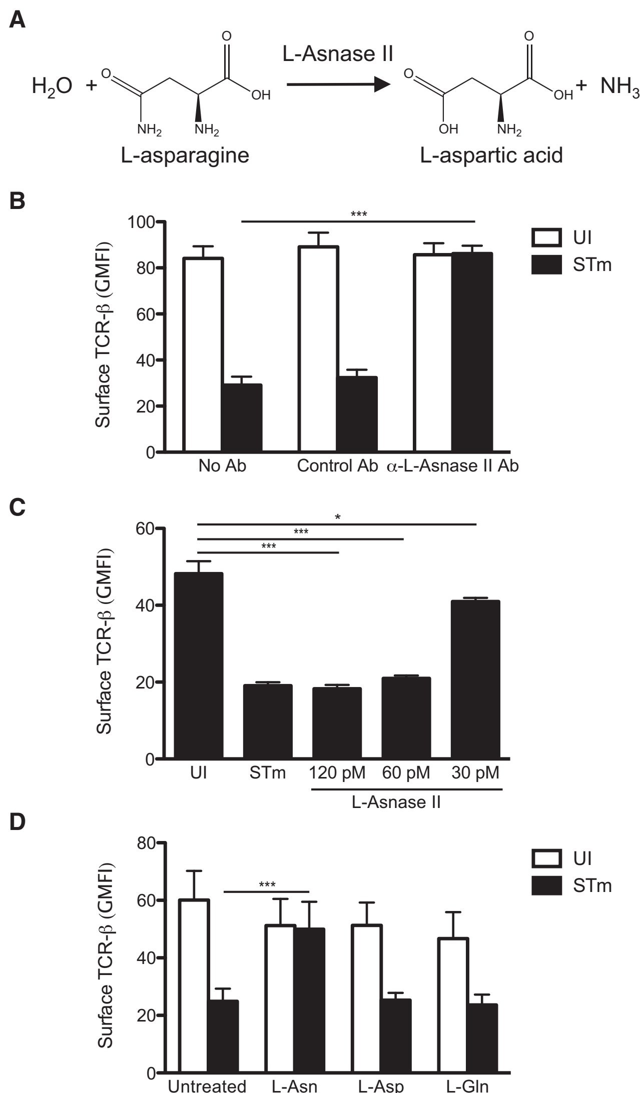


Figure 3. L-Asparaginase II Encoded by *STM3106* Is Necessary and Sufficient to Cause Downmodulation of the TCR

(A) *STM3106* encodes L-Asparaginase II, an extracytoplasmic enzyme that catalyzes the hydrolysis of L-asparagine to L-aspartic acid and ammonia.

(B) Expression of surface TCR-β by CD25+ T cells incubated with filtered medium harvested from T cells left uninfected (UI) or infected with WT *S*. Typhimurium (STm). This medium had been subjected to immunodepletion using L-Asparaginase II-specific antibody (α-L-Asparaginase II Ab), control antibody (Control Ab), or no antibody (No Ab); $p < 0.0001$.

(C) Expression of surface TCR-β by CD25+ T cells left UI, cultured with STm, or treated with purified L-Asparaginase II; $p = 0.0018$.

(D) Expression of surface TCR-β by untreated, L-asparagine-treated (L-Asn), L-aspartate-treated (L-Asp), or L-glutamine-treated (L-Gln) CD25+ T cells left UI or cultured with STm; $p < 0.0001$. Data show the mean with SEM from at least three independent experiments. See also Figure S2.

chloramphenicol (100 μg/ml) for 2 hr. To generate bacterial growth curves, *S*. Typhimurium were grown aerobically at 37°C in LB broth for 18 hr. The resulting overnight cultures were diluted 1:100 in LB broth and grown aerobically at 37°C to stationary phase (~7 hr). The optical density at 600 nm (OD_{600nm}) was measured every 30 min.

Targeted, kanamycin-resistant multi- and single-gene deletion mutants were constructed using the lambda red recombinase method (Datsenko and Wanner, 2000) as described previously (Santiviago et al., 2009). Multi-gene deletion mutants were designed to avoid genes known to be essential for growth on rich medium (M.M. and H.A.-P., unpublished data). Each mutation removed a variable number of genes depending on the nature of the surrounding genes. For mouse infection experiments, mutations were moved from *S*. Typhimurium strain 14028 into *S*. Typhimurium strain IR715 by P22-mediated transduction.

Mouse Experiments

Survival assays and organ burden assays were performed using 8- to 10-week-old C57BL/6J and 129X1/SvJ mice (The Jackson Laboratory). These strains of mice have been used as model hosts to study acute and persistent infections with *Salmonellae*, respectively (Tsolis et al., 2011). Mice were inoculated intragastrically with either WT *S*. Typhimurium strain IR715 or isogenic, *ΔSTM3106 S*. Typhimurium (5×10^5 colony forming units [cfu] for C57BL/6J mice and 5×10^7 cfu for 129X1/SvJ mice) suspended in 0.1 ml of PBS. F1 (C57BL/6J × 129X1/SvJ) hybrid mice were used to determine whether the presence of *STM3106* in *trans* complemented the colonization defect of *ΔSTM3106 S*. Typhimurium in liver, because 129X1/SvJ mice were not commercially available in a timely fashion. To minimize concerns about plasmid loss over time, mice were inoculated intravenously with 5×10^3 cfu of WT *S*. Typhimurium strain IR715, isogenic *ΔSTM3106 S*. Typhimurium, or isogenic *ΔSTM3106 S*. Typhimurium carrying plasmid pWSK29-*STM3106*, and bacterial loads per liver were determined after 10 days of infection. Tenfold serial dilutions of the inoculum were plated on LB agar to determine the inoculum titer. The mice were monitored for survival and were euthanized when they became moribund, or at the termination of the experiment (30 days and 60 days after infection for C57BL/6J mice and 129X1/SvJ mice, respectively). At various times after infection, target organs were harvested from 129X1/SvJ or F1 (C57BL/6J × 129X1/SvJ) hybrid mice and the bacterial burden per gram of tissue was quantified by plating for cfu on LB agar containing nalidixic acid (50 μg/ml), kanamycin (60 μg/ml), or carbenicillin (100 μg/ml). The Institutional Animal Care and Use Committee at Stony Brook University approved all of the animal studies.

T Cell Enrichment and T Cell Assays

Splenocytes harvested from C57BL/6J mice were used as a source of T cells. Following treatment of splenocytes with ACK lysing buffer (Invitrogen) to lyse red blood cells, CD90.2-conjugated MACS microbeads and magnetic separation columns (Miltenyi Biotec) were used for the enrichment of T cells. Enriched populations of T cells were suspended in RP-10 (i.e., RPMI 1640 medium; Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals), L-Gln, HEPES, and 50 μM 2-ME) and used in T cells assays.

S. Typhimurium-induced or L-Asparaginase II-induced downmodulation of TCR-β, suppression of T cell blastogenesis, and inhibition of cytokine production was measured by flow cytometry. Briefly, T cells were seeded at 1×10^5 cells per well (96-well format) in tissue culture plates coated with 5 μg/ml of anti-CD3ε (clone 145-2C11; BioLegend). T cells were cultured in the absence or presence of bacteria at a multiplicity of infection of ~50 or purified L-Asparaginase II. After 2 hr of incubation at 37°C/5% CO_2, T cells were pelleted by centrifugation and resuspended in medium supplemented with penicillin/streptomycin (2%) and gentamicin (50 μg/ml), killing all bacteria within 2 hr (data not shown). After an additional 18–20 hr of incubation at 37°C/5% CO_2, T cells were harvested, stained, and analyzed by flow cytometry. T cells treated with conditioned medium or purified L-Asparaginase II were incubated for 18–20 hr in the presence of antibiotics, as described above. Where noted, medium was supplemented with chloramphenicol (100 μg/ml), L-asparagine (10 mM), L-aspartate (10 mM), or L-glutamine (10 mM).

S. Typhimurium-induced inhibition of T cell proliferation was measured by flow cytometry. Briefly, T cells enriched from mouse spleens were labeled with 5 μM CFSE (Invitrogen) and seeded at 1×10^5 cells per well (96-well format) in tissue culture plates coated with 5 μg/ml of anti-CD3ε. CFSE is distributed evenly among daughter cells with each round of cell division, resulting in a measurable reduction in cell fluorescence (Parish et al., 2009). T cells were left untreated or infected with bacteria at a multiplicity of infection of ~50. After 2 hr of incubation at 37°C/5% CO_2, T cells were pelleted by centrifugation and resuspended in medium supplemented with

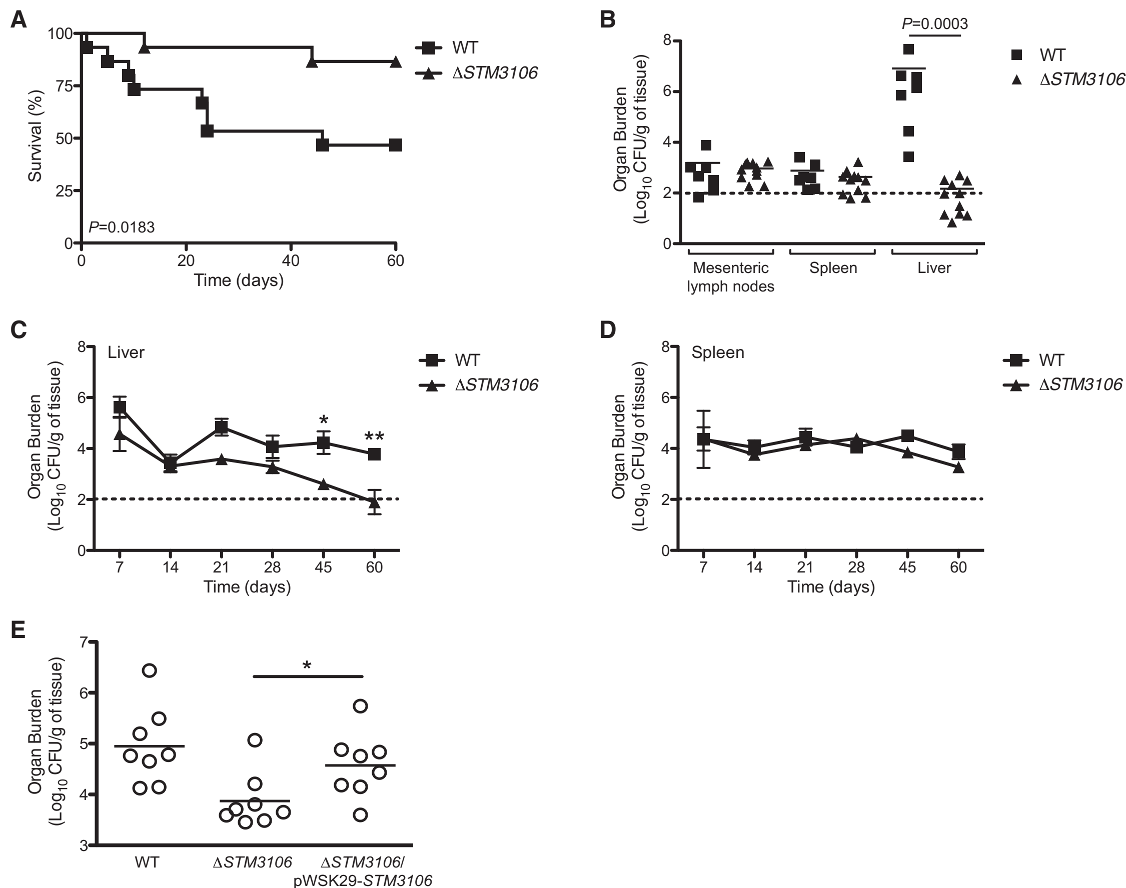


Figure 4. An *S.* Typhimurium *ansB* Mutant Unable to Express L-Asparaginase II Is Attenuated for Virulence in Mouse Models of Infection
(A) Survival of 129X1/SvJ mice (n = 15 per group) inoculated intragastrically with 5 × 10^7 cfu of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium; p = 0.0183.
(B) Bacterial loads per gram of tissue harvested from 129X1/SvJ mice (n = 15 per group) 60 days after intragastric inoculation with 5 × 10^7 cfu of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium; p = 0.0003 for liver. At the time of harvest, eight mice infected with WT *S*. Typhimurium and four mice infected with *ΔSTM3106 S*. Typhimurium had succumbed.
(C and D) Bacterial loads per gram of liver (C) and spleen (D) tissue harvested from 129X1/SvJ mice (n = 5 per group per time point) at various times after intragastric inoculation with 5 × 10^7 cfu of WT (squares) or *ΔSTM3106* (triangles) *S*. Typhimurium; p = 0.0001 for liver and p = 0.2905 for spleen.
(E) Bacterial loads per gram of liver tissue harvested from F1 (C57BL/6J × 129X1/SvJ) hybrid mice (n = 8 per group) 10 days after intravenous inoculation with 5 × 10^3 cfu of WT *S*. Typhimurium, *ΔSTM3106 S*. Typhimurium, or *ΔSTM3106 S*. Typhimurium carrying a derivative of pWSK29 encoding *STM3106* (*ΔSTM3106*/pWSK29-*STM3106*); p = 0.0111. Data show the mean with SEM. See also Figure S3.

penicillin/streptomycin (2%) and gentamicin (50 μg/ml). After an additional 3 days of incubation at 37°C/5% CO_2, T cells were harvested, stained, and analyzed by flow cytometry.

Immunodepletion

Conditioned medium (1.75 ml) from T cells cultured in the absence or presence of *S*. Typhimurium was passed through a 0.22 μm filter. The resulting samples were aliquoted into 250 μl fractions that were precleared using 25 μl of protein A-coupled sepharose beads (Sigma), with agitation. After 1 hr of incubation at 4°C, the beads were pelleted by centrifugation and the supernatants were collected. Where noted, 22.5 μg of polyclonal rabbit anti-*Escherichia coli* L-Asparaginase II antibody or rabbit anti-mouse IgG control antibody (both Abcam) was added to the precleared supernatants. L-Asparaginase II of *E. coli* is 96% identical to L-Asparaginase II of *S*. Typhimurium at the amino acid level, and T cells treated with purified L-Asparaginase II of *E. coli* (Sigma), like T cells treated with purified L-Asparaginase II of *S*. Typhimurium, expressed levels of surface TCR-β comparable to the levels expressed by T cells cultured with WT *S*. Typhimurium (Figure S2G). After overnight incubation at 4°C with rotation, 25 μl of protein A-coupled sepharose beads were added to each sample. After an additional 3 hr of incubation at 4°C with agitation, the beads were pelleted by centrifugation and the resulting supernatants were used in T cell assays.

Statistical Analysis

Statistical analysis of survival assays and organ burden assays was performed using the log-rank test and one-tailed, nonparametric Mann-Whitney test or two-way ANOVA, respectively. Statistical analysis of all other assays was performed using a two-tailed paired t test (invasion assay) or repeated-measures (randomized-block) one-way ANOVA with Bonferroni's multiple-comparison post test, comparing selected pairs of columns. Statistical analysis was performed using Prism 5.0b (GraphPad Software), and p values < 0.05 were considered to be statistically significant. Asterisks indicate statistically significant differences (***p < 0.001, **p < 0.01, *p < 0.05).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.10.018.

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