Listeria monocytogenes induces T cell receptor unresponsiveness through pore-forming toxin listeriolysin O
Listeria monocytogenes Induces T Cell Receptor Unresponsiveness through Pore-Forming Toxin Listeriolysin O

Nelson O. Gekara,1,a Natalia Zietara,1 Robert Geffers,2 and Siegfried Weiss1
1Molecular Immunology and 2Department of Cell Biology, Helmholtz Center for Infection Research, Braunschweig, Germany

Background. The success of many pathogens relies on their ability to circumvent the innate and adaptive immune defenses. How bacterial pathogens subvert adaptive immune defenses is not clear. Cholesterol-dependent cytolsins (CDCs) represent an expansive family of homologous pore-forming toxins that are produced by more than 20 gram-positive bacterial species. Listeriolysin O (LLO), a prototype CDC, is the main virulence factor of Listeria monocytogenes.

Methods. We employed flow cytometric and microarray techniques to analyze the effect of LLO on T cell activation in vitro and in vivo.

Results. In vivo and in vitro proliferation of CD4+ T cells upon T cell receptor (TCR) activation was highly diminished in the presence of LLO or wild-type L. monocytogenes but not in the presence of LLO-deficient L. monocytogenes. This block in T cell proliferation was specific to T cell activation via the TCR and not by phorbol 12-myristate 13-acetate–ionomycin, which bypasses the proximal TCR signaling event. The results of microarray analysis suggest that LLO-induced T cell unresponsiveness is due to the induction of a calcium–nuclear factor of activated T cells–dependent transcriptional program that drives the expression of negative regulators of TCR signaling.

Conclusion. These findings provide important insights into how bacterial toxins silence adaptive immune responses and thus enable prolonged survival of the pathogen in the host.

Successful pathogens have evolved myriad strategies to evade host defenses. Listeria monocytogenes has long served as a model pathogen for elucidating many aspects of host immune responses. Listeriolysin O (LLO), the main virulence factor of L. monocytogenes, is a pore-forming toxin that is structurally and functionally related to several cholesterol-dependent cytolsins (CDCs) produced by more than 20 gram-positive bacterial species, including Bacillus anthracis (anthrolysin O), Streptococcus pyogenes (streptolysin O), Streptococcus pneumoniae (pneumolysin O), and Streptococcus suis (suilysin).

These toxins trigger a broad range of cellular responses that greatly influence pathogenesis. For example, in the early stages of L. monocytogenes infection, LLO is known to trigger the production of various proinflammatory mediators by innate immune cells such as mast cells, neutrophils, and macrophages that contribute to rapid bacterial clearance [1, 2]. Whereas the bulk of infectious load is normally cleared by these early innate immune responses, sterile clearance from the host is contingent on the generation of an effective T cell–mediated immunity. Thus, strains of mice such as nude and SCID that lack T lymphocytes become chronic carriers after infection [3, 4]. Therefore, overcoming this type of host response is probably a daunting challenge in the effort of L. monocytogenes to prolong survival in the host.

In the present study, we show that LLO suppresses antigen-induced T cell activation. LLO-induced immune suppression is due to a block in the proximal T
cell receptor (TCR) signaling. Treatment of T cells with LLO resulted in decreased levels of CD3ζ, which is crucial for proximal TCR signaling events. Congruently, T cell unresponsiveness induced by LLO could be overcome by phorbol 12-myristate 13-acetate (PMA)-ionomycin, which activates T cells by bypassing the proximal TCR signaling. Global gene expression analysis shows that LLO induces several nuclear factor of activated T cells (NFAT) target genes involved in negative modulation of TCR signaling. These findings highlight one of the potential mechanisms by which *L. monocytogenes* and probably other CDC-producing gram-positive bacteria suppress or delay the adaptive immune system in their effort to establish a niche in the host.

**MATERIALS AND METHODS**

**Mice.** C57BL/6 OT I and OT II mice were bred and maintained under specific pathogen-free conditions at the animal facility of the Helmholtz Center for Infection Research. Mice were cared for and experimental procedures were performed under the approval of the local authority, Lower Saxony State Office for Consumer Protection and Food Safety (LAVES).

**Reagents and antibodies.** Anti-CD3ε (clone, 500 A2) was purified in our laboratory. Rabbit anti–mouse CD3ζ (FL-163) antibody was purchased from Santa Cruz Biotechnology. Anti–mouse CD4-allophycocyanin, anti-CD62L, and anti-CD69, were purchased from BD Biosciences. LLO was purified from *Listeria innocua* as described elsewhere [12]. PMA and ionomycin were purchased from Sigma. An Apoptosis Kit was purchased from BD Pharmingen.

**Primary cells, cell lines, antibodies, and evaluation of necrosis and apoptosis.** A5 T cell hybridomas, which express a green fluorescent protein (GFP) reporter under control of a basal NFAT promoter, have been described elsewhere [14]. Ovalbumin-specific CD4+ T (OT II) cells were isolated from basal NFAT promoter, have been described elsewhere [14]. Ovalbumin-specific CD4+ T (OT II) cells were isolated from spleens and mesenteric lymph nodes of transgenic mice and purified using CD4 negative isolation kits (Dynal) according to the manufacturer’s instructions. To monitor proliferation, OT II cells were stained with 1 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) for 10 min at 37°C according to the manufacturer’s protocol. Necrosis and necrosis of cells was evaluated using the BD Pharmingen apoptosis assay kit according to the manufacturer’s instructions.

**In vivo and in vitro analysis of T cell proliferation.** For in vivo experiments, C57BL/6 mice were immunized by injecting 1 mg of ovalbumin together with 50 μg of lipopolysaccharide by the intraperitoneal route. After 24 h, each mouse was intraperitoneally administered ≥1 × 10^6 CFSE-labeled OT II T cells, and 2.5–3 d later, mice were killed and cells from the peritoneum, spleen, and mesenteric lymph nodes were isolated and stained with anti-CD4-allophycocyanin. The proliferation of CD4+ T cells was determined by flow cytometric measurement of CFSE levels in such cells. For in vitro TCR activation, 96-well plates coated overnight with a solution of 100 μg/mL hamster anti–mouse CD3 (clone, 500 A2) were seeded with CFSE-labeled OT II T cells at a density of 1 × 10^5 per well. Alternatively, CFSE-labeled OT II T cells were incubated in the presence of 50 nmol/L PMA plus 1 μmol/L ionomycin. After 3 d of incubation, such cells were analyzed for CFSE levels by means of flow cytometry.

For stimulation of OT I T cells with antigen-presenting cells (APCs), splenic conventional dendritic cells were plated at 2 × 10^6 cells per well, then loaded for 1 h with 250 μg/mL ovalbumin, washed thoroughly (3 times), and then resuspended in complete medium containing 2 × 10^5 CFSE-labeled OT I T cells. After 2.5 d, the proliferation of the OT I T cells was analyzed.

**Exposure of cells to bacteria or LLO and intracellular calcium measurements.** Cells were incubated with either *L. monocytogenes* or mutant LLO-deficient *L. monocytogenes* (Δhly) (multiplicity of infection, 20) or treated with LLO (0.25 μg/mL) for 3 h. It should be noted that this concentration of LLO was used in all the experiments described in this study. This concentration of LLO was chosen following a careful titration in which it was found to be the optimal dose that triggers signals such as protein tyrosine phosphorylation and calcium signaling without killing cells [12]. Intracellular calcium levels upon bacterial or LLO exposure were measured as described elsewhere [12].

**Statistical analysis.** Data in the text and figures are expressed as the mean (± standard deviation). Statistical comparisons were performed using the Student *t* test. Results for which *P* < .05 were considered to be statistically significant.

**Microarray studies.** Primary CD4+ T cells treated with LLO (0.25 μg/mL) or ionomycin (1 μmol/L) for 3 h or left untreated were lysed, and the total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA was processed and analyzed by the array facility of the Helmholtz Center for Infection Research. Gene expression levels were determined by MOE430 GeneChips (version 2.0; Affymetrix) according to the standard protocols recommended by the manufacturer. Signal intensities were calculated using the rate monotonic analysis algorithm, and the GeneSpring GX software suite (version 10; Agilent Technologies) was applied for statistical analysis. Excel software (version 2007; Microsoft) and Genesis software (version 5.1) [42] were used for clustering algorithm data analysis and visualization. Data discussed here have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE19337.

**RESULTS**

*L. monocytogenes inhibits in vivo proliferation of CD4 T cells through LLO.* While analyzing the effect of bacterial virulence factors on T cells, we noted that CD4+ OT II T cells that were...
Figure 1. Inhibition of in vivo proliferation of T cells by *Listeria monocytogenes* (L.m.) via listeriolysin O (LLO). Carboxyfluorescein succinimidyl ester (CFSE)–labeled OT II CD4+ cells were intraperitoneally injected into control (ctrl) mice (A), mice immunized with ovalbumin (ova) (B), and mice immunized with ovalbumin followed by injection with colony-forming units of *L. monocytogenes* (C) or *L. monocytogenes* Δlyt (D). Alternatively, OT II T cells were pretreated with a sublethal dose of 0.25 μg/mL LLO in vitro for 3–4 h and washed thoroughly to remove unbound toxin before intraperitoneal injection into ovalbumin-immunized mice (E). Three days later, the proliferation of cells from peritoneums, spleens, and mesenteric lymph nodes of mice were analyzed. F, Representative percentages of proliferated cells calculated from 3 mice per group. Results are representative of 2 independent experiments. Data are expressed as the mean (± standard deviation). *P < .05; **P < .001. G, Propidium iodide (PI) or annexin V–fluorescein isothiocyanate (FITC) staining in CD4+ T cells 3 h after incubation with *L. monocytogenes* (multiplicity of infection, 20), LLO (0.25 μg/mL), or neither. The percentage of necrotic and apoptotic cells is indicated in the top box. As evident from the control cells, the depicted minor loss of cell viability observed in the LLO-treated T cells was due more to spontaneous cell death during the cell isolation procedure than to LLO toxicity.
Listeriolysin O–Induced TCR Unresponsiveness

Figure 2. Overcoming listeriolysin O (LLO)–induced T cell unresponsiveness by interleukin 2 (IL-2) or phorbol 12-myristate 13-acetate (PMA)–ionomycin. Carboxyfluorescein succinimidyl ester–labeled OT II T cells were pretreated with 0.25 μg/mL LLO or left as untreated controls (ctrl) for 3–4 h, then stimulated in vitro with plate-bound anti-CD3 or anti-CD3 plus exogenous recombinant IL-2 (A). In parallel, LLO-pretreated cells were also stimulated with 1 μmol/L ionomycin (iono) plus 50 nmol/L PMA. The proliferation of cells was then analyzed by flow cytometry 3 d later. B, Representative percentages of proliferated cells calculated from triplicate stimulations. Results are representative of 2 independent experiments. Data are expressed as the mean (± standard deviation). *P < .005.

adoptively transferred into ovalbumin-immunized mice exhibited strongly diminished proliferation in the presence of L. monocytogenes (Figures 1C and 1F). However, such cells proliferated normally in the presence of the LLO-deficient Δhly mutant (Figures 1D and 1F). This suggested that L. monocytogenes inhibits T cell proliferation via LLO-dependent mechanisms. LLO has previously been reported to interfere with antigen presentation [5–8]. However, the fact that the mice were already immunized with ovalbumin prior to the simultaneous inoculation of L. monocytogenes and ovalbumin-specific T cells suggested that the impaired T cell proliferation was not due to defects in APCs but rather due to LLO-induced signals in T cells. To further verify this idea, OT II T cells were transiently exposed in vitro for 3–4 h to a nonlethal dose of LLO, then washed thoroughly to remove unbound toxin before intraperitoneal inoculation into ovalbumin-immunized animals. Consistent with the idea that signals triggered by LLO in T cells impair subsequent TCR activation, as depicted in Figures 1E and 1F, such cells were impaired in proliferation.

Because high doses of L. monocytogenes or LLO are known to cause cell death by necrosis or apoptosis [9–11], T cells preexposed to L. monocytogenes or nonlethal doses of LLO were also analyzed by propidium iodide and annexin V staining. Such treatments were not found to result in significant loss of cell viability (Figure 1G). This is consistent with our recent observations that cells exposed to nonlethal doses of LLO, such as those used in this study, do rapidly repair toxin-induced membrane damage and recover without significant loss of viability [12]. However, it is important to emphasize, independent of those results, that rather than measure the absolute number of T cells (which, in principle, could make the distinction between impaired T cell proliferation and toxin-induced cell death impractical), throughout this study, we have used the CFSE-based system to monitor viable cells for the rounds of divisions or percentage of such cells that had undergone division. This way, we ensured that any cell death could not influence the proliferation readout.

**LLO-induced T cell unresponsiveness can be overcome by interleukin 2 (IL-2) or PMA-ionomycin.** One of the hallmarks of T cell unresponsiveness, or anergy, is that it can be reversed by the addition of exogenous IL-2. To test whether LLO-induced T cell unresponsiveness fits into this category, we resorted to in vitro stimulation with plate-bound anti-CD3 with or without exogenous recombinant IL-2. LLO-induced inhibition of OT II T cell proliferation following anti-CD3 stimulation was effectively overcome by IL-2 supplementation (Figure 2). Next, we ad-
Figure 3. Overcoming listeriolysin O (LLO)–induced T cell unresponsiveness by interleukin 2 (IL-2). CD4+ T cells from wild-type C57BL/6 mice were labeled with carboxyfluorescein succinimidyl ester and treated with 0.25 μg/mL LLO for 3–4 h or left as untreated controls (ctrl), then washed thoroughly. Cells left unstimulated (unstim) were added to uncoated wells. Cells for stimulation were added to wells coated with anti-CD3 antibody (anti-CD3) or coated with anti-CD3 and supplemented with 50 U/mL IL-2. After 3 d, the proliferation of cells was analyzed by flow cytometry.

dressed whether LLO could also inhibit PMA-ionomycin–induced T cell proliferation. PMA-ionomycin treatment triggers mitogen-activated protein kinases and calcium-NFAT signaling cascades, leading to productive activation of T cells, independent of the proximal TCR-CD3 signaling events. In contrast to TCR-CD3 stimulation, LLO-pretreated T cells proliferated normally in response to PMA-ionomycin (Figure 2), suggesting that the anergic state in such cells was due to anomalies in the proximal TCR signaling events. The results also provide an additional control that emphasizes the fact that the diminished response of LLO-pretreated cells following TCR triggering was not due to loss of viability. Similarly, LLO was found to impair the proliferation of polyclonal CD4+ T cells from wild-type C57BL/6 mice following stimulation with plate-bound anti-CD3 (Figure 3) or CD8+ OT I T cells stimulated with ovalbumin-pulsed APCs (Figure 4). Thus, LLO-induced T cell unresponsiveness can be generalized.

To gain more insight into the phenotypic changes triggered by LLO in T cells, we analyzed such cells for T cell activation cell surface molecules. Normally, T cell activation is accompanied by the up-regulation of CD69 but down-regulation of CD62L. Although LLO-treated cells were unresponsive to stimulation by TCR, they were paradoxically found to exhibit a typical T cell activation cell surface expression profile (Figure 5A). LLO-pretreated T cells showed no alteration in surface TCR levels as estimated by anti-CD3ε staining (Figure 5B). However, intriguingly, such cells exhibited decreased levels of CD3δ, which plays a crucial role in proximal TCR signaling (Figure 5B).

The transcription factor NFAT, which senses intracellular calcium elevation, has been implicated in T cell anergy [13]. Therefore, we tested for the induction of the calcium-NFAT signaling pathway in T cells. When added to the A5 T cell hybridomas, L. monocytogenes and LLO were found to elicit a strong increase in intracellular calcium levels (Figure 5C and data not shown). Accordingly, when A5 cells (which express a GFP reporter under the control of a basal NFAT promoter [14]) were tested, L. monocytogenes and LLO were found to induce NFAT-controlled expression of GFP, whereas L. monocytogenes Δlyt failed to do so (Figures 5D–5F).

Calcium signaling and aggregation of lipid rafts are among the main mechanisms by which LLO triggers host responses [12, 15–17]. However, if LLO is pretreated with cholesterol, it
Figure 5. Typical T cell activation cell surface profile but low intracellular levels of CD3ζ in listeriolysin O (LLO)–treated T cells. A, Induction of a typical T cell surface expression profile by LLO. OT II CD4⁺ T cells treated with LLO or left untreated for 3–4 h were washed, stained with antibodies for surface expression of CD69 and CD62L, and analyzed by flow cytometry. B, Normal expression levels of surface T cell receptor but down-regulation of intracellular levels of CD3ζ in LLO-treated T cells. CD4⁺ T cells treated with LLO or left untreated were fixed in 3.7% paraformaldehyde and then permeabilized in 0.1% triton before being stained for intracellular CD3ζ. C, LLO-dependent increases in intracellular calcium levels. A5 T cell hybridomas were labeled with Indo-1 acetoxymethyl ester and stimulated by treatment with LLO. Calcium levels were estimated by flow cytometry. D–F, Induction of nuclear factor of activated T cells (NFAT)–controlled expression of green fluorescent protein (GFP) by L. monocytogenes and LLO but not by L. monocytogenes Δhly. A5 T cell hybridomas that express GFP under the control of NFAT promoter were incubated for 3 h with a multiplicity of infection of 20 of Listeria monocytogenes (L.m.) (D), L. monocytogenes Δhly (E), or 0.25 μg/mL LLO (F). Levels of GFP expression were measured by flow cytometry. AU, arbitrary units; Ca²⁺, calcium ions.
Figure 6. Similarities between the transcriptional program in T cells induced by listeriolysin O (LLO) and that induced by ionomycin. The transcriptional program induced by LLO involves the up-regulation of negative modulators of T cell receptor signaling. RNA was prepared from uninduced primary CD4+ T cells or from CD4+ T cells stimulated for 3 h with 0.25 mg/mL LLO or 1 μmol/L ionomycin (iono). Gene transcription profiles were evaluated using Affymetrix oligonucleotide arrays. A. Hierarchical cluster analysis of genes with a difference of >1 log-fold in inducible gene expression levels in stimulated T cells compared with levels in unstimulated controls (ctrl). B. Genes with expression levels that were altered by >1 log-fold in response to LLO or ionomycin treatments clustered into 8 panels by means of the self-organizing map algorithm on the basis of kinetic expression pattern. The number of clustered genes and the corresponding percentages of all altered genes are indicated inside each panel. C. Expression profiles of highly altered genes selected and grouped into 4 categories on the basis of function. A more comprehensive list of functionally clustered genes is shown in Figure 7. Casp3, caspase 3; Casp8, caspase 8; Casp12, caspase 12; Cblc, Casitas B-lineage lymphoma c; Cd24a, CD24a antigen; Cd62l, selectin, lymphocyte (SeII); Dusp3, dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related); Dusp15, dual specificity phosphatase-like 15; Dusp18, dual specificity phosphatase 18; Egr2, early growth response 2; Egr4, early growth response 4; Ifnar2, interferon (alpha and beta) receptor 2; Ifngr2, interferon gamma receptor 2; Il10ra, interleukin 10 receptor, alpha; Il2rb, interleukin 2 receptor, beta chain; Il4ra, interleukin 4 receptor, alpha; Ncam1, neural cell adhesion molecule 1; Ncam2, neural cell adhesion molecule 2; Nfatc1, nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1; Ptp4a1, protein tyrosine phosphatase 4a1; Ptpns1, protein phosphatase, non-receptor type substrate 1; Ptprc, protein tyrosine phosphatase, receptor type, C; Ptpg, protein tyrosine phosphatase, receptor type, G; Ptpj, protein tyrosine phosphatase, receptor type, J; Rnf11, ring finger 11; Rnf110, ring finger 110; Smad3, MAD homolog 3 (Drosophila); Socs2, suppressor of cytokine signaling 2; Socs4, suppressor of cytokine signaling 4; Stat1, signal transducer and activator of transcription 1; Stat4, signal transducer and activator of transcription 4; Stat5a, signal transducer and activator of transcription 5a; Stat5b, signal transducer and activator of transcription 5b; Stat6, signal transducer and activator of transcription 6; Tob2, transducer of ERBB2, 2; Traf6, TNF receptor-associated factor 6; Ubb, ubiquitin B; Ubc, ubiquitin C; Vcam1, vascular cell adhesion molecule 1.
looses its pore-forming and calcium-inducing properties [12] but not the ability to signal via aggregation of lipid rafts [16, 17]. To determine the potential contribution of rafts aggregation to LLO-induced TCR unresponsiveness, T cells pretreated with cholesterol-pretreated LLO were also tested. In contrast to active LLO, cholesterol-pretreated LLO did not induce TCR unresponsiveness (data not shown), which suggests that LLO-induced TCR unresponsiveness was most likely due to the calcium-inducing property of LLO.

**LLO up-regulates negative modulators of TCR signaling.**

Next, we performed global gene expression analysis to evaluate the transcriptional changes induced by LLO in T cells and compared it with that induced by ionomycin, a well-characterized agent that induces T cell anergy via the calcium-NFAT pathway [13]. From the microarrays, we identified 10,982 genes with expression levels in T cells that were altered by ≥1.5 log₂-fold by LLO or ionomycin (Figure 6A). Genes with similar expression patterns were clustered into 8 panels (Figure 6B). This analysis revealed ≥64.3% similarity between LLO- and ionomycin-induced gene expression patterns, which suggests that the bulk of the gene regulation by LLO in such cells was via calcium-NFAT signaling, with the rest (35.7%) being most likely due to pathways activated by other mechanisms such as rafts aggregation. Therefore, for further evaluation, we mainly focused on those genes with similar patterns of expression in LLO- and ionomycin-treated cells. Of these, 47.5% were up-regulated by both stimuli, whereas 16.8% were down-regulated (Figure 6B). For further analysis, genes with known identity were grouped into functional clusters, namely, cell surface molecules, transcription modulators, components of proteolytic pathways, phosphatases, and molecules related to apoptosis (Figures 6C and 7).

The surface molecules up-regulated by LLO or ionomycin included CD24a and adhesion molecules Ncam1, Ncam2, and Vcam1. Consistent with the data from flow cytometry (Figure 5A), CD62L was down-regulated (Figure 6C). Several cytokine receptors such as interleukin 2rβ, interleukin 4rα, interferon αr2, and interferon γr2 and members of Stat family of transcription factors, which mediate cytokine signaling, were also down-regulated in LLO- or ionomycin-treated cells (Figure 6C). On the other hand, interestingly, several transcription factors known to support T cell anergy, such as NFAT, Egr2, Egr4, Smad3, and Tob2 [13, 18–22], were up-regulated in LLO- or ionomycin-treated cells (Figure 6C). Moreover, several components of proteolytic degradation pathways, such as suppressors of cytokine signaling (Socs), caspases, and E3 ligases, which are also known to impair T cell activation [23–27], were up-regulated (Figure 6C). Protein tyrosine phosphatases (eg, CD45 and CD148) and dual specificity phosphatases (Dusp3, Dusp15, and Dusp18, which is also involved in negative regulation of TCR [28–31]) were also up-regulated by LLO or ionomycin (Figure 6C). On the other hand, analysis of various genes known to regulate apoptosis, for example, the Bcl-2 family members (Bcl2, Bax, Bid, and Bax), the caspases (Casp1, Casp4, and Casp8), and the granzymes, provided no clear pattern of gene regulation in support of a pro-apoptotic program (Figure 7F). These results may also suggest that the cell apoptosis that is often associated with LLO does not occur via a specific pro-apoptotic gene transcriptional program. Whichever the case, it appears from these data that LLO induces specific transcriptional up-regulation of negative regulators, leading to the induction of T cell anergy.

**DISCUSSION**

Effective control of bacterial pathogens such as *L. monocytogenes* calls for a coordinated activation of both the innate and adaptive immune responses. Innate immunity is crucial for clearing the bulk of the bacterial load in the early phase of infection, whereas T cell–mediated immunity is indispensable for sterile bacterial clearance and long-term protection against reinfection [3, 4]. LLO, the main virulence factor, is a key modulator of a plethora of host responses that greatly influences the outcome of infection. LLO is a key trigger of several innate immune responses, including cytokine production, that lead to neutrophil mobilization and hence rapid clearance of bacteria in the early phase of infection [1, 2]. Many of the innate antibacterial pro-inflammatory responses triggered by LLO have been shown to be due to calcium signaling [12]. In the present study, we show that triggering of T cells by LLO impairs their subsequent activation via the TCR. Paradoxically, the data presented here indicate that LLO-induced T cell anergy is due to the calcium-NFAT signaling pathway, which suggests that activation of this pathway by bacteria has opposite effects on the innate and adaptive immune responses.

How can these contradictory effects of LLO be explained in terms of the bacterial strategy for survival in the host? The most successful pathogens are those that colonize and maintain prolonged survival in the host without killing it. Thus, the idea that LLO activates the innate but suppresses the adaptive immune responses probably illustrates how the bacteria strike a fine balance between limiting overwhelming bacterial growth, which would kill the host, and preventing sterile clearance by T cell–mediated immunity.

T cell anergy is generally considered to be a result of incomplete T cell activation (ie, subthreshold T cell activation). Indeed, as judged from the cell surface profile of T cell activation...
markers, LLO alone does activate T cells, albeit partially. T cell anergy induction as a result of partial T cell activation is in fact consistent with previous observations indicating that initial T cell activation during L. monocytogenes infection, as judged by the up-regulation markers such as CD69, is antigen-independent [34]. The data provided here suggest that this partial triggering of T cells that results in anergy is akin to that by ionomycin, which induces several NFAT target genes that act in concert to impair the proximal TCR signaling, among other processes. Some of the target molecules for such impairment include CD3γ, which is involved in the early TCR signaling events. The idea that LLO-induced T cell unresponsiveness is due to a block in the proximal TCR signaling events is further emphasized by the fact that LLO-induced T cell unresponsiveness can be overcome by bypassing the proximal TCR signaling events by means of PMA-ionomycin.

Direct induction of T cell anergy is probably one of many mechanisms by which LLO could attenuate T cell-mediated immunity. Previous studies from our laboratory showed that LLO does interfere with antigen presentation in such a manner as to generate altered major histocompatibility complex (MHC) peptide complexes that, arguably, as a result of partial TCR triggering, could induce antigen-specific T cell anergy [7, 8]. Such form of anergy is, of course, quite different from that induced directly by LLO, as shown in this study. As judged by the impaired proliferative responses of polyclonal T cells following anti-CD3 stimulation, it is clear that T cell anergy induced via direct T cell triggering by LLO is polyclonal.

The relative contribution of antigen-specific versus polyclonal forms of T cell anergy in aiding the escape of L. monocytogenes from T cell-mediated immunity remains to be determined at the present time. Although these forms of T cell anergy are not mutually exclusive, it is likely that they are activated at different stages during infection. Polyclonal anergy induced directly in T cells by LLO probably represents the frontline strategy for delaying the generation of T cell responses against a broad range of listerial antigens. On the other hand, considering the lag period required for the generation of tolerogenic MHC peptides during infection [7, 8], antigen-specific T cell anergy should be more relevant in the later phase of infection. Irrespective of the putative chronological order by which these mechanisms are harnessed, the present findings, together with the previous findings, illustrate that LLO is a key player in the immune escape strategies by L. monocytogenes aimed at attenuating T cell-mediated immunity. Apparently these mechanisms are not sufficient to completely shut down the generation of T cell-mediated immunity during L. monocytogenes infection. L. monocytogenes is characterized by the up-regulation of L. monocytogenes–specific T cell responses, some of which are in fact targeted at LLO-derived epitopes [35]. Thus, it seems that T cell anergy induced by LLO merely serves to delay T cell responses to give the bacteria more leverage in its quest to establish a niche in the host. This is consistent with the transient nature of all the other immune escape mechanisms that, to our knowledge, have been described to date. As already mentioned, the evolutionary aim of any pathogen is not to decimate the host but to coexist with it. Therefore, it is conceivable that extreme attenuation of the immune system would only result in short-term gains but catastrophic long-term consequences for the pathogen: the death of the host. Such is probably the scenario in immunocompromised persons.

The mechanism of T cell anergy induction described here may not be limited to L. monocytogenes. Many of the features of LLO such as modulation of intracellular calcium in target cells are common not only to bacteria that produce CDCs but also to other bacterial species that produce unrelated toxins [36]. Thus, the likelihood that this form of T lymphocyte interference may extend to other bacterial pathogens should now be investigated. In addition to impairing the ability of the immune system to contain bacterial infection, immune suppression caused by such bacterial toxins is likely to predispose patients to other disease-causing agents. Impaired antibody responses are known to occur during the early phase of infection [37]. Given the crucial role of T cell help in the generation of antibody responses, the possibility that such impaired humoral responses are also due to toxin-induced T cell suppression also cannot be eliminated. Finally, many CDCs are currently under consideration in various vaccination strategies as either delivery proteins or vaccine candidates [38–41]; therefore, the discovery that LLO does suppress T cell-mediated immunity is very important in the context of present and future strategies for vaccine development and therapy.

References


