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Cytosolic expression of SecA2 is a prerequisite for long-term protective immunity

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Summary

Induction of efficient adaptive T cell-mediated immunity against the intracellular bacterium *Listeria monocytogenes* requires its successful invasion of host cell cytosol. However, it is not clear whether its cytosolic escape and growth are sufficient to induce T cell-mediated clearance and protection upon secondary infection. To investigate this issue, we have searched for mutants that do not induce long-term protective immunity yet invade the cytosol of infected cells. We found that mice immunized with *L. monocytogenes* lacking the SecA2 ATPase, an auxiliary protein secretion system present in several Gram-positive pathogenic bacteria, mounted a robust cytolytic IFN-γ-secreting CD8$^+$ T cell response but were not protected against a secondary challenge with wild-type (wt) bacteria. Furthermore, CD8$^+$ T cells from mice immunized with *secA2*$^-$ bacteria failed to transfer protection when injected into recipient mice demonstrating that they were unable to confer protection. Also, *secA2*$^-$ and wt *L. monocytogenes* spread to the same myeloid-derived cell types *in vivo* and SecA2 deficiency does not interfere with intracytosolic bacteria multiplication. Therefore, cytosol invasion is not sufficient for inducing secondary protective responses and induction of memory CD8$^+$ T cells mediating long-term antibacterial protective immunity is dependent upon SecA2 expression inside the cytosol of host cells *in vivo*.

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Introduction

Deciphering the basis of protective immunological memory against intracellular pathogens is a field of intense investigations, ultimately aiming at improving vaccination strategies. To gain insight into the mechanisms by which intracellular pathogens induce protective responses, it is important to understand which bacterial factors are critical for the development and the maintenance of long-term T cell-mediated protective responses.

Many studies have identified virulence factors contributing to establishment of long-lived protective immunity against these pathogens (Fields *et al*., 1986; Berche *et al*., 1987; Clemens and Horwitz, 1995; Sturgill-Koszycki *et al*., 1996; Guo *et al*., 1997; Segal *et al*., 1998; VanCott *et al*., 1998; Vogel *et al*., 1998; Coers *et al*., 1999; Noben-Trauth, 2000; Noss *et al*., 2000; Ramachandra *et al*., 2001; Agrawal *et al*., 2003; Neild and Roy, 2003; Raupach *et al*., 2003). Persistent intracellular bacteria such as *Mycobacterium tuberculosis* or *Legionella pneumophila* establish a vacuolar niche inside the cytosol of infected cells where they can replicate actively. *M. tuberculosis* arrests the maturation of phagosomes to maintain them as habitable environment (Sturgill-Koszycki *et al*., 1994; 1996; Clemens and Horwitz, 1995; 1996). Similarly, *Legionella* bacteria create an endoplasmic reticulum-derived vacuole by expressing their *Dot/icm* (defective for organelle trafficking) virulence genes (Segal *et al*., 1998; Vogel *et al*., 1998; Coers *et al*., 1999). The products of these genes allow injection of bacterial proteins inside infected cells' cytoplasm which then prevent the transport of phagosome-containing bacteria to the lysosomes. In several Gram-negative bacteria, specialized secretion pathways are therefore crucial in promoting colonization of host tissues and introduction of antigenic proteins to the host cell cytosol (Segal *et al*., 1998; Vogel *et al*., 1998; Coers *et al*., 1999; Stathopoulos *et al*., 2000). Thus, in the course of infection with Gram-negative as well as with Gram-positive bacteria, effective T cell response occurs only when bacteria can express such

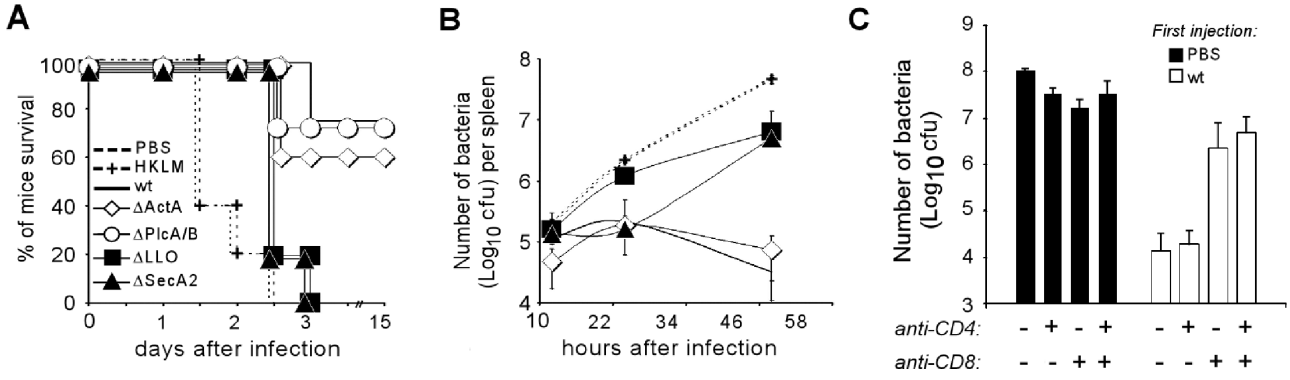


Fig. 1. *secA2*$^-$ bacteria do not induce long-term CD8$^+$ T cell-mediated protective immunity. Six- to 8-week-old BALB/cByJ mice were immunized intravenously with PBS, 10^9 HKLM or $0.1 \times LD_{50}$ of live bacteria (5000 wt, 5×10^8 *llo*$^-$, 10^6 *actA*$^-$, *secA2*$^-$ and *plcA/B*$^-$).
A. Mice (five per group) were challenged with 8×10^5 wt bacteria 1 month later and survival was monitored for 2 weeks.
B. Mice (five per group) were infected with 3×10^5 wt bacteria and bacteria titers in the spleen and the liver were measured 9, 24 and 48 h later.
C. Mice (three per group) were injected with PBS or immunized with 5000 wt bacteria. Mice were injected 30 days later with either 100 μg of anti-CD8β or anti-CD8α (data not shown) intraperitoneally, and/or 200 μg of anti-CD4 for three consecutive days. Mice were challenged 1 day later with 3×10^5 wt bacteria. The numbers of viable bacteria were measured 2 days later in the spleen.
B and C. Data show the number of bacteria (mean ± SD) in one out of three experiments.

essential virulence factors (Noss *et al*., 2000; Ramachandra *et al*., 2001; Neild and Roy, 2003) and therefore establish a successful infectious cycle inside host cell cytosol.

Listeria monocytogenes is a facultative intracellular bacterium that causes severe food-borne infections in immunocompromised patients (Taege, 1999; Portnoy *et al*., 2002). In contrast to intracellular persistent vacuolar bacteria, *L. monocytogenes* escapes primary phagocytic vacuoles and rapidly grows inside the cytosol of host cells to establish a productive infectious cycle. *L. monocytogenes* cytoplasmic escape and multiplication is dependent upon the pore-forming toxin listeriolysin O (LLO) (Gaillard *et al*., 1986), which acts by dissolving primary phagocytic vacuoles. In this setting, both primary and secondary protective CD8$^+$ T cell responses have been well characterized and suggest to require intracytosolic bacterial growth (Berche *et al*., 1987). Along similar lines, immunization of mice with heat-killed *L. monocytogenes* (HKLM), which cannot escape phagocytic vacuoles, do not confer protective immunity (von Koenig *et al*., 1982; Lauvau *et al*., 2001). Thus, infection of mice with a sublethal dose (5×10^3) of live cytosolic invasive wild-type (wt) bacteria induces CD8$^+$ T cell differentiation into memory cells (Busch *et al*., 1998) that mediate long-lived immunological protection against otherwise lethal doses (> 10^5) of bacteria (Mielke *et al*., 1989; Lauvau *et al*., 2001).

While it is widely accepted that the development of protective secondary T cell responses depends upon access of bacterial antigens inside the cytosol of host cells (Shen *et al*., 1998), and requires cytosolic invasive primary infections (Berche *et al*., 1987; VanCott *et al*., 1998; Dixon *et al*., 2000; Noben-Trauth, 2000; Noss *et al*., 2000; Ramachandra *et al*., 2001; Agrawal *et al*., 2003; Neild and Roy, 2003; Raupach *et al*., 2003), it is still unclear whether these events are sufficient. To address this issue, we have searched for mutants of *L. monocytogenes* that escape and grow inside the cytoplasm of infected cells but fail to induce protective secondary responses. We found that *L. monocytogenes* deficient for SecA2, an auxiliary protein secretion system, impacts the development of antibacterial long-term protective immunity.

Results

secA2$^-$ *bacteria do not induce a protective secondary response against* L. monocytogenes

To search for bacterial mutants that do not induce a protective secondary response against wt *L. monocytogenes*, mice were inoculated with $0.1 \times LD_{50}$ of 25 different mutants of this bacterium (Fig. 1 and Table S1). Immunized animals were infected 1 month later with 8×10^5 or 3×10^5 wt bacteria respectively, and monitored for both survival and bacterial titers in the spleen and the liver (Fig. 1). Control animals were injected with PBS, heat-killed (von Koenig *et al*., 1982) or live wt, *actA*$^-$ (Kocks *et al*., 1992) or *plcA/B*$^-$ (Smith *et al*., 1995) bacteria. As expected, mice immunized with HKLM and naïve mice exhibited 1000-fold more bacteria at 1.5 days than mice immunized with wt, *actA*$^-$ or *plcA/B*$^-$ bacteria, and rapidly succumbed to the infection (Fig. 1A and data not shown). Among the 25 mutants, six including one mutant lacking the master regulator of *Listeria* virulence

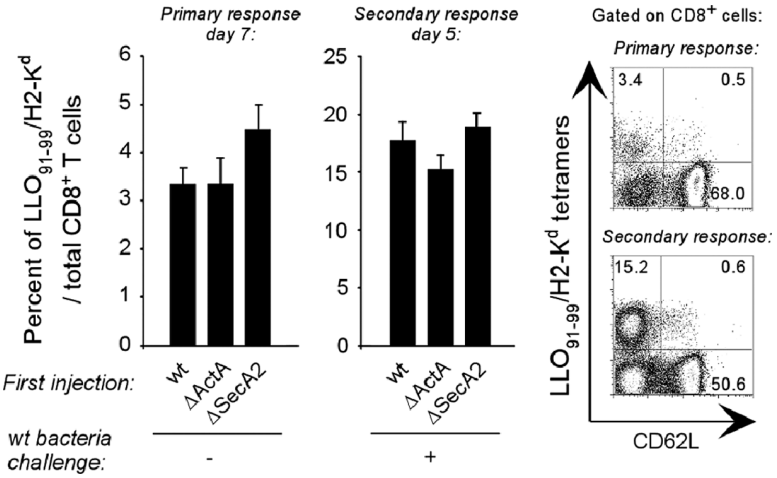


Fig. 2. *secA2*$^-$, *actA*$^-$ and wt bacteria induce comparable bacteria-specific CD8$^+$ T cell primary and secondary expansion. Mice (three per group) were infected with $0.1 \times LD_{50}$ of *secA2*$^-$ (10^6), *actA*$^-$ (10^6) or wt (5000) bacteria and challenged or not 1 month later with 3×10^5 wt bacteria. Secondary challenged mice were treated with ampicillin (25 mg per mouse) to allow survival of non-protected animals. The frequency of CD62L^{low} H2-K^d/LLO$_{91-99}$-specific CD8$^+$ T cells among total CD8$^+$ T cells was measured 7 and 5 days after primary and secondary infection respectively. Data show mean frequency ± SD in one out of two experiments giving the same result. A representative FACS profile is shown after gating on live CD8$^+$ T cells.

gene expression (PrfA) (Leimeister-Wachter *et al*., 1990) and three mutants lacking LLO (Gaillard *et al*., 1986; Berche *et al*., 1987) failed to induce protection. These results were expected because neither *PrfA*$^-$ nor *llo*$^-$ mutants escaped to the cytosol, a prerequisite to induce long-term protective immunity. Interestingly, mutants lacking SecA2 (Lenz and Portnoy, 2002) did not control secondary infection with wt bacteria and exhibited almost comparable increase in bacterial counts over time as compared with mice injected with PBS, HKLM, or *llo*$^-$ bacteria (Fig. 1B). These results show that primary infection with *secA2*$^-$ bacteria did not induce a protective secondary response and further suggest that the lack of this dedicated secretion system interferes with the development of long-term protective immunity. As expected, complementation of the *secA2*$^-$ mutant by reintroducing the wt secA2 gene restored the phenotype of wt bacteria and induced immunity in immunized animals (data not shown). To further investigate this phenomenon, we have compared the immune response induced by *secA2*$^-$, *actA*$^-$ and wt bacteria. We have chosen *actA*$^-$ bacteria because they exhibited the same LD_{50} of 10^7 in BALB/c mice than *secA2*$^-$ bacteria (Goossens and Milon, 1992; Smith *et al*., 1995; Lenz and Portnoy, 2002).

Primary and secondary CD8$^+$ T cell responses are comparable in mice immunized with secA2$^-$, actA$^-$ *and wt bacteria*

Previous results have shown that the long-term maintenance of the protective response against *L. monocytogenes* is dependent on CD8$^+$ T cells (Mielke *et al*., 1989; Lauvau *et al*., 2001). To confirm these data, mice immunized with wt bacteria were depleted of either CD8$^+$ T cells, CD4$^+$ T cells or both (Fig. 1C). As expected, CD8$^+$ T cells, but not CD4$^+$ T cells, were required for protection. We next analysed bacteria-specific CD8$^+$ T cells in mice immunized with *secA2*$^-$, *actA*$^-$ or wt bacteria. Mice exhibited comparable frequencies of endogenous primary LLO$_{91-99}$/H2-K^d-tetramer-specific CD8$^+$ T cells (3–4%) among CD3$^+$CD8$^+$ T cells (Fig. 2A). Similarly, no difference between the different groups were observed following secondary infection (15–17%). Therefore, CD8$^+$ T cells from mice immunized with *secA2*$^-$ bacteria were not impaired in their ability to proliferate *in vivo* upon primary or secondary infection as compared with those from mice immunized with *actA*$^-$ or wt bacteria.

Memory CD8$^+$ T cells from mice immunized with secA2$^-$ *bacteria do not confer protection*

We next assessed whether CD8$^+$ T cells from mice immunized with *secA2*$^-$, *actA*$^-$ and wt bacteria were functionally different. The frequencies of CD8$^+$ T cells that secreted IFN-γ or expressed granzyme B were similar in all groups (Fig. 3A and B). To investigate whether these cells were equally capable of conferring protective immunity, mice were primarily immunized with *secA2*$^-$, *actA*$^-$ or wt *L. monocytogenes* or injected with PBS and secondary challenged with wt bacteria 1 month later. Mice were sacrificed 6 h after the challenge infection and CD8$^+$ T cells were sorted by flow cytometry and transferred into naïve recipients. Animals were subsequently infected with 3×10^5 wt bacteria, and viable bacteria in the spleen and the liver were enumerated 48 h later (Fig. 3C and data not shown). Mice that received CD8$^+$ T cells from mice immunized with *actA*$^-$ or wt bacteria exhibited 4.2- and 5.3-fold less bacteria in the spleen and in the liver respectively, as compared with those that received CD8$^+$ T cells from mice immunized with *secA2*$^-$ bacteria or injected with PBS. Taken together, these results demonstrated that memory CD8$^+$ T cells from mice immunized with *secA2*$^-$ *L. monocytogenes* were impaired in their ability to confer protection.

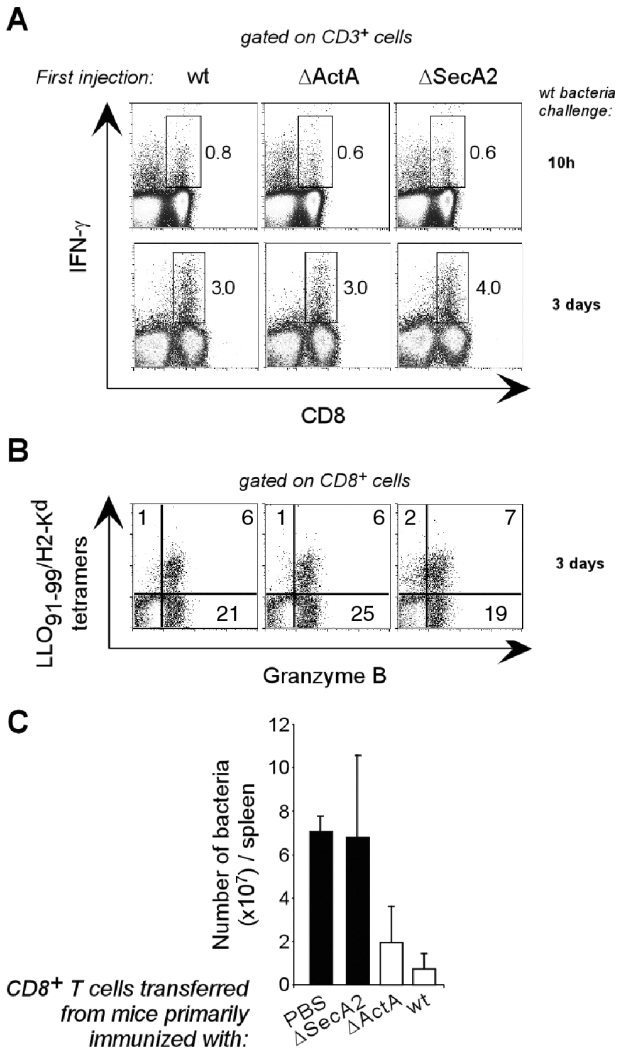
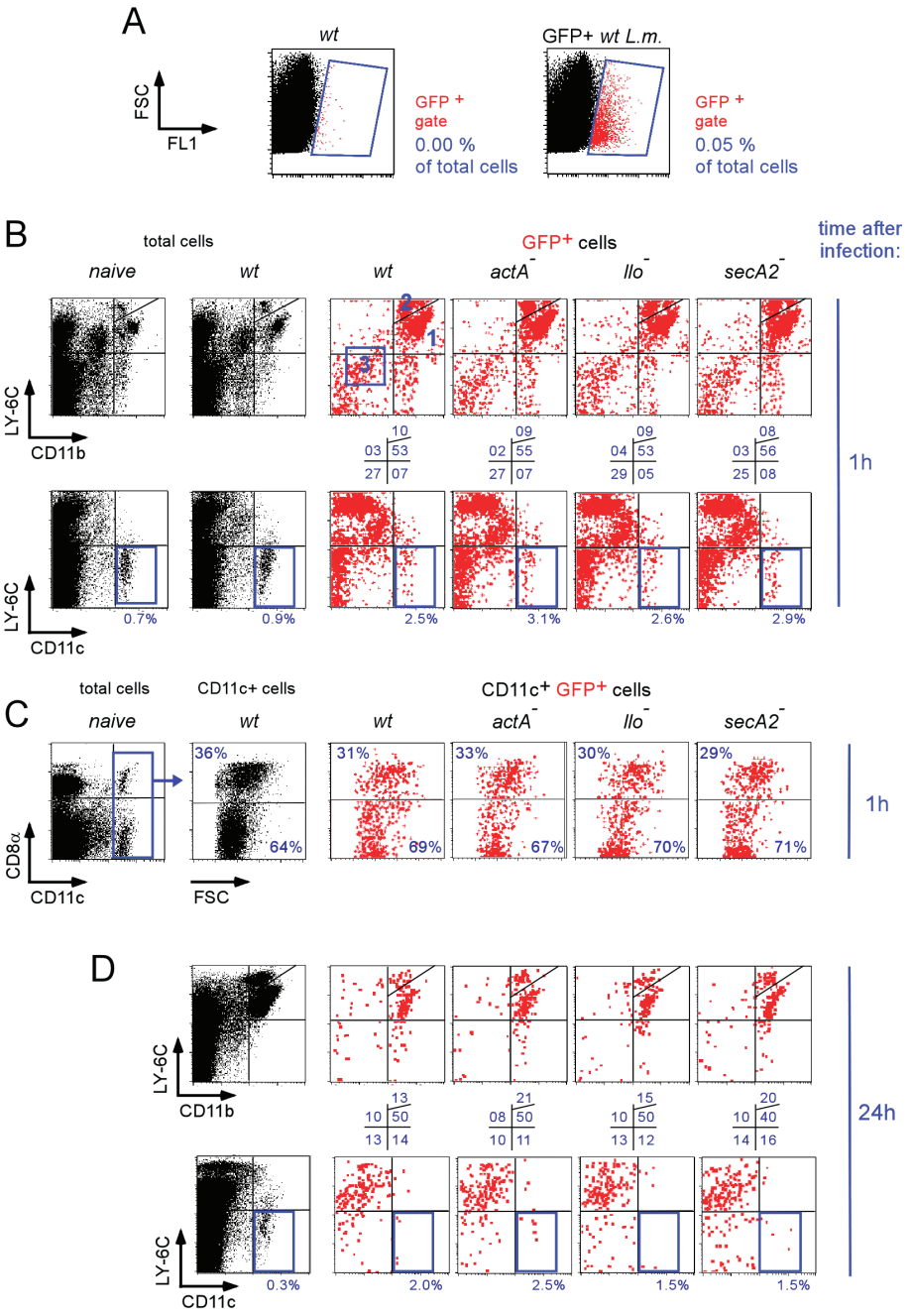


Fig. 3. CD8$^+$ T cells from mice immunized with *secA2*$^-$ bacteria do not confer protective immunity. Mice primarily immunized with *secA2*$^-$, *actA*$^-$ or wt bacteria were challenged 1 month later with 3×10^5 wt bacteria. Spleen cells were harvested 10 h or 3 days after secondary challenge.
A. Cells were restimulated *in vitro* for 6 h with 10^{-9} M of LLO_{91-99} peptide and stained both for CD3ε and CD8α cell surface markers and for intracellular IFN-γ.
B. Spleen cells were stained with H2-K^d/LLO_{91-99} tetramers, CD8α cell surface marker and for intracellular granzyme B.
A and B. Data show representative FACS profiles in one out of three experiments.
C. Six hours after secondary challenge, 20×10^6 CD8$^+$ T cells from mice immunized with *secA2*$^-$, *actA*$^-$, wt bacteria or PBS were sorted by flow cytometry and transferred to naïve recipient mice (two per group) that were subsequently infected with 3×10^5 wt bacteria. Spleen cells were plated 48 h later and bacteria enumerated. Data show mean bacteria cfu ± SD in one out of two experiments.

secA2$^-$, llo$^-$, actA$^-$ *and wt bacteria spread to the same cell types* in vivo

To further investigate why *secA2*$^-$ bacteria failed to induce protective immunity, we have generated erythromycin-resistant bacteria expressing high levels of green fluorescent proteins (GFP). These bacteria were readily detected in infected spleen cells by fluorescence-activating cell sorting (FACS) (Fig. S1A) and by confocal microscopy (Fig. S1B). To determine whether GFP was stably expressed *in vivo*, mice were infected with GFP$^+$ bacteria and live splenocytes sorted by flow cytometry according to their level of fluorescence. Cells were lysed, plated onto Petri dishes, and bacterial colony-forming units (cfu) enumerated (Fig. S1C). GFP^{bright} cells (G4) accounted for 3.5% of GFP$^+$ cells and 0.07% of all cells. GFP^{bright} cells also contained 99% of all bacteria demonstrating that almost all live bacteria expressed GFP *in vivo*. In an alternative experiment, splenocytes from infected mice were prepared and plated on Petri dishes with or without erythromycin. Identical numbers of bacterial CFU were observed on both plates and all bacterial clones expressed GFP further confirming that GFP-expressing bacteria were highly stable *in vivo* (data not shown).

To determine whether *secA2*$^-$, *actA*$^-$ and wt bacteria spread to different cell types *in vivo*, mice were injected with various numbers of bacteria and splenocytes were analysed 1 h later for the presence of GFP$^+$ cells. GFP$^+$ cells were readily detected in spleen only when high numbers ($> 10^8$) of bacteria were used (data not shown). GFP$^+$ splenocytes were analysed for expression of various markers including F4/80, Gr1, CD11c, B220, CD49b, CD80, CD86, MHC-II, CD11b and Ly-6C that allow to distinguish neutrophils (CD11b^{high}Ly-6C$^{med/high}$), TNF/iNOS-producing dendritic cells (Tip-DCs) (CD11b$^{med/high}$Ly-6C^{high}) (Serbina *et al*., 2003), macrophages (CD11b^{med}Ly-6C^{med}) and conventional CD11c^{high} DCs (Serbina and Pamer, 2006) (Fig. 4 and data not shown). One hour after infection, wt and mutant GFP$^+$ bacteria were similarly distributed in neutrophils (53–56%), Tip-DCs (8–10%), macrophages (< 5%) and CD11c^{high} DCs (2.0–3.0%) (Fig. 4B). This latter result was confirmed by analysing GFP$^+$ cells by electron microscopy (EM) (Fig. S2). Therefore, live bacteria were mainly found in CD11b- and Gr1-expressing myeloid-derived cells early after infection. Because CD8α$^+$ CD11c^{high} DCs are more potent initiators of CD8$^+$ T cell-mediated immunity following *L. monocytogenes* infection (Belz *et al*., 2005), we have further analysed the distribution of *secA2*$^-$, *actA*$^-$ and wt bacteria in CD8α$^+$ and CD8α$^-$ DC subsets (Shortman and Liu, 2002) (Fig. 4C). GFP-expressing wt and mutant bacteria exhibited the same tropism and were present in both CD8α$^+$ (29–31%) and CD8α$^-$ (69–71%) CD11c^{high} DCs. Similar experiments were performed in mice infected with $0.1 \times LD_{50}$ of each bacteria for 24 h. As observed at 1 h after infection, *secA2*$^-$, *actA*$^-$ and *llo*$^-$ mutants as well as wt bacteria were similarly distributed in neutrophils (40–50%), Tip-DCs (13–21%) and CD11c^{high} DC (1.5–2%) (Fig. 4D and Fig. S3).



E

	wt	*actA*⁻	*llo*⁻	*secA2*⁻	
Number of GFP⁺ cells / 10^6 live splenocytes:	3875	3721	2441	3663	1h
	638	63	0.02	42	24h
Percentage of GFP⁺ cells among CD11c+ cells:	4.01	4.17	3.84	4.16	1h

Fig. 4. *secA2*⁻, *actA*⁻, *llo*⁻ and wt GFP⁺ bacteria are similarly distributed among cell types. Mice (four per group) were infected with (A, B, C, E) 5×10^8 bacteria for 1 h or (D, E) with $0.1 \times LD_{50}$ bacteria for 24 h. Spleen cells were analysed by flow cytometry upon staining with mAbs against CD11b, Ly-6C, CD11c and CD8α.

A. Data show representative FACS profiles and the gate used to sort GFP⁺ cells (red).

B–D. Data show representative FACS profiles after gating on total or GFP⁺ live cells. GFP⁺ cells are shown in red. Similar results were found in three independent experiments. In (A, B, D) 10^6 cells were collected and in (C) 5000 CD11c^{high} cells were collected. Gates '1', '2' and '3' in (B) corresponding to Tip-DCs, neutrophils and macrophages are shown in blue.

E. The number of GFP⁺ cells per 10^6 splenocytes and the frequency of GFP⁺ cells among CD11c^{high} cells are shown.

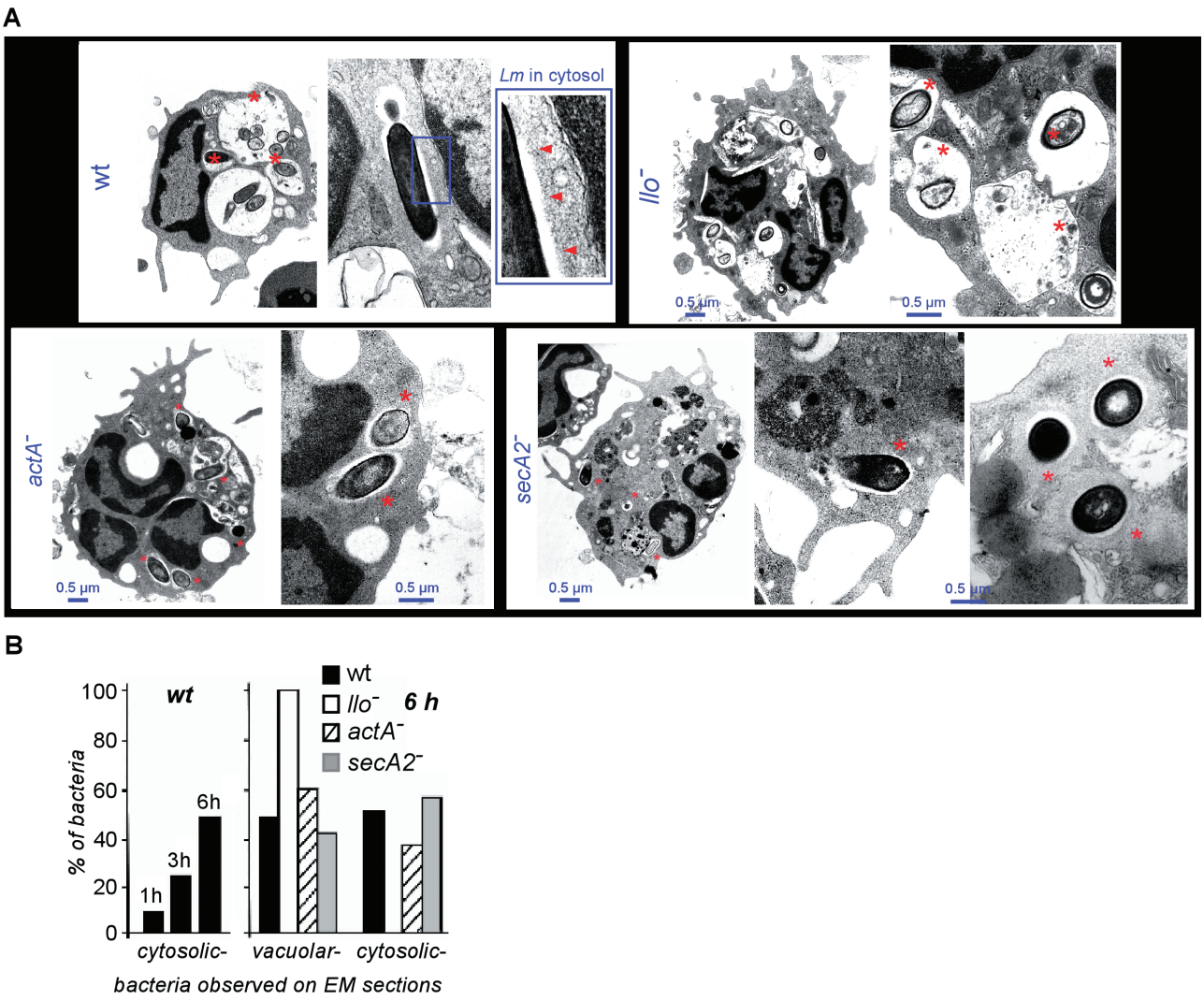


Fig. 5. *secA2*⁻, *actA*⁻ and wt, but not *llo*⁻*L. monocytogenes*, localize inside the cytosol of host cells *in vivo*. Mice (three per group) were infected with wt, *llo*⁻, *actA*⁻ and *secA2*⁻ bacteria for 6 h and GFP⁺ spleen cells were sorted and analysed by EM.
A. Data show low and high magnification views for the indicated bacteria. *llo*⁻ bacteria were trapped inside phagocytic vacuoles. *secA2*⁻, *actA*⁻ or wt bacteria are free inside the cytosol of infected cells with no vacuolar membranes around them.
B. More than 100 distinct cells were analysed at the indicated times after infection (left panel) or 6 h after infection (right panel). Data show the frequencies of vacuolar or cytosolic wt (filled bars), *llo*⁻ (empty bars), *actA*⁻ (dashed bars) or *secA2*⁻ (grey bars) bacteria in spleen cells.

Although the frequency of CD11c^{high} DCs among GFP⁺ was identical 1 and 24 h after infection, the total number of CD11c^{high} DCs was lower at 24 h as compared with 1 h after infection. Furthermore, while the frequency of GFP⁺ cells among splenocytes was similar for all bacteria 1 h after infection, this frequency varied substantially between < 0.001% for *llo*⁻, 0.004% for *secA2*⁻ and 0.006% for *actA*⁻ and 0.06% for wt bacteria at 24 h, correlating with the rate of multiplication of these bacteria *in vivo* (Fig. 4E). Therefore, the impaired ability of *secA2*⁻ bacteria to promote the development of a secondary protective response did not result from an altered tropism of these bacteria *in vivo*.

secA2⁻ *bacteria invade host cell cytosol* in vivo

To investigate whether *secA2*⁻ bacteria grow inside the cytosol of infected cells *in vivo*, GFP⁺ cells were purified from mice infected with GFP-expressing *secA2*⁻ bacteria and analysed by EM (Fig. 5A). As observed in previous reports using *in vitro* infected cell lines, wt and *actA*⁻ bacteria were found in the cytosol of infected splenocytes with no vacuolar membrane around them. In contrast, *llo*⁻ bacteria were always localized within vacuoles in all cell types including neutrophils and monocytes/macrophages that accounted for the vast majority of infected cells at that time. Analysing more than 200

distinct cells from mice infected with wt bacteria showed that 12% were localized in the cytoplasm 1 h after infection (Fig. 5B), a result in agreement with those reported *in vitro* (Tilney and Portnoy, 1989). This proportion increased to 24% and 51% 3 and 6 h after infection respectively. In contrast to *llo*$^-$ bacteria, *secA2*$^-$, *actA*$^-$ and *plcA/B*$^-$ bacteria escaped primary phagocytic vacuoles and were localized in the cytosol 6 h after inoculation (Fig. 5B).

SecA2 ATPase deficiency impairs the secretion of a subset of bacterial proteins that are exported to the cytosol of infected cells (Lenz and Portnoy, 2002). Therefore, we have investigated whether secretion of LLO was altered in *secA2*$^-$ as compared with wt bacteria (Fig. S4). Pellet, culture supernatant and SDS-soluble fraction of log-phase grown bacteria were analysed by Western blot using anti-LLO and anti-SecA1 rabbit sera. Both LLO and SecA1 were expressed at similar levels in wt and *secA2*$^-$ bacteria respectively.

We also found that *secA2*$^-$, *actA*$^-$ and *plcA/B*$^-$ mutants exhibited similar growth curves *in vivo* with a rapid increase in bacterial numbers during the first 12–15 h followed by a rapid decrease thereafter (Fig. S5). Similar data were obtained when mice were given gentamicin over the course of this experiment demonstrating that the impaired ability of *secA2*$^-$ bacteria to induce a potent protective secondary response is unlikely to result from an impaired persistence of *secA2*$^-$ bacteria inside infected cells.

Taken together, our results demonstrate (i) that cytosol invasion is not sufficient for the development of protective immunity to secondary *L. monocytogenes* infection and (ii) that the SecA2 ATPase is required for successful induction of long-term protective immunity against *L. monocytogenes.*

Discussion

Our study provides two main findings on which steps of the *L. monocytogenes* infectious cycle are critical for the development of antibacterial protective secondary responses: we show that cytosolic invasive infections, although required, are not sufficient, and that expression of the SecA2 ATPase by cytoplasmic *L. monocytogenes* is necessary for long-term, CD8$^+$ T cell-mediated protection.

For most intracellular bacteria, previous reports have demonstrated that establishment of a successful cytosolic invasive infection is a prerequisite for inducing protective immunity. In the case of *L. monocytogenes*, escape and multiplication of the bacteria inside the cytosol was believed to be sufficient for inducing protective T cell-mediated memory (Berche *et al*., 1987). Our current results reveal an additional step in this process that depends on the cytosolic expression of the SecA2 auxiliary secretion system.

In Gram-negative pathogens, the SecA ATPase belongs to the general secretory pathway and provides translocating energy for a set of bacterial-derived proteins. An auxiliary SecA2 protein secretion system was identified in several major Gram-positive pathogenic bacteria, including *L. monocytogenes*, *M. tuberculosis* and *Bacillus anthracis* (Braunstein *et al*., 2001; Lenz and Portnoy, 2002). These Gram-positive bacteria contain at least two SecA genes, SecA1 and SecA2 (Lenz and Portnoy, 2002) that have been associated with the virulence and the ability of these bacteria to cause disease (Braunstein *et al*., 2003; Lenz *et al*., 2003). In both *L. monocytogenes* and *M. tuberculosis*, SecA2 promotes pathogenesis by modulating the secretion of virulence proteins such as autolytic enzymes and superoxide dismutases (Braunstein *et al*., 2003; Lenz *et al*., 2003). SecA2 is a soluble protein that does not exhibit any transmembrane domain (Sharma *et al*., 2003). Therefore, it is likely that its effect on the development of protective immunity involves one or several of the SecA2-secreted substrate proteins. As in the case of lipopolysaccharides, it is conceivable that SecA2-dependent proteins contain evolutionary conserved pathogen-associated molecular patterns. For instance, the p60 and *N*-acetylmuramidase autolytic enzymes of *L. monocytogenes*, which secretion is largely dependent upon SecA2, digest bacteria walls and release muramyl peptides into host cell cytoplasm. These peptides may further bind NOD intracellular pattern recognition receptors (Chamaillard *et al*., 2003; Girardin *et al*., 2003a,b) that could trigger adequate signalling pathways inside DC for protective T cell priming (Iwasaki and Medzhitov, 2004).

It is noteworthy that a dedicated secretion system found in several Gram-positive pathogenic bacteria strongly impacts the development of protective T cell memory against *L. monocytogenes.* Recent studies have suggested that ribosomal proteins and superoxide dismutase are the only conserved SecA2-dependent substrates translocated in both *L. monocytogenes* and *M. tuberculosis* (Braunstein *et al*., 2003; Archambaud *et al*., 2006). It remains to be determined if any of the other SecA2-secreted proteins may share common structural or sequence features and whether our finding can be generalized to other pathogenic Gram-positive bacteria.

Finally, although CD8$^+$ T cells from mice infected with *secA2*$^-$ bacteria are cytolytic and secrete IFN-γ, expression of these effector functions is not sufficient to protect against a secondary bacterial infection. This result is relevant in the context of which effector functions should be measured to predict vaccine efficacy.

Experimental procedures

Mice and bacteria

Six- to 8-week-old BALB/c mice were used in all experiments (Janvier). Stocks of wt 10403s and its mutant derivatives *secA2*$^-$, *actA*$^-$, *llo*$^-$, *plcA/B*$^-$, were prepared from isolated bacterial clones selected out of the spleen of infected mice twice. Selected bacteria were frozen at −80°C after growth to log-phase in Broth Heart Infusion medium (BHI, Sigma). HKLM were prepared as previously described (Lauvau *et al*., 2001).

Determination of bacteria titers inside the spleen, the liver or flow-purified cells from infected mice

Organs dissociated on metal screens or flow-purified cells were recovered in water/0.1% X-100 triton (Sigma) and further plated onto BHI media plates. Successive serial dilutions were performed in this buffer to determine bacterial cfu.

Quantification of LLO synthesis

Bacteria were grown to log phase in LB broth, then fractionated as previously described (Lenz and Portnoy, 2002). Briefly, bacteria were pelleted by centrifugation, and supernatant proteins were precipitated with TCA. Whole cell lysates were prepared by incubating TCA-treated bacteria with lysozyme to digest the cell wall. Protein fractions were dissolved in SDS running buffer (62.5 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 5% beta-mercaptoethanol, 0.1% bromophenol blue) and used for SDS-PAGE. Bacterial pellets, supernatant or SDS-soluble fractions were analysed by Western blotting using an antiserum against LLO. We have typically loaded 10 μl per lane which correspond to the amount of LLO produced by 10^9 log phase bacteria of each type.

Generation of GFP-expressing bacteria

Five micrograms of PEG-purified pNF8 (Fortineau *et al*., 2000) was electroporated into wt 10304s or *llo*$^-$, *actA*$^-$ or *secA2*$^-$ bacteria. Transfected bacteria were further selected onto BHI plates with 10 μg ml^{-1} erythromycin and checked their GFP expression using a FACScalibur cytofluorometer.

Confocal tissue sections

Spleens were fixed in 0.1 M phosphate buffer/1% paraformaldehyde (PFA) at 4°C for 3 h and further dehydrated in this buffer completed with 30% sucrose overnight. Then, organs were frozen in OCT (DAKO) on dry ice and 15 μm spleen sections (Cryomicrotome Leica) were stained with alexa647-phalloidin (Molecular Probe). Sections were further analysed by confocal microscopy on a Leica laser scanning confocal microscope equipped with a DM-IRBE inverted microscope and an Argon-Krypton laser.

Preparation of organ suspensions

Organs were cut in very small pieces and incubated (37C, 30 min) into HBSS (Gibco) medium containing 4000 U ml^{-1} of collagenase IV (Roche) and 0.1 mg ml^{-1} of DNase (Sigma). Red blood cells were lysed for 2–3 min in 0.17 M NH_4Cl/0.017 M Tris pH 7.4.

Flow cytometry

A total of 3–5 × 10^6 cells were stained using directly coupled fluorescent mAb combinations in 100 μl PBS 0.5% BSA 0.02% NaN_3 (FACS buffer) and further collected on a FACScalibur cytofluorometer (Becton Dickinson, BD). The following mAbs were purchased from BD Pharmingen: anti-CD8α (53-6.7)-fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein or allophycocyanin (APC), anti-CD3ε-FITC (145-2C11), anti-CD62L-APC (MEL-14), anti-CD11c-PE (HL3), anti-CD11b-PE or APC (M1/70), anti-Gr1-APC (Ly-6G), anti-B220-PE (RA3-6B2), anti-CD49b-PE (DX5), anti-I-Ad-PE (AMS-32.1), anti-Ly-6C-FITC (AL-21). Anti-Granzyme B-APC (mouse and human) and Anti-F4/80-FITC were purchased from Caltag (TebuBio). For flow-cell purifications, similar cell-staining conditions were used and cells were sorted on a FACSvantage SE cell sorter (Becton Dickinson). H2-K^d/$LLO_{91–99}$ tetramers coupled to PE were obtained from the NIH tetramer core facility.

Intracellular cytokine staining

Splenocytes were incubated for 4–6 h in RPMI1640 5% FCS with 2 μg ml^{-1} Golgi Plug (BD Pharmingen) at 37°C, 5% CO_2, except for granzyme B staining. Cells were further washed in FACS buffer, stained for cell surface markers before fixation in PBS/1% PFA for 15–20 min on ice and permeabilized for 30 min using a saponin-based buffer (1X Perm/Wash, BD Pharmingen in FACS buffer) containing the intracellular staining mAbs (anti-IFN-γ-APC XMG1-2, BD Pharmingen). After final fixation in PBS/1% PFA, cells were analysed on a FACScalibur cytofluorometer. No signal was detectable with the isotype control (rat IgG_1-APC, BD Pharmingen).

EM sample preparation

Flow-sorted cells were primarily fixed with 1.6% glutaraldehyde in 0.1 M phosphate buffer pH 7.5, then at 4°C with 1% osmium tetroxyde in 0.1 M cacodylate buffer pH 7.5. Cell pellets were further washed with distilled water and incubated for 2 h with 0.5% water/uranyl acetate at room temperature in the dark. After several more washes in water, pellets were dehydrated in increasing acetone series and embedded in epoxy resin. Blocks were then conventionally thin-sectioned (80 nm thick) and contrasted for the observation on a Philips CM12 electron microscope.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. GFP-expressing *L. monocytogenes* are bright, stable and more than 99% are detected inside infected organs in vivo. (A) Flow-cytometry analysis of GFP expression on pNF8-electroporated bacteria grown to log phase in BHI medium. (B) Intracellular localization of wt GFP^+ bacteria on spleens cryosections of mice infected for 24 hours. (C) More than 99% of live bacteria are recovered from the cells that express GFP: $1–2 \times 10^6$ spleen cells mice for 24 hours were sorted by flow-cytometry both on forward side scatter (FSC) and on GFP intensity. We set different gates from G1 to G4 which include 30% (for G1 and G2), 38% (G3) and 2% (G4) of total spleen cells. G4 was set to 1 to 2% of total spleen cells which represent the lower limit to ensure that sorted cells will be viable. The number of bacteria CFU recovered from the cells purified in each gate was determined by plating onto BHI plates. These numbers are shown on the top of each bar. Experiments were performed twice with 3 mice/group and gave identical results.

Fig. S2. *L. monocytogenes*-infected spleen cell types *in vivo* as seen by electron microscopy (EM).

Pellets of GFP^+ spleen cells from mice infected with 10^9 wt or mutant bacteria after 1, 3 and 6 h were treated for EM observation. While the very dark bacteria are alive, light grey to white or ghostly bacteria are being degraded. Red stars target bacteria. The distinct cell types that contain bacteria include neutrophils (1), eosinophils (2), dendritic cells (3) and monocytes/macrophages (4).

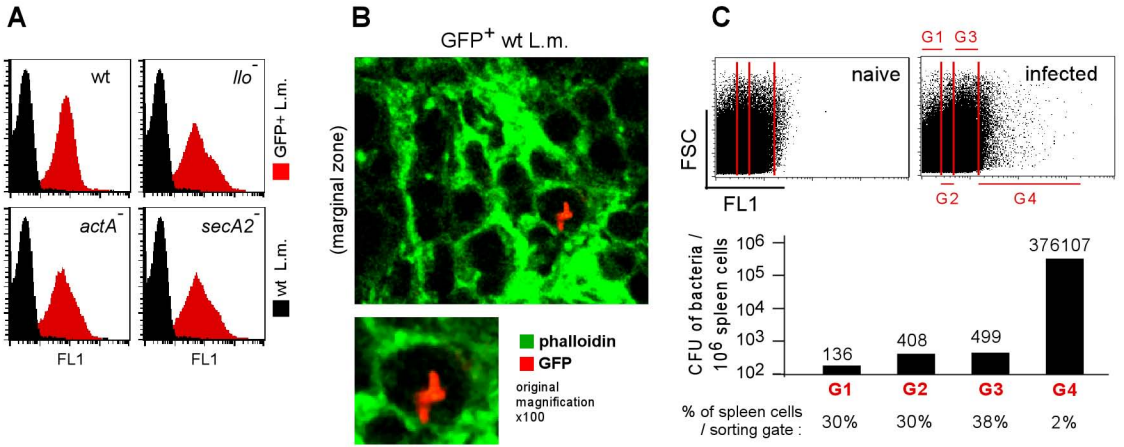
Fig. S3. Cell surface phenotype of infected spleen cells 24 h after wt or mutant bacteria infection. Gating on GFP+ cells demonstrates that $Gr1^+CD11b^+$, $CD11c^{low}$, $F4/80^+$ cells are the main infected cell type.

Fig. S4. *secA2*$^-$ and wt bacteria secrete identical levels of LLO. Organisms were grown to log phase in LB broth, then pelleted by centrifugation (P), and supernatant (SN) proteins were precipitated with TCA. Whole cell lysates were prepared by incubating TCA-treated bacteria with lysozyme to digest the cell wall. Protein fractions were dissolved in SDS running buffer and used for SDS-PAGE. Bacterial pellets (P), supernatant (SN) or SDS soluble fractions (SDS) were analysed by Western Blotting using an anti-serum against LLO or SecA1 as a control.

Fig. S5. Kinetics of bacterial growth and clearance inside spleen of primary infected mice. Mice were infected with $0.1 \times LD_{50}$ of live bacteria (5000 wt, 10^9 *llo*$^-$, 10^6 *actA*$^-$, *secA2*$^-$ and *plcA/B*$^-$), their spleen harvested at different time points after injection and plated onto Petri dishes to determine the number of CFU. For gentamycin treatment, mice were injected i.p with 2 mg one hour after infection and every 24 h.

Table S1. *Listeria monocytogenes* mutants injected.

This material is available as part of the online article from http://www.blackwell-synergy.com



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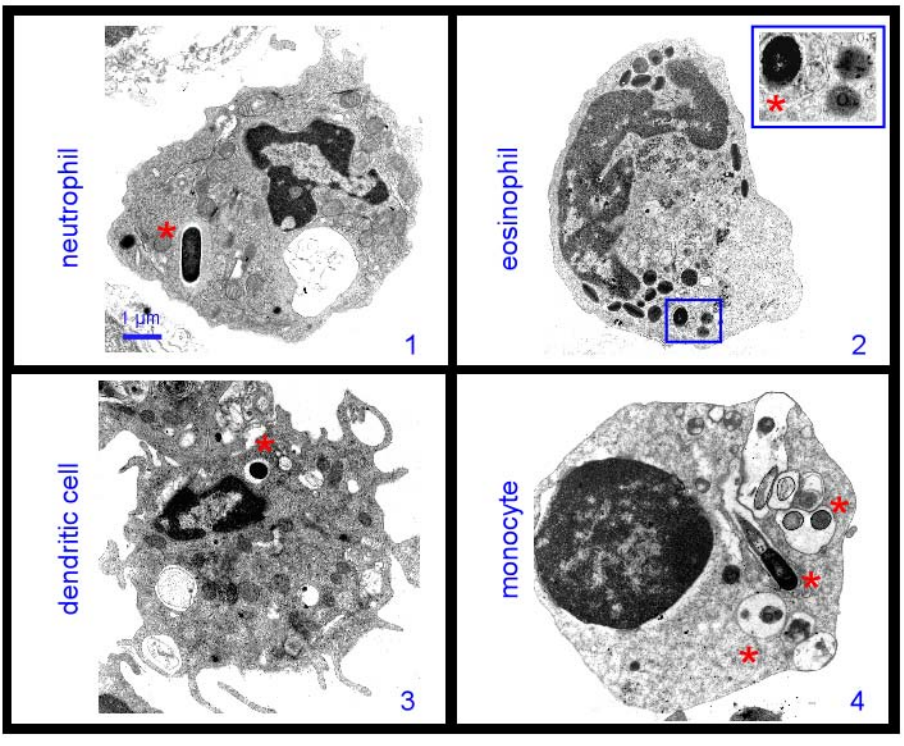

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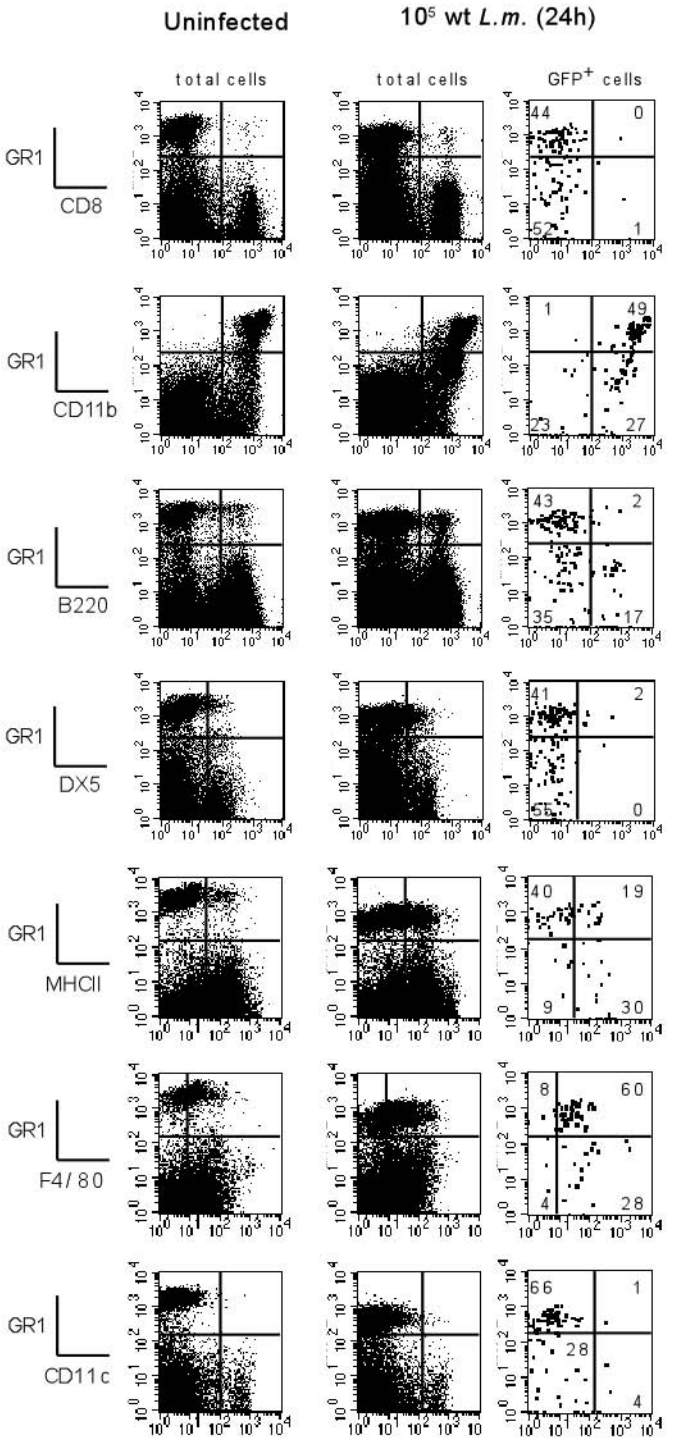

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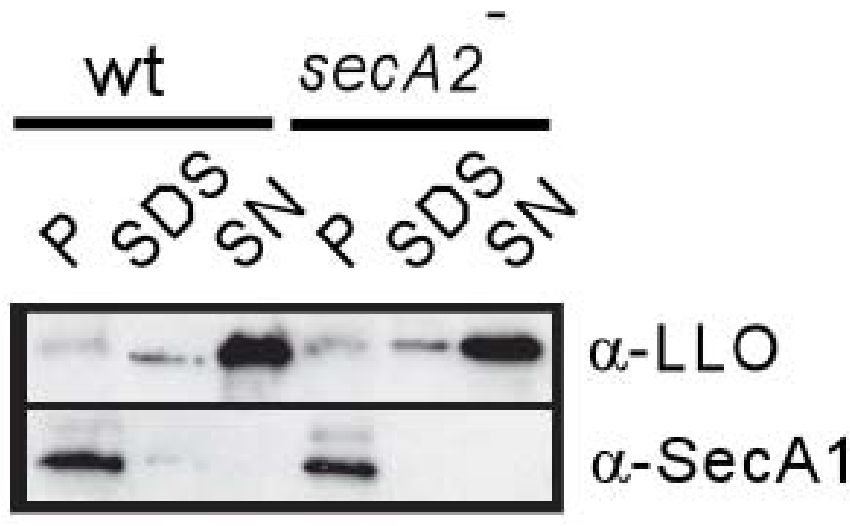

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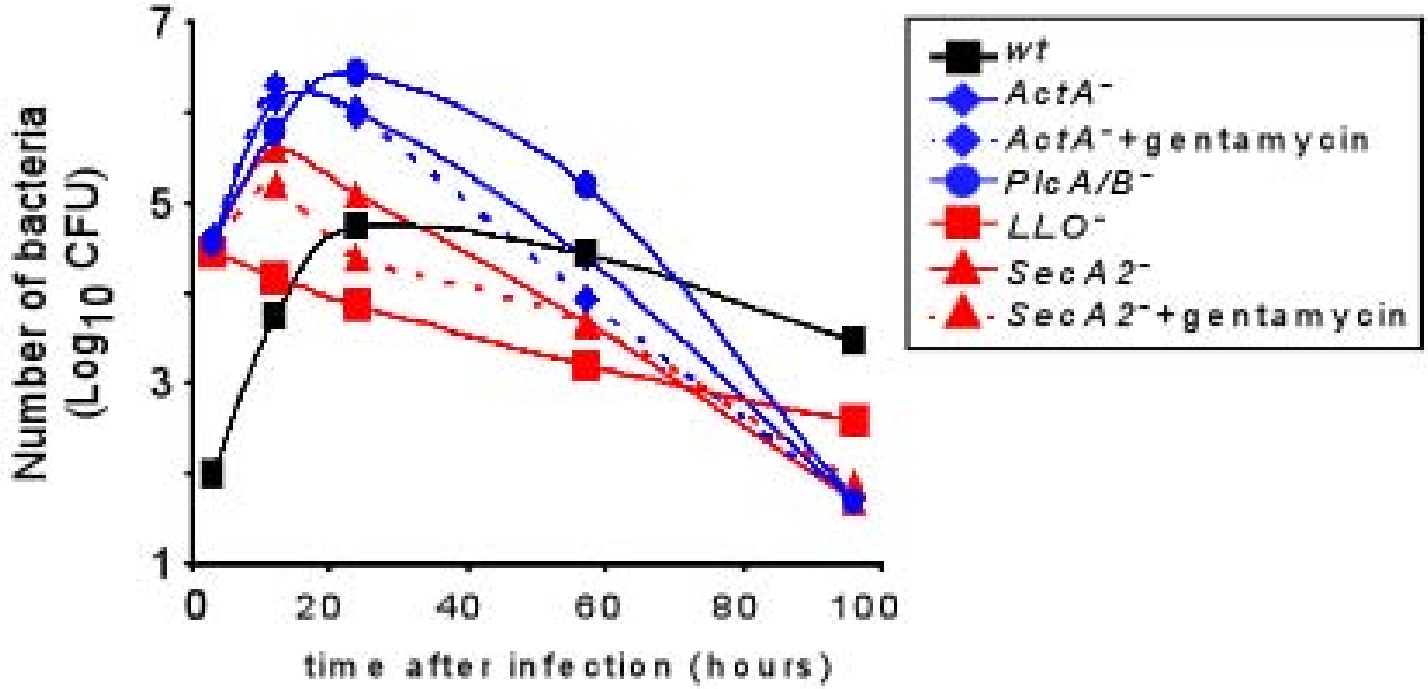

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