CHAPTER 1

Multiple Effector Mechanisms Induced by Recombinant *Listeria monocytogenes* Anticancer Immunotherapeutics

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Abstract *Listeria monocytogenes* is a facultative intracellular gram-positive bacterium that naturally infects professional antigen presenting cells (APC) to target antigens to both class I and class II antigen processing pathways. This infection process results in the stimulation of strong innate and adaptive immune responses, which make it an ideal candidate for a vaccine vector to deliver heterologous antigens. This ability of *L. monocytogenes* has been exploited by several researchers over the past decade to specifically deliver tumor-associated antigens that are poorly immunogenic such as self-antigens. This review describes the preclinical studies that have elucidated the multiple immune responses elicited by this bacterium that direct its ability to influence tumor growth.

I. INTRODUCTION

Listeria monocytogenes is a gram-positive facultative intracellular bacterium responsible for causing listeriosis in humans and animals (Lecuit, 2007; Lorber, 1997; Vazquez-Boland *et al.*, 2001). *L. monocytogenes* is able to infect both phagocytic and nonphagocytic cells (Camilli *et al.*, 1993; Gaillard *et al.*, 1987; Tilney and Portnoy, 1989). Due to its intracellular growth behavior, *L. monocytogenes* triggers potent innate and adaptive immune responses in an infected host that results in the clearance of the organism (Paterson and Maciag, 2005). This unique ability to induce efficient immune responses using multiple simultaneous and integrated mechanisms of action has encouraged efforts to develop this bacterium as an antigen delivery vector to induce protective cellular immunity against cancer or infection. This review describes the multiple effector responses induced by this multifaceted organism, *L. monocytogenes*.

II. MOLECULAR DETERMINANTS OF *L. monocytogenes* VIRULENCE

To survive within the host and cause the severe pathologies associated with infection such as crossing the intestinal, blood-brain, and feto-placental barriers, *L. monocytogenes* activates a set of virulence genes. The virulence genes of *L. monocytogenes* have been identified mainly through biochemical and molecular genetic approaches. The majority of the genes that are responsible for the internalization and intracellular growth of *L. monocytogenes* such as *actA, hly, inlA, inlB, inlC, mpl, plcA,* and *plcB* are regulated by a pluripotential transcriptional activator, PrfA (Chakraborty *et al.*, 1992; Freitag *et al.*, 1993; Renzoni *et al.*, 1999; Scortti *et al.*, 2007). Thus, *prfA* defective *L. monocytogenes* are completely avirulent as they lack the ability to survive within the infected host's phagocytic cells such as dendritic cells (DC), macrophages, and neutrophils (Leimeister-Wachter *et al.*, 1990; Szalay *et al.*, 1994).

A. Virulence factors associated with *L. monocytogenes* invasion

A set of *L. monocytogenes* surface proteins known as invasins interact with the receptors present on host cell plasma membranes to subvert signaling cascades leading to bacterial internalization. The internalins (InlA and InlB) were the first surface proteins that were identified to promote host cell invasion (Braun *et al.*, 1998; Cossart and Lecuit, 1998; Lecuit *et al.*, 1997). InternalinA is a key invasion factor that interacts with the epithelial cadherin (E-cadherin), which is expressed on the surface of epithelial cells and thus promotes epithelial cell invasion and crossing of the gastro-intestinal barrier. The efficiency of the interaction between InlA with its receptor E-cadherin is variable in different mammalian hosts. For example, mice are resistant to intestinal infection with *L. monocytogenes* because of a single amino acid difference between mouse and human E-cadherin (Lecuit *et al.*, 1999). InlA is also suggested to be important for crossing the maternofetal barrier since E-cadherin is expressed by the basal and apical plasma membranes of synciotrophoblasts and villous cytotrophoblasts of the placenta (Lecuit *et al.*, 1997, 2001). However, the precise role of InlA in crossing the fetoplacental barrier remains to be demonstrated since, feto-placental transmission occurs in mice that lack the *inlA* receptor and also occurs in guinea pigs that are infected with an *inlA* deletion mutant *L. monocytogenes* (Lecuit *et al.*, 2001, 2004).

InternalinB promotes *L. monocytogenes* entry into a variety of mammalian cell types including epithelial cells, endothelial cells, hepatocytes, and fibroblasts. The hepatocyte growth factor receptor (Met/HGF-R) has been identified as the major ligand for InlB and is responsible for causing the

entry of *L. monocytogenes* into nonphagocytic cells (Bierne and Cossart, 2002). Met belongs to the family of receptor tryosine kinases, one of the most important families of transmembrane signaling receptors expressed by a variety of cells. The activation of Met by InlB is also species specific; indeed InlB fails to activate rabbit and guinea pig Met, but activates human and murine Met (Khelef *et al.*, 2006). *In vivo* virulence studies in mice have shown that InlB plays an important role in mediating the colonization of *L. monocytogenes* in the spleen and liver (Gaillard *et al.*, 1996). InlB is also considered important for crossing the fetoplacental barrier due to the observation that in the absence of InlB, InlA expressing *L. monocytogenes* invaded placental tissue inefficiently (Lecuit *et al.*, 2004). It has also been suggested that InlB is involved in crossing the blood-brain barrier as InlB is necessary for *in vitro* infection of human brain microvascular endothelial cells (Greiffenberg *et al.*, 1998).

Twenty four additional internalins are present in the *L. monocytogenes* genome and could potentially contribute to host cell invasion (Dramsi *et al.*, 1997). It is plausible that these internalins might cooperate with each other in order to facilitate entry into host cells, for example, InlA mediated entry is enhanced in the presence of InlB and InlC. However, additional studies are required to understand the contributions of each internalin and how these proteins participate in the bacterial entry to establish the successful infection of various cell types.

In addition to the internalins, several other proteins such as Ami, Auto, and Vip are also implicated in the ability of *L. monocytogenes* to enter host cells. In the absence of InlA and InlB, it has been shown that Ami digests the *L. monocytogenes* cell wall and mediates the adherence of a $\triangle$*inlAB* bacterial strain to mammalian cells (Milohanic *et al.*, 2001). Auto is another autolysin that regulates the bacterial surface architecture required for adherence (Cabanes *et al.*, 2004). Vip is a cell wall anchored protein that is involved in the invasion of various cell lines. The endoplasmic reticulum resident chaperone gp96 has been identified as a cellular ligand for this protein (Cabanes *et al.*, 2005). Thus, these *L. monocytogenes* cell surface proteins contribute to the ability of *L. monocytogenes* to infect multiple cell types.

B. *L. monocytogenes* survival in the macrophage

Upon infection of host cells such as macrophages and DC, a majority of the bacteria are killed in the phagolysosome of the host cell with less than 10% of the *L. monocytogenes* escaping into the host cell cytosol. This escape from the phagolysosome is mediated by the expression of Listeriolysin O (LLO), a pore forming hemolysin, which is the product of the *hly* gene and phospholipases (PlcA and PlcB) (Fig. 1.1). LLO is the first identified major virulence factor of *L. monocytogenes* and is a member of the cholesterol-dependent cytolysin family (CDC) (Portnoy *et al.*, 1992a,b; Tweten, 2005).

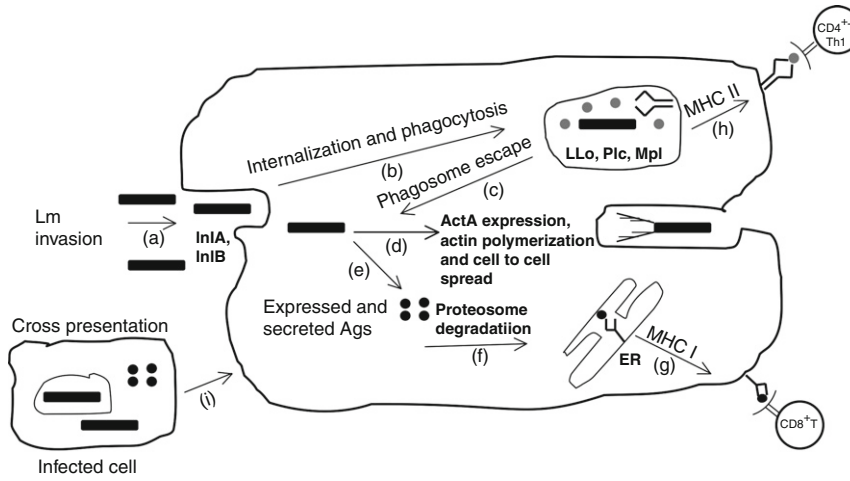

FIGURE 1.1 Intracellular growth of *L. monocytogenes* in an antigen-presenting cell and antigen presentation. Internalization of *L. monocytogenes* on the host cell is mediated by phagocytosis in macrophages but in other host cells such as epithelial and endothelial cells it requires invasins such as InlA and InlB (a). After cellular entry *L. monocytogenes* escape the phagolysosome by secreting Listeriolysin O (LLO), phospholipase (Plc), and metalloprotease (Mpl) resulting in the lysis of the vacuolar membrane, releasing the bacteria in the host cytosol (b and c). Cytosolic bacteria express protein ActA that polymerizes actin filaments and mediates cell to cell spread of *L. monocytogenes* (d). Cytosolic antigens produced after *L. monocytogenes* escape from phagosome are degraded by the proteosome to antigenic epitopes and presented by MHC class I molecules (e, f, and g). Bacterial antigens inside the phagosome are processed as exogenous antigens and epitopes are presented on the membrane surface in the context of MHC class II molecules (h). An alternate route for antigen presentation involves cross presentation with the antigens derived from an *L. monocytogenes* infected cell (i).

LLO binds to the host cell membrane initially as a monomer but then forms oligomers composed of up to 50 subunits, which are inserted into the membrane to form pores of diameter ranging 200–300Å (Walz, 2005). The function of LLO is very crucial for the cellular invasion of *L. monocytogenes* in both phagocytic and nonphagocytic cells.

After entry into the cytosol, another *L. monocytogenes* secreted protein called ActA enables bacterial propulsion in the cytosol leading to the invasion of neighboring uninfected cells by a process called cell to cell spreading (Alvarez-Dominguez *et al.*, 1997; Suarez *et al.*, 2001). In the cytoplasm, *L. monocytogenes* replicates and uses ActA to polymerize host cell actin to become motile enabling spread from cell to cell (Dussurget *et al.*, 2004; Fig. 1.1). As a result, the deletion of *actA* from *L. monocytogenes* results in a highly attenuated bacterium and thus establishes that ActA is a major virulence factor.

III. IMMUNE RESPONSE TO *L. monocytogenes* INFECTION

A. Innate immunity

Innate immunity plays an essential role in the clearance of *L. monocytogenes* and control of the infection at early stages. Mice deficient in T and B cell responses, such as *SCID* and *nude* mice, have normal early resistance to sublethal *L. monocytogenes* infection. However, SCID and nude mice eventually succumb to infection because complete clearance of *L. monocytogenes* requires T-cell mediated immunity (Pamer, 2004). Upon systemic inoculation of *L. monocytogenes*, circulating bacteria are removed from the blood stream primarily by splenic and hepatic macrophages (Aichele *et al.*, 2003). In the spleen, the bacteria localize within macrophages and DC of the marginal zone, between the white and red pulp (Conlan, 1996). Within the first day of infection, these cells containing live bacteria migrate to the T-cell zones in the white pulp, establishing a secondary focus of infection and attracting neutrophils. Interestingly, this process has been associated with lymphocytopenia in this compartment (Conlan, 1996), as T cells undergo apoptosis induced by the *L. monocytogenes* infection in an antigen-independent manner (Carrero and Unanue, 2007).

Both macrophages and neutrophils have essential roles in controlling *L. monocytogenes* infection at early time points. Recruitment of monocytes to the site of infection is an important characteristic of *L. monocytogenes* infection. In the liver, the Kupffer cells clear most of the circulating bacteria. As early as 3 h after systemic injection, *L. monocytogenes* can be found inside the Kupffer cells, followed by granulocyte and mononuclear cell infiltration and formation of foci of infection (Mandel and Cheers, 1980). Neutrophils are rapidly recruited to the site of infection by the cytokine IL-6 and other chemo-attractants, which secrete IL-8 (Arnold and Konig, 1998), CSF-1 and MCP-1. These chemokines are important in the inflammatory response and for attracting macrophages to the infection foci. In the following few days, granulocytes are gradually replaced by large mononuclear cells and within 2 weeks the lesions are completely resolved (Mandel and Cheers, 1980). Further studies have shown that mice depleted of granulocytes are unable to control *L. monocytogenes* infection (Conlan and North, 1994; Conlan *et al.*, 1993; Czuprynski *et al.*, 1994; Rogers and Unanue, 1993). In murine listeriosis, *L. monocytogenes* replicates inside hepatocytes, which are lysed by the granulocytes recruited to the infection foci, releasing the intracellular bacteria to be phagocytosed and killed by neutrophils (Conlan *et al.*, 1993). Although neutrophils are very important in fighting *L. monocytogenes* infection in the liver, depletion of neutrophils does not significantly change the infection course in the spleen (Conlan and North, 1994). Interestingly, mice

depleted of mast cells have significantly higher titers of *L. monocytogenes* in the spleen and liver and are considerably impaired in neutrophil mobilization (Gekara *et al.*, 2008). Although not directly infected by *L. monocytogenes*, mast cells can be activated by the bacteria and rapidly secrete TNF-α and induce neutrophil recruitment (Gekara *et al.*, 2008).

At the cell surface, toll like receptors (TLRs) play a role in the recognition of *L. monocytogenes*. TLRs are important components of innate immunity, recognizing conserved molecular structures on pathogens, and signaling through adaptor molecules, such as MyD88, to induce NF- κB activation and transcription of several proinflammatory genes. NF-κB is a heterodimeric transcription factor composed of p50 and p65 subunits and activates several genes involved in innate immune responses. Mice lacking the p50 subunit of NF-κB are highly susceptible to *L. monocytogenes* infections (Sha *et al.*, 1995).

In particular, TLR2 seems to play a role during *L. monocytogenes* infection because mice deficient in TLR2 are slightly more susceptible to listeriosis (Torres *et al.*, 2004). TLR2 recognizes bacterial peptidoglycan, lipoteichoic acid, and lipoproteins present in the cell wall of gram-positive bacteria, including *L. monocytogenes*. TLR5, which binds bacterial flagellin, however, is unlikely to be involved in *L. monocytogenes* recognition since flagellin expression is downregulated at 37 °C for most *L. monocytogenes* isolates. In addition, TLR5 is not required for innate immune activation against this bacterial infection (Way and Wilson, 2004).

The presence of unmethylated CpG dinucleotides in the bacterial DNA also has stimulatory effects on mammalian immune cells. CpG motifs present in bacterial DNA act as pathogen associated molecular patterns (PAMPs) (Hemmi *et al.*, 2000; Tsujimura *et al.*, 2004) interacting with TLR-9 to trigger an innate immune response in which lymphocytes, DC, and macrophages are stimulated to produce immunoprotective cytokines and chemokines (Ballas *et al.*, 1996; Haddad *et al.*, 1997; Hemmi *et al.*, 2000; Ishii *et al.*, 2002; Tsujimura *et al.*, 2004).

Although TLRs are important in bacterial recognition, a single TLR has not been shown to be essential in innate immune responses to *L. monocytogenes*. On the other hand, the adaptor molecule MyD88, which is used by signal transduction pathways of all TLRs, except TLR-3, is critical to host defense against *L. monocytogenes* and infection with *L. monocytogenes* is lethal in MyD88-deficient mice. Additionally, $MyD88^{-/-}$ mice are unable or severely impaired in the production of IL-12, IFN-γ, TNF-α, and nitric oxide (NO) following *L. monocytogenes* infection. MyD88 is not required for MCP-1 production and monocyte recruitment following *L. monocytogenes* infection but is essential for IL-12 and TNF-α production and monocyte activation (Serbina *et al.*, 2003). The NOD-LRR receptor interacting protein 2 (RIP2) kinase, identified as

immediately downstream of NOD-1, is also required for full signaling through TLR2, 3, and 4. Mice deficient in RIP2 are impaired in their ability to defend against *L. monocytogenes* infection and have decreased IFN-γ production by NK and T cells, which is partially attributed to a defective interleukin-12 signaling (Chin *et al.*, 2002). In addition, Portnoy and associates have recently shown that cytosolic Listerial peptidoglycans generated in the phagosome induce IFN-β in macrophages by a TLR-independent, NOD-1 dependent pathway (Leber *et al.*, 2008).

Overall, several components of the innate immune response participate in early defenses against infection with *L. monocytogenes*. Although there is a critical role of innate immunity in listeriosis, complete eradication of wild type *L. monocytogenes* requires antigen-specific T cell responses against this pathogen.

B. Cellular immune responses

Earlier studies using the mouse as a model of *L. monocytogenes* infection clearly demonstrated the cell mediated nature of the immune responses to the bacterium (Mackaness, 1962). Subsequently, it has been shown that *L. monocytogenes* elicits both class I and class II MHC responses that are essential for controlling infection and inducing long term protective immunity (Ladel *et al.*, 1994).

1. MHC class Ia and Ib restricted T cell responses to *L. monocytogenes*

L. monocytogenes specific $CD8^+$ T cell responses fall into two groups: One recognizes peptides generated by cytosolic degradation of secreted bacterial proteins (class Ia MHC); the other recognizes short hydrophobic peptides that contain *N*-formyl methionine at the amino terminus (class Ib MHC).

MHC-class Ia restricted peptide antigens derived from *L. monocytogenes* are generated from the degradation of secreted proteins (Finelli *et al.*, 1999). *In vitro* labeling studies have shown that *L. monocytogenes* secretes a limited number of proteins into the cytosol of the host cell (Villanueva *et al.*, 1994). Bacterially secreted proteins in the cytosol of macrophages are rapidly degraded by proteosomes. Some secreted proteins such as p60 and LLO are rapidly degraded because their amino termini contain destabilizing residues as defined by the N-end rule (Schnupf *et al.*, 2007; Sijts *et al.*, 1997). LLO is also degraded in a proteosome-dependent fashion as it contains a PEST-like sequence (Decatur and Portnoy, 2000). LLO and p60 are the most antigenic of the secreted proteins in terms of induction of a $CD8^+$ T cell response. On the other hand, ActA has enhanced stability in the cytosol as it contains

a stabilizing amino acid at the amino terminus (Moors *et al.*, 1999). The rapid proteosome mediated degradation of a potentially toxic protein such as LLO enhances host cell survival and generates peptide fragments that enter the MHC class I antigen processing pathway.

MHC class Ia restricted T cell responses to *L. monocytogenes* reach peak frequencies approximately 8 days after intravenous inoculation (Busch *et al.*, 1998). The magnitude of T cell responses that are generated for specific antigenic peptides is independent of the quantity or the duration of *in vivo* antigen presentation. This finding is supported by experiments in which mice were treated with antibiotics to curtail the duration of the infection (Badovinac *et al.*, 2002; Mercado *et al.*, 2000). Despite significant differences in the number of viable bacteria and inflammatory responses, the expansion and contraction of $CD8^+$ T cells is similar in mice treated with antibiotics 24 h after infection and in mice that are untreated, indicating that T cells are programmed during the first few days of infection (Wong and Pamer, 2001). This is consistent with *in vitro* studies of *L. monocytogenes* specific $CD8^+$ T cell proliferation, which showed that transient antigen presentation is followed by prolonged proliferation and do not require further exposure to antigen (Wong and Pamer, 2001). This suggests that innate immune responses that occur after the first 24 h of infection have a very small impact on the kinetics and magnitude of $CD8^+$ T cell responses. The reason for antigen independent proliferation of $CD8^+$ T cells remain unclear, although one hypothesis is that antigen independent T cell proliferation is driven by cytokines such as IL-2. However, studies by Wong *et al.* (Wong and Pamer, 2001) showed that endogenous IL-2 production by $CD8^+$ T cells is required for Ag-independent expansion following TCR stimulation *in vitro*, but not *in vivo*. Thus, there are other factors in addition to IL-2 that regulate antigen-independent proliferation of $CD8^+$ T cells *in vivo*.

The magnitude of *in vivo* $CD8^+$ T cell responses following *L. monocytogenes* infection is also influenced by the cytokines IFN-γ and perforin. *L. monocytogenes* infection of mice deficient in both IFN-γ and perforin results in an increased magnitude of *L. monocytogenes* specific $CD8^+$ T cell responses, and shifting of the immunodominance hierarchy (Badovinac and Harty, 2000). This suggests that neither perforin nor IFN-γ is absolutely necessary for the development of anti-*L. monocytogenes* immune responses.

L. monocytogenes infection of mice lacking MHC class Ia molecules induces $CD8^+$ T cell immunity equivalent to that seen in normal mice. These $CD8^+$ T cells are restricted by MHC class Ib. H2-M3 MHC class Ib molecules selectively bind peptides with *N*-formyl methionine at the N-terminus. H2-M3 restricted T cells are cytolytic and produce IFN-γ and TNF-α and can mediate protective immunity (Finelli *et al.*, 1999).

Transfer of H2-M3 restricted CTL into TAP (transporter for antigen presentation) deficient mice confers partial protection, indicating that TAP dependent and TAP independent antigen processing pathways are operative. Processing and presentation of *L. monocytogenes N*-formyl-methionine peptides by infected cells are poorly defined. In uninfected cells, most H2-M3 molecules remain in the ER because endogenous *N*-formyl-peptides are scarce. Some *L. monocytogenes* derived *N*-formyl-peptides are bound by gp96 prior to association with H2-M3. The number of *L. monocytogenes* specific H2-M3 T cells peak 5–6 days post infection (Finelli *et al.*, 1999). Contraction of H2-M3 restricted T cells results in the generation of a pool of memory cells, but they only have some of the characteristics of traditional memory cells. When rechallenged with a second *L. monocytogenes* infection, these cells upregulate surface expression of activation markers, but do not proliferate. This suppression of proliferation is mediated by the expansion of the MHC class Ia response, which limits available DC for antigen presentation. However, these cells do play a role in the control of primary infection since, H2-M3 knock out mice have a defect in bacterial clearance suggesting that early expansion and IFN-γ production by these cells cannot be compensated by other T cell subsets. Recently, it was demonstrated that MHC class Ib-restricted T cells also help in the enhancement of Ag-specific $CD4^+$ T cell responses (Chow *et al.*, 2006).

Infection of mice intraperitoneally with *L. monocytogenes* has been shown to cause a site-specific induction of γ/δ T cells in the peritoneal cavity (Skeen and Ziegler, 1993). However, no changes are observed in the splenic or lymph node T cell populations after these injections. Moreover, when peritoneal T cells from *L. monocytogenes*-immunized mice are restimulated *in vitro*, the induced γ/δ T cells exhibited a greater expansion potential than the α/β T cells. Significant increase in peritoneal $CD3^+$ cells expressing the γ/δ T cell receptor is observed for 8 days after *L. monocytogenes* injection and the population remains elevated for 6–7 weeks. Both, the induced γ/δ T cells or γ/δ T cells from the normal mice were not found to express $CD4^+$ or $CD8^+$ on the cell surface. The modifications that abrogate the virulence of *L. monocytogenes* such as heat killed *L. monocytogenes* or *hly* negative mutants, also results in elimination of the inductive effect for γ/δ T cells. The *in vivo* depletion of either α/β or γ/δ T cells using a monoclonal antibody in mice results in an impairment in resistance to primary infection with *L. monocytogenes*. However, the memory response is virtually unaffected by the depletion of γ/δ T cells, supporting the hypothesis that this T cell subset forms an important line of defense in innate, rather than adaptive immunity to *L. monocytogenes* (Skeen and Ziegler, 1993).

2. Class II MHC restricted T cells responses

In addition to $CD8^+$ T cell responses, infection with *L. monocytogenes* results in the generation of robust $CD4^+$ T cell responses. Expansion of $CD4^+$ T cells has been shown to be synchronous with the expansion of $CD8^+$ T cells (Skoberne *et al.*, 2002). During the course of infection, $CD4^+$ T cells produce large amounts of Th1 cytokines that are thought to contribute to clearance of *L. monocytogenes*. Immunization with *L. monocytogenes* results in the activation of $CD4^+$ T cells that coexpress dual cytokines such as IFN-γ and TNF-α on day 6 post infection and triple positive cells, TNF-α^+ IFN-γ^+ IL-2^+ on day 10–27 (Freeman and Ziegler, 2005), indicating the generation of memory $CD4^+$ T cell responses. Adoptive transfer studies using *L. monocytogenes* specific $CD4^+$ and $CD8^+$ T cells have shown that $CD4^+$ T cell-mediated protective immunity requires T-cell production of IFN-γ, whereas $CD8^+$ T cells mediate protection independently of IFN-γ (Harty and Bevan, 1995; Harty *et al.*, 1992). It is probable that production of IFN-γ from $CD4^+$ T cells activates macrophages to become more bactericidal, which is supported by *in vitro* studies showing that treatment of macrophages with IFN-γ prevents bacterial escape from the phagosome (Portnoy *et al.*, 1989).

3. Cell-mediated immune responses to heat-killed and irradiated *L. monocytogenes*

T cells primed with live *L. monocytogenes* undergo prolonged division, become cytolytic and produce IFN-γ. By contrast, infection with heat-killed *L. monocytogenes* does not induce a protective immune response. For years, one hypothesis to explain this finding was that killed bacteria do not enter the cytosol of macrophages following phagocytosis, thereby resulting in insufficient antigen presentation. Surprisingly, the immunization of mice with heat-killed *L. monocytogenes* results in the proliferation of antigen specific $CD8^+$ T cells, but does not induce full differentiation of the primed T cells into effector cells (Lauvau *et al.*, 2001). Therefore, T cells that are primed with heat-killed *L. monocytogenes* undergo attenuated division and do not acquire effector functions. In contrast, infection with live bacteria provides a stimulus that remains highly localized and induces T-cell differentiation. On the other hand, irradiated *L. monocytogenes* efficiently activates DC and induces protective T cell responses when used for vaccination (Datta *et al.*, 2006). Therefore, irradiated bacteria could serve as a better vaccine platform for recombinant antigens derived from other pathogens, allergens, and tumors when compared to heat-killed *L. monocytogenes*. However, infection with live *L. monocytogenes* provides the most potent stimulus that remains highly localized and induces T cell differentiation.

IV. RECOMBINANT L. monocytogenes AS A VACCINE VECTOR

L. monocytogenes has been used as a vaccine vector to generate cell mediated immunity against a wide range of viral or tumor antigens such as influenza nucleoprotein, LCMV nucleoprotein, HPV16 E7, HIV gag, SIV gag and env, tyrosinase-related protein (Trp2), high molecular weight melanoma associated antigen (HMW-MAA), ovalbumin, prostrate specific antigen (PSA), and HER-2/neu (Gunn *et al.*, 2001; Ikonomidis *et al.*, 1994; Shahabi *et al.*, 2008; Singh *et al.*, 2005).

A. Construction of recombinant *L. monocytogenes* strains

A variety of viral and tumor antigens such as HPV16E7, HER-2/neu, HMW-MAA, NP, and PSA that are expressed by *L. monocytogenes* as a fusion protein with LLO have been shown to generate antigen specific $CD4^+$ and $CD8^+$ T cell responses in mice. These antigens can be expressed in *L. monocytogenes* by an episomal or chromosomal system. Plasmid based strategies have the advantage of multicopy expression but rely on complementation for the maintenance of the plasmid *in vivo* (Gunn *et al.*, 2001). Chromosomal integration techniques involve either allelic exchange into a known chromosomal locus (Mata *et al.*, 2001) or a phage-based system, which utilizes a site-specific integrase to stably integrate plasmid into the genome (Lauer *et al.*, 2002). Most of the episomal expression systems are based on fusion of the antigen of interest to a nonhemolytic fragment of *hly* (truncated LLO) (Gunn *et al.*, 2001). The retention of plasmid by *L. monocytogenes in vivo* is achieved by the complementation of the *prfA* gene from the plasmid in a *prfA* mutant *L. monocytogenes* background (Gunn *et al.*, 2001). A *prfA* mutant *L. monocytogenes* (XFL7) cannot escape the phagolysosome and is destroyed by host cell macrophages and neutrophils. Thus, due to the lack of intracellular growth, a *prfA* mutant *L. monocytogenes* cannot deliver and present antigenic peptides to the immune cells. Including a copy of *prfA* in the plasmid ensures the *in vivo* retention of the plasmid in *L. monocytogenes* strain XFL7 (Pan *et al.*, 1995a, b). An alternate approach described by Verch *et al.* is based on the retention of a plasmid (pTV3) by complementation of D-alanine racemase in both *Escherichia coli* and *L. monocytogenes* strains that are deficient in D-alanine racemase and D-alanine amino transferase *in vitro* and *in vivo* (Verch *et al.*, 2004). The plasmid pTV3 is devoid of antibiotic resistance and therefore, this recombinant *L. monocytogenes* strain expressing a foreign antigen is more suitable for use in the clinic (Verch *et al.*, 2004).

B. LLO and ACTA as adjuvants in *L. monocytogenes* based immunotherapy

The genetic fusion of antigens to a nonhemolytic truncated form of LLO results in enhanced immunogenicity and *in vivo* efficacy (Gunn *et al.*, 2001; Singh *et al.*, 2005). The immunogenic nature of LLO has been attributed to the presence of PEST sequences close to the N-terminus of the protein that targets LLO for ubiquitin proteosome mediated degradation (Sewell *et al.*, 2004a). Removal of the PEST sequence from LLO used in the fusion constructs partially abrogates the ability of vaccine to induce full tumor regression in mice (Sewell *et al.*, 2004a). Recently, Schnupf *et al.* (2007) have shown that LLO is a substrate of the ubiquitin-dependent N-end rule pathway, which recognizes LLO through its N-terminal Lys residue. The N-end rule pathway is an ubiquitin-dependent proteolytic pathway that is present in all eukaryotes. Thus, the fusion of antigens to LLO may facilitate the secretion of an antigen (Gunn *et al.*, 2001; Ikonomidis *et al.*, 1994), increase antigen presentation (Sewell *et al.*, 2004a), and help to stimulate the maturation of DC (Peng *et al.*, 2004).

Fusion of LLO to tumor antigens in other immunotherapeutic approaches such as viral vectors (Lamikanra *et al.*, 2001) and DNA vaccines (Peng *et al.*, 2007) also enhances vaccine efficacy. Studies using DNA based vaccines have demonstrated that genetic fusion of antigens to LLO is essential for this adjuvant effect as there is a difference in the therapeutic efficacy of chimera or bicistronic vaccines (Peng *et al.*, 2007). However, high levels of specific $CD4^+$ T cell immune responses for the passenger antigen are obtained using bicistronic expression of LLO and antigen (Peng *et al.*, 2007). Recently, Neeson *et al.* (2008) have shown that LLO has adjuvant properties when used in the form of a recombinant protein. In this study, the chemical conjugation of LLO to lymphoma immunoglobulin idiotype induces a potent humoral and cell-mediated immune response and promoted epitope spreading after lymphoma challenge. Thus, LLO is a global enhancer of immune responses in various vaccination studies.

The reasons why LLO potentiates immune responses are only partially understood. LLO is a potent inducer of inflammatory cytokines such as IL-6, IL-8, IL-12, IL-18, and IFN-γ (D'Orazio *et al.*, 2006; Nomura *et al.*, 2002; Yamamoto *et al.*, 2006) that are important for innate and adaptive immune responses. Since, a related pore-forming toxin, anthrolysin, is reported to be a ligand of Toll-like receptor 4 (TLR4) (Park *et al.*, 2004), the proinflammatory cytokine-inducing property of LLO may be a consequence of the activation of the TLR4 signaling pathway (Park *et al.*, 2004). In addition to $CD8^+$ T cell responses, LLO also modulates $CD4^+$ T cell responses. LLO is capable of inhibiting a Th2 immune response by

shifting the differentiation of antigen-specific T cells to Th1 cells (Yamamoto *et al.*, 2005, 2006). Due to the high Th1 cytokine-inducing activity of LLO, protective immunity to *L. monocytogenes* is induced when mice are immunized with killed or avirulent *L. monocytogenes* together with LLO, whereas protection is not generated in mice immunized with killed or avirulent *L. monocytogenes* alone (Tanabe *et al.*, 1999). These results demonstrate that LLO potentiates a strong Th1 response, leading to highly effective cell mediated immunity.

In addition to LLO, the proline-rich listerial virulence factor ActA also contains PEST-like sequences. To test whether ActA could also act as an adjuvant, an *L. monocytogenes* strain was constructed that secreted a fusion protein of the first 390 residues of ActA, which contains four PEST sequences, fused to HPV-16 E7 (Sewell *et al.*, 2004b). This strain enhanced immunogenicity and *in vivo* efficacy, similar to LLO, and was effective at eliminating established E7 expressing tumors in wild type mice (Sewell *et al.*, 2004b) and mice transgenic for E7 (Souders *et al.*, 2007).

V. THE PLEIOTROPIC EFFECTS OF *L. monocytogenes* ON THE TUMOR MICROENVIRONMENT

A. Protective and therapeutic tumor immunity

A number of tumor antigens associated with various types of cancer have shown promise as a target for immunotherapy using *L. monocytogenes* based vaccine strategies. For example, preclinical studies using a recombinant *L. monocytogenes* strain expressing HPV16 E7 has demonstrated both prophylactic and therapeutic efficacy against E7 expressing tumors (Gunn *et al.*, 2001). In addition, *L. monocytogenes* vaccine strains expressing fragments of HER-2/neu are able to induce anti-Her2/neu CTL responses in mice with prolonged stasis in tumor growth (Singh *et al.*, 2005). Very recently, Advaxis has described a recombinant *L. monocytogenes* expressing PSA, *L. monocytogenes*-LLO-PSA that induced the regression of more than 80% of tumors expressing PSA (Shahabi *et al.*, 2008). HMW-MAA, also known as melanoma chondrotin sulfate proteoglycan, is overexpressed on over 90% of the surgically removed benign nevi and melanoma lesions, basal cell carcinoma tumors of neural crest origin and some forms of childhood leukemia and lobular breast carcinoma lesions (Chang *et al.*, 2004). In addition, HMW-MAA is expressed at high levels on both activated pericytes and pericytes involved in tumor angiogenic vasculature (Campoli *et al.*, 2004; Chang *et al.*, 2004). Maciag *et al.* (2008) have shown that recombinant *L. monocytogenes* expressing LLO-HMW-MAA used to target pericytes present within the tumor vasculature has potent antiangiogenic effects in the tumors that express HMW-MAA. The

recombinant *L. monocytogenes* expressing HMW-MAA not only destroyed the cells that support tumor formation such as pericytes but also impacted on the frequency of tumor-infiltrating lymphocytes. *L. monocytogenes* based vaccines have also been studied in melanoma models using TRP-2 as the target antigen (Bruhn *et al.*, 2005). Tumor protection induced by *L. monocytogenes*-TRP2 was long lasting and therapeutic, conferring tumor protection against both tumor subcutaneous tumors and metastatic tumor nodules in the lungs (Bruhn *et al.*, 2005).

The detailed analyses of the T cell responses generated by recombinant *L. monocytogenes* suggest that both $CD4^+$ and $CD8^+$ T cells are important for the regression of established tumors and protection against subsequent challenge in some models (Fig. 1.2). In addition to generating CTLs against the tumor specific antigens, immunization with recombinant *L. monocytogenes* can also impact the growth of tumors that do not contain vaccine epitopes, presumably by means of epitope spreading (Liau *et al.*, 2002). Epitope spreading refers to the development of an immune response to epitopes distinct and non cross-reactive with the disease-causing epitope (Fig. 1.2). This phenomenon is thought to occur following the release of antigens from the tumor cells killed by vaccine induced T cells. These antigens are then phagocytosed by APCs and presented to naïve T cells of different specificities. Epitope spreading correlates with tumor regression in patients undergoing immunotherapy and could therefore, potentially be harnessed to broaden the immune responses to unidentified tumor antigens in the context of therapeutic vaccines (Liau *et al.*, 2002).

B. *L. monocytogenes* promotes a favorable intratumoral milieu

For immunotherapies to be effective vaccination must result in robust generation of a high number of cytolytic T cells followed by their significant infiltration into the tumor microenvironment. Thus, the major challenge in developing a cancer vaccine is not only to generate the right T cells but also to create conditions for them to migrate, infiltrate, and eliminate tumor cells.

Studies from the Paterson lab have suggested that *L. monocytogenes* vaccines are effective agents for tumor immunotherapy because they result in the accumulation of activated $CD8^+$ T cells within tumors (Hussain and Paterson, 2005). While the reasons for this accumulation of $CD8^+$ cells in tumors is not known, Hussain *et al.* have speculated that it may be due to the ability of the vaccine to induce a specific chemokine profile in the $CD8^+$ cells (Hussain and Paterson, 2005). Specifically, studies have shown that the PEST region of LLO is required for the high numbers of $CD8^+$, antigen-specific TILs, which are in turn critical for vaccine efficacy (Sewell *et al.*, 2004a).

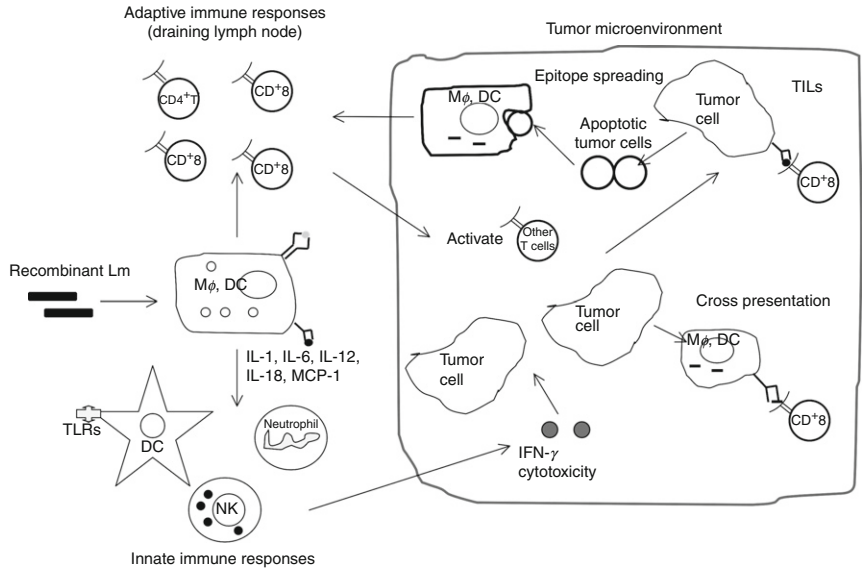

FIGURE 1.2 Multiple effects of *L. monocytogenes* based immunotherapy on the tumor microenvironment. Recombinant *L. monocytogenes*, which is expressing and secreting a target antigen, will be taken up by an antigen presenting cell (APC) such as a macrophage or dendritic cell. This will result in the activation of innate immune responses resulting in the production of various cytokines such as IL-1, IL-6, IL-12, IL-18, and chemokines such as MCP-1 that will attract other immune cells such as dendritic cells, neutrophils, and NK cells to the site of infection. The amplification of immune responses and secretion of these inflammatory cytokines will influence the tumor microenvironment directly or indirectly resulting in the lysis of tumor cells. Additionally, proteins secreted by recombinant *L. monocytogenes* will gain entry into both class I and class II MHC pathways for $CD8^+$ and $CD4^+$ T cell responses. The $CD8^+$ T cells specific for the tumor antigen will lyse the tumor cells presenting the antigen due to their cytotoxic activity. Additionally, recombinant *L. monocytogenes* has the ability to elicit an immune response to epitopes distinct and non cross-reactive with, the disease-causing epitope, referred to as epitope spreading. In this process, the antigens released from the dying tumor cell are taken up by an APC. The mature APC will present those tumor cell antigens to naive $CD8^+$ T cells in the draining lymph node with the activation and expansion of T cells to tumor antigens not shared by the *L. monocytogenes* vaccine. These $CD8^+$ T cells may infiltrate into the tumors and this cycle may continue. Also, there will be cross presentation of the antigens derived from a dying tumor cell to other $CD8^+$ T cells.

Due to its unique life cycle, *L. monocytogenes* also triggers a potent $CD4^+$ T cell response in addition to the cell mediated $CD8^+$ T cell response. Accordingly, tumor specific $CD4^+$ helper cells are produced and migrate to the tumor, similar to CTLs (Beck-Engeser *et al.*, 2001; Pan *et al.*, 1995a) following *L. monocytogenes* vaccination (Fig. 1.2). The fact that

$CD4^+$ cells can lyse antigen/MHC-II expressing tumor cells (Echchakir *et al.*, 2000; Neeson *et al.*, 2008; Ozaki *et al.*, 1987; Yoshimura *et al.*, 1993) is of little consequence since most tumors only express MHC class I molecules. Therefore, the ability of $CD4^+$ T helper cells to promote rejection of MHC-II negative tumors likely occurs via the production of paracrine factors or cytokines (Beck-Engeser *et al.*, 2001; Greenberg, 1991). In fact, the $CD4^+$ T cell response to *L. monocytogenes* infection has been shown to be primarily of the Th1 type with production of the antitumoral cytokines IFN-γ, TNFα, and IL-2.

In addition to targeting exogenous antigens, *L. monocytogenes* vaccines have also been shown to break tolerance in a transgenic mouse model for E6/E7 (Sewell *et al.*, in press; Souders *et al.*, 2007) and HER-2/neu (Singh and Paterson, 2007b). *L. monocytogenes*-based constructs expressing E7 such as *L. monocytogenes*-LLO-E7 and *L. monocytogenes*-ActA-E7 are able to impact the growth of autochthonous tumors that arise in E6/E7 transgenic mice (Sewell *et al.*, in press; Souders *et al.*, 2007). However, the tumor-regression and CTL responses observed following vaccination in transgenic mice was weaker than that observed in the wild type mice. Similarly, in HER-2/neu transgenic mice, all of the *L. monocytogenes* vaccines are capable of slowing or halting the tumor growth despite the fact that $CD8^+$ T cells from the transgenic HER-2/neu mice are of lower avidity than those that arise from the wild-type mice. *L. monocytogenes*-based HER-2/neu constructs also delayed the appearance of spontaneous tumors in the transgenic HER-2/neu mice (Singh and Paterson, 2007b). Interestingly, the tumors that emerged had developed mutations within the CTL epitopes of the HER-2/neu protein. These mutations resided in the exact regions that were targeted by the *L. monocytogenes*-based vaccines suggesting that the rate of generation of escape mutants is a significant factor in the efficacy of these vaccines (Singh and Paterson, 2007a). Based on these findings, it appears that *L. monocytogenes* can overcome tolerance to self antigens and expand autoreactive T cells by activating cells that are usually too low in number and avidity, leading to antitumor responses.

As well as adaptive T cell immunity, multiple cytokines released during innate immune phases play a role in the ability of *L. monocytogenes* to function as an effective immunotherapeutic agent. IFN-γ, for example, plays an especially important role in effective *L. monocytogenes* antitumor responses. Although the majority of IFN-γ is produced by NK cells, $CD4^+$ T-helper cells may also contribute to the IFN-γ levels (Beatty and Paterson, 2001). Using a tumor that is insensitive to IFN-γ (TC1mugR), Dominiecki *et al.* (2005) have shown that *L. monocytogenes* vaccines require IFN-γ for effective tumor regression. Interestingly, the authors demonstrate that IFN-γ is specifically required for tumor infiltration of lymphocytes but not for trafficking to the tumor

(Dominiecki *et al.*, 2005). Additionally, IFN-γ can inhibit angiogenesis at the tumor site in the early effector phase following vaccination (Beatty and Paterson, 2001).

C. Effect of *L. monocytogenes* vaccination on regulatory T cells in the tumors

The accumulation of T regulatory cells (Tregs) represents a formidable challenge to traditional cancer immuno-therapeutics. Frequently, the tumors have evolved to exploit the suppressive properties of these regulatory cells in order to promote their growth and persistence within the host. Furthermore, vaccine strategies may be hampered by their inability to prevent Treg accumulation within tumors. *L. monocytogenes* based vaccines; however, seem to function by decreasing the population of Tregs in the tumors.

L. monocytogenes based vaccines, which express antigen-LLO fusion proteins, have been shown to uniquely prevent large infiltrates of Tregs within tumors. For example, immunization with recombinant *L. monocytogenes*-LLO-E7 fusion protein resulted in fewer Tregs ($CD4^+$, $CD25^+$ cells) in the tumors when compared to recombinant *L. monocytogenes*-E7 that secretes the antigen, but not the LLO-antigen fusion protein (Hussain and Paterson, 2004). Interestingly, immunization with a nonspecific recombinant *L. monocytogenes* expressing LLO-irrelevant antigen vaccine also results in the reduction of Tregs within tumors (Shahabi *et al.*, 2008). The reduction in Tregs, however, was further enhanced when the vaccine was antigen specific suggesting that the mechanism is at least partially antigen-dependent for a maximal effect (Nitcheu-Tefit *et al.*, 2007; Shahabi *et al.*, 2008). Interesting, there is no effect on the population of Tregs in spleens, implying that *L. monocytogenes* selectively reduces Tregs within the tumors. This is an important observation since other therapies (including antibody-mediated depletion of Tregs) that targets Tregs are associated with extensive side effects in humans. *L. monocytogenes*-LLO based vaccines thus may seem superior to other vaccine strategies due, at least in part, to their ability to inhibit Tregs accumulation only within the tumors. Coexpression of LLO with other antigens in different bacterial vectors also enhances the efficacy of the vaccines through the inhibition of Tregs (Nitcheu-Tefit *et al.*, 2007). The combination of *L. monocytogenes* ability to induce MHC I, MHC II pathways coupled with the fact that LLO is a very potent immunogenic molecule likely may have important implications for antitumor vaccination strategies in humans.

D. Implication of the immune response to *L. monocytogenes* infection: *L. monocytogenes* within the tumor

Adaptive immune cells clearly play an important role in modulating the microenvironment of tumors following *L. monocytogenes* vaccination. However, in addition to $CD4^+$ and $CD8^+$ T cells, a number of other regulatory factors can be found within tumors of *L. monocytogenes* vaccinated mice. Most strikingly, *L. monocytogenes* itself can be found within the tumor for up to 7 days while being cleared from the spleen and the liver after just 3 days (Huang *et al.*, 2007; Paterson *et al.*, unpublished data). The persistence of *L. monocytogenes* within tumors suggests that immune responses to the infection itself at the site of the tumor, independent of antigen-specific effects, may play a role in the potent antitumoral effect of these vaccines. For example, macrophages activated by *L. monocytogenes* may home to the tumor and secrete a variety of tumoricidal cytokines including IL-6, IL-12, IL-1, and TNFα. In addition, infected macrophages would serve as a source of LLO which in turn induces a Th1-type cytokine profile with secretion of the proinflammatory cytokines IL-12, IL-18, IFNγ as well as IL-1, IL-6, and TNFα. Interestingly, in *L. monocytogenes* based vaccines, partial depletion of macrophages has no effect on the tumor recall response after vaccination (Weiskirch *et al.*, 2001). In addition to macrophages, mast cells are activated by *L. monocytogenes* and are required to clear the bacteria from the spleen and the liver. Once activated, mast cells secrete TNF-α and induce neutrophil recruitment. Neutrophils are known to play an essential role in controlling *L. monocytogenes* infection at early time points. Once activated, neutrophils secrete IL-8, CSF-1, and MCP-1. These cytokines in turn attract and activate additional macrophages and propagate the antitumoral effects of these cells. It is conceivable that all of these cells could be recruited to tumors and aid in *L. monocytogenes* vaccine efficacy.

VI. CONCLUSIONS AND FUTURE PROSPECTS

Several aspects of *L. monocytogenes* make it a uniquely attractive vaccine vector candidate as compared to other live vectors such as vaccinia virus, *Salmonella*, *Shigella*, *Legionella*, *Lactococcus*, and *Mycobacterium* (BCG), since *L. monocytogenes* can be grown under standard BSL2 laboratory conditions and genetic manipulation of this organism is well-established allowing construction of recombinant vaccine strains. In addition, a single recombinant *L. monocytogenes* strain can be manipulated to express multiple gene products using plasmid or chromosomal systems. There is extensive knowledge about the life cycle, genetics, and immunological characteristics of *L. monocytogenes*. This provides a rationale for the design of potent,

specific and safe vaccine platforms. Results with the various attenuated strains have been very promising and therefore, safety issues are being well addressed.

The ability of *L. monocytogenes* to generate strong innate and adaptive immune responses in the periphery and tumor microenvironment has been exploited for the design of suitable vaccines. Combination of *L. monocytogenes* with other vaccine strategies such as protein, DNA, or peptide coated DC in a heterologous prime-boost strategy could also significantly improve the immune responses for therapeutic studies. Much work remains to be done to identify the combination regimens necessary to obtain optimal responses. In addition, vaccination strategies exploiting epitope spreading may enhance the efficacy of antitumor immune responses.

Preclinical studies to evaluate the efficacy of *L. monocytogenes* based vaccines have demonstrated potent and protective immune responses in mouse models. These aspects provide the foundation for testing these vaccines in clinical trials in humans. Recently, Advaxis Inc., a New Jersey based biotechnology company performed a phase I clinical trial using its *L. monocytogenes* based construct expressing the tumor antigen, HPV16-E7 (Lovaxin C) in end stage cervical cancer patients. Lovaxin C was shown to be well tolerated in most patients who received an IV dose and displayed a dose dependent pattern of side effects. A phase II study to evaluate the efficacy of Lovaxin C in the US is currently being discussed with the Food and Drug Administration.

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