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Lipoteichoic acid promotes macrophage expansion

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ABSTRACT

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Skin is colonized with various microorganisms such as bacteria, and wound healing processes are important to protect skin from the harsh environment. When an injury occurs, multiple biological pathways are needed to activate wound healing processes. During wound healing process, macrophages play important roles in the innate immunity and tissue regeneration, which has been suggested to have beneficial role in wound healing. It has been known that Gram-positive bacteria are the most common microorganism in hospitalized patients with skin infection. Gram-positive bacteria express lipoteichoic acid (LTA) which is involved in the regulation of various immune responses. Despite the importance of LTA, its immunological functions in wound healing process have been poorly studied. In this study, the effect of LTA on wound healing process through the induction of macrophage proliferation was investigated.

To measure macrophage proliferation, CFSE-labeled macrophage cell line RAW 264.7, THP-1, BV-2, bone marrow-derived macrophage (BMDM) from

wild-type and Toll-like receptor 2 (TLR2)-deficient C57BL/6 mice were stimulated with LTA, Pam2CSK4, or *Escherichia coli* lipopolysaccharide (Ec.LPS) and the macrophage proliferation was determined by flow cytometry. To measure cell cycle-related proteins, RAW 264.7 cells were stimulated with LTA, Pam2CSK4 or Ec.LPS and protein expression levels were determined by Western blotting. To examine cell cycle progression, RAW 264.7 cells were stimulated with LTA, Pam2CSK4 or Ec.LPS and DNA contents were measured by flow cytometry. To examine c-Myc expression, RAW 264.7 cells were stimulated with LTA, Pam2CSK4, or Ec.LPS and protein level was measured by Western blotting. To examine peritoneal macrophage proliferation, mice were intraperitoneally administrated with PBS or LTA. Then, peritoneal cells were collected from mouse peritoneal cavity and the peritoneal macrophage proliferation was measured by flow cytometry. In order to measure the effect of LTA on wound healing process, skin wounds were created in the mouse dorsal skin under sterile conditions with 8-mm biopsy punch. After creating the wounds, PBS or LTA was treated and each of the wound areas was measured by using Image J. Changes of the wound areas were expressed as a percentage of the initial wound areas. To examine macrophage expansion in wound area, single cells were separated from wound area and various cell population including macrophage, neutrophil and T cells were measured by flow cytometry. To determine wound healing markers including MIP-1α, CXCL2, MMP-2, MMP-9 and IL-6, pro-inflammatory mediators including iNOS and IL-1β, and anti-inflammatory mediators including IL-4, IL-10, and IL-13, inflammatory macrophage-related genes such as CD86 and TNF-α, tissue macrophage-associated genes such as CD206, Ym-1, and FIZZ-1, the wounds were treated with PBS or LTA and the mRNA expression level was measured by real-time PCR analysis.

LTA-increased proliferation of various macrophages, including RAW 264.7, THP-1, BV-2, and BMDMs. LTAs from other Gram-positive bacteria including

Lactobacillus plantarum and *Streptococcus pneumoniae* also elicited similar effect on the macrophage proliferation. LTA up-regulated the expression of cell cycle-related proteins including CDK2, CDK6, and cyclin D1/D3 and increased cell cycle progression by up-regulating G2/M phases. However, LTA failed to induce the proliferation of BMDM from TLR2-deficient mouse. Moreover, LTA up-regulated c-Myc expression and the inhibitor specific to c-Myc decreased proliferation of the LTA-induced macrophage and cell cycle-related genes. The LTA-induced proliferation and phosphorylation of c-Myc were suppressed in the presence of inhibitors including ERK, JNK, or PI3K. LTA induced phosphorylation of GSK-3β which decreased c-Myc stability, and this effect was decreased by inhibition of PI3K. Remarkably, Pam2CSK4 or Ec.LPS up-regulated IFN-β mRNA expression but not in those stimulated with LTA. Furthermore, IFN-β down-regulated the LTA-induced macrophage proliferation in a dose-dependent manner and LTA-increased c-Myc level was also decreased by IFN-β stimulation in a dose-dependent manner. Moreover, inhibitory effect of IFN-β on the LTA-induced macrophage proliferation and c-Myc expression was recovered by type I IFN receptor-blocking antibody. To clarify LTA-induced macrophage proliferation *in vivo*, mice were intraperitoneally administered with PBS or LTA for 2 days. LTA-administrated mice exhibited increased $F4/80^{high}Ly6c^{low}$, $F4/80^{+}BRdU^{+}$ and $F4/80^{+}/Ki67^{+}$ peritoneal macrophages, representing local tissue macrophages proliferation. In addition, improved wound healing process were observed in mice treated with LTA. Furthermore, wound healing-related genes such as MMP-2, MMP-9 and IL-6 expression were increased by LTA, but macrophage recruitment factor, MIP-1α, and neutrophil recruitment factor, CXCL2, were decreased. Interestingly, when the wound was treated with LTA, pro-inflammatory factors such as iNOS, and IL-1β, were decreased in a time-dependent manner. However, anti-inflammatory factors such as IL-10, IL-13, and IL-4 were increased in the presence of LTA in the wound area. Interestingly, in the presence of LTA in the wound area, only macrophage expansion has shown. Furthermore,

inflammatory macrophage-related genes such as CD86 and TNF-α were decreased in LTA-treated wound compared with PBS-treated wound, but the tissue macrophage-associated genes such as CD206, Ym-1, and FIZZ-1 were increased, which indicated that LTA increases tissue macrophage expansion.

The present study demonstrates that LTA improves wound healing process through up-regulating the proliferation of macrophages by promoting cell cycle progression via enhancing c-Myc stability and TLR2-signaling pathway. These results provide an insight into the role of LTA in wound healing, which could be used in therapeutics to promote wound healing process after injury.

Keywords: Gram-positive bacteria, Lipoteichoic acid, Macrophage proliferation, Tissue macrophage, Toll-like receptor 2, Wound healing

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Abbreviations

LPS	Lipopolysaccharide
LTA	lipoteichoic acid
CDK	cyclin-dependent kinases
BMDM	bone marrow-derived macrophage
Th	T helper cell
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor-1
TGF-β	transforming growth factor-beta
NO	nitric oxcide
TNF-α	tumor necrosis factor alpha
IL	Interleukin
MIP-1α	macrophage inflammatory protein-1 alpha
PGN	Peptidoglycan
PAMP	pathogen associated molecular patterns
TLR	Toll-like receptor
TIR	Toll/IL-1 receptor
MyD88	myeloid-differentiation antigen 88
MAPK	mitogen-activated protein kinases
ERK1/2	extracellular signal-regulated kinase 1/2
JNK	c-Jun N-terminal kinases
PI3K	phosphoinositide 3-kinase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
CD	cluster of differentiation
MBP	mannose binding protein
MCP-1	monocyte chemoattractant protein-1
MD-2	differentiation factor 2

MIP-1α	macrophage inflammatory protein-1 alpha
MyD88	myeloid differentiation primary response gene 88
PAFR	platelet activating factor receptor
PI	propidium iodide
CHX	Cycloheximide
WT	wild type
PMA	phorbol myristate acetate
GSK-3β	glycogen synthase kinase 3 beta

Chapter I. Introduction

1.1. Bacterial cell wall component

1.1.1. Cell wall Structure of Gram-positive and Gram-negative bacteria

Gram-positive bacterial cell walls are mainly composed of peptidoglycan (PGN) which consist of a linear sugar chain of alternating *N*-acetylmuramic acid and *N*-acetylglucosamine, linked by peptide bridges to form an arrangement with large molecular structure and surround the cytoplasmic membrane [1]. In addition, they comprise of other important components such as glycolipid lipoteichoic acid anchored in the cytoplasmic membrane and lipoprotein which is embedded in the bacterial cell wall [2]. In case of Gram-negative bacteria, they contain a thinner layer of PGN than Gram-positive bacteria adjacent to the cytoplasmic membrane. Furthermore, their inner and outer membrane consist of lipopolysaccharide (LPS), phospholipids, and proteins. LPS is also termed endotoxin, which is composed of an O-linked polysaccharide bound to the lipid A moiety through the major polysaccharide [3]. LPS and lipid A are prominent features of Gram-negative bacteria, which show most potent of inducing inflammatory responses [4]. Lipid A consists of a mono- or biphosphorylated disaccharide backbone and acetylated with fatty acids [5]. Moreover, different bacteria express different structures of LPS or lipid A with many phosphorylation, fatty acid and acyl chains compositions [5].

1.1.2. Immune responses against bacteria

Innate immune system is important for host defense during bacterial infection and plays an essential role in the recognition and induction of pro-inflammatory responses against bacteria. On the other hand, adaptive immune response is responsible for removal of bacteria in the late phase of infection and arise of immunological memory response [6]. The innate immune responses are based on physical or chemical barriers against infection, as well as different immune cells recognizing pathogens and inducing antimicrobial immune responses. The innate immune response is initiated with recognition of a particular structure of pathogens which is evolutionarily conserved. Pathogen-associated molecular patterns (PAMPs) are molecules which are associated with groups of pathogens, recognized by innate immune cells via pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) [7].

Various immune cells such as antigen-presenting dendritic cells (DCs), phagocytic macrophages, granulocytes and cytotoxic natural killer (NK) cells have an important role in innate immune responses to recognize microbes in early infection. Furthermore, they have the ability to activate complement, phagocytosis, and autophagy by activation of PRRs [8-10]. Upon PAMP recognition, PRRs are expressed in the cell surface and induce pro-inflammatory factors such as IL-6, IL-8, TNF-α and NO through intracellular signaling cascade [9, 11, 12]. When inflammation is occurred by bacteria, it leads to an increased local blood flow and the cells migrate into the tissue. The

main cell types in initial phases of an inflammatory response are neutrophils, which are recruited into the infected tissue. They also have surface receptors for the recognition of bacteria and complement to engulf and destroy the invading bacteria [13]. After the neutrophil influx, recruited monocytes rapidly differentiate into macrophages known as inflammatory cells and which are further involved in lymphocyte activation [14]. Macrophages have phagocytosed effect on bacteria and become activated to stimulate T lymphocytes. Furthermore, macrophages are specialized in presenting antigen to T lymphocytes to initiate an adaptive immune response [15].

In previous reports, TLR2 is a major receptor of Gram-positive bacteria which plays an important role in detection of Gram-positive bacteria via recognition of bacterial PAMP, including lipoprotein, PGN and lipoteichoic acid (LTA) [16]. Previous reports evidenced that TLR2 is essential for inducing immune responses against Gram-positive bacteria. For example, challenge with *Streptococcus pneumoniae* or *Staphylococcus aureus* in TLR2-deficient mice, which susceptibility of those bacteria is increased compared to wild-type mice [17]. Furthermore, bacterial CpG DNA is one of the important PAMP of Gram-positive bacteria and is recognized via TLR9 [18]. To achieve a successful inflammatory response, Gram-positive bacteria also need to be recognized by nucleotide-binding oligomerization domain-containing protein (NOD)2 and the NOD-like receptor family, pyrin domain-containing (NALP)1 in inflammasome and muramyl-dipeptide (MDP) [19]. In case of Gram-negative

bacteria, LPS binds to extracellular of TLR4 with co-receptors, CD14 and MD2, followed by TLR4 signaling cascade [20]. In addition, flagellin is also a part of some Gram-negative bacteria which strongly induce immune cell activation via TLR5 [21]. Finally, PGN from Gram-negative bacteria are recognized by NOD1 and NOD2 [22]. (Figure 1)

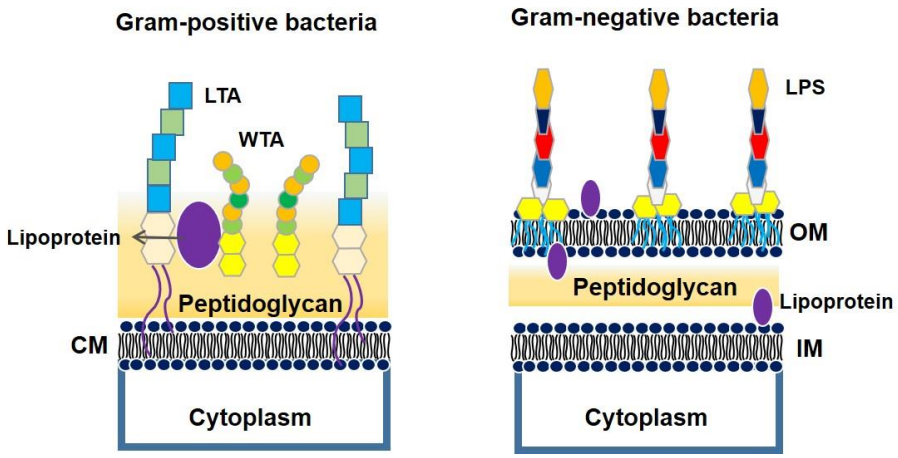

Figure 1. Bacterial cell wall structure. Gram-positive bacterial cell wall has a thicker PGN than Gram-negative bacterial PGN with a single cytoplasmic membrane. Gram-positive bacteria express teichoic acids (TA) as major polysaccharides. TA are covalently anchored to PGN and LTA are anchored to cytoplasmic membrane (CM). In contrast, Gram-negative bacterial cell wall has a thin PGN with an inner (IM) and outer membrane. They majorly express LPS in the outer membrane (OM).

1.2. Lipoteichoic acid (LTA)

1.2.1. Structure of LTA

LTA is an alditol phosphate-containing polymer attached to cytoplasmic membranes via glycolipid anchors [23]. In general, LTA can be classified into five groups depending on the chemical structures of the repetitive backbone. Type I LTA is usually comprised of disaccharide-containing glycolipids linked to repeating polyglycerophosphate units [poly (Gro-P)] and it is expressed in most of Gram-positive bacteria such as *S. aureus, Bacillus subtilis, Enterococcus faecalis,* and *Lactobacillus plantarum* [24]. In case of *S. aureus* and *B. subtilis*, it is connected to the C-6 of the non-reducing glycosyl and within the glycolipid anchor gentiobiosyldiacylglycerol [24]. Type IV LTA contains pseudopentasaccharide 2-acetamino-4-amino-2,4,6-trideoxygalactose, glucose, and ribitolphosphate and is trailed by two *N*-acetylgalactosamine moieties. Furthermore, type II, III, and V LTAs have similar structures, but the repeating units of the backbone structure are slightly different from each other. For example, type II LTA contains a repeating α-Gal(1-6)-α-Gal(1-3)-Gro-P unit of the backbone, whereas type III LTA contains α-Gal(1-6)-Gro-P repeating unit of the backbone. Lastly, type V LTA contains α-$_{D}$-*N*-acetylglucosamine (GlcNAc)-α-$_{D}$-GlcNac repeating units [23] (Figure 2).

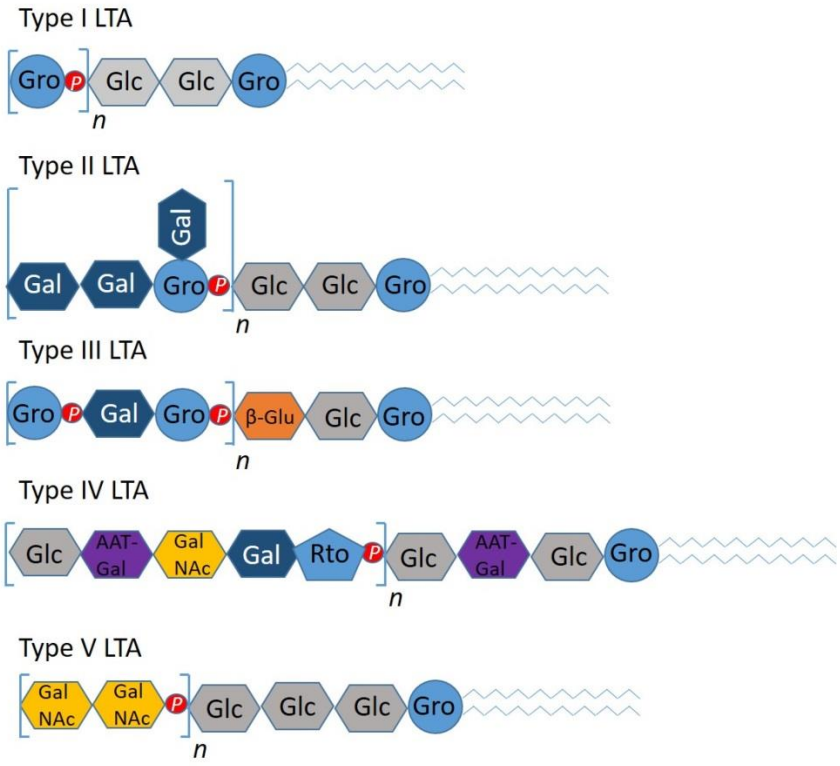

Figure 2. Schematic LTA structure from various Gram-positive bacteria (modified from Kang, SS. *et al.* [23]). Type I LTA structure as found in *S. aureus* and *B. subtilis.* Type II LTA structure as found in *Lactococcus garvieae*. Type III LTA structure as found in *Clostridium innocuum*. *S. pneumoniae* and *C. difficile* contain Type IV and Type V LTA structure respectively. LTAs are linked to glycolipids. "*n*" is marked as repeating unit of each LTA. Gro; glycerol, Glc; glucose, Gal; galactose, β-glu; β-glucosamine, AAT-Gal; 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose, GalNAC; N-acetylgalactosamine, Rto; ribitol, GlcNAc; *N*-acetylglucosamine, *P*; phosphate

1.2.2. Physiological functions of LTA

LTA has various important functions in bacterial physiology. For example, LTA plays an essential role in controlling bacterial division, growth, binding and protection against environmental stress [25, 26]. In previous reports, when the bacterial LTA is deficient, bacterial growth, cell division, septum formation, and PGN level were decreased in various Gram-positive bacteria such as *S. aureus, B. subtilis, B. anthracis*, and *Listeria monocytogenes* [27-29]. Indeed, previous reports suggest that LTA has a key role in bacterial autolysins, and that enzymes require LTA for cleaving PGN during cell division. For example, when *B. subtilis* is lack of LTA, it becomes incapable of assembling at the division site. In addition, LTA has an important role in protecting bacteria from environmental stress such as osmotic pressure. Therefore, LTA is essential in the Gram-positive bacterial physiology. Moreover, LTA has the ability to modulate bacterial susceptibility against antimicrobial peptides or enzymes. Previous studies suggest that D-alanine residues have the ability to decrease vancomycin and autolytic enzymes susceptibility in *S. aureus* [30]. Targeting the polyglycerophosphate moiety of LTA with monoclonal antibodies shows increased mortality in a murine *S. aureus* peritonitis model.

1.2.3. The role of LTA in immune responses

LTA is considered as the most important virulence factor owing to its involvement in the induction of inflammatory responses. In previous reports,

LTA is necessary for the development of diseases such as sepsis [31]. Classically, LTAhas been reported to trigger a cell signaling cascade through TLR2-signaling pathway [20]. In response to LTA, Toll-IL-1 resistance (TIR) domain-containing adapter protein (TIRAP) and intracellular adapter proteins-myeloid differentiation antigen 88 (MyD88) are needed to the TLR2 receptor signaling pathway that is necessary to activate NF-κB and cytokine transcription. Furthermore, LTA is recognized by various co-receptors such as CD14, mannose-binding protein (MBP), L-ficolin, and CD36 and further facilitates LTA-mediated immune responses [32].

According to previous reports, LTA has an important role in the pathogenesis of pneumonia, meningitis, and endocarditis, for being a stimulator of the respiratory burst and inducer of nitric oxide (NO), cytokines, and chemokine including IL-6, IL-8, or TNF-α [11, 33]. LTA from *S. aureus* induces the production of inflammatory mediators including IL-1β, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-1α [34-36]. Furthermore, LTA and peptidoglycan synergistically induce inflammatory responses [37]. In addition, other Gram-positive bacterial LTAs are also associated with inflammatory responses. *S. pneumoniae* LTA induces NO production via TLR2 through platelet-activating factor receptor (PAFR) [38]. *E. faecalis* LTA also induces NO production by stimulating TLR2 in macrophage [39]. However, recent studies demonstrate that LTA also has the ability to inhibit immune function. For example, *L. plantarum* LTA inhibits

Pam2CSK4-induced IL-8 in intestinal epithelial cells [40]. *S. aureus* LTA has the ability to decrease IL-8 expression which is induced by LPS [41]. *L. plantarum* LTA also inhibits LPS-induced TNF-α production in THP-1 cells through down-regulation of MAPK and NF-κB activation [42]. Similarly, *E. faecalis* LTA has the ability to down-regulate LPS-induced IL-8 production through IRAK-M-dependent pathway [43].

1.3. Skin

1.3.1. Skin and microbial infection

Skin is essential for the first line of defense as a physical barrier against microbial infection [44]. Because skin is always colonized with various microorganisms, it has the ability to secret sebaceous fluid and fatty acids to inhibit growth of pathogens and the deterring of colonization by pathogenic microorganisms [45]. When pathogenic microorganisms penetrate into skin integumentary barrier, the infecting organisms cause inflammatory response, resulting in tissue damage [46]. In North America, skin and soft tissue infections occur in approximately 7-10% of hospitalized patients [47]. Furthermore, skin infection could be the cause of death in severely ill patients, yet treatment of chronic and complex wounds is still a difficult task which put a burden on the health care system [46]. Among the various microorganisms that colonize skin, bacteria are the most typical ones. They increase the number when the integumentary barrier is disrupted due to, for example, bite wounds, scratches, burns and surgery, and initiate to colonize, resulting in folliculitis, furuncles, or carbuncles in the epidermis. They initially colonize different layers of the skin architecture such as epidermis, dermis, subcutaneous and muscle fascia [45]. Meanwhile, infection can be divided into two types. The first type of infection has the ability to replicate rapidly, and this includes bacteria, virus, fungi, and protozoa. In this state, T helper 1 (Th1) cells induce

antimicrobial type 1 immune response to eliminate various pathogen to produce pro-inflammatory responses [48]. However, when these pro-inflammatory responses are out of control, it could have a harmful effect to host such as tissue damage [49]. In the second type of infection such as helminth and other metazoan parasites or when type 1 immune response is too self-damaging or ineffective, distinct type 2 immune response dominates, which has a close association with many aspects of wound healing [50]. In addition, Th1 immunity is characterized by interferon-γ (IFN-γ) production evolved to regulate innate immune responses, while Th2 cells produced cytokines such as IL-4, IL-10, and IL-13 to promote alternative macrophage activation, eosinophil maturation and recruitment [48, 51, 52]. According to previous reports, it is suggested that type 2 immune response evolved to direct the wound healing process not only by repairing and reconstructing tissues but also by destroying and expulsion of invaded microorganisms [50]. For example, in a wound, insulin-like growth factor-1 (IGF-1) and transforming growth factor-β (TGF-β) are stimulators for the host defense [53]. Both IGF-1 and TGF-β play important roles in wound healing by activating epidermal cells and fibroblasts to mediate angiogenesis, chemoattractant macrophages and fibroblasts [54, 55].

1.3.2. Skin and host immune response

The skin immune system promotes innate and adaptive immune responses to protect host against foreign antigens. For example, keratinocytes produce a large number of chemokines and cytokines by various stimuli including kinetic

and thermal trauma and neuropeptides to activate innate immune cells in the skin, such as dendritic cells and macrophages [56, 57]. These immune cells express various inducible mediators to recruit additional immune cells from the blood. In addition, both the epithelial cells and resident innate immune cells express PRRs to recognize specific pathogen components, which is important in triggering downstream activation cascades [58]. TLRs are transmembrane glycoproteins which contain an ectodomain of leucine-rich motifs. This signaling cascade to activate NF-κB is a key factor that express immune responses such as cytokines and chemokines [59].

In addition, there are various cell types in human skin which also express TLRs. In the epidermis, keratinocyte, fibroblast and various resident immune cells including monocyte/macrophage and T and B lymphocytes have been shown to express functional TLRs [58]. TLR expression is important in identifying subsets of various cells in skin with specific functions. For example, macrophage activation through TLRs results in expression of pro-inflammatory cytokines and antimicrobial peptides, enhance bacterial killing, and improve wound healing process [60-62]. Keratinocytes activate TLR2 by bacterial component, PGN, and LTA results in NF-κB activation and IL-8 production. Furthermore, keratinocyte growth factor, TGF-α, which expressed in wound healing process, is up-regulated by TLR5 and TLR9 activation [63]. Langerhans cells express TLR2 and TLR3 to produce IL-6, IL-8 and TNF-α [64]. Taken together, there are significant TLR expression and function of

various cells in skin which appear to play an important role in immune responses against microorganisms.

1.4. Wound healing

1.4.1. Wound healing process

When an injury occurs, various activation in wound area must be coordinated to restore tissue integrity and homeostasis [65]. In addition, the blood coagulation cascade and the inflammatory pathways also need to be activated [66]. In this moment, many types of immune cells such as neutrophils, monocytes, macrophages, and lymphocytes, endothelial cells, keratinocytes and fibroblasts are involved in wound healing [67]. These various cells undergo marked changes in phenotype and gene expression which lead to cell migration, differentiation and proliferation. In skin tissue, the wound healing process can be sub-divided into three overlapping but distinct stages [65].

The first stage of wound healing -inflammation- occurs immediately after an injury. To remove dead and devitalized tissues and to prevent infection, inflammatory pathways and immune system are needed. Hemostasis is completed by the platelet plug formation, followed by a fibrin matrix to infiltrate the cells [68]. Neutrophils are then recruited to the wound area for activation of complement, the degranulation of platelets and bacteria clearance [69]. The second stage of wound healing -proliferation occurs 2-10 days after the injury. The first event of this second stage is the migration of keratinocytes to the inner layer of the skin [65]. Then the new blood vessels are formed and fibroblasts and macrophages replace the fibrin matrix with granulation tissue

[70]. Later, some of the fibroblasts differentiate into myofibroblasts to help bridge the gap in the wound [71]. Finally, keratinocytes restore the barrier function of the epithelium [72]. The third stage of wound healing -remodeling – begins two weeks after injury. For dermal regeneration, most of endothelial cells, macrophages, and myofibroblasts go through apoptosis or exit from the wound [65]. The remaining tissue is mostly composed of extracellular matrix proteins and a few remaining cells such as epidermal cells, endothelial cells, fibroblasts and macrophages produce metalloproteinase to remodel a cellular matrix from type III collagen backbone (Figure 3).

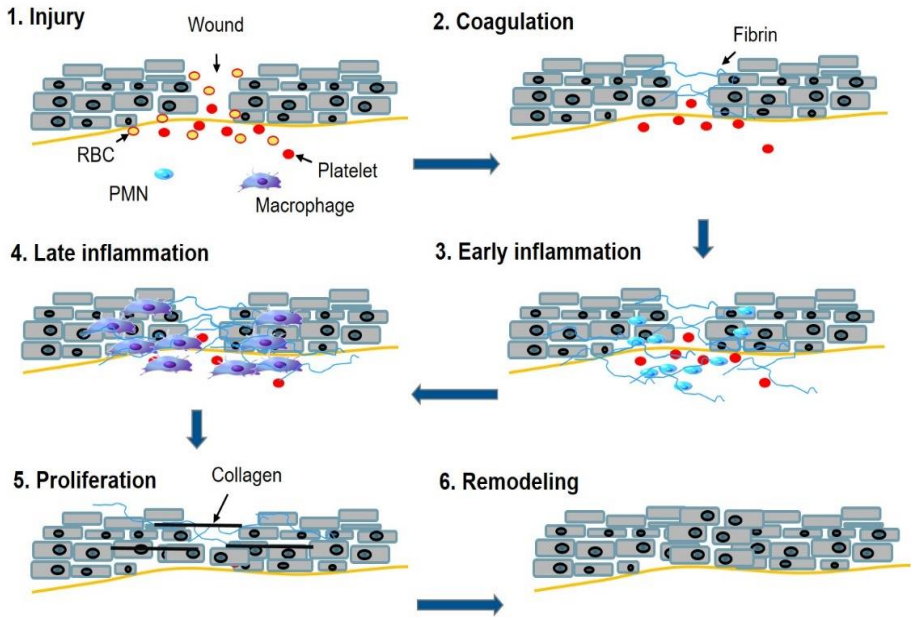

Figure 3. Wound healing process. Schematic representation of the different phases of the wound healing process. Upon initial injury, wound healing is initiated within a few minutes. After stop of bleeding with platelets, the inflammation stage is triggered. As a result of the coagulation stage, fibrin is matured into granulation tissue. During the inflammatory stage, neutrophils and macrophages enter the wound area, facilitating debridement of injured ECM. When proliferation phase is initiated after the inflammation stage, fibroblast migrates into the wound becoming myofibroblasts. The myofibroblasts secrete collagen which contributes to epithelization, resulting in wound healing.

1.4.2. Wound healing and inflammation

During wound healing process, inflammation is an important part of the regular wound healing process. Inflammation has essential role in the clearance of contaminating microorganisms, by various immune cells such as macrophages, neutrophils, pro-inflammatory cytokines, NO and other mediators [65, 73]. Early actions in the innate immune response, neutrophils are recruited to induce antimicrobial peptides including cathelicidin andLL-37 in humans [74]. Neutrophils are recruited by CXC chemokines that are produced by macrophages, keratinocytes and fibroblasts. These immune responses are need to kill bacteria and to prevent the formation of biofilms, however, prolonged inflammation may cause a chronic state of wound. In addition, prolonged inflammation leads to increased proteases induction that down-regulate ECM and growth factors. Furthermore, abnormal inflammation hampers the wound entering the proliferation phases to assist wound healing [75]. For example, TNF-α is a pleiotropic cytokine produced by various cells such as macrophages, T cells and keratinocytes. TNF-α can act as beneficial or harmful mode in wound healing process [76]. It has an ability to induce cytotoxicity against bacteria and fibroblast proliferation, however, an increased level of TNF-α leads to decrease induction of granulation tissue, TGF-β production and collagen disposition. High level of TNF-α is leading to extreme inflammation resulting in impaired wound healing process [76-78]. Therefore, wound healing process needs to switch from inflammation to repair. One of the major event is

a change in the phenotype of macrophages from an inflammatory phenotype, such as M1 macrophages secreting IL-1, TNF-α, and other cytokines, to anti-inflammatory phenotype, such as M2 macrophages producing TGF-β, IL-10 and MMPs. When the M1 macrophages are involved in inflammation during skin injury, M2 macrophages induce anti-inflammatory to promoting wound closure [79].

1.5. Macrophage

1.5.1. Macrophage

Macrophages are versatile phagocytic cells which originate from hematopoietic stem cells in the bone marrow [80]. Macrophages are particularly flexible phagocytic-monocytic cells involved in various functions in disease and health. They are important component of innate immunity and are mainly involved in host defense and immunity against invading pathogens including bacteria, virus, fungi and parasites [81-83] and also have an essential role in tissue remodeling, tissue repair, and maintenance of homeostasis [79, 84]. In general, macrophages can increase the number of cells in infection site by recruiting monocytes from bone marrow, which then differentiate into macrophages. However, recent studies demonstrated that in case of an acute infection or where leukocyte traffic is restricted, macrophages locally proliferate rather than recruiting monocytes and play an important role in defending against pathogen infection [85]. Indeed, under the growth factors effect, macrophages could proliferate. However, in the presence of microbial factors, cytokines or inflammatory factors block macrophage proliferation and initiate to induce pro-inflammatory cytokines. During inflammation, activated macrophages not only remove invaded pathogens via phagocytosis but also presenting the antigens through major histocompatibility complex (MHC) molecules to lymphocytes resulting in lymphocyte activation, and finally, macrophages die through apoptosis when

the stimulation is absence.

1.5.2. Macrophage location and diverse functions

Macrophages are located in various organs where they perform particular functions (Table 1). Their functions differ depending on where they reside in. For example, macrophages present in the spinal cord and brain, known as microglia, support active immune defense in the central nervous system [86]. Alveolar macrophages also have the ability to clear the infectious, toxin, or allergic particles to protect barriers of the respiratory tract [87]. Likewise, peritoneal macrophages which present in the peritoneum and Kupffer cells in liver act as a confront infectious agents [88]. Furthermore, macrophages in the central nervous system (CNS) are derived from the yolk-sac, independent of blood monocytes [89].

Macrophages can be further categorized into subpopulations by their functional phenotype and location. When macrophages are activated by microbial antigens or cytokines such as IFN-γ, they are classified as M1-type macrophages or 'Classically activated macrophages' [36]. Such macrophage activation relies on IFN-γ to prime, and additional stimulation which is either TNF-α, or TLR ligand signaling through MyD88 and NF-κB activation. In addition, M1 macrophages express a high level of MHC, which is important to eliminate intracellular pathogens, and induce NO and pro-inflammatory cytokines such as IL-6, IL-12 and IL-1β. On the other hand, M2-type

macrophages or ‘Alternatively activated macrophages’ promote anti-inflammatory responses and modulate immunoregulatory properties [36]. Furthermore, previous report suggested that IFN-γ down-regulate mannose receptor expression, but IL-4 which increases endocytosis and down-regulates induction of pro-inflammatory cytokines, lead to the alternative activation state of macrophages. IL-4 treated macrophages are known to be better able to phagocytose and kill protozoan including *Trypanasoma cruzi*, and increase Th2 type immune responses to eliminate invading helminth including *Leishmania Mexicana*. Moreover, this type of macrophages initiates tissue repair by releasing anti-inflammatory cytokines such as IL-10 to promote wound healing (Figure 4).

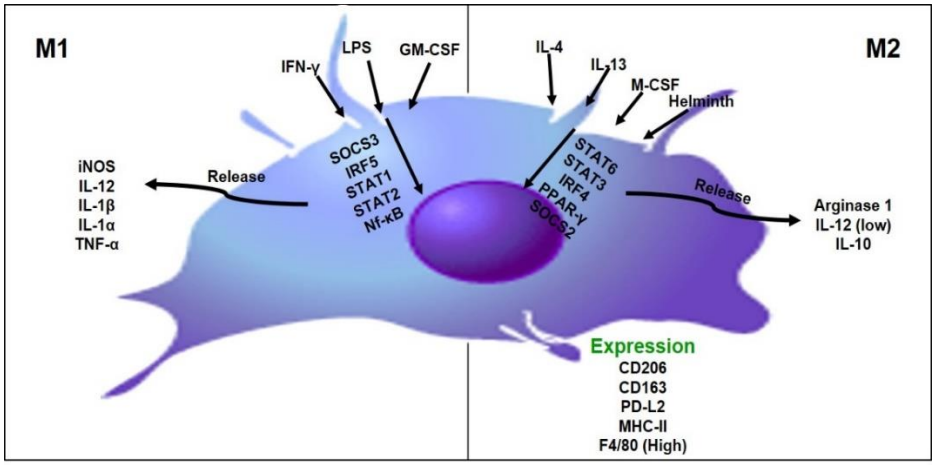

Figure 4. M1/M2 macrophage polarization and immunological functions. In the presence of LPS, IFN-γ, or granulocyte macrophage colony-stimulating factor (GM-CSF), macrophages polarize into M1 phenotype-expressing macrophage and produce pro-inflammatory mediators such as iNOS, IL-12, IL-1β, IL-1α, and TNF-α. In contrast, when macrophages are stimulated with IL-4, IL-13, macrophage colony-stimulating factor (M-CSF), or helminth, they induce an alternative activation of M2 phenotype and produce anti-inflammatory cytokine such as IL-10.

Table 1. Macrophage location and their functions.

Name	Location	Functions	References
Macrophage	Eye	Vascular remodeling	[90]
Alveolar macrophage	Lung	Immune surveillance	[91]
Kupffer cells	Liver	Clearance of debris from blood	[92]
Macrophage (pancreas)	Pancreas	Islet development	[93]
Crypt macrophage	Intestine	Immune surveillance	[94]
Macrophage (ovary)	Ovary	Steroid hormone production and ovulation	[95]
Microglia	Brain	Neuronal survival, repair	[96]
Langerhans cells	Epidermis	Immune surveillance	[44]
Macrophage (kidney)	Kidney	Ductal development	[97]
Bone marrow-derived macrophage	Bone marrow	Erythropoiesis; stem cell niche	[98]
Osteoclast	Bone	Bone remodeling	[99]

1.5.3. Macrophage proliferation

M-CSF is the major growth factor which regulates differentiation, proliferation, and survival of macrophages and their precursors, and also has synergistic effect with other cytokines such as IL-3 or GM-CSF to facilitate differentiation or proliferation of macrophages [100]. In addition, macrophages are able to proliferate in various tissues in inflammatory state. For example, after helminth infection or IL-4 treatment, a high number of alternative activation macrophages in tissues appear to be expanded by local proliferation [85, 101]. Other report suggested that IL-4 complex injection through peritoneal cavity led to an intense macrophage proliferation [102]. Moreover, macrophage proliferation and their numbers are decreased in IL-4-/- and IL-4Rα-/- mice in various infection model, underlining the major role of IL-4 in driving macrophage proliferation during infection. Remarkably, macrophage proliferation was not restricted to the cavities but was found in spleen, lung and liver [103]. Moreover, transplant $CD45.2^{+}Msr1^{-/-}$ and $CD45.1^{+}$ chimera bone marrow cells into irradiated $Ldlr^{-/-}$ mice revealed a role of scavenger receptor A in macrophage proliferation [104]. Oxidized low-density lipoprotein have the ability to induce macrophage proliferation through scavenger receptor A [105]. Furthermore, during wound healing process, macrophages play an important role. Previous reports suggested that collagen secretion was increased by ATP-vesicle-induced macrophage proliferation in a wound area, resulting in an increased wound healing process [106]. Also, M-CSF accelerates skin wound

healing by macrophage proliferation [107]. Nevertheless, various stimuli or growth factors which drive macrophage proliferation, the principal mechanisms for proliferation of macrophages by bacterial cell wall components are not yet recognized.

1.5.4. Macrophage proliferation through TLR signaling pathway

TLR signaling pathway has been shown to regulate macrophage proliferation and survival during host infection or wound healing. In order to activate transcription factors, mitogen-activated protein kinases (MAPK) signaling acts as a major regulator in TLR signaling pathway. There are four groups of particularly controlled MAPKs in mammalians: extracellular signal-regulated kinase 1/2 (ERK1/2), p38 proteins, c-Jun N-terminal kinases (JNK), and ERK5 [108]. In previous reports, MAPKs are shown to have the ability to regulate various inflammatory responses as well as cell proliferation. For example, M-CSF induces macrophage proliferation through RAF-1/ERK-1/2 signaling pathway [109]. When the ERK signaling pathway is inhibited, c-Myc de-phosphorylation is rapidly induced. Subsequently, proliferation is decreased [110].

Furthermore, phosphoinositide 3-kinase (PI3K) is another direct interaction molecule, such as MyD88. Previous report suggested that major transcriptional factor NF-κB activation is decreased when the PI3K signaling pathway is inhibited during TLR2 activation [111]. Furthermore, PI3K signaling pathway

has the ability to regulate cyclin D1, β-catenin, and c-Myc transcription factors. During macrophage proliferation, activation of MAPKs is critical [112]. The activation of MAPKs is further responsible for phosphorylating many transcription factors including fos, Jun, and c-Myc [113, 114]. Among these transcription factors, c-Myc is the most important factor for cell proliferation as it has the ability to control cell cycle, growth and apoptosis [115]. The c-Myc dimerizes with Max to bind E-box element of target gene promoter and causes histone acetyltransferase complexes recruitment for target gene transcription [116]. Although c-Myc has a short-life span, ERK and PI3K/GSK-3β kinase signaling cascade results in Ser62 phosphorylation of c-Myc, which in turn increases the c-Myc stabilization [117]. In addition, c-Myc induces cyclin A, cyclin D, and CDK2 expression and represses the expression of p21 proteins and these proteins regulate each step of the cell cycles and control the cell growth [118, 119].

1.5.5. Macrophage and wound healing

Macrophages are mononuclear phagocytes which are recruited from the blood and bone marrow in inflammatory conditions or tissue repair statement [120]. In particular, macrophages have an important role in homeostasis and tissue re-modelling as well as immune regulation [67, 121, 122]. In previous reports, macrophages are found to be critical for wound healing, as they produce several elements to stimulate fibroplasia and angiogenesis [123]. In addition, depletion of macrophages delayed wound healing process in the guinea pig model [124].

When the macrophages were depleted in mouse, wound closure was delayed, formation of granulation tissue was decreased, and vascular endothelial growth factor level was decreased [125].

In case of inflammation, macrophages are recruited to the wound area and polarize into alternative activation phenotypes by cytokines, oxidants, lipid and growth factors which are released by the same macrophages [122]. These cells have the ability to remove neutrophil from the wound and they take on anti-inflammatory phenotype. Furthermore, macrophage also produce cytokines such as IL-6, IL-1 and TNF-α or growth factors which influence keratinocyte migration, proliferation, and re-epithelialisation [126, 127]. Macrophages express anti-inflammatory mediators such as IL-1 receptor antagonist, IL-10, and TGF-β [128]. This phenomenon seems to be a requisite process to facilitate a switch from inflammation to proliferation in the wound healing progress. These macrophages can then modulate their responses to a healing phase in the wound area. During the wound healing process, the molecule produced by macrophages that has the most important influence in wound healing is TGF-β, which is produced by several cell types during wound healing process. Nevertheless, macrophages are the main source of production. TGF-β induces chemotactic for fibroblasts, differentiates into myofibroblast and produce collagen [102].

1.6. Aim of the present study

The colonization of various microorganisms on skin, bacteria in particular, suggest the importance of wound healing process in the prevention of the wound infection against bacteria through restoring an intact epidermal barrier. During wound healing process, macrophages play important roles in the innate immunity and improve the wound healing process. Therefore, it is important to identify the molecular targets controlling the wound healing process and understand the mechanisms of macrophage expansion in the wound area. LTA is one of the most important Gram-positive bacterial cell wall components which has the ability to control immune responses, yet their immunological role and their function in wound healing process are not well known. The aim of the present study is to investigate the role of LTA in wound healing process, particularly its impact on macrophage expansion.

Chapter II. Materials and methods

2.1. Materials & Methods

2.1.1. Mice

Approval of animal ethics for all experiments was obtained from the Institutional Animal Care and Use Committee of Seoul National University (SNU-100526-4). C57BL/6 mice were purchased from Orient Bio (Seoul, Korea). TLR2-deficient C57BL/6 mice were kindly provided by Dr. Shizuo Akira (Osaka University, Osaka, Japan). Mice were contained free conditions of pathogens with food and water *ad libitum* under a 12-hour day/night cycle.

2.1.2. Reagents and chemicals

Highly-pure and structurally-intact LTA from *S. aureus* (Sa.LTA), *L.plantarum* (Lp.LTA), and *S. pneumoniae* (Sp.LTA) were prepared as previously described [129]. Pam2CSK4 was purchased from EMC Microcollections (Tuebingen, Germany). Ultra-pure *E. coli* LPS (O111:B4) (Ec.LPS) was purchased from Invivogen (San Diego, CA, USA). Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) was purchased from Molecular Probes (Carlsbad, CA, USA). Cell Cycle Regulation Antibody Sampler Kit was purchased from Cell Signaling (Beverly, MA, USA). Antibody specific to c-Myc was purchased from Upstate Biotechnology (Lake Placid, NY, USA). Antibodies specific to phospho (Ser62)-c-Myc, and phospho (Ser62/Thr58)-c-Myc were purchased

from Abcam (San Francisco, CA, USA). Inhibitors of mitogen-activated protein (MAP) kinases including PD98059, SP600159, and SB203580 were purchased from Calbiochem (Darmstadt, Germany). Antibody specific to IFN-α/β receptor 1 was purchased from BD Biosciences (Franklin, NJ, USA). PerCP-conjugated anti-mouse F4/80 antibody, APC-conjugated anti-mouse Ly6C antibody and FITC-conjugated anti-mouse 5-Bromo-2`-deoxyuridine (BrdU) antibody were purchased from eBioscience (San Diego, CA, USA), Recombinant IFN-β is purchased from Peprotech (Rocky Hill, NJ, USA). Protein G agarose was purchased from Milipore (Bedford, MA, USA). All other reagents including propidium iodide (PI), cycloheximide (CHX), PI3K inhibitor LY294002, and BrdU were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.1.3. Cell line culture

The macrophage cell line RAW 264.7, BV-2, and THP-1 were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37℃ in a 5% CO_2 humidified incubator.

2.1.4. Preparation of bone marrow-derived macrophages (BMDM)

To prepare BMDM, 7 to 12-week-old C57BL/6 mice were used. After bone

marrow cells were obtained from tibiae and femurs, the cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a presence of 20 ng/ml of GM-CSF for 6 days to differentiate into M1 macrophage. In case of M2 macrophage differentiation, the cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin in a presence of 20 ng/ml of M-CSF for 6 days. Then, the adherent cells were used as BMDMs.

2.1.5. Proliferation assay

RAW 264.7, BV-2, phorbol myristate acetate (PMA)-differentiated THP-1 cells, and BMDMs were labeled with CFSE (2 μM) at 37℃ for 15 min. The CFSE-labeled cells were washed with PBS and suspended with complete DMEM medium. Then, the cells (2×10^5 cells/ml) were plated on 6-well culture plates at 37℃ overnight, after which they were stimulated with Sa.LTA (1 or 10 μg/ml), Lp.LTA (1 or 10 μg/ml), Sp.LTA (1 or 10 μg/ml), Pam2CSK4 (1 μg/ml) or Ec.LPS (1 μg/ml) for 48 h. Proliferation of the cells were determined by flow cytometry.

2.1.6. Cell cycle analysis

RAW 264.7 cells (2×10^5 cells/ml) were plated on 24-well plates in 1 ml culture medium at 37℃ overnight. The cells were stimulated with LTA (10 μg/ml), Pam2CSK4 (1 μg/ml) or Ec.LPS (1 μg/ml) at 37℃ for 48 h. Then, the cells

were fixed with 70% ethanol and stained with PI solution (40 μg/ml) for 20 min. The cell cycle of the cells was determined by flow cytometry.

2.1.7. Western blotting

RAW 264.7 cells (2×10^5 cells/ml) were plated on 6-well plates at 37℃ overnight. Then, the cells were treated with indicated stimuli for 6, 12, or 24 h. Next, the cells were washed with phosphate-buffered saline (PBS) and were lysed using cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% SDS, and 1% sodium deoxycholate). The cell lysates were separated by 10% SDS–PAGE and electro-transferred to a PVDF membrane using a transfer system (Bio-Rad, CA, USA). The membrane was blocked with 5% skim milk in tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) at room temperature for 1 h and incubated with each of the antibodies at 4℃ overnight. The membrane was washed with TBST and was incubated with HRP-conjugated antibody in the blocking buffer at room temperature for 1 h. After washing, the immuno-reactive bands were detected with ECL reagents (Amersham Biosciences, Princeton NJ, USA) using the LAS-1000 system (Fujifilm, Tokyo, Japan).

2.1.8. Immunoprecipitation

RAW 264.7 cells were stimulated with 10 μg/ml LTA for 24 h and treated with MG132 (20μM) for 3 h before harvest. After the cells were lysed, the lysates were immunoprecipitated with antibody specific to ubiquitin at 4℃ overnight.

The immune complexes were collected with Protein G-agarose and were washed three times with distilled water. The bound proteins were eluted with SDS-sample buffer by boiling at 95℃ for 5 min and subjected to Western blotting.

2.1.9. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW 264.7 cells (2×10^5 cells/ml) were plated on 6-well culture plates at 37℃ overnight. The cells were stimulated with LTA (10 μg/ml), Pam2CSK4 (1 μg/ml) or Ec.LPS (1 μg/ml) at 37ºC for 6 h. The mRNA expression of IFN-β and β-actin were determined by RT-PCR as described previously [130]. An equal amount of RT-PCR products was loaded on a 1.5% agarose gel and was visualized by ethidium bromide staining with a gel documentation system (Bio-Capt X-press, Vilber-Lourmat, Marne La Vallee, France).

2.1.10. Peritoneal macrophage proliferation

Mice were intraperitoneally injected with PBS (100 μl), LTA (50 μg/100 μl), Pam2CSK4 (5 μg/100 μl) or Ec.LPS (5 μg/100 μl) for 48 h. After injection, peritoneal cells were collected from mouse peritoneal cavity and the cells were stained with PerCP-conjugated anti-F4/80 and APC-conjugated anti-Ly6c. Then $F4/80^{high}$ $Ly6c^{-}$ cells were measured by flow cytometry. For BrdU labelling of proliferation cells *in vivo*, the mice were intraperitoneally injected with PBS (100 μl), LTA (50 μg/100 μl), Pam2CSK4 (5 μg/100 μl) or Ec.LPS (5 μg/100 μl) for 48 h and before 12 h experimental end-point, 100 μl of 10

mg/mL BrdU in PBS was injected. The peritoneal cells were collected from mouse peritoneal cavity. The cells were stained with PerCP-conjugated anti-mouse F4/80 and antibody FITC-conjugated anti-mouse BrdU antibody. Then $F4/80^{+}BrdU^{+}$ cells were measured by flow cytometry.

2.1.11. Mouse skin wound healing assay

Seven- to twelve-week-old female mice were used for experiments. After intraperitoneal administration of avertin for mouse anesthesia, full-thickness skin wounds were made using 8 mm biopsy punch under sterile conditions after shaving the dorsal hair. Two wounds per mice were made and then the wounded skins were separated with silicon isolator and treated with PBS or LTA as indicated. After treatment, each wound was digitally photographed and quantified by using Image J.

2.1.12. Isolation of single cells from wound area

Wound skins from mice were minced into small pieces and digested with dispase solution including RPMI 1640 culture media containing with 10% heat-inactivated FBS (HyClone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 1mg/ml dispase (Roche Diagnostics, Indianapolis, IN) and 10 mg/ml gentamicin sulfate (Sigma-Aldrich, St. Louis, MO, USA) at 4℃ overnight. Then, the solid tissues were collected and incubated with enzyme cocktail including RPMI 1640 culture media containing with 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin,100 μg/ml streptomycin, 25

mg of hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), 25 mg of collagenase (Sigma-Aldrich, St. Louis, MO, USA), 30 mg of DNAse I (Roche Diagnostics, Indianapolis, IN, USA), 25 mg of magnesium chloride hexahydrate (Sigma-Aldrich, St. Louis, MO, USA), and 5 mg/ml gentamicin sulfate at 37℃ for 2 h. Then, dispase solution and enzyme cocktail were passed through a 70 μm cell strainer. Then the cells were collected and were used for flow cytometry analysis.

2.1.13. Real-time RT-PCR

To examine the expression of wound healing-related genes in dorsal skin samples, real-time PCR analysis was performed as described previously [131]. Total RNA was extracted from the wounded skin samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The 3 μg of total RNA was reverse transcribed into cDNA using random hexamers (Promega Corporation, Madison, WI, USA) and reverse transcriptase (Promega). Real-time semi-quantitative PCR was performed with SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Shiga, Japan) using an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a specific primer set (Table 2). To determine each mRNA level, each mRNA copy number was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using $2^{-\Delta\Delta Ct}$ method, and the values were subsequently compared to those of the control group.

2.1.14. Statistical analysis

All experiments were performed at least three times. Statistical significance was measured using a student t-test and differences were considered significant when *, $P < 0.05$ by comparing the experimental group with the control group.

Table 2. Sequences of the primers used for real-time RT-PCR and RT-PCR

Transcript	Sequence
IL-1β	F: 5'-GAAATGCCACCTTTTGACAGTG-3' R: 5'-CTGGATGCTCTCATCAGGACA-3'
IL-6	F: 5'-GAGTCCTTCAGAGAGATACAGAAAC-3' R: 5'-TGGTCTTGGTCCTTAGCCAC-3'
IL-10	F: 5'-GGACAACATACTGCTAACCGAC-3' R: 5'-CCTGGGGCATCACTTCTACC-3'
TNF-α	F: 5'-CCCTCACACTCAGATCATCTTCT-3' R: 5'-GCTACGACGTGGGCTACAG-3'
IL-4	F: 5'-GTACCAGGAGCCATATCCACG-3' R: 5'-CGTTGCTGTGAGGACGTTT-3'
IL-13	F: 5'-GCCAAGATCTGTGTCTCTCCC-3' R: 5'-ACTCCATACCATGCTGCCG-3'
Arginase I	F: 5'-GTGGGGAAAGCCAATGAAGAG-3' R: 5'-TCAGGAGAAAGGACACAGGTTG-3'
Ym-1	F: 5'-AGAAGCAATCCTGAAGACACC-3' R: 5'-GCATTCCAGCAAAGGCATAG-3'
Fizz-1	F: 5'-CGTGGAGAATAAGGTCAAGGAAC-3' R: 5'-CAACGAGTAAGCACAGGCAG-3'
CD86	F: 5'-GCACGGACTTGAACAACCAG-3'

	R: 5'-CCTTTGTAAATGGGCACGGC-3'
CD206	F: 5'-TGTGGAGCAGATGGAAGGTC-3' R: 5'-TGTCGTAGTCAGTGGTGGTTC-3'
MIP-1α	F: 5'-TGAATGCCTGAGAGTCTTGG-3' R: 5'-TTGGCAGCAAACAGCTTATC-3'
CXCL2	F: 5'-CCCTGGTTCAGAAAATCATCCAAA-3' R: 5'-TTTGGTTCTTCCGTTGAGGGAC-3'
iNOS	F: 5'-CGCTTGGGTCTTGTTCACTC-3' R: 5'-GGTCATCTTGTATTGTTGGGCTG-3'
MMP-2	F: 5'-CGATGTCGCCCCTAAAACAG-3' R: 5'-CTTGAGGGTATCTTTCAGCACAAA-3'
MMP-9	F: 5'-TCTTCCCCAAAGACCTGAAAAC-3' R: 5'-GCCCGGGTGTAACCATAGC-3'
IFN-β	F: 5’-TCCAAGAAAGGACGAACATTCG–3 R: 5’-TGAGGACATCTCCCACGTCAA–3’
β-actin	F: 5’-GTGGGGCGCCCCAGGCACCA–3’ R: 5’-CTCCTTAATGTCACGCACGAT TTC–3’

F, forward primer; *R*, reverse primer

Chapter III. Results

3.1. LTA effect on macrophage proliferation and its mechanism

3.1.1. LTA increases macrophage proliferation.

In order to determine whether bacterial Sa.LTA is able to induce various macrophage proliferation, CFSE-labeled RAW 264.7 cells were stimulated with Sa.LTA, Pam2CSK4 or Ec.LPS for 48 h and proliferation was measured by flow cytometry. Sa.LTA increased the proliferation of RAW 264.7 cells in a dose-dependent manner (Fig. 5A). However, when the cells were stimulated with Pam2CSK4 or Ec.LPS, proliferation of RAW 264.7 cells was not increased. Next, in order to further examine the effect of macrophage proliferation by other bacterial LTAs, RAW 264.7 cells were stimulated with Sa.LTA, Lp.LTA, or Sp.LTA for 48 h and macrophage proliferation was measured by flow cytometry. This result showed that macrophage proliferation was increased by all LTAs tested including Sa.LTA, Lp.LTA and Sp.LTA in a dose-dependent manner. (Fig. 5E). To examine the effect of Sa.LTA on other macrophage proliferation, human macrophage cell line (THP-1), microglial cell line (BV-2), and BMDM were stimulated with Sa.LTA for 48 h and each cell proliferation was measured by flow cytometry. Macrophage proliferation was also increased by Sa.LTA in these types of macrophages (Fig. 5B-D). These

results suggest that Sa.LTA enhances proliferation of macrophages.

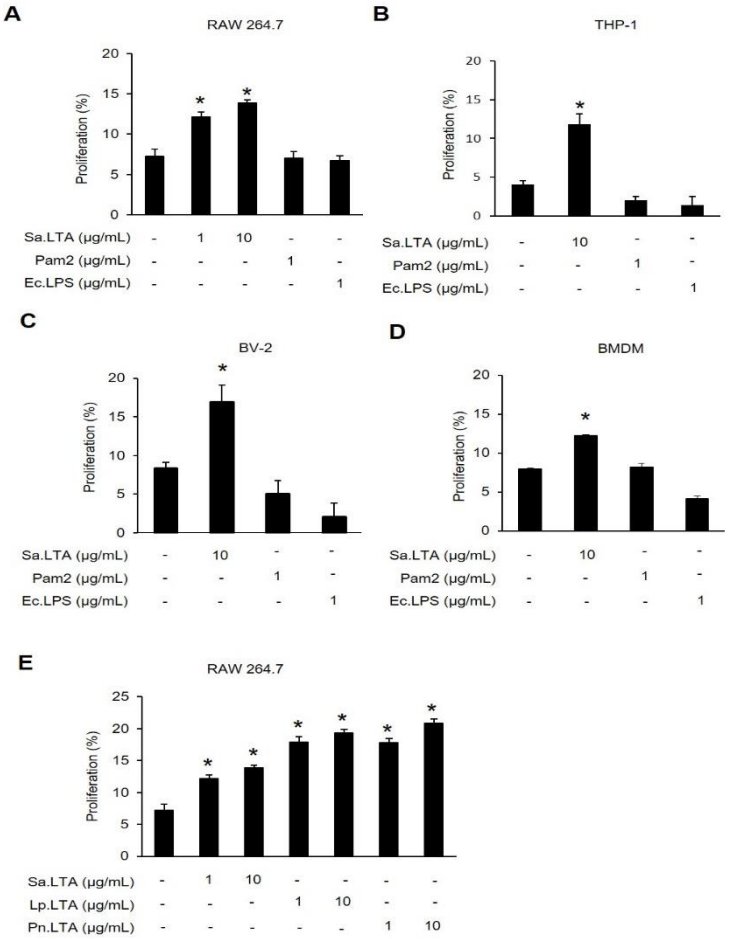

Figure 5. LTA increases macrophage proliferation. (A) CFSE-labeled RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 1 or 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 48 h. (B) CFSE-labeled THP-1 (2 x 10^5 cells/ml), (C) BV-2 (2 x 10^5 cells/ml), and (D) BMDMs cells (2 x 10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 48 h. Then the cell proliferation was analyzed by flow cytometry. (E) CFSE-labeled RAW 264.7 cells were stimulated with 1 μg/ml, or 10 μg/ml of Sa.LTA, Lp.LTA, or Pn.LTA for 48 h. Percentages represent the proliferated cells from the analyzed population. *$P < 0.05$, compared with non-treatment control group.

3.1.2. LTA increases cell cycle progression via induction of cell cycle-related genes

Activation of various cyclin/cyclin-dependent kinase (CDK) complexes is essential for the process of cell cycle [119]. To examine whether Sa.LTA increases the expression of cell cycle-related proteins, RAW 264.7 cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS for 6, 12, and 24 h and the proteins were subjected to Western blotting. As shown in Figure 13, With the Sa.LTA stimulation, expression of cyclin D1, cyclin D3, CDK2, or CDK6 was increased in a time-dependent manner (Fig. 6A). These results indicate that Sa.LTA increases process of cell cycle by augmenting the expression of the cell cycle related proteins such as cyclin D1, cyclin D3, CDK2, and CDK6 in RAW 264.7 cells. To examine whether Sa.LTA disturbs cell cycle progression in RAW 264.7 cells, the cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS for 48 h and each of the cell cycle phases was measured by flow cytometry. This results showed that when the cells were stimulated with Sa.LTA, S and G2/M phases were increased (Fig. 6B). These results indicate that Sa.LTA increases process of cell cycle by augmenting the expression of the cell cycle-related proteins.

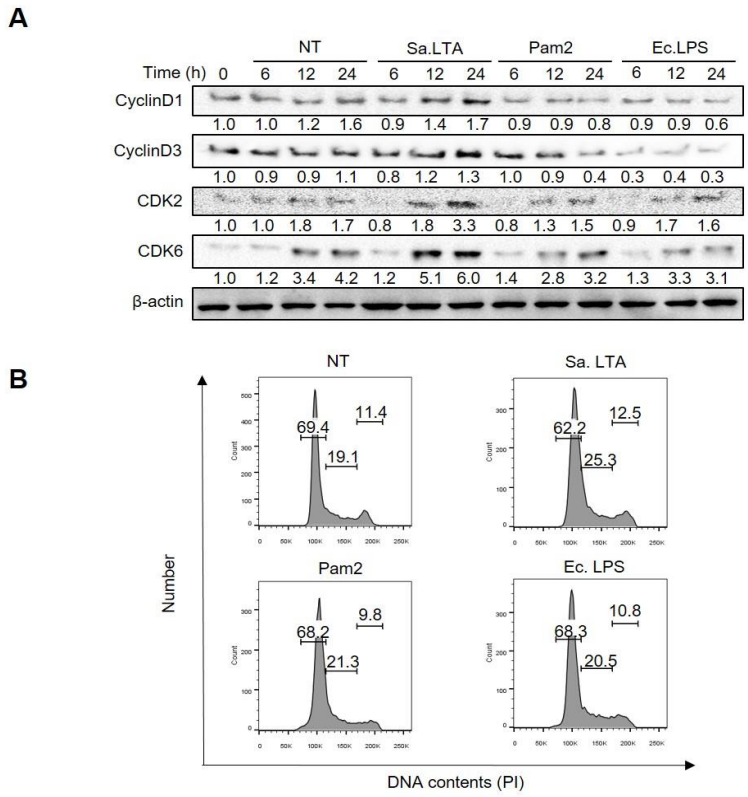

Figure 6. LTA increases the expression of cell cycle-related genes and cell cycle process by up-regulating S and G2/M phases. (A) RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for the indicated time periods. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to cyclin D1, cyclin D3, CDK2, CDK6, or β-actin. The fold increase in proteins expression were determined by Image J. (B) RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 48 h. Then the cells were fixed and stained with propidium iodide (PI). The cell cycle distribution was derived by DNA content analysis using flow cytometry. The percentage of the cells in each phase of the cell cycle was obtained from the flow cytometry data. The results shown are representative of three independent experiments. NT; no treatment

3.1.3. LTA increases c-Myc expression.

c-Myc is involved in the controls of cell proliferation by increasing cyclin D and CDKs expression [132, 133]. To examine the effect of Sa.LTA on the expression of c-Myc, RAW 264.7 cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS for 6, 12, and 24 h. c-Myc expression was subjected to Western blotting. As shown in Figure 14, the expression of c-Myc and phospho (Ser62) c-Myc were increased by Sa.LTA in a time-dependent manner. However, Pam2CSK4 or Ec.LPS did not induce c-Myc expression or phospho (Ser62) c-Myc (Fig. 7). These result indicates that Sa.LTA increases c-Myc and phosphor (Ser62) c-Myc expression in macrophages.

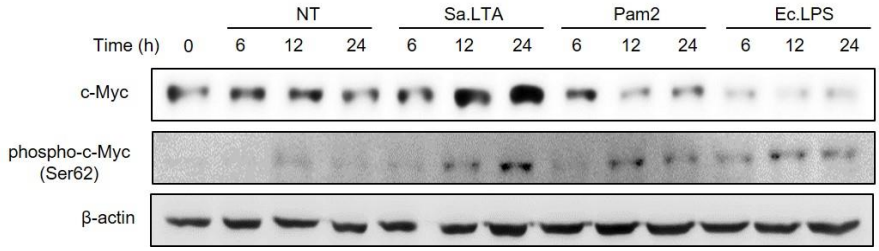

Figure 7. LTA increases c-Myc expression in protein level. RAW 264.7 cells (2×10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for the indicated time periods. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to c-Myc, phospho-c-Myc (Ser62), or β-actin. The results shown are representative of three independent experiments. NT; no treatment

3.1.4. LTA enhances macrophage proliferation by enhancing c-Myc expression

To examine whether c-Myc is a key regulator of Sa.LTA-induced macrophage proliferation, the cells were pretreated with c-Myc inhibitor for 30 min and were stimulated with Sa.LTA for 48 h. Macrophage proliferation was measured by flow cytometry. This results showed that c-Myc inhibitor inhibited Sa.LTA-induced macrophage proliferation in a dose-dependent manner (Fig 8A). Furthermore, Sa.LTA-induced cell cycle-related genes were inhibited in the presence of c-Myc inhibitor in a dose-dependent manner (Fig. 8B). These results imply that c-Myc is essential for Sa.LTA-induced macrophage proliferation.

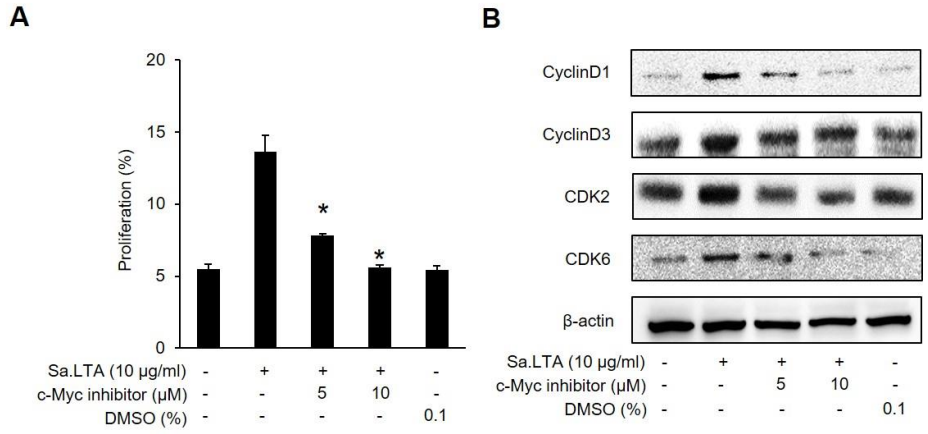

Figure 8. LTA enhances macrophage proliferation by enhancing c-Myc expression. (A) CFSE-labeled RAW 264.7 cells (2 x 10^5 cells/ml) were pre-treated with indicated concentrations of c-Myc inhibitor for 1 h followed by stimulation with 10 μg/ml Sa.LTA for an additional 48 h. Then, the cell proliferation was analyzed by flow cytometry. Data is representative of three independent experiments. Percentages represent the proliferated cells from the analyzed population. *, $P < 0.05$. (B) RAW 264.7 cells were pre-treated with indicated concentrations of c-Myc inhibitor for 1 h followed by stimulation with 10 μg/ml Sa.LTA for an additional 24 h. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to cyclin D1, cyclin D3, CDK2, CDK6, or β-actin.

3.1.5. LTA increases c-Myc protein stability

It is well known that c-Myc is induced by mitogenic signals and is down-regulated by growth-inhibitory signals. Furthermore, c-Myc expression is tightly regulated at multiple stages including mRNA stability, translation, and protein stability [134]. Ubiquitin-proteasome pathway is a major regulating factor in controlling c-Myc stability [135]. To examine whether an increase of c-Myc results from its increased stability, RAW 264.7 cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS in the presence of cycloheximide (CHX) and the protein expression were measured by Western blotting. This results showed that c-Myc was not degraded in the presence of Sa.LTA. However, c-Myc was rapidly degraded in the presence of Pam2CSK4 or Ec.LPS (Fig. 9A and 9B). Therefore, these results indicate that Sa.LTA increases c-Myc stability.

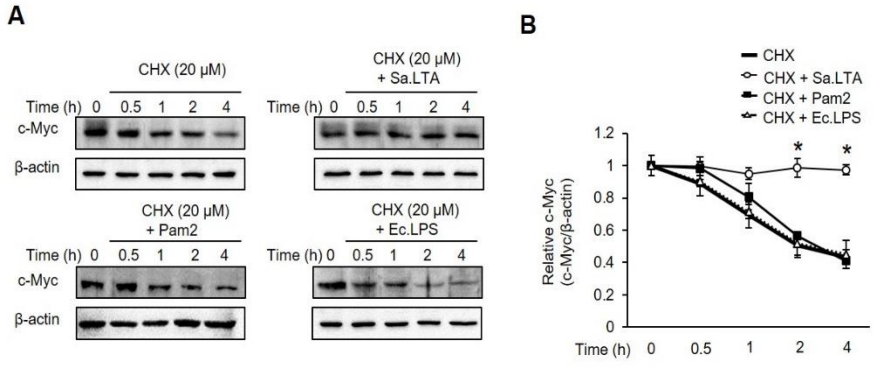

Figure 9. LTA increases c-Myc protein stability. (A) RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS in the presence of cycloheximide (CHX) for the indicated time periods. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to c-Myc or β-actin. (B) The c-Myc expression was normalized to β-actin by densitometry. Data is representative of three independent experiments.

3.1.6. LTA increases c-Myc stability through down-regulating c-Myc ubiquitination

It is known that c-Myc is short-lived protein which has approximately 30 min half-life in growing cells and is degraded by ubiquitin-proteasome system [135]. Therefore, it is possible that Sa.LTA could affect c-Myc-ubiquitin binding. In order to investigate whether Sa.LTA down-regulates c-Myc ubiquitination, the cells were stimulated with Sa.LTA for 24 h and before the experimental end-point, proteasome inhibitor, MG132 were treated for 6 h. Then, the cell lysates were subjected to immunoprecipitation with anti-ubiquitin followed by Western blotting with anti-c-Myc. As shown in Figure 10, c-Myc-ubiquitination were decreased by Sa.LTA but not with Pam2CSK4 or Ec.LPS. These results indicate that Sa.LTA has the ability to increase c-Myc stability by decreasing Myc-ubiquitination.

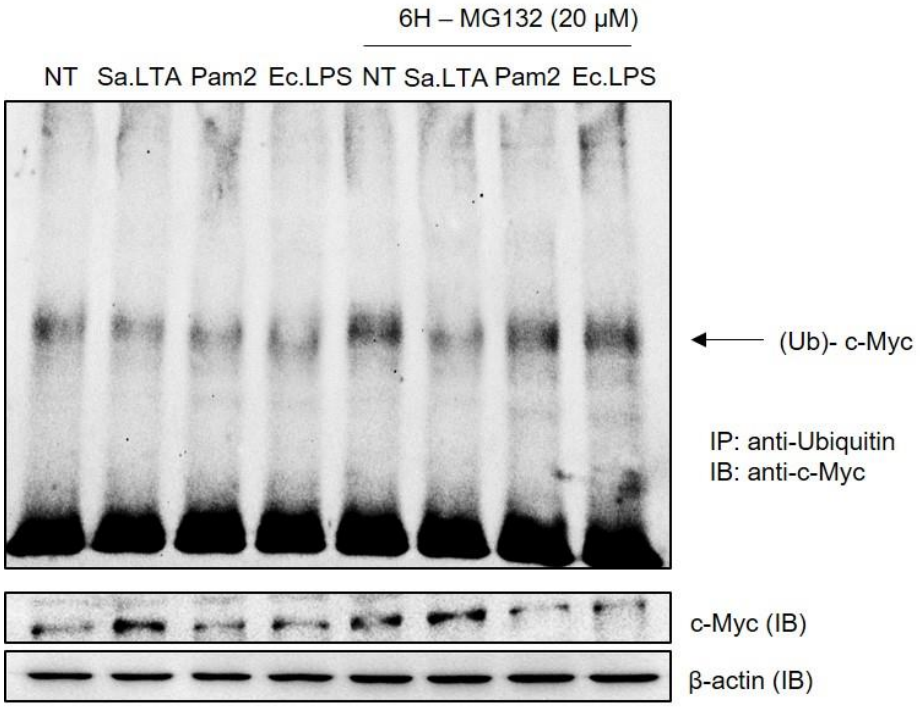

Figure 10. LTA decreases c-Myc ubiquitination. RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 24 h and were treated with or without MG132 for 6 h before harvest. After the cells were lysed, the lysates were subjected to immunoprecipitation (IP) with anti-ubiquitin conjugated beads, followed by Western blotting using specific antibodies to c-Myc or β-actin. NT; no treatment, IP; immunoprecipitation, IB; immunoblotting

3.1.7. TLR2 is required for the LTA-induced macrophage proliferation

It is well known that LTA activates TLR2 and their downstream signaling [11, 34, 136]. To investigate whether TLR2 is essential for Sa.LTA-induced macrophage proliferation, BMDMs differentiated from wild-type (WT) or TLR2-deficient bone marrow cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS for 48 h. Then, the cells were harvested to measure macrophage proliferation using flow cytometry analysis. As shown in Figure 11, Sa.LTA increased WT-BMDM proliferation, but not TLR2-deficient-BMDM. Therefore, these results indicate that TLR2 is required for the Sa.LTA-induced macrophage proliferation.

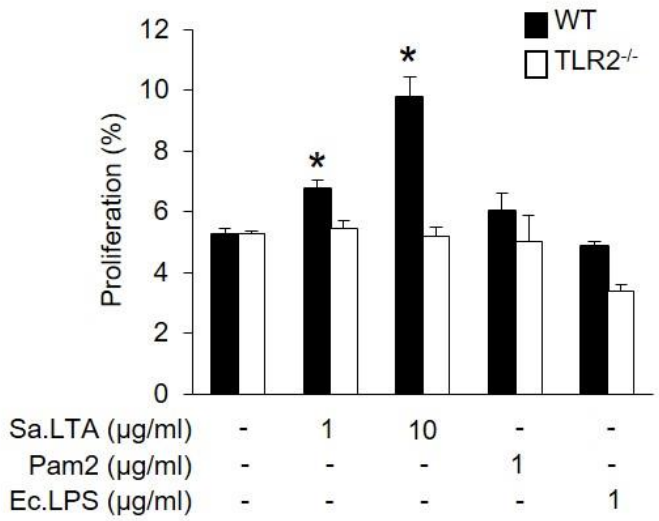

Figure 11. TLR2 is required for LTA-induced macrophage proliferation. BMDMs from wild-type or TLR2-deficient mice (n = 3) were stained with CFSE. The cells were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 48 h. Then, the cell proliferation was analyzed by flow cytometry. Percentage represents the proportion of dividing cells. *P < 0.05, compared with non-treatment control group. Data is representative of three independent experiments.

3.1.8. MAPK and PI3K signaling pathways are important for LTA-induced macrophage proliferation

Phosphorylation of c-Myc is important for increasing c-Myc stability and it is regulated by MAPKs and PI3K/GSK-3β-dependent pathway [137-139]. To investigate whether MAPKs and PI3K were involved in the Sa.LTA-induced macrophage proliferation, RAW 264.7 cells were pre-treated with inhibitors including PD98059, SP600125, SB203580, and LY294002 (selective inhibitors of ERK, JNK, p38, and PI3K) for 1h followed by stimulated with Sa.LTA for additional 48 h. As shown in Figure 12, Sa.LTA-induced macrophage proliferation was decreased in the presence of ERK, JNK or PI3K inhibitors in a dose-dependent manner, but not in the presence of p38 inhibitor (Fig. 12A). Furthermore, Sa.LTA-induced c-Myc and phospho (Ser62) c-Myc were also decreased in the presence of inhibitors blocking ERK, JNK, or PI3K (Fig. 12B). Next, to further investigate whether Sa.LTA induces GSK-3β phosphorylation, RAW 264.7 cells were pre-treated with inhibitors including PD98059, SP600125, SB203580, and LY294002 for 1h followed by stimulated with Sa.LTA for additional 30 min. In this result, Sa.LTA increases expression of GSK-3β phosphorylation. Sa.LTA-induced GSK-3β phosphorylation was inhibited by only LY294002 pre-treatment (Fig. 12C). Collectively, these results indicate that ERK, JNK, and PI3K/GSK-3β signaling pathways are essential for Sa.LTA-induced macrophage proliferation.

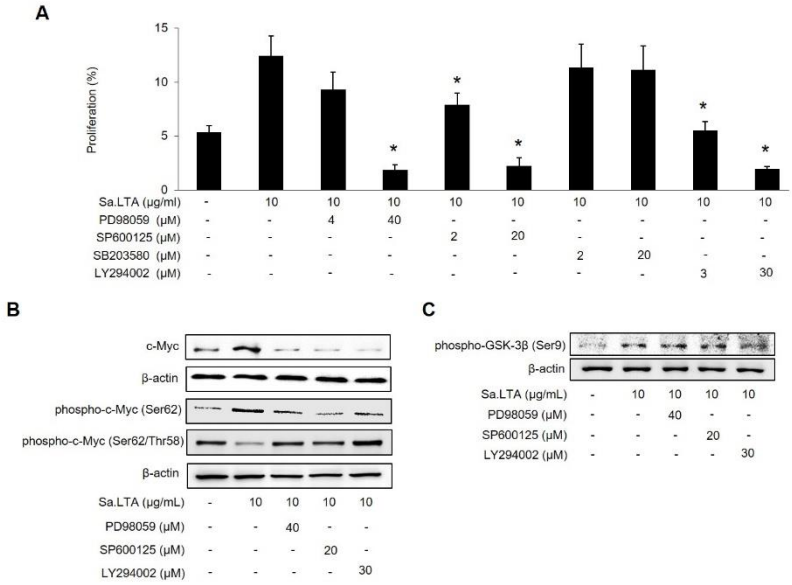

Figure 12. MAPK and PI3K signaling pathways are important for LTA-induced macrophage proliferation. (A) CFSE-labeled RAW 264.7 cells (2 x 10^5 cells/ml) were pre-treated with indicated concentrations of PD98059, SP600125, SB203580, or LY294002 for 1 h followed by stimulation with 10 μg/ml Sa.LTA for an additional 48 h. Then, proliferation was analyzed by flow cytometry. Percentages represent the proliferated cells from the analyzed population. *, $P < 0.05$, compared with non-treatment control group. Data is representative of three independent experiments. (B) RAW 264.7 cells (2 x 10^5 cells/ml) were pre-treated with indicated concentration of PD98059, SP600125, or LY294002 for 1 h followed by stimulation with 10 μg/ml Sa.LTA for an additional 24 h. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to p-c-Myc (Ser62), p-c-Myc (Ser62/Thr58), c-Myc, or β-actin. (C) RAW 264.7 cells (2 x 10^5 cells/ml) were pre-treated with indicated concentration of PD98059, SP600125, or LY294002 for 1 h followed by stimulation with 10 μg/ml Sa.LTA for an additional 30 min. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to p-GSK-3β or β-actin.

3.1.9. IFN-β decreases LTA-induced macrophage proliferation and c-Myc expression

IFN-β is known to have an important role in regulating cell and c-Myc expression [140]. In addition, TLR2 and TLR4 agonists have the ability to induce IFN-β [141, 142]. Therefore, IFN-β might be essential for TLR ligand-induced macrophage proliferation. To examine the mRNA expression of IFN-β, RAW 264.7 cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS for 6 h and the mRNA expression levels were measured by RT-PCR analysis. In this result, the expression of IFN-β was increased in the presence of Pam2CSK4 and Ec.LPS, but not Sa.LTA (Fig. 13A). To confirm whether IFN-β has the ability to decrease Sa.LTA-induced macrophage proliferation, RAW 264.7 cells were stimulated with recombinant IFN-β in the presence of Sa.LTA and macrophage proliferation was measured by flow cytometry analysis. As shown in Figure 13B, IFN-β down-regulated the Sa.LTA-induced macrophage proliferation in a dose-dependent manner. Furthermore, to measure the effect of IFN-β on Sa.LTA-induced c-Myc expression, RAW 264.7 cells were stimulated with recombinant IFN-β in the presence of Sa.LTA. Figure 20C shows that Sa.LTA-induced c-Myc level was also decreased by IFN-β stimulation in a dose-dependent manner. Collectively, these results indicate that IFN-β is a key regulator of Sa.LTA-induced macrophage proliferation.

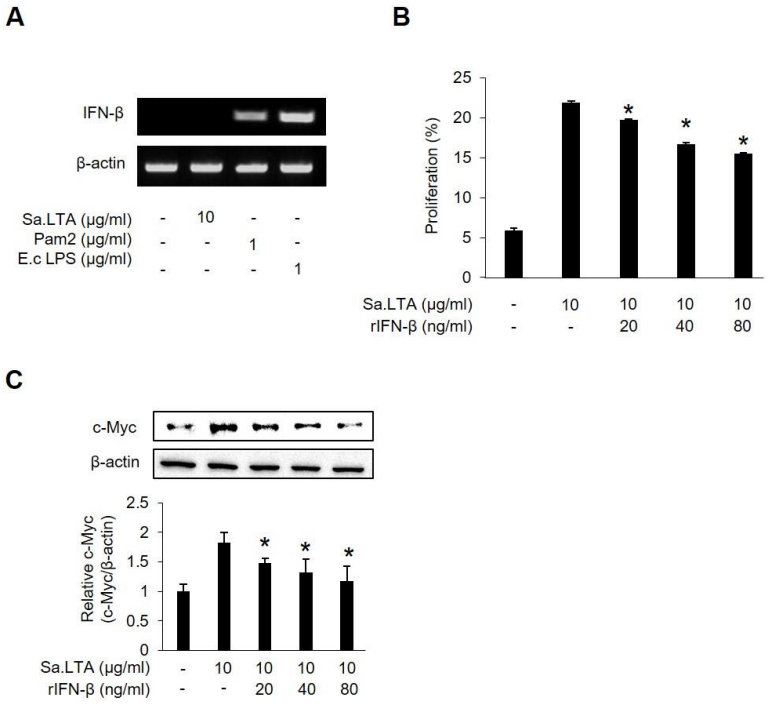

Figure 13. IFN-β decreases LTA-induced macrophage proliferation and c-Myc expression (A) RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 6 h. At the end of stimulation, the cells were collected and IFN-β mRNA were measured by RT-PCR. (B) CFSE-labeled RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/mL Sa.LTA in the presence of 20, 40, or 80 ng/ml of recombinant IFN-β for 48 h. Then, the cell proliferation was analyzed by flow cytometry. Percentages represent the proliferated cells from the analyzed population. *, $P < 0.05$, compared with non-treatment control group. (C) RAW 264.7 cells (2 x 10^5 cells/ml) were stimulated with 10 μg/mL Sa.LTA in the presence of 20, 40, or 80 ng/ml of recombinant IFN-β for 24 h. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to c-Myc or β-actin. Data is representative of three independent experiments.

3.1.10. IFN-β is a key regulator of macrophage proliferation

Next, to confirm the effect of IFN-β on Sa.LTA-induced macrophage proliferation, the cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS in the presence of type I IFN receptor blocking antibody and macrophage proliferation was measured by flow cytometry. The results showed that the inhibitory effect of IFN-β on Sa.LTA-induced macrophage proliferation was recovered by type I IFN receptor blocking antibody. Furthermore, when the cells were stimulated with Pam2CSK4 or Ec.LPS in the presence of type I IFN receptor blocking antibody, the inhibitory effect of macrophage proliferation was recovered (Fig. 14A). Next, to measure c-Myc expression, the cells were stimulated with Sa.LTA, Pam2CSK4, or Ec.LPS in the presence of type I IFN receptor blocking antibody and c-Myc expression was measured by Western blotting. As shown in Figure 14B, the inhibitory effect of IFN-β on Sa.LTA-induced c-Myc expression was recovered by type I IFN receptor blocking antibody. Furthermore, when the cells were stimulated with Pam2CSK4 or Ec.LPS in the presence of type I IFN receptor blocking antibody, the inhibitory effect on c-myc expression was also recovered. Collectively, these result indicate that IFN-β has the ability to inhibit Sa.LTA-induced macrophage proliferation and difference between Sa.LTA and Pam2CSK4 might be due to their varying IFN-β expression ability.

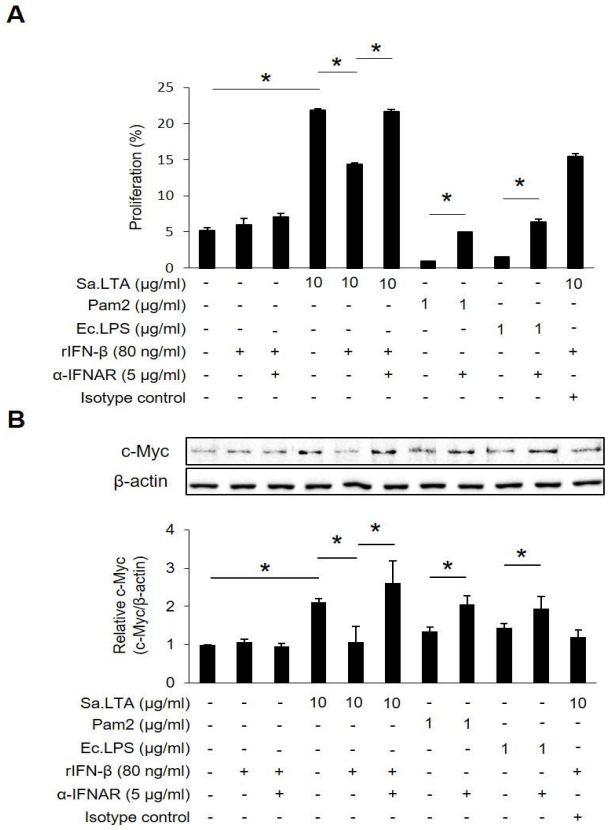

Figure 14. IFN-β is a key regulator of macrophage proliferation. (A) CFSE-labeled RAW 264.7 cells (2 x 10^5 cells/ml) were pre-treated with recombinant IFN-β and were co-stimulated with or without anti-IFNAR Ab for 30 min followed by stimulation with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 48 h. Then, the cell proliferation was analyzed by flow cytometry. (B) RAW 264.7 cells (2 x 10^5 cells/ml) were pre-treated with recombinant IFN-β and were co-stimulated with or without anti-IFNAR Ab for 30 min. Then, the cells were stimulated with 10 μg/ml Sa.LTA, 1 μg/ml Pam2CSK4, or 1 μg/ml Ec.LPS for 48 h. After the cells were lysed, the lysates were subjected to Western blotting using specific antibodies to c-Myc or β-actin. *, $P < 0.05$, compared with non-treatment control group. Data is representative of three independent experiments.

3.1.11. LTA increases F4/80^{high}Ly6c^{low} macrophage proliferation in mice peritoneum

Previous reports suggest that when leukocyte traffic is restricted, macrophages locally proliferate rather than monocytes are recruited from bone marrow [85]. Furthermore, tissue macrophages are known to express F4/80^{high} and Ly6c^{low} [85]. Therefore, to confirm if LTA increases macrophage expansion, mice were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h. At day 2 after administration, the cells were collected from the peritoneum. As shown in Figure 15, F4/80^{high}Ly6c^{low} (tissue macrophage) population of peritoneal cells was increased in Sa.LTA-administrated mice. However, Pam2CSK4- or Ec.LPS-administrated mice exhibited increased F4/80^{low}Ly6c^{high} (monocyte) population (Fig. 15A and 15B). These results indicate that LTA increases tissue macrophage population in mice peritoneum.

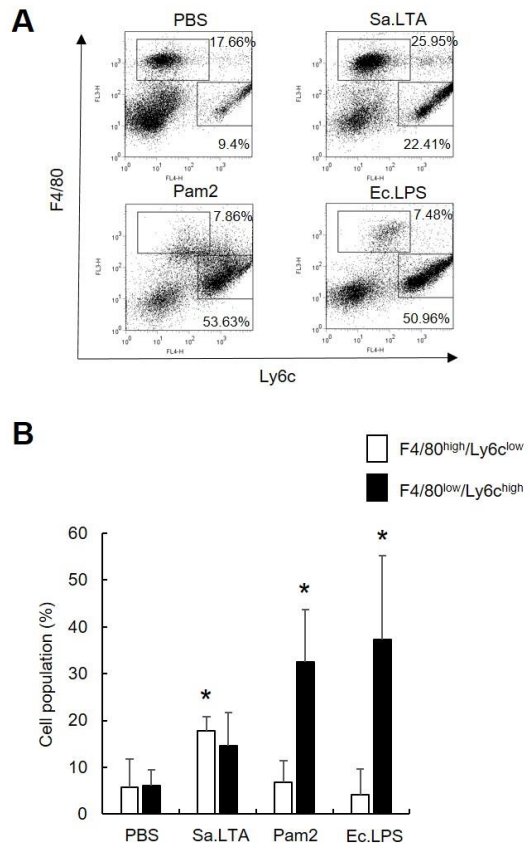

Figure 15. LTA increases F4/80^{high}Ly6c^{low} peritoneal macrophages proliferation. (A and B) Mice (*n* = 5 per group) were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h. On day 2 after administration, the cells were collected from the peritoneum. The cells were stained with PerCP-conjugated anti-mouse F4/80 and APC-conjugated anti- mouse Ly6C and were analyzed by flow cytometry to measure a change in the peritoneal macrophage population. Data representative data of three independent experiments. $*P < 0.05$, compared with non-treatment control group.

3.1.12. LTA induces tissue macrophage expansion in mouse peritoneum

To confirm if Sa.LTA increases tissue macrophage expansion, mice were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h. After 2 days, the cells were collected from the peritoneum and tissue macrophage markers including CD206, PD-L2 and MHCII were measured by using flow cytometry. As shown in Figure 10, F4/80-positive macrophages that express CD206, PD-L2 and MHCII were increased in peritoneal macrophage from Sa.LTA-administrated mice, but not in peritoneal macrophage from Pam2CSK4- or Ec.LPS-administrated mice (Fig. 16). Collectively, these results indicate that Sa.LTA increases tissue macrophage expansion in mice peritoneum.

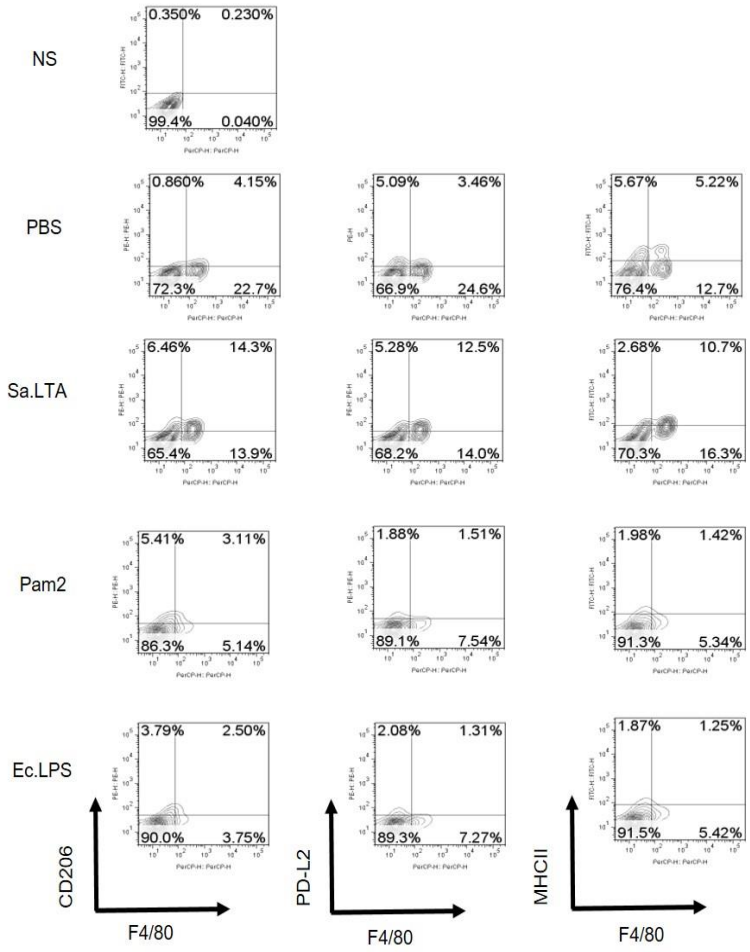

Figure 16. LTA induces tissue macrophage expansion in mouse peritoneal. Mice (*n* = 5 per group) were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h. After 2 days, the cells were collected from the peritoneum. Expression of CD206, PD-L2, and MHCII on the F4/80 macrophages were analyzed by flow cytometry. The results shown are representative of three independent experiments. NS; no staining

3.1.13. LTA increases peritoneal macrophage proliferation

Ki67 and BrdU are known as principal markers for mitotic cells. In addition, Ki67 is used to detect dividing cells for the whole duration of their mitotic process [143]. Proliferating macrophages in peritoneum were reported to express both Ki67 and BrdU markers [144]. Then to examine the Sa.LTA-induced macrophage proliferation ability in mice peritoneum, mice were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h. After injection, peritoneal cells were collected from mouse peritoneal cavity and macrophage proliferation markers were measured by using flow cytometry. For BrdU labelling of proliferation cells *in vivo*, the mice were intraperitoneally injected with PBS (100 μl), Sa.LTA (50 μg/100 μl), Pam2CSK4 (5 μg/100 μl) or Ec.LPS (5 μg/100 μl) for 48 h and before 12 h experimental end-point, 100 μl of 10 mg/mL BrdU in PBS was injected. As shown in Figure 11, $F4/80^{+}Ki67^{+}$ cells (Fig. 17A) and $F4/80^{+}BrdU^{+}$ (Fig. 17B) cells were increased in Sa.LTA-administrated mice. However, Pam2CSK4 or Ec.LPS did not increase $F4/80^{+}Ki67^{+}$ cells and $F4/80^{+}BrdU^{+}$ cells population in mice peritoneal. Collectively, these result indicate that Sa.LTA increases macrophage proliferation in mice peritoneum.

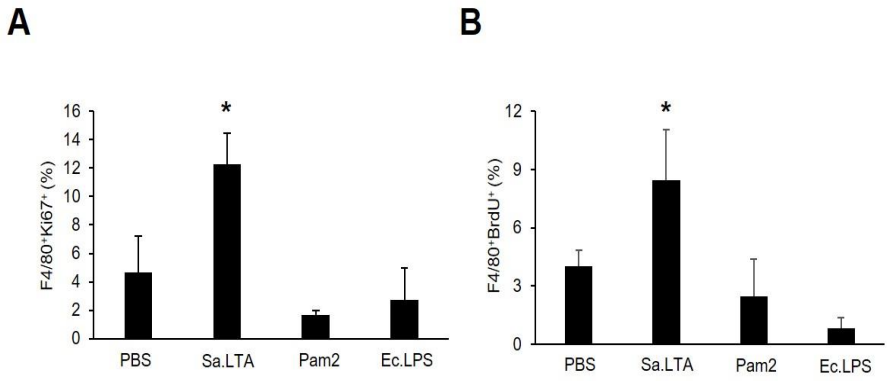

Figure 17. LTA increases peritoneal macrophage proliferation. (A) Mice (*n* = 5 per group) were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h. After injection, peritoneal cells were collected from mouse peritoneal cavity and the cells were stained with PerCP-conjugated anti-mouse F4/80 and FITC-conjugated anti-mouse Ki67 and were analyzed by flow cytometry to determine $F4/80^{+}Ki67^{+}$ cells. (B) Mice (*n* = 3 per group) were intraperitoneally administrated with 100 μl PBS, 50 μg/100 μl Sa.LTA, 5 μg/100 μl Pam2CSK4, or 5 μg/100 μl Ec.LPS for 48 h and before 12 h the experimental end-point, 100 μl of 10 mg/mL BrdU in PBS was injected. After injection, peritoneal cells were collected from mouse peritoneal cavity and the cells were stained with PerCP-conjugated anti-mouse F4/80 and FITC-conjugated anti-mouse BrdU and were analyzed by flow cytometry to determine $F4/80^{+}BrdU^{+}$ cells. Data is representative of three independent experiments. $*P < 0.05$, compared with non-treatment control group.

3.2. Effect of LTA on wound healing process

3.2.1. LTA improves wound healing process

In previous reports, *S. aureus* has been recognized as important bacteria which cause disease through skin infections. This study finds that LTA from *S. aureus* possibly has the ability to promote wound healing process through macrophage proliferation and therefore could be used as a therapeutic molecule. However, molecules from pathogenic bacteria might not be suitable for use as therapeutic molecule. Meanwhile, previous reports also find that lactic acid bacteria including *L. plantarum* have the ability to accelerate wound healing [145, 146]. Therefore, in this study, LTA from *L. plantarum* was used in the in-vivo model.

To determine the effect of LTA on wound healing process, C57BL/6 mice were wounded on the dorsal surface (Fig. 18A). After creating the wounds, PBS (20 μl) or *L. plantarum* LTA (Lp.LTA) (50 μg/20 μl) was treated in the wound areas and re-treated at 2 and 4 days after wounding. To measure the wound healing process, the wound closure was monitored every two days until the end of the experiment. As shown in Figure 18, when the wound was treated with Lp.LTA, the average size of the wound area was decreased by approximately 27% more than the size of the PBS-treated wound at six days after wounding. Lp.LTA-treated wound was almost completely closed at 10 days after wounding, whereas the PBS-treated wound was not closed at 10 days after wounding (Fig. 18B and 18C). These results indicate that Lp.LTA improves the

wound healing process in the mouse wound healing model.

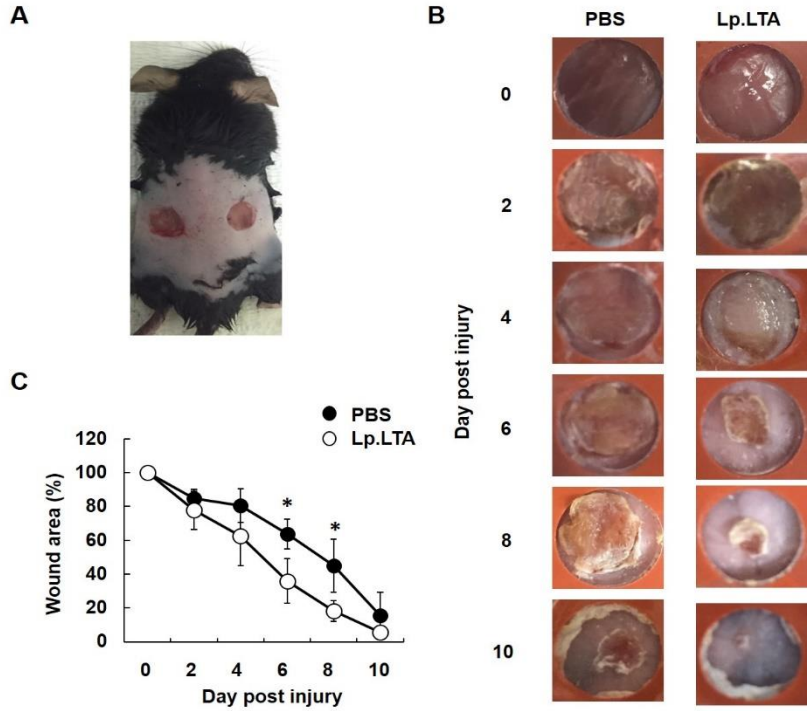

Figure 18. LTA improves wound healing process. (A) Anesthetized mice were shaved the dorsal hair and full-thickness skin wounds were made using 8 mm biopsy punch under sterile conditions. Day 0 picture was taken immediately after wounding in mouse dorsal skin. (B) Skin wounds were created in the mouse dorsal skin under sterile conditions with 8 mm biopsy punch. After creatin the wounds (n = 5 per group), PBS (20 μl) or Lp.LTA (50 μg/20 μl) was treated in the wound areas and re-treated at 2 and 4 days after wounding. After treatment, wound closure was pictured every 2 days until the 10th day. (C) Wound size analysis of mouse skin dorsal wounds. Percentages represent the wound area. Data is a representative data of three independent experiments. *, $P < 0.05$, compared with PBS-treatment control group.

3.2.2. LTA increases wound healing-related genes expression in wound area

Next, to investigate wound healing-related factors that are related to the neutrophil, macrophage recruitment, extracellular matrix (ECM), and pro-reepithelialisation, wounds were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at two and four days after wounding. After treatment, wound tissues were harvested at two days, four days, or six days after wounding. Then MIP-1α, CXCL2, MMP-2, MMP-9, and IL-6 mRNA expression levels were measured by using real-time RT-PCR analysis. In Figure 5, the expression levels of ECM and pro-reepithelialisation related genes such as MMP-2, MMP-9 and IL-6 were increased in the presence of Lp.LTA during wound healing process in a time dependent manner (Fig. 19C-19E). However, macrophage recruitment factor, MIP-1α expression of Lp.LTA-treated wound did not show any change, whereas, PBS-treated wound exhibited increased MIP-1α expression. Furthermore, Lp.LTA-treated wound showed a decreased of the neutrophil recruitment factors, CXCL2 expression in a time-dependent manner. In contrast, CXCL2 expression was time dependently increased when the wound was treated with PBS (Fig. 19A and 19B). These results indicate that Lp.LTA improves wound healing process through induction of wound healing factors including extracellular matrix (ECM) and pro-reepithelialisation, but not neutrophil or macrophage recruitment factors.

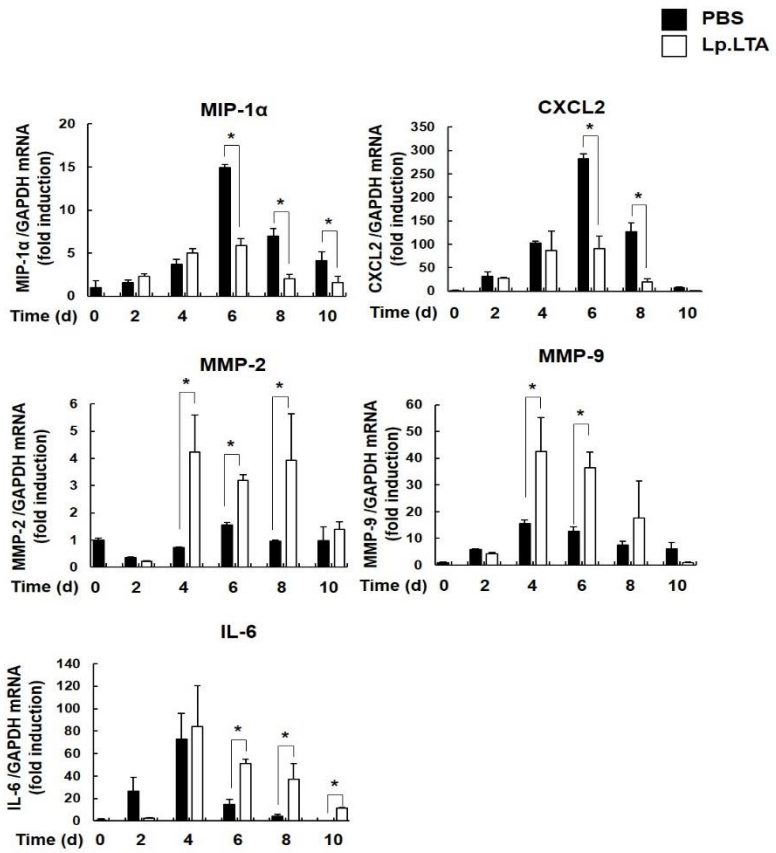

Figure 19. LTA increases wound healing-related genes expression in wound area. (A-E) Skin wounds were created in the mouse dorsal skin under sterile conditions with 8 mm biopsy punch (n = 5 per group). After creating the wounds, the wound areas were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 and 4 days after wounding. After treatment, the wound tissues were harvested and total RNA was prepared using TRIzol reagents. After cDNA synthesis using random hexamers, relative mRNA expression levels of wound healing factors (MIP-1α, CXCL2, MMP-2, MMP-9, and IL-6) were measured by real time RT-PCR and results were normalized to GAPDH expression as a control. Data is representative data of three independent experiments. *P < 0.05, compared with non-treatment control group.

3.2.3. LTA decreases pro-inflammatory gene expression in wound area

According to previous reports, when a wounds occurs, recruitment of neutrophils and macrophages in wound area induces pro-inflammatory cytokines such as TNF-α, iNOS, and IL-1β [67]. These factors have the ability to attenuate tissue regenerative response in mice [121]. Therefore, to examine pro-inflammatory factors in mRNA level, the wounds were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 and 4 days after wounding. After treatment, the wound tissues were harvested in 2, 4, and 6 days after wounding. Then, pro-inflammatory factors such as iNOS and IL-1β were measured by real-time RT-PCR analysis. As shown in Figure 20, the pro-inflammatory factors including iNOS and IL-1β were increased when the wound was treated with PBS. However, when the wound was treated with Lp.LTA, iNOS and IL-1β were not increased than PBS-treated wound. In contrast, anti-inflammatory factor such as IL-4, IL-10 and IL-13 expression was increased by Lp.LTA treatment compare with PBS-treated wound. These results demonstrate that Lp.LTA decreases pro-inflammatory responses but increases anti-inflammatory responses in a wound area.

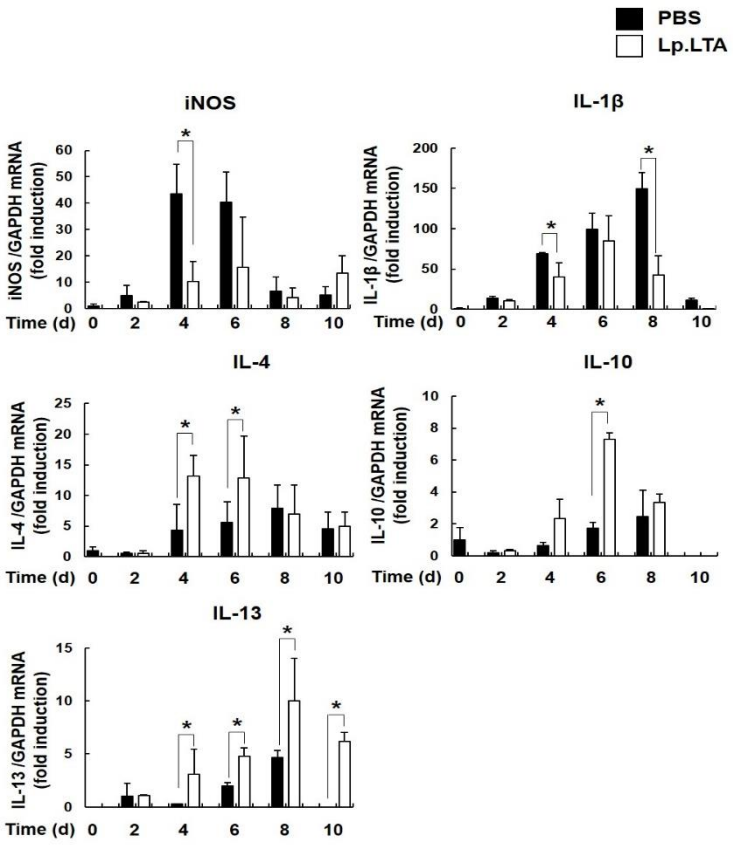

Figure 20. LTA decreases pro-inflammatory gene expression in wound area. Skin wounds were created in the mouse dorsal skin under sterile conditions with 8 mm biopsy punch (n = 5 per group). After creating the wounds, the wound areas were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 and 4 days after wounding. After treatment, the wound tissues were harvested and total RNA was prepared using TRIzol reagents. After cDNA synthesis using random hexamers, relative mRNA expression levels of (A and B) pro-inflammatory factors such as iNOS and IL-1β and (C–E) anti-inflammatory factors such as IL-4, IL-10, and IL-13 were measured by real time RT-PCR and results were normalized to GAPDH expression as a control. Data representative data of three independent experiments. *$P < 0.05$, compared with non-treatment control group.

3.2.4. LTA increases macrophage expansion in wound area

Previous report suggests that macrophages have an essential role in tissue re-modelling as well as immune regulation [147]. To further examine if Lp.LTA increases macrophage expansion in a wound area, the wounds were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 and 4 days after wounding. Then, the wound tissues were harvested and enzymatically digested to obtain single cell. Population of macrophages, neutrophils, and T cells was measured by using flow cytometry. As shown in Figure 21, when Lp.LTA was treated wound area exhibited similar number of neutrophils and T cells in the wound area compared with the cells of PBS-treated wound area. However, Lp.LTA-treated wound showed an increased number of compared with the cells of PBS-treated wound area. This result indicates that Lp.LTA increases macrophage expansion in wound area.

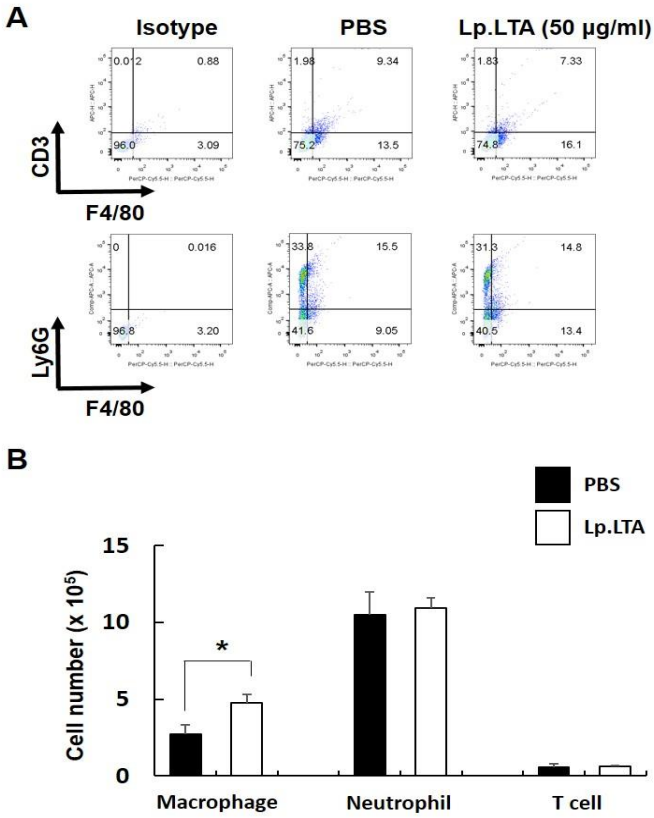

Figure 21. LTA increases macrophage expansion in wound area. Skin wounds were created in the mouse dorsal skin under sterile conditions with 8 mm biopsy punch (n = 5 per group). The wound areas were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 days after wounding. After treatment, the wound tissues were isolated and enzymatically digested to obtain single cell. (A) Then, the cells were stained with PerCP-conjugated anti-mouse F4/80, APC-conjugated anti- mouse, APC-conjugated anti- mouse CD3 and were analyzed by flow cytometry to determine population change of macrophages, neutrophils and T cells, respectively. (B) The total number of macrophages, neutrophils, and T cells were counted. Data is a representative data of five independent experiments. *, $P < 0.05$, compared with PBS-treatment control group.

3.2.5. LTA increases tissue macrophage-associated gene expression in wound area

It is well known that inflammatory and tissue macrophages responses perform opposing activities of killing and repairing [102, 148]. Furthermore, tissue macrophages decrease inflammatory macrophage recruitment in a wound area [149]. Therefore, it is possible that the decrease of pro-inflammatory factors in the Lp.LTA-treated area could be due to the Lp.LTA increasing tissue macrophage in the wound area. To confirm this, the wounds were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 and 4 days after wounding. Inflammatory macrophage associated genes such as CD86 and TNF-α and tissue macrophage associated genes such as CD206, YM-1, and FIZZ-1 in wound area were measured by using real-time RT-PCR analysis. As shown in figure 22, CD86 and TNF-α mRNA levels were decreased in Lp.LTA-treated wound compared with PBS-treated wound, (Fig. 22A and 22B). Furthermore, the tissue macrophage associated genes such as CD206, Ym-1, and FIZZ-1 mRNA levels were increased more than those in the PBS-treated wound (Fig. 22C-22E). These results indicate that Lp.LTA increases tissue macrophage in wound area.

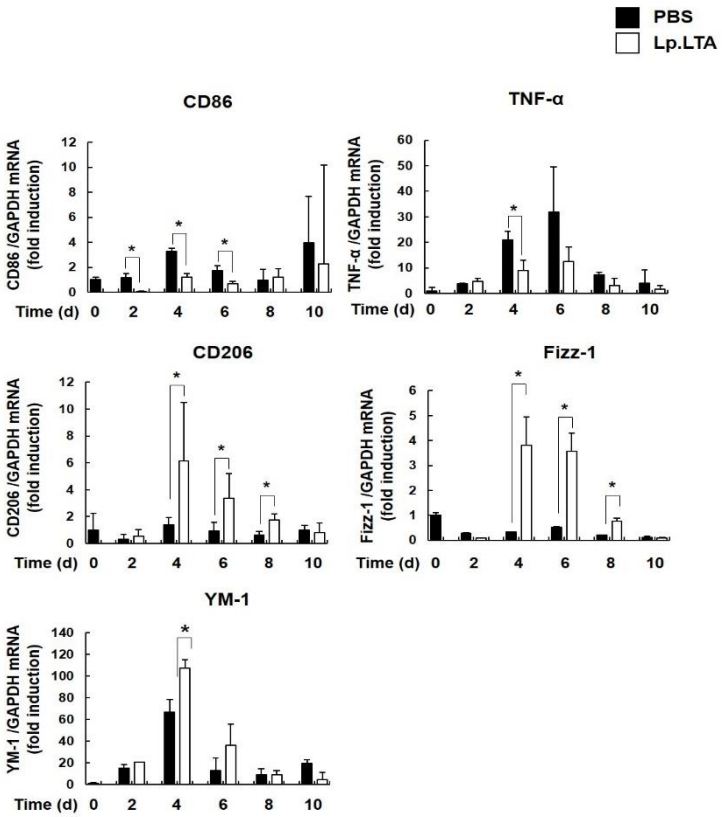

Figure 22. LTA increases tissue macrophage-associated genes expression in wound area. Skin wounds were created in the mouse dorsal skin under sterile conditions with 8 mm biopsy punch (n = 5 per group). The wound areas were treated with PBS (20 μl) or Lp.LTA (50 μg/20 μl) and re-treated at 2 and 4 days after wounding. After treatment, the wound tissues were harvested and total RNA was prepared using TRIzol reagent. After cDNA synthesis using random hexamers, relative mRNA expression levels of (A and B) inflammatory macrophage-associated genes (CD86 and TNF-α) and (C-E) tissue macrophage-associated genes (CD206, YM-1, and FIZZ-1) were measured by real time RT-PCR and results were normalized to GAPDH expression as a control. Data representative data of three independent experiments. *, $P < 0.05$, compared with non-treatment control group.

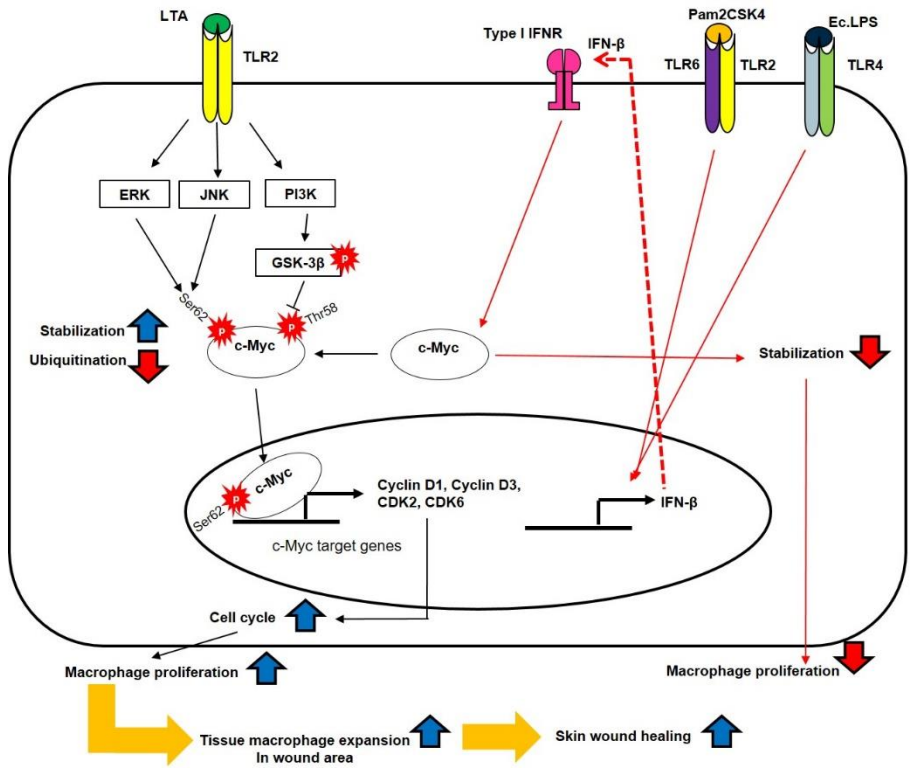

Figure 23. Schematic illustration of the proposed action mechanism of LTA-induced macrophage proliferation contributing to wound healing. LTA improves wound healing progression to bind TLR2, followed by activation of MAPK which phosphorylates Ser62 of c-Myc, while Thr58 of c-Myc phosphorylation is blocked by PI3K/GSK-3β activation. Phosphorylated c-Myc results in down-regulation of ubiquitination and increases c-Myc stability. Cell cycle related genes such as cyclin D1/D3, CDK2/6 are increased by c-Myc, which processes cell cycle to increase macrophage proliferation. Meanwhile, Pam2CSK4 or Ec.LPS induces IFN-β, which down-regulates the c-Myc stability and macrophage proliferation.

Chapter IV. Discussion

Macrophage proliferation is one of the most important immune defense mechanisms. In addition, macrophages play an essential role in the innate immune responses by performing wound healing. [85]. This study demonstrated that LTA could promote macrophage proliferation by increasing c-Myc stability via TLR2. LTA also increased macrophage proliferation in both *in vitro* and *in vivo* model system. Moreover, the present study identified three action mechanisms for the LTA-induced macrophage proliferation: (i) induction of cell cycle process and the expression of cell cycle-related proteins such as cyclin D1, cyclin D3, CDK2, and CDK6, (ii) enhanced stability of c-Myc via down-regulation of ubiquitination, and (iii) TLR2, ERK, JNK, and PI3K/GSK-3β-dependent signaling pathways (Figure 22). Taken together, these results suggest that LTA might be an important Gram-positive bacterial component that induces macrophage proliferation which would contribute to wound healing process.

An increased expression of c-Myc in macrophages is important in the wound healing process. In this study, it was demonstrated that LTA increased c-Myc expression in macrophages. According to previous reports, c-Myc has the ability to control the induction of wound healing factors such as MMP-9, VEFG and TGF-β in macrophages [150]. In the present study, it was observed that when LTA was treated in a wound area, expression of the wound healing factors

MMP-9 and MMP-2 was increased. Also, it was found that proliferation of the cells, which was critical part of the wound healing process, increased more when the cells were co-cultured with macrophages that highly expressed c-Myc, compared to when the cells were co-cultured with macrophages that expressed a low level of c-Myc [151]. In addition, c-Myc has a key role in immuno-regulatory functions by alternative activated macrophage expansion, resulting in promotion of angiogenesis and wound healing [152]. Collectively, these results demonstrate that LTA-induced c-Myc expression in macrophages is one of the key players that facilitate wound healing process. In this study, LTA promoted macrophage proliferation through c-Myc. This result is in agreement with previous studies showing that macrophage growth factor M-CSF increases macrophage proliferation in RAF-1/ERK/c-Myc dependent manner and up-regulates CDK2 and cyclin D1 activities [153]. Oxidized low-density lipoprotein (oxLDL) induces macrophage proliferation by inducing ERK phosphorylation in BMDM. This study also showed that LTA increased macrophage proliferation via activation of ERK, JNK and PI3K through increasing c-Myc stability. Furthermore, LPS and IFN-γ decrease M-CSF induced c-Myc expression, which further block M-CSF induced macrophage proliferation [109]. The results of the present study also exhibited that Ec.LPS or Pam2CSK4 did not induce macrophage proliferation and c-Myc expression in macrophages. Collectively, these results demonstrated that c-Myc has essential role in macrophage proliferation.

This study showed that LTA induced F4/80^{high} macrophage expansion in mice peritoneum. According to previous reports, recruited macrophages and tissue macrophage express different surface phenotype, which are F4/80^{low} and F4/80^{high}, respectively. [85, 154]. Furthermore, previous report suggests that F4/80^{high} peritoneal macrophage proliferation is increased during helminth infection. For example, the rodent filarial nematode *Litomosoides sigmodontis* induces F4/80^{high} macrophage proliferation in mice peritoneal rather than macrophage recruitment. They also suggest that thioglycollate injection increases F4/80^{+} macrophages, but these accumulated macrophages express a lower level of F4/80 than local macrophages [85]. It is already well known that IL-4 has the ability to increase macrophage proliferation. In previous reports, IL-4 signals directly induce local macrophage proliferation which express F4/80^{high} in mice peritoneum [101]. In addition, Ki67 and BrdU are proliferative markers of macrophages. Consistent with these findings, the present study also showed that LTA induced an increase in macrophages that expressed the proliferation markers, Ki67 and Brdu. Collectively, these results indicate that LTA has the ability to increase tissue resident macrophage proliferation which contributes to wound healing process. Previous reports suggest that tissue macrophage is a key regulator of wound healing process. For example, when the tissue macrophage is depleted by hydrocortisone acetate (0.6 mg/g body weight), clearance of neutrophil, fibrin, erythrocytes and other debris is decreased and consequently the wound healing is delayed [124]. Furthermore,

in early stage of wound healing, angiogenesis and myofibroblast differentiation are delayed in macrophage depleted mice. During the mid-stage of wound healing, macrophages are important for stabilization of vascular structures and transition of granulation [155]. When the wounded skin was treated with M-CSF, wound healing of the skin was accelerated by macrophage proliferation [156]. IL-4 directly induces tissue-resident macrophage proliferation which could have an effect on immune regulation and pro-wound repair [101]. In addition, delayed wound healing by depletion of macrophage is recovered by peritoneal macrophage transfusion [157]. Collectively, these results suggest that LTA-induced macrophage expansion in a wound area might be an important factor in accelerating wound healing process.

LTA might have the ability to increases tissue macrophage expansion but not recruitment of macrophage from blood. This study showed that LTA increased tissue macrophage markers such as CD206, YM-1, and Fizz-1, but decreased inflammatory macrophage markers such as CD86 and TNF-α in a wound area. Some possible hypotheses regarding this discrepancy can be made that most of tissue macrophages originate mainly from circulating monocytes and inflammatory macrophages have the ability to increase their number by proliferation [158]. Furthermore, tissue macrophages have the ability to regulate inflammatory macrophage infiltration. According to previous reports, when tissue macrophages are initially depleted, a large number of macrophages is recruited [159]. Another possible explanation is that c-Myc is a key regulator

of macrophage proliferation, but circulating macrophages express low levels of the c-Myc compared with M2 macrophages [150]. In this study, it was observed that c-Myc is essential for LTA-induced macrophage proliferation. However, according to previous report, macrophages have the ability to change their phenotype in specific circumstances. Therefore, it is possible that recruited macrophages could be differentiated into tissue macrophages by LTA treatment. Previous reports find that macrophages have the capacity to change their phenotype according to different stimulators. For example, tissue macrophages induced by exposure to IFN-γ or in combination with LPS can be polarized to express inflammatory phenotype markers [149]. Similarly, macrophages treated with IL-4 can be re-polarized to express anti-inflammatory phenotype markers [36]. However, this study did not find that LTA had the ability to exhibit phenotypic plasticity. Further studies are needed to elucidate the role of LTA on macrophage differentiation.

This study demonstrated that IFN-β is a key regulator of TLR ligand-induced macrophage proliferation. Unlike LTA, Pam2CSK4 and Ec.LPS induce IFN-β expression in RAW 264.7 cells. In addition, LTA-induced macrophage proliferation and c-Myc expression were down-regulated by IFN-β treatment. These results can be explained by previous reports that IFN-β reduces a steady level of c-Myc protein by decreasing c-Myc stability [160]. Furthermore, LPS induces IFN-β expression in RAW 264.7 cells through phosphorylation of STAT-1 [161]. TLR2 ligand, Pam2CSK4 also induces IFN-β via MyD88-IRF1

and –IRF7 pathways [142]. Moreover, when type I IFN receptor is blocked by neutralizing antibody against type I IFN receptor, both Ec.LPS and Pam2CSK4 increase c-Myc expression and cell proliferation [162]. Indeed, this study showed that when type I IFN receptor was blocked, induction of decreased macrophage proliferation and c-Myc expression by Pam2CSK4 or Ec.LPS were recovered. Thus, these results demonstrated that Pam2CSK4 or Ec.LPS did not induce macrophage proliferation because of the induction of IFN-β expression in macrophages. However, it was not found as to what makes the difference in the expression ability of IFN-β while both LTA and Pam2CSK4 were recognized by TLR2. IFN-β expression appears to be critical in the wound healing process. Previous reports suggest that IFN-β decreases epithelial cell proliferation via protein kinase C pathway [163] and increases c-Myc proteolysis in macrophages, resulting in the arrest of the macrophage proliferation [160]. Indeed, the present study showed that LTA-induced macrophage proliferation and c-Myc expression was down-regulated in the presence of IFN-β. Furthermore, when the IFN-β was locally injected into a wound area, the wound healing process was delayed [164]. In addition, it was found that LPS or bacterial lipoprotein had the ability to induce IFN-β in macrophages. For example, when the macrophages were treated with LPS, IFN-β expression was increased through STAT-1 pathway. Furthermore, LPS-induced IFN-β production accelerated the induction of iNOS [161]. Ligands for TLR2/1 including Pam3CSK4 and TLR2/6 including Pam2CSK4 have the

ability to induce IFN-β production in BMDM [142]. In this study, it was also found that Ec.LPS or Pam2CSK4 induced IFN-β expression in RAW 264.7 cells. Thus, this difference in the expression of IFN-β could be linked to the fact that LTA promotes the wound healing process whereas LPS or lipoprotein does not.

This study observed that LTA-induced macrophage proliferation via ERK, JNK, and PI3K signaling pathway. According to previous reports, ERK and JNK signaling could decrease c-Myc ubiquitin-mediated degradation through c-Myc Ser62 phosphorylation, resulting in stabilization of c-Myc [114]. M-CSF induces macrophage proliferation through RAF-1/ERK-1/2 signaling pathway. When the ERK signaling pathway is inhibited, c-Myc de-phosphorylation is rapidly induced and proliferation is also decreased [110]. In addition, Ceramide 1-phosphate, known as a mitogen for fibroblasts, increases expression of cyclin D1, c-Myc and macrophage proliferation through ERK1/2, JNK and PI3K/GSK-3β signaling pathways [165]. Therefore, these results suggest that MAPK and PI3K signaling pathways are important for LTA-induced macrophage proliferation.

The present study showed that LTA decreased inflammatory factor expression in a wound area, which would be crucial for the promotion of wound healing process. These results are in agreement with the previous report that inflammatory factors such as iNOS, IL-1βand TNF-α delay wound healing process. Previous studies suggested that nitric oxide have the ability to down-

regulate collagen formation and wound contraction in distinct ways in wound healing model [73]. When IL-1β signaling pathway is inhibited by neutralizing antibody in a wound area, wound healing is improved [166]. In case of TNF-α, it has the ability to down-regulate collagen synthesis, resulting in a delay of wound healing [167]. Furthermore, these inflammatory factors have the ability to decrease macrophage proliferation. For example, urea-induced inhibition of NO production enhances macrophage proliferation by down-regulation of NO-mediated apoptosis [168]. Collectively, these results demonstrate that macrophage expansion in wound area and wound healing process might be affected by the decreased inflammatory factor expression by LTA in a wound area. This study observed that LTA increased expression of anti-inflammatory factor in a wound area. Previous reports have already suggested that anti-inflammatory factors such as IL-4, and IL-13 have the ability to increase wound healing process. For example, *in vivo* treatment with IL-4 decreases wound size and increases type I collagen in ligament healing [169]. Moreover, IL-13 regulates leukocyte infiltration and M2-like macrophage differentiation, which alters wound healing process [170]. In addition, IL-13 also has the ability to increase macrophage proliferation and to induce alternative macrophage activation during inflammation state [171]. Collectively, these results demonstrate that the expression of LTA-induced anti-inflammatory factor might promote wound healing process.

The relationship between bacterial cell wall component and wound healing

process are crucial for understanding bacterial pathogenesis and host immunity. This study showed that LTA induces macrophage proliferation by increasing c-Myc stability. Furthermore, LTA promotes wound healing process through expansion of tissue macrophages in a wound area. These results provide an insight into the role of LTA in wound healing, which could be used in therapeutics to expedite wound healing process after injury.

Chapter V. References

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국문 초록

목적

일반적으로 피부는 세균을 포함한 다양한 미생물이 서식 하고 있는 것으로 알려져 있으며 상처 치유는 세균으로부터 상처를 보호하고 감염 예방을 돕는다. 상처가 생기면 다양한 신호전달에 의하여 상처 치유 과정이 진행되는데 그 중에서도 특히 대식세포는 초기 면역반응 및 조직 재 생성, 그리고 상처 치유를 돕는 다양한 세포들을 증식시키고 활성화시킨다고 알려져 있다. 한편 그람 양성균은 피부 감염을 일으키는 대표적인 세균이고 그람 양성균의 세포벽 구성 물질인 리포테이코익산 (LTA)은 다양한 면역반응을 유도 혹은 억제한다고 알려져 있지만 현재까지 상처 치유에서의 LTA의 역할은 정확하게 규명되어 있지 않다. 따라서 본 연구에서는 LTA가 대식세포의 증식을 통하여 상처 치유에 미치는 영향에 대한 연구를 진행하였다.

실험 방법

대식세포의 증식을 측정하기 위하여 다양한 대식세포 세포주 인 RAW 264.7, THP-1, BV-2 그리고 쥐의 골수세포에서 분화 된 대식세포를 carboxyfluorescein succinimidyl ester (CFSE) 로 염색 후, 증식에 따른 CFSE의 형광도 감소를 유세포분석기를 통하여 측정하였다. LTA에 의한 대식세포 증식에 톨유사수용체 (Toll-like receptor, TLR) 2의 역할을 규명하기 위하여 야생형 쥐와 TLR2-결여 쥐의 골수세포를 얻어 대식세포로 분화 후 증식에 따른 CFSE의 형광도 감소를 유세포분석기를 통하여 비교 분석하였다.

대식세포주의 세포주기 및 세포주기 관련 인자를 확인하기 위하여 RAW 264.7 세포를 LTA 처리 후, propidium iodide 로 DNA를 염색 후, 유세포분석기를 통하여 측정하였고, 세포주기 관련 인자 cyclin-dependent kinase (CDK) 2, CDK6, cyclin D1, 그리고 cyclin D3의 단백질 발현 정도를 Western blotting 법을 이용하여 측정하였다. 세포의 증식을 조절하는 대표적인 전사 인자인 c-Myc의 발현 정도를 Western blotting 법을 이용하여 측정하였다. LTA에 의 한 c-Myc의 안전성의 변화를 측정하기 위하여 RAW 264.7 세포를 cycloheximide (CHX)를 처리 후, LTA에 의한 c-Myc의 안전성 변화를 Western blotting 법을 이용하여 측정하였다. 또한 c-Myc의 안정성에 영향을 미치는 중요 인자인 유비퀴틴화 (ubiquitination) 정도를 측정하기 위하여 RAW 264.7 세포를 LTA 처리 후, MG132를 마지막 3시간 동안 동시 처리하여 얻은 단백질을 면역침강반응 (Immunoprecipitation) 법을 통하여 확인 하였다. 복강 내 대식세포의 증식을 측정하기 위하여 쥐 복강으로 LTA를 처리 후, 복강 내 세포를 얻어 유세포분석기를 통하여 측정하였다. LTA가 상처 치유에 어떻게 영향을 미치는지 알아보기 위하여 쥐의 등 부위에 좌 · 우 2개의 창상을 8 mm 크기로 유발 한 후 각각 phosphate-buffered saline (PBS)와 LTA를 처리하였다. 그 후 상처가 치유되는 과정을 2일 간격으로 사진을 촬영, Image J 프로그램을 이용하여 상처 치유 정도를 분석 하였다. 상처 부위의 면역세포의 분포를 분석하기 위하여 상처 부위의 세포들을 분리하여 대식세포, 호중구세포 및 T 세포의 분포 및 세포 수를 유세포분석기를 통하여 측정하였다. 상처 부위의 상처 치유 정도를 측정하기 위하여 대표적인 상처 치유 지표인 interleukin-6 (IL-6), macrophage inflammatory protein-1alpha (MIP-1α), CXCL2, matrix metalloproteinase-2 (MMP-2), 그리고 MMP-9 와,

염증성 매개인자인 inducible nitric oxide synthase (iNOS), IL-1β, 염증성 대식세포 지표인 cluster of differentiation 86 (CD86), tumor necrosis factor-alpha (TNF-α) 항염증성 매개인자 IL-10, IL-13, 그리고 IL-4 혹은 조직 대식세포 지표인 CD206, Ym-1, found in inflammatory zone-1 (FIZZ-1) 의 발현 정도를 실시간 중합효소 연쇄반응 (Real time RT-PCR) 측정법을 이용하여 각각 확인하였다.

결과

LTA에 의한 대식세포 증식의 증가는 다양한 대식세포주인 RAW 264.7, THP-1, BV-2, 그리고 골수세포에서 분화 된 대식세포 또한 증식하는 것을 확인 하였고, 또한 이러한 능력이 다양한 그람 양성균 (*Lactobacillus plantarum, Streptococcus pneumonie, Staphylococcus aureus*) 유래 LTA에서 공통적으로 일어나는 현상임을 확인 하였다. LTA는 세포 주기를 조절하는 단백질인 CDK2, CDK6, cyclin D1, 그리고 cyclin D3의 단백질 수준에서의 발현을 시간 의존적으로 증가 시켰고, 세포 주기 또한 S기와 G2/M기를 증가 시켜 세포 주기의 회전을 증가 시켰다. 반면, LTA에 의한 대식세포의 증식 증가가 톨유사수용체-2-결여 쥐 유래 대식세포에서는 확인 할 수 없었다. LTA는 세포의 증식을 조절하는 핵심 인자인 c-Myc 의 안전성을 높여 발현을 증가 시켰고, 대식세포 증식의 증가 및 c-Myc의 발현은 ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinases), 그리고 PI3K/GSK-3β (phosphoinositide 3-kinase/glycogen synthase kinase 3 beta) 의존적으로 조절됨을 확인 하였다. 한편, 또 다른 TLR 리간드인 Pam2CSK4 및 lipopolysaccharide (LPS)는 LTA와 다르게 IFN-β을 발현 하였고 IFN-β는 농도 의존적으로 LTA에 의한

대식세포 증식 및 c-Myc 의 발현을 감소시켰으며, Type I IFN receptor를 저지 한 결과, IFN-β에 의해 감소된 의존적으로 LTA에 의한 대식세포 증식 및 c-Myc 의 발현을 회복시켰으며, Pam2CSK4 및 LPS에 의해 감소된 대식세포의 증식 또한 회복하는 것을 확인하였다. 또한 LTA에 의해서 복강 내 다양한 세포들 중 증식 능력을 가진 조직 대식세포의 증가를 확인하였다. 쥐의 상처 치유과정에서 LTA를 처리한 상처가 PBS를 처리한 상처보다 치유되는 속도가 빨랐다. 또한 상처 치유에 연관된 지표 IL-6, MMP-2, 그리고 MMP-9, 또한 PBS를 처리 한 상처보다 LTA를 처리한 상처에서 발현 정도가 높게 나타났다. 반면 대식세포를 불러들이는 인자인 MIP-1α 와 호중구를 불러들이는 인자 CXCL2는 LTA 처리 상처에서 발현이 낮아지는 것을 확인 할 수 있었다. 염증성 매개 인자인 iNOS 와 IL-1β의 발현은 LTA를 처리한 상처부위에서 낮게 발현함을 확인하였고, 그와 반대로 항 염증성 매개인자 IL-10, IL-13, 그리고 IL-4는 LTA 를 처리한 상처 부위에서 PBS 처리 한 상처 부위보다 높게 발현하였다. LTA 처리에 의한 다양한 세포들의 분포 변화를 확인 한 결과 다른 세포와는 다르게 대식세포의 증가를 확인하였다. 또한 염증성 대식세포의 지표인 TNF-α 및 CD86의 발현은 LTA를 처리한 상처부위에서 낮게 발현함을 확인하였고, 그와 반대로 조직 대식세포의 지표인 CD206, Ym-1, 그리고 FIZZ-1는 LTA 를 처리한 상처 부위에서 PBS 처리 한 상처 부위보다 높게 발현하는 것으로 보아 상처 부위에 LTA 처리 시, 조직 대식세포의 증가를 확인 할 수 있었다. 결론적으로 LTA는 조직 대식세포의 증식을 증진 시키는 것을 알 수 있었다.

결론

이상의 연구결과들로부터 다음과 같은 결론을 얻을 수 있었다. LTA는 피부 상처에 처리 시, M2 대식세포의 증가로 인해 상처 치유를 증가 시키는 것을 확인하였다. 또한 LTA에 의한 대식세포의 증가는 c-Myc 의존적으로 톨유사수용체-2를 경유하여 일어나는 것을 확인 하였다. 결론적으로 이를 통하여 LTA는 상처 치유에 의한 염증 완화 및 상처 치유를 촉진 시키는 치료제로 활용 될 수 있다.

주요어: 그람 양성 세균, 리포테이코익산, 대식세포 증식, 조직 대식세포. 톨유사수용체-2, 상처 치유

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