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Lipoteichoic Acid Suppresses Effector T Cells Induced by *Staphylococcus aureus*-Pulsed Dendritic Cells

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Lipoteichoic acid (LTA), uniquely expressed on gram-positive bacteria, is recognized by Toll-like receptor 2 (TLR2) on not only antigen-presenting cells but also activated T cells. Therefore, it is reasonable to assume that LTA is acting on T cells. However, little is known about the effect of LTA on T-cell regulation. In the present study, we investigated the immunomodulatory effects of LTA on $CD4^+$ T cells. Effector $CD4^+$ T cells, induced after co-culture with *S. aureus*-pulsed dendritic cells, produced high levels of interferon-γ, CD25, CD69, and TLRs 2 and 4. When effector $CD4^+$ T cells were treated with LTA, the expressions of the membrane-bound form of transforming growth factor (TGF)-β and forkhead box P3 increased. Coincidently, the proliferation of effector $CD4^+$ T cells was declined after LTA treatment. When TGF-β signaling was blocked by the TGF-β receptor 1 kinase inhibitor, LTA failed to suppress the proliferation of effector $CD4^+$ T cells. Therefore, the present results suggest that LTA suppresses the activity of effector $CD4^+$ T cells by enhancing TGF-β production.

Keywords: Lipoteichoic acid, immune tolerance, regulatory T cells, effector $CD4^+$ T cells, TGF-β

Introduction

Toll-like receptors (TLRs) acting as an initiator of the innate immune response in antigen-presenting cells (APCs) is well known [3]. TLRs are one of the first sensors to recognize pathogen-associated molecular patterns and trigger signal transduction to induce effector molecules, including inflammatory cytokines and chemokines [1].

Lipopolysaccharide (LPS), a representative virulence factor of gram-negative bacteria, activates both MyD88-dependent and -independent pathways upon recognition by TLR4 [1]. Lipoteichoic acid (LTA), a cell wall component of gram-positive bacteria, is sensed by TLR2 [10], initiating only the MyD88-dependent signaling pathway mainly *via* mitogen-associated protein kinase and nuclear factor kappa-B (NF-κB) [2] to produce proinflammatory cytokines such as interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) [25].

Numerous studies have examined TLR-mediated immune responses in APCs, including dendritic cells [18, 23, 29], yet only recently a few reports suggested that T lymphocytes also express TLR2 and TLR4 [9, 19]. Very low levels of TLRs are expressed on naïve T cells, whereas their expression increases in response to T-cell receptor (TCR) stimulation [19]. Effector $CD4^+$ T cells triggered with Pam3CSK4, a TLR2 ligand, induced increases in the cell expansion and production of cytokines, including IL-2, IFN-γ, and TNF-α [19]. In addition, the bacterial lipoprotein, another TLR2 ligand, attenuates the suppressive function of regulatory T cells (Tregs) by downregulating forkhead box P3 (Foxp3) expression [22]. However, other studies have reported conflicting results that the TLR2 ligand increases the suppressive activity through the Tregs [6, 32]. Therefore, these studies clearly left a margin for further study to define the role of TLR2 on T cells.

Tregs suppress CD4 and CD8 T cells *via* a membrane-bound form of TGF-β in a cell-to-cell contact-dependent manner [34]. TGF-β1 latency-associated peptide (LAP) forms complexes with membrane-bound TGF-β, which can be released as an active form after cleavage by serum

proteinases such as plasmin, which increases during the inflammatory response [26, 35] and is used as a marker for the detection of membrane-bound TGF-β [35].

In this study, we generated effector $CD4^+$ T cells after co-culture with *S. aureus*-pulsed DCs to examine the impact of LTA treatment on (i) TLR2 expression, and (ii) proliferation and activation, and (iii) the effect of LTA-induced LAP expression in the effector $CD4^+$ T cells.

Materials and Methods

Reagents

Staphylococcus aureus was obtained from the American Type Culture Collection (Manassas, VA, USA), highly purified, and its LTA was produced as previously described [13]. LPS was purchased from Sigma-Aldrich (USA) and SB239063 (p38 inhibitor chemical reagent) from Calbiochem (USA). Recombinant human granulocyte-macrophage stimulating factor (rhGM-CSF) and interleukin-4 (rhIL-4) were purchased from R&D Systems (USA). Anti-human CD3, CD4, CD25, CD69, TLR2, TLR4, IFN-γ, IL-13, TGF-β1 LAP, and Foxp3 antibodies were from BD Biosciences (USA). Complete medium containing RPMI-1640 Glutamax medium, fetal bovine serum, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) was purchased from Invitrogen (USA). A T-cell activation/expansion kit was purchased from Miltenyi Biotec (USA), and anti-human CD2/CD3/CD28 antibody-complex beads (Miltenyi Biotec) were formulated according to the manufacturer's instructions.

Preparation of Human Monocyte-Derived DCs

All experiments involving human blood were approved by the Institutional Review Board of Seoul National University (IRB No. 1107/001-006). Normal adult blood samples were anonymously provided by the Blood Center of the Korean Red Cross. Peripheral blood mononuclear cells (PBMCs) were isolated from human adult blood by density gradient centrifugation (410 ×*g* for 25 min) using Ficoll-Paque Plus (Amersham Bioscience, UK). $CD14^+$ monocytes (purity >95%) were isolated from PBMCs using a magnetic bead-based positive selection kit (BD Biosciences). The isolated $CD14^+$ monocytes were cultured in complete medium containing rhIL-4 (500 U/ml) and rhGM-CSF (800 U/ml) for 6 days to generate immature DCs; the medium was changed every 3 days. Immature DCs stimulated with LPS (0.1 μg/ml), LTA (2 μg/ml), or ethanol-killed *S. aureus* (10^7 CFU/ml) for 2 days became mature [31].

Characterization of $CD4^+$ T Cells Co-Cultured with Antigen-Pulsed DCs

$CD4^+$ T cells, isolated from PBMCs using a human CD4 T cell (negative) isolation kit (Miltenyi Biotec), were co-cultured with 1×10^5 antigen-pulsed (with LPS, LTA, or *S. aureus*) DCs for 3 days. The cells were stained with anti-human CD4 together with either anti-human CD25 and CD69 antibodies as early activation markers or with anti-human TLR2, TLR4, or IFN-γ antibodies.

For intracellular staining of IFN-γ, the co-cultured cells were incubated with GolgiStop reagent (BD Biosciences) for the last 10 h of culture. After washing with PBS, the cells were stained with anti-human CD4 antibody, permeabilized with Cytofix/Cytoperm solution (BD Biosciences), stained with anti-human IFN-γ antibody, and then analyzed using flow cytometry (FACSCalibur, BD Biosciences).

To examine the proliferation of $CD4^+$ T cells, the cells were labeled with CFSE and cultured with LPS-, LTA- or *S. aureus*-pulsed DCs for 3 days. Then, the cells were stained with anti-human CD4 antibody and examined for their expansion using flow cytometry.

Isolation of Effector $CD4^+$ T Cells After Co-Cultured with Mature DCs

Effector $CD4^+$ T cells were isolated after co-culture with $CD4^+$ T cells with *S. aureus*-pulsed DCs for 3 days, using anti-human CD4 antibody-conjugated magnetic beads (BD Biosciences).

Isolated cells were stained with anti-human CD3 and CD4 antibodies for 15 min and their purity was determined (above 97%, data not shown) by flow cytometry.

Characterization of Effector $CD4^+$ T Cells Treated with LTA or LPS

Effector $CD4^+$ T cells were treated with LTA (1, 2, or 4 μg/ml) or LPS (0.1 μg/ml) for 2 days, and the cells were stained with anti-human CD25, IFN-γ, IL-13, Foxp3, and TGF-β1 LAP antibodies for 15 min to examine their expression profiles. Then, $CD25^+$ cells were gated and analyzed to determine the changes of IFN-γ^+, IL-13^+, $Foxp3^+$, or TGF-β1 LAP^+ $CD4^+$ T cells using flow cytometry.

Proliferation of Effector $CD4^+$ T Cells

To measure the proliferation, effector $CD4^+$ T cells were stimulated with LTA with/without a TGF-β R1 kinase inhibitor (Calbiochem). Briefly, the cells (1×10^5 /well) were cultured on 96-well plates and stimulated with anti-human CD2/CD3/CD28 antibody-complex beads (1:20 bead to cell ratio) together with 2 μg/ml LTA for 3 days. In some inhibitor tests, the cells were pretreated with a TGF-β R1 kinase inhibitor for 1 h before the LTA treatment. After the culture, 20 μl of MTT (Sigma-Aldrich) solution (5 mg/ml in PBS) was added to each well and the plates were incubated for an additional 4 h at 37°C in a humidified incubator with 5% CO_2. After removing the supernatant, the blue formazan product was dissolved in 200 μl of dimethyl sulfoxide per well for 5 min. Changes in color were measured using a microplate reader (Molecular Device, USA).

p38 Signaling for TGF-β1 LAP Induced by LTA Treatment

To identify the role of p38 signaling in effector $CD4^+$ T cells treated with LTA to induce TGF-β1 LAP, effector $CD4^+$ T cells were pretreated with SB239063 (p38 inhibitor) for 1 h, followed by

addition of LTA (2 μg/ml) and incubation for a further 2 days. Then, the cells were stained with anti-human CD25 and TGF-β1 LAP antibodies for 15 min. The $CD25^+$ cells were gated and the proportion of TGF-β1 LAP^+ cells analyzed using flow cytometry.

Statistical Analysis

All data are presented as means ± standard deviations for each treatment group. Statistical significance was evaluated using a 2-tailed paired *t*-test. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Induction of Effector $CD4^+$ T Cells After Co-Culture with *S. aureus*-Pulsed DCs

DCs have the best ability for presenting antigenic molecules to T lymphocytes. In the previous study, we observed that LTA, LPS, or ethanol-killed *S. aureus* induced maturation of DCs, which can activate $CD4^+$ T cells though presenting antigenic molecules [31]. After recognizing antigenic molecules loaded on the major histocompatibility complex (MHC) of mature DCs, $CD4^+$ T cells are activated, producing some cytokines such as IFN-γ, IL-2, and IL-4. In addition, the surface expression of activation markers, such as CD25 and CD69, increased [14]. To generate effector T cells *in vitro*, human $CD4^+$ T cells were co-cultured with LTA-, LPS-, or *S. aureus*-pulsed DCs. As shown in Fig. 1A, $CD4^+$ T cells co-cultured with LTA- or LPS-pulsed DCs produced slightly higher IFN-γ levels compared with that of control $CD4^+$ T cells co-cultured with immature DCs, whereas $CD4^+$ T cells co-cultured with *S. aureus*-pulsed DCs produced about 8-fold higher IFN-γ than that in the immature DC control. A high percentage of $CD4^+$ T cells with $CD25^+$ and/or $CD69^+$ were also detected when co-cultured with *S. aureus*-pulsed DCs, compared with that of other groups (Fig. 1B). Next, we measured the proliferation of CFSE-labeled $CD4^+$ T cells co-cultured with LTA-, LPS-, or *S. aureus*-pulsed DCs. Similar to Fig. 1A, greater proliferation was detected in $CD4^+$ T cells co-cultured with *S. aureus*-pulsed DCs than those of the other $CD4^+$ T-cell groups (Fig. 1C). These results show that although LTA and LPS can induce the maturation of DCs, LTA- or LPS-pulsed DCs could not activate $CD4^+$ T cells as compared with *S. aureus*-pulsed DCs.

A recent study suggested that effector $CD4^+$ T cells stimulated by TCR ligation express more TLRs compared with naïve $CD4^+$ T cells [19].

In contrast to LPS or LTA stimulation, inactivated *S. aureus* and its antigenic molecules, including enterotoxin B and peptidoglycan, increased the induction and proliferation of antigen-specific $CD4^+$ T cells [20, 24, 33], suggesting that *S. aureus* could not only activate DCs but also directly stimulate TCR signaling in T cells. Therefore, we examined whether the expression of TLRs are changed on $CD4^+$ T cells when co-cultured with *S. aureus*-pulsed DCs, since LTA- or LPS-pulsed DCs had no effect to activate $CD4^+$ T cells. The results showed that the expressions of TLR2 and TLR4 were higher (Fig. 1D), coincident with an increased level of CD25 (Fig. 1E), on $CD4^+$ T cells co-cultured with *S. aureus*-pulsed DCs than the $CD4^+$ T cells co-cultured with immature DCs. It was noteworthy that the expressions of TLR2 and TLR4 were increased in anti-CD3 antibody-activated $CD4^+$ T cells when compared with that on control $CD4^+$ T cells (data not shown). These results suggest that activated effector $CD4^+$ T cells that had been co-cultured with *S. aureus*-pulsed DCs express TLR2 and TLR4.

Differentiation of LTA- or LPS-Treated Effector $CD4^+$ T Cells

To investigate the direct impact of LTA or LPS, we isolated $CD4^+$ T cells using magnetic beads after co-culturing T cells with *S. aureus*-pulsed DCs. Since the dose of LTA or LPS used to pulse DCs in the present study had a minimal activating effect on T cells ([31] and Fig. 1), effector $CD4^+$ T cells co-cultured with *S. aureus*-pulsed DCs were used for further study. No apparent apoptosis was detected in isolated $CD4^+$ T cells after the stimulation (data not shown). The cells were then gated on CD25, the early activation marker, and IFN-γ, IL-13, TGF-β1 LAP, and Foxp3 expressions were analyzed. IFN-γ and IL-13 productions were not changed in LTA- and LPS-treated effector $CD4^+$ T cells compared with control cells, whereas TGF-β1 LAP and Foxp3 expression increased by approximately 2-fold in LTA-treated effector $CD4^+$ T cells but not in LPS-treated cells (Fig. 2). These results indicate that LTA could induce the activation and/or differentiation of Tregs.

Suppression of Effector $CD4^+$ T-Cell Proliferation by TGF-β1 LAP Induced by LTA Treatment

Next, we treated effector $CD4^+$ T cells with various concentrations (0, 1, 2, and 4 μg/ml) of LTA to further validate the TGF-β1 LAP production in effector $CD4^+$ T cells. TGF-β1 LAP production increased to the greatest extent when 2 μg/ml LTA was added (Fig. 3A), whereas the soluble TGF-β1 level remained unchanged (data not shown). We treated the cells with a specific p38 inhibitor prior to the LTA treatment to identify the signaling pathway that underlies TGF-β1 LAP expression in effector $CD4^+$ T cells, because p38 signaling has been implicated in TGF-β1

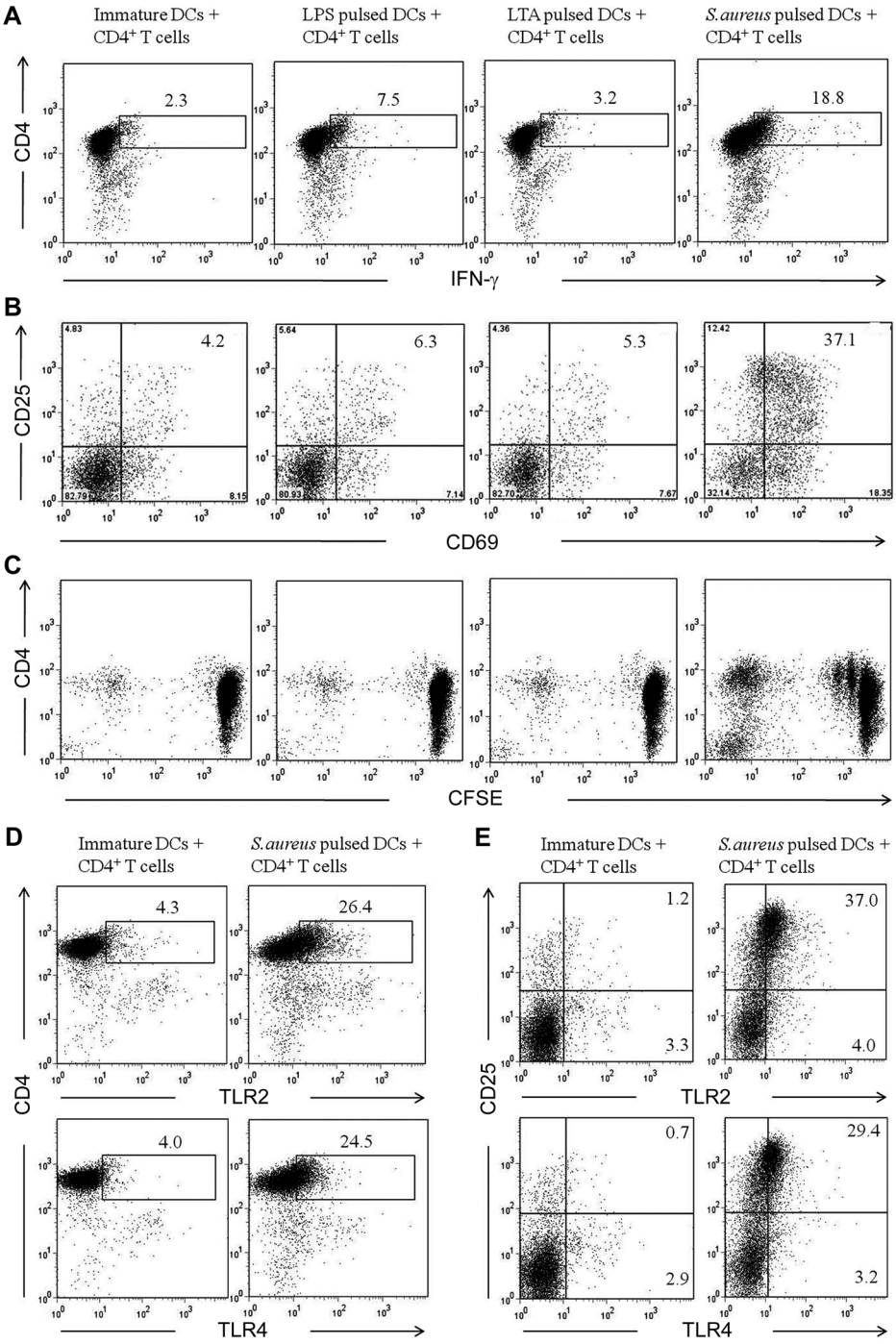

Fig. 1. *Staphylococcus aureus*-pulsed dendritic cells induce the expression of TLRs 2 and 4 on effector $CD4^+$ T cells.

Immature DCs were stimulated with 2 μg/ml of lipoteichoic acid (LTA), 0.1 μg/ml lipopolysaccharide (LPS), or ethanol-killed *S. aureus* (1×10^7 CFU/ml) for 2 days and then cultured with naïve $CD4^+$ T cells for an additional 3 days. Then, the cells were stained with (**A**) anti-human CD4 and interferon (IFN)-γ, or (**B**) CD25 and CD69 antibodies. (**C**) LTA-, LPS- or *S. aureus*-pulsed DCs were co-cultured with CFSE-labeled naïve $CD4^+$ T cells for 3 days and then stained with anti-human CD4 antibody. Additionally, $CD4^+$ T cells co-cultured with *S. aureus*-pulsed DCs were stained with (**D**) anti-human CD4, TLR2, and TLR4 antibodies, or (**E**) anti-human CD25, TLR2, and TLR4 antibodies. Then, $CD4^+$ cells were analyzed for (**A**) IFN-γ production, (**B**) T-cell activation, (**C**) proliferation, and (**D**), (**E**) the expression of TLR2 and TLR4 using flow cytometry. Data are representative of three independent experiments with similar results.

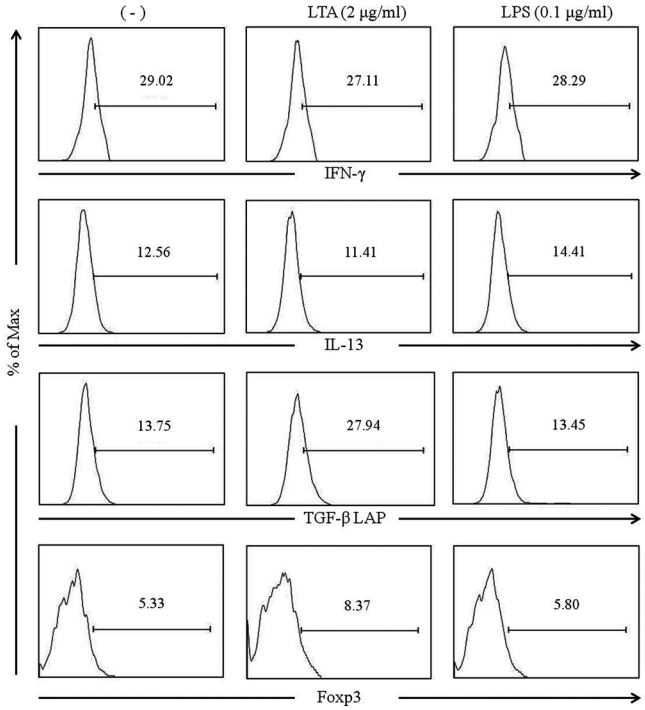

Fig. 2. LTA induces TGF-β1 LAP and Foxp3 production in effector CD4+ T cells.

Activated CD4+ T cells were isolated after co-culture of naïve CD4+ T cells with *Staphylococcus aureus*-pulsed DCs using anti-human CD4 antibody conjugated to magnetic beads. Then, the cells were stained with anti-human CD3 and CD4 antibodies to measure CD3+CD4+ T cell purity. Isolated CD4+ T cells were stimulated with LTA (2 μg/ml) or LPS (0.1 μg/ml) for 2 days and then stained with anti-human CD25, IFN-γ, IL-13, TGF-β1 LAP, or Foxp3 antibodies. The stained cells were gated to CD25+ and analyzed by flow cytometry. Data are representative of three independent experiments with similar results.

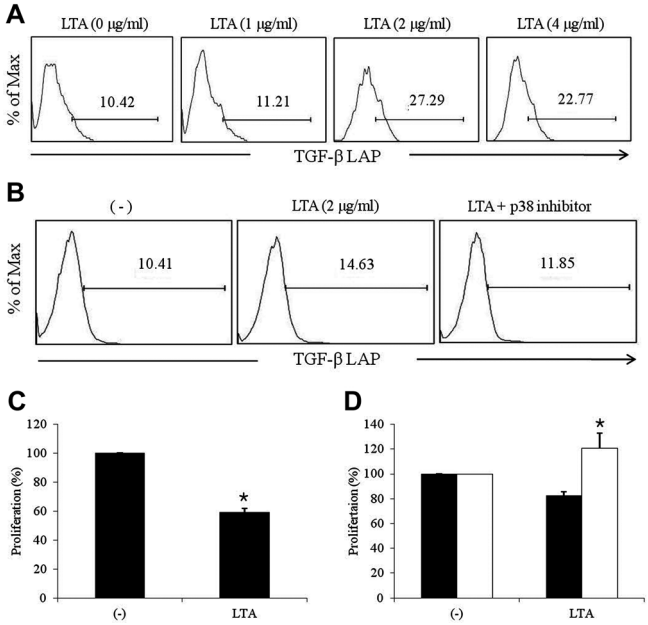

Fig. 3. LTA-induced TGF-β1 LAP regulates effector CD4+ T-cell proliferation.

(**A**) Activated CD4+ T cells were isolated after co-culture of naïve CD4+ T cells with *Staphylococcus aureus*-pulsed DCs using anti-human CD4 antibodies conjugated to magnetic beads. Effector CD4+ T cells were stimulated with various LTA concentrations (0, 1, 2, or 4 μg/ml) for 2 days. (**B**) Effector CD4+ T cells were incubated with 10 μM of the p38 inhibitor SB239063 for 1 h and treated with LTA (0 or 2 μg/ml) for an additional 2 days. The cells were stained with anti-human CD25 and TGF-β1 LAP antibodies. Then, the cells were gated to CD25+ and the proportion of TGF-β1 LAP+ cells was determined by flow cytometry; results are presented as a histogram. (**C**) The proliferation of effector CD4+ T cells stimulated with LTA (0 or 2 μg/ml) and anti-human CD2/CD3/CD28 antibody-complex beads was measured using the MTT assay. (**D**) The effect of pretreatment with a TGF-β R1 kinase inhibitor (filled bars indicate without inhibitor and empty bars with), and the proliferation of effector CD4+ T cells stimulated with LTA (0 or 2 μg/ml) or anti-human CD2/CD3/CD28 antibody-complex beads was measured using the MTT assay. Proliferation levels are expressed as a percentage of the color intensity that resulted from LTA (2 μg/ml) treatment relative to that of untreated controls. *Indicates statistically significant at $p < 0.05$ compared with the control.

production in effector T cells [21]. As a result, the LTA-induced production of TGF-β1 LAP was decreased in effector CD4+ T cells compared with that in cells that were not treated with the p38 inhibitor (Fig. 3B).

TGF-β1 LAP and soluble TGF-β1 are important sources of the cytokines that suppress T-cell proliferation and differentiation into Tregs [35]. Therefore, we treated effector CD4+ T cells for 3 days with LTA and low-dose anti-CD3/CD2/CD28 antibody-complexed beads (1:20 bead-to-cell ratio) to examine the ability of TGF-β1 LAP to regulate T-cell expansion. The results showed that the proliferation decreased significantly ($p < 0.05$) when cells were treated with LTA (Fig. 3C). Next, the cells were pretreated with a TGF-β R1 kinase inhibitor before LTA treatment, and the proliferation of effector CD4+ T cells was examined. Interestingly, blocking the TGF-β receptor did not inhibit the proliferation of cells treated with LTA ($p < 0.05$), compared with that of untreated cells (Fig. 3D). These results indicate that p38 signaling is responsible for LTA-induced TGF-β LAP, which inhibits the expansion of effector CD4+ T cells.

Discussion

The major findings of our study were (i) *S. aureus*–pulsed DCs caused increased expressions of TLR2 and TLR4 on activated $CD4^+$ T cells, (ii) LTA, but not LPS, induced an increase of TGF-β1 LAP and Foxp3 in effector $CD4^+$ T cells, and (iii) LTA-mediated TGF-β1 LAP production through p38 signaling inhibited effector $CD4^+$ T cell proliferation.

LPS is an endotoxin derived from gram-negative bacteria that induces sepsis in infected hosts. In 1998, TLR4 was discovered as an LPS-recognition receptor that induces the expression of proinflammatory cytokines [28]. LTA, on the other hand, is a surface molecule expressed uniquely on gram-positive bacteria [15]. A critical role for TLR2 signaling was demonstrated in TLR2-deficient mice that are highly susceptible to invasion by gram-positive bacteria [36]. The direct effect of TLR signaling in T lymphocytes has been reported, in which treatment with TLR2 ligands induces the activation and survival of T cells [12, 16]. Intriguingly, TLR2 signaling in Tregs is also suggested to induce the proliferation and functional activation of Tregs [27]. Accordingly, these findings led us to hypothesize that LTA might be involved in not only the action of antigen-presenting cells but also in direct regulation of effector T cells through the TLR signaling pathway. To clarify this, we examined the TLR expression, cytokine production, and proliferation in activated $CD4^+$ T cells after LTA treatment.

We observed that $CD4^+$ T cells activated with *S. aureus*-pulsed DCs increased the production of IFN-γ, the surface expression of CD25 and CD69, and the proliferation, compared with those of $CD4^+$ T cells activated with LTA- or LPS-pulsed DCs. Although it has been suggested that TLR1, 2, 3, 4, 5, 7, and 9 mRNA are induced when human T cells are activated [8], poly I:C (a TLR3 ligand) or CpG (a TLR9 ligand) fail to stimulate activated or memory T cells [19]. When we measured the expression levels of TLR2 and TLR4, they were increased on the effector $CD4^+$ T cells co-cultured with *S. aureus*-pulsed DCs. It is possible that the effector $CD4^+$ T cells could directly interact with TLR2 or TLR4 ligands. Therefore, we examined whether LTA or LPS can influence the production of cytokines and transcript factors.

A recent study showed that heat shock protein 60 (HSP60), known as a TLR2 ligand that enhances the suppressive activity of Tregs, inhibits the proliferation of $CD4^+CD25^-$ T cells [37]. To test whether LTA could enhance Tregs activity, effector T cells were treated with LTA, and then the expression levels of IFN-γ, IL-13, TGF-β1 LAP, and Foxp3 were measured. We observed that LTA increased TGF-β1 LAP and Foxp3 expressions in effector $CD4^+$ T cells, whereas it did not increase IFN-γ and IL-13. Interestingly, LPS did not increase TGF-β1 LAP or Foxp3 in effector $CD4^+$ T cells. LPS has no effect on the function of Tregs [7, 19, 32], which suggested that the lack of a co-receptor, such as CD14, could interfere with and/or reduce the LPS recognition in $CD4^+$ T cells. It is possible that the balance between Myd 88-dependent (by LPS and LTA) and -independent (by LPS) signaling plays a critical role on this, since LTA acts towards tolerance, whereas LPS acts towards inflammatory responses. Furthermore, it has been shown that negative regulators induced by LPS [4] are different from those by LTA [17].

Foxp3 and TGF-β1 are key molecules in differentiation and proliferation of Tregs [30]. Foxp3 is the transcriptional factor that induces the differentiation of Tregs producing TGF-β [34] and TGF-β1 LAP [26] which cause a suppressive feature. We demonstrated that LTA increased TGF-β1 LAP production in a dose-dependent manner on effector $CD4^+$ T cells through p38 signaling. Furthermore, we showed that the significant suppression of effector $CD4^+$ T cell proliferation after LTA treatment is dependent on TGF-β signaling. Our findings suggest that LTA suppressed effector $CD4^+$ T cell proliferation by enhancing TGF-β1 LAP production.

The suppressive effect of LTA on the proliferation of $CD4^+$ T cells could be a type of immune-evading strategy to increase the viability and infectivity of *S. aureus*. The immune-escaping mechanism by intracellular bacteria has been well defined, including the cytotoxicity of toxin(s) to host cells and protein A-mediated resistance to phagocytic activity [11].

Indeed, it has been demonstrated that TLR ligands downregulate T-cell proliferation by enhancing the suppressive function of Tregs [5, 7, 32]. Furthermore, the p38 signaling pathway is involved in TGF-β1 production and the suppressive functions of Tregs [35]. Therefore, we investigated the relationship between LTA and the p38 signaling pathways for the induction of TGF-β1 LAP in effector $CD4^+$ T cells. Inhibition of the p38 pathway led to decreased TGF-β1 LAP production, indicating that p38 mediates LTA-induced TGF-β1 LAP production in effector $CD4^+$ T-cells.

In conclusion, effector $CD4^+$ T cells expressed TLR2 and TLR4 after LTA treatment, and LTA, but not LPS, treatment suppressed effector $CD4^+$ T cell proliferation by enhancing TGF-β1 LAP production through the p38 signaling pathway.

Acknowledgments

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