J. Microbiol. Biotechnol. (2017), 27(4), 709–717
https://doi.org/10.4014/jmb.1611.11072

jmb

Dendritic Cell-Mediated Mechanisms Triggered by LT-IIa-B_5, a Mucosal Adjuvant Derived from a Type II Heat-Labile Enterotoxin of *Escherichia coli*

Chang Hoon Lee[1,2*], George Hajishengallis[3], and Terry D. Connell[4*]

[1]*Bio & Drug Discovery Division, Center for Drug Discovery Technology, Korea Research Institute of Chemical Technology (KRICT), Daejeon, Republic of Korea*

[2]*Immunotherapy Convergence Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), Daejeon 34114, Republic of Korea*

[3]*Department of Microbiology, School of Dental Medicine, The University of Pennsylvania, Philadelphia, PA 19104, USA*

[4]*Department of Microbiology & Immunology and The Witebsky Center for Microbial Pathogenesis and Immunology, The Jacob's School of Medicine and Biomedical Sciences, The University at Buffalo, State University of New York, Buffalo, NY 14214, USA*

Received: November 29, 2016
Revised: January 29, 2017
Accepted: January 31, 2017

First published online
February 1, 2017

**Corresponding authors*
C.H.L.
Phone: +82-42-860-7414;
Fax: +82-42-861-4246;
E-mail: Lee2014@krict.re.kr
T.D.C.
Phone: +716-829-3364;
Fax: +716-829-2158;
E-mail: connell@buffalo.edu

pISSN 1017-7825, eISSN 1738-8872

Mucosal tissues are the initial site through which most pathogens invade. As such, vaccines and adjuvants that modulate mucosal immune functions have emerged as important agents for disease prevention. Herein, we investigated the immunomodulatory mechanisms of the B subunit of *Escherichia coli* heat-labile enterotoxin type IIa (LT-IIa-B_5), a potent non-toxic mucosal adjuvant. Alternations in gene expression in response to LT-IIa-B_5 were identified using a genome-wide transcriptional microarray that focused on dendritic cells (DC), a type of cell that broadly orchestrates adaptive and innate immune responses. We found that LT-IIa-B_5 enhanced the homing capacity of DC into the lymph nodes and selectively regulated transcription of pro-inflammatory cytokines, chemokines, and cytokine receptors. These data are consistent with a model in which directional activation and differentiation of immune cells by LT-IIa-B_5 serve as a critical mechanism whereby this potent adjuvant amplifies mucosal immunity to co-administered antigens.

Keywords: LT-IIa-B_5, mucosal vaccine adjuvant, dendritic cells, immune cell migration, lymph node

Introduction

A number of important human pathogens invade the body through mucosal surfaces. In these cases, the mucosal immune system acts as the first line of defense against infection [1]. Development of vaccines that stimulate the immune system on those mucosal surfaces is critical for generating immune protection against those mucosal pathogens [2]. A confounding feature of the mucosal immune system, however, is a well-developed immune tolerance mechanism [3], which likely evolved to avoid the generation of unwarranted immune responses to commonly encountered environmental Ags [4]. As a consequence, it is difficult to evoke strong mucosal immune responses solely by administering Ag to a mucosal site. To overcome this immunological barrier, Ags are commonly administered in the presence of a strong mucosal adjuvant. Two of the earliest mucosal adjuvants described belong to the type I subfamily of heat-labile enterotoxins, which includes cholera toxin of *Vibrio cholerae* and LT (referred herein as LT-I) of *Escherichia coli*, and their respective non-toxic B subunits [5–8]. Recently, the powerful mucosal adjuvant properties of a second subfamily of heat-labile enterotoxins were described. LT-IIa is a type II heat-labile enterotoxin of *E. coli* [5, 8], which when delivered simultaneously with an Ag by the mucosal route (*e.g.*, intranasal or oral), amplifies

Ag-specific primary and memory immune responses on the mucosal surface and in the systemic circulation [9, 10]. Similar augmented Ag-specific immune responses were observed when Ags were mucosally delivered in the presence of LT-IIa-B_5, the non-toxic B subunit of LT-IIa [8].

Understanding the mechanisms by which mucosal adjuvants trigger strong memory immune responses to co-administered Ags is critical for development of successful vaccines against serious human mucosal pathogens. In this study, we investigated the mucosal adjuvant properties of LT-IIa-B_5 using genome-wide gene expression profiling, specifically in regard to the responses to dendritic cells (DCs), a cell type that has been shown to react in vitro and in vivo to the pentamer [8, 11]. Using a mouse mucosal immunization model, we evaluated the effect of LT-IIa-B_5 on migration of DCs to the lymph nodes and changes in expression of the CC chemokine receptors CCR7, CCR1, CCR2, and CCR5 and of co-stimulatory molecules and inflammatory cytokines. Analysis of these DC-mediated changes will provide new insights into the underlying immune mechanisms that are augmented by LT-IIa-B_5 and suggest new strategies that can be employed to develop mucosal vaccines to enhance Ag-specific immune responses on mucosal surfaces.

Materials and Methods

Mice

Female wild-type and DO11.10 mice [12] expressing T cell receptors specific to the ovalbumin (OVA) epitope (323–339) on the BALB/c background were purchased from The Jackson Laboratory (USA). All mice were used at 6 to 8 weeks of age. Animal experiments were approved by the Institutional Animal Care and Use Committee at The University at Buffalo (IACUC No. MIC01010Y).

Cloning and Purification of LT-IIa-B_5

Methods for the cloning and purification of His-tagged LT-IIa-B_5 were reported previously [8]. In brief, the plasmid pHN1.1 encoding LT-IIa-B_5 was introduced into *E. coli* DH5 F′Kan (Life Technologies, USA). Expression of LT-IIa-B_5 by the transformant was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside to log-phase cultures. LT-IIa-B_5 was extracted from the cells using isotonic shock and purified by nickel affinity chromatography (Qiagen, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using polyclonal antibodies against the LT-IIa holotoxin were used to confirm the homogeneity of the purified LT-IIa-B_5. The purified protein was analyzed for endotoxin by use of an end-point quantitative *Limulus* amoebocyte lysate assay (Charles River Endosafe, USA). The level of endotoxin in the purified preparations of LT-IIa-B_5 was 0.003 ng/μg protein, which is considerably below the level required to evoke an immune response in cells.

Migration Assay

Mice were treated with 15 μl of phosphate-buffered saline (PBS) or 100 μg of OVA in PBS (Sigma-Aldrich, USA) in the presence or absence of 10 μg of LT-IIa-B_5. The final volume was divided between both external nares. After 3 days, the cervical lymph nodes (CLN) were removed and digested for 1 h in PBS containing 1 mg/ml collagenase D and 0.5 mg/ml DNase I. Total cells from the CLN were resuspended in FACS buffer (PBS containing 2% bovine serum albumin) by forcing the tissue through a 40 μm nylon cell strainer (BD Biosciences, USA). Cells from the mouse CLN were stained with a phycoerythrin-conjugated anti-mouse CD11c antibody (clone N418; BioLegend, USA) and an allophycocyanin-conjugated anti-mouse I-A/I-E antibody (clone M5/114.15.2; BioLegend, USA) with an Fc-blocking anti-mouse CD16/32 antibody (clone 93; BioLegend, USA). The cells were then washed and resuspended in FACS buffer. The number of $CD11c^+MHCII^+$ DCDC was determined using a FACSCalibur four-color flow cytometer (BD Biosciences, USA) with flow cytometry counting beads (Thermo Fisher Scientific, USA).

Generation of Bone Marrow-Derived DC

Cells from the bone marrow flushed from the femurs and tibiae of 6 to 8 weeks old BALB/c mice were cultured in 6-well plates at 1×10^6 cells/ml in complete RPMI-1640 (Mediatech, USA) that was supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 1% HEPES, 1% sodium pyruvate, 1% non-essential amino acids (Invitrogen, USA), 10 ng/ml recombinant murine IL-4 (eBioscience), and 20 ng/ml recombinant murine granulocyte macrophage-colony stimulating factor (BioLegend, USA). The culture medium was replaced on day 3 and the cells were used on day 6, at which point over 90% were $CD11c^+$ (data not shown). Non-adherent $CD11c^+$ DCs were sorted on day 6 using anti-CD11c-coated magnetic beads, according to the manufacturer's directions (Miltenyi Biotech, Germany). Sorted $CD11c^+$ DCs were used for subsequent experiments.

Microarray and Data Analysis

Purified DCs (5×10^6) in 10 ml of complete RPMI culture medium were incubated for 6 h at 37°C with PBS alone (untreated) or PBS containing 1 μg/ml of LT-IIa-B_5. Total RNA was isolated from the DCs using Trizol (Invitrogen, USA). Analyses were conducted using an Affymetrix whole-genome expression microarray (Mouse genome 430 2.0 array). Data were summarized and normalized with the robust multi-average method implemented in Affymetrix Expression Console software. The results of the gene-level robust multi-average analysis were exported and used for the differentially expressed gene analysis. Statistical significance of the expression data was determined using the local pooled error test and fold change, in which the null hypothesis was that no difference existed among groups. The false discovery rate was controlled by

adjusting the *p*-values using the Benjamini-Hochberg algorithm. For a differentially expressed gene set, hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene-enrichment and functional annotation analyses for the significant probe list were performed using DAVID (http://david.abcc.ncifcrf.gov/home.jsp). All data analyses and visualization of differentially expressed genes were conducted using R 3.0.2 software (http://www.r-project.org).

Statistical Analysis

Analysis of variance was used for multiple comparisons. Unpaired-*t* tests were performed to analyze differences between two groups. Statistical analyses were performed using the Prism software package (GraphPad Software, USA). Differences were considered significant at $p \leq 0.05$.

Results

Gene Expression Profile of DCs Regulated by LT-IIa-B_5, and between-Group Analysis of the Microarray Data

Transcriptional analyses were performed using 99% pure $CD11c^+$ DCs (data not shown) to investigate changes in the genome-wide transcription levels between untreated and LT-IIa-B_5-treated cells. The analysis identified 732 genes that were upregulated by ≥2-fold and 1,028 genes that were downregulated by ≥2-fold in LT-IIa-B_5-treated DCs in comparison to untreated DCs. To understand the biological significance of these microarray data, we further analyzed those differentially expressed genes using functional enrichment analysis with the DAVID database, a Gene Ontology (GO) software tool (http://david.abcc.ncifcrf.gov/home.jsp). Because GO (http://www.geneontology.org/) annotations are important for investigation of functional significance from large dataset, we followed these annotations based on the GO consortium (3) and found 101 significant GO terms, the majority of which were associated with triggering immune response and defense response. The top GO term was immune response. The 113 LT-IIa-B_5-mediated differentially regulated genes associated with the immune response GO term are shown in Table 1. Among 1,760 genes significantly differentially regulated in DCs by LT-IIa-B_5, 79 immune response-associated genes (4.48% from total differentially regulated by LT-IIa-B_5) were upregulated and 34 immune response-associated genes (1.9% from total differentially regulated by LT-IIa-B_5) were downregulated (Figs. 1A and 1B). Furthermore, in the top 10 GO terms, four terms (immune response, response to wounding, inflammatory response, cytokine-cytokine receptor interaction) were related with immune regulation directly (data not shown). These results strongly suggest

Table 1. Immune response-associated genes significantly differentially regulated by LT-IIa-B_5.

Genes	Fold change	Genes	Fold change	Genes	Fold change
AF251705	-4.0	Enpp1	-2.2	Mx2	3.9
Bcl3	3.0	Enpp2	4.5	Myo1f	-4.6
Btla	53.7	Exo1	-2.0	Ncf1	2.1
C1qa	-3.7	Faim3	2.6	Nfkb2	2.6
C1qb	-5.5	Fcgr1	-6.6	Oas1a	2.2
C1qc	-4.0	Fcgr2b	-2.1	Oas1b	2.8
Cblb	2.1	Gbp2	2.5	Oas3	5.7
Ccl2	2.3	Gbp3	2.7	Oasl1	15.7
Ccl3	5.3	Gbp6	2.7	Oasl2	6.1
Ccl4	3.5	Gpr183	2.0	Osm	2.3
Ccl6	-2.8	H2-DMb1	-2.0	P2ry14	-2.2
Ccr2	-13.2	Hc	3.7	Pag1	-7.3
Ccr5	-3.0	Icam1	2.3	Polm	2.1
Ccr7	2.1	Ifih1	3.8	Procr	8.4
Cd14	8.8	Ifng	10.4	Ptx3	5.2
Cd1d1	2.7	Il10	10.9	Rnf125	4.8
Cd28	-3.2	Il12a	46.4	Rnf19b	3.1
Cd300a	-10.2	Il12b	113.4	Rsad2	15.6
Cd300lb	-29.8	Il15	4.1	Serping1	2.4
Cd300ld	-3.3	Il1a	27.5	Sp110	2.4
Cd300lf	-11.2	Il1b	107.2	Susd2	4.9
Cd70	12.5	Il1f6	3.5	Swap70	2.1
Cfb	5.8	Il1f9	4.0	Tgtp1	2.5
Cfp	-2.6	Il1r1	2.3	Tlr13	-5.1
Clec4a2	-26.3	Il1rn	3.8	Tlr4	-2.6
Clec4e	7.0	Il23a	110.0	Tlr7	-2.7
Clec4n	-3.3	Il6	10.8	Tnf	12.8
Clec5a	-8.8	Irf7	21.9	Tnfrsf1b	2.3
Clec7a	-2.6	Irf8	4.1	Tnfsf10	2.1
Csf2	15.1	Irgm1	2.9	Tnfsf11	5.2
Csf3	38.4	Klhl6	-3.4	Tnfsf12	-4.1
Cxcl1	73.3	Lat2	-5.0	Tnfsf13b	-2.3
Cxcl10	25.4	Lif	4.4	Tnfsf4	3.3
Cxcl2	26.7	Lta	4.7	Tnfsf9	6.9
Cxcl3	4.7	Ly96	2.5	Traf3ip2	2.4
Cxcl5	37.2	Malt1	2.5	Ung	-2.6
Cxcl9	7.0	Mr1	-2.1		
Dhx58	4.4	Mx1	3.2		

that transcriptional regulation of DC triggered by LT-IIa-B_5 is highly associated with immune responses.

Selectively Regulated Expression of DC Chemokine Receptors and Enhanced Homing of These Cells into the Lymph Nodes Triggered by LT-IIa-B_5

The expression pattern of DC chemokine receptors activated by LT-IIa-B_5 was selectively regulated, which likely enhanced the cells' homing capacity into the draining lymph nodes. DCs treated with LT-IIa-B_5, but not untreated DCs, transformed into a mature DC phenotype by significantly increasing the expression of CCR7, a critical chemokine receptor for homing to the lymph nodes [11, 13] (Fig. 2A). In addition, DCs activated with LT-IIa-B_5 showed reduced expression levels of CCR1, CCR2, and CCR5, which are inflammatory chemokine receptors that facilitate migration of inflammatory immune cells, such as T lymphocytes and monocytes, into inflamed peripheral tissues [14]. Downregulation of these inflammatory chemokine receptors and the subsequent upregulation of CCR7 likely induce migration of DCs into the draining lymph nodes.

The homing process of DCs after exposure to Ags in peripheral tissues is a critical step for triggering Ag-specific adaptive immune responses orchestrated by T lymphocytes, and is initiated by the migration of Ag-priming DCs into the lymph nodes. To confirm our hypothesis of an association between the selectively regulated expression pattern of chemokine receptors and homing to the draining lymph nodes by DCs, we investigated the number of DCs in the CLN after intranasal administration of LT-IIa-B_5 into naïve BALB/c mice. Mice without prior exposure to LT-IIa-B_5 were intranasally administered OVA in the absence and presence of LT-IIa-B_5. After 3 days, the number of DCs in the CLN was significantly increased in the mice that had received OVA + LT-IIa-B_5 in comparison with mice that had received only OVA in the absence of the mucosal adjuvant (Fig. 2B).

LT-IIa-B_5-Mediated Transformation of DCs by Selectively Regulating Expression of Co-Stimulatory Molecules and Cytokines

Critical changes in the expression of relevant genes in immature bone marrow-derived DCs were observed upon treatment with LT-IIa-B_5. In our previous study, we also found that CD40, CD80, and CD86 were increased in an in vivo mice model after intranasal administration of LT-IIa-B_5 [11]. Co-stimulatory molecules such as CD40, CD80, and CD86 that are required for robust Ag-priming of naïve and central memory T cells were significantly enhanced [15]. Thus, we want to confirm expression of CD40, CD48, and CD86 in our microarray data. As a result, we found the similar results that CD40, CD80, and CD86 were increased in DCs treated with LT-IIa-B_5 (Fig. 3A). In addition, changes in the expression levels of the intercellular adhesion molecules (ICAMs) of DCs were evident. Specifically, the transcriptional levels of ICAM1 were increased, while the levels of ICAM2 and ICAM4 were decreased in DCs activated by LT-IIa-B_5 (Fig. 3A). Moreover, we found that another adhesion molecule, TGFβi, was significantly downregulated by DCs activated with LT-IIa-B_5 (Fig. 3A). Thus, we found that LT-IIa-B_5 selectively regulated the transcriptions of various adhesion molecules, including ICAM1, 2, 3, and TGFβi. Activating DCs with LT-IIa-B_5 elicited a dramatic increase in the transcription of numerous inflammatory cytokines, including tumor necrosis factor-alpha (TNFα), IL-1α, IL-1β, IL-2, IL-6, and IL-12 (Fig. 3B). In contrast, transcription levels of transforming growth factor-beta (TGFβ)1 and TGFβ2 were not significantly changed (Fig. 3B). Furthermore, DCs activated with LT-IIa-B_5 reduced their sensitivity to the anti-inflammatory cytokine TGFβ by decreasing the expression of TGFβ receptors 1 and 2 (Fig. 3C). Significantly increased transcription levels of

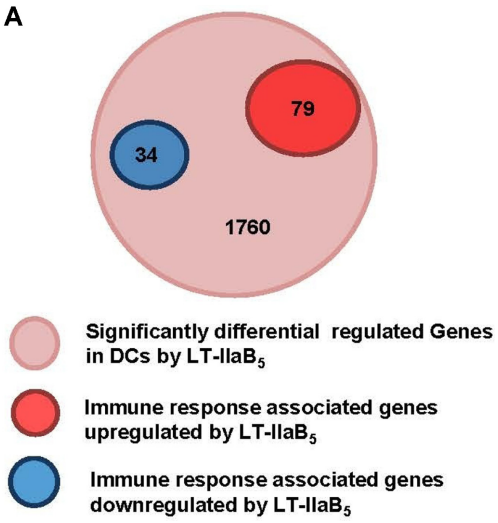

Fig. 1. LT-IIa-B_5 regulates a broad range of genes associated with immune response.

(**A**) Venn diagrams displaying the number of upregulated (red circles, 79 genes) and downregulated (blue circles, 34 genes) genes associated with immune response among the significantly differentially regulated genes in dendritic cells (DCs) by LT-IIa-B_5 (pink circles, 1,760 genes). (**B**) Heat map representation of the expression levels of immune response-associated genes that were changed by more than 2-fold after LT-IIa-B_5 stimulation of three independent bone marrow-derived DC experiments.

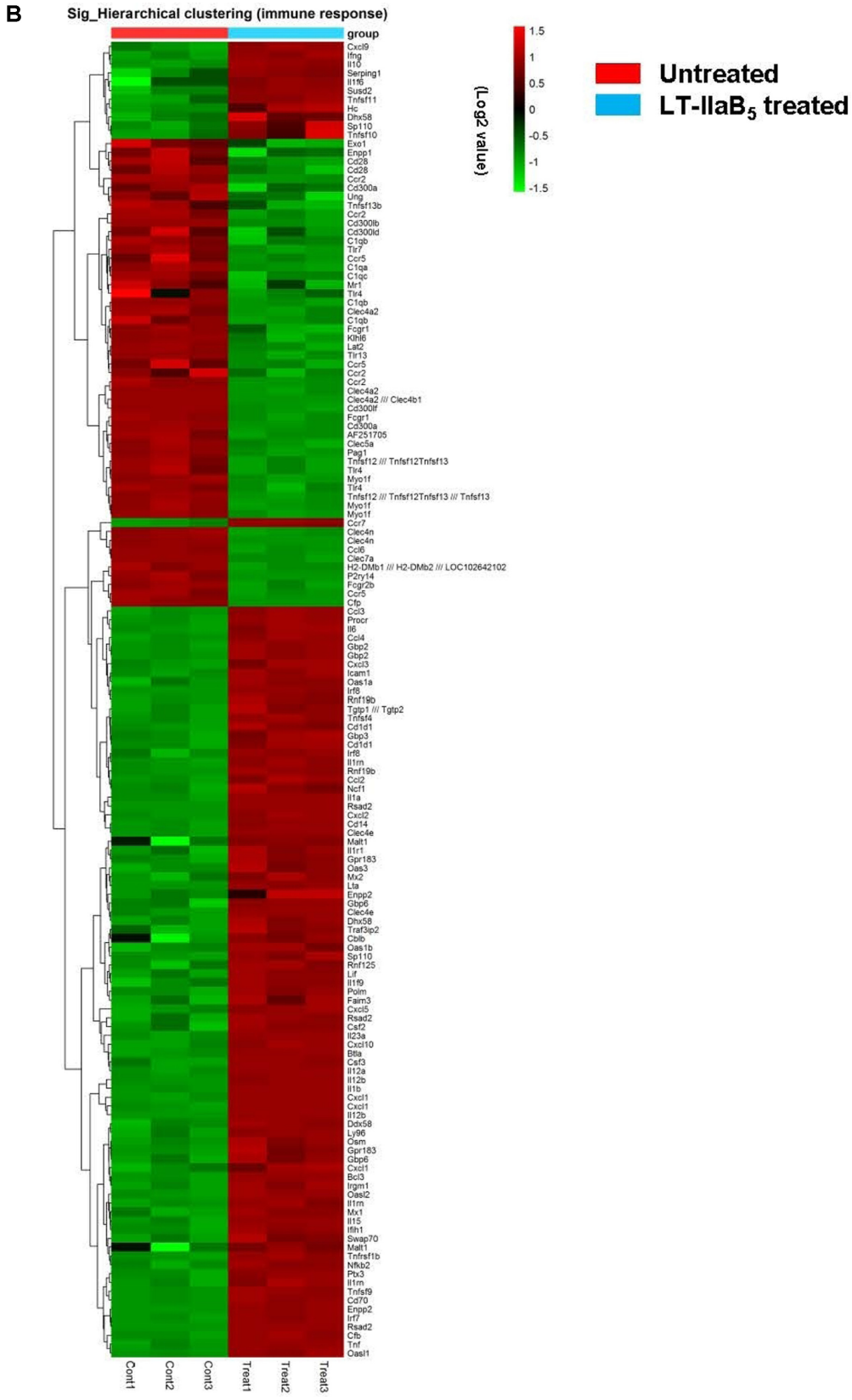

Fig. 1. Continued

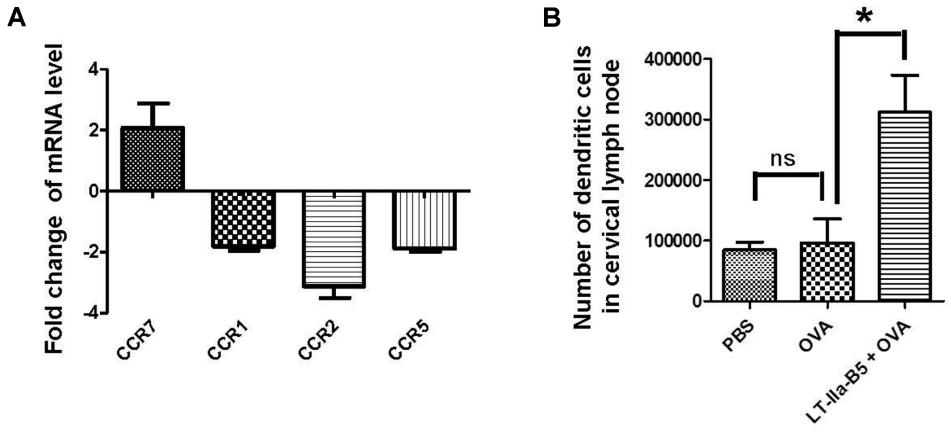

Fig. 2. Selectively regulated expression of chemokine receptors on dendritic cells (DCs) by LT-IIa-B_5, and enhanced homing capacity of DCs in draining lymph nodes.

(**A**) Fold changes (log_2 value) in the mRNA levels of genes encoding CCR1, CCR2, CCR5, and CCR7 on bone marrow-derived DCs activated by LT-IIa-B_5 compared with untreated cells. All groups showed significant differences from untreated cells at $p < 0.05$ (unpaired Student's *t*-test, $n = 3$). Data are expressed as means ± SD. (**B**) The number of DCs in the cervical lymph nodes of female BALB/c mice administered ovalbumin (OVA) + LT-IIa-B_5 intranasally compared with mice given only OVA. Data are expressed as means ± range. Data are from two separate experiments with a total of four mice per treatment group. ns: not significant; *$p < 0.05$ (unpaired Student's *t*-test).

chemokine genes CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL3, CXCL5 , CXCL9, CXCL10, and CXCL16 were also observed in our microarray (Fig. 3D).

Discussion

Most pathogens invade the human body through mucosal tissues. Thus, there has been increasing efforts to develop vaccines that evoke strong pathogen-specific mucosal immune responses on the mucosal surfaces to interdict pathogens at an early stage of infection [16–18].

In this study, we performed a genome-wide transcriptional profile analysis to investigate the mechanisms by which LT-IIa-B_5, a potent mucosal adjuvant, regulates DCs, an immune cell type that orchestrates important cellular responses. Although several studies have described the properties by which LT-IIa-B_5 enhances mucosal immune responses to co-administered Ags [7, 16], few studies have evaluated the molecular and genetic mechanisms that drive immune augmentation by LT-IIa-B_5. Herein, we employed a genome-wide analysis to determine the transcriptional responses of DCs that were engendered by exposure to LT-IIa-B_5. We found that LT-IIa-B_5 regulated a broad array of gene sets in DCs. Globally, LT-IIa-B_5 upregulated transcription of 732 genes and downregulated transcription of 1,028 genes.

To better understand the functional meaning of the changes in gene expression fostered by LT-IIa-B_5, we analyzed the expression profiles obtained from microarray data of untreated and LT-IIa-B_5-treated DCs using a GO software tool. This analysis revealed that 79 genes, 4.48% from total genes differentially regulated in DCs by LT-IIa-B_5, were upregulated, and 34 genes, 1.9% from total genes differentially regulated in DCs by LT-IIa-B_5, were downregulated, each of which is involved in immune response. Furthermore, we found that LT-IIa-B_5 enhanced the lymph node-homing capacity of DCs by selective regulation of chemokine receptors. Specifically, transcription of CCR7 was highly increased by treatment of DCs with LT-IIa-B_5, while expression of genes encoding CCR1, CCR2, and CCR5 was decreased. These in vitro observations and their implications for adjuvant activity were supported by the in vivo migration assay. Indeed, after intranasal administration of LT-IIa-B_5, the number of DCs in mice was increased dramatically in the proximal draining CLN. In our previous report [11], we showed that enhanced migration of DCs into draining CLN after intranasal administration of LT-IIa-B_5 was dependent on CCR7, using CCR7 knockout mice, which did not show increased migration of DCs after administration of LT-IIa-B_5. In this study, we suggest that those LT-IIa-B_5-triggered migration of DCs was not mediated only by the increase of CCR7 on DCs, but also selective regulation of multiple chemokine receptors like CCR1, 2, 5 and adhesion molecules like ICAM1, 2, 4, and TGFβi. DCs exposed to LT-IIa-B_5 exhibited robust changes in transcription of various gene sets that

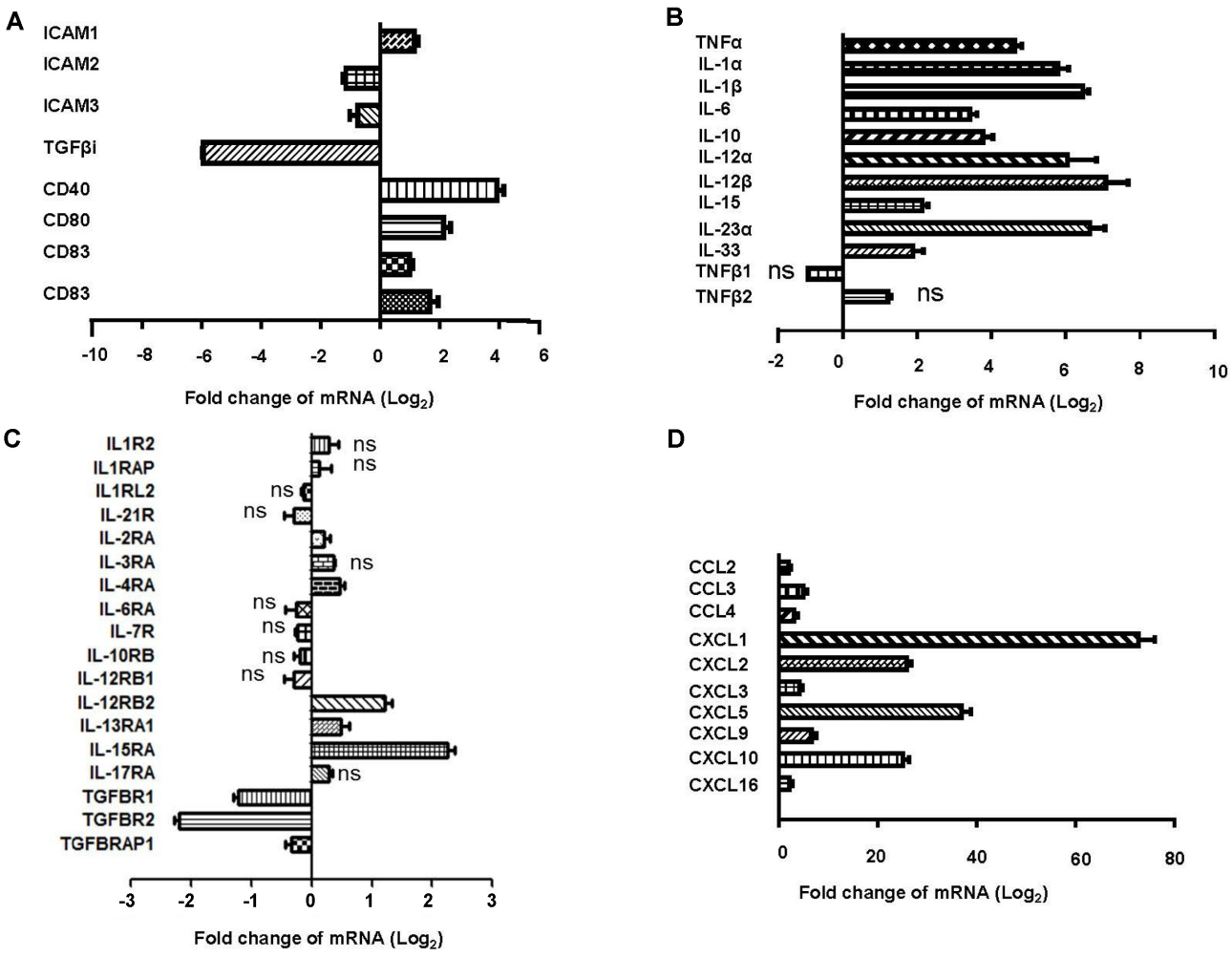

Fig. 3. Selectively regulated expression of co-stimulatory molecules and cytokines of mature dendritic cells (DCs) differentiated by LT-IIa-B_5.

(**A**) Fold changes ($\log_2$ value) in mRNA levels of ICAM1, 2, and 4, TGFβi, and CD40, 80, 83, and 86, of bone marrow-derived DCs activated by LT-IIa-B_5 compared with untreated cells. All groups were significantly different from untreated cells at $p < 0.05$ (unpaired Student's *t*-test). (**B**) Fold changes ($\log_2$ value) in mRNA levels of cytokines of bone marrow-derived DCs activated by LT-IIa-B_5 compared with untreated cells. All groups were significantly different from untreated cells at $p < 0.05$ except groups indicated as not significant (ns) (unpaired Student's *t*-test). (**C**) Fold changes ($\log_2$ value) in mRNA levels of cytokine receptors on bone marrow-derived DCs activated by LT-IIa-B_5 compared with untreated cells. All groups were significantly different from untreated cells at $p < 0.05$ except groups indicated as ns (unpaired Student's *t*-test). Data are expressed as means ± SD ($n = 3$). (**D**) Fold changes ($\log_2$ value) in mRNA levels of chemokines of bone marrow-derived DCs activated by LT-IIa-B_5 compared with untreated cells. All groups were significantly different from untreated cells at $p < 0.05$ (unpaired Student's *t*-test).

likely regulate other tissues and immune cells in nearby environments. Specifically, we found differential transcription of several ICAMs in DCs activated by LT-IIa-B_5. Transcription of ICAM1 was significantly increased, whereas the transcription of ICAM 2 and 4 was decreased. Markey *et al.* [19] reported that ICAM1 was a critical component for the immunological synapse between dendritic and T cells. Thus, we suggest that LT-IIa-B_5 can enhance the immunological synapse formation between dendritic and T cells via increased expression of ICAM1 to boost Ag-specific T cell activation. These findings suggested that ICAM1 might be a critical molecule for promoting strong T cell–DC synapse formation and subsequently boosting Ag-specific T cell activation. Such sophisticated regulation of multiple chemokine receptors and adhesion molecules of DCs might be required for their migration into draining lymph nodes after their exposure to LT-IIa-B_5.

Furthermore, the increased expression of pro-

inflammatory cytokines including TNFα, IL-1α, IL-1β, IL-2, IL-6, and IL-12, and chemokines including CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL3, CXCL5, CXCL9, CXCL10, and CXCL16 observed, in part, explains the strong adaptive immune responses that are driven by LT-IIa-B_5. In contrast, the transcription of the anti-inflammatory cytokines TGFβ1 and TGFβ2 by LT-IIa-B_5 was not changed (Fig. 3B), indicating a directional activation or differentiation mechanism by LT-IIa-B_5 on DCs. TGFβ1 and TGFβ2 have critical roles in differentiating regulatory T cells to downregulate immune responses. Furthermore, DCs activated with LT-IIa-B_5 reduced their sensitivity to the anti-inflammatory cytokine TGFβ by decreasing the expression of TGFβ receptors 1 and 2. In contrast to the reduction of TGFβ receptor expression, LT-IIa-B_5 sensitizes DCs to several critical inflammatory cytokines, including IL-12 and IL-15, by increasing transcription of IL-12 receptor beta 2 and IL-15 receptor alpha. These directional changes in cytokine expression and cytokine receptor expression patterns in DCs activated with LT-IIa-B_5 mediate, at least in part, an acceleration of directional differentiation for priming Ags, and boost the immune system efficiency by minimizing signals by TGFβ and regulatory T cells that downregulate immune responses. Furthermore, these changes on cytokine signaling might affect the expression of adhesion molecules like TGFβI and ICAM1, 2, 3, which might be critical for optimal migration or synapse formation with T cells. Those changes on DCs are likely critical for LT-IIa-B_5 in triggering the strong immune responses to co-administered Ags that have been observed in various studies [7, 11].

Taken together, our results indicate that the adjuvant mechanism of LT-IIa-B_5 is likely a driving force for the differentiation of DCs into the morphotypes required to evoke strong immune responses. This conclusion is based on the capacity of LT-IIa-B_5 to upregulate the migration, cytokine expression, chemokine expression, and the sensitivity of DCs themselves to immunomodulatory mediators. Overall, the results of this transcriptional study revealed an important profile for the DC-mediated adjuvant mechanism triggered by LT-IIa-B_5. Genome-wide analysis is a useful and powerful tool for revealing the potential mechanisms underlying the adjuvant properties of LT-IIa-B_5 and other mucosal adjuvants, and for suggesting avenues for productive experimentation to dissect these immune mechanisms. In conclusion, the current genome-wide analysis broadens our understanding of the immunological processes triggered by LT-IIa-B_5 and potentially other immunomodulatory molecules, which will likely facilitate development of better mucosal vaccines that incorporate these powerful adjuvants.

Acknowledgments

This research was supported by a grant from The John R. Oishei Foundation awarded to T.D.C., Public Health grants R0rk DE13833 and R56 DE13833 awarded to T.D.C. and DE017138 awarded to G.H., and the National Research Council of Science & Technology (NST) grant from the Korea government (MSIP) (No. CRC-15-02-KRIBB) awarded to C.L.

References

1. Holmgren J, Czerkinsky C. 2005. Mucosal immunity and vaccines. *Nat. Med.* **11:** S45-S53.
2. Willimann K, Legler DF, Loetscher M, Roos RS, Delgado MB, Clark-Lewis I, *et al*. 1998. The chemokine SLC is expressed in T cell areas of lymph nodes and mucosal lymphoid tissues and attracts activated T cells via CCR7. *Eur. J. Immunol.* **28:** 2025-2034.
3. van Ginkel FW, Nguyen HH, McGhee JR. 2000. Vaccines for mucosal immunity to combat emerging infectious diseases. *Emerg. Infect. Dis.* **6:** 123-132.
4. Commins SP. 2015. Mechanisms of oral tolerance. *Pediatr. Clin. North Am.* **62:** 1523-1529.
5. da Hora VP, Conceicao FR, Dellagostin OA, Doolan DL. 2011. Non-toxic derivatives of LT as potent adjuvants. *Vaccine* **29:** 1538-1544.
6. Isaka M, Yasuda Y, Mizokami M, Kozuka S, Taniguchi T, Matano K, *et al.* 2001. Mucosal immunization against hepatitis B virus by intranasal co-administration of recombinant hepatitis B surface antigen and recombinant cholera toxin B subunit as an adjuvant. *Vaccine* **19:** 1460–1466.
7. Langridge W, Denes B, Fodor I. 2010. Cholera toxin B subunit modulation of mucosal vaccines for infectious and autoimmune diseases. *Curr. Opin. Investig. Drugs* **11:** 919-928.
8. Lee CH, Nawar HF, Mandell L, Liang S, Hajishengallis G, Connell TD. 2010. Enhanced antigen uptake by DCs induced by the B pentamer of the type II heat-labile enterotoxin LT-IIa requires engagement of TLR2. *Vaccine* **28:** 3696-3705.
9. Boberg A, Gaunitz S, Brave A, Wahren B, Carlin N. 2008. Enhancement of epitope-specific cellular immune responses by immunization with HIV-1 peptides genetically conjugated to the B-subunit of recombinant cholera toxin. *Vaccine* **26:** 5079-5082.
10. Harrod T, Martin M, Russell MW. 2001. Long-term persistence and recall of immune responses in aged mice after mucosal immunization. *Oral Microbiol. Immunol.* **16:** 170-177.
11. Lee CH, Masso-Welch P, Hajishengallis G, Connell TD. 2011. TLR2-dependent modulation of dendritic cells by LT-

IIa-B_5, a novel mucosal adjuvant derived from a type II heat-labile enterotoxin. *J. Leukoc. Biol*. **90:** 911-921.

12. Murphy KM, Heimberger AB, Loh DY. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+$TCRlo thymocytes in vivo. *Science* **250:** 1720-1723.
13. Saeki H, Moore AM, Brown MJ, Hwang ST. 1999. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature DCs from the skin to regional lymph nodes. *J. Immunol.* **162:** 2472-2475.
14. Gouwy M, Struyf S, Berghmans N, Vanormelingen C, Schols D, Van Damme J. 2011. CXCR4 and CCR5 ligands cooperate in monocyte and lymphocyte migration and in inhibition of dual-tropic (R5/X4) HIV-1 infection. *Eur. J. Immunol.* **41:** 963-973.
15. Leitner J, Grabmeier-Pfistershammer K, Steinberger P. 2010. Receptors and ligands implicated in human T cell costimulatory processes. *Immunol. Lett.* **128:** 89-97.
16. Lundgren A, Leach S, Tobias J, Carlin N, Gustafsson B, Jertborn M, *et al*. 2013. Clinical trial to evaluate safety and immunogenicity of an oral inactivated enterotoxigenic *Escherichia coli* prototype vaccine containing CFA/I overexpressing bacteria and recombinantly produced LTB/CTB hybrid protein. *Vaccine* **31:** 1163-1170.
17. Miller T, Fanton M, Nickelson S, Mason H, Webb S. 2012. Safety and immunogenicity of bacterial and tobacco plant cell line derived recombinant native and mutant *Escherichia coli* heat-labile toxin in chickens. *Avian Pathol.* **41:** 441-449.
18. Sun M, He C, Cong Y, Liu Z. 2015. Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. *Mucosal Immunol.* **8:** 969-978.
19. Markey KA, Gartlan KH, Kuns RD, MacDonald KP, Hill GR. 2015. Imaging the immunological synapse between DCs and T cells. *J. Immunol. Methods* **423:** 40-44.