Published in final edited form as:

J Microbiol Biotechnol. 2017 April 28; 27(4): 709–717. doi:10.4014/jmb.1611.11072.

Dendritic cell-mediated mechanisms triggered by LT-IIa-B₅, a mucosal adjuvant derived from a type II heat-labile enterotoxin of *Escherichia coli*

Chang Hoon Lee^{1,2,*}, George Hajishengallis³, and Terry D. Connell^{4,*}

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

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

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

⁴Department 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, USA

Abstract

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-B₅), a potent non-toxic, mucosal adjuvant. Alterations in gene expression in response to LT-IIa-B₅ 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-B₅ 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-B₅ serves as a critical mechanism whereby this potent adjuvant amplifies mucosal immunity to co-administered Ags (Ag).

Keywords

LT-IIa-B₅; mucosal vaccine adjuvant; dendritic cells; immune cell migration; lymph node

*Corresponding authors: Chang Hoon Lee: Bio & Drug Discovery Division, Center for Drug Discovery Technology, Korea Research Institute of Chemical Technology, 141 Gajeongro, Yuseong, Daejeon 305-600, Republic of Korea. Tel: +82-42-860-7414; Fax: +82-42-861-4246; Lee2014@kRICT.re.kr. Terry D. Connell: Department of Microbiology & Immunology and The Witebsky Center for Microbial Pathogenesis and Immunology, The University at Buffalo, State University of New York, Buffalo, NY, USA. Tel: 716-829-3364; Fax: 716-829-2158; connell@buffalo.edu.

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 [7]. Development of vaccines that stimulate the immune system on those mucosal surfaces is critical for generating immune protection against those mucosal pathogens [22]. A confounding feature of the mucosal immune system, however, is a well-developed immune tolerance mechanism [21], which likely evolved to avoid the generation of unwarranted immune responses to commonly encountered environmental Ags [2]. 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 [4, 8, 9, 11]. 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* [4, 11], which when delivered simultaneously with an Ag by the mucosal route (e.g., intranasal or oral), LT-IIa amplifies Ag-specific primary and memory immune responses on the mucosal surface and in the systemic circulation [1, 6]. Similar augmented Ag-specific immune response were observed when Ags were mucosally delivered in the presence of LT-IIa-B₅, the non-toxic B subunit of LT-IIa [11].

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 B₅ using genome-wide gene expression profiling, specifically in regards to the responses to dendritic cells (DC), a cell type that has been shown to react *in vitro* and *in vivo* to the pentamer [10, 11]. Using a mouse mucosal immunization model, we evaluated the effect of LT-IIa B₅ on migration of DC 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-B₅ 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 [16] expressing T cell receptors specific to the ovalbumin (OVA) epitope (323–339) on the BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME, 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 number = MIC01010Y).

Cloning and purification of LT-IIa-B₅

Methods for the cloning and purification of His-tagged LT-IIa-B₅ were reported previously [11]. In brief, the plasmid pHN1.1 encoding LT-IIa-B₅ was introduced into *E. coli* DH5 F'Kan (Life Technologies, Gaithersburg, MD, USA). Expression of LT-IIa-B₅ by the transformant was induced by the addition of 1 mM isopropyl-β-D-thiogalactoside to log-phase cultures. LT-IIa-B₅ was extracted from the cells using isotonic shock and purified by nickel affinity chromatography (Qiagen, Valencia, CA, 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-B₅. The purified protein was analyzed for endotoxin by use of an end-point quantitative *Limulus* amoebocyte lysate assay (Charles River Endosafe, Charleston, SC, USA). The level of endotoxin in the purified preparations of LT-IIa-B₅ 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 phosphate-buffered saline (PBS) or 100 μg OVA in PBS (Sigma-Aldrich, St. Louis, MO, USA) in the presence or absence of 10 μg LTIIa-B₅. 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, San Jose, CA, USA). Cells from the mouse CLN were stained with a phycoerythrin-conjugated anti-mouse CD11c antibody (clone N418; BioLegend, San Diego, CA, USA) and an allophycocyanin (APC)-conjugated anti-mouse I-A/I-E antibody (clone M5/114.15.2; BioLegend) with an Fc-blocking anti-mouse CD16/32 antibody (clone 93; BioLegend). 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) with flow cytometry counting beads (Thermo Fisher Scientific, Waltham, MA, 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, Herndon, VA, USA) that was supplemented with 10% fetal bovine serum, 50 μM 2-mercaptoethanol, 1% HEPES, 1% sodium pyruvate, 1% non-essential amino acids (Invitrogen, Grand Island, NY, USA), 10 ng/mL recombinant murine interleukin IL-4 (eBioscience), and 20 ng/mL recombinant murine granulocyte macrophage-colony stimulating factor (BioLegend). 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⁺ DC were sorted on day 6 using anti-CD11c-coated magnetic beads, according to the manufacturer's directions (Miltenyi Biotech, CITY, STATE). Sorted CD11c⁺ DC were used for subsequent experiments.

Microarray and data analysis

Purified DC (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-B₅. Total RNA was isolated from the DC using Trizol (Invitrogen). 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 was 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 (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, San Diego, CA, USA). Differences were considered significant at *P* = 0.05.

Results

Gene expression profile of DC regulated by LT-IIa-B₅, and between-group analysis of the microarray data

Transcriptional analyses were performed using 99% pure CD11c⁺ DC (data not shown) to investigate changes in the genome-wide transcription levels between untreated and LT-IIa-B₅-treated DC. The analysis identified 732 genes that were upregulated by 2-fold and 1,028 genes that were downregulated by 2-fold in LT-IIa-B₅-treated DC in comparison to untreated DC. To understand the biological significance of these microarray data, we further analyzed those differentially expressed genes using functional enrichment analysis with DAVID database, a gene ontology software tool (<http://david.abcc.ncifcrf.gov/home.jsp>). Because gene ontology (GO, <http://www.geneontology.org/>) annotations are important for investigation of functional significance from large dataset, we followed Gene ontology annotations based on Gene ontology consortium (3) and found significant 101 gene ontology terms and the majority of gene ontology terms were associated with triggering immune response and defense response. Top gene ontology term was immune response. LT-IIa-B₅ differentially regulated 113 genes associated with immune response (Table 1). Among 1760 genes that were significantly differentially regulated in DCs by LT-IIa-B₅, 79 immune-response associated genes, (4.48 % of total genes differentially regulated in DCs by LT-IIa-B₅) were upregulated by LT-IIa-B₅, whereas 34 immune-response associated genes (1.9 % of total genes differentially regulated in DCs by LT-IIa-B₅) were downregulated by LT-IIa-B₅ (Figure 1A, B). Furthermore, in top ten gene ontology terms, four (immune response,

response to wounding, inflammatory response, cytokine-cytokine receptor interaction) were related to immune regulation directly (data not shown). These results strongly suggest that transcriptional regulation of DC triggered by LT-IIa-B₅ 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-B₅

The expression pattern of DC chemokine receptors activated by LT-IIa-B₅ was selectively regulated, which likely enhanced the cells' homing capacity into the draining lymph nodes. DC treated with LT-IIa-B₅, but not untreated DC, transformed into a mature DC phenotype by significantly increasing the expression of CCR7, a critical chemokine receptor for homing to the lymph nodes [10, 18] (Fig. 2A). In addition, DC activated with LT-IIa-B₅ 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 [5]. Downregulation of these inflammatory chemokine receptors and the subsequent upregulation of CCR7 likely induces migration of DC into the draining lymph nodes.

The homing process of DC 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 DC 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 DC, we investigated the number of DC in the CLN after intranasal administration of LT-IIa-B₅ into naïve BALB/c mice. Mice without prior exposure to LT-IIa-B₅ were intranasally administered OVA in the absence and presence of LT-IIa-B₅. After 3 days, the number of DC in the CLN was significantly increased in the mice that had received OVA + LT-IIa-B₅ in comparison to mice that had received only OVA in the absence of the mucosal adjuvant (Fig. 2B).

LT-IIa-B₅-mediated transformation of DC by selectively regulating expression of co-stimulatory molecules and cytokines

Critical changes in the expression of relevant genes in immature bone marrow-derived DC were observed upon treatment with LT-IIa-B₅. In our previous study, we also found that CD40, CD80, and CD86 were increased in an *in vivo* mouse model after intranasal administration of LT-IIa-B₅ [10]. 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 [12]. Thus, we set out to confirm expression of CD40, CD48, and CD86 in our microarray data. Indeed, we similarly found that CD40, CD80 and CD86 were increased in DCs treated with LT-IIa-B₅ (Fig. 3A). In addition, changes in the expression levels of the intercellular adhesion molecules (ICAMs) of DC were evident. Specifically, the transcriptional levels of ICAM1 were increased, while the levels of ICAM2 and ICAM4 were decreased in DC activated by LT-IIa-B₅ (Fig. 3A). Also, we found that another adhesion molecule, TGFβ₁ was significantly downregulated by DC activated with LT-IIa-B₅ (Fig. 3A). Thus, it appears that LT-IIa-B₅ selectively regulated the transcriptions of various adhesion molecules including ICAM1,2,3 and TGFβ₁. Activating DC with LT-IIa-B₅ elicited

a dramatic increase in the transcription of numerous inflammatory cytokines, including tumor necrosis factor- α (TNF α), IL-1 α , IL-1 β , IL-2, IL-6, and IL-12 (Fig. 3B). In contrast, transcription of transforming growth factor- β (TGF β)1 and TGF β 2 were not significantly changed (Fig. 3B). Furthermore, DC activated with LT-IIa-B₅ reduced their sensitivity to the anti-inflammatory cytokine TGF β by decreasing the expression of TGF β receptors 1 and 2 (Fig. 3C). Significantly increased transcription of 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 [13, 15, 20].

In this study, we performed a genome-wide transcriptional profile analysis to investigate the mechanisms by which LT-IIa-B₅, a potent mucosal adjuvant, regulates DC, an immune cell type that orchestrates important cellular responses. Although several studies have described the properties by which LT-IIa-B₅ enhances mucosal immune responses to co-administered Ags [9, 13], few studies have evaluated the molecular and genetic mechanisms that drive immune augmentation by LT-IIa-B₅. Herein, we employed a genome-wide analysis to determine the transcriptional responses of DC that were engendered by exposure to LT-IIa-B₅. We found that LT-IIa-B₅ regulated a broad array of gene sets in DC. Globally, LT-IIa-B₅ upregulated transcription of 732 genes and downregulated transcription of 1,028 genes, respectively.

To better understand the functional meaning of the changes in gene expression fostered by LT-IIa-B₅, we analyzed the expression profiles obtained from microarray data of untreated and LT-IIa-B₅-treated DC using a gene ontology software tool. This analysis revealed that 79 genes, 4.48 % from total genes differential regulated in DCs by LT-IIa-B₅ were upregulated and 34 genes, 4.48 % from total genes differential regulated in DCs by LT-IIa-B₅ were downregulated, each of which is involved in immune response. Furthermore, we found that LT-IIa-B₅ enhanced the lymph node-homing capacity DC by selective regulation of chemokine receptors. Specifically, transcription of CCR7 was highly increased by treatment of DC with LT-IIa-B₅, while expression of genes encoding CCR1, CCR2, and CCR5 was decreased. These *in vitro* observations and their implications for adjuvanticity were supported by the *in vivo* migration assay. Indeed, after intranasal administration of LT-IIa-B₅, the number of DC in mice was increased dramatically in the proximal draining CLN. In our previous report, we showed that enhanced migration of DCs into cervical draining lymph nodes after intranasal administration of LT-IIa-B₅ was dependent on CCR7 using CCR7 knock out mice, which did not show increased migration of DCs after administration of LT-IIa-B₅. In this study, we suggest that those LT-IIa-B₅ triggered-migration of DCs was not mediated only by increased of CCR7 on DCs, but also selective regulation of multiple chemokine receptors like CCR1, 2 5, and adhesion molecules like ICAM1, 2,4 and TGF β i. DC exposed to LT-IIa-B₅ exhibited robust changes in transcription of various gene sets that

likely regulate other tissues and immune cells in nearby environments. Specifically, we found differential transcription of several ICAMs in DC activated by LT-IIa-B₅. Transcription of ICAM1 was significantly increased, whereas the transcription of ICAMs 2 and 4 was decreased. Markey et al. [14] reported that ICAM1 was a critical component for the immunological synapse between dendritic and T cells. Thus, we suggest that LT-IIa-B₅ 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. This sophisticated regulation of multiple chemokine receptors and adhesion molecules of DCs might be required for migration of DCs into draining lymph nodes after their exposure to LT-IIa-B₅.

Furthermore, increased expression of pro-inflammatory cytokines including TNF α , IL-1 α , IL-1 β , IL-2, IL-6, and IL-12, and chemokines including CCL2, CCL3, CCL4, CXCL1, CXCL2, CXCL3, CXCL5, CXCL9, CXCL10, and CXCL16 is anticipated, in part, to explain the strong adaptive immune responses that are driven by LT-IIa-B₅. In contrast, the transcription of the anti-inflammatory cytokine TGF β 1 and TGF β 2 by LT-IIa-B₅ was not changed (Fig. 3B), indicating a directional activation or differentiation mechanism by LT-IIa-B₅ on DC. TGF β 1 and TGF β 2 have critical roles in differentiating regulatory T cells to downregulate immune responses. Furthermore, DC activated with LT-IIa-B₅ reduced their sensitivity to the anti-inflammatory cytokine TGF β by decreasing the expression of TGF β receptors 1 and 2. In contrast to the reduction of TGF β receptor expression, LT-IIa-B₅ sensitizes DC 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 DC activated with LT-IIa-B₅ mediate, at least in part, an acceleration of directional differentiation for priming Ags and boost the immune system efficiency by minimizing signals by TGF β and regulatory T cells that downregulate immune responses. Furthermore, these changes on cytokine signaling might affect the expression of adhesion molecules like TGF β I 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-B₅ in triggering the strong immune responses to co-administered Ags that have been observed in various studies [9, 10].

Taken together, our results indicate that the adjuvant mechanism of LT-IIa-B₅ is likely a driving force for the differentiation of DC into the morphotypes required to evoke strong immune responses. This conclusion is based on the capacity of LT-IIa-B₅ to upregulate the migration, cytokine expression, chemokine expression, and the sensitivity of DC 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-B₅. Genome-wide analysis is a useful and powerful tool for revealing the potential mechanisms underlying the adjuvant properties of LT-IIa-B₅ 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-B₅ 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 R01 DE13833 and R56 DE13833 awarded to T.D.C. and by the Public Health grant DE017138 awarded to G.H.

References

1. Boberg A, Gaunitz S, Brave A, Wahren B, Carlin N. Enhancement of epitope-specific cellular immune responses by immunization with HIV-1 peptides genetically conjugated to the B-subunit of recombinant cholera toxin. *Vaccine*. 2008; 26:5079–5082. [PubMed: 18514370]
2. Commins SP. Mechanisms of oral tolerance. *Pediatr Clin North Am*. 2015; 62:1523–1529. [PubMed: 26456448]
3. Consortium GO. Gene ontology consortium: going forward. *Nucleic Acids Res*. 2015; 43:1049–1056.
4. da Hora VP, Conceicao FR, Dellagostin OA, Doolan DL. Non-toxic derivatives of LT as potent adjuvants. *Vaccine*. 2011; 29:1538–1544. [PubMed: 21163247]
5. Gouwy M, Struyf S, Berghmans N, Vanormelingen C, Schols D, Van Damme J. CXCR4 and CCR5 ligands cooperate in monocyte and lymphocyte migration and in inhibition of dual-tropic (R5/X4) HIV-1 infection. *Eur J Immunol*. 2011; 41:963–973. [PubMed: 21381021]
6. Harrod T, Martin M, Russell MW. Long-term persistence and recall of immune responses in aged mice after mucosal immunization. *Oral Microbiol Immunol*. 2001; 16:170–177. [PubMed: 11358539]
7. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. *Nat Med*. 2005; 11:S45–S53. [PubMed: 15812489]
8. Isaka M, Yasuda Y, Mizokami M, Kozuka S, Taniguchi T, Matano K, Maeyama J, Mizuno K, Morokuma K, Ohkuma K, Goto N, Tochikubo K. 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*. 2001; 19:1460–1466. [PubMed: 11163669]
9. Langridge W, Denes B, Fodor I. Cholera toxin B subunit modulation of mucosal vaccines for infectious and autoimmune diseases. *Curr Opin Investig Drugs*. 2010; 11:919–928.
10. Lee CH, Masso-Welch P, Hajishengallis G, Connell TD. TLR2-dependent modulation of dendritic cells by LT-IIa-B5, a novel mucosal adjuvant derived from a type II heat-labile enterotoxin. *J Leukoc Biol*. 2011; 90(5):911–921. [PubMed: 21791597]
11. Lee CH, Nawar HF, Mandell L, Liang S, Hajishengallis G, Connell TD. Enhanced antigen uptake by DCs induced by the B pentamer of the type II heat-labile enterotoxin LT-IIa requires engagement of TLR2. *Vaccine*. 2010; 28:3696–3705. [PubMed: 20332049]
12. Leitner J, Grabmeier-Pfistershammer K, Steinberger P. Receptors and ligands implicated in human T cell costimulatory processes. *Immunol Lett*. 2010; 128:89–97. [PubMed: 19941899]
13. Lundgren A, Leach S, Tobias J, Carlin N, Gustafsson B, Jertborn M, Bourgeois L, Walker R, Holmgren J, Svennerholm AM. 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*. 2013; 31:1163–1170. [PubMed: 23306362]
14. Markey KA, Gartlan KH, Kuns RD, MacDonald KP, Hill GR. Imaging the immunological synapse between DCs and T cells. *J Immunol Methods*. 2015; 423:40–44. [PubMed: 25967948]
15. Miller T, Fanton M, Nickelson S, Mason H, Webb S. Safety and immunogenicity of bacterial and tobacco plant cell line derived recombinant native and mutant *Escherichia coli* heat-labile toxin in chickens. *Avian Pathol*. 2012; 41:441–449. [PubMed: 22928883]
16. Murphy KM, Heimberger AB, Loh DY. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCR α thymocytes in vivo. *Science*. 1990; 250:1720–1723. [PubMed: 2125367]
17. Paun A, Danska JS. Immuno-ecology: how the microbiome regulates tolerance and autoimmunity. *Curr Opin Immunol*. 2015; 37:34–39. [PubMed: 26460968]

18. Saeki H, Moore AM, Brown MJ, Hwang ST. 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.* 1999; 162:2472–2475. [PubMed: 10072485]
19. Su F, Patel GB, Hu S, Chen W. Induction of mucosal immunity through systemic immunization: phantom or reality? *Hum Vaccines Immunother.* 2016; 12:1070–1079.
20. Sun M, He C, Cong Y, Liu Z. Regulatory immune cells in regulation of intestinal inflammatory response to microbiota. *Mucosal Immunol.* 2015; 8:969–978. [PubMed: 26080708]
21. van Ginkel FW, Nguyen HH, McGhee JR. Vaccines for mucosal immunity to combat emerging infectious diseases. *Emerg Infect Dis.* 2000; 6:123–132. [PubMed: 10756145]
22. Willimann K, Legler DF, Loetscher M, Roos RS, Delgado MB, Clark-Lewis I, Baggiolini M, Moser B. 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.* 1998; 28:2025–2034. [PubMed: 9645384]

Figure 1. LT-IIa-B₅ regulates a broad range of genes associated with immune response
 (A) Venn diagrams displaying the number of LT-IIa-B₅-mediated upregulated (greater than 2-fold, red circles, gene number = 79) and downregulated (greater than 2-fold, blue circles, gene number = 34) genes from significantly differential regulated genes in DCs by LT-IIa-B₅ (pink circles, gene number = 1760). (B) Heat map representation of the expression levels of immune response associated genes that were changed by more than 2-fold after LT-IIa-B₅ stimulation of three independent bone marrow-derived DC experiments.

Figure 2. Selectively regulated expression of chemokine receptors on DC by LT-IIa-B₅, and enhanced homing capacity of DC in draining lymph nodes

(A) Fold changes (log₂ value) in the mRNA levels of genes encoding CCR1, CCR2, CCR5, and CCR7 on bone marrow-derived DC activated by LT-IIa-B₅ compared to 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 $\pm$ SD. (B) The number of DC in the cervical lymph nodes of female BALB/c mice administered OVA (OVA) + LT-IIa-B₅ intranasally compared to mice given only OVA. Data are expressed as means $\pm$ 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).

Figure 3. Selectively regulated expression of co-stimulatory molecules and cytokines of mature DC differentiated by LT-IIa-B₅

(A) Fold changes (log₂ value) in mRNA levels of ICAM1, 2, and 4, TGFβi and CD40, 80, 83, and 86, of bone marrow-derived DC activated by LT-IIa-B₅ compared to untreated cells. All groups were significantly different from untreated cells at $P < 0.05$ (unpaired Student's t -test). (B) Fold changes (log₂ value) in mRNA levels of cytokines of bone marrow derived-DC activated by LT-IIa-B₅ compared to 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 (log₂ value) in mRNA levels of cytokine receptors on bone marrow-derived DC activated by LT-IIa-B₅ compared to 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 $\pm$ SD ($n = 3$). (D) A) Fold changes (log₂ value) in mRNA levels of chemokines of bone marrow-derived DC activated by LT-IIa-B₅ compared to untreated cells. All groups were significantly different from untreated cells at $P < 0.05$ (unpaired Student's t -test).

Table 1
Immune response-associated genes significantly differentially regulated by LT-IIaB₅.

Gens	Fold change	Gens	Fold change	Gens	Fold change
AF251705	-4.01175	Enpp1	-2.1522	Mx2	3.916443
Bcl3	3.02105	Exo1	4.53841	Myo1f	-4.59249
Btla	53.67841	Enpp2	-2.06988	Ncf1	2.070582
Clqa	-3.70588	Faim3	2.60413	NfkB2	2.619926
Clqb	-5.53338	Fcgr1	-6.59223	Oas1a	2.19973
Clqc	-4.00127	Fcgr2b	-2.11102	Oasl1b	2.77037
Cblb	2.141765	Gbp2	2.51167	Oas3	5.682778
Ccl2	2.274228	Gbp3	2.71714	Oas1	15.67486
Ccl3	5.26489	Gbp6	2.725306	Oas2	6.074723
Ccl4	3.524096	Gpr183	2.006543	Osm	2.25035
Ccl6	-2.78426	H2-DNb1	-2.03814	P2ry14	-2.24387
Cer2	-13.1665	Hc	3.722043	Pag1	-7.29287
Cer5	-3.03347	Icam1	2.28312	Polm	2.059059
Cer7	2.132664	Ifih1	3.838744	Procr	8.441738
Cd14	8.792162	Ifng	10.4368	Ptx3	5.160679
Cd1d1	2.742892	Il10	10.86072	Rnf125	4.79437
Cd28	-3.2446	Il12a	46.37081	Rnf19b	3.08599
Cd300a	-10.1548	Il12b	113.3806	Rsad2	15.61375
Cd300lb	-29.7869	Il15	4.121315	Serping1	2.401721
Cd300ld	-3.27123	Il1a	27.46101	Sp110	2.371898
Cd300lf	-11.1645	Il1b	107.1949	Susd2	4.873823
Cd70	12.49258	Il1f6	3.464392	Swap70	2.077384
Cfb	5.84534	Il1f9	4.006716	Tgtp1	2.475449
Cfp	-2.56648	Il1r1	2.339572	Tlr13	-5.05463
Clec4a2	-26.2842	Il1rn	3.801351	Tlr4	-2.5738
Clec4e	7.037697	Il23a	110.0505	Tlr7	-2.73101
Clec4n	-3.299	Il6	10.75864	Tnf	12.77939
Clec5a	-8.80265	Ilr7	21.91071	Tnfrsf1b	2.29976

Gens	Fold change	Gens	Fold change	Gens	Fold change
Clec7a	-2.55836	Irf8	4.086535	Tnfsf10	2.090967
Csf2	15.145	Irgm1	2.865748	Tnfsf11	5.232337
Csf3	38.36961	Klhl6	-3.41192	Tnfsf12	-4.14
Cxcl1	73.29714	Lat2	-5.04746	Tnfsf13b	-2.3057
Cxcl10	25.36828	Lif	4.364953	Tnfsf4	3.273475
Cxcl2	26.72429	Lta	4.765535	Tnfsf9	6.972128
Cxcl3	4.717775	Ly96	2.460622	Traf3ip2	2.392669
Cxcl5	37.1544	Malt1	2.551815	Ung	-2.55582
Cxcl9	7.014457	Mr1	-2.12122		
Dhx58	4.416773	Mxl	3.232579		