A Heat-labile Enterotoxin B Subunit Mutant (LTB26) of *Escherichia coli* Enhances Mucosal Immune adjuvanticity via Increased BCR Activity

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Abstract

Heat-labile enterotoxin B subunit (LTB) of *Escherichia coli* is a potential mucosal immune adjuvant for its safety. However, the weaker adjuvant activity fails to meet the clinical requirement. Here, one of LTB mutant numbered LTB26 is constructed with enhanced mucosal immune adjuvanticity than that of LTB wild type (LTB). The transcriptome analysis data suggest that LTB26 enhances mucosal immune adjuvanticity via increased expression of BCR and MHC II^+ molecular. Furthermore, LTB26 can promote both Th1 and Th2 cell mediated immunity via upregulated expression of IL-4 and IFN-γ. Flow cytometry analysis confirms that LTB26 significantly upregulates the activity of antigen presenting cells (DCs and mature macrophage) compared with LTB and LTB57 mutant. The result demonstrates that LTB26 is a potent mucosal immune adjuvant meeting clinical requirement. The GM1 ganglioside (GM1) binding activity of LTB57 is higher than that of LTB26; instead, the immune adjuvanticity of LTB57 is lower than that of LTB26. The

result highlights that the immune adjuvanticity of LTB and its mutant are not positively associated with GM1 affinity, which upends decades understanding of the relationship of LTB adjuvanticity and GM1-binding affinity.

Introduction

Heat-labile toxin (LT) of Enterotoxigenic *Escherichia coli* (ETEC) belongs to the members of the AB5 bacterial toxin family and consists of five B subunit (LTB) with a single catalytically active A subunit (LTA)[1]. LTA has toxic effects of ADP-ribosylating activity and LTB binds to the cell membrane with GM1 ganglioside (GM1) following delivery LTA into cells inducing infectious diarrhea[2]. LT has been reported as a potent mucosal immune adjuvant in 1988[3]. However, it fails to meet the clinical requirement for its toxicity[2, 4]. LTB has mucosal immune adjuvanticity without toxicity[2]. Nevertheless, LTB is also not applicable for weaker adjuvanticity comparing with LT. Therefore, the construction of non-toxic

LT has been a research hotspot in recent years[4, 5, 6, 7, 8]. However, there is little report to develop adjuvanticity-enhanced LTB adjuvant.

In this study, we designs four LTB mutants covered the T-and B-cell epitopes of LTB [4, 9]. One of the most enhanced adjuvant mutants numbered LTB26 is detecting from four mutants (LTB26, LTB34, LTB57, and LTB85). In addition, we find that the immune adjuvanticity of LTB and its mutants are not positively associated with its GM1-binding affinity[10]. This report provides a promising LTB26 adjuvant in future.

Results

The characteristic of LTB mutants. The pET32a-LTB, pET32a-LTB26, pET32a-LTB34, pET32a-LTB57 and pET32a-LTB85 were confirmed by sequencing and the products of the five proteins were detected by SDS-PAGE. Then, the proteins were purified using BeaverBeads™ His-tag protein purification kit (Beaverbio, China) and were detected a band of 30 KD protein using SDS-PAGE (Fig.1A). The

protein concentration of LTB was 3.412 μg/μl, LTB26, 3.215 μg/μl, LTB34, 3.325 μg/μl, LTB57, 3.011 μg/μl, and LTB85, 1.375 μg/μl, respectively.

Compared with LTB, LTB26 was mutated from I26T27E28L29C30 to M26S27N28 and from E32Y33H34 to T32I33N34, and meanwhile deleted L29C30 residues (Fig.1B). In turn, LTB34 was mutated from N35T36 Q37I38Y39T40I41N42D43K44 to L35S36L37S38N39S40T41I42N43 Y44 (Fig.1B), LTB57 was mutated from E57M58 to F57Y58 and from I60I61T62F63 to E60F61H62H63 (Fig.1C), and LTB85 was mutated from I86E87R88M89K90D91 to Y86V87E88F89H90H91 (Fig.1C).

LTB26 significantly enhances mucosal immune response. The serum VP8 specific IgG levels of LTB+VP8, LTB26+VP8 and LTB34+VP8 were significantly higher than that of the VP8 control group (Fig.2A, $p<0.01$). However, the serum IgG level of LTB34+VP8 vaccination was slightly higher than that of LTB+VP8 treatment, but lower than that of LTB26+VP8 vaccination (Fig.2A, $p<0.01$). On the contrary, the serum

IgG levels of LTB57+VP8 and LTB85+VP8 vaccinations were lower than that of LTB+VP8 groups (Fig. 2A). Similarly, the variation trends of lung mucosal VP8 specific sIgA were consistent with that of serum IgG among the LTB+VP8 and the other LTB mutants vaccination on 21 d post-vaccination ($p<0.001$) (Fig. 2B). LTB26 also elicited the highest mucosal immune response compared with LTB and LTB34, respectively. Thus, LTB26 was the most active adjuvant mutant to elicit robust systematic and mucosal immune responses in this study.

The GM1-binding affinity of LTB mutants no positive association with its adjuvant activity. Previous study suggested that LTB adjuvant positively associated with its GM1-binding affinity[9, 10]. Thus, the GM1-binding activity of the four LTB mutants was tested in study. The GM1-binding affinity was decreased gradually LTB57, LTB26, LTB34, LTB85, and LTB (Fig. 2C, D). However, the adjuvanticity was decreased successively LTB26, LTB34, LTB, LTB57 and LTB85. Therefore, LTB57 had the highest GM1-binding affinity with the lowest adjuvanticity.

Instead, LTB26 obtained the highest adjuvanticity with a weaker GM1-binding affinity (Fig. 2D). These upended decades of belief that LTB adjuvant activity was proportional to GM1-binding affinity[10]. Thus, the highlight of this work suggested that the adjuvanticity of LTB was not positively associated with GM1-binding affinity.

The transcriptome analysis. According to GM1-binding analysis and immune data, LTB26 and LTB57 were selected as two LTB mutant adjuvants to analyze the differential gene expression. Compared with the group of PBS treated mice, 375 differentiation genes were identified (240 upregulation and 135 downregulation) in the group of LTB+VP8 treatment mice, and 654 differentiations (523 upregulation and 131 downregulation) in the group of LTB26+VP8 treatment. Similarly, 725 differentiations (610 upregulation and 115 downregulation) in the group of LTB57+VP8 treatment, and 890 differentiations (746 upregulation and 144 downregulation) in the group of VP8 alone treatment, respectively (Table 1).

The Gene ontology (GO) pathway analysis suggested that LTB26 activated several B cell related signal pathways, APC (antigen presenting cell) pathways and MHC II pathway (Fig. 3A). Instead, the LTB57 just upregulated inflammatory related signal pathway (Fig. 3B). However, LTB preferred to activate inflammatory response, upregulate TNF and IL-10 expression (Fig. 3C). The results indicated that LTB26 acted adjuvant via enhancing MHC II related APCs activation and B cell related signal pathway activation (Fig. 3A).

Compared with the expression of immune-related cluster of differentiation (CD) genes, the LTB26 upregulated thirteen B cell associated CDs (CD79a, CD79b, CD19, CD22, CD14, CD37, CD38, CD40, CD48, CD52, CD72, CD74 and CD180), one macrophage marker (CD68) and one common lymphocyte marker (D300c2). Among the fifteen upregulated genes, about 86.7% belonged to B cell associated CDs (Table 2). Of course, LTB upregulated two T cell (and NK cell) marker (CD2, CD8a), one macrophage marker (CD68) and one monocyte, macrophage and dendritic

cell common marker (CD14). However, B cell marker was no variation in LTB treatment (Table 2). Similarly, LTB57 upregulated three T cell associated CDs (CD47, CD63, and CD164), one lymphocyte homing receptor (CD44), one lymphocyte differentiation marker (CD52), one leukocyte marker (CD53), one neutrophils marker (CD177) and one mast cell marker (D300ld3), respectively (Table 2). The T cell related CDs accounted about 37.5%. However, LTB57 was lost the ability of BCR related activation.

More importantly, CD79a, CD79b and CD19 were the major component of BCR, which indicated that LTB26 functioned adjuvanticity via BCR pathway. The variation of BCR downstream genes showed that LTB26 upregulated the expression of BCR downstream genes more than 2-fold. They were following from Syk, Plc-γ, Ras (Rasa3 and Rras), ERKs (Map3k1 or ERK1), Mapk1ip1l (ERK2), Map4k1 (ERK4)) to transcriptional factors Jund and Atf6b (Table 3). However, LTB57 significantly upregulated the expression of Bcl-10 and the downstream of

NFκB inhibitors (Nfκbiz, Nfκbia, and Nfκbib). Otherwise, the upregulation of Mapk6, Akt3 and two transcriptional factors (Egr-1 and Crebrf) did not contribute to adjuvant activity of LTB57 (Supplementary table 2). Unexpectedly, LTB only upregulated Jun expression (half-level of Jund of LTB26) and significantly enhanced the expression of two NFκB inhibitors (Nfκbia and Nfκbiz) (Table 3). The result suggested that the inhabitation of NFκB activity inducing adjuvanticity impair in LTB57 vaccination.

Quantitative real-time PCR (q-PCR) verification. In order to verify the transcriptome data and confirm the above-mentioned APCs, MHC II and BCR pathways, CD8a, CD79a, CD4, IL-1β, IL-4, TNF-α, Jun, Junb, Jund, H2-Ab1, and NFκB1 of PBMC (peripheral blood mononuclear cell) were tested by quantitative real time polymerase chain reaction PCR(q-PCR). The result indicated that ratio of CD4, CD8a, CD79a, IL-4, Jun, H2-Ab1, and NFκB1 were significantly upregulated in LTB26 compared with LTB and LTB57, respectively. The increased expression of IL-4 suggested that

LTB26 activated Th2 cells response. Meanwhile, the expression of IL-1β, Jund, CD68, TNF-α and Junb were significantly down regulated in LTB26 treatment (Fig. 3D). The result confirmed the transcriptome analysis that LTB26 activated BCR and MHC II pathways and decreased inflammatory response via downregulation of IL-1β and TNF-α.

IL-10 was also commonly produced in Th2 cells and regarded as an anti-inflammatory cytokine and stimulated the proliferation of B cells[11, 12]. Meanwhile, IFN-γ made Naive $CD4^+$ T cells differentiate to Th1 cells[13]. Therefore, IFN-γ, IL-10 and IL-21were also detected by qPCR. Compared with LTB and LTB57 adjuvant, the LTB26 treatment mice were significantly increased expression of IL-10 and IL-21 in lymphocytes (Fig. 3E, F, $p<0.001$). Because IL-21 played a critical role in T cell-dependent B cell activation[14, 15], the result suggested that LTB26 increased the function of T, B and DCs (dendritic cells). Similarly, the expression of IFN-γ was significantly increased in LTB26 and LTB57 treatment mice compared with the LTB adjuvant, indicating LTB26 also activated Th1 cells (Fig. 3G,

p<0.001). However, there were no significantly change between LTB26 and LTB57 treatment (Fig. 3G, p>0.05). Taken together, LTB26 significantly upregulated both Th1 and Th2 cells activation.

The characteristic of FCM (flow cytometry) of PBMCs. Peripheral blood was sampled to confirm the function of B cell, T cell and APCs elicited by LTB and the two mutants at 24 h of nasal vaccination. The proportion of $CD19^{+}CD45R^{+}$ B cells in BPMCs were 17.82±0.61% (PBS), 24.91 ± 0.82% (LTB26+VP8), 20.08 ± 0.73% (LTB57+VP8), 21.57 ± 1.24% (LTB+VP8) and 20.32 ± 0.61% (VP8), respectively. Compared with VP8 treatment, LTB26+VP8 and LTB+VP8 treatment were significantly increased the proportion of $CD19^{+}CD45R^{+}$ B cells in BPMCs than that of LTB57+VP8 vaccination (Fig. 4A, p<0.05, P<0.01), respectively. Meanwhile, there were no significant differences between LTB57+VP8, and VP8 vaccination (Fig. 4A, p>0.05). However, the proportion of $CD19^{+}CD45R^{+}$ B cell in groups of LTB26+VP8 and LTB+VP8 were no significantly difference (Fig. 4A, p>0.05).

As to the proportion of $CD3e^+$ $NK1.1^+$ T cells in PBMCs, there were no significant variations among the groups of LTB26+VP8 (8.22±0.60%), LTB57+VP8 (7.47±0.51%), and LTB+VP8 (8.34±0.48%) compared with VP8 treatment (7.71±0.63%), respectively (Fig. 4B, $p>0.05$).

The proportion of $CD11c^+CD11b^+IA/IE^+$ DCs in BPMCs were 0.76 ± 0.01% (PBS), 1.23 ± 0.03% (LTB26+VP8), 0.91 ± 0.01% (LTB57+VP8), 1.08±0.06% (LTB+VP8), and 0.88±0.03% (VP8), respectively. The proportion of $CD11c^+CD11b^+IA/IE^+$ DCs were significantly increased in LTB26+VP8 treatment compared with that of LTB57+VP8, LTB+VP8, and VP8 treatments (Fig. 5, $P<0.001$, $P<0.01$, $P<0.001$), respectively. That means LTB26 upregulated DCs activation than its wild type.

The F4/80 antigen as a major macrophage marker is expressed on mature macrophages and a subpopulation of DCs (APCs)[16]. $F4/80^+$ cells were major MHC II^+ mature macrophages with APC function; therefore, the proportion of $F4/80^+$ cells in $CD11c^+CD11b^+IA/IE^+$ DCs was a very

important functional indicator of DCs. In this study, the proportion of $F4/80^+$ cells in DCs were 1.85±0.11% (PBS)，3.61±0.37% (LTB26+VP8)，2.33±0.10% (LTB57+VP8)，2.79±0.26% (LTB+VP8)，0.28±0.11% (VP8), respectively. Compared with LTB26+VP8 treatment, the proportion of $F4/80^+$ cells in DCs were significantly decreased in LTB57+VP8, LTB+VP8, and VP8 treatments (Fig. 5, $P<0.01$，$P<0.05$，$P<0.01$), respectively. That meant LTB26 were significantly upregulated MHC II^+ APCs (macrophages and a subpopulation of DCs) activation than its wild type ($p<0.05$). Summarily, FCM analysis also confirmed the GO data that LTB26 activated MHCII APCs function and increased B cell activation in line with LTB (wild type).

Immunohistochemical staining test. IHC was performed to verify the transcriptome and FCM data. IHC of extent and intensity (EI) score of 0-3 were considered low expression and EI score >3 were considered high expression. The EI values of splenic CD11b were 5.5, 2.875, 2.125, and 1.875 in groups of LTB26+VP8, LTB57+VP8, LTB+VP8, and VP8,

respectively. The result suggested that CD11b were significantly upregulated more than 2-fold by LTB26 than that of LTB57, LTB and VP8 treatments (Fig.9). While the EI values of splenic CD45 were 3, 1.125, 1.25, and 1.375 in groups of LTB26+VP8, LTB57+VP8, LTB+VP8, and VP8, respectively. The result suggested that CD45 were significantly upregulated more than 2-fold by LTB26 than that of LTB57, LTB and VP8 treatments (Fig. 6). Of course, the level of CD45 was slightly decreased in LTB26+VP8 treatment than that of CD11b. The EI values of splenic CD4 were 4.125, 3.75, 1.625, and 1.875 in groups of LTB26+VP8, LTB57+VP8, LTB+VP8, and VP8, respectively. The result suggested that CD4 were significantly upregulated more than 2-fold by LTB26 and LTB57 than that of LTB treatments (Fig. 6). Nonetheless, the EI value of LTB26 treatment was higher than that of LTB57.

NLRP3 was one of NOD-like Receptors (NLRs) and played a crucial role in Alum-based adjuvant immune by activating of NLRP3 inflammasome and IL-1β production via MAPK signaling pathway[17]. However, the

expression of NLRP3 in nasal tissue of LTB, LTB26 and LTB57 adjuvant mice was lower than that of in VP8 treatment (Fig. 7, $p<0.01$). The result indicated that LTB and its mutants functioned mucosal adjuvant with independent on NLRs activation.

Discussion

Mucosal immunity was one of the important ways to protect the body from the invading pathogens[4, 18, 19]. However, mucosal adjuvant research lagged behind vaccine development and resulted in vaccines failure in some case[20, 21]. Therefore, it was important to develop safety and high effective mucosal adjuvants to enhance the effectiveness of mucosal vaccines.

Mucosal immune-based vaccines were generally weak in immune response and difficult to achieve immune protection[22]. Thus, mucosal immune cell targeting strategies had been developed to enhance the effectiveness of mucosal vaccines by targeting to specific receptors of mucosal cells (e.g. M cell, APCs)[23]. LTB26 has the potential to target mucosal cells via GM1 receptor.

LT could increase the permeability of epithelial cells, regulate the differentiation of B cells and regulate the production of T cells through mucosal immunity[2, 9]. However, the toxicity of LT prevented its application in human. In recent years, non-toxic LTB adjuvant had been widely studied[6, 9, 24, 25, 26].

The binding specificity of LTB to GM1 was associated with adjuvant activity. Previous studies reported that the property of LTB adjuvant was dependent on GM1-binding affinity [10]. The LTB (G33D, numbered G54 in this study for counting the N-terminal signal peptide) mutant might lose the immune adjuvant activity for failing bind to GM1[10]. However GM1 binding sites also existed in other epitopes of LTB such as 51E, Ile58, 58I and 91K[9]. Therefore, we had mentioned that beyond GM1-binding, G33 of LTB was a crucial antigenic determinant residue located in the B- and T-cell epitope region (residue S26 to G45)[4]. Scientist supposed that G33 residue was crucial for the binding affinity of LTB to GM1 and LTB

(G33D) mutant lost its GM1-binding affinity and oral immune adjuvant activity[27].

Even so, we had deduced that LTB (G33D) destroyed the function of the key residue of LTB antigenic determinant (residue S26 to G45) rather than the GM1-binding affinity[4]. Therefore, the integrity of LTB epitope was more important than the GM1-binding affinity[4]. This hypothesis has been verified that LTB57 (57-63 aa) mutation (F57Y58E60F61H62H63) was located in the central T- and B-cell epitope and significantly impaired the adjuvanticity. However, it was separated two residues from the downstream of the typical GM1-binding residue of G33 (numbered G54 in this study) (Fig.1B, C) [4, 27, 28]. Intriguingly, this octapetide (M26S27 N28--T32I33N34) mutation was not only impaired the GM1 affinity, but also enhanced the GM1-binding capacity. Similarly, LTB85 (86-91 aa) mutation was also located in the central T- and B-cell epitope[4], and this hexapetide (Y86V87E88F89H90H91) mutation was not impaired the GM1 affinity, too. Even though the K91 of LTB57 (K91H) was a

conserved GM1-binding residue[4, 9]. However, both LTB57 and LTB85 lost their adjuvanticity for destroying the structure of T- and B-cell epitope of LTB (Fig.1C).

Instead, LTB26 (26-34 aa) mutation was located in the N-terminal decapeptide region (A22P23Q24S25I26T27E28L29C30S31) which was crucial for competent pentameric B-subunit assembly and stabilization (Fig.1B)[29]. However, the octapetide (M26S27N28--T32I33N34) mutation did not affect the B-subunit pentamerization and enhanced the affinity of GM1. The residue of Y39 of LTB was involved in stabilizing the GM1-binding[4, 9]. The LTB34 (35-44 aa) mutation also did not affect the adjuvanticity. Conversely, the decapeptide mutation (L35S36L37S38N39 S40T41I42N43Y44) let LTB57 obtain higher GM1-binding affinity than LTB (Fig.1B)[4]. It was suggested that Y39N did not damage LTB GM1-binding affinity.

Adjuvant could induce host-derived damage-associated molecular patterns (DAMPs) or recognizing pathogen-associated molecular patterns (PAMPs)

to enhance antigen-specific immune responses via APCs (DCs) or macrophages[30]. DCs sensed and phagocytized invading pathogens and activated naïve T cells. Then they acted as a major link between innate and acquired immunity to determine the polarization of T cell responses into different effector subtypes[31 32]. In this study, LTB26 adjuvant significantly increased the proportion of MHC II^+ DCs, $F4/80^+$ cells in $CD11c^+CD11b^+IA/IE^+$ DCs and $CD19^+CD45^+$ B cells in peripheral blood, which indicated that LTB26 enhanced the processing of vaccine (antigen) via DCs activation.

Cytokines were important immune regulators. LTB26 was capable of orchestrating upregulated IL-4, IL-10 and IL-21 expression, significantly, compared with LTB and LTB57 (Fig. 3D, 3E, 3F). Generally, B cells and $CD8^+$ T cells were the responders to IL□21 by IL□21R[15]. $CD4^+$ T cells were main producers of IL□21, while TCR stimulation increased $CD4^+$ T cell expression of IL□21R as a positive feedback. IL-10 stimulated cytotoxicity of $CD8^+$ T cells and IFN-γ expression[33].

IL-4 promoted humoral immunity and IFN-γ promoted cell-mediated immunity[34]. LTB, LTB26 and LTB57 significantly upregulated the expression of IFN-γ and TNF-α compared with VP8 alone treatment, but there were no differentiation among LTB, LTB26 and LTB57 (Fig. 3G). Thus, LTB26 promoted both Th1 and Th2 cells mediated immunity via upregulating IL-4 and IFN-γ expression, which was different from Alum-based vaccine (IL-4 only). This reconciled the data obtained from FCM and transcriptome analysis.

Material and Methods

LTB mutants design. Full-length LTB DNA was cloned from EC44815 strain and constructed pET32a-LTB plasmid as our previously report[25]. The four LTB mutants were designed to mutate some amino acids located in the B- and T-cell epitope region[4]. The amino acid was numbered from the first M (Met) of signal peptide in LTB. The four LTB mutants were also constructed into plasmid pET32 at *Bam*H I /*Sal* I site, respectively. Full-length of hRV VP8 DNA (GenBank: L34161) was commercially

synthesized (Sangon, Shanghai, China) and inserted into plasmid pET32 at *Bam*H I/*Sal* I site. The recombinants of pET32-LTB, pET32-LTB26, pET32-LTB34, pET32-LTB57, pET32-LTB85 and pET32-VP8 were expressed in *E. coli* BL21 cells and purified with BeaverBeads™ His-tag protein purification kits (Beaver, Suzhou, China). The endotoxin was removed using ToxinEraserTM resin (Genscript, Nanjing, China) and the protein concentration was measured by the BCA protein assay kit (Genscript, Nanjing, China) as previous described[25].

Animal and Immunization. Balb/c mice of 3-4 weeks (male) were bred in the experimental animal center of Chongqing Medical University and divided into four groups. Six groups of mice (n=6) were nasal vaccinated with VP8, LTB+VP8, LTB26+VP8, LTB34+VP8, LTB57+VP8, LTB85+ VP8, and 20 μl PBS, respectively, after anesthetizing with chloral hydrate (0.5 mL/100 g). The VP8, LTB and four LTB mutants were each added 10 μg/mouse respectively. The mice were intranasal boosted twice on 7, and 14 day with same method after first vaccination. This study was carried out

in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments at the Chongqing Medical University (SYXK2012-0001, 2013-03-11). All surgery was performed under sodium pentobarbital anesthesia, and euthanized by cervical dislocation.

Immune assay. Blood samples were individually collected from immunized mice by tail bleeding on days 0, 7, 14, and 21 for the analysis of systemic VP8 specific antibodies (n=6). Fresh fecal pellets were individually collected and lyophilized from the same mice on days 0, 7, 14, and 21. The samples of bronchial mucosal washing were washed from euthanized mice on day 21 after third vaccination (n=6). All the samples were treated as previously described[24]. The supernatants of bronchial mucosal washing were analyzed for VP8-specific sIgA to evaluate the mucosal immune response. The samples collected on 48 h post-vaccination and 21 day (7 day post-third-vaccination) were analyzed

with horseradish peroxidase (HRP)-labeled goat anti-mice IgG and goat anti-mice IgA (1.0 μg/mL, Boster, Wuhan, China) by ELISA, respectively, as previously described[24]. Endpoint titers were determined as the dilution of each sample showing a 2.1-fold higher absorbance level of 450 nm as compared to that of the negative control samples. Average OD_{450} values for the animals were calculated.

GM1-binding analysis. The GM1-binding affinity of LTB and its four mutants were determined using GM1-ELLSA (enzyme-linked immunosorbent assay) assay[35]. The wells of microplate were coated with 200 μL (2.0 μg/ml and 10 μg/ml, respectively) of GM1 (Qilu pharma, Shandong, China) or PBS, respectively, at 48□ overnight. Plates were washed three times with 500 μl PBS to remove uncombined GM1. Subsequently, plates were blocked by the addition of 200 μl PBS containing 1.0 % BSA at 37□ for 30 min. Then, plates were washed again as described above. Finally, plates were incubated with 1000 ng/well LTB, LTB26, LTB34, LTB57, LTB85, and PBS, respectively, at 37□ for 2 h,

followed by washing as described above. 100 µl of mouse anti-His-tag antibody (1:1000, BioVision, USA) was incubated at 37□ for 2 h, followed by washing as described above. 100 µl of HRP conjugated goat anti-mouse IgG(H+L) secondary antibody (1:1000, Boster, China, cat# BA1051) was incubated at 37□ for 2.5 h, followed by washing four times. Finally, added 100 µl of TMB (tetramethylbenzidine) to each well and incubated at 37□ for 5 min. Then the reaction was stopped by 200 µl H_2SO_4 (2.0 mol/l). The OD_{450} was read by microplate Reader.

Transcriptome analysis of peripheral lymphocyte. According to animal immune data, the peripheral lymphocytes were sampled in groups of LTB, LTB26+VP8 (the highest immune adjuvanticity with middle GM1-binding capacity), LTB57+VP8 (the highest GM1-binding affinity with lowest immune adjuvanticity), VP8 (in PBS), and PBS treatment mice after 24 hours vaccination. Total RNA was extracted using TRIzol Reagent (Invitrogen, USA) according the manufacturer manual. The RNA quality were measured using a NanoDrop 2000 spectrophotometer

(Thermo Fisher, USA) at 260/280 nm and agarose gel electrophoresis. Then RNA quantity was measured using Qubit2.0 Fluorometer. mRNA sequencing was performed on Illumina Hiseq platform (Illumina, USA) with 12 G bps and 10 M reads (Genminix Informatic Ltd., Shanghai, China).

The differentially expressed genes were selected as having more than 2-fold difference between their geometrical mean expression in the compared groups and a statistically significant P-value (<0.05) by analysis of DEseq2. The GO analysis on differentially expressed genes was performed with an R package: cluster profiler using a P<0.05 to define statistically enriched GO categories. Pathway analysis was used to determine the significant pathway of the differential genes according to Kyoto Encyclopedia of Genes and Genomes Database (http://www.genome.jp/kegg/).

Peripheral blood qPCR analysis. Total RNA was isolated as previous described. RNA reverse transcription was performed using the TaqMan

RNA Reverse Transcription kit (Thermo Fisher, USA). qPCR was performed using SYBR Green Supermix (Bio□Rad, USA) and the iCycler iQ Real□Time PCR system (Bio□Rad, USA). The reactions were run as follow: denaturation at 95˚C for 10 min followed by 50 cycles of 95˚C for 10 sec, 55˚C for 10 sec and 72˚C for 5 sec; 99˚C for 1 sec; 59˚C for 15 sec; 95˚C for 1 sec; then cooling to 4˚C. Relative mRNA expression was normalized against the endogenous control, GAPDH, using the 2□ΔΔCt method. The primers used in the current study were listed as table (Supplementary table 1).

Flow Cytometry. PBMCs from heparinized blood were isolated with Ficoll-Paque. The PBMC samples were stained with 5 μl of anti-mouse CD3e-PerCP/cy5.5 (Biolegend, USA, cat# 100327), NK1.1-PE (Biolegend, USA, cat# 108707),CD19-PE (Biolegend, USA, cat#115511), CD45R/B220-APC (Biolegend, USA, cat# 103207), CD11c-APC (Biolegend, USA, cat# 117309), CD11b-FITC (Biolegend, USA, cat# 101205), F4/80-PE (Biolegend, USA, cat# 123109), and

IA/IE-PerCP/cy5.5 (Biolegend, USA, cat# 107625), respectively, for 30 min at RT in the dark. The cell were stained with 5 µl of PerCP/cy5.5-Armenian Hamster-IgG (Biolegend, USA, cat#400931), PE-Rat-IgG2a (Biolegend, USA, cat#400508), PE-Rat- IgG2b (Biolegend, USA, cat#400211), APC-Rat-IgG2a (Biolegend, USA, cat#400511), APC-Rat-IgG1 (Biolegend, USA, cat#401903), FITC-Rat-IgG2b (Biolegend, USA, cat#400634), PerCP/cy5.5-Rat IgG2b (Biolegend, USA, cat#400631), and respective isotype control for 30 min at RT in the dark, respectively. Then washed twice with 500 µl PBS and assessed by four-colored flow cytometry. Then measured the percentages of $CD3e^{+}NK1.1^{+}$ NK cells, $CD19^{+}CD45R^{+}$ B cells and $CD11c^{+}CD11b^{+}F40^{-}80^{+}IA/IE^{+}$ DCs (dendritic cells) and the fluorescence intensity (MFI) of cell finally.

Immunohistochemistry. Spleen played an important role for lymphocyte migration and immune response after receiving antigen stimulation. Therefore, CD4, CD11b, and CD45 of splenic lymphocyte were tested

after 24h poster vaccination. Haematoxylin and eosin staining and antibody labeling were performed on 4-μm tissue sections as described previously[24]. Antibodies(rabbit) against CD11b, CD45 and CD4 were incubated overnight at 4℃. Antibodies (goat) against IgG (rabbit) were used for labeling for 2 h at room temperature. Images of histology and immunohistochemistry were taken with a Nikon Eclipse E600 and Nikon DS-Ri1 camera or with a Digital Slide Scanner (20 × magnification).

The expressions of CD11b, CD45 and CD4 were quantified using a visual grading system based on the extent of staining as previously described[36]. Briefly, percentage of positive spleen cells (extent of staining) was graded on scale from 0 to 3: 0, none; 1, 1-30%; 2, 31-60%; 3, 41-60%. The intensity of staining was graded on a scale of 0-3: 0, none; 1, weak staining; 2, moderate staining; 3, strong staining. The combination of extent (E) and intensity (I) of staining was obtained by the product of E times I called EI varying from 0 to 9 for each spot. For statistical analysis, EI score of 0-3

were considered low expression and EI score >3 were considered high expression.

Acknowledgements

Financial support from Chongqing Medical University.

Contributions

Y.M. conceived of the paper; Q.S. and Q.W. contributed equally and performed main experiments; S.C. and S.G. performed some experiments; Y.M., Q.S., F.S. and T.L. contributed to write the paper.

Competing interests

No competing interests.

Supplementary material: Supplementary table 1 qPCR primers used in this study.

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Table 1. The profiles of differential genes summary

Groups	Upregulation gene numbers	downregulation gene numbers	Total variation gene numbers
LTB+VP8 vs PBS	240	135	375
LTB26+VP8 vs PBS	523	131	654
LTB57 +VP8 vs PBS	610	115	725
VP8 vs PBS	746	144	890

Table 2. The variation of CDs expression in LTB26 and other groups

CDs	LTB+VP8	LTB26+VP8	LTB57+VP8	Adjuvant Significant	Cell type
CD2	2.0110114	N	2.235971319	-	T cell, NK cell
CD14	2.1038286	N	2.825551073	-	monocyte, macrophage, dendritic cell
CD8a	2.1593616	N	n	-	T cell, NK cell
CD68	2.0262211	2.666606105	n	+	macrophage
CD79b	n	4.669013871	n	+	B cell antigen receptor (BCR)
CD79a	n	2.166643886	n	+	BCR
CD48	n	2.404601005	n	+	T cell
CD40	n	2.243108146	n	+	B cell
CD180	n	2.771753281	n	+	B cell
CD74	n	2.811559737	n	+	B cell
CD72	n	2.546204783	n	+	B cell
CD38	n	2.521370117	n	+	B cell
CD22	n	2.982325847	n	+	Mature B cell
CD19	n	3.040386195	n	+	primitive & mature B cell
CD37	n	2.566581649	n	+	B cell
CD300c2	n	2.458761325	n	-	monocytes, neutrophils, partial T & B cell
CD52	n	2.525736598	2.242208379	-	B cell
CD44	n	N	2.058549419	-	B1 cell, IgM>IgG
CD164	n	N	2.137254166	-	T cell
CD63	n	N	2.115720638	-	T cell
CD53		N	2.25028684	-	leukocyte
CD177		N	7.451954307	-	neutrophils
CD47		N	2.041258354	-	T cell
CD300ld3		N	2.117187532	-	mast cells

*:n, no differentiate; +, LTB26 vs LTB and LTB57 with significant adjuvanticity; -, no significant adjuvanticity;

Table 3. The variation of BCR downstream genes

genes	LTB+VP8	LTB26+VP8	LTB57+VP8	Adjuvant Significant
Syk	n	2.9179075	2.8384351	-
Plcg2	n	**2.7476057**	n	+
Cdc42ep5	n	4.1154716	7.770395047	-
Rras	n	**2.357993**	n	+
Rasa3	n	**2.2090269**	n	+
Map3k1	n	**2.115917**	n	+
Mapk1ip1l	n	**2.1197696**	n	+
Map4k1	n	**2.2946296**	n	+
Mapk6	n	n	2.2102702	-
Jun	2.2490814	n	n	+
Jund	n	**5.183886**	n	+
Bcl-10	n	n	2.0637835	-
Nfkbia	3.8505929	n	5.6544579	-
Nfkbib	n	n	2.0815464	-
Nfkbiz	3.3298047	n	6.6704407	-
Rasgrp3	n	n	2.0318061	-
Atf1	n	n	2.1382357	-
Atf6b	n	**2.0878157**	n	+
Crebrf	n	n	2.2254203	-
Egr1	n	n	3.2136266	-
Foxo1	n	**2.5975225**	n	+
Akt3	n	n	2.4481639	-

*:n, no difference; +, LTB26 vs LTB and LTB26 vs LTB57 with significant adjuvanticity; -, no significant adjuvanticity;

Figures legends

Figure 1. The construction of LTB mutants. (A) LTB and the mutants were purified and detected in SDS-PAGE. Lane 1: standard proteins; lane 2-6: LTB, LTB26, LTB34, LTB57, and LTB85; (B) Alignment of LTB26 and LTB34 with LTB; (C) alignment of LTB57 and LTB85 with LTB. The mutations were highlighted with black background.

Figure 2. Immune responses and GM1-binding affinity analysis

(A) Serum IgG titer on post-vaccination of 7 d, 14 d and 21 d, respectively; (B) Lung mucosal sIgA titer on the third robust post-vaccination; (C) 1000 ng/well LTB, LTB26, LTB34, LTB57, LTB85, and VP8 were incubated with 2.0 μg/ml GM1; (D) 1000 ng/well LTB, LTB26, LTB34, LTB57, LTB85, and VP8 were incubated with 10 μg/ml GM1; ***, $p<0.001$; *, $p<0.01$; #, $p>0.05$.

Figure 3. GO analysis the adjuvant related signal pathways and qPCR test.

(A)Compared with LTB57, LTB26 significantly activated APCs (MHC II), B cell proliferation, BCR activation and immunoglobulin mediated immune responses, respectively. (B) Compared with LTB, LTB26 significantly activated Th1 immune response, TNF and IL-10 expression, and immune response. (C) LTB 57 did not active APCs (MHC II) and B cell related signal pathways, but upregulated inflammatory response. (D) Cd79a, H2-Ab1, IL-4, CD4, NκkB1, Cd8a, and Jun were significantly upregulated by LTB26. IL-1β and TNFα were significantly downregulated by LTB26. S26: LTB26; S57: LTB57. (E-G) LTB26 significantly upregulated IL-10 and IL-21expression, respectively. LTB26 and LTB57 were significantly upregulated IFN-γ expression than that of VP8, but there were no differences each other. ****, $p<0.001$; ***, $p<0.01$; #, $p>0.05$.

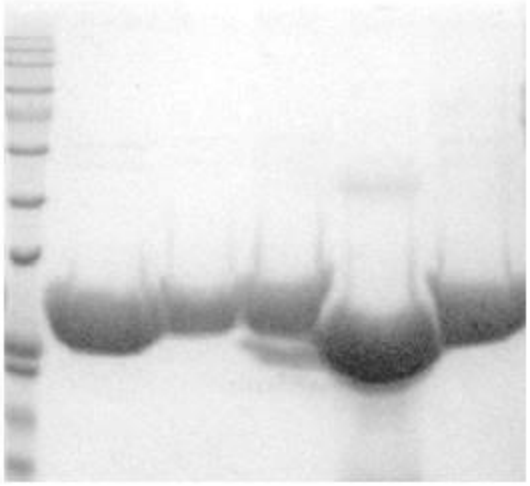
Figure 4. FCM analysis of B cells and NK cells. (A) LTB26 significantly activated CD45R+CD19+ B cells. (B) LTB26 significantly activated CD45R+CD19+ B cells. (b) NK1.1 cells were no variations among LTB, LTB26, LTB57 and VP8 alone treatment. **, $p<0.001$; *, $p<0.01$; #, $p>0.05$.

Figure 5. FCM analysis of DCs and F4-80 macrophage cells. LTB26 significantly activated $CD11^{+}$DCs and F4-80 macrophage cells. LTB57 was similar to VP8 alone treatment. ***, $p<0.001$; **, $p<0.01$; #, $p>0.05$.

Figure 6. IHC analysis of CDs. CD11b, CD45 and CD4 were significantly upregulated more than 2-fold by LTB26. ****, $p<0.001$, **, $p<0.01$; #, $p>0.05$.

Figure 7. IHC analysis of NLRP3. NLRP3 was significantly downregulated by LTB26. *$p<0.01$.

A



B

Protein Sequences

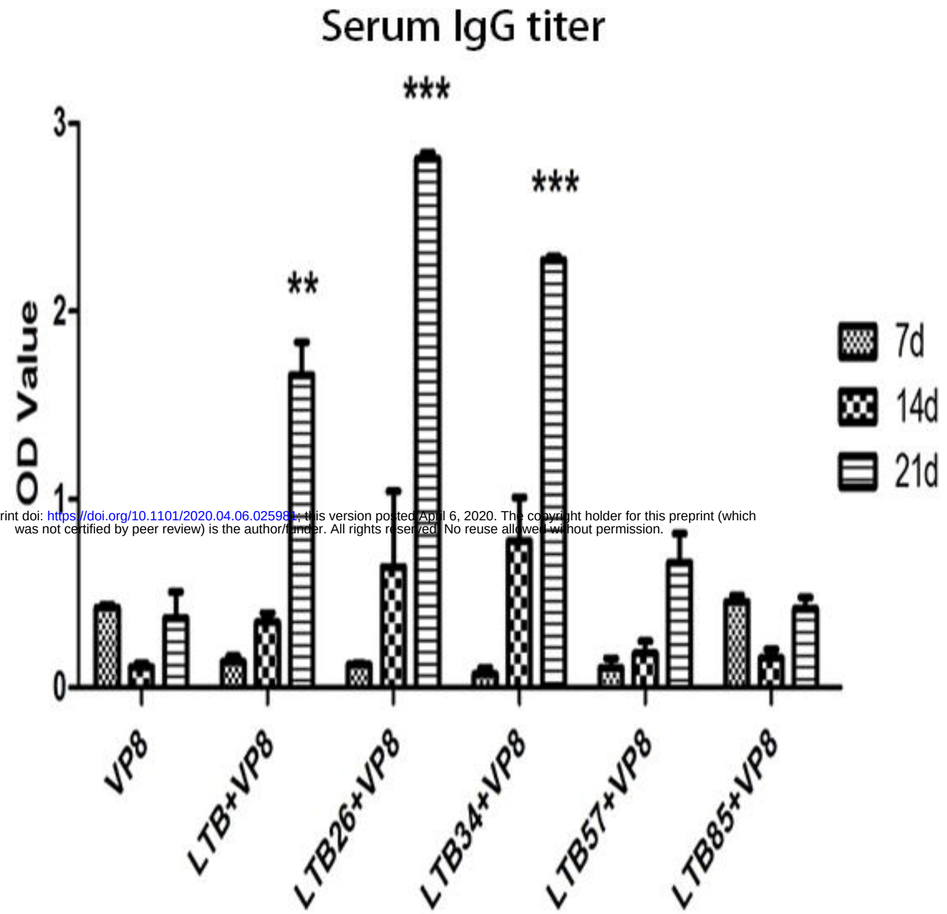
Species/	26	30	35	40	44
1. LTB	GA PQSITELCSEYHNTQIYTINDKIL				
2. LTB26	-SPQSMSN--STINNTQIYTINDKIL				
3. LTB34	-SPQSITELCSEYHLSLSNSTINYIL				
4. LTB57	-SPQSITELCSEYRNTQIYTINDKIL				
5. LTB85	-SPQSITELCSEYRNTQIYTINDKIL				

C

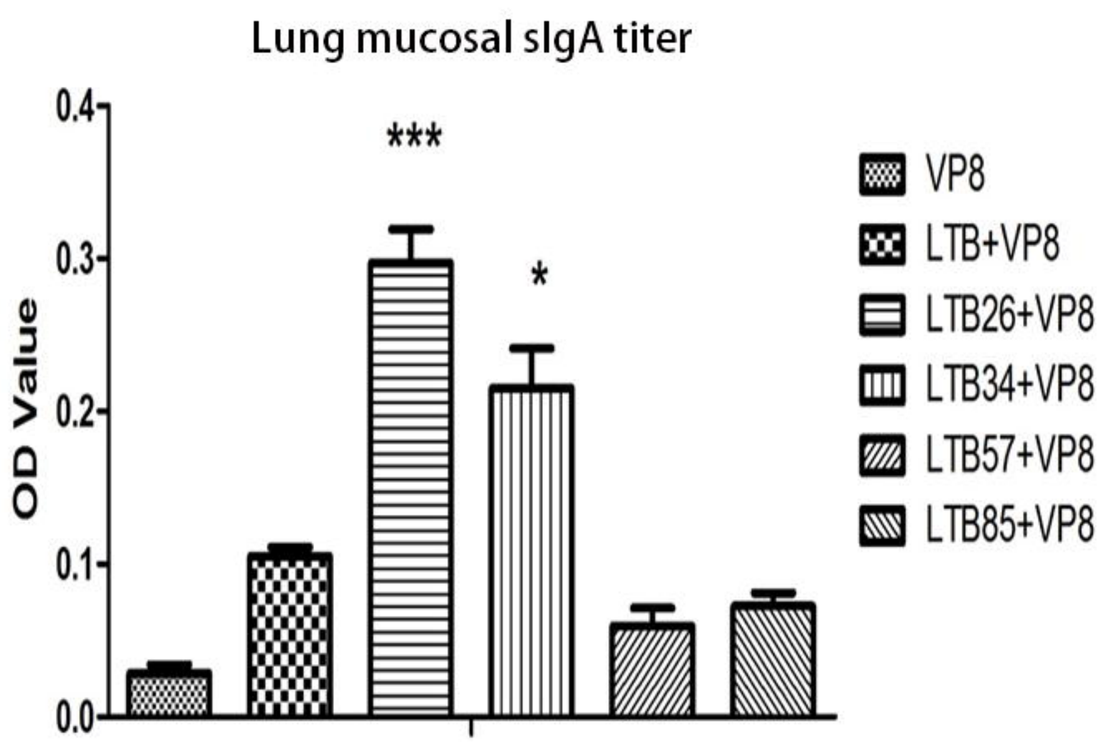
Protein Sequences

Species/	57	60	63	86	90
1. LTB	SYTESMAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDT				
2. LTB26	SYTESMAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDT				
3. LTB34	SYTESMAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAIERMKDT				
4. LTB57	SYTESMAGKRFYVEFHHKSGATFQVEVPGSQHIDSQKKAIERMKDT				
5. LTB85	SYTESMAGKREMVIITFKSGATFQVEVPGSQHIDSQKKAYVEFHHT				

A



B



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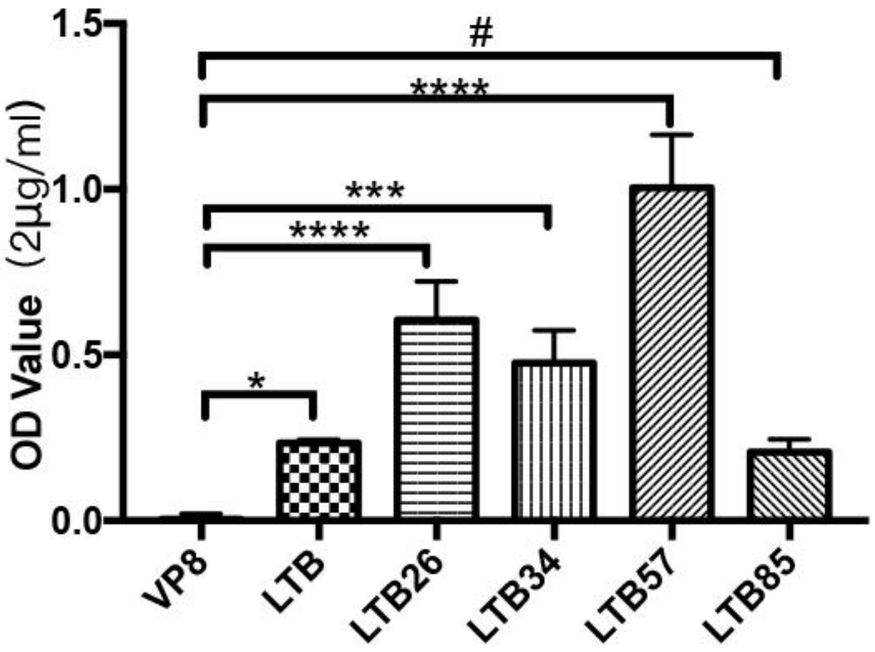

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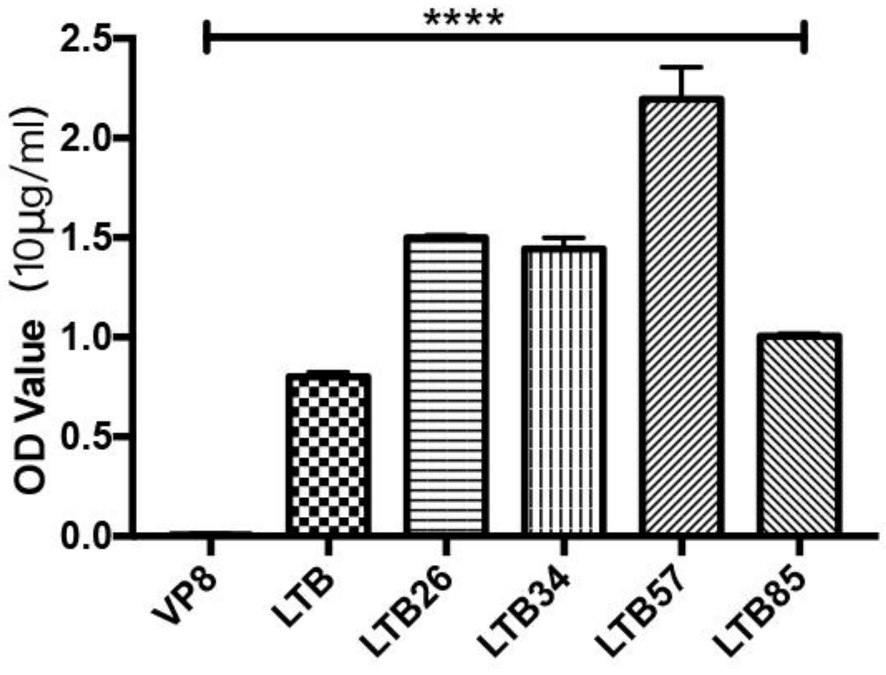

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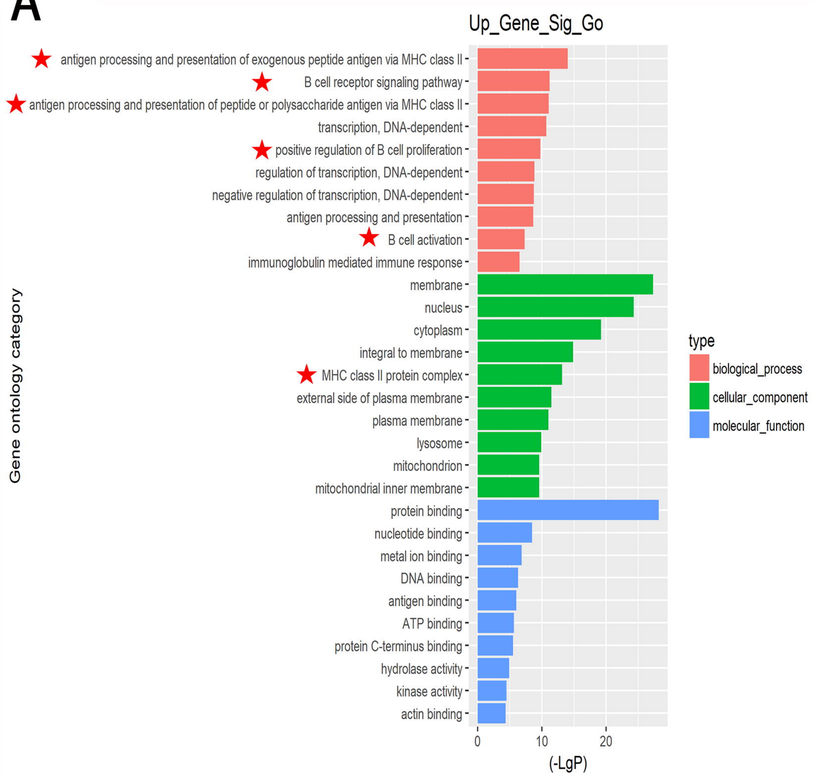
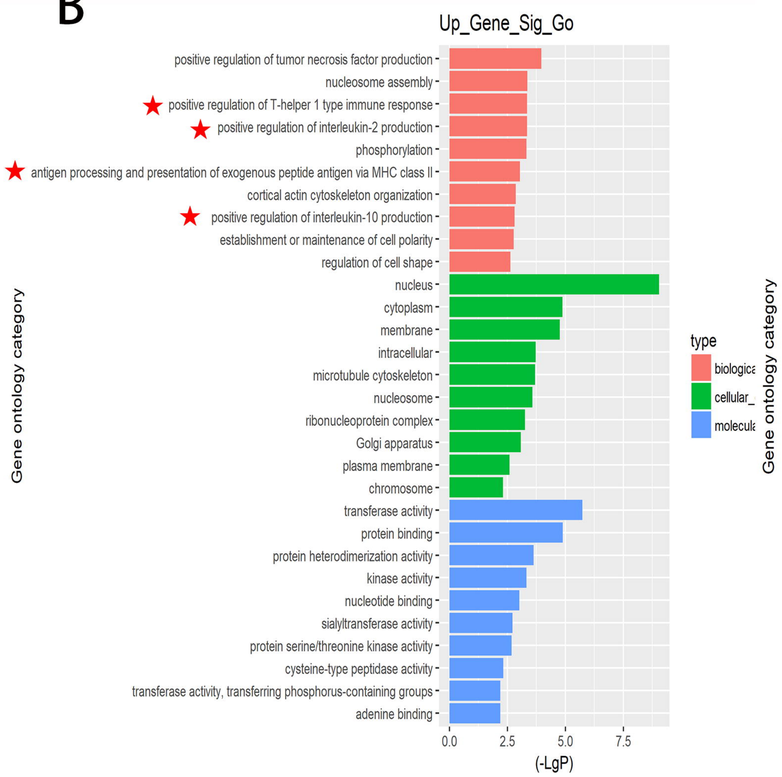


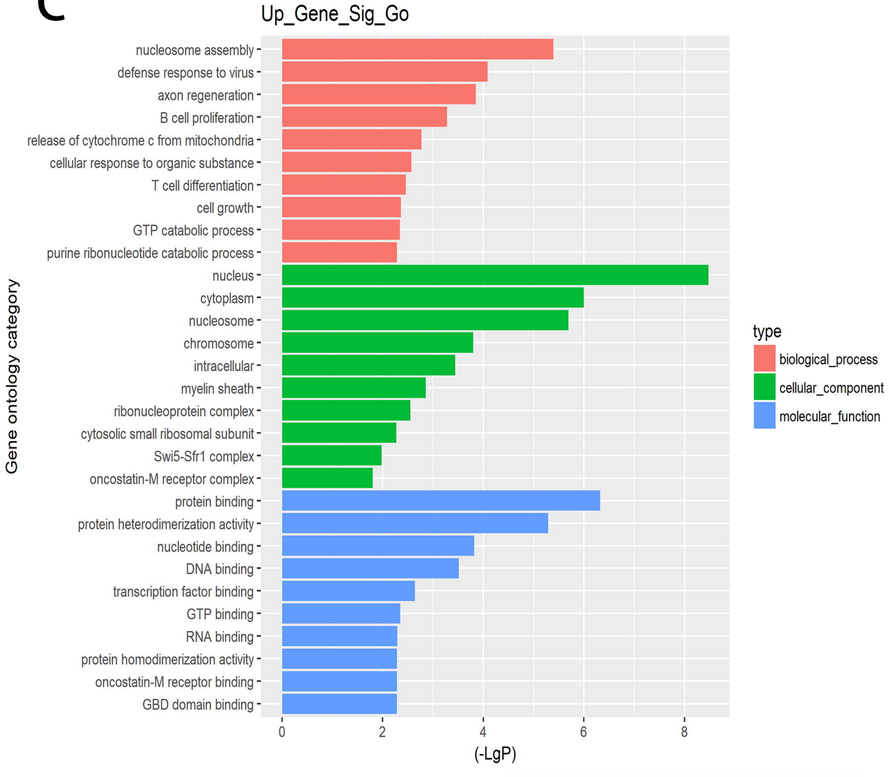
LTB26+VP8 vs LTB57+VP8

B



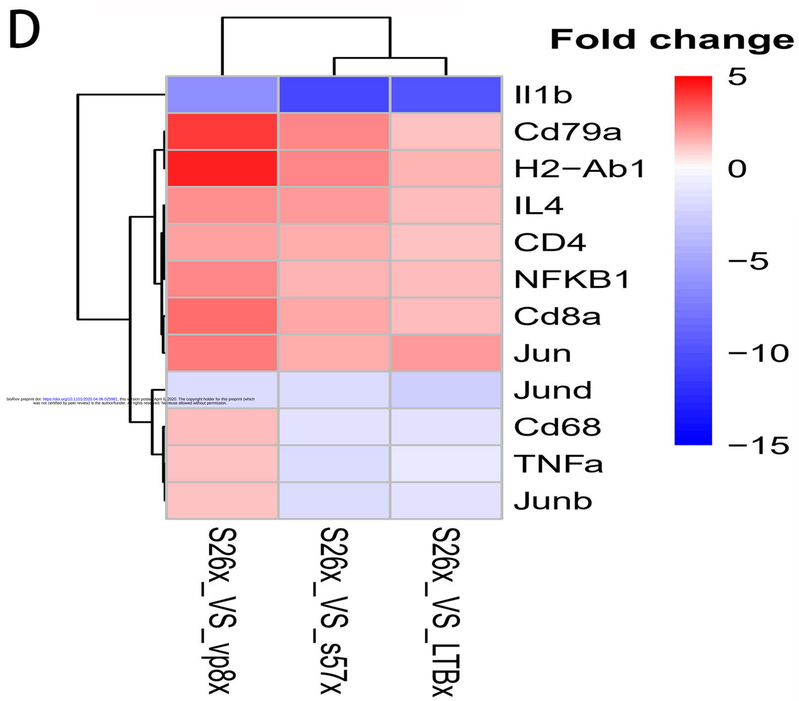
LTB26+VP8 vs LTB+VP8

C



LTB57+VP8 vs LTB+VP8

D



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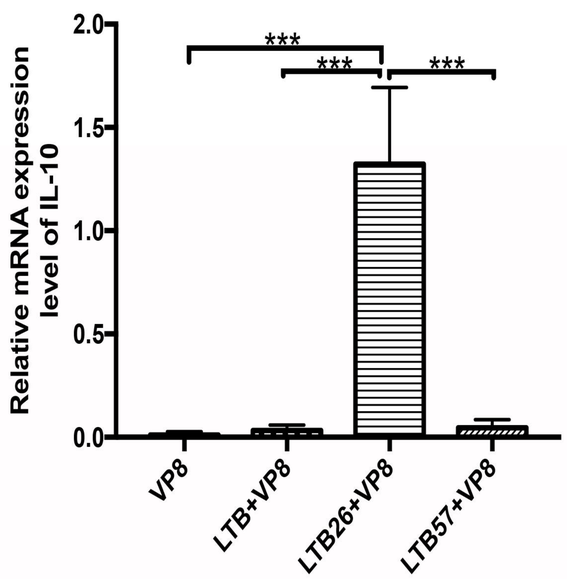

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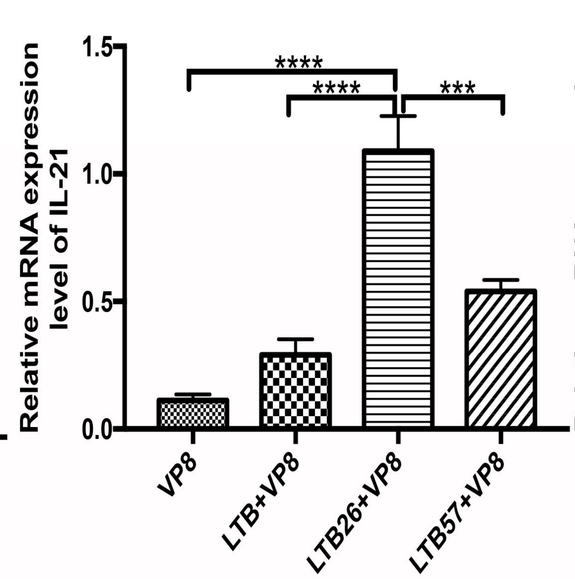

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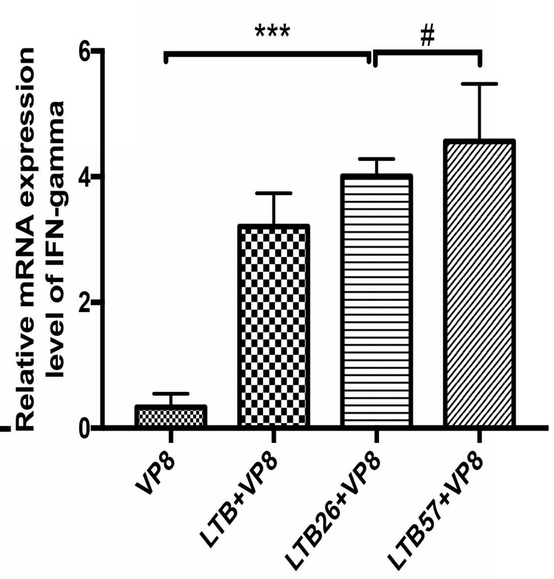
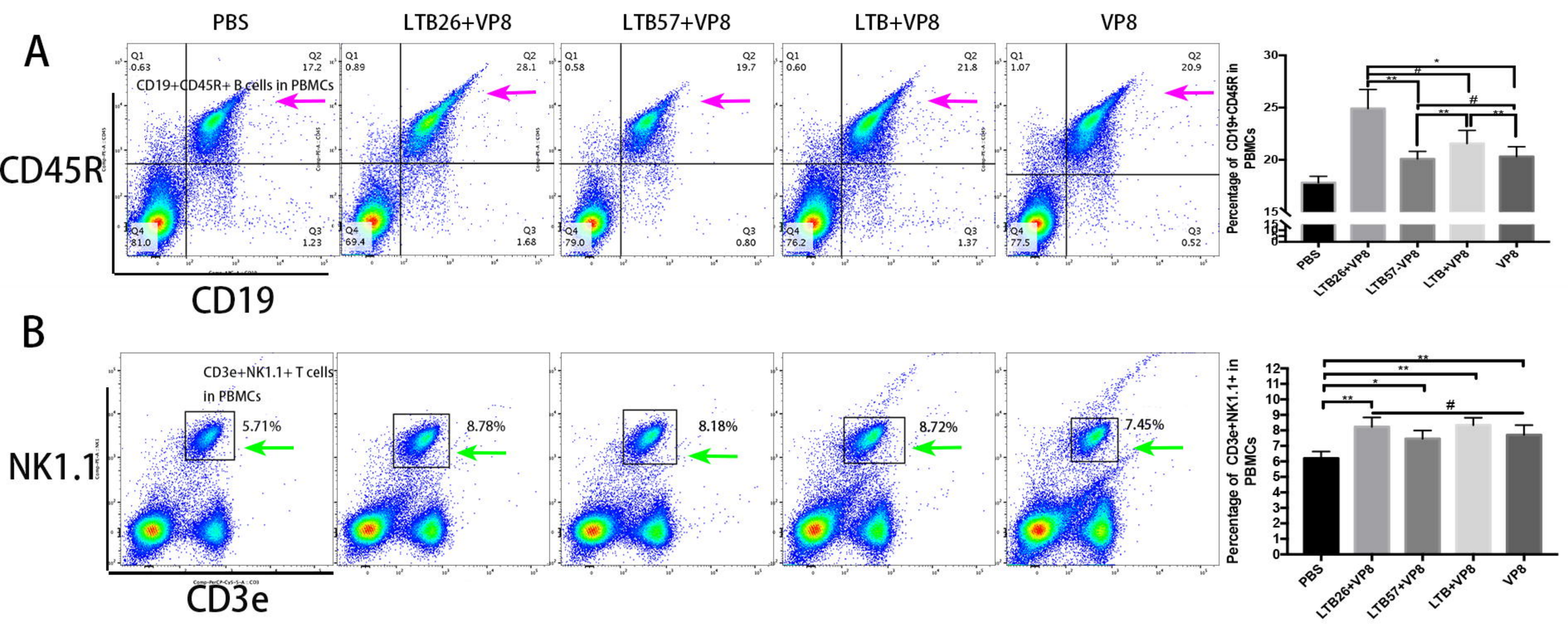



A
PBS
LTB26+VP8
LTB57+VP8
LTB+VP8
VP8
CD45R
CD19
CD19+CD45R+ B cells in PBMCs
Q1 0.63
Q2 17.2
Q4 81.0
Q3 1.23
Q1 0.89
Q2 28.1
Q4 69.4
Q3 1.68
Q1 0.58
Q2 19.7
Q4 79.0
Q3 0.80
Q1 0.60
Q2 21.8
Q4 76.2
Q3 1.37
Q1 1.07
Q2 20.9
Q4 77.5
Q3 0.52
Percentage of CD19+CD45R in PBMCs
PBS
LTB26+VP8
LTB57-VP8
LTB+VP8
VP8
B
NK1.1
CD3e
CD3e+NK1.1+ T cells in PBMCs
5.71%
8.78%
8.18%
8.72%
7.45%
Percentage of CD3e+NK1.1+ in PBMCs
PBS
LTB26+VP8
LTB57+VP8
LTB+VP8
VP8

PBS

LTB26+VP8

LTB57+VP8

LTB+VP8

VP8

CD11c+DC cells in PBMCs

0.86%

1.51%

0.97%

1.24%

0.99%

SSC

CD11c-APC

CD11B+IA/IE+ in CD11c+DC cells

Q1 7.30 Q2 88.6 Q4 1.86 Q3 2.29

Q1 4.63 Q2 84.9 Q4 3.03 Q3 7.41

Q1 4.53 Q2 94.2 Q4 0.52 Q3 0.78

Q1 10.7 Q2 83.0 Q4 2.98 Q3 3.29

Q1 8.85 Q2 87.4 Q4 1.15 Q3 2.56

IA/IE-PercP/Cy5.5

CD11b-FITC

F4-80+ in CD11c+CD11b+IA/IE+ DC cells

PBS

LTB26+VP8

LTB57+VP8

LTB+VP8

VP8

Count

0 10^3 10^4 10^5

F4-80 (MHC II)-PE

Percentage of $CD11c^+$ $CD11b^+IA\text{-}IE^+$ in PBMCs

1.5

1.0

0.75

0.50

0.25

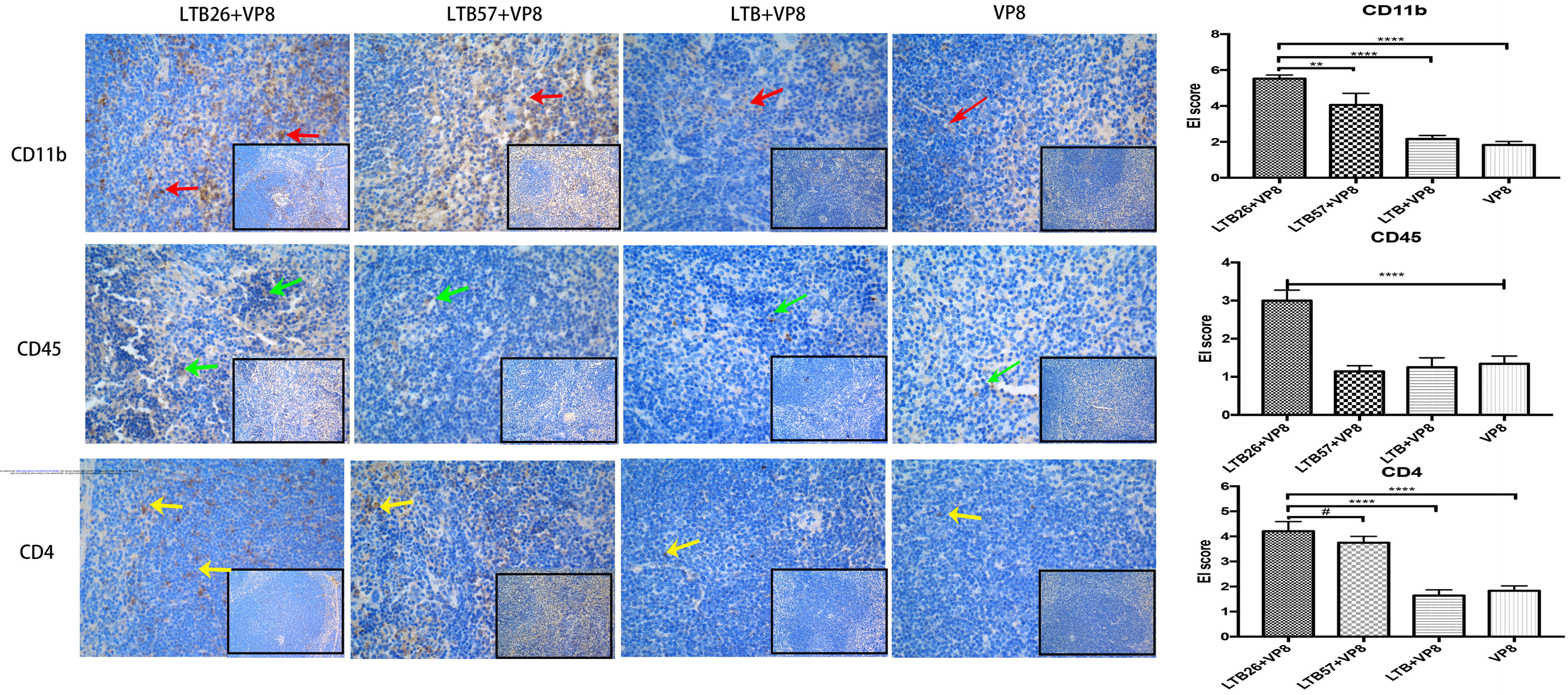
0.00

PBS LTB26+VP8 LTB57-VP8 LTB+VP8 VP8

Percentage of $F4\text{-}80^+$ in $CD11b^+IA\text{-}IE^+$ $CD11c^+$ DC cells(%)

6 5 5 4 3 2 1 0

PBS LTB26+VP8 LTB57-VP8 LTB+VP8 VP8


LTB26+VP8
LTB57+VP8
LTB+VP8
VP8
CD11b
CD45
CD4
CD11b
EI score
****
****
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LTB57+VP8
LTB+VP8
VP8
CD45
EI score
****
LTB26+VP8
LTB57+VP8
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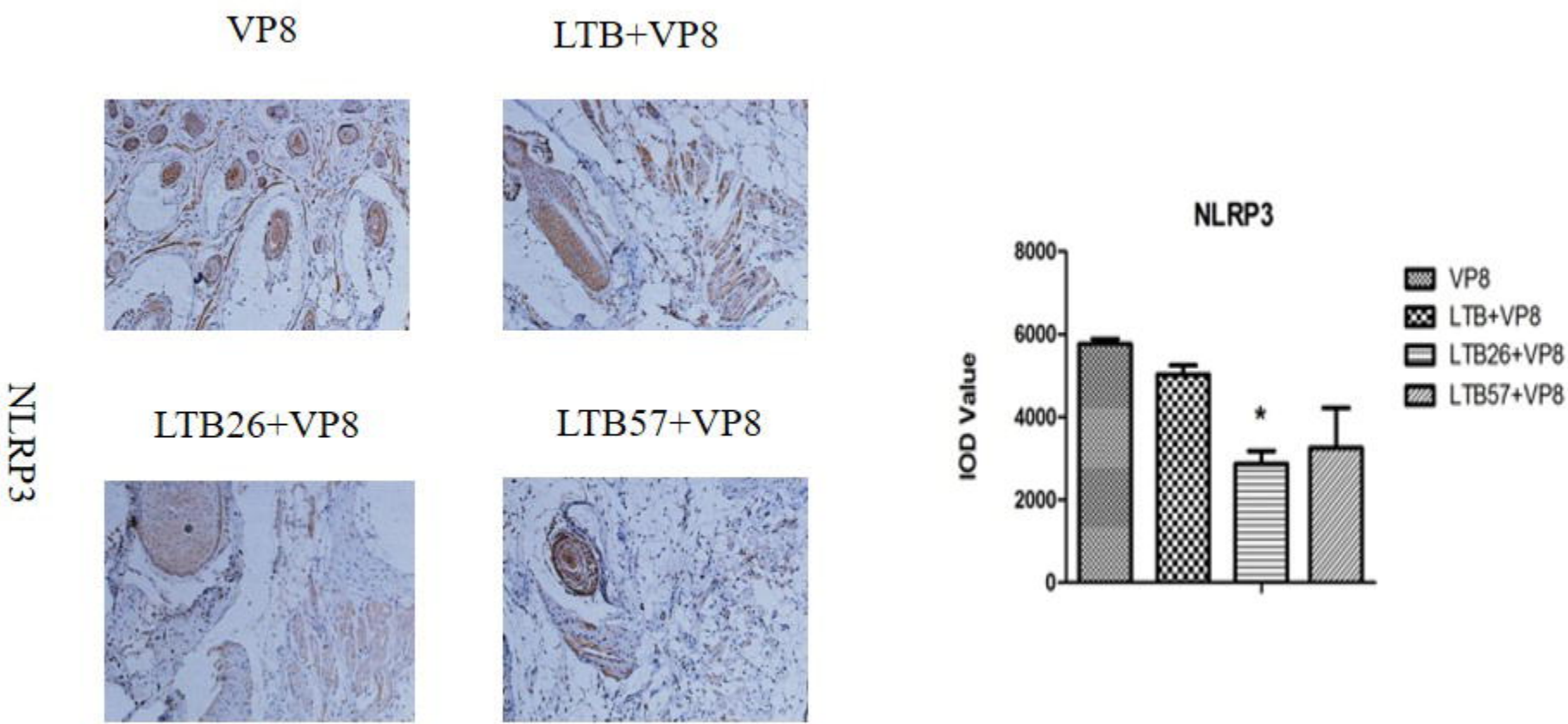
#
LTB26+VP8
LTB57+VP8
LTB+VP8
VP8


VP8
LTB+VP8
NLRP3
LTB26+VP8
LTB57+VP8
NLRP3
IOD Value
8000
6000
4000
2000
0
*
VP8
LTB+VP8
LTB26+VP8
LTB57+VP8