Transdermal entry of yeast components elicits transient B cell-associated responses in skin-draining lymph nodes

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Abstract

Immune responses to non-pathogenic yeasts induced within the draining lymph node remain to be understood. In this study, we have investigated the changes in lymphocytes and their activity in skin-draining lymph nodes in response to transdermally injected zymosan (component of the yeast cell wall). Zymosan elicited the transient increase of B cell number and activation status without affecting the capacity for proliferation. The increased B cell content in the regional lymph nodes was likely due to the reduction of B cell egress from the tissue and in part the increase of homing from the circulation. Zymosan also upregulated the inflammatory cytokines, such as IL-1β, IL-6, IL-12, and IFNγ, regulatory cytokines IL-10 and TGFβ, and lymphoid chemokine CXCL13. Among these, the expression of IL-12 and IL-10 was markedly high in B cells. Altogether, these findings demonstrate a unique B cell-associated response to non-pathogenic yeast component in the draining lymph nodes. This will provide insights into the clinical and healthcare applications of non-pathogenic beneficial microbes.

Keywords: B cells, Skin-draining lymph node, Yeast, Zymosan

1. Introduction

Humans have long been in close and continuous contact with a variety of yeasts in the environment. Yeasts are single-celled fungi that can be used in the production of various fermented foods or drugs and are of importance in civilization and culture in human history [1-4]. However, some yeasts are pathogenic including symbiotic yeasts that cause opportunistic infections in immunocompromised individuals [5-9]. Mycobiota have gained importance owing to their role in the development of diseases induced by the dysbiosis of the fungal community [10]. A large number of non-pathogenic yeasts constitute the microbial environment; however, the interaction between these harmless yeasts and the human body remains to be understood.

Yeast-derived components trigger immune response [6, 7, 11, 12]. β-glucans are a major group of polysaccharides in the yeast cell wall that induce the activation of immune cells via cell surface receptors, dectin-1 and TLR2, thereby activating NF-κB signaling and stimulates the gene expression of cytokines/chemokines [6, 7, 13-16]. Zymosan is an insoluble component of the cell wall that comprises β-glucans in the baker's yeast *Saccharomyces cerevisiae* and induces a variety of immune cells [15, 17-19].

Along with fermented foods, the human body is continuously exposed to live, dead, or fragmented yeasts through various routes. Although inhalation and the respiratory tract is the most common mode of entry [20], damaged or wounded skin is also a popular route [8] wherein skin-draining lymph nodes (sdLNs) are the sites of response. However, the type and extent of responses in sdLNs induced by non-pathogenic yeasts or their components remain to be understood. Yeasts are extensively used in vaccines, adjuvants, and drug delivery carriers/vectors [21-24]; thus, a detailed understanding of the responses elicited in sdLNs against yeasts and their components is important to effectively utilize these microbes in healthcare.

In this study, we examined sdLN responses after the subcutaneous injection of zymosan into mice as a model to study the entry of yeast via the skin. Zymosan transiently activated non-proliferative B cell responses and increased cellularity in the regional sdLNs. Zymosan also augmented the expression of several inflammatory and regulatory cytokines and chemokines. These findings provide insights into the physiological response of the body to non-pathogenic yeasts and their use in healthcare in the future.

2. Materials and Methods

2.1. Mice

C57BL/6JJcl mice were purchased from CLEA Japan. Mice were housed and crossed under specific pathogen-free conditions in the animal facility of Niigata University. All animal procedures were approved by the Committee on Animal Research at Niigata University. Mice aged 7–12 weeks were used for experiments. An independent experiment (n=1) represented the use of a single mouse or a

pair of mice.

2.2. Preparation of zymosan and subcutaneous injection

Zymosan A (Fujifilm Wako Pure Chemical) was dissolved in phosphate-buffered saline (PBS) to a final concentration of 5 mg/ml and boiled for 1 h followed by chilling to room temperature for 1 h. The zymosan A solution was washed 3 times with ice cold PBS after centrifugation at 300×*g* at 4°C for 10 min and resuspended in PBS at 5 mg/ml. Zymosan solution was used in the injection after diluting to 1 mg/ml with PBS. Mice were subcutaneously injected (fore and hind footpads and flanks) with 0.4 ml (0.4 mg) of zymosan or sterile PBS.

2.3. Cell isolation and flow cytometry

Single cell suspensions were prepared by harvesting sdLNs (brachial, inguinal, and popliteal nodes), cutting into small fragments, and digesting with 1 mg/ml collagenase D and 0.1 mg/ml DNase I (Roche Diagnostics) as described [25] followed by counting the total cell numbers using a hemocytometer. The cells were then stained with fluorescently labeled antibodies as follows: fluorescein isothiocyanate-, phycoerythrin-, or biotin-conjugated monoclonal antibodies for CD3 (145-2C11), B220 (RA3-6B2), CD4 (N318), CD8 (53-6.7), CD5 (53-7.3), CD69 (H1.2F3), MHC class II (M5/114.15.2), CD86 (GL1), and CD138 (281-2) were purchased from eBioscience. Data were acquired using the FACSCalibur flow cytometer (BD Biosciences) and analyzed using CellQuest (BD Biosciences) and FlowJo. Dead cells were excluded using forward scatter and propidium iodide staining data.

2.4. Magnetic cell sorting (MACS)

B or T cells were enriched from total lymph node cells by MACS using the B Cell Isolation Kit or Pan T Cell Isolation Kit II (Miltenyi Biotec), respectively, as per the kit instructions. Briefly, 1×10^7 cells in 60 µl of MACS buffer was incubated with 20 µl microbead-conjugated antibody cocktail for 15–20 min at 4˚C followed by incubating with 20 µl of anti-microbead antibody for 15–20 min at 4˚C. After washing twice with MACS buffer, the cells were passed through the MACS Separator using an LS column (Miltenyi Biotec). To enrich the non-lymphocyte fraction, B and T cells were removed from the total lymph node cells using biotinylated-antibodies against CD3, CD5, and B220. Purity of B cells, T cells, and non-lymphocytes were confirmed to be >98% by flow cytometry.

2.5. Cell culture and stimulation

Total lymph node cells or isolated B cells were cultured at 6×10^5 cells/0.2 ml of 8% fetal calf serum-containing RPMI-1640 medium supplemented with penicillin/streptomycin, L-glutamine, and 50 µM 2-mercaptoethanol in round-bottom 96-well plates. For *in vitro* stimulation, cultured cells were

incubated for 24 h with 20 μg/ml zymosan.

2.6. Cell proliferation

Lymphocytes isolated from the spleen were incubated with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) for 25 min at 37°C and washed twice with PBS. Mice were intravenously injected with 2×10^7 CFSE-labeled cells first and subcutaneously injected with zymosan 18 h later. Lymphocytes were harvested from sdLNs at 72 h and stained with antibodies against CD3 or B220, and CD69 for flow cytometry.

Total lymph node cells or isolated B cells were labeled with CFSE and cultured in round-bottom 96-well plates in the presence or absence of 50 μg/ml zymosan or 25 μg/ml lipopolysaccharide for 72 h; the cells were then stained for flow cytometry. The proliferation module of the FlowJo software was used to determine the percentage of cells that divided at least once based on the CFSE signal.

2.7. Inhibition of lymphocyte homing into lymph nodes

Anti-CD62L (MEL-14) antibody was purified from a hybridoma culture supernatant. Mice were intraperitoneally injected with or without 150 μg anti-CD62L antibody [26] followed by subcutaneous injection with zymosan or PBS. After 24 h, cells were isolated from sdLNs and total cell numbers were counted using a hemocytometer. The cells were then stained with anti-CD3 and anti-B220 antibodies for flow cytometry.

2.8. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from sdLNs or isolated cells using TRIzol reagent (Invitrogen) and cDNA was synthesized using the Superscript III First-Strand Synthesis System (Invitrogen). Quantitative PCR was performed using the LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche) and LightCycler (Roche). Primers used were as follows: IL-1β, 5′-AGTTGACGGACCCCAAAAG-3′ and 5′-TGTCCTCATCCTGGAAGGTC-3′; IL-6, 5′-GAGGATACCACTCCCAACAGACC-3′ and 5′-AAGTGCATCATCGTTGTTCATACA-3′; TNFα, 5′-ATGAGGACAGAAAGCATGA-3′ and 5′-AGTAGACAGAAGAGCGTGGT-3′; IL-12p35, 5′-GATGACATGGTGAAGACGGC-3′ and 5′-AGGCACAGGGTCATCATCAA-3′; IFNγ, 5′-TTCTTCAGCAACAGCAAGGC-3′ and 5′-TCAGCAGCGACTCCTTTTCC-3′; IL-4, 5′-AACGAGGTCACAGGAGAAGG-3′ and 5′-TCTGCAGCTCCATGAGAACA-3′; IL-10, 5′-TGGCCCAGAATCAAGGAGC-3′ and 5′-CAGCAGACTCAATACACACT-3′; TGFβ, 5′-TGCGCTTGCAGAGATTAAAA-3′ and 5′-CTGCCGTACAACTCCAGTGA-3′; CXCL13, 5′-TGGCTGCCCCAAAACTGA-3′ and 5′-TGGCACGAGGATTCACACAT-3′; CCL19, 5′-CTGCCTCAGATTATCTGCCAT-3′ and 5′-AGGTAGCGGAAGGCTTTCAC-3′; CCL21a, 5′-ATCCCGGCAATCCTGTTCTC-3′ and 5′-

GGTTCTGCACCCAGCCTTC-3′; and GAPDH, 5′-GCCAAGGTCATCCATGACAACT-3′ and 5′-GAGGGGCCATCCACAGTCTT-3′. mRNA levels of the test genes were normalized to the expression of GAPDH.

2.9. Immunohistochemistry

Isolated sdLNs (axially, brachial, inguinal, and popliteal nodes) were fixed with 0.05% phosphate buffer containing 0.075 M L-lysine (pH 7.4), 0.01 M $NaIO_4$, and 1% paraformaldehyde at 4 °C for 24 h. After fixation, tissues were equilibrated gradually with 10%, 20%, and 30% sucrose in PBS at 4 °C, embedded in OTC compound (Sakura Finetechnical), and frozen at −80 °C. Frozen sections (10 µm) were made using a cryostat (Leica Biosystems) and post-fixed with cold acetone for 3 min. Sections were stained with antibodies to B220 (RA3-6B2; eBioscience), laminin (rabbit polyclonal; LSL) and CXCL13 (BAF470; R&D Systems), and mounted with Permafluor mountant (ThermoFisher Scientific). The specimens were examined using a FV1200 confocal microscope (Olympus). Digital images were prepared using FV10-ASW (Olympus) and Adobe Photoshop CS6 (Adobe Systems).

2.10.Statistical analysis

Statistical analysis was performed with GraphPad Prism 7 software using Mann-Whitney *U*-test when comparing two groups and one-way or two-way ANOVA with multiple comparisons tests when comparing more than two groups. P values <0.05 were considered statistically significant.

3. **Results**

3.1. Transdermal injection of zymosan induces a transient increase in B cells in sdLNs

To determine the physiological responses to yeast that entered through the transdermal route, mice were subcutaneously injected with zymosan or control (PBS) followed by flow cytometric analysis of the immune cells in the sdLNs. The total cell number in sdLNs increased by ~2-fold 24 h after the zymosan injection and rapidly reduced thereafter (Fig. 1A). The proportion of B cells increased by ~20% after 24 h and decreased within 96 h of injecting zymosan, while the proportion of T cells decreased at 24 h and were partially restored 96 h after injecting zymosan (Fig. 1B–D). B cell number increased markedly in response to zymosan at 24 h, while T cell number was only marginally changed (Fig. 1E and F). The ratio of $CD4^+$ and $CD8^+$ T cells did not change within 96 h of zymosan treatment (data not shown). These findings suggest that B cells in sdLNs are transiently responsive to the transdermal entry of zymosan.

3.2. Zymosan transiently activates B cells in sdLNs

Since there was a transient increase in the number of B cells in the sdLNs induced by zymosan, we wanted to elucidate the mechanism involved. To confirm whether lymphocyte activation was induced

by zymosan, we determined the expression of the early activation marker CD69. Zymosan significantly induced CD69 expression in ~30% of B cells in the sdLNs after 24 h, but CD69 was downregulated within 96 h of zymosan injection (Fig. 2A and C), indicating a rapid response to zymosan in B cells. In contrast, although a fraction of T cells constitutively expressed CD69 in untreated conditions, CD69 did not significantly increase upon zymosan treatment as compared to the expression in B cells (Fig. 2B and D). To ascertain if the B cell response to zymosan was direct, cultured lymph node cells or isolated B cells were stimulated with zymosan *in vitro*. A fraction of B cells expressed CD69 (Fig. 2E and F), demonstrating that B cells directly respond to zymosan. In comparison with CD69, however, the expression of other activation markers such as MHC class II and CD86 was not significantly changed upon zymosan injection (Fig. 3), suggesting that zymosan induces a limited activation status in B cells.

3.3. Zymosan induces non-proliferative B cell activation in sdLNs

Next, we investigated the change in B cell proliferation by zymosan. We subcutaneously injected zymosan to mice that was in advance adoptively transferred with CFSE-labeled B cells. Despite the clear induction of CD69 expression, the dilution of CFSE signals was undetectable both in $CD69^+$ and $CD69^-$ fractions (Fig. 4), suggesting that B cells did not proliferate *in vivo*. Next, we checked the stimulation of CSFE-labeled lymph node cells or isolated B cells by zymosan *in vitro*. We detected low amounts of B cell proliferation in presence of zymosan especially in $CD69^+$ fraction (Fig. 5), suggesting that zymosan can directly induce proliferation in a small fraction of B cells. In contrast, lipopolysaccharide markedly induced the proliferation of CFSE-labeled B cells with a high expression of CD69 (Fig. 5). However, zymosan did not stimulate $CD138^+$ plasma cell differentiation *in vitro* (data not shown). These findings suggest that transdermally injecting zymosan transient activates non-proliferative B cell in sdLNs.

3.4. Zymosan inhibits the egress of B cells from sdLNs

The transient increase in B cells in sdLNs could be attributed to increased homing of circulating lymphocytes into lymph nodes or decreased egress from the lymph nodes. To test this, mice were intravenously injected with anti-CD62L antibody to block the homing of blood lymphocytes to lymph nodes [26, 27] followed by injecting zymosan. Anti-CD62L antibody markedly reduced the total number of lymphocytes in sdLNs by ~1/3 as those in untreated samples (Fig. 6A). Even in the homing-suppressed condition, zymosan significantly augmented lymphocytes in sdLNs by primarily increasing the number of B cells; the increased cell number was equivalent to ~2/3 of the cell increase in zymosan-treated sdLNs without CD62L blockage (Fig. 6B). These suggest that zymosan-mediated increase in lymphocyte content in sdLNs is mainly due to the reduction of egress from the tissue, although some fraction (~1/3) of cell increase is mediated by the enhancement of homing.

3.5. Zymosan augments the expression of inflammatory cytokines, IL-10, and CXCL13 in sdLNs

To further explore the response induced by zymosan in sdLNs, we analyzed the expression of cytokines and chemokines after 24 h of injecting zymosan by quantitative PCR analysis. The expression of typical inflammatory cytokines, IL-1β and IL-6, clearly increased in response to zymosan (Fig. 7A). We also observed an increase in the expression of Th1 cytokines, IL-12 and IFNγ, while that of a Th2 cytokine, IL-4, was unaltered (Fig. 7A). The expression of immunoregulatory cytokines, IL-10 and TGFβ, also increased (Fig. 7A). Among the lymphoid chemokines assayed, CXCL13 was upregulated, whereas CCL21a, CCL19, and CXCL12 were reduced (Fig. 7B). These findings show that the transdermal injection of zymosan induces the unique expression profiles of immune mediators in sdLNs.

To identify the cell types responsible for these changes, we determined the expression of these cytokines and chemokines in isolated B cell, T cell, and non-lymphocyte fractions (Fig. 7C). Non-lymphocyte fraction showed increased levels of IL-1β and IL-6. Upregulated IL-12p35 expression was primarily detected in B cells and non-lymphocytes, while T cells showed an increased expression of IFNγ. Interestingly, B cells showed an increase in the levels of IL-10 and TGFβ. Thus, zymosan differentially regulates cytokine expression in a variety of immune cells in sdLNs.

3.6. Zymosan directly stimulates the expression of cytokines in B cells

We wanted to ascertain if B cells directly respond to zymosan by the increase in cytokine gene expression. We cultured isolated B cells in the presence of zymosan followed by quantitative PCR to analyze the gene expression profiles. We observed a significant increase in IL-1β, IL-12p35 and IL-10 expression in isolated B cells (Fig. 7D). Therefore, zymosan directly stimulates some cytokine expression in B cells.

3.7. Zymosan induces the expansion of B cell follicles in sdLNs

Finally, we examined tissue alteration in sdLNs after zymosan injection using immunohistochemistry. Zymosan induced clear enlargement of lymph nodes compared with the control after 24 h (Fig. 8). In particular, expansion of B cell follicles was remarkable in comparison with other areas, which is likely to reflect the increased content of B cells. CXCL13-producing follicular stromal cells also appeared to be increased with the expansion of follicular areas. This observation is consistent with upregulated expression in CXCL13 gene in zymosan-treated sdLN. Together, zymosan induces structural alterations in the regional lymph nodes mainly through the change of B cell content.

4. Discussion

The transdermal entry of yeast cell wall component, zymosan, induced characteristic immune

responses in sdLNs. We observed a transient alteration in the B cell composition and content; a fraction of these B cells showed increased expression of CD69 without significant changes to proliferation. Zymosan stimulated CD69 expression in isolated B cells *in vitro*, suggesting that B cells respond directly to zymosan.

The inhibition of lymphocyte homing into lymph nodes did not largely affect zymosan-mediated increase of B cells, suggesting that zymosan reduces the egress of B cells from regional lymph nodes, although some minor fraction of cell increase was likely due to the augmentation of homing from the circulation. In general, inflammatory stimuli rapidly halt the egress of lymphocytes from lymph nodes, a process termed as “shutdown” [28, 29]. The cell surface induction of CD69 on lymphocytes reduces responsiveness to sphingosine-1-phosphate (S1P) by facilitating the internalization of specific receptor $S1P_1$, thereby inhibiting lymphocyte egress from efferent lymph and promoting their retention in the lymph nodes [30-32]. Owing to the coordinated increase in CD69 expression in B cells, this could have caused the increase in the numbers of B cells in sdLNs upon injecting zymosan. Moreover, the zymosan-induced increase in expression of the B cell attractant CXCL13 is consistent with B cell retention in sdLNs, although the mechanism by which zymosan stimulates CXCL13 expression in stromal cells remains to be elucidated.

We also detected specific patterns of cytokine expression in sdLNs in response to zymosan. Increased expression of IL-1β and IL-6 in the non-lymphocyte fraction indicated the induction of specific inflammatory responses. In addition, increased levels of IFNγ in T cells suggested that zymosan enhanced cytotoxic T cell responses. IL-12 expression was also markedly increased in B cells, which is in accordance with previous studies that show the capacity of B cells to express this cytokine [33, 34]. Zymosan injection also increased IL-10 and TGFβ expression in sdLNs. Notably, these immunoregulatory cytokines were increased in the B cell fraction. We speculate that zymosan stimulates a counter response mediated by regulatory B cells [35].

Humans have long coexisted with non-pathogenic yeasts that are not particularly harmful to healthy individuals. However, the immune system has the capacity to eliminate any microbe since they are foreign substances. The immune system induces the regulatory arm of response to harmless non-commensal microbes since an extreme response to non-pathogens harms the body. The induction of IL-10 and TGFβ in B cells by zymosan possibly reflects such a reaction: some inflammatory cytokines are induced along with the expression of inhibitory cytokines to balance any potentially harmful effects.

Elucidating the properties of yeast components involved in inducing specific immune responses could be advantageous for the development of novel strategies for vaccination or drug delivery vectors and enhancing the nutritional value of foods. Thus, further explorations are needed to understand the role of yeasts in host physiological responses in the future.

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Conflict of interest

The authors declare no conflict of financial interest.

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Figure legends

Figure 1.

Subcutaneously injected zymosan increases overall cellularity and B cells in skin-draining lymph nodes (sdLNs).

(A) Time-dependent changes in total cell number in sdLNs after injecting zymosan or PBS. Mean ± standard deviation (SD), n=5. **: $p<0.01$, ****: $p<0.0001$ (one-way ANOVA with Dunnett's post-test).

(B) Flow cytometric analysis for determining the composition of B and T cells in sdLNs. Cells isolated from sdLNs at the indicated times after zymosan or PBS injection were stained for B220 and CD3.

(C–F) The change of the percentages (C, D) and numbers (E, F) of B ($B220^{+}CD3^{-}$) and T cells ($CD3^{+}B220^{-}$) in sdLNs. Mean ± SD, n=5. *: $p<0.05$, **: $p<0.01$, ****: $p<0.0001$ (one-way ANOVA with Dunnett's post-test).

Figure 2.

Zymosan transiently activates B cells in sdLNs.

(A, B) Flow cytometric analysis of CD69 expression in B (A) and T cells (B) in sdLNs after subcutaneously injecting zymosan. Cells isolated from sdLNs at the indicated times after zymosan injection were stained for B220 (A) or CD3 (B) as well as CD69.

(C, D) Changes in CD69 expression in B cells and T cells in sdLNs. Percentages of $B220^+CD69^+$ (C) or $CD3^+CD69^+$ (D) cells among the total lymph node cells are shown. Mean ± SD, n=5. *: $p<0.05$, ****: $p<0.0001$ (one-way ANOVA with Dunnett's post-test).

(E, F) CD69 expression in B cells in the presence or absence (phosphate-buffered saline; PBS) of zymosan in the total lymph node cell (E) or isolated B cell (F) *in vitro* cultures for 24 h. Percentages of $B220^+CD69^+$ cells are shown in the lower graphs. Mean ± SD, n=6. **: $p<0.01$ (Mann-Whitney *U*-test).

Figure 3.

A limited activation state of B cells in response to zymosan.

(A-C) Flow cytometric analysis of the expression of CD69 (A), MHC class II (B), and CD86 (C) in B cells in sdLNs after zymosan injection. Cells isolated from sdLNs at 24 h after zymosan injection were stained for B220 and other markers. Expression of the indicated markers in $B220^+$-gated cells are shown in histograms. Mean ± SD, n≧4. *: $p<0.05$ (Mann-Whitney *U*-test).

Figure 4.

Zymosan does not induce B cell proliferation *in vivo*.

(A, B) CFSE analysis of B cell proliferation in sdLNs after 72 h of subcutaneously injecting zymosan or PBS. (A) Detection of transferred $CFSE^+$ cells in total lymph node cells. (B) CFSE intensities in $B220^+CD69^-$- and $B220^+CD69^+$-gated fractions in dot plots (A) are shown in histograms. CFSE dilutions were virtually undetectable in the presence or absence of zymosan injection. Representative result of 4 experiments is shown. CSFE diluted cells (%): PBS, 1.14 ± 0.67; Zym, 1.49 ± 1.26. Statistically no significant difference was detected between PBS and zymosan (Mann-Whitney *U*-test).

Figure 5.

Zymosan induces proliferation in a fraction of B cells *in vitro*.

(A-F) CFSE analysis of the total lymph node cell (A-C) or isolated B cell (D-F) cultures in the presence of zymosan, lipopolysaccharide (LPS), or PBS for 72 h. (A, D) Profiles of CD69 expression (upper) and CFSE signal in $B220^+$-gated cells (lower). (B, E) CFSE intensities in total $B220^+$, $B220^+CD69$- and $B220^+CD69^-$-gated cells in dot plots (A, D) are shown in histograms. (C, F) Percentages of cells in each fraction are shown. Mean ± SD, n=4. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$ (one-way ANOVA with Dunnett's post-test).

Figure 6.

Egress block is a major cause of increasing number of B cells in sdLNs after injecting zymosan.

(A–C) The numbers of total (A), B (B), and T cells (C) in sdLNs in the presence or absence of anti-

CD62L antibody and subcutaneously injected zymosan or PBS were determined by cell counting and flow cytometry. Mean ± SD, n=4. **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$ (two-way ANOVA with Tukey's post-test).

Figure 7.

Zymosan induces a unique cytokine and chemokine expression profile in sdLNs.

(A, B) Quantitative reverse transcript-polymerase chain reaction for the expression of cytokines (A) and chemokines (B) in sdLNs after 24 h of subcutaneously injecting zymosan or PBS. The cytokine and chemokine levels were normalized to that of GAPDH. Mean ± SD, n≧4. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$ (Mann-Whitney *U*-test).

(C) The expression of cytokines in B cells (B), T cells (T), and non-lymphocyte (nL) fractions isolated from sdLNs (LN).

(D) Cytokine expression in isolated B cells stimulated with zymosan *in vitro*.

Figure 8.

Zymosan induces the expansion of B cell follicles in sdLNs

(A, B) Fluorescent immunohistochemistry of brachial (A) and popliteal (B) lymph nodes after 24 h of subcutaneously injecting zymosan or PBS. Tissue sections were stained for B220, CXCL13, and laminin. Boxed regions in the middle images for CXCL13 are magnified in the right panels. Images are the representatives of more than 6 lymph nodes from 2 mice in each treatment.

Figure 1

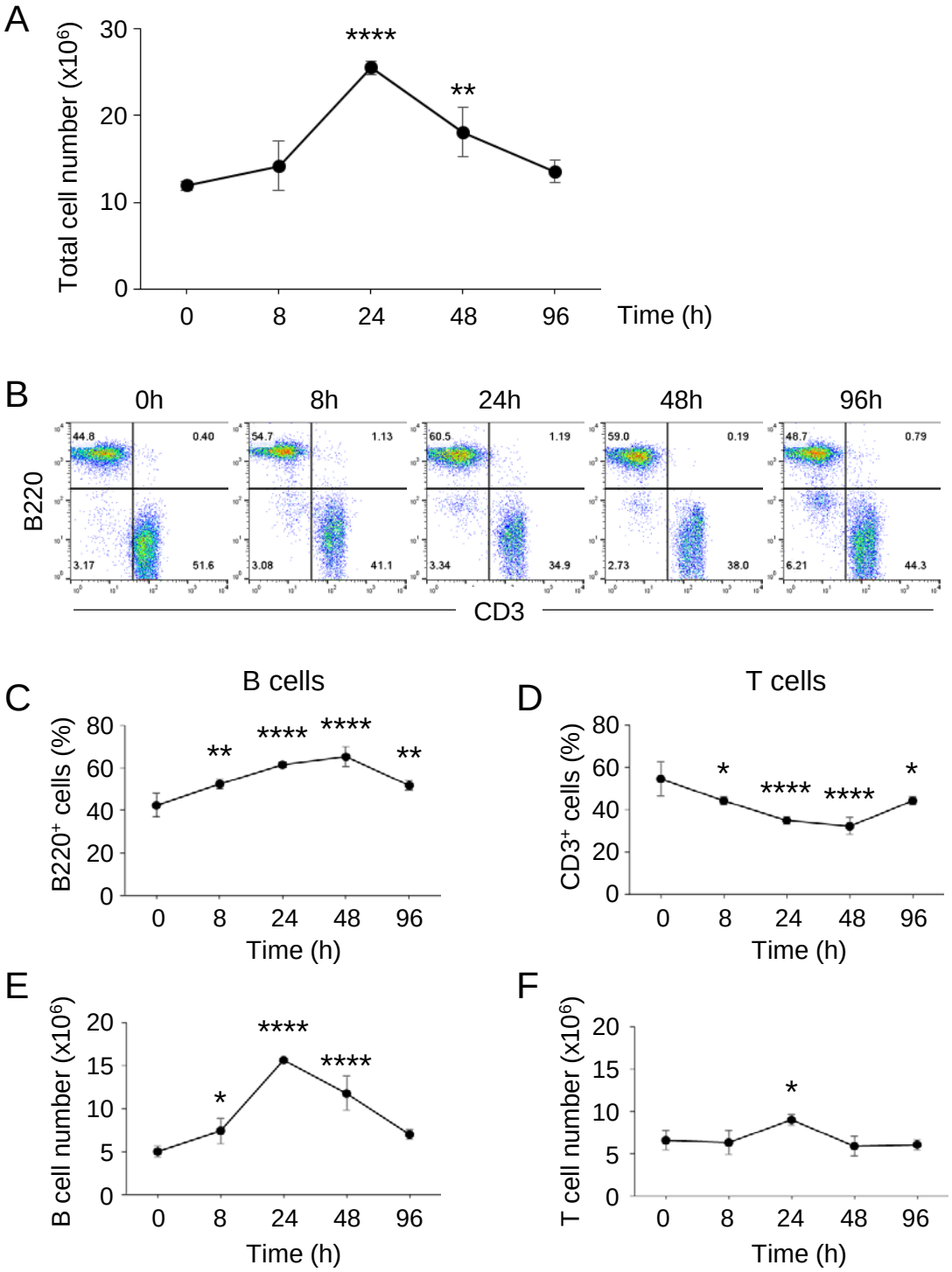

A
Total cell number (x10⁶)
30
20
10
0
****
**
0
8
24
48
96
Time (h)
B
0h
8h
24h
48h
96h
B220
CD3
44.8
0.40
3.17
51.6
54.7
1.13
3.08
41.1
60.5
1.19
3.34
34.9
59.0
0.19
2.73
38.0
48.7
0.79
6.21
44.3
C
B cells
B220⁺ cells (%)
80
60
40
20
0
**
****
****
**
0
8
24
48
96
Time (h)
D
T cells
CD3⁺ cells (%)
80
60
40
20
0
*
****
****
*
0
8
24
48
96
Time (h)
E
B cell number (x10⁶)
20
15
10
5
0
*
****
****
0
8
24
48
96
Time (h)
F
T cell number (x10⁶)
20
15
10
5
0
*
0
8
24
48
96
Time (h)

Figure 2

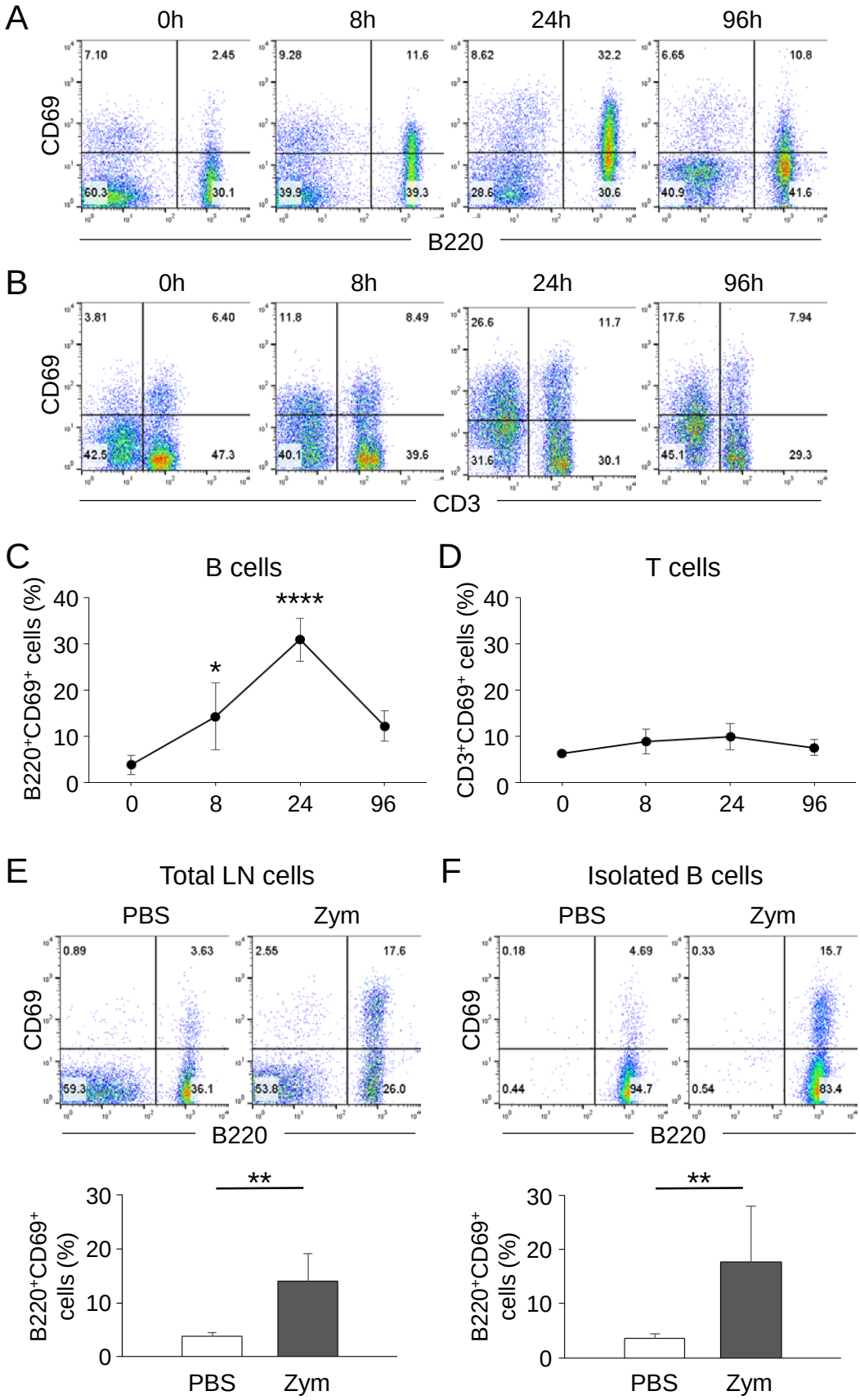

A
0h
8h
24h
96h
CD69
B220
7.10
2.45
60.3
30.1
9.28
11.6
39.9
39.3
8.62
32.2
28.6
30.6
6.65
10.8
40.9
41.6
B
CD69
CD3
3.81
6.40
42.5
47.3
11.8
8.49
40.1
39.6
26.6
11.7
31.6
30.1
17.6
7.94
45.1
29.3
C
B cells
B220+CD69+ cells (%)
****
*
D
T cells
CD3+CD69+ cells (%)
E
Total LN cells
PBS
Zym
CD69
B220
0.89
3.63
59.3
36.1
2.55
17.6
53.8
26.0
B220+CD69+ cells (%)
**
F
Isolated B cells
0.18
4.69
0.44
94.7
0.33
15.7
0.54
83.4

Figure 3

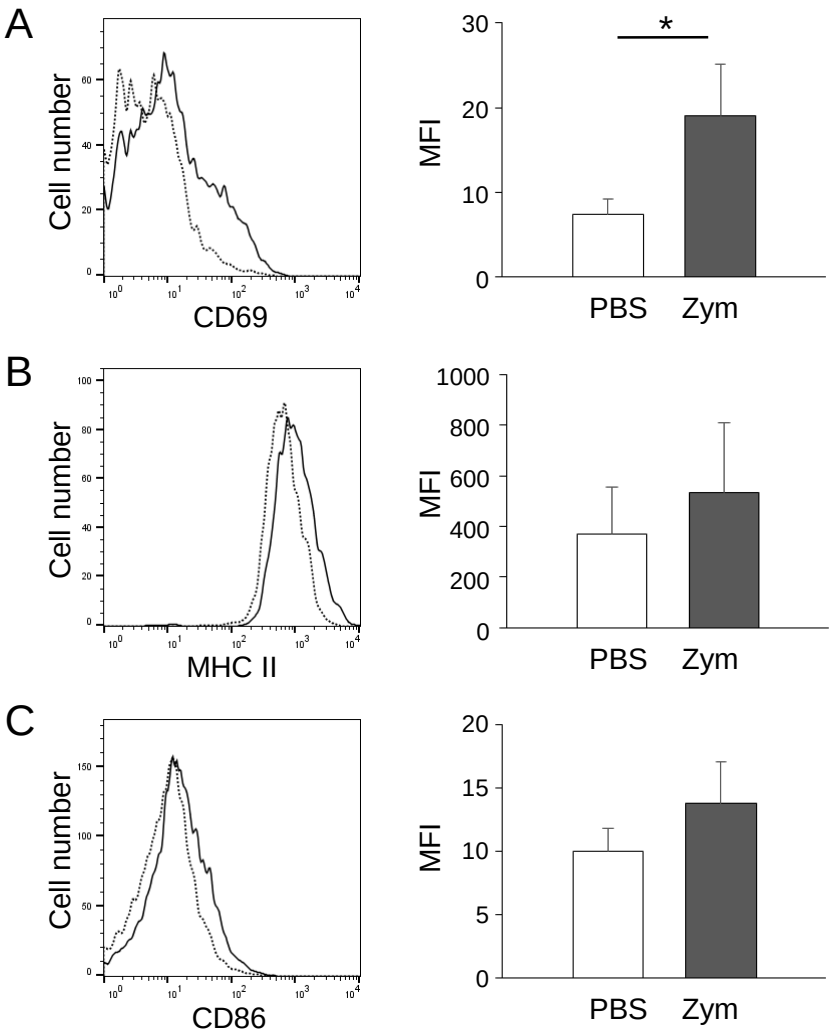

A
Cell number
CD69
MFI
*
PBS
Zym
B
Cell number
MHC II
MFI
PBS
Zym
C
Cell number
CD86
MFI
PBS
Zym

Figure 4

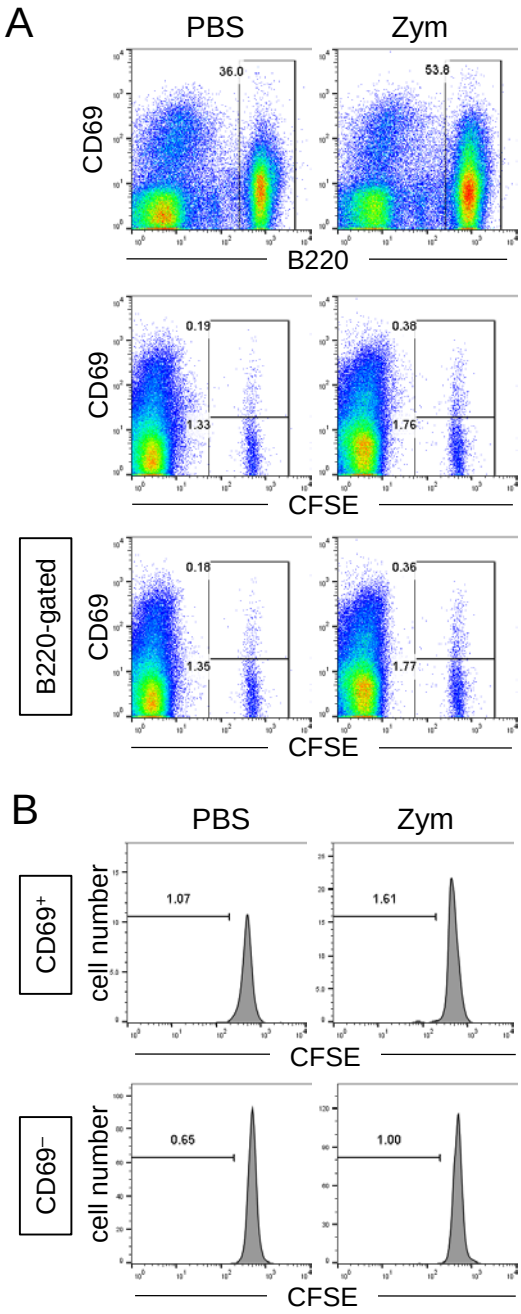

A
PBS
Zym
CD69
36.0
53.8
B220
CD69
0.19
1.33
0.38
1.76
CFSE
B220-gated
CD69
0.18
1.35
0.36
1.77
CFSE
B
PBS
Zym
CD69+
cell number
1.07
1.61
CFSE
CD69−
cell number
0.65
1.00
CFSE

Figure 5

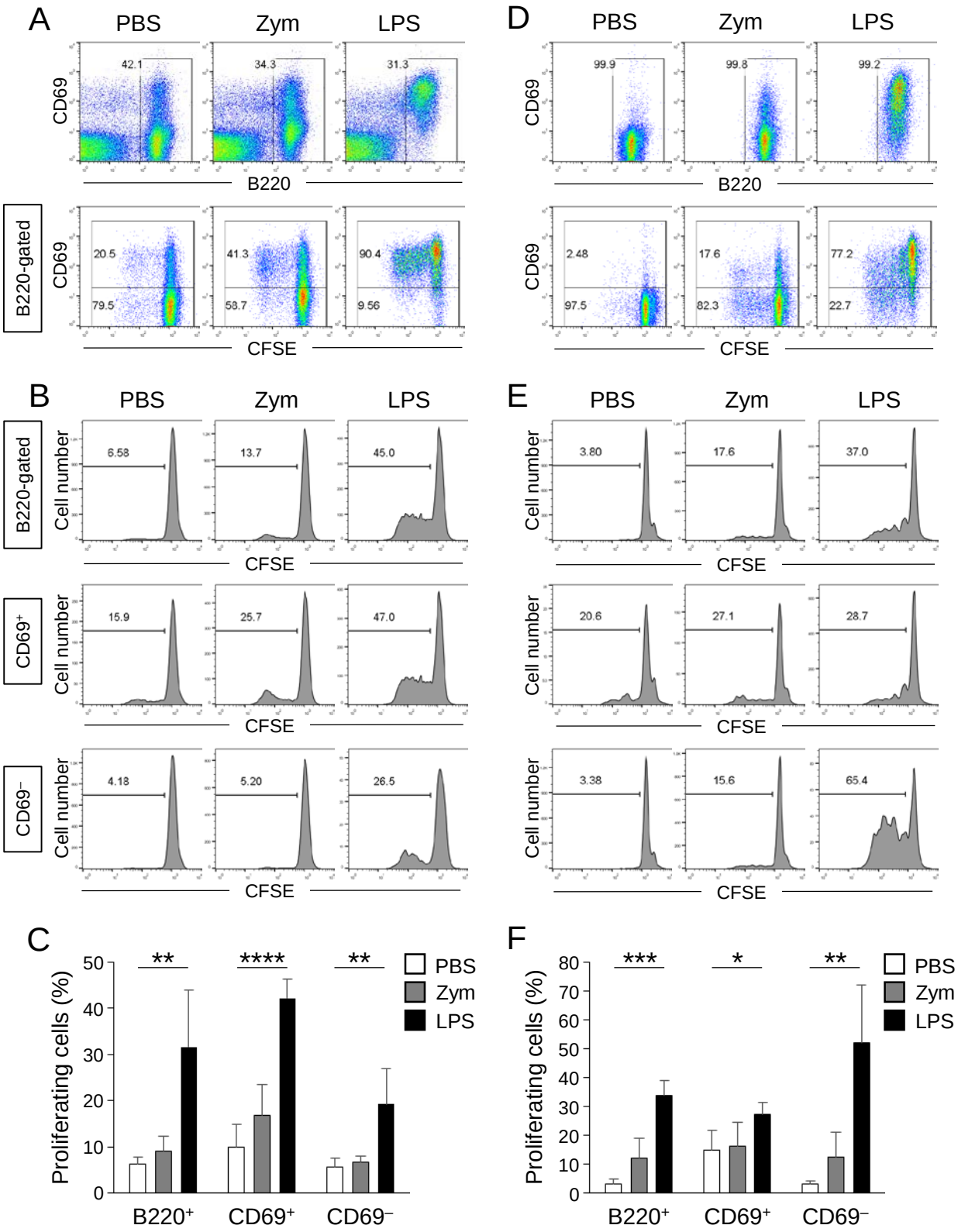

A
PBS
Zym
LPS
CD69
B220
42.1
34.3
31.3
B220-gated
CD69
CFSE
20.5
79.5
41.3
58.7
90.4
9.56
D
PBS
Zym
LPS
CD69
B220
99.9
99.8
99.2
CD69
CFSE
2.48
97.5
17.6
82.3
77.2
22.7
B
PBS
Zym
LPS
B220-gated
Cell number
CFSE
6.58
13.7
45.0
CD69+
15.9
25.7
47.0
CD69−
4.18
5.20
26.5
E
PBS
Zym
LPS
Cell number
CFSE
3.80
17.6
37.0
20.6
27.1
28.7
3.38
15.6
65.4
C
Proliferating cells (%)
50
40
30
20
10
0
**
****
**
PBS
Zym
LPS
B220+
CD69+
CD69−
F
Proliferating cells (%)
80
70
60
50
40
30
20
10
0
***
*
**
PBS
Zym
LPS
B220+
CD69+
CD69−

Figure 6

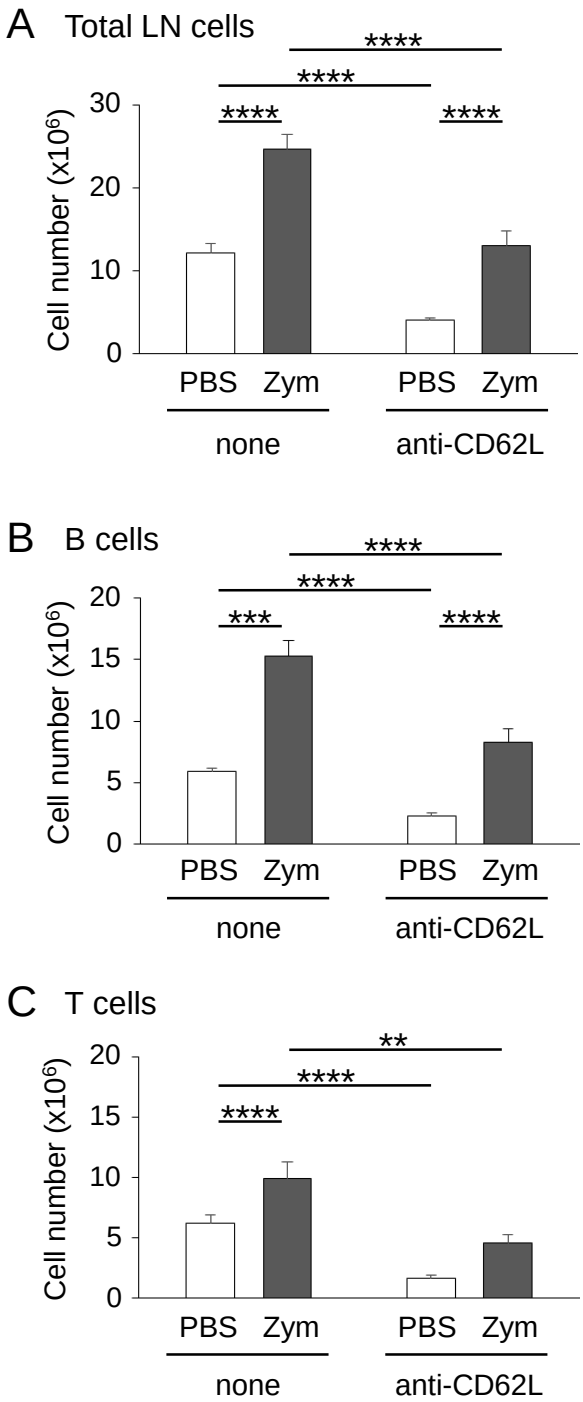

A
Total LN cells
****
****
****
****
30
20
10
0
Cell number (x10^6)
PBS
Zym
PBS
Zym
none
anti-CD62L
B
B cells
****
****
***
****
20
15
10
5
0
Cell number (x10^6)
PBS
Zym
PBS
Zym
none
anti-CD62L
C
T cells
**
****
****
20
15
10
5
0
Cell number (x10^6)
PBS
Zym
PBS
Zym
none
anti-CD62L

Figure 7

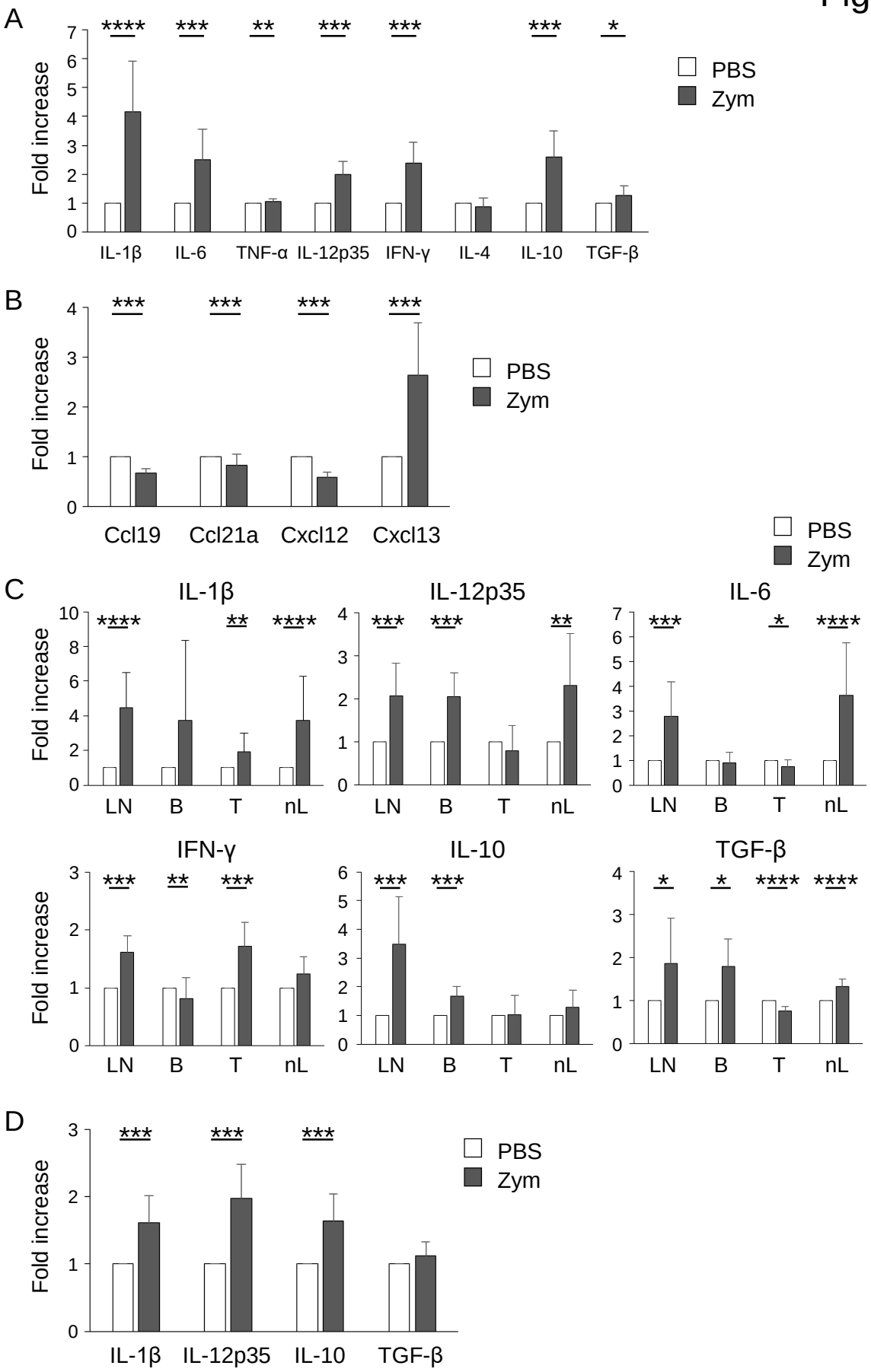
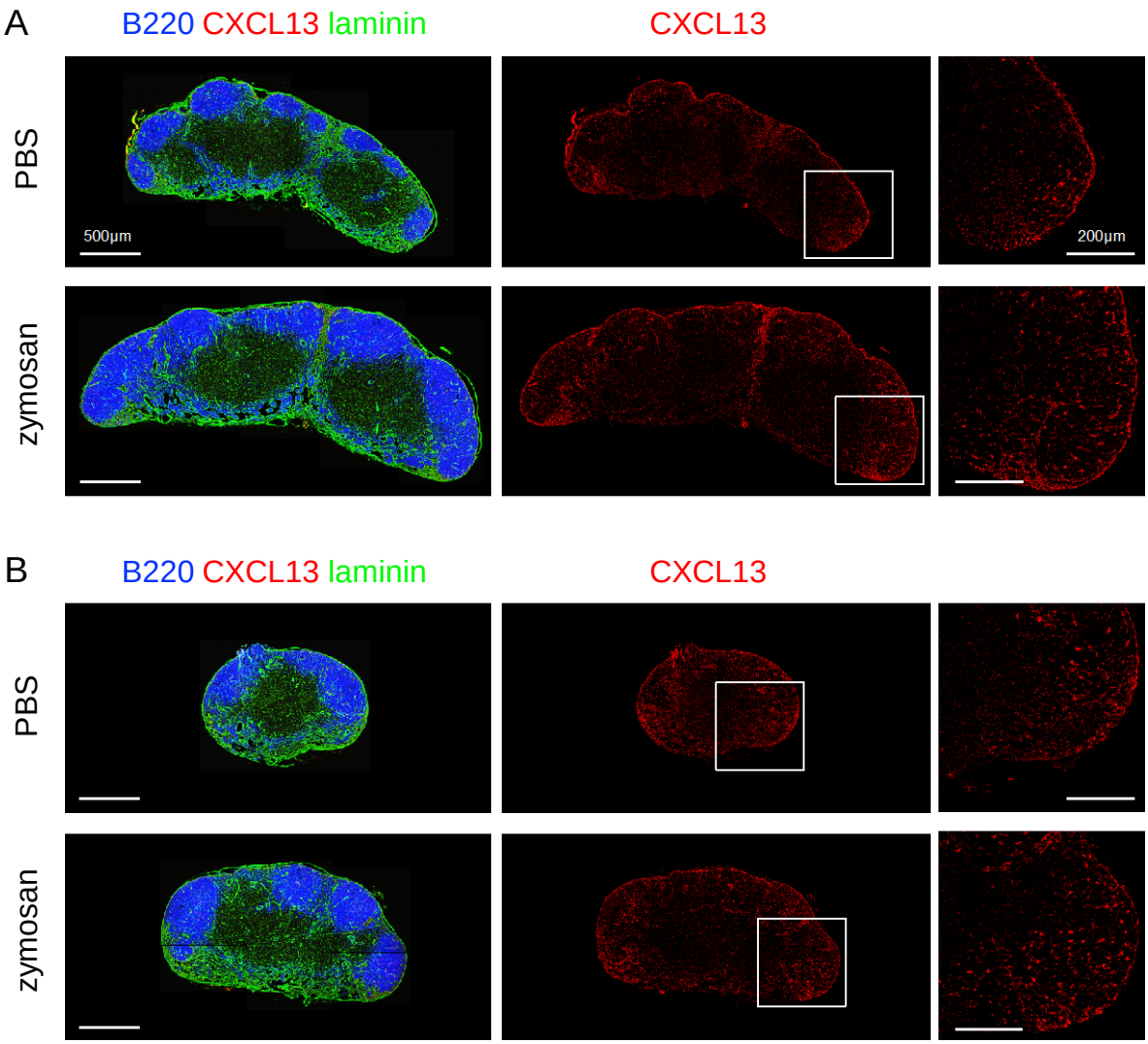

A
Fold increase
PBS
Zym
IL-1β
IL-6
TNF-α
IL-12p35
IFN-γ
IL-4
IL-10
TGF-β
B
Ccl19
Ccl21a
Cxcl12
Cxcl13
C
IL-1β
IL-12p35
IL-6
IFN-γ
IL-10
TGF-β
LN
B
T
nL
D

Figure 8


A
B220 CXCL13 laminin
CXCL13
PBS
zymosan
500μm
200μm
B
B220 CXCL13 laminin
CXCL13
PBS
zymosan