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Lipopolysaccharide (LPS) Enhances Antibody Responses in c-Jun N-Terminal Kinase 1 (JNK1)- and JNK2-deficient B Cells

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Abstract

Lipopolysaccharide (LPS) activates polyclonal B cells and antibody production independent of T cells. Treatment of murine B cells with LPS caused immediate phosphorylation of JNK1 and JNK2, which was further enhanced by the addition of interleukin-4 (IL-4). Whether LPS-induced JNK phosphorylation is involved in polyclonal antibody production by B cells was examined using B cells from JNK1-deficient ($JNK1^{-/-}$) and $JNK2^{-/-}$ mice. B cell development in both $JNK1^{-/-}$ and $JNK2^{-/-}$ mice was comparable to that of wild-type (WT) mice. The LPS-induced proliferative responses of $JNK1^{-/-}$ B cells, as assessed by ^{3}H-thymidine incorporation, were higher than those of WT B cells over the range of doses tested, suggesting that JNK1 negatively regulates B cell proliferative responses following stimulation with LPS. In contrast, $JNK2^{-/-}$ B cell proliferative responses were slightly increased in a narrow range of doses (around 5 μg/ml), while they were slightly decreased at low and high concentrations of LPS (0.15 and 50 μg/ml). Production of IgG1 and IgG2a polyclonal antibodies in responses to LPS plus IL-4 was increased in both $JNK1^{-/-}$ and $JNK2^{-/-}$ B cells relative to WT B cells, whereas IgM production was substantially decreased. LPS-induced IgG2b production was substantially enhanced in $JNK2^{-/-}$ B cells, but unaltered in $JNK1^{-/-}$ B cells relative to WT B cells. These results suggest that JNK1 and JNK2 negatively regulate B cell isotype switching, but positively affect B cell proliferative responses following stimulation with LPS and/or IL-4. Because LPS is a component of gram-negative bacteria, these results have implications for understanding B cell behaviour after bacterial invasion as well as the molecular mechanisms underlying LPS-induced polyclonal B cell activation.

Introduction

Lipopolysaccharide (LPS), a major cell wall component of Gram-negative bacterial organisms, potently activates polyclonal B cells to proliferate and differentiate into antibody-forming cells[1)2)]. LPS-activated B cells undergo isotype switching under the influence of T cells or their derived cytokines and co-stimulators. LPS in combination with IL-4 and IFN-γ induces isotype switching to IgG1 and IgG2a[3-5)], respectively, suggesting that isotype switching depends on the cytokine milieu. LPS binds to the complex formed by Toll-like receptor4 (TLR4) with MD-2 and CD14[6)], activating a signal transduction cascade that includes nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases (MAPKs)[6)7)]. The MAPKs comprise extracellular-signal-regulated kinases (ERKs), p38MAPKs, and c-Jun NH_2-terminal kinases (JNKs)[8)9)]. The JNKs include JNK1 and JNK2, which are expressed ubiquitously, and JNK3, which is present

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mainly in brain and testis[10][11]. Recent analyses using mice deficient in JNK genes have demonstrated that differential expression of JNKs may play a crucial role in multiple tissues, including T cells[12]. CD4-positive ($CD4^+$) T cells from JNK1-deficient ($JNK1^{-/-}$) mice display increased production of IL-4 and IL-10[13], resembling a T-cell helper 2 (Th2) phenotype. Production of IFN-γ, a hallmark of the Th1 phenotype, is severely impaired in $JNK2^{-/-}$ $CD4^+$ T cells[14], suggesting that JNK1 and JNK2 play non-redundant roles in T cell immune responses.

To assess the role of JNK1 and JNK2 in B cells, we employed the T cell-independent B cell activator LPS and examined whether LPS-induced B cell proliferation and differentiation are affected in $JNK1^{-/-}$ and $JNK2^{-/-}$ B cells. The proliferative responses of B cells to LPS were moderately increased in $JNK1^{-/-}$ B cells, with a minor change in $JNK2^{-/-}$ B cells. In addition, production of IgG1 and IgG2a in response to LPS plus IL-4 was somewhat enhanced in both $JNK1^{-/-}$ and $JNK2^{-/-}$ B cells, with concomitant reduction of IgM generation. The present findings suggest that JNK1 and JNK2 function as modulators of immune responses in B cells as well as T cells.

Materials and methods

Mice

$JNK1^{-/-}$, $JNK2^{-/-}$, and WT mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). The mice were backcrossed to C57 BL/6J for 6 generations. Breeding and maintenance were carried out under specific pathogen-free conditions at Tokyo Medical University and followed the guidelines of the Ethical Committee of Animal Experiments of Tokyo Medical University.

Isolation and culture of B cells

For isolation of B lymphocytes, single-cell suspensions prepared from spleen were treated with ACK solution (0.83% NH_4Cl, 9 vol : 0.17% Tris-HCl buffer, 1 vol) to remove red cells and then isolated by negative selection using a B cell isolation kit (Military Biotech, Bergisch Gladbach, Germany). Briefly, the red cell-depleted spleen cells were incubated with biotin-conjugated anti-CD43, anti-CD4, and anti-Ter-119, followed by incubation with magnetic microbeads conjugated with anti-biotin. After washing, the labeled cells were isolated using autoMACS Pro (Military Biotech). Purified B cells (1×10^5/well) were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 2-mercaptoethanol (2-ME) in the presence or absence of LPS (Sigma, St. Louis, MO, USA) or LPS plus IL-4 (Peprotech Inc., Rocky Hill, NJ, USA). In some experiments, Baf-3 cells overexpressing MAPK kinases 7 (MKK7)-JNK1 or MKK7-JNK2[15] (Cao et al. unpublished observation) were used to confirm the specificity of the antibodies (Abs) used in this study.

Western blotting

Western blotting was performed as previously described[16]. Briefly, samples (40 μg/lane) were separated by SDS-PAGE, then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After washing, membranes were blotted with primary Abs : anti-JNK1, anti-JNK2 (Santa Cruz Inc., Santa Cruz, CA, USA), anti-phospho-JNK (Cell Signaling Tech. Inc., Danvers, MA, USA), and actin (Santa Cruz Inc.). Bound primary Abs were then detected with HRP-conjugated goat anti-rabbit IgG (Fc) (1 : 2,000) (ICN Pharmaceuticals Inc., Aurora, OH, USA). After several washings, membrane-bound HRP-conjugated Abs were visualized with ECL (GE Healthcare, Buckinghamshire, UK).

Flow cytometric analysis of surface markers

Flow cytometric analysis was done as previously described[17]. Briefly, cells (1×10^6/sample) were stained with anti-CD3-conjugated with fluorescein isothiocyanate (anti-CD3-FITC), anti-IgM-FITC, anti-CD21-PE, anti-CD23-FITC, anti-CD45R (B220)-APC, or anti-IgM-Percp-cy5.5 (BD Pharmingen, San Diego, CA, USA) on ice for 30 min, followed by flow cytometry (FACSCalibur, Nippon Becton Dickinson Company Ltd., Tokyo, Japan).

^{3}H-thymidine uptake

Cells were cultured with or without various concentrations of LPS for the indicated number of days and were pulsed with 1 μCi (1 mCi=37 MBq) tritiated thymidine (^{3}H-TdR) for the final 6h. The cells were harvested and counted by a liquid scintillation counter.

CFSE cell division analysis

B cells were labeled with 1 μM carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Carlsbad, CA, USA) in PBS with 0.1% FBS for 5 min at room temperature. The labelling reaction was quenched by addition of a 10-fold volume of PBS/0.1% FBS, and the cells were washed twice with cold RPMI-1640 medium to remove excess CFSE. FACS analysis enabled gating on individual CFSE generation of B cells.

Quantitation of Ig by enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed by a modified procedure as previously described[18]. Briefly, ELISA plates were coated with 2 μg/ml anti-mouse IgM, anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgG2b (Southern Biotech, Birmingham, AL, USA) in a total volume of 50 μl. After incubation at 4°C overnight, the supernatant was discarded, followed by several washes with a buffer (0.05% Tween 20 in PBS). The plates were pre-incubated with a blocking buffer (0.25% bovine serum albumin (BSA) (Sigma) and Tween 20 in PBS) at 37°C for 1h. After

several washes, samples were added to the plates, followed by overnight incubation at 4°C. After several washes, alkaline phosphatase-labeled goat IgG (Southern Biotech) specific for mouse IgM, IgG1, IgG2a, or IgG2b was added and then incubated at 4°C overnight. Color detection was done using p-nitrophenyl phosphate for 30 min. The concentrations of IgM, IgG1, IgG2a, and IgG2b were determined on the basis of standard curves from standard antibody preparations (Assay Designs, Ann Arbor, MI, USA).

Statistical analysis

Data were expressed as the means±SD of several independent experiments. Statistical significance was determined by Student's *t* test ; a difference of $p<0.05$ was considered to be significant.

Results

LPS induces phosphorylation of both JNK1 and JNK2 in murine B cells

To assess whether LPS in combination with IL-4 induces JNK activation in B cells, B cells were cultured with 10 μg/ml LPS, 10 ng/ml IL-4, or 10 μg/ml LPS plus 10 ng/ml IL-4 for 30 min and assayed for JNK phosphorylation using Abs specific for phospho-JNK. Addition of LPS immediately resulted in substantial levels of phosphorylation of the 54 kDa protein, with moderate phosphorylation of the 46 kDa isoform (Fig. 1A). Phosphorylation of both the 54 kDa and 46 kDa proteins was higher after combined stimulation with LPS and IL-4 than after stimulation with either alone. Levels of JNK1 and JNK2 were unaltered by stimulation with LPS and IL-4. The specificity of the Abs used in this experiment was confirmed using Baf-3 cells expressing MKK7-JNK1 and MKK7-JNK2. The anti-JNK1 Abs detected MKK7-JNK1, but not MKK7-JNK2, which was specifically visualized with the anti-JNK2 Ab (Fig. 1B). These results suggest that LPS induced phosphorylation of both JNK1 and JNK2, and that it was further enhanced by the addition of IL-4.

JNK protein expression in B cells from JNK1- and JNK2-deficient mice

To analyze the role of JNKs in the antibody responses in vivo, homozygous mutant mice were generated by intercrossing heterozygotes, which were determined by PCR using genomic DNA. The major JNK1 and JNK2 proteins found in spleen B cells from WT mice corresponded to the 46 kDa and 54 kDa isoforms, respectively (Fig. 2). The JNK1-deficient B cells were confirmed to be defective in JNK1 protein expression, with JNK2 levels comparable to those of WT B cells. Likewise, JNK2 protein was confirmed to be absent in the JNK2$^{-/-}$ B cells. These results suggest that homozygous JNK1$^{-/-}$ and JNK2$^{-/-}$ B cells were defective in JNK1 and JNK2 protein expression, respectively.

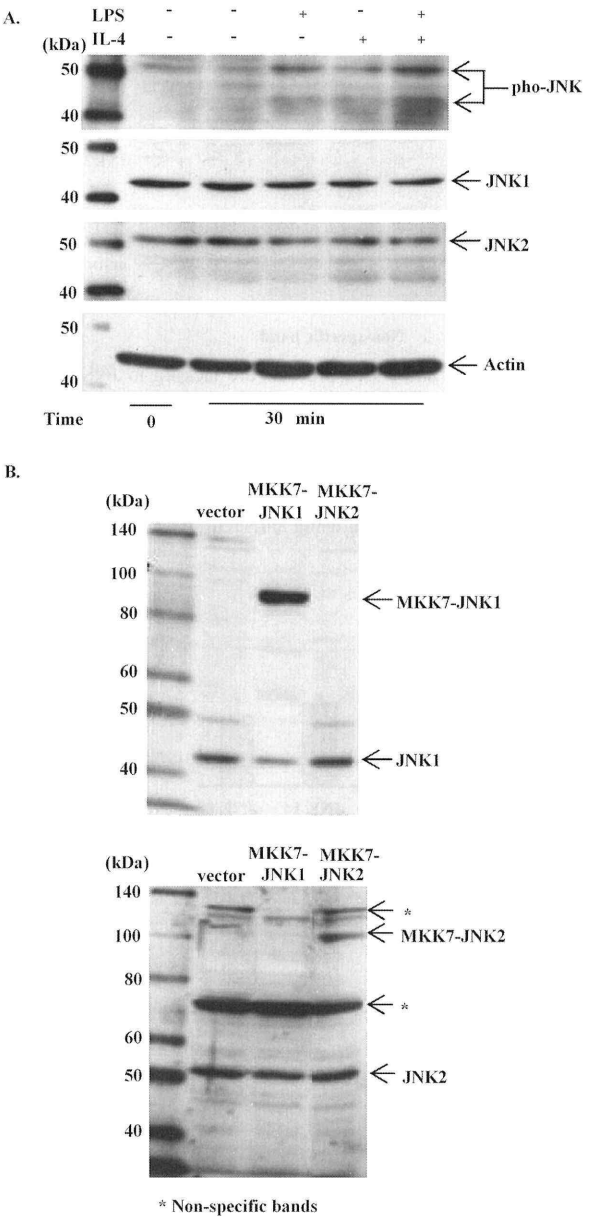

Fig. 1 JNK1 and JNK2 are phosphorylated in response to LPS and LPS plus IL-4. B cells were stimulated with or without 10 μg/ml LPS or LPS in combination with IL-4 (10 ng/ml) for 30 min, followed by assay for phosphorylation of JNKs using phospho-specific JNK Abs (A). Levels of JNK1 and JNK2 were also evaluated using anti-JNK1 and anti-JNK2 Abs (A), the specificity of which was confirmed using Baf-3 cells expressing MKK7-JNK1 and MKK7-JNK2, respectively (B).

Lymphocyte development is not impaired

To determine the roles of JNK1 and JNK2 in lymphocyte development, spleen cells were stained with anti-CD3 and anti-IgM for identification of T cells and B cells, respectively. No differences between the percentages of CD3$^+$ T cells and IgM$^+$ B cells were observed in JNK1$^{-/-}$, JNK2$^{-/-}$, and WT spleen cells (Fig. 3A), as re-

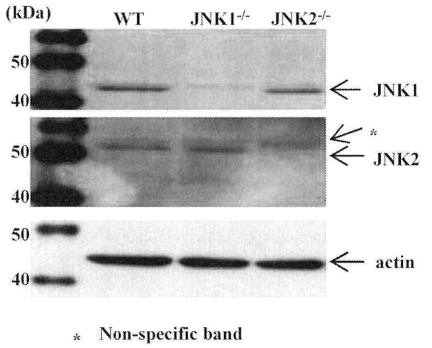

Fig. 2 JNK1 and JNK2 proteins are lacking in JNK1$^{-/-}$ and JNK2$^{-/-}$ B cells, respectively. B cells from JNK1$^{-/-}$, JNK2$^{-/-}$, and control WT mice were assayed for JNK1 and JNK2 expression by Western blotting. As a control, the levels of actin were also evaluated by Western blotting using anti-actin Abs.

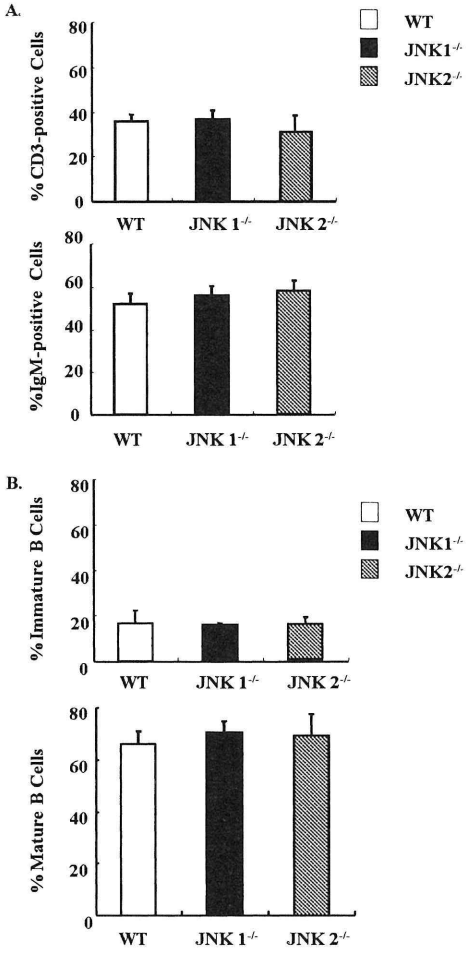

Fig. 3 Lymphocyte development in JNK1$^{-/-}$ and JNK2$^{-/-}$ mice is unaltered. Spleen cells were stained with anti-CD3-FITC and anti-IgM-FITC mAb (A), followed by flow cytometric analysis. IgM^{+} B cells were also stained with anti-CD21 and anti-CD23 for quantification of immature (CD21^{-}CD23^{-}) and mature (CD21^{+}CD23^{+}) B cells, respectively (B). Results are shown as means ±SD from three independent experiments.

ported previously[14]. Analysis of differentiation markers (B220, IgM, CD21, CD23) that characterize the maturational stage of B cells[19)20)] did not reveal differences among JNK$^{-/-}$, JNK2$^{-/-}$, and WT mice (Fig. 3B). These results suggest that lymphocyte development is not perturbed in JNK1$^{-/-}$ and JNK2$^{-/-}$ mice.

LPS-induced proliferative responses in JNK1- and JNK2-deficient B cells

To assess the roles of JNK isoforms in B cell proliferation, JNK1$^{-/-}$, JNK2$^{-/-}$, and WT B cells were cultured with the indicated concentrations of LPS for three days. LPS-induced B cell proliferative responses, as assessed by ^{3}H-TdR incorporation (Fig. 4A) and CFSE labelling (Fig. 4B), were considerably increased in JNK1$^{-/-}$ B cells compared with controls. The enhanced LPS-induced proliferative responses in JNK1$^{-/-}$ B cells were not due to alteration of the kinetics of the LPS-induced proliferative responses (Cao et al., unpublished observation), suggesting that JNK1 negatively regulates LPS-induced proliferation. JNK2$^{-/-}$ B cells showed a somewhat different pattern of LPS-induced proliferation, with a slightly decreased response at the lower and higher doses (Fig. 4A). The proliferative responses of JNK2$^{-/-}$ B cells in response to 10 μg/ml LPS were comparable to those of the control, as assessed by ^{3}H-TdR incorporation and CSFE-labelling (Figs. 4A and 4B). In contrast, the LPS-induced proliferation at 5 μg/ml was somewhat increased in JNK2$^{-/-}$ B cells. Thus, JNK1 and JNK2 differentially regulate LPS-induced B cell proliferation.

LPS modulates antibody responses in JNK1- and JNK2-deficient B cells

To assess the role of JNKs in polyclonal antibody responses, JNK1$^{-/-}$, JNK2$^{-/-}$, and control B cells were cultured with 10 μg/ml LPS in the presence or absence of IL-4 for seven days, followed by assays for antibody production by ELISA. LPS-induced IgG1 generation was slightly increased, and IgG1 generation was further moderately augmented in both JNK1$^{-/-}$ and JNK2$^{-/-}$ B cells by the addition of IL-4 (Fig. 5). Interestingly, LPS-induced enhancement of the IgG2b response, representing a Th1-like phenotype, was found in JNK2$^{-/-}$ B cells (Fig. 5). Likewise, a moderate increase in IgG2a production in response to LPS plus IL-4 was detected in JNK2$^{-/-}$ B cells, although it was not significant in JNK1$^{-/-}$ B cells. Concomitant with the increased production of IgG1 and/or IgG2a/IgG2b in both JNK1$^{-/-}$ and JNK2$^{-/-}$ B cells, LPS-induced IgM production was substantially decreased, suggesting that JNK1 and JNK2 regulate isotype switching. These results suggest that JNK1 and JNK2 modulate the LPS-induced polyclonal antibody responses to a marked extent.

Discussion

B cell proliferation and antibody generation in B cells

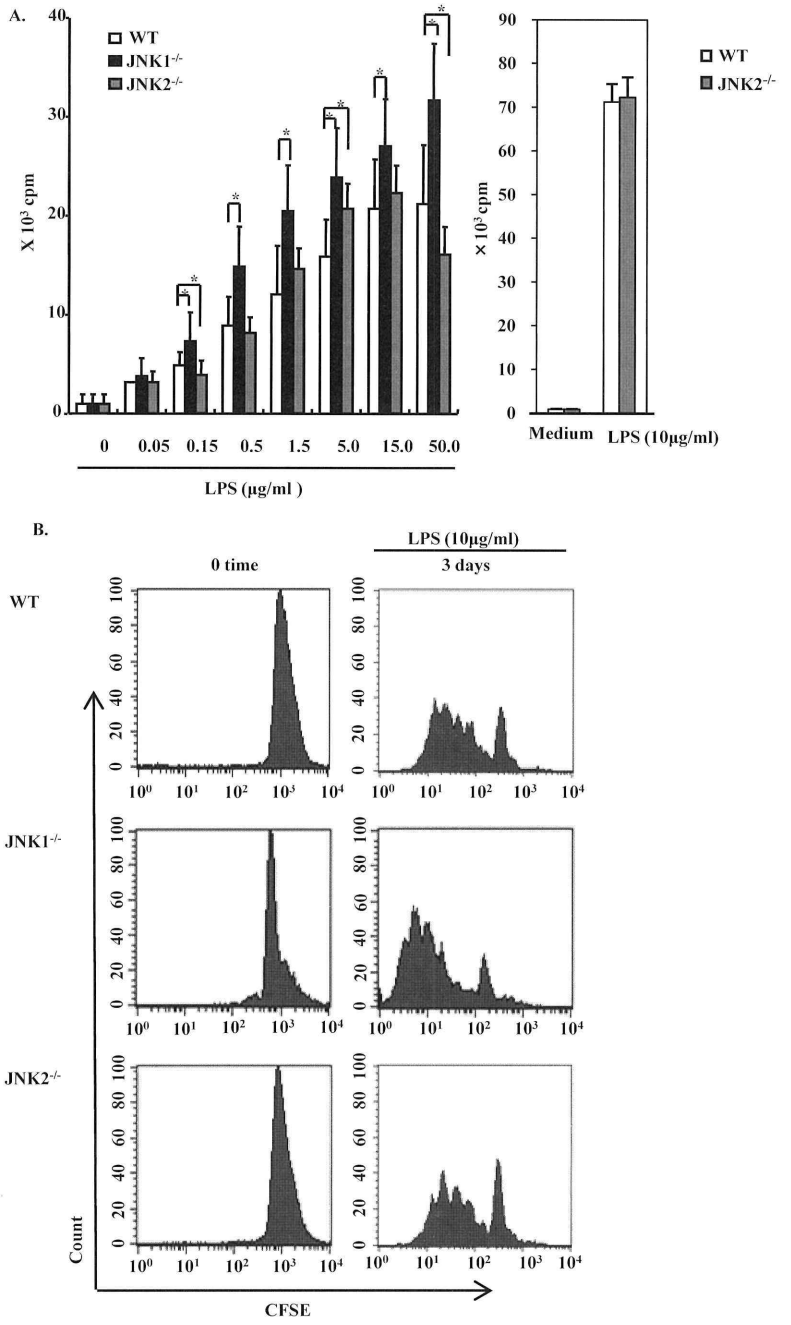

Fig. 4 LPS-induced proliferation is enhanced in JNK1$^{-/-}$ B cells, whereas it depends on LPS dose in JNK2$^{-/-}$ B cells. JNK1$^{-/-}$, JNK2$^{-/-}$, and control WT B cells were cultured with various concentrations of LPS for 3 d and pulsed with ^{3}H-TdR for final 6 h before harvesting (A). The LPS-stimulated B cells were also evaluated by CFSE-labelling method (B), as described in Materials and Methods. Results are shown as means±SD from three independent experiments. *Significantly different from controls.

are controlled by several factors including CD40L, cytokines, and/or TLR signals[21)22)]. IL-4 directs IgG1 and IgE antibody production[4)], whereas IFN-γ and/or IL-27 favor IgG2a antibody isotype switching[3)5)23)]. In addition, CpG-ODN, a ligand for TLR9, stimulates B cells to generate IgG2a with concomitant inhibition of IgE synthesis through the activation of transcription factor T-bet[24-26)]. LPS stimulates IgG2a/2b and IgG3[27)] production by B cells, and the addition of IL-4 stimulates IgG1[1)4)]. In the present study, we examined whether JNK isoforms modulate B cell function after stimulation with the polyclonal B cell activator LPS, a ligand for TLR4.

LPS-induced IgG2a production in the presence of IL-4 was moderately enhanced in JNK2$^{-/-}$ B cells, compared with that of the control (Fig. 5), although JNK2 regulates production of IFN-γ, a hallmark of Th1 cytokine, in CD4^{+} T cells[14)], favoring IgG2a production[3)5)14)]. These results suggest that JNK2 controls IgG2a produc-

tion depending on cell lineages and/or cytokine milieu. Moreover, LPS-induced IgG2b generation was enhanced in JNK2$^{-/-}$ B cells compared with those of JNK1$^{-/-}$ and control B cells (Fig. 5). Concomitantly, IgM generation was substantially inhibited in JNK2$^{-/-}$ B cells, suggesting that JNK2 negatively regulates isotype switching to IgG isoforms. Interestingly, JNK2$^{-/-}$ mice also showed an enhanced NP-specific IgG2a production after immunization with T cell-independent antigen NP-Ficoll (Takada et al. unpublished observation). Thus, it is possible that JNK2 regulates antibody responses in response to bacteria and viral invasion through TLRs.

LPS-induced proliferation, as assayed by ^{3}H-TdR incorporation, was also moderately enhanced in JNK1$^{-/-}$ B cells relative to the control during the entire period tested (Figures 4A and Cao and Takada et al. unpublished observation). The number of cell divisions was moderately increased in JNK1$^{-/-}$ but not JNK2$^{-/-}$ B cells relative to WT B cells after stimulation with 10 μg/ml LPS (Fig. 4B). Thus, it is reasonable to speculate that enhancement of the LPS-induced proliferation of JNK1$^{-/-}$ B cells was due to increased cell division rather than cell survival. In contrast, JNK2$^{-/-}$ B cell proliferation in response to LPS was moderately increased only at 5 μg/ml, with minor decreases at lower and higher concentrations. Further studies are required to understand the LPS dose-response of JNK2$^{-/-}$ B cell proliferation in a physiological setting. Together, JNK1 and JNK2 differentially regulate LPS-induced B cell proliferative responses.

The molecular mechanisms underlying the increased isotype switching in JNK1$^{-/-}$/JNK2$^{-/-}$ B cells remain unclear. CpG-ODN and IL-27/LPS induce IgG2a production through activation of transcription factor T-bet, with a concomitant decline in IgG1 and IgE synthesis[23)24)]. However, both IgG2a and IgG1 productions were augmented in JNK1$^{-/-}$ and JNK2$^{-/-}$ B cells after stimulation with LPS plus IL-4, suggesting that T-bet alone does not account for the enhancement of the LPS-induced antibody production. Because JNK1 activation results in activation of transcription factor AP-1[10)28)], determination of how AP-1 modulates antibody formation in B cells is of interest. Further studies are needed to resolve the molecular mechanisms by which JNKs regulate isotype switching as well as proliferation in B cells.

JNKs might contribute to the prevention of bacterial or viral infection through production of antibodies as well as pro-inflammatory cytokines[29)30)]. Although bacterial and viral invasion has to be blocked, an excessive response that damages the host must be avoided. In the present study, we clearly demonstrate that JNKs negatively regulate antibody production in response to the TLR4 agonist LPS, helping to prevent an excessive im-

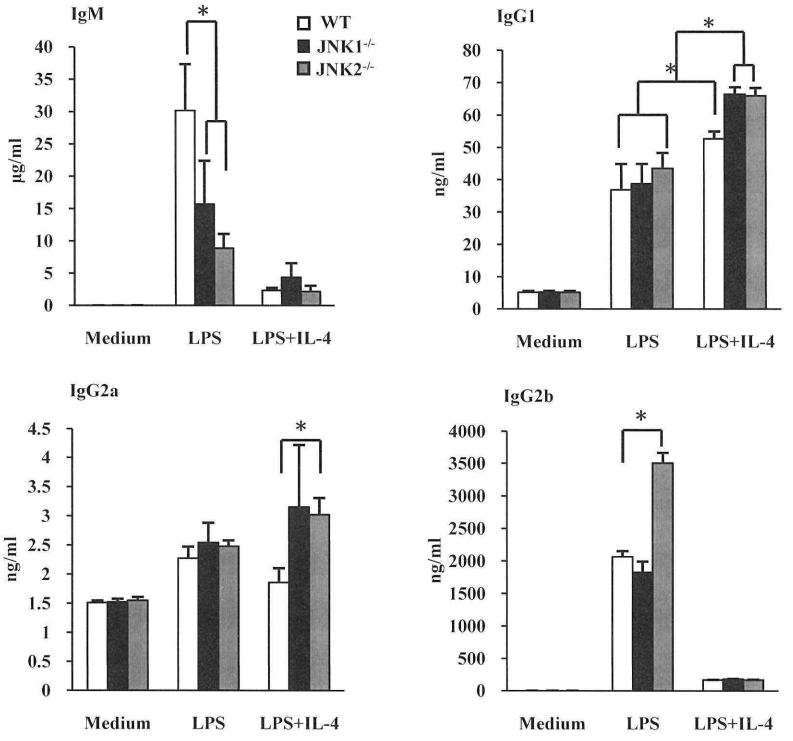

Fig. 5 The production of IgG1 and IgG2a/IgG2b in response to LPS and/or IL-4 is enhanced, with concomitant decreases in LPS-induced IgM production in both JNK1- and JNK2$^{-/-}$ B cells. The JNK1$^{-/-}$, JNK2$^{-/-}$, and control WT B cells were cultured with IL-4, LPS, or LPS plus IL-4 for 7 d, and culture supernatants were assayed for production of IgM, IgG1, IgG2a, and IgG2b. Results are shown as means ± SD from three independent experiments. *Significantly different from controls.

mune response. These observations are valuable for understanding the mechanisms of polyclonal antibody responses, and also the defense mechanisms against microbial invasion.

Acknowledgments

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JNK1 及び JNK2 欠損 B 細胞におけるリポ多糖体（LPS）誘導性抗体産生の増強

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リポ多糖体（lipopolysaccharide, LPS）はマウス B 細胞を T 細胞非依存的に活性化することが知られている。B 細胞を LPS で刺激すると JNK1 及び JNK2 のリン酸化が誘導され、IL4 添加によってこのリン酸化はさらに増強された。本研究では、JNK 活性化が LPS 誘導性抗体産生に関与しているか否かを JNK1 及び JNK2 欠損マウス由来 B 細胞を用いて検討した。JNK 欠損マウスでは野生型マウスと同様な B 細胞成熟パターンを示した。JNK1 欠損 B 細胞における LPS 誘導性 B 細胞増殖は増強されたが、JNK2 欠損 B 細胞では中程度の刺激（5 μg/ml）でのみ軽度な上昇が観察され、低濃度及び高濃度（50 μg/ml）の LPS 刺激ではむしろ抑制された。LPS 及び IL-4 刺激によって誘導される JNK1 及び JNK2 欠損 B 細胞の IgG1 及び IgG2a 応答はコントロール B 細胞に比べて増強されたが、IgM 応答は有意に減少した。また、JNK2 欠損 B 細胞における LPS 誘導性 IgG2b 産生は増強されたが、JNK1 欠損 B 細胞ではコントロール B 細胞と同程度であった。以上の結果より、JNK1 及び JNK2 が LPS と IL-4 共刺激による B 細胞増殖やアイソタイプスイッチを調節していることが示唆された。LPS はグラム陰性細菌の構成成分であることから、本研究の成果は細菌侵入後の B 細胞応答や多クローン性 B 細胞活性化の分子機序を解析する上で有用な情報となるであろう。

〈キーワード〉 JNK1、JNK2、抗体産生、多クローン性 B 細胞活性化