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Suppressive effect of reactive oxygen species on CD40-induced B cell activation

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Abstract **Reactive oxygen species (ROS) produced by the innate immune system work as effectors to destroy pathogens and to control cellular responses. However, their role in the adaptive immune response remains unclear. Here we studied the effect of exogenous ROS on CD40-induced B cell activation. H_2O_2 treatment inhibited CD40-induced immunoglobulin production of B cells, DNA binding of NF-κB, IκBα degradation and IKK phosphorylation. On the other hand, H_2O_2 treatment did not induce obvious B cell death after 30 min of stimulation. Although the ligation of anti-CD40 antibody was not disturbed by H_2O_2, TRAF2 recruitment to CD40 was inhibited. These results suggest that exogenous ROS play a negative role in CD40 signaling during B cell activation.**

Keywords: B cell; ROS; CD40 Signaling; TRAF2

1. Introduction

When pathogens invade host, macrophages are activated and produce pro-inflammatory cytokines, chemokines, growth factors, and proteases [1]. Besides, the activated macrophages produce reactive oxygen species (ROS) such as H_2O_2 and other metabolites by the respiratory burst [2]. The produced H_2O_2 subsequently affect other cell types, such as T cells and B cells, and impact immune responses. Activated B cells have been shown to produce ROS and the role of endogenous ROS in B cell function has been studied [3–5]. However, less attention was paid for the exogenous ROS for B cell activation and function.

CD40 belongs to the TNFR superfamily and is expressed on a variety of cells, such as B cells, DCs, and epithelial cells [6–8]. In B cells, the ligation of CD40 delivers activation signal that lead to proliferation, differentiation, Ig production, and cytokine secretion [9,10]. CD40 signaling is initiated by the interaction of CD40 with specific TNFR-associated factor (TRAF) molecules. Among the TRAF family, TRAF2, 3, 5, 6 were the most extensively studied in CD40 signal transduction [11–14]. Especially, TRAF2 is known to be important for B cell maturation and activation [15,16].

Nuclear factor κB (NF-κB) was firstly identified in B cells as a key transcription factor for IgG production. Each NF-κB/Rel protein forms different homo- or heterodimers with other members of the family. The most typical NF-κB complex is a p50/p65 heterodimer. NF-κB is largely sequestered in the cytoplasm through its association with an inhibitor of κB (IκB). Upon stimulation, the IκB proteins are phosphorylated by the IκB kinases (IKK-α, -β, -γ), following ubiqutinylation, and then are degraded in a proteasome dependent manner. The degradation of IκB thereby allows the NF-κB complex to translocate into the nucleus [17].

ROS are known to involve in many important cellular events, which include the activation of transcription factor, cell proliferation, and apoptosis. They also act as second messengers in the signal transduction pathway of various receptors, including TNF family receptors [18–23]. The CD40-induced endogenous ROS production is important for the downstream signal events that lead to the activation of JNK, NF-κB, and interleukin (IL)-6 secretion in B cells [4]. However, many studies were done using antioxidants such as ROS scavenger and NADPH inhibitor in different cell types. Several studies also revealed that chemicals itself affect cellular responses. For example, Hayakawa et al. reported that NAC, which is a well-known antioxidant, directly affect TNF receptor signaling pathway independent of its antioxidant character [24]. Therefore, it is important to investigate the direct effect of ROS on cellular events without using antioxidants.

In this study, we investigated the effect of exogenous ROS on primary B cell activation, especially its role in CD40 signaling-mediated NF-κB activation.

2. Materials and methods

2.1. Mice

Female BALB/c mice (5–8 weeks) were purchased from Charles River Laboratories Japan (Yokohama, Japan). These mice were maintained in the Animal Research Center at the University of Occupational and Environmental Health under specific pathogen-free conditions. All animal experiments were performed according to the guideline for the care and use of animals approved by the University of Occupational and Environmental Health.

2.2. Cell preparation and culture

B cells from spleen cells were negatively selected by B cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions, and cells were 97 ~ 99% B220 positive. A20 cells, mouse B lymphoma cell line, were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were cultured in humidified 5% CO_2/95% air at 37 °C in RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal

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bovine serum (Bio Whittaker, Walkersville, MD), 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco, Grand Island, NY).

2.3. Reagents and antibodies

Hydrogen peroxide was purchased from Wako (Osaka, Japan). *N*-acetylcysteine (NAC) was purchased from Sigma (St. Louis, MO). NAC was resolved in RPMI 1640 medium and the solution was adjusted to pH 7.0 by 8 N NaOH. Anti-mouse CD40 antibody (MAB440) was obtained from R&D Systems (Minneapolis, MN). Anti-NF-κB p65 antibody (sc-372 X), anti-Stat5 antibody (sc-835 X), anti-PU.1 antibody (sc-352 X), anti-rabbit HRP antibody (sc-2030), anti-TRAF2 antibody (sc-876), anti-IκBα antibody (sc-371) and Protein A/G PLUS-Agarose (sc-2003) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-IKKα(Ser180)/β(Ser181) antibody (#2681), anti-IKKα antibody (#2682) and anti-IKKβ antibody (#2684) were obtained from Cell Signaling Technology (Danvers, MA). Anti-β-actin antibody (A5441) was purchased from Sigma (St. Louis, MO).

2.4. Proliferation assay

B cells were stimulated with anti-CD40 antibody (1 μg/ml) with or without several concentrations of H_2O_2 for 3 days. The cells were labeled with 0.5 μCi of tritiated thymidine ([^{3}H]-TdR; specific activity 6.0 Ci/mmol; Amersham, Aylesbury, UK) for the last 16 h and were harvested with the aid of a semiautomated cell harvester (Abe Kagaku, Chiba, Japan). The amount of radioactivity incorporated into the DNA in the cells as measured with a liquid scintillation counter (Aloka, Tokyo, Japan). The results were expressed as the mean count per min (cpm) of [^{3}H]-TdR incorporated by the cells with S.D. in quadruplicate cultures.

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as previously reported [25]. Briefly, rabbit anti-mouse IgG capture antibody (Medical & Biological Laboratories, Nagoya, Japan) was coated into 96-well plate at 37 °C for 2 h. After the blocking with 1% BSA–PBS, culture supernatants of B cells were incubated in duplicate in the plate at 37 °C for 1 h. Subsequently, peroxidase-conjugated anti-mouse IgG Fc fragment-specific antibody (Jackson ImmunoResearch, West Grove, PA) was incubated at 37 °C for 1 h for IgG detection. Finally, 2-2′-Azino-bis (3-ethylbenzo-thiazoline-6-sulfoic acid) diammonium salt (ABTS) (Sigma, St. Louis, MO) substrate solution was added to the plate at room temperature. Color development was detected at 405 nm. IgM was detected using mouse IgM ELISA quantitation kit (Bethyl, Montgomery, Texas), according to the manufacturer's protocol.

2.6. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [26]. B cells were lysed with 100 μl of RIPA lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail) and used as whole cell extracts. The nuclear extracts were prepared by nuclear extraction kit (Sigma) according to the manufacturer's protocol. Murine NF-κB oligonucleotides: 5′-AGTTGAGGGGACTTTCCCAGGC-3′, derived from the Igκ enhancer region, was purchased from Promega (Madison, WI). In brief, 10 μg of extracts were pre-incubated for 20 min at room temperature in 15 μl of buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM β-mercaptoethanol, 4% glycerol, 40 mM NaCl) containing 0.1 μg of poly(dI-dC) and oligonucleotide labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and [γ-^{32}P]ATP (3000 Ci/mmol, Amersham Pharmacia Biotech, Litle Chalfont, UK). Protein–DNA complexes were resolved in 4% TBE polyacrylamide gels and analyzed with or without a specific antibody.

2.7. Flow cytometry

B cell morphology was analyzed by EPICS XL flow cytometry (Beckman Coulter, Fullerton, CA). Parameters in the histogram were side scatter and forward scatter. To study the binding affinity of anti-CD40 antibody to B cells, anti-CD40 antibody was conjugated with FITC before use. B cells were incubated with FITC-conjugated anti-CD40 antibody in the presence or absence of H_2O_2 (0.1 mM) for 30 min. After washing cells with PBS, FITC-positive cells were counted by EPICS XL flow cytometry.

2.8. Western blot and immunoprecipitation

Equivalent amounts of protein (10 μg) were resolved in SDS–PAGE gel, transferred and immobilized onto nitrocellulose membranes (Amersham, Aylesbury, UK), and probed with appropriate primary and secondary antibodies. Immunodetection was accomplished using a chemiluminescence detection system (Alpha Innotech, San Leandro, CA). For immunoprecipitation, samples were incubated with immunoprecipitation antibody and Protein A/G PLUS-Agarose for 2 h. After washing three times with cold PBS, the precipitated proteins were eluted with sample buffer (0.5 M Tris, 10% SDS and bromophenol blue in 50% glycerol).

2.9. Statistics

All experiments were repeated more than three times and representative results are shown in figures. Statistical analysis was performed using Student's *t*-test. A confidence level of <0.05 was considered significant.

3. Results

3.1. Exogenous ROS (H_2O_2) inhibited B cell proliferation and Ig production

First, the effects of H_2O_2 on B cell proliferation were investigated by DNA synthesis with thymidine uptake. Fig. 1A shows that CD40 stimulation induced B cell proliferation, which is inhibited by H_2O_2 treatment (0.25 mM and 0.1 mM). Next, IgG and IgM production in B cells was studied. At 5 days after CD40 stimulation, cell culture supernatants were harvested and assayed. ELISA showed that anti-CD40 antibody induced IgG and IgM production, which was dramatically inhibited by H_2O_2 (0.25 mM and 0.1 mM) (Fig. 1B). The cell viability after H_2O_2 treatment was studied by counting cell numbers. As shown in Fig. 1C, H_2O_2 treatment (0.25 mM and 0.1 mM) did not markedly induce cell death after 30 min of stimulation. Moreover, high concentration of H_2O_2 (0.5 mM) also had no effect on B cell viability at 30 min of stimulation (data not shown). At 5 days after stimulation, about 50% of B cells were alive by CD40 stimulation, which was decreased to 23% in the presence of H_2O_2. Although there were still enough cells to produce Ig at day 5, Ig productions in H_2O_2-treated B cells were completely abolished. Therefore, these results indicate that the decreased Ig production by H_2O_2 treatment is not only due to fewer cell number, but also due to weakened B cell ability.

3.2. Exogenous ROS inhibited CD40-mediated NF-κB activation

NF-κB is known as a critical transcription factor in B cells leading to proliferation, differentiation, germinal center formation, isotype switching of Ig genes, and cytokine secretion [27]. Then, we investigated the effect of ROS on NF-κB activation. B cells were stimulated with anti-CD40 antibody for 30 min, and the nuclear extraction was applied for EMSA using a radiolabeled oligonucleotide, which contains the NF-κB DNA binding site of immunoglobulin κ enhancer. Consistent with previous report, anti-CD40 antibody induced rapid NF-κB activation (Fig. 2A, lane 4). This protein–DNA complex was supershifted by anti-p65 antibody (Fig. 2A, lanes 5 and 9), but not Stat5 or PU.1 antibodies (Fig. 2A, lanes 10 and 11), indicating that this complex contains NF-κB molecules. CD40-induced DNA binding activity of NF-κB was inhibited by H_2O_2 (Fig. 2A, lanes 6 and 7). Because H_2O_2 treatment of 30 min did not induce cell death (Fig. 1C), the inhibited NF-κB

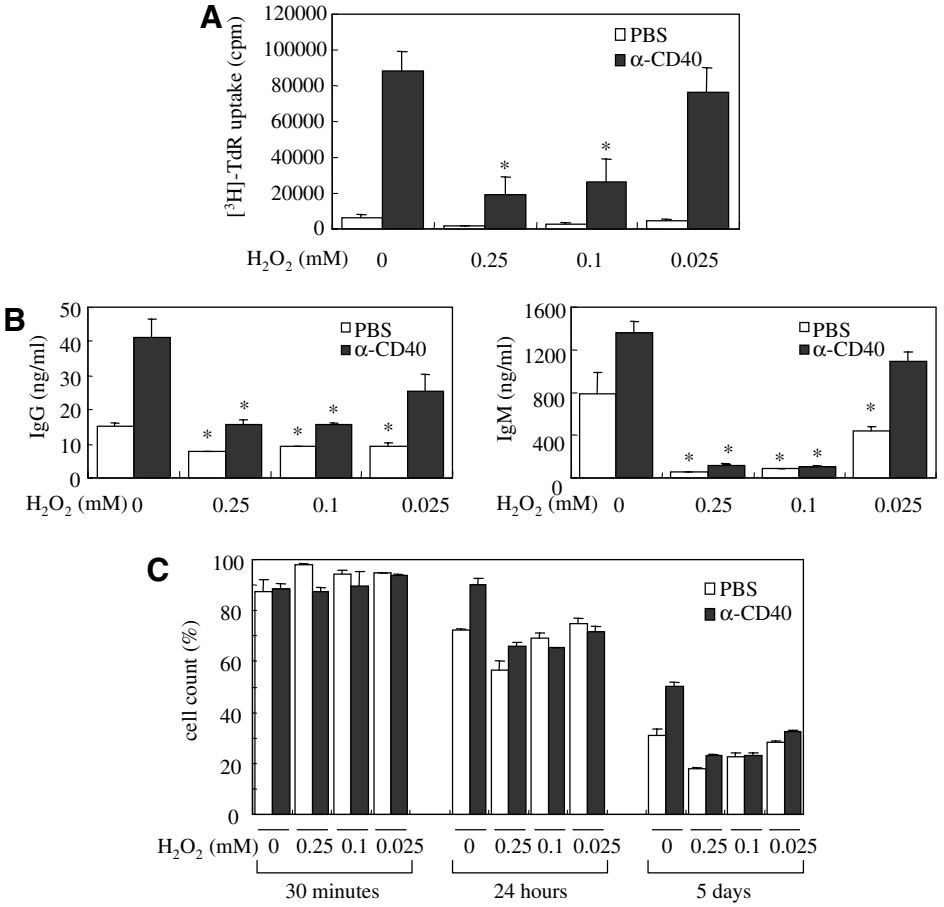

Fig. 1. CD40-induced IgG production was inhibited by H_2O_2. (A) B cells (2×10^5/well) were stimulated with anti-CD40 antibody with or without H_2O_2 (from 0.25 mM to 0.025 mM) for 3 days. The cells were labeled with [^{3}H]-TdR for the last 16 h and then harvested. The amount of [^{3}H]-TdR, which was incorporated by the cells, was counted. Results are expressed as mean cpm of [^{3}H]-TdR uptake and S.D. from quadruplicate cultures. (B) B cells (3×10^6/ml) were stimulated with anti-CD40 antibody in the presence or absence of H_2O_2 for 5 days. IgG or IgM productions in culture supernatants were assayed by ELISA. (C) After the indicated stimulations, a cell suspension was mixed with 0.1% trypan blue solution and the viable cells were counted. Cell viability was expressed as a percent ratio of the starting cell numbers. Results are representative of at least three similar experiments. * Significantly decreased from the 0 mM H_2O_2 control ($P < 0.05$).

activation was not due to decreased cell viability. These results indicate that CD40 signaling induces NF-κB activation in primary B cells, which is disturbed by exogenous ROS.

It is shown that endogenous ROS in B cell line act as signal mediators and subsequently affect cell function, using antioxidant such as *N*-acetylcysteine (NAC) [4]. To study the effect of endogenous ROS to our assay system, we added NAC in the stimulation of B cells. As shown in Fig. 2B (lane 4), CD40-induced NF-κB activation was enhanced by NAC treatment. Surprisingly, NAC itself also induced DNA binding activity of NF-κB (lane 2), which was observed until 12 h after stimulation. By flow cytometry, we also found that NAC itself induced B cell blast formation (Fig. 2C). These results indicate a unique role of NAC on NF-κB activation, independent of its antioxidant character. Therefore, it is important to study the direct effect of ROS on cell function without chemicals such as antioxidant.

3.3. IκBα degradation and IKK activation were inhibited by ROS

Upon stimulation, IκBα is phosphorylated, ubiquitinylated and degraded, resulting in the nuclear translocation of p50 and p65 [17]. To study the mechanism by which H_2O_2 inhibits NF-κB activation, the kinetics of IκBα degradation after stimulation was investigated. As shown in Fig. 3A, CD40-induced IκBα degradation was observed at 15 min after stimulation and started to recover at 30 min. However, the presence of H_2O_2 absolutely abrogated CD40-induced IκBα degradation. Stimulus-induced IκB degradation is controlled by IκB kinase (IKK), which phosphorylates two N-terminal serines [28,29]. Therefore, the effect of H_2O_2 on IKK activation was studied by Western blot. Anti-CD40 antibody induced IKKα/β phosphorylation after 30 min stimulation (Fig. 3B, lane 4), which is inhibited by H_2O_2 treatment (lanes 5 and 6), although H_2O_2 slightly induced IKK phosphorylation in the absence of anti-CD40 antibody (lane 3). These results suggest that H_2O_2 blocks CD40 signaling from upstream of IKK and IκBα, and subsequently inhibited NF-κB activation.

3.4. TRAF2 recruitment to CD40 was disturbed by ROS

Next, we examined the possibility that H_2O_2 interrupts the antibody binding with CD40 on B cells. FITC-conjugated anti-CD40 antibody was employed to test the CD40 liga-

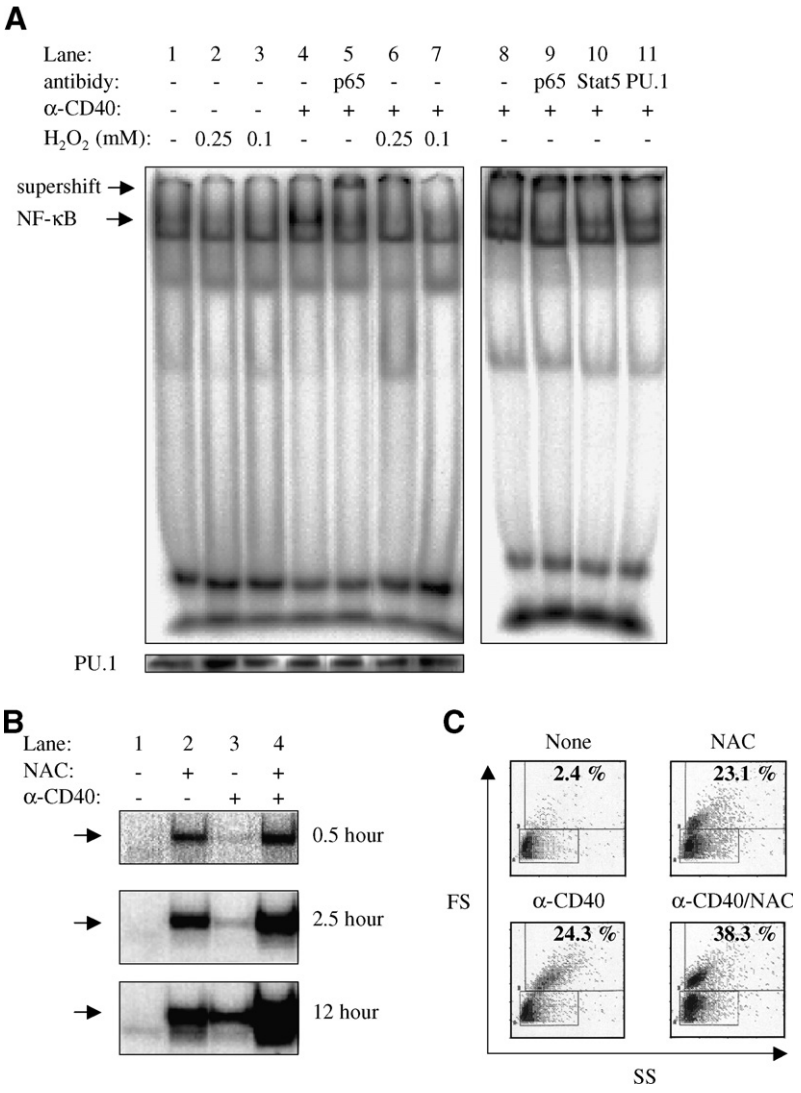

Fig. 2. CD40-induced NF-κ B activation was inhibited by H_2O_2. (A) B cells were stimulated with anti-CD40 antibody and different concentrations of H_2O_2 for 30 min, then nuclear extracts of B cells were subjected to EMSA using a radiolabeled oligonucleotide, which contains the NF-κB DNA binding site of immunoglobulin κ enhancer (Ig-κB). The lower arrow indicates the protein–DNA complex, and upper arrow indicates supershift. Lower panel shows Western blot result with anti-PU.1 antibody, which indicates the loading of nuclear protein. (B) Purified splenic B cells were stimulated with anti-CD40 antibody (1 μg/ml), in the presence or absence of NAC (10 mM), for the indicated time period. Whole cell extracts were applied for EMSA using Ig-κB oligonucleotide. The arrow indicates protein–DNA complex. Results are representative of at least three similar experiments. (C) B cells (2×10^6/ml) were stimulated with anti-CD40 antibody (1 μg/ml) for 24 h in the presence or absence of NAC (10 mM), morphology of B cells were analyzed by flow cytometry. The *x*-axis and *y*-axis indicate side scatter (SS) and forward scatter (FS), respectively. The number represents the cell population in the upper rectangle region.

tion. After 30 min incubation, the binding of anti-CD40 antibody to B cells was not interrupted by H_2O_2 (Fig. 4A).

Finally, the recruitment of TRAF2 to CD40 after H_2O_2 treatment was examined because CD40-mediated signal transduction is primarily initiated by the recruitment of specific TRAF molecules [11–14]. Fig. 4B shows that anti-CD40 antibody induced TRAF2 recruitment to CD40 (lane 2) in primary B cells, which was inhibited in the presence of H_2O_2 (lane 3), without changing CD40 expression level. Furthermore, in mouse B lymphoma cell line, A20 cells, which TRAF2 constitutively binds to CD40 molecules (Fig. 4B, lane 4), H_2O_2 was found to strip TRAF2 from CD40 (lane 5). The failure of TRAF2 recruitment to CD40 explained the inhibitory effect of H_2O_2 on NF-κB activation pathway. These results demonstrate that H_2O_2 disturbs CD40 signaling through interrupting TRAF2 recruitment, without affecting ligand binding.

4. Discussion

Infection of microorganisms activates the NADPH oxidase of neutrophils and monocytes, resulting in the production of large amounts of reactive oxygen species (ROS) [30]. These ROS are important for killing microorganisms or infected cells. However, the release of ROS also affects surrounding cells and tissues, including immune cells. In this study, we investigated the effect of exogenous ROS on B cells and found that ROS inhibited CD40-induced B cell activation, especially through interrupting TRAF2 recruitment.

In the studies about involvement of ROS in cellular events, antioxidants such as NAC were commonly used [5,31]. However, the unique effects of chemicals on cells were reported. Hayakawa et al. showed that NAC affected NF-κB activation by lowering the affinity of receptor to TNF, independent of

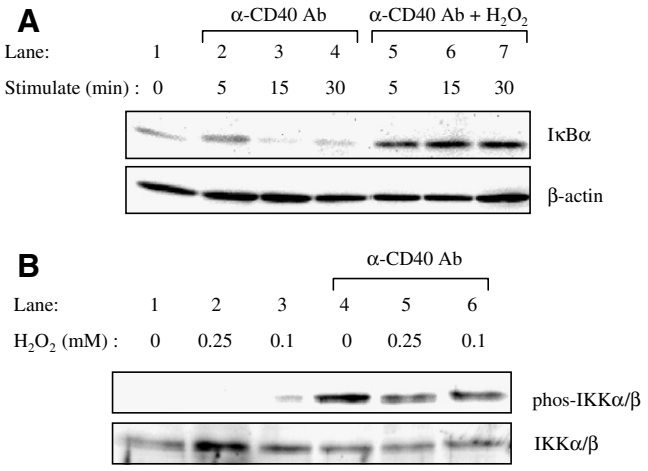

Fig. 3. CD40-mediated IκBα degradation and IKK activation was inhibited by H_2O_2. (A) B cells were stimulated with anti-CD40 antibody in the presence or absence of H_2O_2 (0.25 mM) for the indicated time period, then whole cell extracts were applied for Western blot of IκBα. β-Actin served to demonstrate equal protein loading. (B) After treatment with anti-CD40 antibody and H_2O_2 (0.25 mM and 0.1 mM) for 30 min, B cells were harvested and whole cell extracts were applied for Western blot. The same membrane was employed for blotting with phospho-IKKα/β and total IKK-α/β. Results are representative of at least three similar experiments.

antioxidative function [24]. Our data also showed that NAC itself but not other antioxidants (Fig. 2B and data not shown) induced DNA binding activity of NF-κB for a long period, indicating its unique character independent of antioxidant effect. Thus, the study of direct effect of ROS on cells is important for interpreting ROS-induced cellular events. Therefore, we performed the experiments using H_2O_2, which is known to be a popular member of ROS and more stable than other ROS.

NF-κB plays a critical role for both the survival and activation of resting B cells, and disruption of NF-κB members has been shown to impair B cell proliferation, survival, and Ig class switching [32–34]. H_2O_2 inhibited NF-κB activation in B cells (Fig. 2A), which may explain the decreased cell viability by H_2O_2 treatment (Fig. 1C).

It is known that several stimulations induce ROS production in B cells [4,35], and the effect of endogenous ROS was studied using antioxidants or NADPH inhibitors [36,37]. Lee et al. reported that ROS led to the activation of JNK and NF-κB in WEHI 231 cells [4,5]. They showed the positive effect of ROS on B cell function by applying inhibitory effect of antioxidant NAC. This different approach, such as using NAC, may explain the inconsistence with our results. Additionally, the different cell types were used, it also may be another reason. Thus, direct ROS treatment or antioxidant treatment should be carefully selected in the studies about ROS function.

Among members of the TRAF family, TRAF2, 3, 5 and 6 were the most extensively studied as mediators in CD40 signaling. TRAF2 was shown to be most important for CD40 signaling [15,38]. Our results showed that TRAF2 recruitment to CD40 was inhibited by H_2O_2 treatment, which led to inefficient IKK phosphorylation, IκBα degradation, NF-κB activation and subsequently decreased B cell activation. It is reported that CD40 stimulation induces TRAF2 degradation [39]. We found that CD40 stimulation slightly reduced TRAF2 expression (Fig. 4B, TRAF2 input, lane 2), which is consistent with the previous reports. However, TRAF2 expression level was

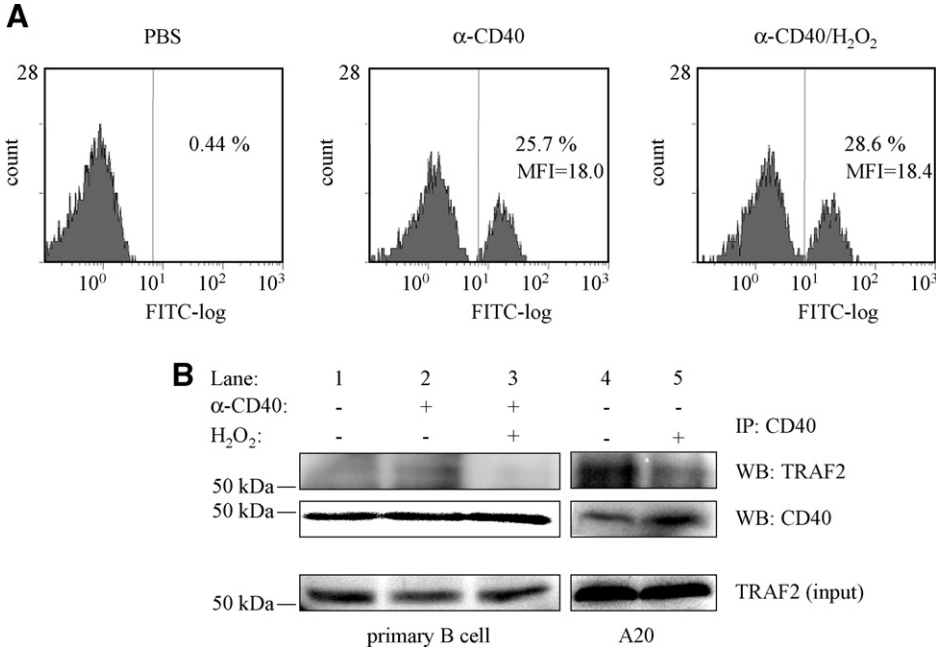

Fig. 4. H_2O_2 disturbed TRAF2 recruitment to CD40, without affecting on ligand ligation. (A) B cells were incubated with FITC-conjugated anti-CD40 antibody, in the presence or absence of H_2O_2, for 30 min. FITC-positive cells were analyzed by flow cytometry and the percentage and MFI (mean fluorescence intensity) of CD40-positive cells were indicated. (B) B cells or A20 cells were stimulated with/without anti-CD40 antibody in the presence or absence of H_2O_2 for 30 min. Whole cell lysate were subjected to immunoprecipitation with anti-CD40 antibody, and subsequently evaluated by Western blot using anti-TRAF2 antibody and anti-CD40 antibody. TRAF2 expression in cell lysate (input) served to demonstrate equal protein loading. Results are representative of at least three similar experiments.

not changed in the presence of H_2O_2 (Fig. 4B, TRAF2 input, lane 3). Since the direct binding of TRAF2 to CD40 is necessary for TRAF2 degradation [39,40], the unchanged TRAF2 level suggests the failure of TRAF2–CD40 association. Hence, H_2O_2 seems to interrupt the binding of TRAF2 to CD40, rather than to promote TRAF2 degradation. Besides the canonical TRAF2-binding site [14], a non-canonical TRAF2-binding domain in the C-terminus of CD40 has been recently reported [41]. Both of these two binding sites are important for CD40 signal transduction and subsequently B cell activation [41,42]. It is still not clear which binding site was involved in the inhibitory effect of H_2O_2 on TRAF2 recruitment, however, the abolished TRAF2 binding to CD40 in IP Western blot (Fig. 4B, lane 3) indicates that H_2O_2 possibly affects recruitment of TRAF2 to both binding sites. However, the exact mechanism by which ROS inhibits TRAF2 recruitment remains unknown, which might involve the conformational changes of membrane, receptors or adaptor molecules.

In this study, the effect of ROS on CD40 signaling was investigated in B cells. Because CD40 belongs to TNF receptor family and is expressed in various cell types, such as monocytes, our finding provides a clue for the further investigation of CD40 and ROS in other cell types. ROS is mainly produced by the activation of innate immune system and affects the adaptive immune responses. Therefore, ROS play an important role in the connection between innate and adaptive immunity.

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