EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 43, No. 2, 101-110, February 2011

# Ligation of CD40 receptor in human B lymphocytes triggers the 5-lipoxygenase pathway to produce reactive oxygen species and activate p38 MAPK

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DOI 10.3858/emm.2011.43.2.012

Accepted 31 December 2010
Available Online 4 January 2011

Abbreviations: DAPI, 4'6'-diamidino-2-phenylindole; 5-LO, 5-lipoxygenase; $cPLA_2$, cytosolic phospholipase $A_2$; DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DN, dominant-negative; DPI, diphenyleneiodonium chloride; ETYA, eicosatetraynoic acid; HEK, human embryonic kidney; IP, immunoprecipitation; NAC, N-acetyl-L-cysteine; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; TRAF, TNF receptor-associated factor; TRITC, tetramethylrhodamine isothiocyanate; WB, western blotting

## Abstract

**Previously, we reported that CD40-induced production of reactive oxygen species (ROS) by NADPH oxidase requires the TNF receptor-associated factor (TRAF) 3, as well as the activities of phosphatidylinositol 3-kinase (PI3K) and Rac1. Here we investigated the possible mechanisms of the production of ROS after CD40 ligation in B cells. We describe an alternative ROS production pathway that is triggered by CD40 ligation, involves 5-lipoxygenase (5-LO), and results in activation of p38 MAPK. Our studies in Raji human B lymphomas revealed that CD40-induced ROS production by 5-LO also requires the activities of PI3K and Rac1. In contrast to the NADPH oxidase pathway, however, TRAF molecules are not required for the CD40-induced ROS production by 5-LO. The association of CD40 with 5-LO is dependent on CD40 ligation in Raji B cells, and co-immunoprecipitation experiments using epitope-tagged proteins transiently expressed in human embryonic kidney 293T cells revealed the role of the regulatory subunit of PI3K, p85, in this association. Collectively, these data suggest a separate pathway for the CD40-induced ROS production in B cells and demonstrate that this pathway requires 5-LO *via* direct association of p85 with both CD40 and 5-LO.**

**Keywords:** antigens, CD40; arachidonate 5-lipoxygenase; B-lymphocytes; p38 mitogen-activated protein kinases; phosphatidylinositol 3-kinase; reactive oxygen species

## Introduction

Reactive oxygen species (ROS) are important regulatory molecules that are implicated in the signaling cascade triggered by numerous growth factors and cytokines, including TNF-$\alpha$ family members (Sattler *et al*., 1999; Aslan and Ozben, 2003; Han *et al*., 2009). Although these highly reactive molecules regulate many important cellular events, including transcriptional factor activation (Lo and Cruz, 1995; Sulciner *et al*., 1996; Bubici *et al*., 2006), cellular proliferation (Irani *et al*., 1997), and apoptosis (O'Donnell *et al*., 1995) in a variety of non-phagocytic cells, the generation of ROS is not yet well characterized. Recently, potential roles of the mitogenic oxidase Nox family NADPH oxidases (Lambeth *et al*., 2000; Petry *et al*., 2010) and/or 5-lipoxygenase (5-LO), which catalyzes the production of leukotrienes and ROS from arachidonic acid (Harrison and Murphy, 1995; Catalano *et al*., 2005; Kim *et al*., 2008), have been demonstrated in the generation of ROS following receptor ligation in various cell types.

CD40, a member of the TNF receptor superfamily, is expressed in a wide range of both immune and non-immune cell types (Elgueta *et al*., 2009; Law and Grewal, 2009). Signals generated through CD40 ligation in B cells are required for T cell-dependent B cell activation and proliferation, isotype switching of Ig genes, upregulation of costimulatory receptors, cytokine secretion, germinal center formation, and memory generation (van Kooten and Banchereau, 2000; Elgueta *et al*., 2009). Signal transduction through CD40 is initiated by binding of trimeric CD40 ligand on the surface of activated T cells (van Kooten and Banchereau, 2000; Elgueta *et al*., 2009). Signaling

pathways that are highly activated by CD40 engagement in B cells include NF-κB, the MAPK p38, and JNK (Beberich *et al*., 1994, 1996; Francis *et al*., 1995; Sutherland *et al*., 1996; Craxton *et al*., 1998). Activation of CD40- dependent signaling pathways is mediated primarily by recruitment of several TNF receptor- associated factor (TRAF) protein family members to the multimerized CD40 cytoplasmic domain (Ishida *et al*., 1996; Pullen *et al*., 1998, 1999; Leo *et al*., 1999). Among members of the TRAF family, TRAF2, 3, 5, and 6 have been most extensively studied as mediators in CD40-activated signal transduction (Ishida *et al*., 1996; Pullen *et al*., 1998, 1999; Leo *et al*., 1999).

Previously, we showed that ROS generation by CD40 ligation in B cells is important for the downstream signaling events that lead to the activation of JNK, p38, and NF-κB, as well as IL-6 secretion (Lee and Koretzky, 1998; Lee, 2003; Ha and Lee, 2004). Furthermore, we demonstrated in the WEHI 231 murine B cell line that CD40- associated TRAF3 mediates the activation of NADPH oxidase and phosphatidylinositol 3-kinase (PI3K) through the recruitment of p40phox and p85, respectively (Ha and Lee, 2004). This result suggests that CD40-stimulated activation events in these B cells require the production of ROS, which are produced after CD40 ligation and serve as secondary messengers.

In the present study, we demonstrate an alternative ROS production pathway that involves 5-LO and is triggered by CD40 ligation. Our studies in Raji human B cells revealed that CD40-induced ROS production by 5-LO also requires the activities of PI3K and Rac1. In contrast to the NADPH oxidase pathway, however, TRAF molecules are not required, but rather, the regulatory subunit of PI3K, p85, mediates the association between CD40 and 5-LO after ligation of CD40. Collectively, these data suggest a separate pathway for CD40-induced ROS production in B cells *via* direct association of p85 with both CD40 and 5-LO.

## Results

### ROS production after CD40 ligation involves 5-LO and PI3K in Raji human B cells

We previously showed that ROS serve as signaling intermediates that follow CD40 ligation and that these CD40-induced ROS production is through NADPH oxidase pathways in both primary splenic B cells and the mouse B cell line, WEHI 231 (Lee and Koretzky, 1998; Lee, 2003; Ha and Lee, 2004). In this study, we extended our investigations in the role of ROS as signaling intermediates following

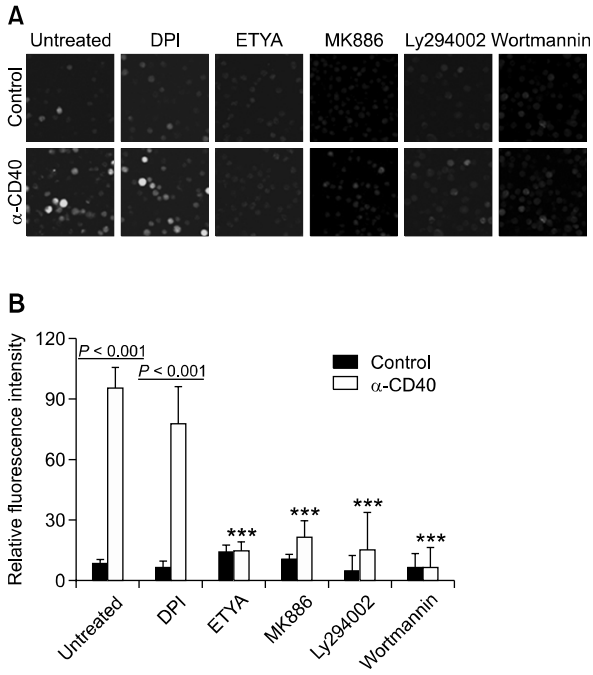


**Figure 1.** ROS production after CD40 ligation in Raji human B cells. (A) The cells ($1 \times 10^6$) that were incubated with 20 μM DCFDA for 15 min were stimulated with either control Ig or anti-CD40 monoclonal Ab (10 μg/ml). When pretreated with the inhibitors, the cells were preincubated in the presence of inhibitors for 30 min before the addition of DCFDA. Representative microscopic fields of 2', 7'-dichlorofluorescein (DCF) fluorescence from the Raji cells after a 20-min stimulation with anti-CD40 in the absence or presence of treatment with several inhibitors (15 μM DPI, 35 μM ETYA, 0.5 μM MK-886, 10 μM LY294002, or 0.1 μM wortmannin). Fluorescence of the cells after stimulation with control Ig or CD40 ligation is shown. Data represent three independent experiments. (B) The DCF fluorescence intensity in Raji B cells after a 20-min stimulation with anti-CD40 is shown. The different treatments are indicated. The numbers of cells ($n$ = 35) on the fluorescence image from all three experiments are given. The data are presented as mean ± SD. Statistical significance was determined by two-way analysis of variance, and the data was subjected to paired t-tests as post-hoc analysis. ***, $P < 0.001$ indicates significantly lower values for cells under CD40 ligation than values for cells either untreated or treated with DPI.

ligation of CD40 in Raji human B cells. First, we investigated the CD40-induced production of intracellular ROS in Raji B cells that were incubated continuously with a redox-sensitive fluorescent probe, 2', 7'-dichloro-dihydrofluorescein diacetate (DCFDA). The representative fluorescent images of the cells after 20 min of stimulation with either control Ig or anti-human CD40 are shown in Figure 1A, and the fluorescence intensity that is produced by CD40-driven ROS is compared quantitatively in Figure 1B. Because the NADPH oxidase and 5-LO pathways function as sources of ROS that are generated by receptor ligation in various non-phagocytic cell types, we investigated whether inhibitors of these enzymes block ROS production in anti-CD40-stimulated Raji B cells. The pre-

incubation of cells with 15 μM diphenyleneiodonium chloride (DPI) for 1 h failed to block ROS production by CD40 ligation in Raji cells (Figures 1A and 1B). In contrast, stimulation with anti-CD40 following preincubation of cells for 1 h with either 35 μM eicosatetraynoic acid (ETYA) or 0.5 μM MK-886 inhibited the anti-CD40-induced ROS production in Raji B cells (Figures 1A and 1B). This result demonstrates that 5-LO, but not NADPH oxidase, is responsible for the CD40-induced ROS production in these cells. A previous report also demonstrated that the 5-LO pathway is required for IL-1β-stimulated ROS production in Raji B cells (Bonizzi *et al*., 1999).

We next investigated signaling events involved in CD40-induced ROS production by 5-LO in Raji B cells. To examine the effects of PI3K antagonists, the cells were preincubated with 10 μM LY294002 or 0.1 μM wortmannin for 1 h before stimulation with anti-CD40. Preincubation with either compound strongly inhibited the CD40-mediated ROS production to basal levels (Figures 1A and 1B). These results indicate that stimulation of ROS production by 5-LO after CD40 ligation requires PI3K activity in Raji B cells.

**Correlation between ROS production and activation of p38 MAPK after CD40 ligation in Raji B cells**

Because CD40 stimulation results in the rapid activation of p38 MAPK (Sutherland *et al*., 1996; Craxton *et al*., 1998) and because low levels of oxidative stress selectively activate p38 (Kurata, 2000), we next determined whether p38 activation is mediated by ROS that are produced in response to CD40 ligation in Raji B cells. Stimulation of the cells with anti-CD40 resulted in the rapid activation of p38 MAPK, and this activation persisted for up to 30 min (Figure 2). Preincubation with an antioxidant, *N*-acetyl-L-cysteine (NAC) (30 mM) strongly diminished CD40-mediated p38 activation, and treatment with 300 μM $H_2O_2$ also activated p38, as assessed by the strong induction of p38 MAPK phosphorylation in Raji B cells (Figure 2A). Preincubation with NAC did not affect much CD40-mediated activation of other MAPK, JNK and ERK (Supplemental Data Figure S1).

Similarly to the CD40-mediated ROS production, precincubation with ETYA or MK886 (Figure 2C) but not with DPI (Figure 2B) inhibited p38 MAPK activation after stimulation with anti-CD40 in Raji B cells. When the cells were preincubated with PI3K antagonists, LY294002 or wortmannin, for 1 h before stimulation with anti-CD40, p38 activation was also inhibited (Figure 2D). Preincubation with inhibitor compounds did not interfere with p38 activation by 0.6 M sorbitol (an osmotic inducer of p38 MAPK) in Raji B cells (Figures 2B-2D). These results, therefore, indicate that ROS generation by 5-LO may play an important role in signaling events that are mediated by CD40 in this cell type.

**Rac, but not TRAF, is a component in the cascade of CD40-mediated ROS production by 5-LO**

Rac, a small GTP-binding protein, plays a role in mediating TNF-α-induced intracellular ROS production *via* the cytosolic phospholipase A2 (cPLA2)-linked cascade (Woo *et al*., 2000). Therefore, we also investigated whether Rac was critical in the signaling cascade involved in ROS production after CD40 ligation in Raji B cells. The intracellular ROS production after stimulation with anti-CD40 was determined in Raji cells transfected transiently with an expression plasmid that encodes a dominant-negative (DN) mutant of Rac1 (N17Rac1) along with an expression plasmid that encodes a red fluorescent protein (pCMVDsRed). ROS-induced fluorescence changes in these cells are shown as relative fluorescence intensities in the cells that were transfected with the control or N17Rac1 plasmid (Figure 3A). When stimulated with anti-CD40, the majority of the vector-transfected cells, but not the N17Rac1-transfected cells, responded by producing ROS. Therefore, Rac likely plays a role upstream in the signaling cascade for the production of ROS by 5-LO after CD40 stimulation in Raji B cells.

Recruitment of specific TRAF molecules to the CD40 cytoplasmic domain that contains direct binding sites for TRAF 2, 3, and 6 primarily initiates CD40-mediated signal transduction in B cells (Ishida *et al*., 1996; Pullen *et al*., 1998, 1999; Leo *et al*., 1999). We have also previously demonstrated that TRAF3 is required for CD40-stimulated ROS production by NADPH oxidase in WEHI 231 murine B cells (Ha and Lee, 2004). We, therefore, examined whether TRAF proteins also play a role in the CD40-induced ROS production by 5-LO in Raji B cells. To determine the effect of each TRAF protein on ROS production after CD40 ligation, we transiently expressed the DN form of each TRAF protein, along with DsRed, in the Raji B cells. These transfected cells were incubated with a DCFDA probe, and the intracellular ROS production was examined after stimulation with anti-CD40 mAb. The relative fluorescence intensities in cells that were transfected with the control or the DN form of TRAF (DNTRAF) plasmids are shown in Figure 3B. Following stimulation with anti-CD40, Raji B cells that had been transfected with DNTRAF DNA (TRAF 2, 3, or 6) produced ROS at

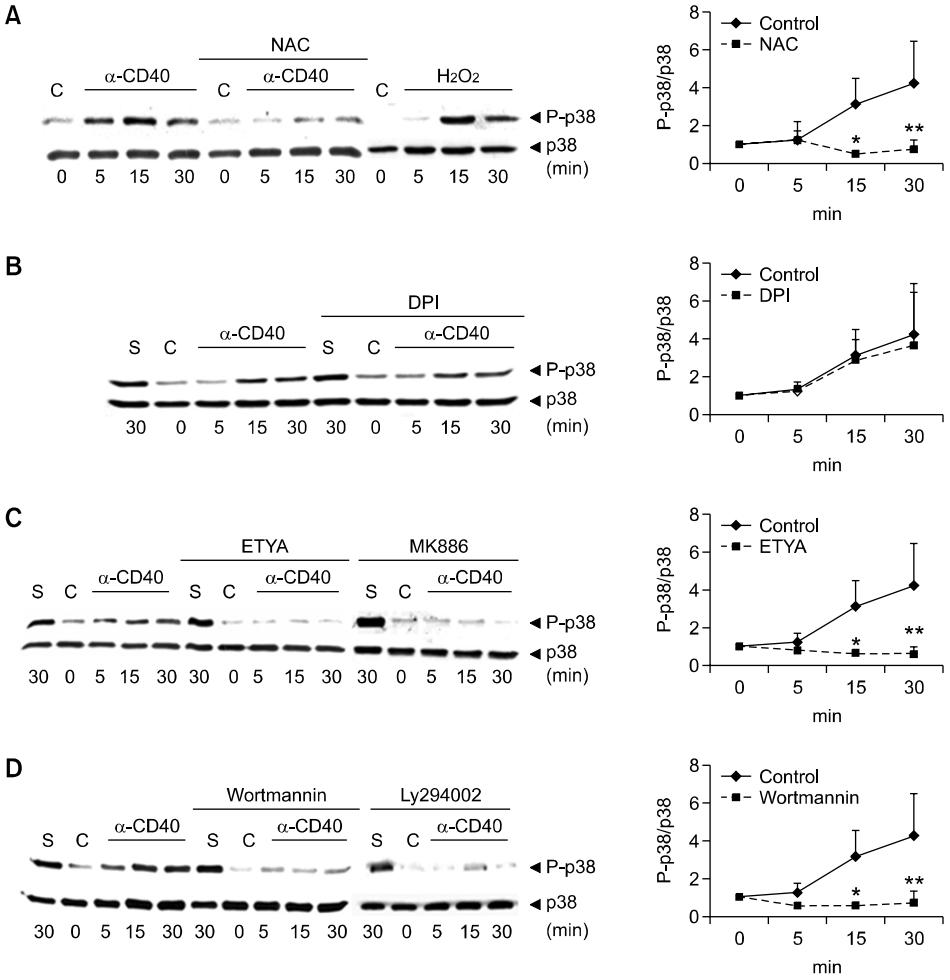


**Figure 2.** Correlation of ROS production and p38 MAPK activation after CD40 ligation. (A) The cells ($5 \times 10^6$) were pretreated with 30 mM NAC for 1 h or were untreated, and then the cells were stimulated with medium containing control Ig (control) or 10 μg/ml anti-CD40 for various times. Some of the untreated cells ($5 \times 10^6$) were also incubated with 300 μM $H_2O_2$ for the indicated times as a positive control for ROS-mediated p38 activation. The lysates from $1 \times 10^6$ cells were subjected to SDS-PAGE and a phospho-p38 (P-p38) WB analysis. The same blot was stripped and re-probed with anti-p38 Ab to insure equal loading of the cell lysates in each lane. (B-D) The cells were pretreated with either 15 μM DPI (B), 35 μM ETYA, or 0.5 μM MK886 (C), 0.1 μM wortmannin, or 10 μM LY294002 (D) or left untreated before CD40 ligation. Cells were also treated with 0.6 M *D*-sorbitol (S) for 30 min as a positive control for p38 activation. The data shown represent three separate experiments. The intensities of the bands in the phospho-p38 and p38 WB were quantitated, and normalized values (P-p38/p38) were calculated. The data shown are the average of the normalized values from three separate WB for each inhibitor treatment. The data are presented as mean ± SD. Statistical significance was determined by two-way analysis of variance, and paired t-tests were used as post-hoc analysis. *, $P < 0.05$ and **, $P < 0.01$ indicate values significantly lower than control at the same time point.

levels similar to those in vector-transfected cells. Therefore, TRAF molecules are not required for ROS production following CD40 ligation in Raji B cells.

### Direct association of p85, the regulatory subunit of PI3K, with CD40 and 5-LO

PI3K and Rac play important roles in CD40-induced ROS production *via* the 5-LO pathway in Raji B cells (Figures 1 and 3A). In addition, TRAF molecules are not required for these signaling pathways (Figure 3B). To determine the molecular associations that are involved in CD40-induced ROS production, co-immunoprecipitation (IP) experiments with CD40, PI3K, and 5-LO were performed in HEK 293T cells using epitope-tagged molecules. CD40 was transiently expressed in combination with 5-LO or p85 in human embryonic kidney (HEK) 293T cells. Analyses of the immune

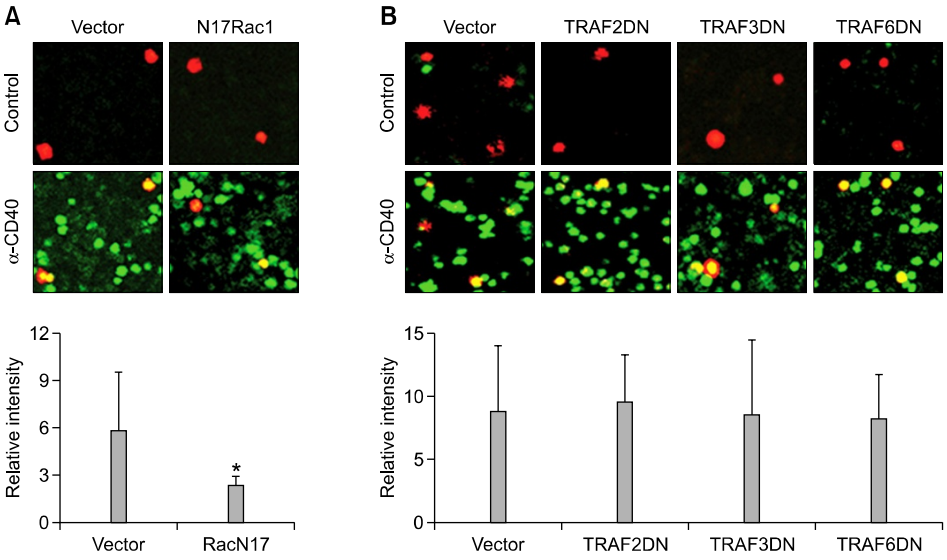


**Figure 3.** Effects of N17Rac1, DNTRAF 2, 3, or 6 on CD40-induced ROS production. The cells were transiently transfected with either empty vector or one of the DN expression plasmids, along with a red fluorescent protein, DsRed. Intracellular ROS production after a 20-min stimulation with anti-CD40 was measured (described in Figure 1). (A) Changes in the fluorescence to yellow, due to the green fluorescence from CD40-induced ROS production, are clearly shown in cells that were doubly transfected with the vector and DsRed plasmids. In the case of the concurrent expression of DsRed and a DN mutant of Rac1 (N17Rac1), however, not all of the cells responded to the CD40 stimulation that led to fluorescence changes. The fluorescence intensity after CD40 ligation relative to the control is shown in cells that were transfected either with a vector or a DN mutant of Rac1 (N17Rac1). The average fluorescence intensity from at least 50 cells from three experiments are presented as the average ± SD; *, $P < 0.001$. (B) The relative fluorescence intensity from ROS production after CD40 ligation for 20 min in cells that were transfected with either vector alone or with each DNTRAF is given as a ratio to control fluorescence. Data obtained as in (A) are presented as the average ± S.D.

complex with anti-CD40 showed that CD40 associates directly with p85 but not with 5-LO (Figure 4A). In reciprocal experiments, CD40 was also detected in the immunoprecipitates with p85 but not with 5-LO (Figure 4A). Immune complexes with isotype control Ig were also analyzed to ensure specific molecular associations (data not shown).

Because CD40 does not associate directly with 5-LO (Figure 4A), we investigated the role of p85 in mediating the CD40 signal to 5-LO. In these experiments, 5-LO was transiently expressed along with p85 in HEK 293T cells. Co-IP experiments revealed the presence of 5-LO in p85 immunoprecipitates and of p85 in 5-LO immunoprecipitates (Figure 4B). These results demonstrated that CD40 directly associates with p85, which associates directly with 5-LO. Furthermore, these results strongly support a role for PI3K in mediating the CD40 signal to the 5-LO pathway. Consequently, this signaling results in the production of ROS after CD40 ligation.

Additionally, immune complexes from Raji B cells that were stimulated with either isotype control Ig or anti-CD40 were examined for molecular associations between CD40, p85, and 5-LO by Western blotting (WB). The association between CD40, p85, and 5-LO was validated in immune complexes following stimulation with anti-CD40 but not after incubation with isotype control Ig (Figure 5A). The colocalization of p85 and 5-LO was also observed at the plasma membrane after CD40 ligation (Figure 5B). Upon co-staining for CD40 and 5-LO, colocalization of these two proteins was observed following CD40 ligation (Figure 5C). These results further support that the recruitment of 5-LO by the association of p85 with the CD40 receptor complex after CD40 ligation results in the production of ROS in Raji B cells.

## Discussion

The TNF receptor superfamily member CD40 is expressed primarily by professional antigen-presenting cells, including B cells, and plays a critical role in T cell-dependent immune responses *via* an interaction between B cells and T cells that express the CD40 ligand (CD40L, gp39) (van Kooten and Banchereau, 1997, 2000; Elgueta *et al*., 2009). Signals generated through CD40 in B cells induce the activation of JNK, p38 MAPK, and NF-κB (Beberich *et al*., 1994, 1996; Francis *et al*.,

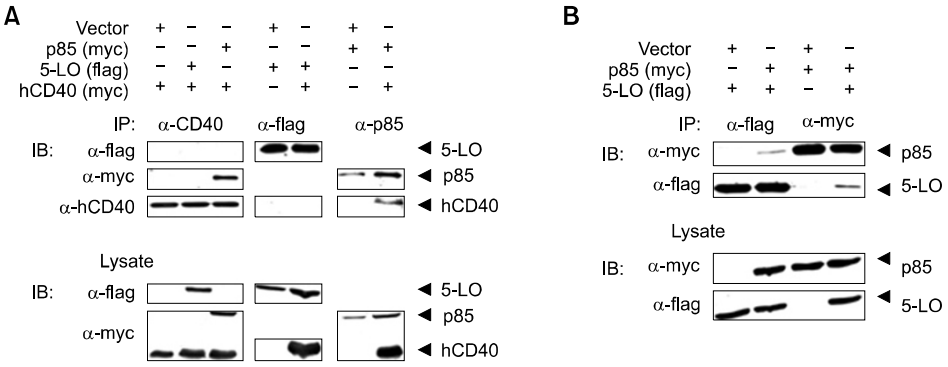


**Figure 4.** Molecular associations in CD40 signaling complexes involved in ROS production. (A) HEK 293T cells were transfected transiently with combinations of plasmids: human CD40 plasmid with myc epitope, hCD40(myc) and either empty vector, p85(myc), or 5-LO(flag); 5-LO plasmid with either empty vector or hCD40; and p85 plasmid with either empty vector or hCD40. Analyses of the anti-CD40 immune complexes show specific associations of CD40 with p85 but not with 5-LO. In reciprocal experiments, CD40 was detected in p85 but not in 5-LO immunoprecipitates. (B) HEK 293T cells were transfected transiently with a combination of p85(myc) and either empty vector or 5-LO(flag) or *vice versa*. Analyses of the immune complexes of the cell lysates with anti-myc Ab show a selective association of p85 with 5-LO. Reciprocal experiments also showed associations of these molecules. The expression of the plasmids was determined by WB of the lysates using Abs against epitope tags. The data shown represent three separate experiments.

1995; Sutherland *et al*., 1996; Craxton *et al*., 1998). Ligation of CD40 also activates PI3K, which plays a crucial role in CD40-induced proliferation and NF-κB activation in B cells (Aagaard-Tillery and Jelinek, 1996; Andjelic *et al*., 2000; Clayton *et al*., 2002). Previously, we suggested that CD40 ligation may be linked to JNK, NF-κB activation, and subsequent IL-6 production *via* the production of ROS (Lee and Koretzky, 1998).

The sources of ROS, which are generated after receptor ligation in various non-phagocytic cells, have yet to be determined. The Nox/Duox family of NADPH oxidases, which are related to phagocyte oxidase, are expressed in various cell types and generate ROS that function in cell signaling, immune function, hypoxic response, and oxidative modification of proteins (Lambeth, 2002). These enzymes may perform different functions in different tissues or within the same cell at different times. The 5-LO activity that requires FLAP for activation (Dixon *et al*., 1990; Miller *et al*., 1990; Reid *et al*., 1990) is also required for ROS production by CD28 stimulation in T lymphocytes (Los *et al*., 1995) and for IL-1β-induced NF-κB-dependent transcription in lymphoid (Bonizzi *et al*., 1997) and endothelial cells (Lee *et al*., 1997). In addition, PI3K and Rac play roles in the TNF-α or platelet-derived growth factor-induced production of ROS (Bae *et al*., 2000; Woo *et al*., 2000).

Our previous reports showed that ROS are generated by CD40 ligation in primary splenic and WEHI 231 B cells and play roles as signaling intermediates in the downstream signaling events (Lee and Koretzky, 1998; Lee, 2003; Ha and Lee, 2004). In WEHI 231 B cells that express only the NADPH oxidase system but not 5-LO, we also demonstrated the role of CD40-associated TRAF3 in the activation of NADPH oxidase, and this activation resulted in CD40-induced ROS production by associations with p40phox and p85 (Ha and Lee, 2004). In this study, we used Raji human B cells for the examination of ROS production after the CD40 ligation, and these cells showed significant expression of both NADPH oxidase and 5-LO as determined by immunoblotting of cell lysates for p67phox (a component of NADPH oxidase) and 5-LO (unpublished data). In contrast to the previous results, the pharmacological inhibitor(s) for 5-LO, but not for NADPH oxidase, selectively inhibited CD40-induced ROS production in Raji B cells. Our study, using pharmacological and molecular tools, also demonstrated that PI3K and Rac act as effector molecules that are possibly required for ROS production after CD40 ligation in Raji B cells (Figures 1 and 3A). We also showed a correlation between ROS production and p38 MAPK activation after CD40 stimulation in Raji B cells (Figure 2). Therefore, we conclude that PI3K and Rac also play essential roles upstream of 5-LO in producing ROS after CD40 ligation, and that ROS then mediate downstream signaling in Raji B cells.

Recruitment of TRAF protein family members to the multimerized CD40 cytoplasmic domain is thought to mediate CD40-dependent signaling pathways (Ishida *et al*., 1996; Pullen *et al*., 1998,

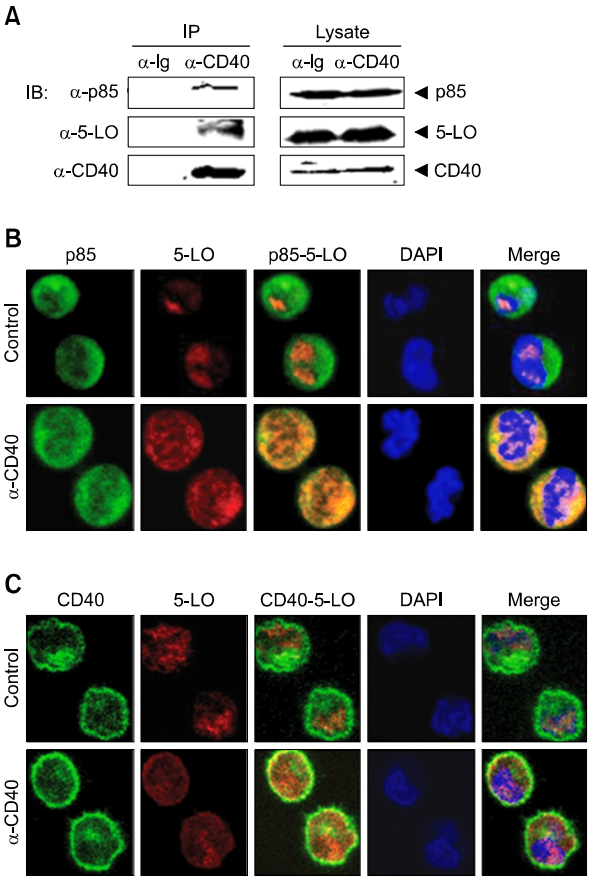


**Figure 5.** Recruitment of 5-LO to the CD40 receptor complex through association with p85 after stimulation in Raji B cells. (A) The lysates from 1 $\times$ $10^7$ Raji B cells that were stimulated either with anti-mouse (control) or anti-CD40 Ig for 10 min were tumbled with GammaBind G sepharose beads. The immune complexes for each stimulation condition were then subjected to SDS-PAGE, which was followed by WB with anti-p85, anti-5-LO, and anti-CD40 Ab. The data represent three independent experiments. (B, C) The Raji B cells, either unstimulated (control) or stimulated with anti-CD40 for 15 min, were labeled for endogenous p85 (green) or CD40 (green) along with 5-LO (red) using anti-p85, anti-CD40, and anti-5-LO, followed by FITC- and TRITC-conjugated secondary Ab, respectively. Cell nuclei were labeled using DAPI (blue). Fluorescence was visualized under a confocal microscope. The merged staining patterns of p85-5-LO and CD40-5-LO are shown in the middle panels. The merged staining patterns of all three colors are shown in the far right panels. Representative images from three separate experiments with similar results are shown.

1999; Leo *et al*., 1999). TRAF3 specifically mediates association of CD40 with p85 and p40phox for ROS production after CD40 ligation in WEHI 231 B cells (Ha and Lee, 2004); however, in Raji B cells, TRAF proteins are not required for CD40 ligation to trigger PI3K and 5-LO activation, which consequently results in ROS production. Moreover, a role for TRAF3 was not found in the association of CD40 with the NADPH oxidase system in Raji B cells, although this association may induce ROS production after CD40 stimulation. Thus, the association of CD40 with TRAF proteins may differ in different cell types and may depend on the expression levels of the proteins especially since the expression level of TRAF3 in Raji cells was relatively low compared to that of other TRAF proteins and was lower than TRAF3 expression in WEHI 231 cells (unpublished data). This finding suggests that the composition and function of a CD40 signaling complex may vary depending on the cellular context.

Direct molecular associations of p85 with CD40 and 5-LO in Raji cells (Figure 5) may indicate an alternative pathway for the production of ROS after CD40 stimulation in B cells. During TNF-α-induced ROS generation, PI3K activates Rac signaling through the cPLA2-linked cascade (Woo *et al*., 2000). Once activated, $cPLA_2$ catalyzes the synthesis of arachidonic acid, and 5-LO metabolizes this compound to leukotrienes. These pathways are involved in Rac signaling for ROS generation. Similarly, PI3K may initiate CD40-induced ROS production in Raji human B cells through the recruitment of 5-LO to the CD40 complex and Rac-mediated activation of $cPLA_2$ to synthesize arachidonic acid concurrently. Therefore, ROS production may be enhanced after CD40 ligation in Raji cells *via* these pathways.

As compared in a schematic diagram (Figure 6), our data collectively demonstrate an alternative mechanism for ROS production after CD40 ligation in B cells *via* the 5-LO pathway, which then mediates downstream p38 MAPK activation. Our data also show that CD40-induced ROS production *via* 5-LO requires the activities of PI3K and Rac1 similarly to ROS production via NADPH oxidase.

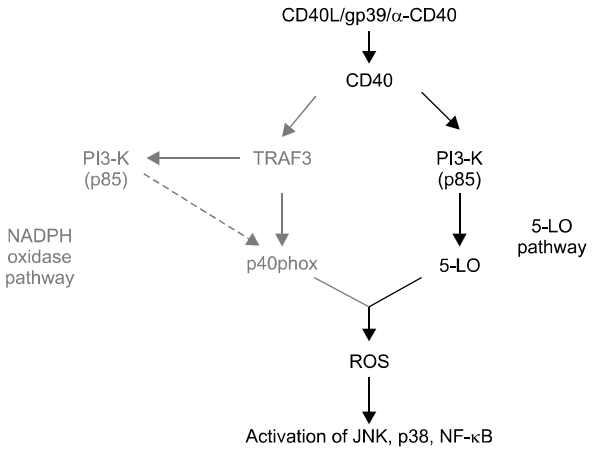


**Figure 6.** A schematic diagram showing the signaling pathways in the CD40-induced ROS production. The solid lines represent direct protein:protein associations and the dashed line represent the presence of intermediary steps. As shown, the NADPH oxidase pathway is presented in gray color.

Unlike the NADPH oxidase pathway, however, the regulatory subunit of PI3K, p85, rather than TRAF proteins directly associates with CD40 and 5-LO to activate the pathway for ROS production. It remains to be studied whether these associations of 5-LO with CD40 play roles in the production of leukotrienes from arachidonic acid by 5-LO after CD40 ligation.

In summary, CD40 ligation signals through PI3K in order to recruit and activate 5-LO and $cPLA_2$ at the same time. Via activation of $cPLA_2$, arachidonic acid is synthesized, and this process may ultimately enhance the production of ROS by 5-LO. Studies are underway to characterize the pathway responsible for the CD40-induced ROS production in various B cell types. These studies will elucidate the mechanism(s) of CD40-induced ROS signaling in B cells as these mechanisms may vary depending upon the differences in species or maturation stages as well as upon the expression levels of the signaling proteins.

## Methods

### Ab and other reagents

Mouse anti-human CD40 monoclonal Ab (5C3) from BD PharMingen (San Diego, CA) was used for the cell stimulation. Anti-CD40 rabbit polyclonal Ab from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) was used for IP, immunofluorescence, and WB. Anti-p38 rabbit polyclonal Ab was also obtained from Santa Cruz Biotechnology, Inc. Anti-5-LO mouse monoclonal Ab (clone 33) from BD Transduction Laboratories (Lexington, KY) was used. Ab against the phosphorylated form of p38 and a secondary Ab, HRP-conjugated anti-rabbit IgG, were purchased from New England Biolabs Ltd. (Beverly, MA). We also used anti-p85 rabbit polyclonal Ab, anti-myc monoclonal Ab (9E10) from Upstate Biotechnology Inc. (Lake Placid, NY) and anti-flag monoclonal Ab (M2) from Sigma Chemical Co. (St. Louis, MO). A secondary Ab, HRP-conjugated anti-mouse IgG, was purchased from Bio-Rad (Hercules, CA). Secondary Ab, FITC-conjugated anti-rabbit IgG and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG, from Jackson ImmunoResearch Laboratories (West Grove, CA) were used for the immunofluorescence staining.

LY294002 and MK-886 were obtained from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). NAC, DPI, ETYA, wortmannin, D-sorbitol, 4'6'-diamidino-2-phenylindole (DAPI), and poly-L-lysine were purchased from Sigma Chemical Co. The DCFDA was obtained from Molecular Probes (Eugene, OR). ECL reagents and Gamma-Bind G Sepharose were purchased from Amersham Pharmacia Biotech Co. (Arlington Heights, IL). The TRIZOL reagent and oligo$(dT)_{12\text{-}18}$ primer were obtained from Life Technologies, Inc. (Grand Island, NY).

### Plasmids

Mammalian expression vectors that encode N17Rac1, the p85 subunit of PI3K (myc), 5-LO (flag), and a DN form of TRAF3 (DNTRAF3) were kindly provided by Drs. T. Finkel (NIH, Bethesda, MD), A. Toker (Harvard Medical School, Boston, MA), O. Rådmark (Karolinska Institute, Sweden), and D. Goeddel (Tularik Inc., CA), respectively. The mammalian DsRed expression plasmid, pCMVDsRed and the plasmids of DNTRAF 2 or 6 were described previously (Ha and Lee, 2004). The human CD40 cDNA was generated by a RT-PCR from Raji B cells, using oligonucleotides 5'-gatcagatctccaccatggttcgtctgcctc-3' and 5'-ccgctcgagctgtctctcctgcac-3', and ligated into pCDNA3.1(+)-myc (Invitrogen, Carlsbad, CA) between the *Bam*HI and *Xho*I sites. DNA sequencing confirmed that the PCR product was completely free of undesired mutations.

### Cell culture and treatments

The human B lymphoma cell line, Raji, was cultured in a RPMI 1640 medium that contained 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 mM glutamine. Cell culture of a HEK 293T cell line and treatments of the Raji cells with pharmacological inhibitors were described previously (Ha and Lee, 2004). The optimal treatment time and concentrations for various inhibitors were determined in our previous experiments (Ha and Lee, 2004).

### Transient expression of plasmid DNA, WB, and IP

Transfection of the Raji B cells and HEK 293T cells was described previously (Ha and Lee, 2004). Determination of p38 MAPK activation, WB, and IP were also performed as described previously (Ha and Lee, 2004).

### Fluorescence measurement of intracellular ROS

CD40-induced ROS production was measured using a fluorescent probe, DCFDA, as described previously (Ha and Lee, 2004). Briefly, after 15 min loading of the cells ($1 \times 10^6$) with 20 μM DCFDA, stimulation with either isotype control Ig (control), or anti-CD40 Ab (10 μg/ml) was followed. Fluorescence intensities were measured under excitation settings at 488 nm and emission at 515-540 nm every 30 s for 30 min using a Zeiss Axiovert 100M inverted microscope that was equipped with a 40X Achroplan objective LD and a Zeiss LSM510 confocal attachment (Zeiss, Oberkochen, Germany).

### Immunocytochemistry

The cells stimulated with either isotype control Ig (control), or anti-CD40 Ab (10 μg/ml) were attached on poly-L-lysine coated slide, fixed with 3.7% paraformaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min. After being blocked with 1% BSA for 1 h, the samples were incubated with anti-5-LO, anti-p85 for 1 h at room temperature, or anti-CD40 Ab overnight at 4°C, followed by a 40-min incubation with FITC- or TRITC-conjugated secondary Ab. After being mounted, the samples were

analyzed using a Zeiss Axiovert 100 M inverted microscope that was equipped with a 100X EC Plan-Neofluar objective and a Zeiss LSM510 META confocal attachment.

### Statistical analysis

The paired Student's *t* test was used to determine the statistical significance of the data. Comparisons between samples were performed using a two-tailed Student's *t* test. Statistics were determined using Prism software (GraphPad Software, Inc.). Values of $P < 0.05$ were considered significant.

### Supplemental data

Supplemental data include a figure and can be found with this article online at http://e-emm.or.kr/article/article_files/SP-43-2-05.pdf.

### Acknowledgements

We thank Drs. Torren Finkel, Alex Toker, Olof Rådmark, and David Goeddel for providing valuable reagents. This work was supported by grants from the Korea Research Foundation (RO4-2004-000-10077-0, 2009-0067361), the Basic Research Program of the Korea Science and Engineering Foundation (R01-2006-000-10429-0), and the National Core Research Center Program of the Ministry of Science & Technology and the Korea Science and Engineering Foundation (R15-2006-020) through the Center for Cell Signaling & Drug Discovery Research at Ewha Womans University. Y.J.H. and H.J.S. were supported in part by the second stage of the Brain Korea 21 Program of the Korea Ministry of Education.

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