# Differential Signaling via Tumor Necrosis Factor-Associated Factors (TRAFs) by CD27 and CD40 in Mouse B Cells

So-Youn Woo[1]*, Hae-Kyung Park[1] and Gail A. Bishop[2,3,4]

*Department of Microbiology[1], College of Medicine, Ewha Womans University, Seoul 158-710, Korea, Department of Microbiology[2], and Department of Internal Medicine[3], The University of Iowa, Veterans Affairs Medical Center[4], Iowa City, IA 52242*

## ABSTRACT

**Background:** CD27 is recently known as a memory B cell marker and is mainly expressed in activated T cells, some B cell population and NK cells. CD27 is a member of tumor necrosis factor receptor family. Like CD40 molecule, CD27 has (P/S/T/A) X(Q/E)E motif for interacting with TNF receptor-associated factors (TRAFs), and TRAF2 and TRAF5 bindings to CD27 in 293T cells were reported. **Methods:** To investigate the CD27 signaling effect in B cells, human CD40 extracellular domain containing mouse CD27 cytoplamic domain construct (hCD40-mCD27) was transfected into mouse B cell line CH12.LX and M12.4.1. **Results:** Through the stimulation of hCD40-mCD27 molecule via anti-human CD40 antibody or CD154 ligation, expression of CD11a, CD23, CD54, CD70 and CD80 were increased and secretion of IgM was induced, which were comparable to the effect of CD40 stimulation. TRAF2 and TRAF3 were recruited into lipid-enriched membrane raft and were bound to CD27 in M12.4.1 cells. CD27 stimulation, however, did not increase TRAF2 or TRAF3 degradation. **Conclusion:** In contrast to CD40 signaling pathway, TRAF2 and TRAF3 degradation was not observed after CD27 stimulation and it might contribute to prolonged B cell activation through CD27 signaling. **(Immune Network 2004;4(3):143-154)**

**Key Words:** B cell, CD40, CD27, TRAF2, TRAF3

## Introduction

B cell can be activated by signals from immunoglobulin (Ig) receptors, cytokines, and cell-to-cell contacts. Among them, CD40/CD154 and CD27/CD70 interactions are important for B cell activation.

Both CD40 and CD27 are the members of the tumor necrosis factor receptor (TNFR) family. TNFR family can be divided into two groups according to adapter molecules binding to cytoplasmic tail of the receptor. TNFR-associated factor (TRAF)-linked group includes CD27, CD30, OX-40 (CD134), 4-1BB (CD137), CD40 and RANK. Death domain (DD)-linked group includes CD95, TNFR1, TRAIL, and DR3 (1).

Correspondence to: So-Youn Woo, Department of Microbiology, Ewha Womans University College of Medicine, Mok-6-dong 911-1, Yang-chun-gu, Seoul 158-710, Korea. (Tel) 82-2-2650-5740, (Fax) 82-2-2653-8891, (E-mail) soyounwoo@ewha.ac.kr

This work was supported by the Intramural Research Grant of Ewha Womans University.

In mammals, six different TRAFs have been identified (2). All TRAFs, except TRAF1, have N-terminal zinc RING finger, zinc finger region, coiled-coil, and C-terminal TRAF domain (3). TRAFs can be aggregated or can be bound to receptors through TRAF domain. N-terminal zinc RING finger and zinc finger region of TRAFs are important for signaling event through NF-κB and AP-1 transcription factors. NF-κB activation is induced by IκB kinase (IKK) activation, which leads to phosphorylation and degradation of IκB followed by translocation of NF-κB into nucleus. AP-1 activation is induced by mitogen-activated protein (MAP) kinases, including JNK/SAPK, ERK, and p38.

CD40 (TNFRSF5) is expressed on the mature B cells, dendritic cells, monocytes, endothelial cells, epithelial cells, but not on the plasma cells. CD40 plays a major role in B cells in T cells dependent class switching, germinal center formation, and memory B cell proliferation (4). Though CD40 does not have possible kinase domain, but it has PXQXT motif,

which can bind TRAFs through this motif, on cytoplasmic tail. CD40 molecule can bind TRAF1, TRAF2, TRAF3, TRAF5 and TRAF6 (5-8). CD40 stimulation leads to increase IgM secretion via TRAF2 and TRAF6, but not by TRAF3. CD40 mediated JNK activation is related mostly to TRAF2, and NF-κB activation is mediated by either TRAF2 or TRAF6 (9). TRAF6 is required for surface molecule up-regulation, IL-6 secretion, isotype switching, and plasma cell differentiation (10).

CD27 (TNFRSF7) is a 50- to 55 kDa type I glycoprotein and is expressed mostly on T cells, NK cells, adult peripheral blood B cells (5～14%) (11), tonsilar B cells, and Epstein-Barr virus transformed cell lines. CD27 is recently known as a memory B cell marker (12). CD27 expression is negative on cord blood cells but CD27+ cells replenish the human splenic marginal zone after 2 years after birth (13). These CD27+ cells express higher level of LFA-1 (CD11a), ICAM-1 (CD54), LFA-3 (CD58) and CD44 (14) than CD27- cells do. In CD27+ B cells, CD27/CD70 interaction augments IgM and IgG secretion (15). Like CD40 molecules, CD27 activates NF-κB and SAPK/JNK via TRAF2, TRAF5 and NF-κB-inducing kinase (NIK) (16). CD27/CD70 interaction in B cells can induce plasma cell differentiation (17). CD27 is reported to bind TRAF2 and TRAF5 in 293 cells by overexpresion study (16), but CD27 and TRAF interaction in B cells has not been reported yet.

Though both CD40 and CD27 share the (P/S/T/A)X(Q/E)E motif for TRAF bindings, CD27- and CD40-derived signals would be different effect in B cells. TRAF degradation induced by CD40 stimulation might be affected by CD27 stimulation in B cells. Because CD70 (CD27 ligand) is expressed later on activated T cells than CD154 (CD40 ligand), it is also possible that CD27 signaling follows that of CD40 to sustain activation state of the B cells as showing similar effect on B cells. Because the simultaneous CD27 and CD40 signaling was never tested before in B cells and signaling event caused by CD27 is not much known so far, it needs to investigate possible interaction between signaling in these two molecules. In the present study, signaling via CD40 and/or CD27 was investigated by using mouse B cell lines, which endogenous express mouse CD40. For selective stimulation of CD40 or CD27, cells were transfected with human CD40 extracellular domain containing cytoplasmic mCD27 tails.

## Materials and Methods

*Cells.* Mouse B cell cell line M12.4.1 (18), and CH12.LX (19, 20) were grown in B cell medium (BCM-10; RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 10μMβ-mercaptoethanol, and penicillin/streptomycin). Transfected cells were cultured in 400μg/ml of G418 (Life Technologies, Rockville, MA). Spodoptera frugiperda (Sf9) cells expressing hCD154 have been described previously (21, 22).

*Antibodies.* Anti-mCD27-PE (Armenian hamster IgG, clone LG.3A10), Hamster IgG1, κ-PE (isotype control, anti-TNP-KLH, clone A19-3), and anti-mCD80 (Rat IgG2a) were purchesed from BD PharMingen (San Diego, CA). Biotin labeled anti-mCD70 (clone FR70, Rat IgG2b, κ), anti-IgE (clone MP5-32C11) and isotype control antibodies were purchased from eBioscience. Mouse IgG1, κ isotype antibody (MO PC-21) was purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-TRAF1 (N-19), rabbit anti-TRAF3 (H-122), goat anti-TRAF5 antibodies (N-20), and anti-JNK1 (FL) antibody were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Rabbit anti-TRAF2 antibody was purchased from MBL (Watertown, MA). Horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (H+L) and goat anti-mouse IgG (H+L) antibodies were purchased from Bio-Rad (Hercules, CA). HRP conjugated rabbit anti-sheep IgG antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-rat IgG (H+L)-FITC (goat, mouse absorbed) and streptavidin-FITC were purchased from Southern Biotechnology Associates (Birmingham, AL). Anti-phospho-SAPK/JNK (Thr183/Tyr185) antibody was purchased from Cell Signaling Technology (Beverly, MA). Anti-actin antibody was from Hybridoma Facilities of University of Iowa. Anti-mCD40 (1C10, rat IgG2a) was from Dr. F. Lund (Trudeau institute, Saranac Lake, NY). Anti-mCD23 (B3B4, rat IgG2a) antibody was from Dr. T. Waldschmidt (University of Iowa, Iowa City, IA). Anti-mCD54 (YN1/1.7.4, rat IgG2b), anti-hCD40 (G28-5, mouse IgG1), and anti-mCD11a (M17/4.4.11.9, rat IgG2a) antibodies were from American Type Culture Collection (Manassas, VA).

*DNA constructs and transfection.* M12.4.1 cells were stimulated with anti-mouse CD40 (1C10) for 3 days to induce CD27 expression and mRNA was prepared with TRIZOL (Gibco) by manufacturer's instruction. Human CD40 extracellular domain and mouse CD27 transmemebrane-cytoplasmic domain hybrid construct was made by the ligation of hCD40 construct, containing SacI restriction enzyme cut site, and mCD27 insert containing SacI and XbaI cut sites from the PCR. Mouse CD27 insert was amplified by mCD27 forward primer (5' - AAA GAA TTC GCA GTT GGG GCT CAG AA - 3') and mCD27 reverse primer (5' - AAA GTC GAC CCG GTC AAG GGT AGA AAG C - 3'). Mouse CD27Δ16 insert was

amplified with mCD27 forward primer (5' - AAA GAA TTC GCA GTT GGG GCT CAG AA - 3') and mCD27Δ16 reverse primer (5' - ATA TCT AGA CTA AGC ACT GCC CTC CTC TTC - 3'). All PCR reactions were done in 94°C 3 minute incubation followed by 35 cycles of 94°C 45 seconds, 60°C 30 seconds, and 68°C 1 minute. After confirmation of the sequences, each construct was transfected into 293T cells with calcium-phosphate methods to check the level of expression. Briefly, $5\times10^5$ cells of 293T cells were plated on a 60-mm culture dish. Total 5μg of plasmid DNA was mixed with 0.5 ml of 2× HBS buffer (10% HEPES, 16% NaCl, 0.74% KCl, 0.2% $Na_2HPO_4$, 2% dextrose, pH 7.05) and 0.5 ml of 125 mM $CaCl_2$. DNA mixture was added to the cells and incubated for 12 hours before changing into 10 % serum containing medium. After 48 hours of transfection, cells were lysed and separated on SDS-PAGE for Western blot. For stable transfection of M12.4.1, and CH12.LX cells, $1\times10^7$ cells were washed with serum-free and antibiotics-free RPMI1640 medium and resuspended with 400μl electroporation medum (5 mM glutathione in RPMI1640). Cells were mixed with 10μg of ScaI cut and purified plasmid, then electroporated on 225 V for 30 mseconds. Cells were plated onto 24-well culture plates on the concentration of $2\times10^4$ cells/well. Stably transfected cells were selected on the medium containing 400μg/ml of G418. The level of expression of hCD40-mCD27 molecules or hCD40-mCD27Δ16 was measured by FITC-labeled anti-hCD40 Ab staining profiles in flow cytometer.

*Cell surface molecules.* M12.4.1 cells and stably transfected cells with hCD40-mCD27 or hCD40-mCD27Δ16, were stimulated with 4μg/ml of anti-hCD40 Ab (G28.5) and/or anti-mCD40 Ab (1C10) for 72 hours. Cells were washed with 0.5% FBS/PBS and stained with FITC-labeled anti-CD11a (LFA-1), anti-CD23 (FcεRII), anti-CD54 (ICAM-1), anti-CD70, anti-CD80 (B7-1) Abs and isotype Abs. Cells were analysed by FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

*IgM secretion assay.* CH12.LX cells and their transfected cells were expressed surface IgM specific to phosphatidylcholine. IgM secreted cells can be counted by lytic plaque on sheep RBC (sRBC) plate (23) in the presence of complement after stimulation of cells with anti-hCD40 or anti-mCD40 Abs. Briefly, cells were cultured for 72 hours with stimulating Abs or antigen (sRBC). Then cells were mixed with sRBC and complement, transferred to chamber slides, and incubated about 30 minutes at 37°C. During this incubation, IgM on CH12.LX cells binds to sRBC and sRBC is lysed by complement fixation.

*TRAF recruitement to Brij 58-insoluble fraction.* To determine TRAF recruitment into Brij 58-insoluble fractions, M12.4.1 cells and hCD40-mCD27 transfected cells ($10^7$ cells/condition) were stimulated for 10 minutes with the combination of anti-hCD40 Ab (G28.5), anti-mCD40 (1C10), or isotype antibodies (MOPC-21 or EM95.3) on the concentration of 10 μg/ml. Cells were lysed in Brij 58 buffer (1% Brij 58, 150 mM NaCl, 20 mM Tris (pH 7.5), 50 mMβ -glycerophosphate) containing protease inhibitors (50 μg/ml aprotinin, 10μg/ml leupeptin, 10μg/ml pepstatin A, 50μg/ml phenylmethylsulfonyl fluoride (PMSF)) and phophatase inhibitor (2 mM $Na_3VO_4$) for 30 minutes on ice. Brij 58-soluble fraction was separated by centrifugation and Brij 58-insoluble pellets were resuspended with sodium dodecyl sulfate (SDS) containing buffer (0.5 % SDS, 1% β-mercaptoethanol, 1% Brij 58, 150 mM NaCl, 20 mM Tris (pH 7.5), 50 mM β-glycerophosphate) followed by brief sonication (Branson Ultrasonics, Dansbury, CT). Brij 58-soluble and -insoluble fractions were separated by SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF (Immobilon-P, Millipore, Bedford, MA)). HRP labeled Ab bands were detected using chemiluminescent detection reagent (Pierce, Rockford, IL).

*Immunoprecipitation.* M12.4.1 or transfected cells ($2\times10^7$) were stimulated by mixing hCD154 (CD40L) expressed *Sf*9 cells or vector transfected *Sf*9 cells ($5\times10^6$) for 10 minutes at 37°C. Cells were lysed with 1% Brij 58 buffer for 30 minutes on ice. Brij 58-insoluble fraction was separated by centrigugation. To dissolve the Brij 58-insoluble fraction, octylglucopyranoside buffer (60 mM octylglucopyranoside, 150 mM NaCl, 20 mM Tris (pH 7.5), 50 mM β-glycerophosphate, 1% Triton X-100, 0.1% SDS) supplemented with protease inhibitors and phosphatase inhibitor, were added onto the pellet and sonicated briefly on ice. After centrifugation, anti-hCD40 Ab (G28.5, 2μg/ml) and 20μl of protein G agarose beads (Sigma) were added to each of the lysates and incubated on rotator for 2 hours at 4°C. Then protein G agarose beads were washed 4 times with lysis buffer and binding proteins on beads were released by adding 2×SDS-PAGE buffer (62.5 mM Tris (pH 6.8), 1% SDS, 15% glycine, 2% β-mercaptoethanol, 0.28% bromophenol blue). Samples were separated on SDS-PAGE and transferred onto PVDF membrane for immunoblot.

*TRAF Degradation assays.* M12.4.1 or transfected cells ($1\times10^6$) were stimulated with anti-hCD40 and/or anti-mCD40 (10μg/ml) for the time course at 37°C. After brief spin, 200μl of 2×SDS-PAGE buffer was added to the cell pellet and sonicated to shear the DNA. Samples were loaded and separated on SDS-PAGE and transferred onto PVDF for blotting. For

anti-actin blot, PVDF membrane was stripped by incubation in strip buffer (62.5 mM Tris (pH 6.8), 2% SDS, and 0.4% β-mercaptoethanol) for 30 minutes at 50°C rotator. PVDF membrane was re-blocked with 5% skim milk/Tris buffered saline/0.05% Tween 20 solution for actin blot. Band densities of the blot were measured by lumimager (LAS-1000, Fujifilm).

*JNK activation.* Total cell lysates were prepared in same manner as in TRAF degradation assay. To decrease the background JNK stimulation, cells were always kept at 37°C during sample preparation. Total cell lysates were separated in SDS-PAGE and transferred to PVDF for anti-phospho-JNK blotting. PVDF membrane was stripped and re-blotted with anti-JNK Ab.

*NF-κB luciferase assay.* Cells ($4\times10^7$) were transfected with a total of 80μg of DNA (76μg of reporter plasmid, 4μg of pRL-Renilla) by electroporation at 200 V for 50 ms in an electroporator (BTX, San Diego, CA). After electroporation, cells were transferred to 30 ml of BCM-20 and incubated at 37°C for 6 h. Cells were washed once in RPMI, and cell viability was determined by trypan blue exclusion. Cells were stimulated as described below (luciferase assay conditions). Viable B cells ($5\times10^5$) were resuspended in 2 ml of BCM-10 with various stimuli in triplicate wells of 24-well tissue culture plates and incubated for 24 h at 37°C. Cells were harvested by centrifugation, and cell pellets were lysed in 100μl of passive lysis buffer (Promega) and stored at -20°C until use. Forty microliters of cell lysate was assayed for both firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, and relative light units (RLU) were measured on a luminometer. RLU from the firefly luciferase was normalized for transfection efficiency to the Renilla luciferase RLU in each lysate (normalized RLU = $RLU_{\text{firefly luciferase}}$/ $RLU_{\text{Renilla luciferase}}$).

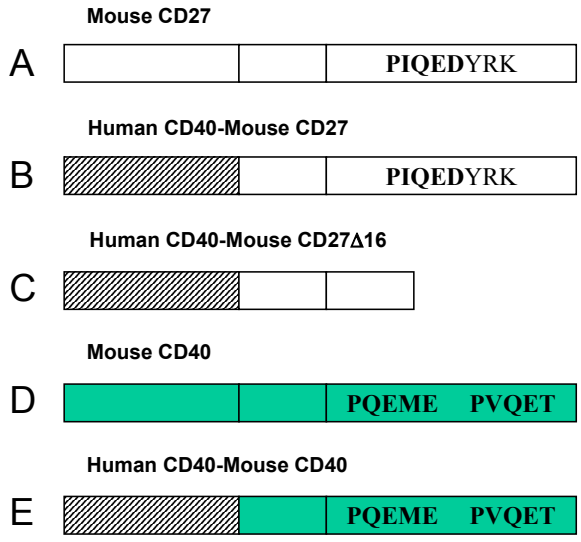


**Figure 1.** Schematic presentation of CD27, CD40, and hybrid molecules. Extracellular domain, transmembrane domain, and cytoplasmic tail are represented as boxes for each constructs. TRAF2 and TRAF5 binding motif (PIQED) is bold-lettered in mCD27 sequence (A and B). Truncated mutant of mCD27 (human CD40-mouse CD27Δ16) is lack of TRAF2/5 binding motif and NF-kB activation sequence, PIQEDYR (C). Mouse CD27 originated sequences, including transmembrane domain and cytoplasmic tail, are depicted as clear boxes in (B) and (C). In mouse CD40 sequence, both TRAF6 binding motif (PQEME) and TRAF2/3/5 binding motif (PVQET) are marked (D and E). Human CD40 extracellular domain is depicted as stripped box (B, C and E).

## Results

*Cells transfected with hCD40-mCD27 and hCD40- mCD 27Δ16.* Because CD70, which is the ligand for CD27, is constitutively expressed on B cells, selective stimulation of CD27 on B cells was needed to observe the effect of CD27 signaling pathway. To investigate the CD27 signaling on B cells, hCD40 extracellular domain and transmembrane-cytoplasmic domain mCD27 molecule as well as distal 16 amino acid deletion mutant (Δ16) hybrid molecules were designed (Fig. 1) to exclude endogenous stimulation of CD27. The PIQEDYR motif of CD27 is required for NF-κB activation, and PIQED is required to bind TRAF2, TRAF3, and TRAF5 (16), which was deleted in Δ16 mutant. Stably transfected M12.4.1 and CH12.LX cells kept high level of expression of hCD40-mCD27 or hCD40-mCD27Δ16 molecules in compared to the parental cell lines (Fig. 2). For all assays in this study, G418 (400μg/ml) was always included for selection.

*Expression of B cell activation markers increased in CD27 stimulation.* To see the surface molecule LFA-1 (CD11a-CD18), CD23, ICAM-1 (CD54), and B7.1 (CD80) expression on B cells after CD27 stimulation, M12.4.1, hCD40-mCD27 and hCD40-mCD27Δ16 transfected cells were stimulated with anti-hCD40 Ab and anti-mCD40 Ab for 72 hours. For comparison with endogenous CD40 signaling, anti-mCD40 Ab (1C10) was added to stimulate mouse CD40 molecules on M12.4.1 cells. Because CH12.LX cells had higher background expression of these surface molecules, M12.4.1 cells were used for this assay. In M12.4.1 cells, mCD40 stimulation induced the increased expression of CD80, CD11a, CD23, CD54, and CD70 (Fig. 3) but anti-hCD40 Ab, or isotype Abs did not induce surface molecule expression. Surface molecule expression was up-regulated by anti-hCD40 Ab in hCD40-mCD27 transfected cells, but not in hCD40-mCD27Δ16 transfected cells. However, CD27 stimulation though anti-hCD40 Ab, and

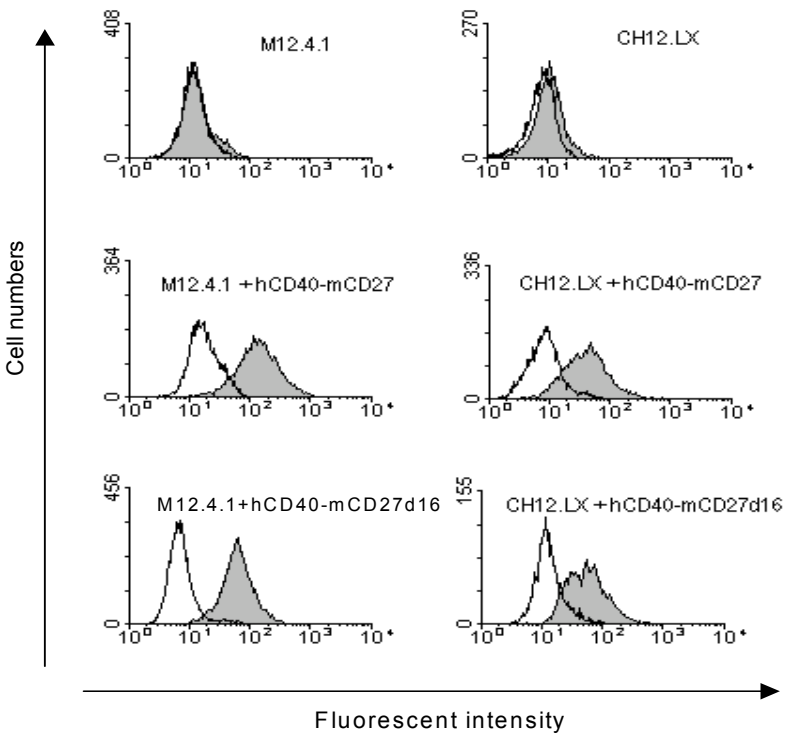


**Figure 2.** Expression of transfected hCD 40-mCD27 and hCD40-mCD27d16 (Δ16) constructs in M12.4.1 and CH12.LX cells. Cells were stained with FITC-labeled anti-hCD40 Ab (G28.5, gray-shadowed) or isotype Ab (MOPC-21, solid line) and were analyzed by flow cytometer. The expression levels of hCD40- mCD27 or hCD40-mCD27d16 were all analyzed all in transfectants used in this study.

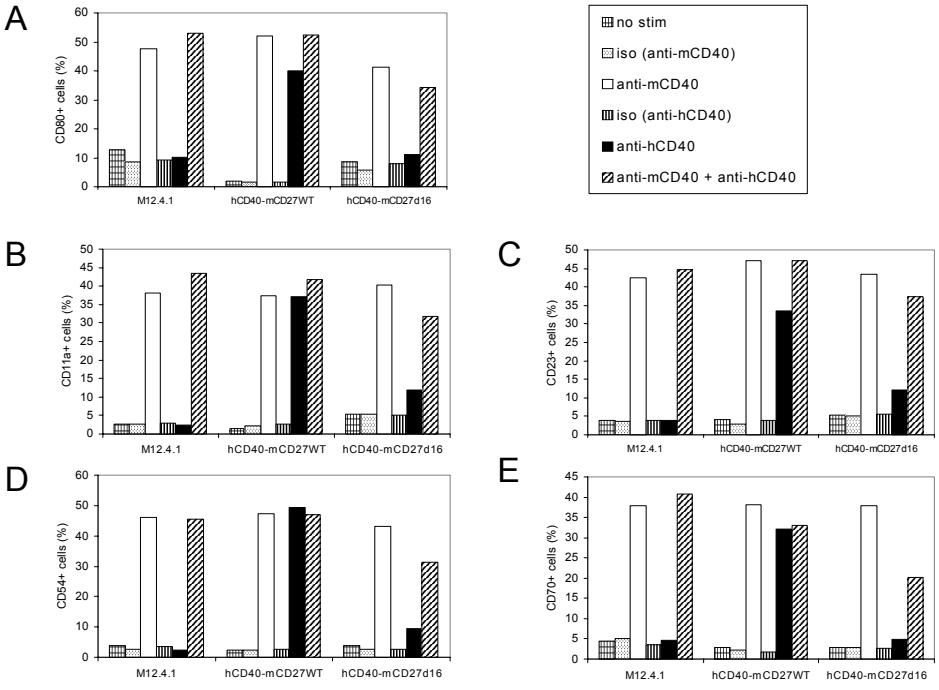


**Figure 3.** CD27 stimulation upregulates surface molecule expression. M12.4.1 cells, transfected hCD40-mCD27 and hCD 40-mCD 27 d16 cells were stimulated with anti-mCD40 Ab (4 mg/ml) or anti-hCD40 Ab (4 mg/ml), including both isotype Abs for 72 hours. Cells were stained with FITC labeled anti-CD80 (A), -CD11a (B), -CD23 (C), CD54 (D), and -CD 70 (E) Abs. The result was the representative of two different subclones (#1 and #2) and two experiments.

CD40 stimulation via anti-mCD40 Ab did not show any additive effect on surface molecule expression in hCD40-mCD27 transfected M12.4.1 cells.

*IgM secretion.* CH12.LX cells, expressing surface IgM and IgD specific to phosphatidylcholine on sheep RBC, were used for the IgM secretion assay in CD27

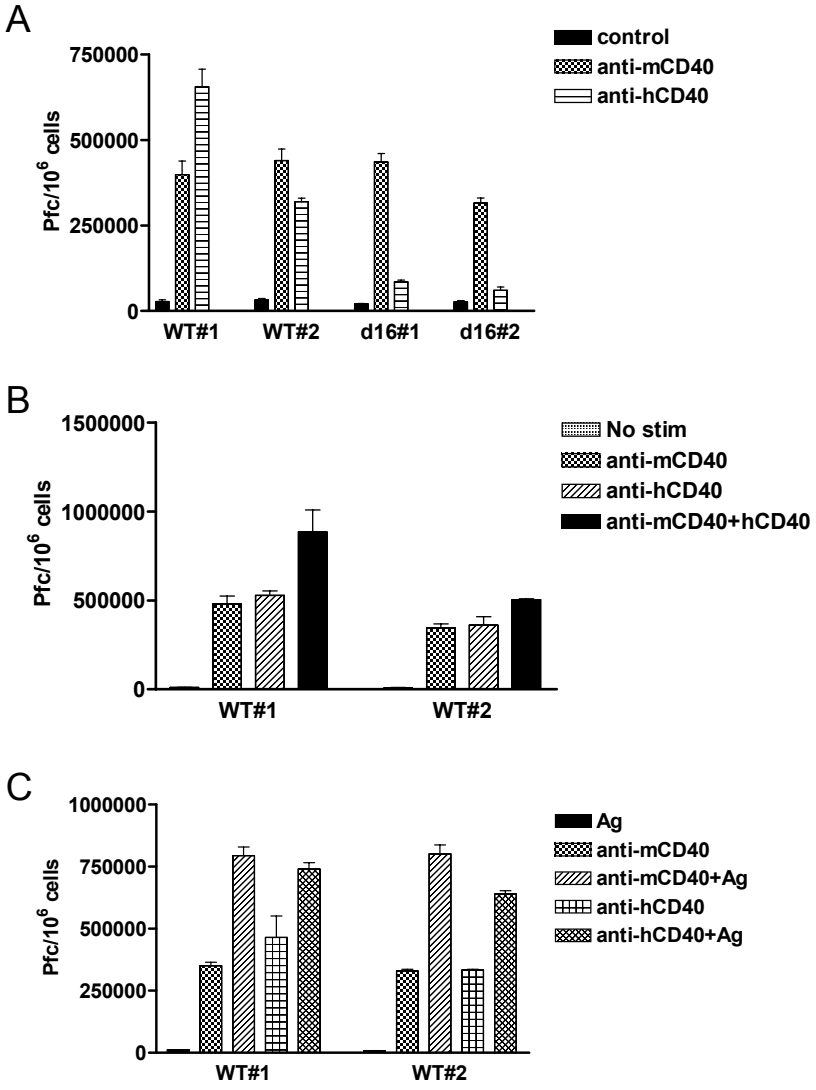


**Figure 4.** Induction of IgM secretion by mCD 40 and hCD40-mCD27. (A) hCD40-mCD27 transfected CH12.LX cells (wild type cytoplasmic tail of mCD27, WT) and hCD40-mCD27d16 transfected CH12.LX cells (Δ16 deleted cytoplasmic tail of mCD27, d16) were stimulated with anti-mCD40 Ab, anti-hCD40 Ab, or isotype Abs. (B) CD27 and CD40 stimulation was induced with adding anti-mCD40 Ab and anti-hCD40 Ab. (C) Transfectants were stimulated with or without antigen (sheep RBC) and anti-mCD40 or anti-hCD40 Abs. Plaque forming cells were counted on sRBC plate. Values represent mean±SD of replicate samples and are representative of four sets of transfected subclones.

or CD40 stimulation. On CD27 stimulation, the number of IgM secreting CH12.LX cells was increased in hCD40-mCD27 transfectant (Fig. 4, A, WT#1 and WT#2), but not in mCD40Δ16 transfectant (Fig. 4, A, d16#1 and d16#2). When the CD40 and CD27 stimulation was delivered simultaneously, additive effect was not shown (Fig. 4, B). Like CD40 stimulation with antigen on CH12.LX cells, CD27 and antigen stimulation induced more of IgM secreting cells (Fig. 4, C) than CD27 stimulation alone.

*TRAF2 and TRAF3 recruitment to Brij 58-insoluble fraction after CD27 stimulation.* To investigate the movement of CD27 molecule and binding TRAFs to lipid raft fraction after CD27 stimulation, M12.4.1 cells and hCD40-mCD27 transfected M12.4.1 cells were stimulated with anti-hCD40 Ab (CD27 stimulation) and/or anti-mCD40 Ab (CD40 stimulation). Cells were lysed in 1% Brij 58 buffer and soluble/insoluble fractions were separated. Brij 58-insoluble fraction contained lipid raft fraction (24) and CD40 was reported to move into raft fraction with TRAF2 and TRAF3 in B cells (25). In M12.4.1 cells, only after mCD40 stimulation TRAF2, and TRAF3 recruitment into Brij 58-insoluble fraction was detected on the immunoblot (Fig. 5, A). In hCD40-mCD27 transfected cells, TRAF2, TRAF3 and hCD40-mCD27 were shifted into Brij 58-insoluble fraction (Fig. 5, B).

*TRAF2 and TRAF3 bind to cytoplasmic tail of CD27.* To confirm TRAF binding to CD27 cytoplasmic tail, co-immunoprecipitation was performed. M12.4.1 cells, hCD40-mCD27 and hCD40-mCD27Δ16 transfected M12.4.1 cells were stimulated with human CD154 (CD40L) expressed Sf9 cells or vector transfected Sf9 cells for 10 minutes. Cells lysed in Brij 58 buffer and insoluble fraction was separated for immunoprecipitation. TRAF2, and TRAF3 bound to CD27 tail in Brij 58 insoluble lysate. TRAF1 and TRAF6 did not bind to CD27 tail in Brij 58 insoluble lysate.

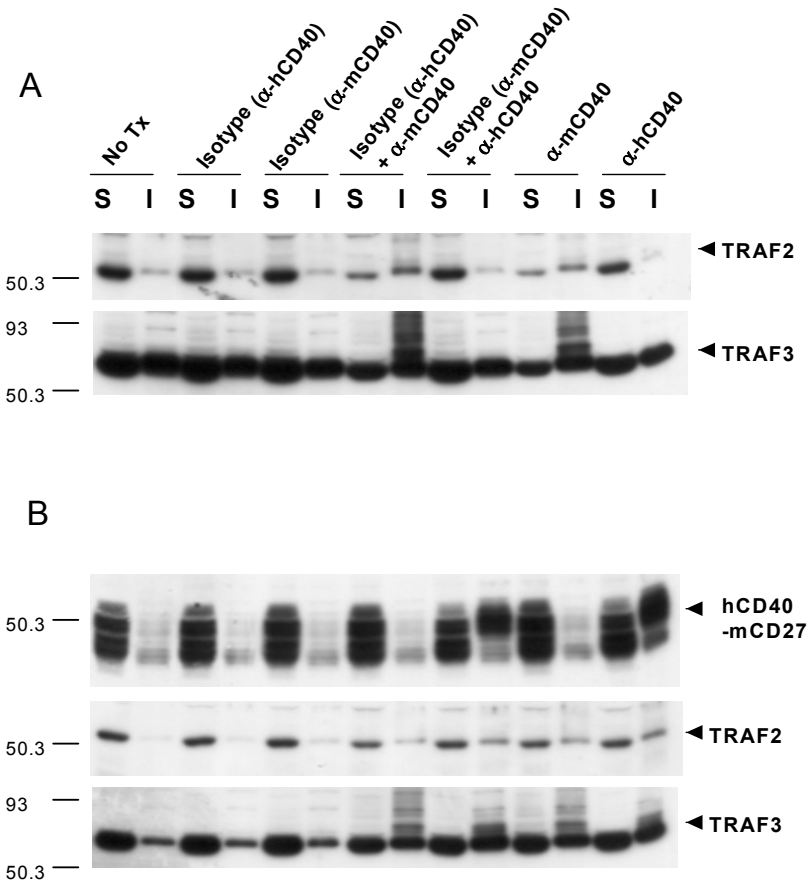


**Figure 5.** TRAF recruitment into Brij 58- insoluble fraction by mCD40 or hCD40-mCD27. M12.4.1 cells (A) and hCD40- mCD27 transfected M12.4.1 cells (B) were stimulated for 10 minutes with anti-mCD40 or anti-hCD40 Abs. Cells were lysed in 1% Brij 58 buffer and Brij-soluble (S)/insoluble (I) fractions were separated as described in Materials and Methods. Cell lysates were separated by SDS-PAGE, transferred, and analyzed by immunoblotting. Both TRAF2 and TRAF3 were moved into Brij 58-insoluble fraction after endogenous mCD40 stimulation (A, lane 8 and lane 12) or after hCD40-mCD27 stimulation (B, lane 8, 10, 12, and 14). Anti-hCD40 polyclonal Ab was used for detection of hCD40-mCD27 hybrid protein (B, lane 1, 3, 5, 7, 9, 10, 11, 13, and 14). Endogenous mCD40 expression was not shown here.

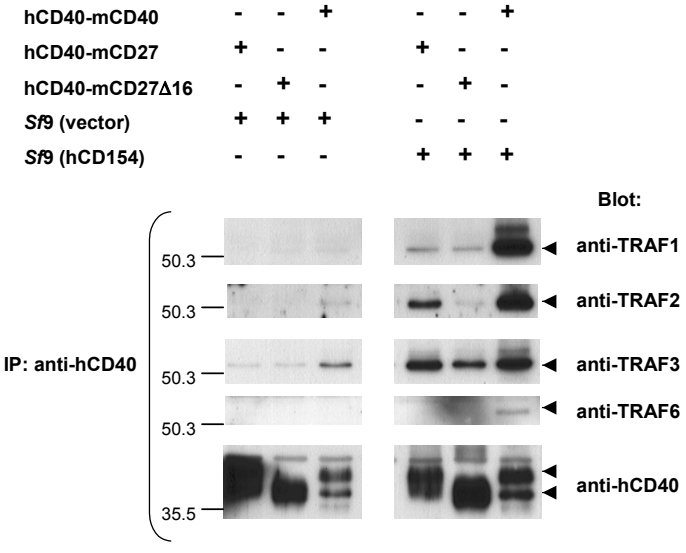


**Figure 6.** CD27 binds to TRAF2 and TRAF3. M12.4.1 cells stably transfected with hCD40-mCD40, hCD40-mCD27, or hCD40-mCD27d16 were stimulated hCD154 expressed S*f*9 cells by inducing cross-linking of extracellular domain of hCD40 (lane 4, 5, and 6), or vector transfected S*f*9 cells as a negative control (lane 1, 2 and, 3) at 37°C for 10 minutes. Cells were lysed with 1% Brij 58 buffer and Brij-insoluble fraction was isolated for immunoprecipitation as described in Materials and Methods. Binding of TRAFs to cytoplasmic tail of mCD40 or mCD27 were analysed by immunoblotting.

Though TRAF3 also bound to truncated mutants of mCD27 tail, the interaction was weaker than to the mCD27 tail (Fig. 6). Human CD40-mCD40 transfectant was used as a positive control for TRAF1, 2, 3 and 6 binding in B cells.

*TRAF2 and TRAF3 degradation.* To detect TRAF2 and TRAF3 degradation on B cells after CD27 or CD40 stimulation, M12.4.1 cells and hCD40-mCD27 trans-

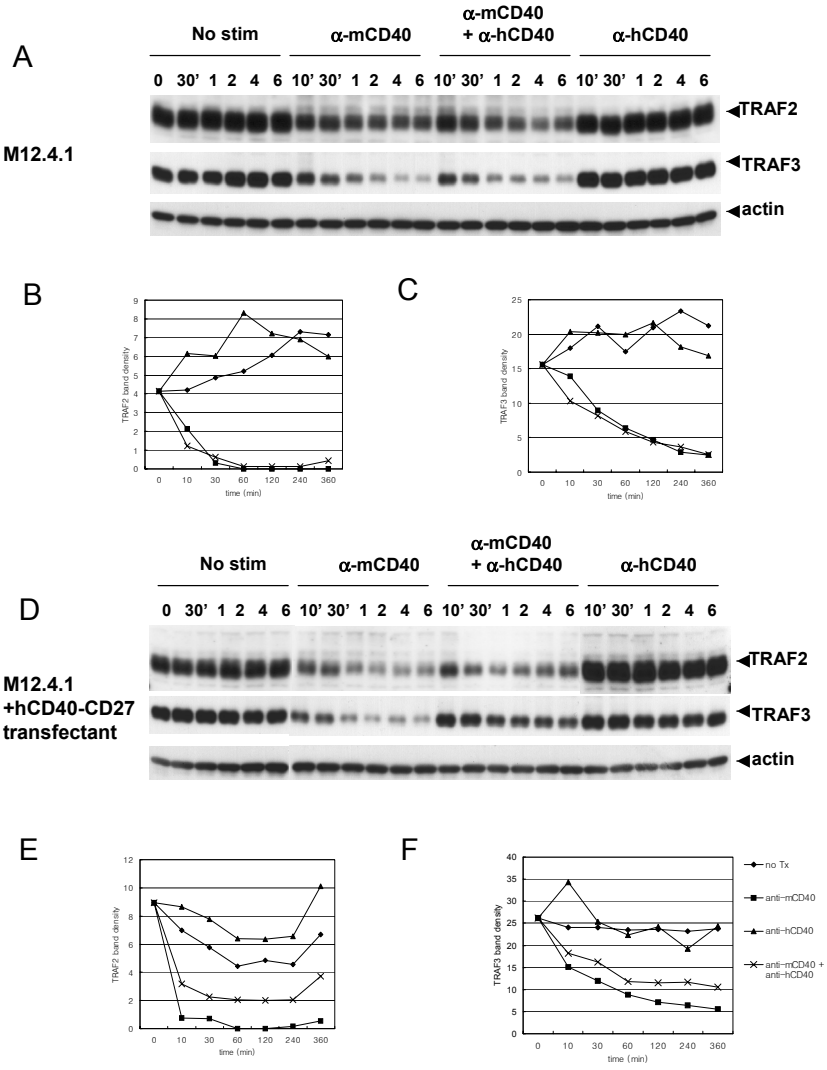


**Figure 7.** TRAF2 and TRAF3 degradation in M12.4.1, and hCD40 -mCD27 transfected M12.4.1 cells. M12.4.1 cells (A, B, and C) and hCD40-mCD27 transfected M12.4.1 cells (D, E, and F)($1\times10^6$ cells) were stimulated with anti-mCD40 and/or anti-hCD40 Abs for the indicated number of minutes or hours (0 min, 10 min, 30 min, 1 hr, 2 hr, 4 hr, and 6 hr). Total cell lysates were prepared as described in Materials and Methods. Bands densities of TRAF2 and TRAF3 were corrected by actin band density (corrected band density = band density of TRAF2 or TRAF3 ×100 / actin band density) for each lane. Plots for TRAF2 (B and E) and TRAF3 (C and F) were the representatives of the degradation result from two different sets of subclones and two experiments.

fectant were stimulated with antibodies and total cell lysated were prepared for immunoblotting. In M12.4.1 cells, CD40 stimulation induced TRAF2 and TRAF3 degradation (Fig. 7, A, B, and C). In hCD40-mCD27 transfected cells, stimulation of CD27 (by anti-hCD 40 Ab) did not induce degradation of TRAF2 or TRAF3 (Fig. 7, D, E, and F). Moreover, CD27 ligation mitigated the TRAF2 and TRAF3 degradation induced by CD40 ligation (Fig. 7, E and F).

*JNK activation.* CD40 stimulation induced strong activation of JNK after 5 or 10 minutes in M12.4.1 cells, and transfectants (Fig. 8, anti-mCD40 lanes). CD27 stimulation also induced activation of JNK in hCD 40-mCD27 transfectant, but not in Δ16 mutant (Fig. 8, anti-hCD40 lanes). In anti-mCD40 and anti-hCD 40 stimulation, JNK activation seemed to sustain even in 30 minutes after the stimulation (Fig. 8, B, anti-mCD40+anti-hCD40 lanes).

*NF-κB luciferase assay.* CD40 stimulation induced strong activation of NF-κB after 10 minutes in M12.4.1 cells, CH12.LX cells and their transfectants (Fig. 9). CD27 stimulation also induced activation of NF-κB in hCD40-mCD27 transfectant, but not as much as in endogenous CD40 stimulation. In Δ16 mutants, NF-κB was not activated. The level of NF-κB activation showed not additive effect in anti-mCD40 and anti-hCD40 stimulation.

## Discussion

B cells were activated via signals from surface Ig receptors, cytokine receptors, and most importantly, surface molecules including CD40 and CD27 on B cell surfaces. CD40 and CD27 are both TNFR family and share similar TRAF binding motif in their cytoplasmic tail. It implies the possible sharing signaling event with CD40 and CD27 or the eventual immune responses in B cells. In CD40 stimulation, signaling was delivered through TRAF molecules but in CD27

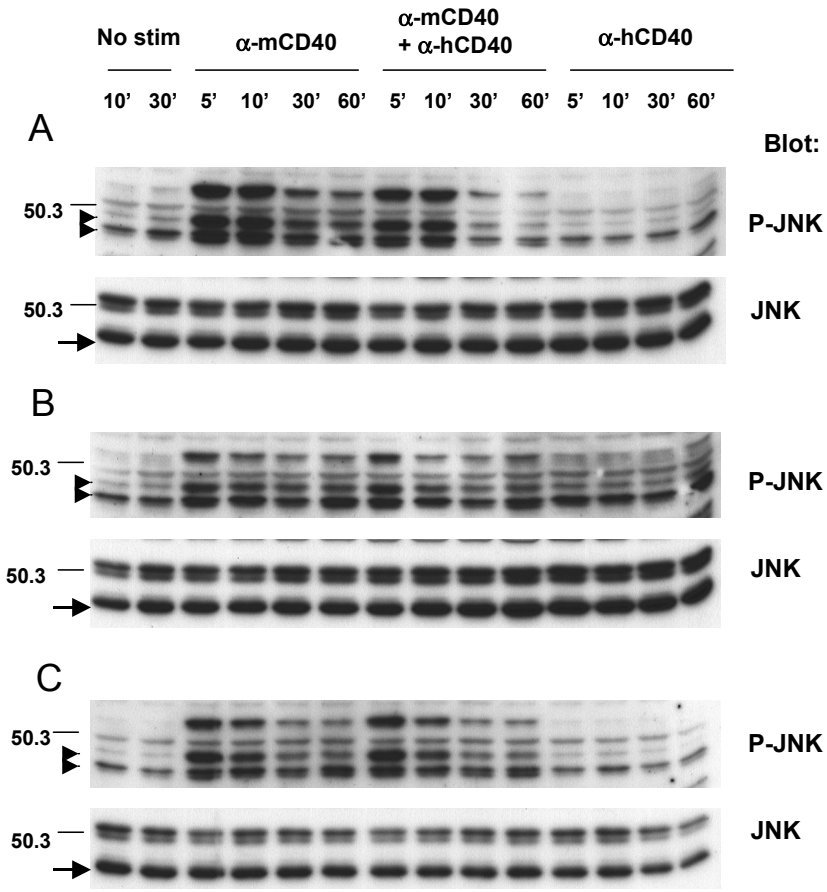


**Figure 8.** JNK activation. M12.4.1 cells (A), hCD40-mCD27 transfected cells (B), and hCD40-mCD27d16 transfected cells (C) were stimulated with anti-mCD40 Ab and/or anti-hCD40 Ab for marked time period (0 min, 5 min, 10 min, 30 min and 60 min). Total cells lysates were prepared as in degradation assay. Phospho-JNK bands were indicated by arrowheads. Total JNK bands were indicated by arrows.

stimulation, however, role of TRAF molecules was not established yet.

CD27, which belongs to the TNF receptor family, have been known to as a general marker for somatically mutated memory B cells (12). CD27 is a type I glycoprotein expressed on T and B cells and B cell maliganancies. The interaction between CD27 and its ligand, CD70, augments IgE secretion by promoting the differentiation of memory B cells into plasma cells (17). Because of induction of IgE secretion requires IL-4 and CD40 stimulation, CD27 might have similar effect on B cells through CD40.

To investigate the role of CD27 and CD40 stimulation on B cells, mouse B cell line M12.4.1 and CH12.LX cells were used. Because both cell lines constitutively express CD70 ligands on their cell surface, hybrid molecules were needed for the selective stimulation of CD40 or CD27. Cells were transfected with hybrid construct of human CD40 extracellular domain containing cytoplasmic mCD27 tails. Using hCD40-mCD27 or hCD40-mCD27Δ16 hybrids constructs transfected mouse B cell lines, CD27 mediated signaling event and possible CD27 binding molecules in the mouse B cell were investigated.

Expressions of LFA-1, ICAM-1, CD23, and CD80 were up-regulated after CD27 stimulation via anti-hCD40 Ab ligation on hCD40-mCD27 transfected M12.4.1 cells but no additive up-regulation effect on simultaneous CD40 stimulation was observed in surface molecule expression. In truncated mutant, mCD27Δ16, surface molecule up-regulation was not observed after anti-hCD40 Ab ligation and it did not affect up-regulation of the surface molecules in mCD40 stimulation (Fig. 3). Because immunoprecipitation result showed that mCD27 bound to TRAF2 and TRAF3 and CD27Δ16 tail bound only to TRAF3 (Fig. 6), it implied that deletion of TRAF2 and TRAF5 binding site or NF-κB activation sequence (Fig. 1) of the CD27 was sufficient to abolish the surface molecule up-regulation, or TRAF3 binding to CD27Δ16 is necessary to down-regulate expression of B cell activation markers. This result from truncation mutant has some consistency with the previous CD40 mutant study, in which showed that modified TRAF2/3 binding site of CD40 molecules abrogated up-regulation of CD23 and ICAM-1 expression (26).

As TRAF3 was reported to block CD40-mediated NF-κB activation in epithelial cells (27), and also block the antibody secretion in B cells (28), TRAF3

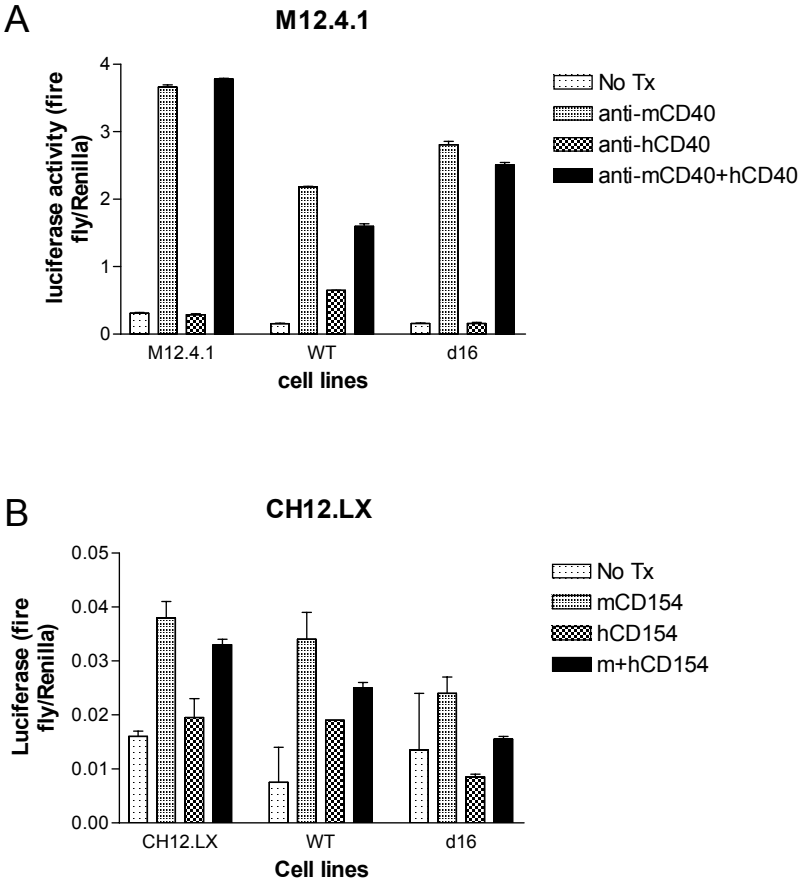


**Figure 9.** NF-κB luciferase assay. (A) M12. 4.1 cells, hCD40-mCD27 transfected cells (wild type cytoplasmic tail of mCD27, WT) and hCD40-mCD27 d16 transfected M12.4.1 cells (Δ16 deleted cytoplasmic tail of mCD 27, d16) were stimulated with anti-mCD40 Ab for endogenous mouse CD40 stimulation, or with anti-hCD40 Ab for mouse CD27 or mouse CD27d16 mediated signaling. (B) CH12.LX cells, hCD40- mCD27 transfected cells, and hCD40- mCD27d16 transfected cells were stimulated with mCD154 transfected S*f*9 cells for endogenous mouse CD40 stimulation, or with hCD154 transfected S*f*9 cells for mouse CD27 or mouse CD27d16 mediated signaling. After transfection, viable B cells ($5\times10^5$) were stimulated transfected Sf9 cells ($1\times10^5$) for 24 h at 37°C. RLU from the firefly luciferase was normalized for transfection efficiency to the Renilla luciferase RLU in each lysate (normalized RLU = $RLU_{\text{firefly luciferase}}/RLU_{\text{Renilla luciferase}}$).

binding CD27Δ16 mutant seemed to have negative effect in B cells. In contrast to the CD27 stimulation induced IgM secretion as in case of CD40 stimulation (Fig. 4), CD27Δ16 inhibit the IgM secretion in transfected CH12.LX cells. This result was similar to the result using dominant negative TRAF2 transfectant, in which TRAF3 had a negative effect on IgM secretion and positive effects of TRAF2 (28).

TRAF2, TRAF3, and CD27 were recruited into lipid rafts after CD27 stimulation as well as in case of CD40 stimulation (Fig. 5). TRAF 2/3 association with CD27 in lipid rafts, and immunoprecipitation result showed that TRAF2, and TRAF3 bound to CD27 but not TRAF1 or TRAF6. Truncated CD27 (Δ16), which was reported not to bind TRAF2 or TRAF5, bound to TRAF3 (Fig. 6). Because both CD40 and CD27 bind to TRAF2 and TRAF3, and use these TRAFs for signaling, CD27 and CD40 stimulation might have similar effect on the cells after stimulation through each receptor in B cell. However, this study showed that CD27 and CD40 signaling effect were different in TRAF2/3 degradation profiles in mouse B cell lines. CD27 stimulation alone did not induce TRAF2/3 degradation (Fig. 7). TRAF2 and TRAF3 are degraded after CD40 stimulation but not in CD27 stimulation. TRAF2 and TRAF3 degradation profile in CD27Δ16 mutants was almost same in M12.4.1 cells (data not shown), in which TRAF2 and TRAF3 only got degraded after anti-mCD40 stimulation and did not show similar blocking effect on TRAF2 or TRAF3 degradation induced by anti-mCD40 Ab. So TRAF3 binding itself did not seem to affect TRAF3 degradation in CD40 and CD27 mediated signaling. On CD40-mediated signaling, TRAF2 degradation requires intact RING domain of TRAF2 and mediated by ubiquitination process (29) but TRAFs degradation process was not much known in TNFR signaling pathway.

CD27 also activate JNK and NF-κB, but it was not confirmed that CD27 signals might amplify or prolong signals evoked by CD40 stimulation on B cells (Fig. 8 and 9). However, it can be postulated that like in latent membrane protein 1 (LMP-1), which plays a critical role in B cell transformation by Epstein-Barr virus (EBV) and appears to mimic a constitutively active CD40, induced signaling in B cells, failure to degradation of TRAFs may attribute to enhancing CD40 signaling process (30).

Because CD27 and CD40 signaling effect was never tested in vivo, it would be interesting to show

CD27 distribution in CD40 transgenic mouse, in which CD40 only can be expressed on B cells. Moreover, simultaneous CD40 and CD27 stimulation effects in B cells should be tested using natural ligand of CD40 or CD27 for endogenous signaling event. In addition, CD27 signaling effect should be investigated in the settings of TRAF3-/- cells to clear the TRAF3 role in CD27 signaling in B cells.

In summary, CD27 signaling is different from CD40 signaling via TRAF2/3 degradation, as well as TRAF3 binding site. Inhibition of TRAF2/3 degradation in CD40 with CD27 stimulation may play a role of enhancing or prolonging CD40 mediated signal or taking over the B cell activation signal from CD40 to CD27. Unlike to CD40, CD27 binds to TRAF2 and TRAF3 on different sequence and that may explain the different signaling effect through CD27 and CD40.

## References


1. Locksley RM, Killeen N, Lenardo MJ: The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 104;487-501, 2001
2. Brink R, Lodish HF: Tumor necrosis factor receptor (TNFR)-associated factor 2A (TRAF2A), a TRAF2 splice variant with an extended RING finger domain that inhibits TNFR2- mediated NF-kappaB activation. J Biol Chem 273;4129-4134, 1998
3. Bradley JR, Pober JS: Tumor necrosis factor receptor- associated factors (TRAFs). Oncogene 20;6482-6491, 2001
4. Calderhead DM, Kosaka Y, Manning EM, Noelle RJ: CD40-CD154 interactions in B-cell signaling. Curr Top Microbiol Immunol 245;73-99, 2000
5. Rothe M, Sarma V, Dixit VM, Goeddel DV: TRAF2- mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science 269;1424-1427, 1995
6. Hu HM, O'Rourke K, Boguski MS, Dixit VM: A novel RING finger protein interacts with the cytoplasmic domain of CD40. J Biol Chem 269;30069-30072, 1994
7. Ishida TK, Tojo T, Aoki T, Kobayashi N, Ohishi T, Watanabe T, Yamamoto T, Inoue J: TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. Proc Natl Acad Sci U S A 93;9437-9442, 1996
8. Ishida T, Mizushima S, Azuma S, Kobayashi N, Tojo T, Suzuki K, Aizawa S, Watanabe T, Mosialos G, Kieff E, Yamamoto T, Inoue J: Identification of TRAF6, a novel tumor necrosis factor receptor-associated factor protein that mediates signaling from an amino-terminal domain of the CD40 cytoplasmic region. J Biol Chem 271;28745-29748, 1996
9. Bishop GA, Hostager BS: Molecular mechanisms of CD40 signaling. Arch Immunol Ther Exp 49;129-137, 2001
10. Ahonen C, Manning E, Erickson LD, O'Connor B, Lind EF, Pullen SS, Kehry MR, Noelle RJ: The CD40-TRAF6 axis controls affinity maturation and the generation of long-lived plasma cells. Nat Immunol 3;451-456, 2002
11. van Lier RA, Borst J, Vroom TM, Klein H, Van Mourik P, Zeijlemaker WP, Melief CJ: Tissue distribution and biochemical and functional properties of Tp55 (CD27), a novel T cell differentiation antigen. J Immunol 139;1589-1596, 1987
12. Klein U, Rajewsky K, Kuppers R: Human immunoglobulin (Ig)$M^+IgD^+$ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 188;1679-1689, 1998
13. Zandvoort A, Lodewijk ME, de Boer NK, Dammers PM, Kroese FG, Timens W: CD27 expression in the human splenic marginal zone: the infant marginal zone is populated by naive B cells. Tissue Antigens 58;234-242, 2001
14. Maurer D, Holter W, Majdic O, Fischer GF, Knapp W: CD27 expression by a distinct subpopulation of human B lymphocytes. Eur J Immunol 20;2679-2684, 1990
15. Kobata T, Jacquot S, Kozlowski S, Agematsu K, Schlossman SF, Morimoto C: CD27-CD70 interactions regulate B-cell activation by T cells. Proc Natl Acad Sci U S A 92;11249-11253, 1995
16. Akiba H, Nakano H, Nishinaka S, Shindo M, Kobata T, Atsuta M, Morimoto C, Ware CF, Malinin NL, Wallach D, Yagita H, Okumura K: CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. J Biol Chem 273;13353-13358, 1998
17. Nagumo H, Agematsu K, Shinozaki K, Hokibara S, Ito S, Takamoto M, Nikaido T, Yasui K, Uehara Y, Yachie A, Komiyama A: CD27/CD70 interaction augments IgE secretion by promoting the differentiation of memory B cells into plasma cells. J Immunol 161;6496-6502, 1998
18. Hamano T, Kim KJ, Leiserson WM, Asofsky R: Establishment of B cell hybridomas with B cell surface antigens. J Immunol 129;1403-1406, 1982
19. Bishop GA, Haughton G: Induced differentiation of a transformed clone of $Ly\text{-}1^+$ B cells by clonal T cells and antigen. Proc. Natl. Acad. Sci. (USA) 83;7410-414, 1986
20. Mercolino TJ, Arnold LW, Haughton G: Phosphatidyl choline is recognized by a series of $Ly\text{-}1^+$ murine B cell lymphomas specific for erythrocyte membranes. J Exp Med 163;155- 165, 1986
21. Bishop GA, Warren WD, Berton MT: Signaling via MHC class II molecules and antigen receptors enhances the B cell response to gp39/CD40 ligand. Eur J Immunol 25;1230-1238, 1995
22. Warren WD, Berton MT: Induction of germ-line γ1 and ε Ig gene expression in murine B cells. IL-4 and the CD40L-CD40 interaction provide distinct but synergistic signals. J Immunol 155;5637-5646, 1995
23. Bishop GA: Requirements of class II-mediated B cell differentiation for class II crosslinking and cAMP. J Immunol. 147;1107-1114, 1991
24. Montixi C, Langlet C, Bernard AM, Thimonier J, Dubois C, Wurbel MA, Chauvin JP, Pierres M, He HT: Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains. EMBO J 17;5334-5348, 1998
25. Hostager BS, Catlett IM, Bishop GA: Recruitment of CD40, TRAF2 and TRAF3 to membrane microdomains during CD40 signaling. J Biol Chem 275;15392-15398, 2000
26. Hsing Y, Hostager BS, Bishop GA: Characterization of CD40 signaling determinants regulating NF-κB activation in lymphocytes. J Immunol 159;4898-4906, 1997
27. Rothe M, Sarma V, Dixit VM, Goeddel DV: TRAF2- mediated activation of NF-kB by TNF receptor 2 and CD40. Science 269;1424-1427, 1995
28. Hostager BS, Bishop GA: Cutting Edge: Contrasting roles of TRAF2 and TRAF3 in CD40-mediated B lymphocyte activation. J Immunol 162;6307-6311, 1999
29. Brown KD, Hostager BS, Bishop GA: Regulation of TRAF2

signaling by self-induced degradation. J Biol Chem in press; 2002
30. Brown KD, Hostager BS, Bishop GA: Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). J Exp Med 193;943-954, 2001