梨花醫大誌 : 第 30 卷 第 1 號 2007
Ewha Med J Vol. 30, No. 1, 2007

CD70 Expression on *Spodoptera Frugiperda(Sf9)* Cells by Baculovirus to Induce CD27 Stimulation in Mouse B Cells

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Sf9 세포주를 이용한 CD70 분자의 발현

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목 적 : 종양괴사인자(tumor-necrosis factor)와 그 수용체의 결합은 여러 종류의 세포에 대해 분화와 세포사멸을 유도하며, 특히 면역세포들의 상호 작용 중 B 림프구와 T 림프구 표면의 종양괴사인자와 수용체의 결합은 다양한 면역반응을 유도한다. 생물학적으로 이상적인 조건으로의 수용체-리간드의 결합을 유도하기 위해 실험적으로 수용체를 자극하는 항체를 사용하거나, 수용체 또는 리간드의 발현을 증가하는 방법을 사용하기도 한다. 그러나 종양괴사인자의 경우는 수용체와 리간드가 각각 삼합체(trimer)를 이루어 결합하기 때문에 항체를 사용하는 것은 효과적인 수용체의 활성을 유도하지 못하는 경우가 많다. 따라서 이 실험에서는 종양괴사인자의 하나인 CD27의 리간드인 CD70을 과발현하여 CD27-CD70의 결합을 유도하고자 하였다.

방 법 : 순수한 리간드만의 발현을 위해 곤충세포인 *Spodoptera frugiperda(Sf9)*세포에 baculovirus를 사용하여 CD70을 클로닝 후 과발현 하였다. 그 후 *Sf9* 세포 표면의 CD70의 발현을 확인하고 마우스 B 세포주인 A20 세포를 사용하여 CD27을 통한 표면항원의 발현 증가를 확인하였다.

결 과 : 마우스 CD70은 baculovirus 벡터를 사용하여 *Sf9* 세포에 감염한 후 96시간 이후에 발현이 나타났으며, A20세포와 CD70을 발현하는 *Sf9*세포의 결합을 통해 CD86, CD14의 증가를 관찰하였다.

결 론 : 종양괴사인자의 수용체-리간드 결합을 유도하기 위해서는 baculovirus를 사용한 Sf9세포의 리간드 발현이 효과적이다.

중심 단어 : 종양괴사인자(TNF) · CD27 · CD70 · *Sf* 9 · Baculovirus.

Introduction

Members of the large and growing tumor-necrosis factor (TNF) receptor (TNFR) superfamily of molecules have a variety of important roles in the regulation of activation and apoptosis of many cell types, but they are particularly important for cells of the immune system[1]. Several family members are expressed on B cells and T cells.

CD40 has important roles in B-cell proliferation and differentiation, immunoglobulin isotype switching, cell-surface molecule upregulation, cytokine production and resistance to apoptosis[2]. B cells also express CD120b (TNFR2), which seems to contribute to the differentiation of B cells and the upregulation of expression of mo-

lecules involved in antigen presentation. CD27 is expressed by a subpopulation of B cells, and it is thought to be a marker of memory cells[3)4)]. Although signals delivered by CD27 overlap with those delivered by CD40, they seem to be most important in the late stages of the humoral immune response. Although ligation of most TNFR-family members has activating effects on B cells, CD30 seems to be a negative regulator of antibody responses, and its expression is increased following signalling through CD40[5)]. B cells also express the pro-apoptotic TNFR-family members CD95 (Fas) and the receptors for TNF-related apoptosis-inducing ligand (TRAIL)[6)]. The most recently described members of the TNFR family that are expressed by B cells are the three distinct receptors for B-cell activating factor (BAFF)-the BAFFR (BAFF receptor), BCMA (B-cell maturation antigen) and TACI (transmembrane activator and calcium-modulating cyclophilin ligand (CAML) interactor)-which transduce signals that regulate B-cell viability and differentiation[7)].

TNFR-TNF interactions lead to the formation of trimers of TNFR on cell surface[8)]. In order to stimulate TNFR molecules in vitro settings, two kinds of tools commonly used are stimulating antibodies or overexpressed TNF proteins on mammalian cells. In the former case, stimulating antibodies, might not deliver physiologic stimulation to TNFR due to lack of formation of trimers. In the latertool, it is suitable to the purpose of formation of TNFR trimer but other unknown expressed proteins on mammalian cells might exert unknown "noise" stimulation through unknown many receptors on cells.

For achievement of selective TNFR-TNF stimulation without "noise" background stimulation, insect cell line based expression system was adopted. In this study, CD 70, which is the ligand of CD27 on B cells and T cells, was expressed on *Spodoptera frugiperda* (*Sf* 9) cells with Baculovirus expression system to induce CD27-CD70 interaction.

Materials and Methods

1. Cells

Mouse B lymphocyte cell line A20 (American Type Culture Collection, TIB-208, BALB/c mouse B cell lymphoma line) were grown in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS ; HyClone, Logan, UT), 10 μM β-mercaptoethanol, and penicillin/streptomycin.

Spodoptera frugiperda (Sf)9 were cultured in Sf-900 II SFM medium (Invitogen, Rockville, MA). Culture media was dispensed in disposable Erlenmeyer flask (500 ml, Corning, Corning, NY) and cultured with constant shaking in orbital shaker (135−150 rpm). Cell density was maintained at the minimum concentration of 3×10^5 cell/ml and incubate cultures at 27±0.5℃ with loose caps. Cryopreservation was at the density of 1×10^7 to 2×10^7cells/ml in cryopreservation medium (100% fresh SFM containing 10% BSA and 7.5% DMSO).

2. Mouse CD70 expressing *Sf* 9 cells

Sf 9 cells expressing mCD70 was made by Bac-to-Bac Baculovirus expression system (Invitrogen). Mouse CD70 mRNA was prepared with Trizol (Invitrogen) from cultured A20 cells. After getting cDNA from mRNA, PCR was done with mCD70F primer (5'-AAA GGA TCC ATG CCG GAG GAA GGT CG-3') and mCD70R primer (5'-AAA AAG CTT TCA AGG GCA TAT CCA CTG AAC-3'). Mouse CD70 insert containing *Bam*HI and *Hind*III cut sites, was cloned into pFastBac1 and insert sequences was confirmed by sequencing analysis (Bioneer, Korea). To generate recombinant bacmid, pFastBac1-mCD70 plasmid was transformed into DH10Bac™, followed by transformants selection on LB agar plate containg kanamycin (50 μg/ml), gentamicin (7 μg/ml), tetracycline (10 μg/ml), 5-bromo-4-chloro-3-indolyl- β-D-galactopyranoside (X-gal, Bioneer, 100 μg/ml), and isopropyl- β-D-thiogalactopyranoside (IPTG, Bioneer, 40 μg/ml). PFastBac1-Gus plasmid was used as a positive control. To verify the insert in the recombinant bacmid in white colonies, PCR was performed with M13 forward primer (5'-GTT TTC CCA GTC ACG AC-3') and M13 reverse primer (5'-CAG GAA ACA GCT ATG AC 3').

For transfecting insect cells, Cellfectin reagent (Invitrogen) was used. After 72 hours after transfection, mouse CD70 expression was confirmed by flow cytometer analysis with PE conjugated anti-mouse CD70 antibody (BD PharMingen, San Diego, CA).

3. Cell stimulation

A20 cells (5×10^6) were stimulated by mixing CD70 expressed *Sf* 9 cells or vector transfected *Sf* 9 cells (5×10^6) for 24 hrs at 37℃. Cells were washed with 0.5% FBS/PBS and stained with FITC-labeled anti-CD80, anti-CD40, and PE-labeled anti-CD27, anti-CD70, anti-CD86 and anti-CD14 Abs and isotype Abs. Cells were analysed by flow cytometer (Becton Dickinson, Mountain View, CA).

Results

1. Cloning of mouse CD70

Before RT-PCR, expression of CD70 on A20 cells was confirmed by flow cytometry analysis (Fig. 1). Insert and vector plasmid were digested with *Bam*HI and *Hind*III (Fig. 2) and ligated. Ligation reaction was transformed into DH5 α and miniprep was done. Extracted

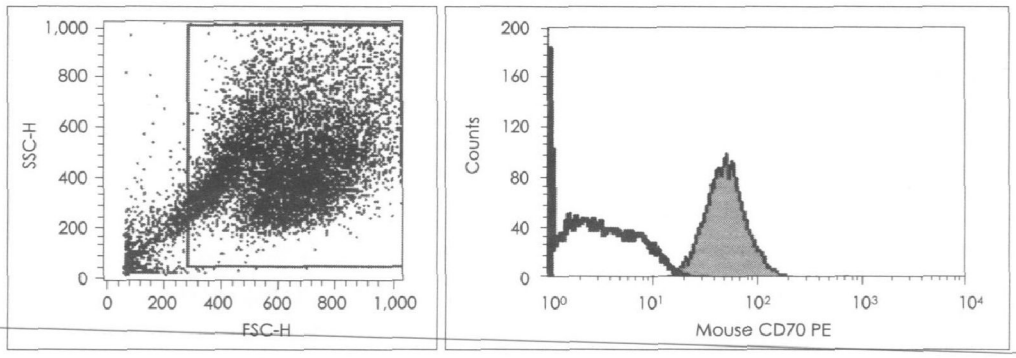

Fig. 1. Flow cytometric analysis of the expression of CD70 on mouse B cell line, A20. CD70 is highly expressed on A20 cells (shaded histogram). Isotype control antibody is shown as thick line.

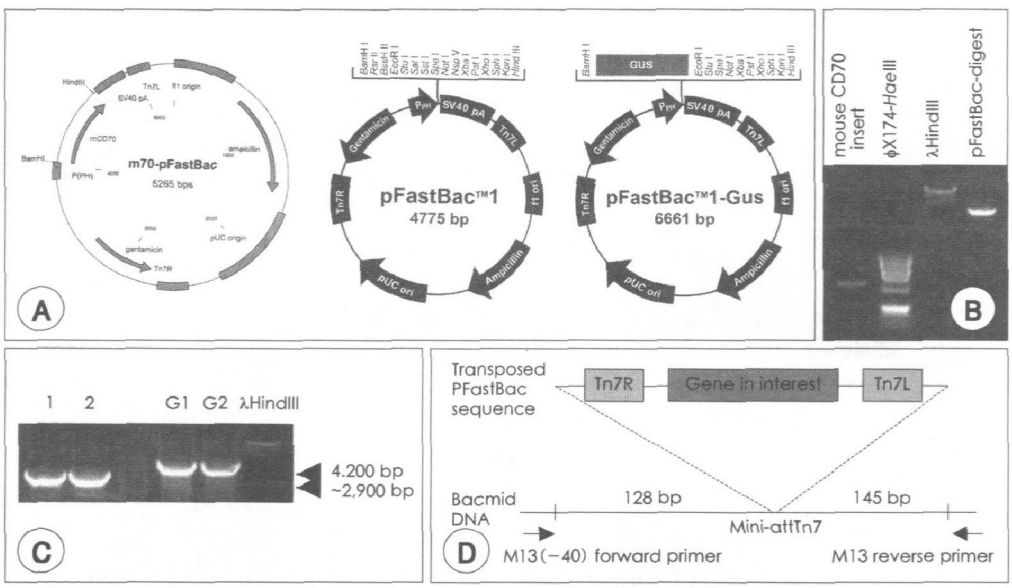

Fig. 2. Vectors and constructs for mouse CD70 cloning. A : pFastBac1 was used for cloning vector, pFastBac-1-Gus construct was for the positive control. Construct of mouse CD70-pFastBac1 is also shown here. B : Insert (mouse CD70) and vector digest are shown. C : Analyzing recombinant Bacmid DNA by PCR. To confirm the inserts from mouse CD70-pFastBac constructs, M13(−40) forward 5'-GTT TTC CCA GTC ACG AC-3' and M13 reverse 5'-CAG GAA ACA GCT ATG AC-3'were used for PCR analysis. Bacmid DNA were isolated and used as templates. The sizes of the PCR products are : Bacmid alone(−300bp), Bacmid transposed with pFastBac1 (−2,300bp+600bp (of mouse CD70), lane 1 and 2), and Bacmid transposed with pFastBac1-Gus (−4,200bp, lane G1 and G2). D : Schematic view of reconbinant bacmid DNA shows the location of M13 primers and gene inserted site.

plamids were sent to sequence analysis (Bioneer, Korea).

2. Transform DH10Bac™ E. coli for transposition into the bacmid

Mouse CD70-FastBac1 construct was transformed to DH10Bac™ *E.coli*. pFastBac-1-Gus, which was a 6,661 bp containing the *Arabidopsis thaliana* gene for β-glucuronidase (Gus), was used as a positive control. Eight of white colonies were selected each and confirmed to contain the recombinant bacmid DNA by PCR (Fig. 2).

3. Transfection

Baculovirus transfected *Sf* 9 cells expressed the m CD70 only after 96 hours (Fig. 3).

4. Interaction with A20 cells

After A20 cells (5×10^6) were stimulated by mixing CD70 expressed *Sf* 9 cells or vector transfected *Sf* 9 cells (5×10^6) for 24 hrs at 37℃, cells were stained with antibodies for CD14, CD27, CD40, CD70, CD80, and CD86 (Fig. 4). CD27 stimulation by mouse CD70 expressed *Sf*9 cells, upregulated CD14 and CD86 expression on A20 cells. Interestingly, levels of CD40 and IgG expression was decreased after CD27 stimulation on A20 cells.

Discussion

In this study, we generated the mouse CD70 expressed *Sf 9* insect cell line, using Baculovirus vector. For the achievement of selective TNFR-TNF stimulation without "noise" background stimulation, insect cell line based expression system was the ideal one. We can apply this insect cell lines to induction of physiologic condition of receptor-ligand interaction between cells.

B lymphocyte can be activated by signals from immunoglobulin (Ig) receptors, cytokines, and cell-to-cell contact. Among them, CD40/CD154 and CD27/CD70 interactions are important for B lymphocyte 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[9]. TNFR-associated factor (TRAF)-linked group includes CD27, CD30, OX-40 (CD134), 4-1BB (CD137), CD40 and

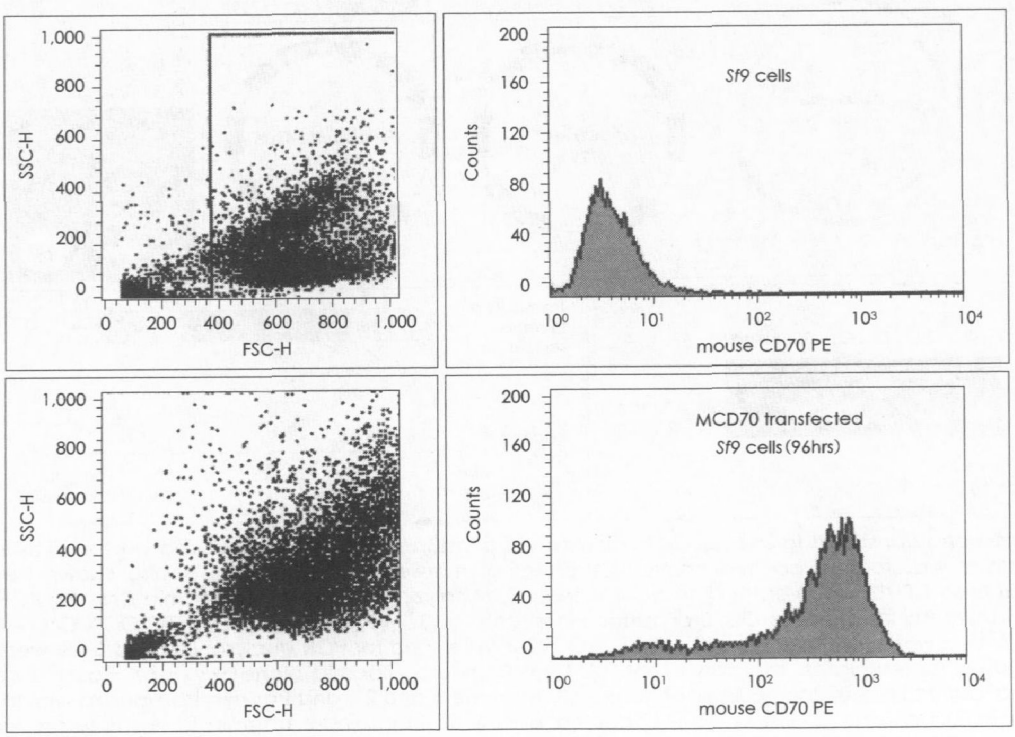

Fig. 3. CD70 expression profile after 96 hours of baculovirus infection. Most of transfected cells express mouse CD 70 on their surface.

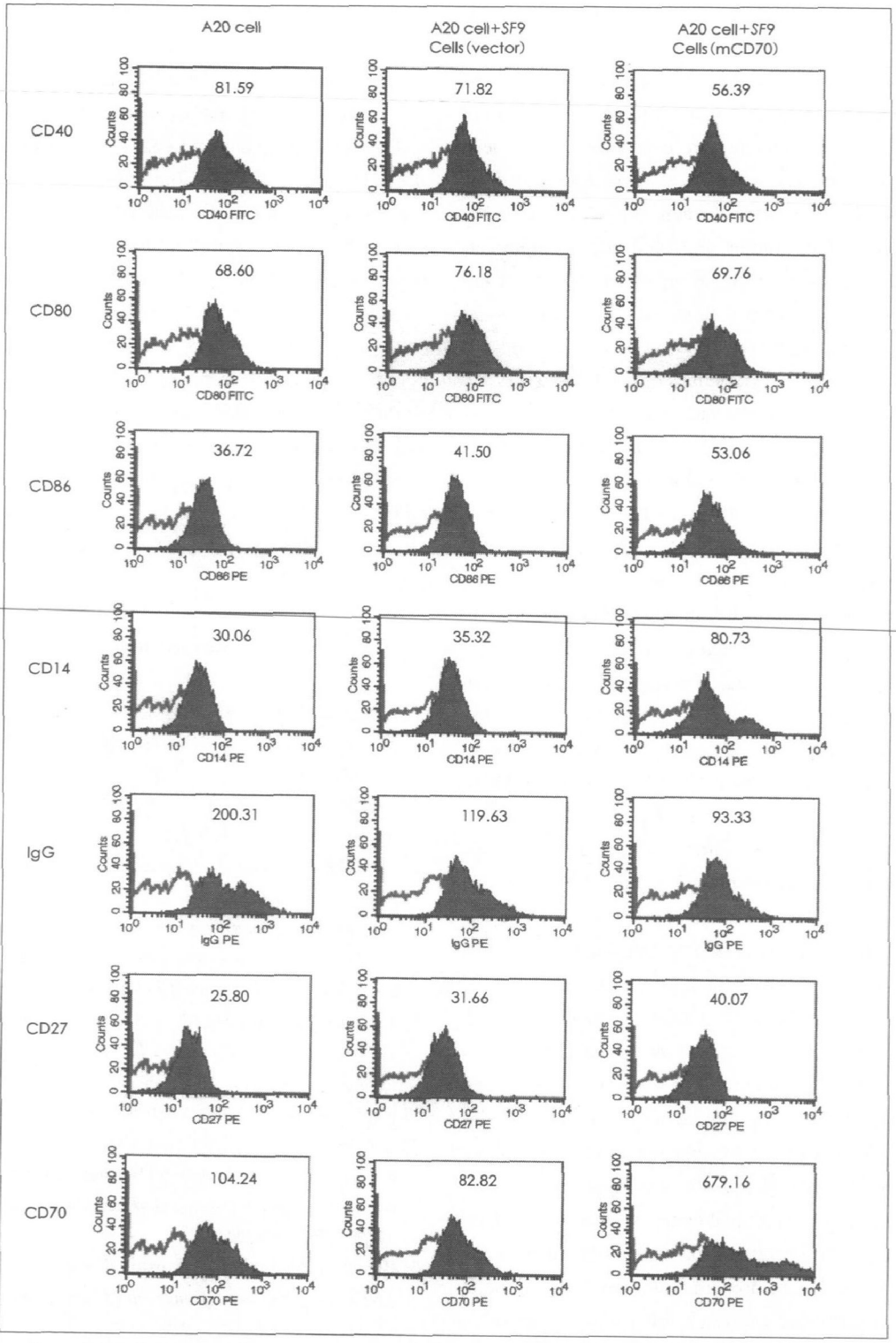

Fig. 4. Expression of CD40, CD80, CD86, CD14, IgG, CD27 and CD70 on A20 cells. Cells were stimulated with CD70 expressed *Sf*9 cells or vector transfected *Sf*9 cells (5×10^6) for 24 hrs at 37℃, and stained with antibodies (shaded histograms). Numbers in the histograms represent mean fluorescent intensity of the binding antibodies. Isotype control antibodies are shown as lines.

RANK. Death domain (DD)-linked group includes CD95, TNFR1, TRAIL, and DR-3.

In mammals, six different TRAFs have been identified[10]. All TRAFs, except TRAF1, have N-terminal zinc RING finger, zinc finger region, coiled-coil, and C-terminal TRAF domain[11]. 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.

CD27 (TNFRSF7) is a 50- to 55kDa type I glycoprotein and is expressed mostly on T lymphocytes, NK cells, adult peripheral blood B lymphocytes (5−14%)[12], tonsilar B lymphocytes, and Ebstein-Barr virus transformed cell lines. CD27 is also known as a memory B lymphocyte marker[13]. CD27 expression is negative on cord blood lymphocytes but CD27+ cells replenish the human splenic marginal zone after 2 years after birth[14]. These CD27+ cells express higher level of LFA-1 (CD11a), ICAM-1 (CD54), LFA-3 (CD58) and CD44[15] than CD 27- cells do. In CD27+ B lymphocytes, CD27/CD70 interaction augments IgM and IgG secretion[16]. Like CD 40 molecules, CD27 activates NF-κB and SAPK/JNK via TRAF2, TRAF5 and NF-κB-inducing kinase (NIK)[17]. CD27/CD70 interaction in B lymphocytes can induce plasma cell differentiation[18]. CD27 is reported to bind TRAF2 and TRAF5 in 293 cells by over-expresion study[17].

CD40 (TNFRSF5) is expressed on the mature B lymphocytes, dendritic cells, monocytes, endothelial cells, epithelial cells, but not on the plasma cells. CD40 plays a major role in B lymphocytes in T lymphocytes dependent class switching, germinal center formation, and memory B lymphocyte proliferation[19]. 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[20-22]. 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[23]. TRAF6 is required for surface molecule up-regulation, IL-6 secretion, isotype switching, and plasma cell differentiation[24].

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 lymphocytes. TRAF degradation induced by CD40 stimulation might be affected by CD27 stimulation in B lymphocytes. 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 lymphocytes as showing similar effect on B lymphocytes. Because in this study, CD27 stimulation down-regulated the CD40 expression on IgG+ A20 cells, it needs to investigate possible interaction or feedback mechanism between these two molecules.

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