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*J Immunol* 2010; 185:6499-6507; Prepublished online 25 October 2010;
doi: 10.4049/jimmunol.0903229
http://www.jimmunol.org/content/185/11/6499

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2010/10/25/jimmunol.0903229.DC1

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Inhibition of Terminal Differentiation of B Cells Mediated by CD27 and CD40 Involves Signaling through JNK

Shuchismita Satpathy,*1,2 Gautam N. Shenoy,*1 Sheetal Kaw,*1 Tushar Vaidya,† Vineeta Bal,*3 Satyajit Rath,*3 and Anna George*3

B cells responding to cognate Ag in vivo undergo clonal expansion that is followed by differentiation into Ab-secreting plasma cells or into quiescent restimulable memory. Both these events occur in the germinal center and require that cells exit from proliferation, but the signals that lead to one or the other of these mutually exclusive differentiation pathways have not been definitively characterized. Previous experiments have shown that signals transduced through the TNFRs CD27 and CD40 at the time of B cell stimulation in vitro or in vivo can influence this cell fate decision by inhibiting terminal differentiation and promoting memory. In this study, we show that the PIQED domain of the cytoplasmic tail of murine CD27 and the adapter molecule TNFR-associated factor 2 are involved in this effect. Using pharmacological inhibitors of signaling intermediates, we identify JNK as being necessary and sufficient for the observed inhibition of terminal differentiation. While JNK is involved downstream of CD40, inhibition of the MEK pathway can also partially restore plasma cell generation, indicating that both signaling intermediates may be involved. We also show that inhibition of induction of IFN regulatory factor 4 and B lymphocyte induced maturation protein 1 are downstream events common to both receptors. The Journal of Immunology, 2010, 185: 6499–6507.

The online version of this article contains supplemental material.

Abbreviations used in this paper:
- AID: Activation-induced deaminase
- AID: Activation-induced deaminase
- BCL-6: B cell lymphoma-6
- BLIMP-1: B lymphocyte induced maturation protein 1
- CD27: CD27 antigen
- CD40: CD40 antigen
- CD44: CD44 antigen
- CXCR4: Chemokine (C-X-C motif) receptor 4
- IFN: Interferon
- JNK: c-Jun NH2-terminal kinase
- MEK: Mitogen-activated protein kinase/ERK kinase
- PI: Propidium iodide
- TNF: Tumor necrosis factor
- TNFR: TNF receptor
- TRAF: TNF receptor-associated factor
- WCL: Whole-cell lysates
- XBP-1: X-box binding protein 1
- pJNK: Phosphorylated JNK

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903229
signaling intermediate downstream of CD27 and that the transcription factors IRF4 and BLIMP-1, but not BTB and CNC homology 1, basic leucine zipper transcription factor 2 (Bach2), are modulated by CD27 ligation. Yet inhibition of plasma cell generation by CD40 ligation involves JNK as well as MEK; however, they appear to lead to modulation of the same transcription factors as are modulated after CD27 ligation. Our data indicate that both TNFRs influence B cell differentiation via similar signaling pathways and that CD27 may prove valuable in the further understanding of lineage commitment after B cell activation.

Materials and Methods

Mice
C57BL/6 and BALB/c mice used in the study were obtained from The Jackson Laboratory (Bar Harbor, ME) and were bred and maintained in the Small Animal Facility of the National Institute of Immunology (New Delhi, India). Mice were used for experiments at 8–12 wk of age. Approval from the institutional animal ethics committee was obtained for all experimental procedures involving animals.

Reagents

The following reagents were used: LPS, Triton X-100, sodium orthovanadate, PMSF, KCl, EDTA (Sigma, St. Louis, MO), Tris base (Amresco, Solon, OH), Blotto (Santa Cruz Biotechnology, Santa Cruz, CA), leupeptin (Boehringer Ingelheim India, Mumbai, India), aprotenin (Merck, India, Mumbai, India), azide-free anti-myc (clone 9E10; Roche Applied Sciences, Mannheim, Germany), anti-CD40 (clone 1C10; eBioscience, San Diego, CA), anti-CD6 (clone 53-6.7; BD Biosciences, San Jose, CA), PE-streptavidin, fluorescein-streptavidin, FITC/PE donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Westgrove, PA), propidium iodide (PI; Sigma), CFSE (Molecular Probes, Eugene, OR), fluorescein/ biotin/P-E-Cy5 anti-B220, FE-anti-CD138, CD44, CD27, CD138, BLIMP-1, Bach2, IRF4 (BD Biosciences or eBioscience), and anti-phospho SAPK/JNK (Th183/Tyr185; Cell Signalling Technology, Danvers, MA). ECL agent and x-ray films were from Amersham Pharmacia Biotech, Little Chalfont, U.K.

Inhibitors used in culture were obtained from Calibiochem or Sigma and included JNK-1, JNK-II, JNK-3 (30–3 μM), rottlerin (10–0.1 μM), LY294002, AG490, SB203580, genistein (all 1–3 mM), acetylcysteine (30–0.3 mM), olomoucine (100–0.1 μM), and PD98059 and caffeine (10–1 μM).

Primers

For cloning full-length (FL) CD27: forward 5′-CGGAAAATTCATGGGCA-TGGGCACACTCTCTCTTA-3′, reverse 5′-CGGATGACGGTGAAGAGCC-GGCCTTCG-3′. For generating myc-tagged FL CD27: forward 5′-GGGGCGTGAGGGGGTACGCTGCGCAGGAGGCTTCTTGGTTAGAGGG-3′, reverse 5′-GGGGGGATGCAAGTGAACTGCCCTCTCT-3′. Myc-tagged FL CD27 and its mutants were PCR-amplified from pPCR-Script Amp containing total cDNA using appropriate forward and reverse primers containing XhoI and BamHI sites, respectively. The amplicons were cloned into pGEM-T Easy (Promega, Madison, WI) and 2 μg plasmid DNA extraction (Qiagen columns), insert checked by digestion with EcoRI and BamHI, and confirmed by sequencing (University of Delhi, South campus facility, New Delhi, India). Myc-tagged FL CD27 and its mutants were PCR-amplified from pPCR-Script Amp containing CD27 DNA using appropriate forward and reverse primers containing XhoI and BamHI sites, respectively. The amplicons were cloned into pGEM-T Easy, the clones obtained were sequenced (LabIndia Systems, New Delhi, India), and the constructs subcloned into the XhoI-BamHI sites of the LNCX2-IRES-EGFP vector.

Vectors

pPCR-Script Amp (Stratagene) and pGEM-T-Easy (Promega, Madison, WI) were used for cloning, and pLNCX2-IRES-enhanced GFP (EGFP) was used for eukaryotic expression. The latter was generated by insertion of the IRES-EGFP cassette with flanking XhoI and NotI sites from pIRE-EGFP (Clontech, Mountainview, CA) into pLNCX2 (Clontech).

Cloning of murine CD27 and its C-terminal deletion mutants

Splenocytes were of murine origin and treated with 4 μg/ml Con A (Sigma) for 48 h, CD27 induction confirmed by flow cytometry, RNA extracted (TRizol; Sigma), and 2 μg total RNA used to synthesize cDNA with oligo-deoxynucleotide using First Strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendations. The 752-bp CD27 cDNA was PCR-amplified from total cDNA using gene-specific primers, with EcoRI and BamHI sites incorporated into the forward and reverse primers, respectively. The amplicon was resolved on an agarose gel, eluted, and purified using QiAquick Gel Extraction kit (Qiagen, Valencia, CA), and blunt-end ligated into an SrfI-digested pGEM-Script vector (PCR-Script Amp cloning kit; Stratagene) by overnight incubation. Competent cells (DH5α, Escherichia coli) were transformed with the ligation mixture and plated on Luria–Bertani agar (Difco Laboratories, Detroit, MI; BD Biosciences) plates containing 100 μg/ml ampicillin (Sigma). Single colonies were screened by plasmid DNA extraction (Qiagen columns), insert checked by digestion with EcoRI and BamHI, and confirmed by sequencing (University of Delhi, South campus facility, New Delhi, India). Myc-tagged FL CD27 and its mutants were PCR-amplified from pPCR-Script Amp containing CD27 DNA using appropriate forward and reverse primers containing XhoI and BamHI sites, respectively. The amplicons were cloned into pGEM-T Easy, the clones obtained were sequenced (LabIndia Systems, New Delhi, India), and the constructs subcloned into the XhoI-BamHI sites of the LNCX2-IRES-EGFP vector.

B cells

Single-cell suspensions of cells from spleen were obtained by mechanical disruption, and erythrocytes in the splenocyte population were lysed by treatment of the cell pellet with Gey’s solution. B cells were enriched by treatment of the cell suspension with biotinylated anti-IgD (Southern Biotechnology, Birmingham, AL) followed by streptavidin microbeads (Miltenyi Biotech, Auburn, CA) or with anti-B220 magnetic beads (Miltenyi Biotech) and separated on MACS columns (Miltenyi Biotech) according to the manufacturer’s instructions. Purified cells were routinely ≥90% pure by flow cytometric analysis.

Cells were stimulated in 96-well flat-bottom plates or in 24- or 6-well plates (BD Falcon, Franklin Lakes, NJ) with 10 μg/ml LPS in 1% FCS in the medium (Irvine Scientific, Santa Ana, CA) or RPMI 1640 (Biological Industries, San Diego, CA) supplemented with 10% FBS, 2 mM l-glutamine, 0.1 mM 2-ME, and penicillin (100 U/ml) and streptomycin (100 μg/ml) (Life Technologies, Carlsbad, CA) in the presence or absence of the indicated amounts of anti-CD40. Wherever necessary, viable cells were isolated on a Ficoll-Hypaque gradient (Cedarlane, Hornby, Ontario, Canada) before further analysis. To assess proliferation, cells were labeled with 10 μM CFSE (Molecular Probes) before stimulation, and 72–96 h later, CFSE dilution was estimated after excluding dead cells with 1 μg/ml PI.

For B cell stimulation, cells were activated with 10 μg/ml LPS for 48 h, washed, and seeded at a density of 5 × 10^6/ml/well of OptiMEM in 6-well plates and transfected with plasmid DNA using TransIT-LT1 or TransIT-Express (Mirus Bio, Madison, WI) transfection reagent, according to the manufacturer’s recommendations. OptiMEM was replaced with complete medium containing LPS 4 h later, and 16 h after transfection, 10 μg/ml anti-myc Ab was added to ligate CD27 on transfected cells. Cells were analyzed by flow cytometry after a further 48 h.

For retroviral transduction of primary B cells, the retroviral packaging cell lines Plat-E (Cell Bioslabs, Cambridge, U.K.) or RetroPack PFP67 (Clonetech) were transfected with 4 μg plasmid DNA using FuGENE 6 (Roche) at a DNA/reagent ratio of 3:2 in OptiMEM in 6-well plates, and medium replaced 12 h later as described earlier. The efficiency of transfection was checked 24 h later and was routinely >60%. Culture medium was replaced at this time, the plates transferred to 32 °C for virus production, and the supernatant harvested 24 h later and filtered to remove cell debris. LPS-activated B cells were spin-infected with virus supernatant in the presence of 8 μg/ml Sequabrene (Sigma) and anti-myc added to ligate CD27 on transfected cells.

Immunoprecipitation

The B cell line A20 was spin-infected with virus expressing FL CD27 or various deletion mutants in the presence of 12 μg/ml Sequabrene, and G418 (1 mg/ml; Sigma) was added 24 h later. Two weeks into selection, more than 95% of cells expressed FL as determined by flow cytometry. The stable transductants were either left unligated or ligated with 10 μg/ml anti-myc for 15 or 30 min. Cells were washed twice with cold PBS after which they were lysed in TKM buffer (1% Triton X-100, 50 mM Tris

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FlowJo software (Tree Star, San Carlos, CA). Data were analyzed with
for 15 min. Abs directed against intracellular markers were then added,
meabilized with 100 μl Cytoperm/Cytofix buffer (BD Biosciences) on ice
for 15 min. Abs directed against intracellular markers were then added,
diluted in Permwash buffer (BD Biosciences). Data were analyzed with
FlowJo software (Tree Star, San Carlos, CA).

Statistical analysis
Data were analyzed by Student t test.

Results
CD27 signaling in primary B cells inhibits plasma cell
generation
We have previously shown that ligation of CD27 on activated B
cells with an Ab inhibits plasma cell generation in vitro and in
vivo (17, 18). To elucidate the mechanisms underlying this
inhibition, we cloned myc-tagged FL CD27 into a bicistronic EGFP-
expressing retroviral vector for transfection or retroviral trans-
duction of primary B cells (see Materials and Methods). The GFP
tag was included to enable detection of transfected cells by flow
cytometry, and the myc tag was included to enable specific liga-
tion of the transfected molecule with an anti-myc Ab. Expression
of myc and CD27 on GFP+ transfectants was confirmed, and rela-
tive levels of CD27 on the transfected cells relative to endoge-
nous levels were found to be high (Fig. 1A, 1B and microscopy
data not shown). The construct was then transfected into primary
B cells stimulated for 48 h with LPS, and 12 h later, anti-myc Ab
was added to ligate transfected CD27 molecules. As shown in Fig.
1C, ligation of transfected FL CD27 led to near-complete abro-
gation of plasma cell generation, as read out by surface expression
of CD138 and of intracellular BLIMP-1. When FL CD27 was
transfected into cells and left unligated, no effect on plasma cell
generation was seen, indicating that overexpression of CD27 had
no deleterious effects (Supplemental Fig. 1). The transfection data
reproduce what we have reported earlier with anti-CD27 Ab and
blots probed with Abs specific for various TRAFs. We found (Fig.
4A) that TRAF2 was recruited to the FL molecule as well as to the
Q/A mutant. Recruitment to the Δ-10 mutant was partial, and there
was no recruitment to the Δ/A mutant.

CD27 signals through JNK in primary B cells
To identify the signaling molecules involved downstream of CD27
ligation, we transfected FL CD27 into primary B cells and ligated
myc in the presence or absence of a range of pharmacological
inhibitors. We reasoned that pharmacological inhibition of a relevant
signaling pathway downstream of CD27 would reverse the effect
of CD27 ligation and lead to equivalent proportions of CD138+ plasma
cells in the GFP+ and GFP− populations. After testing a wide range
of inhibitor concentrations and identifying a range that had no ef-
fect on LPS-mediated cell proliferation or differentiation (data
not shown), we found that addition of JNK-II inhibitor led to a
dose-dependent reversal of the effect of CD27 ligation (Fig. 4B).
Other inhibitors tested (rottlerin, AG1478, bisindolylmaleimide-I,
LY294002, AG490, SB203580, genistein, N-acetyl cysteine, olo-
moucine, and PD98059) had no effect and failed to restore LPS-
induced plasma cell generation at concentrations that did not oth-
erwise affect B cell activation, proliferation, or differentiation
(Supplemental Fig. 3). Ligation of FL CD27 on retrovirally trans-
duced B cells also led to inhibition of induction of IRF4, and the
induction was restored when ligation was done in the presence of
JNK inhibitor (Fig. 4C).

STATICAL ANALYSIS
Data were analyzed by Student t test.

RESULTS
CD27 signaling in primary B cells inhibits plasma cell
generation
We have previously shown that ligation of CD27 on activated B
cells with an Ab inhibits plasma cell generation in vitro and in
vivo (17, 18). To elucidate the mechanisms underlying this
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gation of plasma cell generation, as read out by surface expression
of CD138 and of intracellular BLIMP-1. When FL CD27 was
transfected into cells and left unligated, no effect on plasma cell
generation was seen, indicating that overexpression of CD27 had
no deleterious effects (Supplemental Fig. 1). The transfection data
reproduce what we have reported earlier with anti-CD27 Ab and
are therefore consistent with the physiological possibility in-
dicated in our earlier data of a role for CD27 in skewing cell fate
determination away from terminal differentiation and toward the
memory lineage (17, 18).

The PIQED region of the CD27 tail is involved in inhibition of
terminal differentiation
Signal transduction downstream of CD27 ligation requires the re-
cruitment of TRAF molecules. The cytoplasmic domain of CD27
contains two putative TRAF-binding domains: a PIQED domain,
which could recruit the activating TRAFs 2, 3, and 5, and an EEFG
domain, which could recruit TRAFs 1 and 2 (19, 20). To elucidate
which of these regions was involved in the signaling in B cells,
we next cloned three deletion mutants into the EGFP-expressing
vector (19) (Supplemental Fig. 2). The Δ-10 mutant contains both
domains, Δ-16 lacks the PIQED domain, and Δ-23 lacks both
PIQED and EEFG domains. The myc tag enabled ligation of the
mutant CD27 molecule with an anti-myc Ab, leaving endogenous
CD27 molecules untouched. All transfected cells expressed GFP
and myc (Fig. 2A and microscopy data not shown). The constructs
were then transfected into primary B cells stimulated for 48 h with
LPS, and 12 h later, anti-myc Ab was added to ligate the trans-
ferred CD27 molecules. Viable cells were isolated on Ficoll-
Hypaque (Cedarlane) gradients 48 h later and stained for B220
and CD138. Plasma cell frequencies were then assessed on gated
GFP+ cells. As shown in Fig. 2B, and as expected from Fig. 1B,
ligation of transfected FL CD27 led to near-complete abrogation
of plasma cell generation. No inhibition was seen after ligation of
the Δ-23 mutant, which lacks both domains, or with the Δ-16
molecule, which lacks only the PIQED domain, indicating that
this region and not the EEFG domain is involved in signaling.
Partial inhibition was seen after ligation of the Δ-10 molecule, which
contains both domains. These data indicate that the PIQED
domain in the cytoplasmic tail of CD27 is likely involved in the
recruitment of factors responsible for signaling events that lead to
inhibition of terminal differentiation. To confirm this, we gener-
ated point mutants in the PIQED domain by mutating residues
P, Q, or D to A. The resultant constructs were cloned into a retro-
viral vector as before and transduced into LPS-stimulated B cells.
Ligation of the Q/A mutant led to abrogation of plasma cell gen-
eration, as with the FL molecule (Fig. 3). However, ligation of ei-
ther the P/A or the D/A mutant had no effect, indicating that these
two residues are critical for recruitment of adapters to the cytosolic
tail of CD27.

TRAF2 is recruited to the CD27 tail
To identify specific TRAFs recruited to the PIQED domain of the
CD27 tail, we transduced the B cell line A20 with the FL CD27
construct and the Δ-10, the Q/A, and the D/A mutants, and stable
lines expressing GFP were generated by selection in G418. Cells
were stimulated and immunoprecipitated with anti-myc Ab and
blots probed with Abs specific for various TRAFs. We found (Fig.
4A) that TRAF2 was recruited to the FL molecule as well as to the
Q/A mutant. Recruitment to the Δ-10 mutant was partial, and there
was no recruitment to the Δ/A mutant.
Inhibition of plasma cell generation by CD40 ligation involves JNK, MEK, and IRF4 but not Bach2

CD40 is the other TNFR expressed on B cells that is known to inhibit terminal differentiation and to enhance memory generation (17, 18, 21). We tested the effect of CD40 ligation on primary B cells by addition of stimulatory anti-CD40 Ab to LPS-stimulated cultures. As seen in Fig. 5, addition of anti-CD40 leads to inhibition of terminal differentiation in a dose-dependent fashion. At the higher concentrations, however, the Ab also induced prolific expansion of B cells (18) (data not shown), and so we used 4 μg/ml for all experiments. We then tested pharmacological inhibitors of signaling intermediates for their ability to reverse the CD40-mediated inhibition, and we found that three different inhibitors of JNK were able to partially restore plasma cell generation (Fig. 6A). As expected, and as seen with CD27 ligation, abrogation of plasma cell generation was accompanied by reduction in levels of intracellular BLIMP-1 (Fig. 7). Proliferation, as assessed by CFSE dilution, was similar in the presence or absence of inhibitor (Supplemental Fig. 4), and similar proportions of live cells were seen in the two groups (45.8 versus 45.2%), indicating that the concentrations of inhibitor used did not inhibit proliferation or induce apoptosis. Various other inhibitors were tested, and apart from the MEK inhibitor PD98059, they had minimal effects on rescue of terminal differentiation in the presence of anti-CD40 (Supplemental Fig. 5A). To show more directly that JNK is involved downstream of the two receptors, we looked for phosphorylation of the enzyme after ligation of CD27 or CD40. As shown in Fig. 6B, JNK is phosphorylated after treatment of primary B cells with anti-CD40 as well as after treatment of A20 cells stably expressing FL CD27 with anti-myc, and this phosphorylation is inhibited in the presence JNK-1. We also confirmed that the inhibitor did not inhibit the phosphorylation of other MAPKs (Supplemental Fig. 4B, 4C).

As seen with CD27 (Fig. 4C), ligation of CD40 also led to lower levels of IRF4 (Fig. 7A, 7B), and addition of JNK-II inhibitor to such cultures partially rescued plasma cell generation as read out by CD138, BLIMP-1, and IRF4 induction. Together, the data indicate that CD27 and CD40 can both signal through JNK to suppress induction of IRF4 and BLIMP-1 and thereby to suppress plasma cell generation. We also looked at intracellular levels of Bach2, a molecule that has been reported to be involved in plasma cell generation (15, 22). We found that Bach2 was downregulated equivalently by 24 h after LPS stimulation in the presence or absence of added anti-CD40 Ab and stayed downregulated at 48, 72, and 96 h. (Fig. 7C and data not shown). Hence, it is unlikely to be involved in negatively influencing terminal differentiation after CD40 ligation. It is also unlikely to be involved downstream of CD27 as the ligation of myc in the transfection and transduction experiments occurs 60 h after LPS stimulation. Together, the data indicate that CD27 and CD40 can both signal through JNK to suppress induction of IRF4 and BLIMP-1 and thereby suppress plasma cell generation.

Discussion

Plasma cell differentiation is initiated after activation of B cells by specific Ags or by LPS. The precise mechanisms leading to plasma cell generation were investigated. After transduction (hatched line, from the GFP+ gate in C). The dotted line represents endogenous levels of CD27 at 96 h (from the GFP+ gate in C). E. Abrogation of plasma cell generation by myc ligation. Primary B cells were transfected with reagent alone, empty vector, or FL CD27 (as labeled), treated with anti-myc Ab and stained for surface CD138 and intracellular BLIMP-1. One experiment representative of three is shown.

FIGURE 1. Expression of cloned myc-tagged, EGFP-expressing FL CD27. A, Coexpression of CD27 and myc with GFP on PT67 cells or LPS-activated B cells respectively transfected with myc-CD27-EGFP vector (CD27) or empty vector (vector). Representative of two independent experiments. B, CD27 levels on ex vivo B cells (shaded histogram) and on B cells stimulated with 10 μg/ml LPS for 48 h. C, Gating for GFP+ and GFP− cells 48 h after retroviral transduction of FL CD27. D, Relative expression of endogenous CD27 on ex vivo B cells (shaded histogram) and on B cells 48 h after stimulation with LPS (unbroken line) both gated as in B, and of retrovirally transduced CD27 96 h into LPS stimulation, but 48 h after transduction (hatched line, from the GFP+ gate in C). The dotted line represents endogenous levels of CD27 at 96 h (from the GFP+ gate in C). E, Abrogation of plasma cell generation by myc ligation. Primary B cells were transfected with reagent alone, empty vector, or FL CD27 (as labeled), treated with anti-myc Ab and stained for surface CD138 and intracellular BLIMP-1. One experiment representative of three is shown.
cell commitment remain unclear, but some transcription factors, especially BLIMP-1, IRF4, and XBP-1, appear to be critical for plasma cell formation and maintenance (23–26). BLIMP-1 has been considered a “master switch” for plasma cell generation (23, 27); however, recent data indicate that it is necessary but not sufficient for the induction (25, 28). IRF4 regulates isotype switching and terminal differentiation by controlling the expression of AID and BLIMP-1, respectively. Thus, restoration of AID in IRF4<sup>−/−</sup> B cells rescues isotype switching, and restoration of Blimp-1 rescues plasma cell generation (28). XBP-1 expands elements of the secretory pathway and enhances the unfolded protein response (29, 30). Vav may be upstream of some of these factors, as mature marginal zone B cells from Vav-null mice show induction of IRF4 but no induction of BLIMP-1 or XBP-1 and fail to undergo terminal differentiation (31). In contrast, the transcription factors BCL-6 and PAX5, which can repress XBP-1 and BLIMP-1, respectively, are downregulated during plasma cell differentiation (32–34). BCL-6 is required for GC development and may be one of the primary molecules that influence differentiation (35–37). There is a considerable amount of cross-talk between these factors. Thus, upregulation of BLIMP-1 represses PAX5, a gene product that maintains B cell identity (38, 39) and also represses BCL-6 (26).

Most of these studies have relied on the use of cell lines or on B cells from mice deficient for various factors, and they have...
provided a wealth of information on the complex regulatory interactions between the transcription factors involved. However, little is known about the cell surface molecules and the signaling intermediates that are involved in initiating this cascade of transcriptional events in normal B cells. Further, little is known about signaling events that determine whether a cell exiting from cycle in the GC will undergo terminal differentiation or become a restimulable memory cell. Previous studies have indicated a role for Ag affinity in controlling this process (40, 41) and also a role for the TNFRs CD27 and CD40 in this cell fate determination (17, 18, 21). The experiments reported here followed from these earlier data, which showed that ligation of CD27 or CD40 with an Ab could specifically inhibit terminal differentiation in vitro (17). Physiological relevance for the finding came from the observation that a single dose of anti-CD27, injected at the time of immunization, led to higher memory cell generation in mice by three independent readouts: limiting dilution assays, adoptive transfer experiments for secondary responses, and the tracking of Ag-specific B cells in vivo over time. As expected, treatment of mice with anti-CD40 Ab also enhanced memory cell frequencies. Significantly, we also showed that secondary responses were higher to the T-independent Ag 4-hydroxy-3-nitrophenylacetyl–Ficoll when immunization was done under cover of anti-CD27 treatment (18). Thus, CD27, at levels expressed on unmanipulated mouse B cells, can trigger signal modulations leading to altered cell-fate commitment. CD40-mediated signals achieve a plethora of effects in B cells, whereas CD27-mediated signals appear to be selectively...
restricted to the alteration of the plasma cell-memory B cell decision. Thus, characterization of CD27-mediated effects will provide a tool for understanding the signals mediated through TNFR family members, such as CD40, for the induction of B cell memory. In pursuit of that aim, we have used a transfection-driven system to express CD27 mutants to understand the signaling intermediates involved in the plasma cell-memory B cell decision alteration effect of CD27 ligation. Identification of the tail regions of CD27 that are likely to be involved in these effects, most likely TRAF recruitment, required transfection/transduction of CD27 tail mutants into primary B cells and the ability to ligate selectively the mutant molecules while leaving the endogenous molecule untouched.

Both molecules signal through the recruitment of TRAFs to their cytoplasmic tail. In B lineage cells, CD40 has been shown to recruit TRAFs 1, 2, 3, 4, 5, and 6 (42), but little is known about TRAFs recruited by CD27 in primary B cells. Two putative TRAF binding domains, PIQED and EEEG, have been identified in the cytoplasmic tail of CD27 (19, 20), and they have been shown, in other cell lineages, to recruit TRAFs 2, 3, and 5. In this study, we cloned and expressed FL CD27 and three deletion mutants that either retained both PIQED and EEEG domains, lacked both domains, or lacked only the PIQED domain, and used them to transfect or virally transduce primary B cells in culture. We found that ligation of the overexpressed FL molecule or the Δ-16 molecule, which lacks the PIQED domain, or the Δ-23 molecule, which lacks both domains, abrogates plasma cell generation, as read out by CD138 and BLIMP-1 staining. However, ligation of the Δ-10 molecule, which contains the PIQED domain, continues to inhibit plasma cell generation, albeit not completely (Fig. 2).
Immunoprecipitation experiments identified TRAF2 as being recruited to the CD27 tail after receptor ligation (Fig. 4). Some level of constitutive association of TRAF2 with CD27 was seen, and this is in keeping with earlier reports indicating that TRAF2 is constitutively associated with many TNFRs including CD40, CD30, and CD27 (19, 43, 44).

A previous study in which CD27 was overexpressed in 293 cells identified a 13 aa region in the cytoplasmic tail containing both EEEG and PIQED domains as necessary for recruitment of TRAF2 and activation of NF-κB after CD27 ligation. However, a construct with 10 C-terminal residues deleted, similar to the Δ-10 construct used in our study, continued to activate NF-κB (19). The incomplete effect seen with the Δ-10 mutant in our study suggests the possibility that additional residues downstream of the PIQED, although not necessary for NF-κB activation, may be required for abrogation of plasma cell generation. Another overexpression study with deletion mutants identified the PIQEDYR domain as necessary for recruitment of TRAF2 and TRAF5 and downstream activation of JNK and NF-κB (20). This raises the further possibility that the incomplete effect of the Δ-10 mutant seen here may be related to unstable interaction of TRAFs with the truncated molecule. We addressed these possibilities by generating point mutants in the PIQED domain for overexpression in B cells and found that CD27 molecules in which either the P or the D residues were mutated were unable to abrogate plasma cell generation (Fig. 3).

To elucidate the signaling intermediates involved downstream of CD27 ligation and TRAF2 recruitment, we attempted to reverse the effect of CD27 ligation and rescue plasma cell generation by adding pharmacological inhibitors at the time of ligation of overexpressed CD27. We chose a range of concentrations of inhibitors that did not affect either B cell proliferation or Ig secretion (data not shown). We found that CD27-mediated inhibition of plasma cell generation was uniquely susceptible to reversal by JNK-II inhibitor. In a parallel series of experiments, we added the same inhibitors to cultures stimulated with LPS in the presence of anti-CD40 Ab and found that JNK-II inhibitor as well as PD98059 could partially rescue plasma cell generation that was abrogated by CD40 ligation. CD40 is known to signal by the JNK and ERK pathways (19, 43–45), and our data indicate that both pathways may be involved downstream of CD40 in determining lineage commitment after activation.

Two other molecules that have been implicated in suppressing plasma cell generation are Bach2 and Mitf. Bach2 is a transcriptional regulator that inhibits plasma cell formation by binding to Maf responding elements in the BLIMP-1 promoter (22), and Mitf is a basic helix-loop-helix leucine zipper protein that inhibits terminal differentiation by inhibiting IRF4 (43–46). We found that intracellular Bach2 was downmodulated by 48 h in LPS-activated B cells regardless of whether anti-CD40 Ab was present or not (Fig. 7), indicating that this transcription factor is not involved in the signaling events downstream of CD40 ligation that lead to inhibition of terminal differentiation. Bach2 is also unlikely to contribute to the observed effect of CD27 ligation as ligation of the molecule in the transfection assays occurs only 60 h after stimulation. IRF4, however, was induced by 72–96 h after LPS activation, and this induction was inhibited if CD27 or CD40 was ligated during activation (Figs. 4, 7). Thus, the two TNFRs inhibit terminal differentiation through activation of JNK and suppression of IRF4 and BLIMP-1. We and others have shown that B and T cell memory responses are enhanced when immunization is accompanied by CD27 or CD40 ligation in vivo (18, 19, 45–48). Because CD27 ligation has the unique effect on B cells of promoting memory at the expense of terminal differentiation, and because JNK signaling is uniquely involved in this effect, our data indicate that intervention strategies involving this molecule may be possible for the rational design of vaccines.

Disclosures

The authors have no financial conflicts of interest.

References