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*J Immunol* 2005; 174:3264-3272; doi: 10.4049/jimmunol.174.6.3264
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Coligation of the B Cell Receptor with Complement Receptor Type 2 (CR2/CD21) Using Its Natural Ligand C3dg: Activation without Engagement of an Inhibitory Signaling Pathway

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C3dg is a cleavage product of the C3 component of complement that can facilitate the coligation of the complement receptor 2 (CR2/CD21) with the BCR via C3dg/Ag complexes. This interaction can greatly amplify BCR-mediated signaling events and acts to lower the threshold for B cell activation. Although previous studies have used anti-CR2 Abs or used chimeric Ags in the context of BCR transgenic mice as surrogate C3d-containing ligands, we have used a physiological form of C3d to study signaling in B cells from wild-type C57BL/6 mice. We find that while CR2-enhanced BCR signaling causes intracellular Ca²⁺ mobilization and total pTyr phosphorylation of an intensity comparable to optimal BCR ligation using anti-IgM Abs, it does so with limited receptor cross-linking. In summary, we demonstrate that CR2-enhanced BCR signaling may proceed not only through activation of inhibitory effectors (such as CD22, Src homology region 2 domain containing phosphatase 1, and SHIP-1) and without total pTyr phosphorylation of an intensity comparable to optimal BCR ligation using anti-IgM Abs, it does so with limited activation of inhibitory effectors (such as CD22, Src homology region 2 domain containing phosphatase 1, and SHIP-1) and without receptor cross-linking. This article must therefore be hereby marked as being an example of a manuscript that has been refereed.

Almost all of the studies published on this subject to date have used cell lines and/or surrogate ligands such as anti-CR2 Ab (14), hen egg lysozyme/C3dg complexes and hen egg lysozyme transgenic mice (12, 15) or anti-CD19 Ab (16) to presumably mimic the effect of CR2-C3dg interactions on BCR signaling. Because of this, the effects of CR2 ligation by its natural ligand have not been explored in detail. In this study, we have used a natural ligand of CR2–C3dg to stimulate B cells isolated from wild-type C57BL/6 mice. We analyzed the contribution of CR2-mediated stimulus alone and in conjunction with BCR ligation to the cumulative activation signal by a number of parameters: intracellular Ca²⁺ influx, total tyrosine phosphorylation (pTyr) in activated B cells, as well as phosphorylation of some key signaling molecules involved in positive (Erk, Lyn, Syk, B cell linker protein (BLNK), CD19) and negative (SHIP-1, Src homology region 2 domain containing phosphatase 1 (SHIP-1), SHP-2, CD22) BCR signal regulation. Importantly, we demonstrate that CR2-BCR coligation does not lead to extensive surface receptor aggregation (i.e., “capping”) as is the case with an optimal anti-BCR stimulus (anti-IgM Ab), and does not engage inhibitory signaling molecules (CD22, SHIP-1, SHP-1, SHP-2) to the same extent, although both stimuli elicit similar Ca²⁺ influx and total pTyr signals. In addition, using single cell analysis we have found that intracellular Ca²⁺ influx triggered by CR2 ligation with C3dg occurs in a capacitative store-operated fashion via depletion-activated ion channels. Finally, we have explored the differences in the capability and dynamics of CR2-mediated stimulation of peripheral B cell subpopulations and demonstrate that CR2 is capable of amplifying BCR signal transduction in each subpopulation of B-2 cells, but not B-1 cells. Taken together, these data confirm the important role of CD19 in

The Journal of Immunology, 2005, 174: 3264–3272.

Declarations of interest: None.
CR2-mediated BCR signal amplification as well as extend our understanding of the molecular details by identifying further targets of CR2-BCR oligation as well as intrinsic differences in complement-dependent and -independent B cell signaling pathways.

**Materials and Methods**

**Animals and cell lines**

C57BL/6- and CD19-deficient (CD19−/−) C57BL/6 mice were obtained from The Jackson Laboratory. CR2-deficient (CR2−/−) C57BL/6 mice were generated (17) and bred in our laboratory. For microscopy and Western blotting experiments, intact splenic B cells were purified using MACS B cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. K46μ (μ-chain IgM-expressing) and CH12 (B-1 phenotype) (18) B lymphoma cell lines were cultured in IMDM (In vitro FlowTechnologies) supplemented with 5% FCS (HyClone), 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM t-glutamine at 37°C in 5% CO₂.

**Reagents**

Abs and their sources are as follows: goat-anti-mouse IgM F(ab)₂, biotin conjugated goat-anti-mouse-IgM F(ab)₂ (Southern Biotechnology Associates); donkey-anti-mouse IgM/C5/5, donkey anti-rabbit IgG/FITC, normal rabbit serum (Jackson ImmunoResearch Laboratories); HRP-conjugated sheep-anti-mouse Ig (Amersham Pharmacia Biotech); anti-CD21-CD35/FITC mAb (7G6), anti-CD19/FITC mAb, anti-CD22 mAb, biotin-conjugated anti-CD19 mAb, anti-B220/APC mAb, anti-CD5/PE mAb, anti-CD1d/PPE mAb, anti-CD24/FITC mAb, streptavidin (sa)/PE (BD Pharmingen); anti-γ-1γ-10 mAb (Upstate Biotechnology); anti-μ-γ-1γ-10 PY20/FITC mAb, rabbit anti-Syk, rabbit anti-phospho-ERK (Santa Cruz Biotechnology); anti-Lyn mAb (BD Transduction Laboratories); and rabbit anti-phospho-BLNK, rabbit anti-phospho-CD19, rabbit anti-phospho-SHIP-1, rabbit anti-phospho-SHIP-2, rabbit anti-SHIP-2 (Cell Signaling Technology). Biotin-conjugated C3dg was produced in our laboratory as described (19). Abs were neutralized using specific Abs in conjunction with ECL (PerkinElmer). For Lyn, signal and anti-IgM/C3dg/sa tetramer binding to C57BL/6 B cells. To provide as strong a Ca²⁺ influx as possible, the experiments were performed at room temperature on BD FACScan Flow Cytometer (BD Biosciences).

**Intracellular Ca²⁺ measurement by microscopy**

Freshly isolated B cells (2 × 10⁶ cells/ml in IMDM supplemented with 2.5% FCS) were incubated with fura-2 AM (1 μM for 30 min at room temperature) and put on poly-t-lysine-coated glass bottom microscope dish (MatTek) for 15 min, allowing the cells to sediment and adhere. The excess of cells was washed away to obtain a monolayer. Time-lapse λ340/380 nm image acquisition was started, and after 5 min the dish was perfused with medium containing the indicated stimulus. Experiments were performed at room temperature. The imaging system is from Intellgent Imaging Innovations and uses a Nikon TE2000 microscope. We used a ratiometric technique for monitoring the intracellular Ca²⁺ time courses in single cells (20). Where noted, single cell Ca²⁺ traces (typically 100–150 cells) were averaged.

**Intracellular Ca²⁺ measurement by flow cytometry**

Isolated murine splenocytes underwent erythrocyte lysis in ammonium chloride-potassium hydrocarbonate-EDTA buffer (Sigma-Aldrich). Cells were washed, incubated with indophenol-1 AM (5 μM for 30 min at 37°C), and stained with fluorescein-labeled Abs as indicated. One minute after data acquisition was started, the stimulus was added. Populations of interest were gated upon intracellular Ca²⁺ influx and analyzed using FlowJo software (Tree Star). Experiments were performed at room temperature on BD LSR Flow Cytometer (BD Biosciences).

**Western blot analysis**

Cells (2 × 10⁶/ml) were treated for indicated times at 37°C with a stimulus as indicated and then immediately lysed in 0.5% CHAPS lysis buffer (50 mM NaCl, 10 mM Tris, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMFS, 10 mM NaF, 0.4 mM EDTA, 1 mM aprotinin, 1 mM antipyrin, and 1 mM leupeptin, Sigma-Aldrich) (pH 7.5). Lysates were kept on ice for 30 min, then centrifuged at 10,000 rpm for 10 min at 4°C. Supernatants were mixed with Laemmli SDS reducing sample buffer (Sigma-Aldrich) and boiled for 5 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and visualized using specific Abs in conjunction with ECL (PerkinElmer). For Lyn, Syk, and SHP-1 immunoprecipitations, lysates were incubated with the indicated specific Ab preconjugated with protein A-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C in CHAPS buffer. Beads were then washed twice, mixed with Laemmli SDS reducing sample buffer and boiled for 5 min, followed by SDS-PAGE and transfer to membranes as above. Polyvinylidene difluoride membranes were stained with 4G10 mAb and developed using ECL. Membranes were then stripped and reprobed for proteins of interest as a loading control. Total pTy7 (4G10 mAb), pBBLNK, pCD19, pSHP-1, pSHP-2, and pERK blots (whole cell lysates) were stained with primary Abs specific to the phosphorylated molecules (no prior immunoprecipitation). In these experiments, loading controls were done with Ponceau S (Bio-Rad) staining as follows: membranes were washed from ECL reagent, incubated with Ponceau S for 10 min. washed again, and scanned for densitometry (whole lanes were boxed and average density was compared between the lanes; obtained values differed by <5% between samples). Densitometry analysis was performed with National Institutes of Health Image software.

**Immunocytochemistry**

Purified splenic B cells were adhered to poly-t-lysine-coated cover glass as above. K46μ and CH12 lymphoma cells were grown on cover glass (no poly-t-lysine) overnight to adhere. Cells were treated with a stimulus for the indicated times, fixed with chilled 4% paraformaldehyde in TBS, permeabilized (0.5% Tween 20, 0.1% FCS, 0.01% saponin in TBS), blocked with SuperBlock (ScyTek), and stained with primary Abs (normal rabbit serum for staining controls), followed by fluorescently labeled secondary Abs and mounted on glass slides in 2% 0-phenylenediamine dehydrochloride (Sigma-Aldrich). Slides were imaged on the above-described microscope.

**Cell proliferation**

K46μ B lymphoma cells were loaded with CFSE (BD Pharmingen) according to the manufacturer’s instructions and cultured for 24 or 48 h in IMDM supplemented with 5% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM t-glutamine at 37°C in 5% CO₂. Proliferation was analyzed on BD FACScan Flow Cytometer (BD Biosciences).

**Results**

**CR2 enhances suboptimal BCR stimulation**

Anti-IgM/C3dg/sa conjugates were generated using a technique previously described (19). Briefly, biotin-conjugated anti-IgM

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**FIGURE 1.** CR2 enhances suboptimal BCR stimulation in a ligand-specific fashion. A, Ca²⁺ responses in C57BL/6 B cells stimulated by anti-IgM/C3dg/sa conjugates (dashed line). Neither suboptimal anti-IgM/sa (black line) nor C3dg/sa alone (gray line) stimulated Ca²⁺ influx. B, Unlike in C57BL/6 B cells (dashed line), anti-IgM/C3dg/sa conjugates have no effect in B cells from CR2-deficient mice (black line). C, Physical association between anti-IgM and C3dg with sa is essential for optimal induction of Ca²⁺ signal. Anti-IgM/C3dg mixture without sa (black line) does not provide as strong a Ca²⁺ stimulus as anti-IgM/C3dg/sa conjugates (dashed line); suboptimal anti-IgM/sa (gray line). D, Correlation between Ca²⁺ signal and anti-IgM/C3dg/sa tetramer binding to C57BL/6 B cells.
F(ab')2 Ab at its optimal concentration (1 μg/ml) was titrated down (data not shown) to determine a concentration in response to which splenic B cells no longer mobilized intracellular Ca2⁺ (suboptimal stimuli, 0.001 μg/ml). Recombinant biotinylated C3dg and sa were titrated to obtain concentrations capable of amplifying this suboptimal BCR stimulus (0.2 and 0.04 μg/ml, respectively). Anti-IgM/C3dg/sa conjugates stimulated Ca2⁺ influx (Fig. 1A); control suboptimal anti-IgM/sa and C3dg/sa complexes had no effect. We also demonstrate that physical association between anti-IgM and C3dg with sa is essential for induction of signaling (Fig. 1C) and that this effect is receptor (CR2) specific as B cells from Cr2⁻/⁻ mice were unresponsive to anti-IgM/C3dg/sa stimulation (Fig. 1B). In the experiment where sa/PE was used to make conjugates (Fig. 1D), we found that as conjugates continued to bind to the cells, at a certain time point Ca2⁺ signal reached its peak and additional binding of anti-IgM/C3dg/sa complex did not further increase Ca2⁺ influx.

Signaling effects of C3dg are CD19-dependent

CR2-mediated BCR signaling amplification has been suggested to depend on CD19 (2, 3). To assess the role of CD19 in B cell activation mediated by CR2 ligation with C3dg in our model system, we stimulated splenic B cells from Cd19⁻/⁻ mice with anti-IgM/C3dg/sa conjugates. Cd19⁻/⁻ B cells demonstrated lower Ca2⁺ responses compared with B cells from C57BL/6 mice when stimulated with 1 μg/ml anti-IgM (optimal concentration for C57BL/6 B cells), which is characteristic for this phenotype (Fig. 2A) (21). We found that conjugates did not at all stimulate Ca2⁺ influx in B cells from Cd19⁻/⁻ mice (Fig. 2B) even though they have identical C57BL/6 levels of CR2 expression (Fig. 2E). In addition, our data indicate that anti-CD19 Ab (in this experiment C3dg/biotin was replaced with anti-CD19/biotin in conjugates) can functionally replace C3dg as anti-IgM/anti-CD19/sa complex provided Ca2⁺ response similar to anti-IgM/C3dg/sa conjugates (Fig. 2C) (control anti-CD19/sa did not stimulate the cells (data not shown)). These results further suggest that CR2-C3dg interaction does not bring any other than CD19-dependent signal amplification molecules into the BCR complex.

To investigate the surface associations of CR2, CD19 and BCR, we preligated CR2 with C3dg/sa complexes (as monomeric C3dg has been shown not to bind well to cell surface (19)) before stimulation with an optimal dose of anti-IgM. Such preligation lowered the Ca2⁺ response (Fig. 2D), suggesting that CR2/C3dg complexes were sequestered away, making fewer CD19 molecules available for signal amplification in cross-linked BCR complexes.

Characterization of C3dg-mediated Ca2⁺ influx

We reasoned that because C3dg has such a profound potentiating effect on Ca2⁺ responses, properties of CR2-mediated Ca2⁺ influx could be different from the one triggered by BCR cross-linking.

**FIGURE 2.** Signaling effects of C3dg are CD19-dependent. A, B cells from CD19-deficient mice (black line) have impaired Ca2⁺ responses to optimal (1 μg/ml) anti-IgM stimulus compared with C57BL/6 (dashed line). B, CD19-deficient B cells do not respond to CR2-mediated stimulus (C57BL6, dashed line; Cd19⁻/⁻, black line). C, Anti-IgM/anti-CD19/sa conjugates induce Ca2⁺ responses in C57BL/6 B cells, therefore functionally replacing CR2 ligation with that of CD19 (anti-IgM/anti-CD19/sa, gray line; anti-IgM/C3dg/sa, black line; 1 μg/ml anti-IgM, dashed line). D, Comparison of Ca2⁺ responses in K46μ B cells to 1 μg/ml anti-IgM (black line) and the same cells incubated with C3dg/sa for 15 min before stimulation (gray line). Pregluation with C3dg inhibits intracellular Ca2⁺ responses induced by BCR cross-linking, most likely by sequestering CR2 and thus CD19 from the BCR signaling complex. E, CR2 expression in CD19-deficient and wild-type C57BL/6 B cells.

**FIGURE 3.** Characterization of C3dg-mediated Ca2⁺ influx. Heterogeneity of single cell Ca2⁺ responses in C57BL/6 B cells stimulated with 1 μg/ml anti-IgM (A) and anti-IgM/C3dg/sa conjugates (B). In Ca2⁺-free medium, CR2-mediated stimulation with anti-IgM/C3dg/sa conjugates provided only a small initial Ca2⁺ rise (gray line) indicating Ca2⁺ release from intracellular stores. C, Extracellular Ca2⁺ influx in Ca2⁺-sufficient medium (black line).
with an optimal dose of anti-IgM. We examined C3d-mediated Ca\(^{2+}\) influx by microscopy in single B cells in an attempt to determine whether or not CR2-mediated Ca\(^{2+}\) responses have a specific pattern. Our data indicate that single cell Ca\(^{2+}\) responses induced by anti-IgM/C3dg/sa conjugates (Fig. 3B) are as heterogeneous as responses triggered by BCR cross-linking with 1 \(\mu\)g/ml anti-IgM (Fig. 3A), and we did not find significant differences in response patterns (neither the latency nor average maximum peak time or value were significantly different between the two (data not shown)). We also demonstrated that, as it is the case for BCR cross-linking, CR2-mediated Ca\(^{2+}\) influx occurs in a capacitative store-operated fashion via generic store-operated channel (SOC) entry channels (Fig. 3C), because in Ca\(^{2+}\)-free medium CR2-mediated stimulus provided only a small initial Ca\(^{2+}\) rise, which reflects Ca\(^{2+}\) release from intracellular stores (this experiment used Ca\(^{2+}\) measurements by flow cytometry).

**Ca\(^{2+}\) responses in subsets of peripheral B cells**

We compared Ca\(^{2+}\) responses to anti-IgM or anti-IgM/C3dg/sa conjugates in immature, mature, and marginal zone B cells (Fig. 4). All of the subpopulations had minor differences in response to 1 \(\mu\)g/ml anti-IgM, but in the case of CR2-mediated stimulus, marginal zone cells gave the most rapid response, mature B cells responded with a comparable amplitude but the peak came much later, and immature cells were the least sensitive.

Peritoneal B-1 cells have been reported to be hyporesponsive (22) and to have signaling properties similar to tolerant B cells (23). Previous studies also suggest that these cells may have impaired CD19 signaling (24). As CR2-BCR coligation appears to be a potent enhancer of BCR signaling, we decided to investigate the relevance of CR2/CD19-mediated signal amplification mechanism in B-1 cells by testing the ability of anti-IgM/C3dg/sa and anti-IgM/anti-CD19/sa conjugates to break unresponsiveness in this subpopulation. Peritoneal exudate B cells from C57BL/6 mice were stimulated with the conjugates and Ca\(^{2+}\) responses of B-2 (B220\(^{+}\)/CD5\(^{−}\)) and B-1 (B220\(^{+}\)/CD5\(^{+}\)) subpopulations compared (Fig. 5A). Although B-1 cells did respond to optimal anti-IgM (though it was lower than B-2 response to the same stimulus),
CR2-dependent phosphorylation of signaling proteins

We examined the pattern of signaling protein phosphorylation in splenic B cells stimulated by anti-IgM/C3dg/SA conjugates to determine whether there are qualitative differences between CR2-dependent and -independent stimuli. Total cell tyrosine phosphorylation was increased (Fig. 6A), and the phosphorylation profile was similar to the one from cells stimulated by 1 μg/ml anti-IgM. Consistent with our previous results, amplification of BCR signaling by anti-IgM/C3dg/SA conjugates required C3dg recognition as splenic B cells from CR2-deficient mice did not have a significant increase in pTyr levels (data not shown). We found increased phosphorylation of Lyn, Syk, Erk, BLNK, and CD19 in C57BL/6 splenic B cells stimulated by anti-IgM/C3dg/SA conjugates (Fig. 6, B–F, correspondingly), which demonstrates involvement of these signaling molecules in CR2-mediated signal amplification pathway. In contrast, negative signal regulatory molecules (SHIP-1, SHP-1, SHP-2, and CD22) were not phosphorylated in cells stimulated with anti-IgM/C3dg/SA conjugates, but were activated in cells stimulated with an optimal dose of anti-IgM (Fig. 7). A comparative analysis of relative phosphorylation of signal enhancers and inhibitors in cells stimulated by anti-IgM (1 μg/ml) or anti-IgM/C3dg/SA conjugates is presented in Fig. 7E. These findings have been confirmed in several experiments for each molecule. Mean phosphorylation increases in cells stimulated by anti-IgM/ C3dg/SA conjugates as compared with mitogenic dose of anti-IgM (as 100%) are the following: Lyn (88.32 ± 6.96%; four experiments), Syk (78.02 ± 1.35%; three experiments), Erk1/2 (84.85 ± 6.0%; eight experiments), BLNK (100.83 ± 6.07%; two experiments), CD19 (91.49 ± 9.92%; three experiments), CD22 (3.48 ± 5.01%; three experiments), SHIP-1 (1.53 ± 3.04%; four experiments), SHP-1 (5.95 ± 3.19%; three experiments), and SHP-2 (10.32 ± 1.79%; three experiments).

CR2-C3dg interaction and BCR capping

We found that in C57BL/6 splenic B cells CR2 mediated stimulus, while being able to provide C2⁺ influx and pTyr signals of an intensity comparable to the one triggered by surface BCR cross-linking with an optimal (1 μg/ml) dose of anti-IgM, did not cause capping typical for BCR cross-linking (Fig. 8, A, E, and I). K46μ cells also had comparable increases in C2⁺ influx (Fig. 8, D, H, L, and P) and pTyr (Fig. 8, C, G, K, and O) levels, but still no BCR capping in the case of conjugates (Fig. 8N). These results may suggest that with CR2-dependent stimulus formation of large signaling complexes like BCR “caps” is not required to deliver the signal of a comparable intensity. Fig. 8, E–H, demonstrates extreme capping in splenic B cells (E) and K46μ cells (F–H) stimulated with a higher dose of anti-IgM (10 μg/ml).

B cell proliferation

Complement is an important factor among antigenic signals received by B cells during the development to survive, mature, and proliferate, or to undergo apoptosis, and CR2 plays an important role in this process (2, 25, 26). Consistent with this effect, we found that anti-IgM/C3dg/SA conjugates increased proliferation in K46μ cells (data not shown).
and by association CD19, is sequestered by preligation with C3dg complexes, fewer of these molecules remain available to interact with the BCR signaling complex. This is consistent with other studies (14, 28), which suggest that independent ligation of CR2 or CD19 leads to sequestration of Lyn, thereby decreasing the magnitude of BCR-induced signaling. This hypothesis is also supported by the finding that the CD19/CR2/CD81 complex may contribute to the partitioning and retention time of BCR molecules into “lipid raft” moieties important for proper signal propagation (29). Finally, real-time analysis of cell binding using fluochrome-labeled anti-IgM/C3dg/sa conjugates also suggests that the enhanced signaling by conjugates was not a consequence of improved binding avidity, as additional binding of anti-IgM/C3dg/sa complexes (up until 600 s) does not further increase Ca\textsuperscript{2+} influx (Fig. 1D).

BRC-mediated activation of phospholipase C and consequent inositol 1,4,5-triphosphate production lead to rapid release of Ca\textsuperscript{2+} from intracellular stores in the endoplasmic reticulum. Store depletion activates Ca\textsuperscript{2+} influx across the plasma membrane via highly selective Ca\textsuperscript{2+} release-activated channels (CRAC) (30, 31), replenishing empty stores, and providing a sustained increase in the concentration of cytoplasmic-free Ca\textsuperscript{2+}. Such influx has been termed capacitative or SOC entry, and is essential for regulating diverse cellular responses to receptor-mediated stimuli. The amplitude and time course of the Ca\textsuperscript{2+} responses as a result of BCR stimulation are important in the activation of transcription factors regulating effector immune function, cell differentiation, proliferation, or death (32). Thus the involvement of costimulatory (CD19, CD21) or inhibitory (CD22) surface molecules, which can modulate the strength and duration of the B cell signaling, can significantly influence cellular outcome. Imaging of individual B cells loaded with intracellular Ca\textsuperscript{2+} indicator fura 2-AM allowed us to analyze single cell Ca\textsuperscript{2+} responses induced by anti-IgM/C3dg/sa conjugates (Fig. 3B). Noteworthy, this kind of data is lost in Ca\textsuperscript{2+} influx analysis by flow cytometry or spectrometry where the read-out represents only an average Ca\textsuperscript{2+} level at a given time point. Single B cells Ca\textsuperscript{2+} responses were extremely heterogeneous and undistinguishable from those induced by BCR cross-linking with mitogenic dose of anti-IgM (Fig. 3A). This may suggest that cross-linked BCRs and CR2-mediated stimulus use the same Ca\textsuperscript{2+} influx mechanism(s). Such a heterogeneity of single cell Ca\textsuperscript{2+} responses may also serve as an indirect evidence for involvement of CRAC as other studies also describe highly heterogeneous single cell responses in T lymphocytes where influx has been established to occur via CRAC (30). We demonstrated that, as is the case for BCR cross-linking with mitogenic dose of anti-IgM, CR2-mediated Ca\textsuperscript{2+} influx occurs in a capacitative store-operated fashion via generic SOC (Fig. 3C), as in Ca\textsuperscript{2+}-free medium, CR2-mediated stimulus provided only a small initial Ca\textsuperscript{2+} rise, which reflects Ca\textsuperscript{2+} release from intracellular stores. Our current data do not, though, provide a direct evidence for the exact type of SOC involved.

The nature and intensity of BCR-induced cell activation plays a critical role in B cell maturation and selection at different stages of development (33). Several reports have shown that BCR-induced signaling is significantly different in mature and immature B cells and have used these discrepancies to explain, in part, the increased sensitivity for tolerance induction in the immature population (34–37). In both representative immature B cell lines and ex vivo isolated immature B cells, variations in effector molecule expression, activation, and kinetics have been observed (38–41). Consistent with previous data, we observed similar levels of CD19 and CR2

Discussion
In this study, we provide a unique way to investigate CR2-mediated stimulation in B cells with a more physiological form of the complement component C3dg. Consistent with published data from our laboratory and others (19, 27), ligand containing our C3dg required a ∼1000-fold less concentration of anti-IgM Ab (1.0 vs. 0.001 μg/ml) to stimulate B cells to a similar extent, as assessed by intracellular Ca\textsuperscript{2+} influx and total tyrosine phosphorylation (Figs. 1A and 6A). Because anti-IgM molecules may have more than one biotin site, biotinylated anti-IgM and C3dg may form complexes of a greater complexity than simple tetramers when conjugated with sa and this may contribute to nonspecific stimulation of cells. To address this concern we used an irrelevant Ab (biotinylated anti-MHC class I) at the same concentration to form conjugates with C3dg and sa. These conjugates had no effect on B cells (data not shown). That anti-IgM/C3dg/sa conjugates did not stimulate B cells from CR2-deficient or CD19-deficient mice (Figs. 1B and 2B) demonstrates the receptor-specificity of the C3dg conjugates and the importance of CD19 in CR2-enhanced BCR signaling, respectively. This latter fact was independently confirmed by the ability of anti-CD19 Ab to functionally substitute for C3dg (in the form of anti-IgM/anti-CD19/ sa conjugates) to stimulate B cells (Fig. 2C). Interestingly, preligation of CR2 with C3dg/ sa complexes before stimulation with an optimal (mitogenic) dose of anti-IgM lowered Ca\textsuperscript{2+} response in C57BL/6 B cells (Fig. 2D). One possible explanation for this effect could be that as CR2,
expression on mature and immature (sic transitional) B cells isolated from the spleen of wild-type C57BL/6 mice (26). Intriguingly, while mature B cells had practically identical Ca\textsuperscript{2+} responses to mitogenic dose of anti-IgM or anti-IgM/C3dg/sa conjugates (Fig. 4), the dynamics of Ca\textsuperscript{2+} response in immature/transitional B cells were quite different. The immature/transitional B cells response to conjugates was considerably slower and of a much lower level of activation molecules directly or indirectly involved in Ca\textsuperscript{2+} influx induced by BCR cross-linking demonstrated comparable increases in phosphorylation of Lyn, Syk, ERK, BLNK, and CD19 in B cells stimulated by anti-IgM/C3dg/sa conjugates or mitogenic dose of anti-IgM (Fig. 6, B–F). Conversely, a similar analysis of inhibitory effectors produced surprising results. Unlike BCR cross-linking with mitogenic dose of anti-IgM, anti-IgM/C3dg/sa conjugates did not initiate comparable phosphorylation/activation of inhibitory signaling molecules, such as CD22, SHP-1, SHP-2, or SHIP-1 (Fig. 7). Normally, these molecules are triggered after BCR ligation as a feedback regulatory network to modulate and limit BCR signaling (43–46). However, the absence of such regulatory elements in response to complement-mediated stimulus may contribute to a lower threshold and more sustained activation of following BCR engagement and represents an important qualitative difference between complement-dependent (anti-IgM/C3dg/sa conjugates) and -independent (BCR cross-linking with mitogenic dose of anti-IgM) stimuli.

We also demonstrate that CR2-mediated stimulus can provide Ca\textsuperscript{2+} and pTyr signals of an intensity comparable to the one triggered by BCR cross-linking with mitogenic dose of anti-IgM but without the extensive BCR capping. In a model cell line, 15 min of stimulation with mitogenic dose of anti-IgM or anti-IgM/C3dg/sa conjugates elicited comparable increases in Ca\textsuperscript{2+} and pTyr levels in response to both stimuli but in the case of CR2-mediated stimulus, no BCR capping was observed (Fig. 8). We believe our results imply that formation of large supramolecular activation complexes (SMAC) (BCR caps in the case of mIgM cross-linking) is not required for CR2-mediated stimulus to deliver the signal of a comparable strength. This is because with the help of CD19 the same level of signal amplification can be achieved in much smaller
store-operated ion channels. Thus, our study has confirmed several
regulated Ca2+
(anti-IgM), CD19)-mediated stimulus. We also demonstrate that C3dg-mediated stimulus in mature, immature, and marginal zone
B cells. Peritoneal B-1 cells were unresponsive to C3dg- (and
C3dg-mediated stimulus in combination with anti-IgM. This may contribute to more sustained complement-mediated signal transfer.

In sum, in this study we have used the natural ligand of CR2 to
analyze C3dg-mediated B cell activation in wild-type C57BL/6 mice. It occurs in a receptor-specific fashion and possesses key features of CD19-mediated BCR signal amplification pathway. We demonstrate that CR2 and CD19 are associated together even out-
side the BCR complex and CD19 appears to be the only signal amplification molecule directly brought by CR2 into the BCR sig-
naling complex. Unlike BCR ligation with an Ag (in our case
substituted by anti-BCR Ab), CR2-mediated stimulus does not re-
quire formation of visible BCR caps/SMACs and does not activate
negative signal regulators. Therefore, it would indicate that unlike BCR
activation, if this is the case then the absence of caps
is not clear how far the TCR signaling analogy can be extrapolated
outside the BCR complex and CD19 appears to be the only signal
regulator because by the time they form most of the
ports in T cell activation studies argue that SMACs may play a
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Acknowledgments

We thank Dr. A. Zweifach (Department of Physiology and Biophysics,
University of Colorado Health Sciences Center, Denver, CO) for helpful
discussions regarding the manuscript and Dr. L. Kulik (Department of
Medicine, University of Colorado Health Sciences Center) for providing
recombinant C3dg.

Disclosures

The authors have no financial conflict of interest.

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