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Cutting Edge: Complement (C3d)-Linked Antigens Break B Cell Anergy

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C3dg adducts of Ag can coligate complement receptor type 2 (CR2; CD21) and the B cell Ag receptor. This interaction significantly amplifies BCR-mediated signals in Ag-naive wild-type mice, lowering the threshold for B cell activation and the generation of humoral immune responses as much as 1000-fold. In this study we demonstrate that CR2-mediated complementation of BCR signals can also overcome B cell anergy. Unlike Ag alone, BCR/CR2 co-stimulation (Ars-CCG/C3dg complexes) of anergic Ars/A1 B cells led to Ca2+ mobilization in vitro and the production of autoantibodies in vivo. Interestingly, the in vivo immune response of anergic cells occurs without the formation of germinal centers. These results suggest that the Ag unresponsiveness of anergic B cells can be overcome by cross-reactive (self-mimicking) Ags that have been complement-opsonized. This mechanism may place individuals exposed to complement-fixing bacteria at risk for autoimmunity. The Journal of Immunology, 2007, 179: 2695–2699.

Greater than 60% of newly produced B cells are autoreactive and must be silenced to prevent autoimmunity (1, 2). Silencing occurs by one of three primary mechanisms. Upon interaction with high avidity Ags, immature B cells undergo receptor editing involving additional V(D)J recombinations and the expression of a receptor with altered specificity (3). If this receptor is innocuous, cells enter the peripheral pool and can participate in immune responses. If editing does not eliminate autoreactivity, the cell may be eliminated by clonal deletion that is thought to involve death by apoptosis (4). Twenty-five to 30% of mature peripheral B cells show signs of receptor editing and thus have escaped autoreactivity by changing receptor specificity (5). The third known silencing mechanism is anergy, which is induced by low avidity binding to self-Ags (6, 7). Anergic cells persist in the periphery for a few days and, despite expressing unoccupied Ag receptors, are unresponsive to Ag stimulation. We have recently shown that ~50% of newly produced B cells are destined to become anergic (8). These cells, although short lived, represent a significant hazard of autoimmunity because they may bind cross-reactive antigenic epitopes on infectious agents that stimulate B cells via innate immune ligands.

The onset of autoimmunity often coincides with infection by pathogenic organisms (9, 10). Although the activation of self-reactive T cells has been attributed to superantigens, epitope-mimicking, epitope spreading, and other mechanisms, little is known regarding the events leading to the failure of B cell tolerance. One major innate immunity factor known to influence B cell responses is complement, particularly the C3dg protein that is one of the cleavage products of the C3 component. Invasive pathogenic organisms spontaneously bind and fix complement. C3dg is a ligand for complement receptor 2 (CR2)3 (CD21) on the B cell surface, and CR2-C3dg interaction greatly amplifies signals elicited by the binding of a C3dg-attached Ag with its specific BCR (11, 12). It was recently shown that C3dg association with collagen enhances its ability to induce arthritis and that CD21 expression by either B cells or dendritic cells is sufficient to support this response (13). In this study we explored whether B cell anergy is susceptible to subversion by components of the innate immune system, in this case complement.

The Ars/A1 murine model of B cell anergy offers an optimal setting in which to study the effects of complement-mediated signaling in anergic B cells (14). Ars/A1 mice express a transgenic (Tg) Ig (μδ/κ) that encodes a canonical anti-p-azophenylarsonate (Ars) BCR. The Ars-specific transgene-encoded BCR cross-reacts with an undefined self-Ag in the bone marrow and periphery that induces anergy. Similar to other well-established murine models of B cell anergy such as HyHEL (15), Ars/A1 B cells that reach later transitional stages are capable of binding Ag; however, their receptors fail to transduce proximal biochemical signaling events as indicated by intracellular Ca2+ mobilization or tyrosine phosphorylation. Phenotypically, the Ars/A1 model is in many aspects similar to the HyHEL model (16). Thus, using this system it is possible to assess the effects of C3dg-associated cross-reactive Ags on B cell anergy.

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3 Abbreviations used in this paper: CR2, complement receptor 2; AFC, Ab-forming cell; AM, acetoxymethyl ester; Ars, p-azophenylarsonate; CCG, chicken γ-globulin; GC, germinal center; PNA, peanut agglutinin; PtdInsP3, phosphatidylinositol 3,4,5-triphosphate; Tg, transgenic.

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In this report we describe the ability of C3dg augmentation of BCR signals to overcome proximal inhibitory mechanisms responsible for the Ag unresponsiveness of anergic B cells.

### Materials and Methods

#### Generation of anti-IgM/C3dg conjugates

Biotin-conjugated C3dg and anti-IgM/C3dg/streptavidin conjugates were generated as described (12). Briefly, biotin-conjugated anti-mouse-IgM F(ab’)2; Ab (anti-IgM) was titrated down from its optimal concentration (1 μg/ml) to the concentration at which splenic B cells no longer mobilized intracellular Ca2+ (suboptimal, 0.001 μg/ml; data not shown). Recombinant biotin-conjugated C3dg and streptavidin were titrated to concentrations capable of amplifying the suboptimal anti-IgM stimulus (0.2 and 0.04 μg/ml, respectively).

#### Animals and reagents

C57BL/6 mice were obtained from The Jackson Laboratory. Ars/A1 mice (B6.Cg-Tg[lgAb36–65]1Wys Tg[lgAb36–71]1Wys) were generated and bred in our laboratory (14). Abs and their sources are as follows: biotin-conjugated goat-anti-mouse-IgM F(ab’)2 (Southern Biotechnology Associates); unlabeled or FITC-conjugated anti-mouse-CD21/CD35 mAb (7G6), FITC-conjugated anti-mouse-CD19 mAb, FITC-conjugated anti-mouse-TCR mAb, labeled or FITC-conjugated anti-mouse-CD21/CD35 mAb (7G6), FITC-labeled or FITC-conjugated anti-mouse-B220 mAb, FITC-conjugated anti-mouse-CD3, and FITC-conjugated anti-mouse-IgM (normal rabbit serum [Jackson ImmunoResearch]; IgMa (RS3.1; American Type Culture Collection), κ (187.1; American Type Culture Collection), and anti-idiotype Ab (17)). Streptavidin was purchased from BioSource International, 10-FITC-labeled goat-anti-mouse-IgM F(ab’)2 (Southern Biotechnology Associates); unlabelled or FITC-conjugated anti-mouse-CD21/CD35 mAb (7G6), FITC-conjugated anti-mouse-CD19 mAb; 1 min after data acquisition was started the gated anti-mouse-B220 mAb; 1 min after data acquisition was started the gated anti-mouse-IgM normal rabbit serum (Jackson ImmunoResearch); IgMa (RS3.1; American Type Culture Collection), κ (187.1; American Type Culture Collection), and anti-idiotype Ab (17). Streptavidin was purchased from BioSource International, and mounted on glass slides in 2% o/pBlock (Scytek), stained with fluorescently labeled specific Abs as indicated, and analyzed for Ars-specific Ab titers using ELISA as described (14). The immunization sera were analyzed for Ars-specific Ab titers using ELISA as described (14). The data were acquired and analyzed with SlideBook software. Imaging Innovations fitted on a Nikon TE2000 epifluorescence microscope.

#### Flow cytometry, proliferation, and intracellular Ca2+ analysis

Isolated murine splenocytes underwent erythrocyte lysis in RBC lysing buffer (Sigma-Aldrich). Cells were washed and stained with fluorescently labeled Abs as indicated. For Ca2+ analysis by flow cytometry, cells were incubated with Indo-1 AM (1 μM) for 30 min at 37°C in medium supplemented with 5% FCS, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at 37°C in 5% CO2. Experiments were performed at room temperature on BD LSR or BD FACscan flow cytometers (BD Biosciences).

#### Immunization, ELISA, and histological analysis

Mice (8–12 wk old) were immunized by a single i.p. injection of 100 μg of Ars-CCG or Ars-CCG-C3dg conjugates in 200 μl of PBS in IFA. Preimmunization (day 0) and postimmunization (days 5, 9, and 14) immunization sera were analyzed for Ars-specific Ab titers using ELISA as described (14). The number of splenic Ab-forming cells (AFCs) was assayed by ELISPOT (18). For histological analysis, mice were sacrificed on days 7 and 16, harvested spleens were snap frozen in OCT embedding medium (Sakura Finetek) on dry ice/ethanol bath and 5-μm cryosections were acquired, air-dried, permeabilized (0.5% Tween 20, 0.1% BSA, and 0.01% saponin in PBS), blocked with Superblock (Scytek), stained with fluorescently labeled specific Abs as indicated, and mounted on glass slides in 2% p-phenylenediamine (Sigma-Aldrich). Specimens were examined on a digital imaging system purchased from Intelligent Imaging Innovations fitted on a Nikon TE2000 epifluorescence microscope. Data were acquired and analyzed with SlideBook software.

### Results and Discussion

#### Complement C3dg enables BCR-mediated Ca2+ mobilization by anergic B cells

Primary splenic B cells isolated from C57BL/6 or Ars/A1 mice were stimulated in a complement-independent (anti-IgM-biotin (1 μg/ml) or anti-IgM-biotin (0.001 μg/ml)/streptavidin; Fig. 1, A and B) or complement-dependent (anti-IgM-biotin (0.001 μg/ml)/C3dg-biotin/streptavidin; Fig. 1C) fashion. Although Ars/A1 cells failed to respond to BCR cross-linking with 1000-fold higher concentration of anti-IgM (1 μg/ml), they were sensitive to the “complement-enhanced” stimulus at a low anti-IgM concentration. Analysis of cytograms revealed that nearly 90% of B cells from the anergic mouse spleen respond to this stimulus; thus, the rise in mean Ca2+ does not reflect a response of some minor population (data not shown). Furthermore, these cells could not be effectively stimulated with even higher oversaturating doses of the stimulus (up to 20 μg/ml; data not shown). Ars/A1 B cells exhibited the high baseline Ca2+ levels typical for anergic cells, reflecting their constant stimulation by a self-Ag (14, 19).

The ability of C3dg-Ag to overcome the anergy of Ars/A1 B cells is not simply due to changes in BCR or other receptor levels. Ars/A1 B cells bind effectively to Ag (Ars) and anti-idiotype Ab (Fig. 1, G and H) and express surface CD19 and complement receptors at levels comparable to those of C57BL6 B cells (Fig. 1, D–F). These data demonstrate that CR2-mediated complementation can override the internal inhibitory mechanisms responsible for the anergic status of peripheral B cells in this model.

**Complement C3dg conjugated to Ag breaks anergy in vivo as evaluated by AFC generation and Ab production**

To assess the effect of C3dg costimulation on in vivo Ab responses by anergic B cells, Ars/A1 and control κTg mice were immunized with Ars-CCG or Ars-CCG-C3dg complexes and total Ars-specific Ig levels were measured in the serum by ELISA (Fig. 2A). Control mice responded to both Ags, with Ars-CCG-C3dg being more immunogenic, particularly on day 14. Ars/A1 mice were not sensitive to Ars-CCG immunization but did respond to Ars-CCG-C3dg with Ab levels resembling those of immunized control mice. In addition, an increase in the number of splenic AFCs was observed in an ELISPOT assay in both Ars/A1 B cells.
Ars/A1 and control mice 1 wk after immunization with Ars-CCG-C3dg (Fig. 2B). The effects of C3dg were prevented by blocking CR2 with anti-CR2 mAb and C3dg (a mix of biotin-conjugated anti-IgM and C3dg without streptavidin) as well as C3dg/streptavidin complexes without anti-IgM were not capable of stimulating Ca\(^{2+}\) influx in C57BL/6 B cells (12). Furthermore, nonaggregated monomeric C3dg does not bind well to B cells (20).

Generation of extra-follicular AFCs in anergic mice after immunization with Ars-CCG-C3dg conjugates

We observed increases in the numbers of AFCs in the spleens of Ars/A1 as well as control mice following immunization with the C3dg-conjugated Ars-CCG complex as compared with Ars-CCG alone (day 7 after immunization; Fig. 3B). The kinetics of AFC generation in the spleen following immunization with Ars-C3d-CCG were different between the Ars/A1 and control kTg mice. In the control mice AFCs were detectable for a longer period following immunization than in Ars/A1 mice, where they were significantly diminished by day 16 (Fig. 3A, left panels). The mean fluorescence of IgM\(^{+}\) cells is shown in Fig. 3A (right panel). Ars/A1 mice exhibited impaired formation of germinal centers (GCs) after immunization with Ars-CCG-C3dg conjugates as compared with control animals or those immunized with Ars-CCG (Fig. 3G; PNA staining for GC). Important is comparison of red staining in the far right top and bottom panels (Ars-CCG-C3dg in Ars vs control mice) in Fig. 3C. These data indicate that the involvement of CR2 in BCR signaling prompts Ars/A1 B cells to generate AFCs (Fig. 2) by a GC-independent mechanism.

To evaluate the effect of complement stimulation on proliferation and expression of CD80 and CD86 activation markers, ex vivo Ars/A1 or control B cells were cultured in the presence of anti-IgM (1 µg/ml) or anti-IgM (0.001 µg/ml)/C3dg/streptavidin. Our results indicate that, unlike control kTg cells, Ars/A1 B cells do not proliferate or up-regulate CD80 and CD86 in response to both stimuli (data not shown).

This report provides the first demonstration of the ability of complement fragments to directly influence intracellular mechanisms responsible for maintaining B cell anergy. Our data indicate that BCR-CR2(C3dg)-complementary signaling can overcome the BCR proximal signaling “defects” responsible for maintaining the unresponsiveness of anergic B cells to Ag. Such complementary signaling triggers immediate (intracellular Ca\(^{2+}\) influx) and long-term (Ab production) manifestations of Ag-induced activation in B cells that cannot be activated by other means. However, certain responses are not restored, e.g., up-regulation of CD86 and proliferation. C3dg signals seem to divert the response toward direct differentiation to Ab-secreting cells.
High baseline intracellular Ca\(^{2+}\) (Fig. 1, A–C, dashed line) is typical of anergic B cells in all experimental models and is due to the constant signals induced by self-Ag binding (19). Naïve B cells undergoing their initial response to an Ag exhibit a rapid rise in intracellular Ca\(^{2+}\), but within a few minutes the levels fall to a plateau that is maintained as long as the Ag is present. This plateau level is equivalent to the high baseline of anergic cells. Thus anergic cells are the physiologic equivalent of chronically Ag-stimulated naïve cells.

Chronic Ag stimulation leads to the activation of inhibitory circuitry and perhaps other mechanisms that limit subsequent responses to immunogenic stimuli. BCR stimulation alone (anti-IgM at 1 \(\mu\)g/ml), which successfully stimulates control B cells, cannot overcome the signal inhibitory mechanisms operative in anergic cells (Fig. 1A). Only when CR2 is recruited into the BCR signaling complex through its interaction with C3dg in the anti-IgM/C3dg complex are these inhibitory mechanisms overridden and robust Ca\(^{2+}\) mobilization seen (Fig. 1C). It is noteworthy that CR2 engagement leads to the recruitment of additional BCR signal-amplifying coreceptors such as CD19 (11, 12). It is important to note that anergy can be subverted by other B cells stimuli such as CD40 ligand (21) and TLR agonists (22). Thus, infection may lead to autoimmunity by the generation of multiple innate effectors as well as by T cell activation.

Although anergic Ars/A1 B cells express modestly (~30%) lower amounts of surface IgM than control cells (Fig. 1D), this difference cannot determine their unresponsiveness to BCR stimulation (Fig. 1A). Based on Ca\(^{2+}\) mobilization, naïve B cells can be activated by Ag/anti-BCR concentrations that occupy >10% of their Ag receptors. Complement/anti-IgM adducts stimulate Ca\(^{2+}\) in both anergic and naïve B cells at concentrations far below (1000 times) BCR saturation. Therefore, it is not the Ag binding but rather a signal transduction defect that is responsible for the anergic state of Ars/A1 B cells. This signaling defect is overcome by CD21 oligation with the BCR. Nevertheless, we cannot completely exclude a possibility that a ~30% difference in IgM expression may have some influence on B cell responses in our model system. Preliminary studies in our laboratory indicate that anergy is mediated in part by activation of the inositol lipid phosphatase SHIP-1 (K. T. Merrell and J. C. Cambier, unpublished observations). SHIP-1 activation leads to the hydrolysis of its substrate phosphatidylinositol 3,4,5-trisphosphate (PtdInsP3), needed for BCR signaling (23, 24). CD19 has been shown to mediate the recruitment and activation of PI3K leading to PtdInsP3 generation (25, 26). Because CD21 is associated with CD19 it is tempting to speculate that Ag/C3dg-mediated CD21-BCR coligation overcomes anergy by enhancing PtdInsP3 production. This possibility is currently under study in our laboratory.

Another possibility is raised by our previous observations (12) demonstrating that, unlike high dose Ag BCR ligation (1 \(\mu\)g/ml anti-IgM), stimulation by subthreshold concentrations of Ag/C3dg adducts (anti-IgM (0.001 \(\mu\)g/ml)/C3dg/streptavidin conjugates), while inducing activation, does not initiate the detectable activation of inhibitory signal regulators such as CD22, SHP-1, SHP-2, or SHIP-1. Typically, these molecules are activated shortly after BCR ligation by high dose Ag as a feedback regulatory mechanism to modulate and limit BCR signaling (27, 28). The absence of activation of such regulatory elements in response to Ag/C3dg is likely responsible in part for the potency of the adducts. This represents an important qualitative difference between high dose Ag and low dose Ag/C3dg stimulation that is likely to play a major role in overcoming the inhibitory mechanisms that normally prevent anergic B cells from activation.

Similar to Ca\(^{2+}\) mobilization, Ab production cannot be induced by the stimulation of anergic Ars/A1 mice with Ag (Ars-CCG) alone (Fig. 2A, open squares). In contrast, mice immunized with Ag/C3dg produced Ars-specific Abs with Ig levels resembling those of control mice immunized with the Ag (Fig. 2A, filled squares). Ag/C3dg also increased Ab production in control mice as compared with immunization with the Ag alone (Fig. 2A). Similar effects were observed in AFC generation (Fig. 2B). Thus, Ag/C3dg conjugates are able to restore the Ig-producing capacity of anergic Ars/A1 B cells to the level of normal B cells in response to the same stimulus. The C3dg-dependent increase in Ab production is clearly receptor (CR2) specific because it can be blocked with anti-CR2 Ab (Fig. 2C).

Our data indicate that anergic Ars/A1 B cells costimulated by Ag and C3dg generate fully functional extrafollicular AFCs, bypassing the GC stage. Ars/A1 mice exhibited impaired formation of GCs after immunization with Ars-CCG-C3dg conjugates as compared with control animals or those immunized with Ars-CCG (Fig. 3C; PNA staining for GC). Our results suggest that C3dg association may render Ag T cell independent, allowing B cells to proceed directly to the Ab-forming stage without the necessity of obtaining T cell help in GCs. Although C3dg-augmented Ag induces an Ab-response in anergic mice, this response appears short lived; the formation of AFCs in anergic mice immunized with complement-conjugated Ag is more transient and diminishes at later time points after immunization (Fig. 3, A and B). Previous studies have shown that anergic B cells are impaired in their ability to elicit T cell help (i.e., poor APCs) (29). We hypothesize that CR2-mediated signaling does not rescue this particular impairment because we did not observe enhanced proliferation or up-regulation of CD86 in anergic B cells stimulated in a complement-mediated fashion consistent with this hypothesis. Our previous results (30) demonstrated up-regulation of CD80 and CD86 in response to BCR/CR2 cross-linking with a mix of anti-IgM and anti-CR2 Abs (whole Ig). The difference could in part be explained by the involvement of Fc receptors, as in the current report we used anti-IgM F(ab')\(^2\)/C3dg conjugates. However, we cannot completely rule out a possibility that differences in the effects seen result from the cross-linking of CR2 with anti-CR2 Abs vs C3dg.

Interestingly, other studies (31) performed in a hen egg lysozyme (HEL) anergy model suggested that signaling through CR2 enhances B cell tolerance and is involved in the negative selection of developing self-reactive B cells. Our results demonstrate that CR2-mediated signaling can break anergy in mature Ars/A1 B cells. One possible explanation might be that complement/Ag costimulation may have different outcomes in B cells at different developmental stages.

In vivo immunopathogenic effects of complement-conjugated Ag in the Ars/A1 anergy model may involve several traditional manifestations of autoimmunity such as glomerulonephritis, arthritis-like symptoms, and other. Such effects represent another important role that complement may play in breaking B cell anergy and are under study in our laboratory.
This study is the first demonstration that B cell anergy can be overcome by complement-opsonized immunogens that cross-react with autoantigens and that the resultant Ab responses are GC independent. Our data demonstrate that complement-mediated signaling pathway can bypass/overcome the intracellular inhibitory mechanism responsible for maintaining clonal energy in B cells. Complement and autoantibodies are known to play an important pathological role in autoimmune disorders such as rheumatoid arthritis, lupus, and myocarditis, but the molecular mechanisms of B cell “receptor tuning” responsible for continuous energizing/desensitizing of potentially autoactive B cells are not known. Our results demonstrate that spontaneous Ab-independent complement deposition on self-tissues and mimetic bacterial pathogens may be an important factor that can disrupt the regulatory mechanism responsible for keeping the potentially harmful autoactive mature B cells from responding to self-Ags.

Disclosures
The authors have no financial conflict of interest.

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