A Critical Role for Complement C3d and the B Cell Coreceptor (CD19/CD21) Complex in the Initiation of Inflammatory Arthritis

Christopher J. Del Nagro, Ravi V. Kolla and Robert C. Rickert

*J Immunol* 2005; 175:5379-5389; doi: 10.4049/jimmunol.175.8.5379

http://www.jimmunol.org/content/175/8/5379

**References**

This article cites 60 articles, 36 of which you can access for free at: http://www.jimmunol.org/content/175/8/5379.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
A Critical Role for Complement C3d and the B Cell Coreceptor (CD19/CD21) Complex in the Initiation of Inflammatory Arthritis

Christopher J. Del Nagro,*†‡ Ravi V. Kolla,*†‡ and Robert C. Rickert2*†

Complement C3 cleavage products mediate the recognition and clearance of toxic or infectious agents. In addition, binding of the C3d fragment to Ag promotes B lymphocyte activation through coengagement of the BCR and complement receptor 2 (CD21). Signal augmentation is thought to be achieved through enhanced recruitment and activation of CD21-associated CD19. In this study we show, using the DBA/1 collagen-induced arthritis (CIA) model, that conjugation of C3d to heterologous type II collagen is sufficient to cause disease in the absence of the mycobacterial components of CFA. Transient depletion of C3 during the inductive phase of CIA delays and lessens the severity of disease, and DBA/1 mice deficient for coreceptor components CD19 or CD21 are not susceptible to CIA. Adoptive transfer experiments revealed that CD21 expression on either B cells or follicular dendritic cells is sufficient to acquire disease susceptibility. Although CD19−/− and CD21−/− mice produce primary Ab responses to heterologous and autologous type II collagen, they are impaired in the ability to activate T cells, form germinal centers, and produce secondary autoantibody responses. These findings indicate that binding of C3d to self-Ags can promote autoimmunity through enhanced Ag retention and presentation by follicular dendritic cells and B cells, respectively. The Journal of Immunology, 2005, 175: 5379–5389.

Infection and inflammation are important contributors to autoimmunity. Both the introduction of foreign Ags cross-reactive with self-constituents as well as the sustained activation of innate immunity can lead to self-reactivity. Accordingly, induced animal models (e.g., experimental autoimmune encephalomyelitis, collagen-induced arthritis (CIA), and adjuvant arthritis) of autoimmunity require mycobacterial components of CFA that cause a local inflammatory reaction, enhance Ag uptake, and the promote TLR-dependent cytokine release. In addition, mycobacteria activate the complement cascade directly via microbial cell wall components (alternate pathway) or indirectly via bound Ab (classical pathway) (1, 2). Early steps in complement activation are distinct for each pathway of induction, but unite in the cleavage of complement C3 into active fragments that function as important chemoattractants (C3a), opsonins (C3b, iC3b, and C3d,g), and precursors to the generation of the chemoattractant C5a and formation of the membrane attack complex (C5b-9) (3). Although complement plays an essential role in the recognition and clearance of extracellular pathogens and toxins, deregulated complement activation during the acute inflammatory response can promote autoimmune disease brought on by the binding of Abs to self-tissues, or deposition of immune complexes in glomeruli and small vessels (4).

In addition to the roles of C3 derivatives as soluble effector molecules, the cleavage product C3d acts during the inductive phase of the Ab response by binding to CD21 on B cells and follicular dendritic cells (FDCs) (5). Specific recognition of C3d-bound Ag results in co-clustering of BCR and CD21 as well as CD21-associated CD19. CD19 functions as a transmembrane adaptor protein for both CD21 and the BCR, and is thought to be responsible for the noted adjuvant effect of C3d on Ag-specific B cells (6, 7). Mice deficient for Cd19, Cr2 (encoding CD21 and CD35), or C3 exhibit impaired production of natural Ab and induced responses to T cell-dependent Ags and some T cell-independent (TI) Ags (8–14). In the case of CD19−/− mice, impaired responses to some TI Ags may be explained by the absence of the marginal zone and B-1 populations combined with attenuated BCR signaling by follicular B cells (15). Cr2−/− mice retain the marginal zone and B-1 cell compartments, but still show quantitative defects in Ab responses to some TI Ags (11, 12, 14). CD19 deficiency presents a more severe phenotype than the loss of CD21, because CD19 transduces signals by CD21 as well as the BCR. However, CD21 exerts an additional CD19-independent role on FDCs to capture and retain opsonized Ags for B cell selection and propagation (16, 17). Together, these findings suggest that C3d deposition on foreign Ag and subsequent recognition by CD19/CD21 are crucial events in the Ab response, and that complement activation must be carefully regulated to prevent autodestructive effects during Ab-mediated inflammation.

Rheumatoid arthritis (RA) is a common chronic inflammatory disease affecting the joints and is correlated with local complement activation (18, 19). In RA, the release of collagen types I (I) and II (II) serves as a biomarker for damaged bone and cartilage, respectively (20). Immunization of susceptible strains of mice with heterologous foreign II leads to production of autoantibodies and...
subsequent initiation of cartilage and subchondral bone similar to human RA (21, 22). DBA/1 mice (H-2b) show a high penetrance of disease after immunization with heterologous CII (23). B cells and CD4+ T cells are required for CIA, which is instigated by the production of arthritogenic anti-CII Abs (24–26). In addition, increasing evidence suggests that B cells play an important role in the recognition, processing, and presentation of native CIA (27, 28).

Disease progression in CIA can be divided into the early lymphocyte-dependent inductive phase and the Ab-dependent effector phase, where it has been shown that transfer of arthritogenic anti-CII Abs is sufficient to cause disease (26, 29, 30). Joint destruction is caused by cartilage-bound, CII-specific Abs or other immune complexes (31) that activate complement- and FcR-bearing inflammatory cells. Indeed, the pathology associated with CIA requires the complement components C3 and C5, and cells bearing C5a receptor and/or FcγR (32–37). C3 cleavage products function as opsonins, chemoattractants, and precursors to C5a formation in the effector phase and are also required for optimal anti-CII titers (33). Several reports have now shown that C3d(g) binding promotes Ab responses to foreign Ags (7, 38, 39); however, the consequences of C3d deposition and CD21-dependent adjuvant effects have not been addressed in CIA or other models of autoimmune disease.

In this study we show that C3d-bound CII is sufficient to cause disease independently of the broadly immunostimulatory effects of CFA that are otherwise required. Consistent with this finding, transient depletion of C3 at the time of immunization with CII in CFA lessens the incidence and severity of CIA. The expression of CD19 on B cells as well as that of CD21 on B cells or FDCs are important for disease susceptibility, indicating that C3d promotes autoimmunity by direct activation of B cells and through increased retention of CII by FDCs. In addition, lymph node cells from primed CD21−/− mice do not efficiently activate CII-specific T cells, indicating that coreceptor function is required for the role of B cells as the primary APC type in this disease. These findings demonstrate that the natural avidity of C3d can induce the production of pathogenic autoantibodies and thus is of relevance to other B cell-dependent autoimmune diseases that are initiated in the context of inflammation.

Materials and Methods

Mice

CD19−/− mice were generated previously (9). CD21−/− mice were provided by Dr. V. M. Holers (University of Colorado Health Science Center, Denver, CO). CD19−/− and CD21−/− mice were backcrossed to DBA/1J wild-type mice (The Jackson Laboratory) for seven generations or more, as indicated in text. All experiments were performed on backcrossed, age-matched, sex-matched, CD19−/− and CD19+/+ or CD21−/− and CD21+/+ mice. CD19−/−, CD19−/+, CD21−/−, CD21−/+, CD21+/− animals with −1 × 107 tail vein-injected bone marrow cells after lethal irradiation (1000 rad) to gauge the efficiency of reconstitution, chimeric mice were screened after 8 wk for the absence or the presence of CD21-expressing cells in the peripheral blood by flow cytometry. All animals were maintained under pathogen-free conditions and handled in accordance with the guidelines set forth by the animal subjects program at University of California-San Diego.

Serum C3 depletion in mice

Mice were given 18 U of cobra venom factor (CVF; Quidel) 24 h before primary or secondary immunization with heterologous bovine CII (BCII). Serum levels of C3 at the time of immunization and 10 days after immunization were measured by ELISA on serum samples collected by retro-orbital bleed. ELISAs for serum C3 levels were performed by coating 96-well plates (BD Biosciences) with serial dilutions of serum, and C3 levels were detected by means of a polyclonal goat anti-mouse C3 IgG antiserum (ICN Biotechnologies), followed by a secondary rabbit anti-goat IgG alkaline phosphatase-labeled reagent (Southern Biotechnology Associates), and p-nitrophenyl phosphate (Southern Biotechnology Associates) substrate for visualization at 405 nm using a Versamax microplate reader (Molecular Devices).

Collagen-induced arthritis

CD21−/− or CD19−/− mice (6–8 wk old) were immunized by intradermal injection at the base of the tail with either 100 μg of BCII (50 μl; Chondrex) or 50 μl of CFA (Sigma-Aldrich) or CFA plus PBS alone. A boost of either 100 μg of BCII in PBS or PBS alone was injected i.p. on day 21 in experiments involving clinical, histopathological, and serological analyses. CD21 chimeric mice were allowed to reconstitute for 8 wk before starting the immunization protocol. The CD3d-bioreagent was created by PCR cloning three copies of murine C3d into the PinPoint vector (Promega), which allows for in vivo biotinylation of C3d at its N terminus in Escherichia coli. Copies of C3d were introduced into PinPoint sequentially using a common 5′ primer (5′-ATCGGGCGCGGGCGCCACACT GATCGTGACCCCCCGAAGGGCTGGG-3′) and distinct 3′ C3d primers encoding Myc (5′‘-TGGCAGATCTCTTTGGACATCC TCTTCTGGCCTCTCTCCTCCGCGGGGAGGTTGAAGGACC TCTCCTCCGCGCCCCGCCGCGGGGAGGTTGAAGGACCT GATCGTGACCCCCCGAAGGGCTGGG-3′), Flag (5′-AAAGTTATCTATCATCTCCTGGTGTATTACCGCT CTCGCTCTCCGCGCCCCGCCGCGGGGAGGTTGAAGGACCT GATCGTGACCCCCCGAAGGGCTGGG-3′), or His (5′- TCAAGTTGCTGTGGTGGTGGGCTCCTCCGCGCCCCGCCGCG GGAGGTTGAAGGACCT GATCGTGACCCCCCGAAGGGCTGGG-3′) epitope tags. The C-terminal His tag was used for batch purification on cobalt beads (Pierce), dialyzed extensively, and found to be endotoxin free. C3d-bio was coupled to in vitro biotinylated (EZ-Link Sulfo-NHS-Biotin; Pierce) BCII in the presence of avidin (Sigma-Aldrich) at a 1:2:1 molar ratio (BCII-bio:avidin:C3d-bio). An amount of C3d-bio equivalent to 100 μg of BCII was emulsified in IFA (Sigma-Aldrich) before injection.

Clinical scoring

A 0–16 clinical scoring method was used to evaluate the induction and severity of arthritis. Each foot was evaluated on a 0–4 scale of severity (0, no inflammation; 1, swelling surrounding the ankle; 2, swelling extending into the upper foot; 3, swelling extending into the lower foot; 4, swelling extending into the toes). The summation of individual evaluation scores for all four feet per animal produced a 0–16 clinical score per mouse. All group scores were taken as the average clinical score for all mice in each group, and SDs were calculated. All clinical evaluations were performed blind to investigators.

Ab titers

Detection of anti-collagen Abs in the sera of mice was performed by standard ELISA. Peripheral blood serum was isolated from mice by retro-orbital bleed before immunization and at 7- to 10-day intervals after immunization. An empirically determined optimal dilution of 1/500 was chosen because it represented 50% maximum binding on the linear portion of the binding curve. Collagen-specific Abs were captured from serum using either 10 μg/ml BCII- or CII-coated (Chondrex) 96-well plates (BD Biosciences) and were detected with alkaline phosphatase-conjugated rat anti-mouse IgG, IgM, IgG2a, IgG2b, IgG1, and IgG3 secondary Abs (Southern Biotechnology Associates) applied at a 1:2000 dilution. p-Nitrophenyl phosphate (Southern Biotechnology Associates) substrate was used for visualization at 405 nm using a Versamax microplate reader (Molecular Devices).

Histology

Mice were euthanized 60 days after primary immunization, and right hind feet were removed for histological examination. Samples were cleaned of skin tissue, fixed for 24 h in Safe-Fix solution (Fisher Scientific), and decalcified for 7 days in decalifier B solution (Fisher Scientific). Samples were paraffin embedded, microtome sectioned along the sagittal axis, and H&E stained (performed by Comparative Biosciences). Ankle sections were visually analyzed for signs of lymphocytic infiltration into the cartilage and bone, cartilage and bone erosion, synovial hyperplasia, and extra-articular inflammation.

Enumeration of lymph node cell populations

Popliteal and inguinal lymph nodes were isolated 11 days after immunization with BCII (immunizations were performed as described above), and single-cell suspensions were prepared. Cells (1 × 106) were stained with 15 μl of cell-permeable anti-CIITA-FITC, anti-B220-allophycocyanin (both from BD Pharmingen), and biotinylated anti-CD4 (eBioscience) diluted in PBS containing 1% FBS (Omega Scientific). Cells were washed with PBS plus 1% FBS and incubated with streptavidin-PE (BD Pharmingen) diluted in PBS.
mice were transiently depleted of C3 by treatment with CVF 1 day before primary and secondary immunizations with BCII. Effective depletion of serum C3 was verified by ELISA at the time of primary immunization with BCII in CFA and secondary immunization with BCII in PBS; C3 levels returned to normal within 10 days (data not shown). We found that C3 depletion at the time of immunization delayed the onset and reduced the clinical severity of CIA compared with immunized mice that did not receive CVF pretreatment (Fig. 1c). Thus, C3 is important during the inductive phase of CIA in addition to its other known effector functions during the inflammatory phase of the disease (33).

**CD21⁻/⁻ and CD19⁻/⁻ mice are protected from CIA**

CD3 binds BCII expressed on B cells and FDCs. On B cells, CD21 is thought to promote activation via recruitment of CD19, whereas on FDCs CD21 is thought to act by passively retaining C3d-bearing Ags and immune complexes (5). To determine the cellular and molecular bases of C3d-dependent autoimmune disease induced by BCII, we examined disease progression in CD21⁻/⁻ and CD19⁻/⁻ mice backcrossed to DBA/1 for seven or 10 generations, respectively. Groups of age- and sex-matched mice were immunized intradermally with BCII/CFA or C3d⁻/⁻-BCII/IFA, and all were boosted with BCII in PBS on day 21. CD21⁻/⁻ mice were found to be resistant to CIA caused by BCII/CFA or C3d⁻/⁻-BCII/IFA immunization (Fig. 2a). Because this failure to develop disease could be due to impaired FDC and/or B cell function, reciprocal bone marrow chimeric animals were generated, immunized with BCII or C3d⁻/⁻-BCII, and evaluated by clinical score. Interestingly, the expression of CD21 on either B cells or radio-resistant FDCs was sufficient to cause disease (Fig. 2b). Because CD19 is thought to be the primary signal-transducing component for CD21 on B cells, we examined disease progression in CD19⁻/⁻ DBA/1 mice. No clinical signs of CIA were observed in BCII-immunized CD19⁻/⁻ DBA/1 mice, which were indistinguishable from mice immunized with CFA alone (Fig. 2c). Thus, both CD19 and CD21 are essential for CIA. CD21 exerts an important action on FDCs, presumably by trapping BCII, whereas on B cells it binds BCII that has become C3d opsonized. CD19 expression on B cells appears to be important for the activation of B cells by C3d-bound and unbound forms of BCII.

**CD19⁻/⁻ and CD21⁻/⁻ mice produce altered and distinct Ab responses to BCII**

Because passive transfer of CII-specific Ab is sufficient to cause arthritis (26, 29, 30), we reasoned that CD19⁻/⁻ and CD21⁻/⁻ DBA/1 mice may be nonsusceptible to CIA because of an impaired humoral response to BCII. Therefore, during the course of clinical evaluation, blood sera were collected at weekly intervals from immunized mice, and BCII-specific Ab titers were measured. Both CD19⁻/⁻ and CD21⁻/⁻ DBA/1 animals displayed reduced IgM and IgG responses to BCII after primary immunization with BCII/CFA, and failed to mount an elevated IgG response upon secondary immunization with BCII (Fig. 3, a and b). Disease incidence was 100% in DBA/1 mice and 0% in DBA/1-backcrossed (F10) CD19⁻/⁻ mice (Fig. 2 and data not shown). CD21⁻/⁻ mice derived from an F1 or F2 intercross of CD21⁻/⁻ mice on the DBA/1 background presented disease at an incidence of 55 or 85%, respectively, consistent with the required coinheritance of multiple DBA/1-derived susceptibility genes (data not shown). Interestingly, CD21⁻/⁻ mice derived from these crosses and found to be arthritic generated high titers of BCII-specific IgG, whereas anti-BCII IgG titers from nonarthritic wild-type animals approximated those of CD21⁻/⁻ animals (Fig. 3b). BCII-specific Ab responses elicited by C3d⁻/⁻-BCII/IFA immunization of CD19⁻/⁻, CD21⁻/⁻,

**Results**

**Immunization with a C3d₃-BCII conjugate is sufficient to cause CIA**

During immunization, complement C3d is rapidly generated and acts as a potent opsonin to promote B cell activation upon Ag corecognition by CD21 and the BCR (5, 7, 38, 39). We conjugated C3d to BCII to determine whether C3d opsonization confers immunogenicity and the induction of autoimmune disease. The native triple-helical structure of CII needs to be maintained to cause disease (21, 40). Therefore, instead of preparing a BCII-C3d fusion protein, murine C3d was produced from a bacterial expression vector that encoded an N-terminal biotin tag fused to three copies of C3d (C3d₃-bio). The C3d₃-BCII conjugate was generated by admixing C3d₃-bio and in vitro biotinylated BCII in the presence of avidin (see Materials and Methods).

To test the pathogenicity of C3d₃-BCII, DBA/1 mice were immunized intradermally with C3d₃-BCII in IFA, which lacks the mycobacterial membrane component of CFA. These animals were directly compared with a group of DBA/1 mice that received an equivalent amount of unconjugated C3d₃-bio/BCII-bio in CFA. All mice received a second i.p. injection of BCII in PBS on day 21. Disease was monitored over a 60-day period according to standard protocols (41). Strikingly, we found that immunization with C3d₃-BCII induced disease of similar clinical severity as that observed in BCII/CFA-treated mice (Fig. 1a). Importantly, multimerized BCII or unconjugated C3d₃-bio/BCII-bio were nonimmunogenic (Fig. 1a), indicating that linkage of C3d to BCII is required for its adjuvancy. Consistent with the clinical scoring, only immunization with C3d₃-BCII/IFA or BCII/IFA induced BCII-specific Ab (Fig. 1b). Of note, BCII in CFA induced a more robust IgG2a response (Fig. 1b), which is probably attributed to the known Th1 bias induced by CFA immunization. These findings demonstrate that C3d fixation to BCII is sufficient to cause autoimmune disease in the absence of coadministered mycobacteria.

Conjugation of C3d to BCII obviates the need for proteolytic processing of C3. To determine whether C3-derived opsonins promote CIA during the early inductive phase of the disease, DBA/1
and DBA/1 mice were of similar kinetics and magnitude to those of BCII/CFA-immunized animals (Fig. 3, c and d).

Although both CD19−/− and CD21−/− mice were protected from developing CIA and exhibited similar impairments in total anti-BCII IgG titers, they differed significantly in the contribution of particular isotypes (Fig. 3, e and f). CD19−/− mice mounted a reduced primary IgM response and a gradual increase in IgG2a/IgG2b titers, and failed to produce any measurable BCII-specific IgG1 Ab (Fig. 3e). CD21−/− mice also exhibited an impaired primary IgM response and produced reduced, but measurable, titers of IgG2a, IgG2b, and IgG1 to BCII (Fig. 3e). Because IgG1 does not efficiently activate complement, shared reductions in BCII-specific IgG2a/b in CD19−/− and CD21−/− mice are probably of greater relevance to the disease. Secondary immunization on day 21 failed to induce elevated levels of BCII-specific Ab in CD19−/− mice and only induced a modest response in CD21−/− mice (Fig. 3, e and f). Thus, the humoral response to BCII immunization is present, but reduced, in DBA/1 mice lacking CD19 or CD21, with a more marked impairment in the secondary response. CD21−/− and CD19−/− mice exhibited quantitative reductions in BCII-specific Ab, but no evidence of arthritis, as measured by joint swelling. Therefore, to ensure that our clinical scoring was consistent with joint pathology, sections of rear ankle tissue of experimental mice were examined 60 days after immunization for histologic signs of arthritic lesions, as revealed by H&E staining (Fig. 4). Bone and cartilage erosion, synovial hyperplasia, pannus tissue formation, and extra-articular inflammation were evident in BCII-immunized CD19−/− DBA/1 mice (Fig. 4, left panels). However, joint tissue sections from CD19−/− DBA/1 mice immunized with BCII appeared normal (Fig. 4, right panels). These findings indicate that the modest levels of anti-BCII IgG induced in CD19−/− animals are not arthritogenic.

Reduced autoantibody production to mouse CII (MCII) in CD19−/− and CD21−/− mice

In the experiments described above, a heterologous source of CII induces autoreactivity and the onset of disease. However, because chronic disease and autodestruction of joint tissues are due to the propagation, activation, and differentiation of B cells reactive to autologous collagen, we assessed autoantibody titers to cartilage-derived MCII. Immunization with BCII/CFA (Fig. 5, a and b) or C3d3-BCII/IFA (Fig. 5, c and d) induced production of MCII-specific IgG in DBA/1 mice. As was the case...
with Ab titers to BCII, both CD19<sup>−/−</sup> (Fig. 5, a and c) and CD21<sup>−/−</sup> (Fig. 5, b and d) mice produced significant, but reduced, titers of anti-MC-II Abs. Arthritic CD21<sup>−/−</sup> mice derived from the F<sub>7</sub> backcross to DBA/1 produced elevated and sustained levels of MCII-specific Abs, whereas nonarthritic CD21<sup>−/−</sup> cohorts produced titers similar to those of CD19<sup>−/−</sup> or CD21<sup>−/−</sup> animals (Fig. 5, b and d). These reduced titers may account for the reduced susceptibility to CIA observed in CD19<sup>−/−</sup> and CD21<sup>−/−</sup> animals.

**CD19<sup>−/−</sup> and CD21<sup>−/−</sup> mice do not produce germinal centers (GCs) in response to BCII immunization**

We have shown that immunization with either C3d<sub>3</sub>-BCII/IFA or BCII/CFA induces the production of pathogenic Abs to MCII. The
The generation of these Abs may arise from the induced differentiation of pre-existing self-reactive B cells. Alternatively, the process of somatic hypermutation, which drives affinity maturation in the GC, may also allow for the generation of B cells that have acquired autospecificities. Provision of T cell help allows for additional B cell differentiation and Ag-driven selection in the GC. In CD19<sup>−/−</sup> mice, GCs are generally absent in the spleen and lymph nodes (8, 9), but can be found in Peyer's patches (42). GCs are reduced in size and frequency in CD21<sup>−/−</sup> mice (11, 12, 43), but the defect is less severe than in CD19<sup>−/−</sup> animals. For these reasons, it was important to evaluate GC formation in CD19<sup>−/−</sup>, CD21<sup>−/−</sup> and DBA/1 mice after CII injection. Cells were isolated from the draining lymph nodes of naive or BCII-immunized (day 11) mice and were analyzed by flow cytometry to enumerate B cells (B220<sup>+</sup>), GC B cells (B220<sup>+</sup>GL7<sup>+</sup>), and Th cells (CD4<sup>+</sup>). No significant change in CD4<sup>+</sup> Th cell number was observed in any of the mice after immunization (data not shown), but the percentage of B cells in the draining lymph nodes of all immunized mice was ~2-fold.

**FIGURE 3.** CD19<sup>−/−</sup> and CD21<sup>−/−</sup> mice produce altered Ab responses to BCII. The production of BCII-specific serum IgG was measured by ELISA. 

- **a** and **c**, CD19<sup>−/−</sup> (●), CD19<sup>−/−</sup> (○), and DBA/1 mice (●); **b** and **d**, CD21<sup>−/−</sup> mice that developed arthritis (●), CD21<sup>−/−</sup> mice that did not develop arthritis (●), CD21<sup>−/−</sup> mice (○), and DBA/1 mice (●). **a** and **b**, Mice were immunized with BCII in CFA or CFA alone. **c** and **d**, Mice were immunized with conjugated C3d<sub>3</sub>-BCII in IFA or unconjugated C3d<sub>3</sub>-bio and C3d<sub>3</sub>-bio in IFA. All animals were boosted on day 21 with BCII in PBS. Results are the mean ± SD of ELISAs performed in triplicate. Data shown are representative of multiple experiments (n = 7–9 mice/group).
higher after immunization (Fig. 6). Interestingly, only DBA/1 mice generated GL7+ GC B cells (Fig. 6, a and b), which were present in the draining lymph nodes, but not in spleen. Immunization with C3d-linked BCII was also shown to promote GC formation in lymph nodes, whereas unlinked BCII controls do not (Fig. 6c). The GC phenotype was confirmed by histologic staining of spleen and lymph node sections with peanut agglutinin lectin (data not shown). Thus, the observed failure to generate GCs is consistent with the weak secondary responses to BCII and MCH class II.

**Impaired activation of BCII-specific T cells from CD19^-/- and CD21^-/- mice**

Generation of pathogenic IgG Abs requires the provision of T cell-derived stimuli, which largely explains the contributions of B and T cells to CIA. However, because the native conformation of CII is required for immunogenicity, it is also apparent that B cells function as key APCs in this disease. Therefore, to determine the contributions of CD19 and CD21 to T cell activation, lymph node cells were isolated from immunized CD19^-/-, CD21^-/-, and DBA/1 mice and assayed for T and B cell proliferation after antigen rechallenge in vitro. Draining lymph node cells were isolated from mice 9 days after immunization with 100 µg of BCII in IFA or CFA alone. Cells were cultured with or without 50 µg/ml native or hdbCII for 60 h, and proliferation was measured by BrdU incorporation. Naive lymph node cells from CFA-immunized wild-type, CD19^-/-, or CD21^-/- mice did not proliferate in response to BCII challenge in vitro (Fig. 7, a and b). Lymph node B and T cells from immunized DBA/1 mice proliferated in response to Ag rechallenge. However, lymph node cells from immunized CD19^-/- and CD21^-/- mice could not be induced to proliferate in response to BCII, but did respond to T and B cell mitogens (Fig. 7, a and b).

To directly evaluate T cell differentiation in terms of cytokine production, in vitro-stimulated lymph node cells from DBA/1, CD19^-/-, and CD21^-/- mice were surface stained for CD4, followed by intracellular staining for IL-2, IFN-γ, and IL-4 production. The percentage of IL-2, IFN-γ, or IL-4-producing cells was enumerated by flow cytometry (Fig. 7, c–e). Only lymph node CD4+ T cells from BCII-primed DBA/1 mice were activated in an Ag-specific manner to become BCII-specific Th effectors, as measured by IL-2, IFN-γ, and IL-4 production upon re-encounter with native BCII. The native conformation of BCII was important for the immunogenicity of BCII, because hdbBCII did not induce cytokine production. Additionally, the stimulation of wild-type T cells with the Ag-nonspecific mitogen Con A led to the production of all three cytokines (Fig. 7, c–e). By contrast, CD4+ T cells from CD19^-/- and CD21^-/- mice did not respond in an Ag-specific manner to BCII re-encounter, consistent with the proliferation data. These findings suggest that the reduced GC and secondary Ab responses observed in BCII-immunized CD19^-/- mice may be attributed at least in part to inefficient Ag-specific T cell activation. In summary, this work underscores the importance of B cells and, specifically, the B cell coreceptor complex in the uptake and presentation of C3d-bound CII for the elicitation of T cell help as a prerequisite for the development of inflammatory arthritis.

**Discussion**

In the current work we demonstrate that mice immunized with C3d-bound BCII in IFA develop an autoimmune condition similar to that observed in mice treated with BCII in CFA. Susceptibility to CIA requires CD19 on B cells as well as CD21 on B cells or FDCs to promote B cell activation and propagation. Impaired activation of CII-specific T cells and failure to form GCs in CD19^-/- and CD21^-/- DBA/1 mice culminate in the inability to produce elevated and sustained levels of BCII- and MCH-specific IgG. Hence, C3d-binding and coreceptor-dependent B cell activation are important determinants for developing CIA.

The enzymatic cleavage of C3 is a highly amplified step in the complement cascade. To avoid autodestruction of host constituents, most C3 products are short-lived or rapidly inactivated. C3d may be an exception to this regulation, because it is a terminal cleavage product. Of relevance, C3d accumulation and excretion are of diagnostic value for autoimmune diseases involving impaired clearance of immune complexes such as RA or lupus (44, 45). To date, the adjuvant activity of C3d has only been studied in the context of Ab responses to foreign Ags. In this study we used the CIA model to demonstrate that the inappropriate deposition of C3d is not only correlative, but can also be causative of B cell-dependent autoimmune disease.

Similar to other models of induced autoimmune disease, the DBA/1 model of inflammatory arthritis requires the use of CFA to activate innate immunity (46). One of the immunostimulatory effects of CFA is complement activation (2), resulting in C5a-dependent recruitment and activation of effector cells that precipitate joint destruction in CIA (32–34, 37). C3^-/- mice and mice with impaired C3 convertase activity also show reduced susceptibility to CIA (33, 47, 48). However, these studies cannot assign the relative importance of C3 in inducing lymphocyte activation in the early asymptomatic stages of the disease vs recruitment of myeloid effector cells during the late symptomatic stages. We found that transient depletion of C3 by pretreatment of mice with CVF significantly decreased the onset and severity of CIA. Because serum C3 levels return to normal long before any clinical symptoms of disease are noted, this finding supports an early role for C3 in the inductive phase of the disease as well as serving as an essential precursor for the biosynthesis of C5a during the effector phase.

Cleavage of C3 generates a soluble C3a fragment and a bound C3b fragment that is further cleaved into the opsonins iC3b and C3dg. The fact that C3d fixation to CII was sufficient to cause disease without an overt role for CFA in eliciting inflammatory mediators indicates a direct role for C3d in the activation of CII-specific B cells and the production of arthritogenic Abs. Indeed, we showed that expression of the C3d receptor CD21 was required.
FIGURE 5. CD19<sup>−/−</sup> and CD21<sup>−/−</sup> mice show decreased production of MCII-reactive autoantibodies. The production of MCII-specific serum IgG was measured by ELISA. a and c, CD19<sup>−/−</sup> (▲), CD19<sup>−/−</sup> (●), and DBA/1 mice (○). b and d, CD21<sup>−/−</sup> mice that developed arthritis (▲), CD21<sup>−/−</sup> mice that did not develop arthritis (●), CD21<sup>−/−</sup> mice (○), and DBA/1 mice (○). a and b, Mice were immunized with BCII in CFA or CFA alone. c and d, Mice were immunized with conjugated C3d<sub>3</sub>-BCII in IFA or unconjugated BCII-bio and C3d<sub>3</sub>-bio in IFA. All animals were boosted on day 21 with BCII in PBS. Results are the mean ± SD of ELISAs performed in triplicate. Data shown are representative of multiple experiments (n = 7–9 mice/group).

FIGURE 6. CD19<sup>−/−</sup> and CD21<sup>−/−</sup> mice do not produce GCs in response to BCII immunization. The percentage of total lymph node cells representing B cells (B220<sup>+</sup>) and GC B cells (B220<sup>+</sup> GL7<sup>+</sup>) 11 days after immunization with BCII in CFA (a and b) or BCII-C3d<sub>3</sub> in IFA (c) is shown. Data shown are representative of three experiments performed with two or more mice per group.
for disease susceptibility, but bone marrow chimera analysis revealed that the expression of CD21 on either B cells or FDCs was sufficient to elevate Ab responses to BCII and allow disease after immunization with BCII/CFA or C3d3-BCII/IFA. The finding of additive, yet distinct, contributions of CD21 expressed on FDCs and B cells is in agreement with responses to other T cell-dependent Ags (17, 49). CD21 is not thought to have a signal-transducing ability for FDCs, but acts to retain opsonized Ags and immune complexes. By contrast, CD21 acts in concert with the BCR to mediate corecognition of C3d(g)-bearing Ags (5). Thus, our findings indicate that the role of CD21 in either promoting B cell activation or retaining Ag by FDCs is sufficient to cause disease.

CD21 is thought to augment B cell activation through recruitment of CD19, which is also a proximal substrate for signal transduction induced by BCR engagement alone. We found that CD19<sup>−/−</sup> mice were not susceptible to CIA after immunization with BCII/CFA or

**FIGURE 7.** Ineffective generation of BCII-specific B and T cells in CD19<sup>−/−</sup> and CD21<sup>−/−</sup> mice. a and b, Draining lymph node cells were isolated from wild-type (WT), CD19<sup>−/−</sup>, and CD21<sup>−/−</sup> mice 9 days after immunization with BCII in CFA or with CFA alone. Lymph node cells were cultured with medium alone, 50 μg/ml BCII, 50 μg/ml hdBCII, 1 μg/ml LPS (a), or 1 μg/ml Con A (b–e) for 48 h in the presence of BrdU. The percentages of proliferating B cells (a; B220<sup>+</sup>) and T cells (b; CD4<sup>+</sup>) were determined by intracellular flow cytometric staining for BrdU. c–e, Draining lymph node cells were isolated from WT, CD19<sup>−/−</sup>, and CD21<sup>−/−</sup> mice 5 days after immunization with BCII in CFA or with CFA alone. Lymph node cells were cultured with medium alone, 100 μg/ml BCII, 100 μg/ml hdBCII, or 1 μg/ml Con A for 48 h. The percentage of CD4<sup>+</sup> lymph node cells producing cytokines was enumerated by intracellular flow cytometry staining for IFN-γ (c), IL-2 (d), or IL-4 (e). * p < 0.005; ** p < 0.01 (significant differences from cells cultured with medium alone).
CD19-binding and effector function may be dependent upon additional B cell differences in anti-CII IgG, qualitative differences in Ab specificity (29, 30), indicating that continued autoantibody production and perpetuation of anti-CII Abs induces a severe yet transient arthritic condition (26, 27). These collective findings further implicate B cells as the primary APC type in this disease.

Although the production of CII-specific Ab is clearly required for CIA, parameters of the anti-CII Ab response relative to the onset and severity of disease are not well characterized. We found a strong correlation between clinical disease and the amount of anti-MCII/BCII IgG titers. Both CD19+/− and CD21+/− mice exhibited reduced anti-CII IgG and were resistant to disease. Anti-CII titers and disease susceptibility were restored in chimeric mice expressing CD21 on B cells or FDCs. Passive immunization with CII in its native conformation (21, 40), and CII-opsonized CII bound to FDCs. Future studies of spontaneous or inflammation-induced binding of C3d to self-Ags will probably reveal the importance of this innate mechanism in the etiology of other B cell-dependent autoimmune diseases.

Acknowledgments

We thank Dr. Gary S. Firestein (University of California-San Diego) for acquainting us with the CIA model and its use, Dr. V. Michael Holers for providing CD21+/− mice and discussing unpublished results, Dr. D. Karp for discussions, and members of the Rickert laboratory for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References