Complement Receptors 1 and 2 Influence the Immune Environment in a B Cell Receptor-Independent Manner

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Complement Receptors 1 and 2 Influence the Immune Environment in a B Cell Receptor-Independent Manner1,2

Amanda C. Jacobson, Janis J. Weis, and John H. Weis3

The CD21/35 proteins are complement receptors implicated in controlling and interpreting activation states of the innate and acquired immune responses. One defect of CD21/35−/− animals is depressed production of Ag-specific IgG3 which we show is evident in vivo but not in vitro. Gene expression profiles obtained from naive wild-type and CD21/35−/− splenocytes demonstrated enhanced expression of inflammatory mediators from CD11b+ splenocytes in the CD21/35−/− animals. Splenocyte populations between wild-type and CD21/35−/− mice were similar except for a moderate increase in GR1+CD31+ immature myeloid cells. Furthermore, depletion of neutrophils and other GR1-expressing cells alleviates elevated inflammatory gene expression in the CD21/35−/− spleen. Complement activation also plays a key role in the differential gene expression observed in the CD21/35-deficient mouse as depletion of C3 or inhibition of C3a receptor signaling within the animal returned inflammatory gene expression within the spleen to wild-type levels. Finally, C3 depletion before immunization allowed for the enhanced production of Ag-specific IgG3 production in the CD21/35−/− mouse compared with mock-depleted animals. These data suggest that the overall environment of the CD21/35−/− spleen is quite different from that of the wild-type animal perhaps due to altered complement convertase activity. This difference may be responsible for a number of the phenotypes ascribed to the deficiency of CD21/35 proteins on B cells and follicular dendritic cells. The Journal of Immunology, 2008, 180: 5057–5066.

The complement system is an important part of the innate immune response and provides a link to adaptive responses. Effector functions of the rapidly generated complement cleavage products include lysis of susceptible microorganisms, opsonization and clearance of immune complexes, and induction of inflammatory response (1, 2). The three characterized pathways of complement activation (classical, alternative, or mannose-binding lectin) result in cleavage of the prominent serum protein C3 into C3a and C3b. Newly cleaved C3b may participate in additional C3 and C5 convertases, generating C3b and C5b as well as the potent inflammatory mediators, anaphylatoxins C3a and C5a. C3a and C5a are important for the development of inflammatory responses by recruiting and activating inflammatory cell types and have also been implicated in the pathogenesis of several chronic and acute inflammatory diseases (2, 3).

Regulation of complement convertase activation is achieved by several soluble and membrane bound complement regulatory proteins such as membrane cofactor protein, decay acceleration factor and CD35 (complement receptor 1; CR1).4 Whereas short membrane bound proteins like membrane cofactor protein and decay acceleration factor inhibit complement activation on host cell surfaces, CD35 functions as a cofactor for factor I-mediated cleavage of C3, resulting in deposition C3d, C3dg, and C3bi (4). These complement breakdown products are the ligands for CD21 (complement receptor 2; CR2). In the mouse, the CD21 and CD35 proteins are encoded by the Cr2 locus and coexpressed on B cells and follicular dendritic cells (FDC; Ref. 5). On the B cell, the CD21/35 proteins are components of the B cell coreceptor complex including CD19, CD81, and Leu13 (6), and the roles of CD21/35 in optimal B cell immune function have been analyzed. Cologation of complement-bound opsonins via CD21/35 and the BCR results in a higher level of cellular activation than BCR cross-linking alone (7, 8) as well as increased localization of the BCR into lipid rafts (9). The CD21/35 proteins can also function in transferring immune complexes from marginal zone B cells to FDCs (10).

Animals lacking the CD21/35 proteins generate modest T cell-dependent and -independent Ab responses with a prominent impairment in IgG3 responses (compared with wild type (WT; Refs. 11–13). Such animals show depressed levels of natural Abs (defined as products of the B-1 subset of B cells) (14, 15) and demonstrate increased sensitivity to lethal infection of Streptococcus pneumoniae (12). Germinal centers in CD21/35−/− mice are sparse and underdeveloped (12, 16, 17). Additionally, CD21/35−/− B cells transplanted into a WT host are lost in the germinal center, a finding that has been attributed to the absence of a CD21/CD35-mediated survival signal (18, 19).

For decades, a role for complement in enhancing Ab responses to vaccination with limiting Ag has been recognized. Mice depleted of functional serum C3 via the administration of cobra venom factor (CVF) demonstrated low immunization-specific responses compared with mock-depleted animals (20). Conversely, some complement breakdown products, such as C3a, have been

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2 The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.
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4 Abbreviations used in this paper: CR1, complement receptor 1; CR2, complement receptor 2; CR3, complement receptor 3; CR4, complement receptor 4; FDC, follicular dendritic cell; CVF, cobra venom factor; 1-Cy3, 1-Cy3 germine transcript; CL48A, C3a receptor antagonist; WT, wild type; TNP, trinitrophenol.

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shown to suppress Ab responses (21, 22), suggesting that dysregulated complement activation and anaphylatoxin production may not only initiate/enhance inflammatory responses but also influence Ab production. We chose to analyze the depressed production of IgG3 products in the CD21/35−/− mouse to determine whether the suppression of isotype switching to IgG3 was due to an intrinsic defect in the B cells themselves, or whether an altered microenvironment of the spleen dictated this response. In this report, we show that B cells from CD21/35−/− mice are fully capable, in tissue culture, of producing IgG3 gene products suggesting the splenic environment of the CD21/35−/− animal is depressing IgG3 isotype switching. Gene expression analysis between WT and CD21/35−/− splenocytes demonstrated a fundamental difference in inflammatory gene expression that was enhanced in the CD21/35−/− mice. The transcriptional profile of the CD21/35−/− mouse as well as IgG3 production could be restored to near WT levels by depleting mice of serum C3. These data suggest that a normal function of CD21/CD35 is to limit the level of complement convertase activity in lymphoid tissues thus restricting the production of complement breakdown products such as the anaphylotoxins C3a and C5a.

Materials and Methods

Mice

Female BALB/c mice were obtained from the National Cancer Institute. CD21/35−/− mice were generated as described (12) and backcrossed 10 times onto BALB/c backgrounds and used for the experiments described. Animals were housed in the Animal Resource Center (University of Utah Health Science Center, Salt Lake City, UT) according to the guidelines of the National Institutes of Health for the care and use of laboratory animals.

Gene expression analysis

Equal amounts of total RNA from the spleens of five naive BALB/c or female 8-wk-old CD21/35−/− mice were pooled into a single sample that was prepared for Affymetrix array hybridization. cDNA was synthesized from 6 μg of total RNA, and each sample was hybridized to a single GeneChip Mouse Genome 430 2.0 Array (Affymetrix). Data from GeneChip Mouse Genome 430 2.0 Array (Affymetrix). Animals were housed in the Animal Resource Center (University of Utah Health Science Center, Salt Lake City, UT) according to the guidelines of the National Institutes of Health for the care and use of laboratory animals.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from spleen tissue using CsCl guanidine extraction. After RBC lysis, single-cell populations of splenocytes were labeled with B220 or CD11b magnetic microbeads (Miltenyi Biotech) and separated according to the manufacturer’s protocol. Total RNA was isolated from cells using Qiagen RNAeasy kit (Qiagen). RT-PCR on 2 μg of total RNA was performed using random primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). cDNA levels were assessed using quantitative PCR on the LightCycler (Roche) as in Ref. 23. β-Actin: forward, 5′-tgaaattgcttgctgctc-3′; reverse, 5′-cttcagcttgggctc-3′, 135 bp. Alox5: forward, 5′-tgatggctggtctcctg-3′; reverse, 5′-ctgtctctgccgtctc-3′, 109 bp. Iox18f: forward, 5′-ctctcagctacagcagcc-3′; reverse, 5′-gcttctctctggcttctc-3′, 150 bp. Lcn2: forward, 5′-acacgaagccagctgtgctg-3′; reverse, 5′-tggcgaagcggagcagagc-3′, 151 bp. Chsp: forward, 5′-cgagagtggctgtgctgagc-3′; reverse, 5′-tggcgaagcggagcagagc-3′, 151 bp. Hsp90: forward, 5′-tgttgcagcgctgtgtgtc-3′; reverse, 5′-ctgctctcagctctgctc-3′, 151 bp. I-Cα: forward, 5′-ggggctctctgtcagctgctc-3′; reverse, 5′-gcctctctctgtcagctgctc-3′, 151 bp. I-Cβ: forward, 5′-ggggctctctgtcagctgctc-3′; reverse, 5′-gcctctctctgtcagctgctc-3′, 151 bp. I-Cγ: forward, 5′-tggcgaagcggagcagagc-3′; reverse, 5′-ctgctctcagctctgctc-3′, 151 bp. C3: forward, 5′-tgagagcttgctgctgctc-3′; reverse, 5′-ctgctctcagctctgctc-3′, 151 bp.

Immunizations and treatments

Mice 6–8 wk old were immunized by i.p. injection of 30 μg of trinitrophenol (TNP)-LPS (Biosearch Technologies.) and boosted 14 days later. Serum was collected from mice by retro-orbital bleeding on days 0, 7, and 15. Neutrophil depletion was achieved by injecting 150 μg of anti-GR1 Ab, clone RB6-8C5 (BD Pharmingen) or rat IgG isotype i.p. as in Ref. 24. Complement was depleted with CVF (Naja naja kaouthia; Quidel). Twelve units of CVF in 0.1 ml of PBS were administered 54 and 48 h before immunization, challenge, or spleen harvest. Inhibition of C3aR signaling was achieved by injecting 30 mg of the C3a receptor antagonist (C3aRA) SB290517 (Calbiochem) per kg in PBS i.p. at time zero and after 16 h. Spleens were harvested 24 h after the last C3aRA injection. ELISAs

Hapten-specific ELISAs were performed as described previously (25). Briefly, Immulon plates were coated with 10 μg/ml TNP-BSA overnight at 4°C. Serum samples were added at dilutions of 1/200-1/600. TNP-specific Abs were detected using HRP anti-mouse Ig (Zymed) or IgG3 (Caltag). Total Ig ELISAs were performed by coating 96-well plates with 5 μg/ml anti-mouse IgM, IgG, and IgA (Zymed) overnight, 4°C. Serial dilutions of serum samples were added, and isotype-specific Ab titers were detected as above. Mouse C3 in sera was measured by ELISA as previously described (26).

Abs and immunofluorescence analysis

Fluochrome-conjugated Abs used to identify cell types within the spleen included: CD21/35 (clone 7G6), Ly-6G (GR1; clone RB6-8C5), CD3ε (clone 145-2C11), and CD11b (clone M1/70; BD Biosciences). B220 (clone RA3-6B2; eBioscience) and F4/80 (Calu6/84). Anti-CD117 was provided by Dr. G. Spangrude (University of Utah, Salt Lake City, UT). Cell staining and analysis was performed as described previously (12).

Cell culture, in vitro stimulations

Single-cell suspensions were made from spleens of 6- to 8-wk-old mice, and RBCs were lysed with ACK lysis buffer. Cells were cultured at 5 × 10⁵ cells/ml in RPMI 1640 supplemented with 10% FBS (Invitrogen Life Technologies), 2 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin for 4 days with or without 30 μg/ml LPS serotype 0111:B4 (Sigma-Aldrich).

Statistical analysis

Data are given as means ± SEM. Student’s t test was used to determine whether there were significant differences between sample means.

Results

CD21/35−/− splenocytes produce IgG3 in vitro

Animals deficient in the CD21/CD35 proteins show decreased IgG responses to low-dose immunizations of T cell-dependent and -independent Ags (12). Production of Ag-specific IgG3 isotypes are suppressed in the CD21/35−/− mouse under most immunization strategies, whereas production of other isotypes (IgG1, IgG2a, and IgG2b) show reduced production at low dose but close to WT levels at high-dose immunizations. We chose to determine whether the selective depression of IgG3 products was due to an intrinsic defect of the CD21/35−/− B cells themselves (and thus should be evident in in vitro cultures) or was due to the splenic microenvironment of the CD21/35−/− mouse. IgG3 Ab production and I-Cγ3 germline transcripts were analyzed in mice immunized with the T cell-independent Ag, TNP-LPS. As expected CD21/35−/− mice produced similar levels of TNP-specific IgM as WT mice but less Ag-specific IgG and distinctly less IgG3 (Fig. 1, A–C). This deficiency in Ig products from the CD21/35−/− mice is not due to strain differences in that these experiments were performed with CD21/35−/− mice on the BALB/c background, whereas those published previously were on C57BL/6 or mixed backgrounds (12). Splenocytes from the CD21/35-deficient mice also produced decreased amounts of the I-Cγ3 germline transcripts necessary for IgG3 isotype switching (27) than WT mice after immunization with TNP-LPS (Fig. 1D). A standard protocol to induce IgG3 isotypes from bulk splenocyte cultures is to incubate such cells with LPS without BCR activation (28–30). When total splenocytes from WT or CD21/35−/− animals were cultured for 4
days with LPS, cells from CD21/35−/− mice proved capable of producing equivalent IgG3 Abs in the supernatant and I-Cy3 splenocytes (Fig. 1, E and F) indicating there is no intrinsic defect in the CD21/35−/− cells that abrogates the ability to produce IgG3.

**Differential gene expression between WT and CD21/35−/− splenocytes**

The conflicting in vivo and in vitro IgG3 Ab response data suggest that the splenic environment of the CD21/35−/− mouse may be altered compared with WT, which in turn could influence immune responses such as Ab production. To investigate differences within the splenic microenvironment between WT and CD21/35−/− mice, gene expression profiles from total spleen RNA of naive WT or CD21/35−/− mice were obtained using Affymetrix GeneChip Microarrays. We chose to analyze total splenocyte transcripts (instead of just B cells products) from naive WT and CD21/35−/− animals to determine whether resting baseline differences in gene expression existed between the heterogeneous cell populations of the entire lymphoid organ.

The microarray data from this analysis indicated that a variety of genes are differentially expressed between WT and CD21/35−/− splenocytes (Table I). The majority of the genes with greater expression in the CD21/35−/− spleen (positive fold changes) can be grouped as inflammatory genes or those involved with platelet activation and innate responses. WT splenocytes showed greater expression of genes (negative fold changes compared with the mutant) involved in leukocyte recruitment and cell activation. In total, the CD21/35−/− samples demonstrated greater expression of 239 genes (with ≥2-fold difference), whereas the WT animals possessed 434 genes with ≥2-fold expression compared with the mutant counterparts (Supplemental Table I).5

We chose a subset of these differentially expressed genes for RT-PCR confirmation by selecting genes from the various subsets of Table I that demonstrated elevated expression in the CD21/35−/− spleen (Fig. 2). Thus arachidonic 5-lipoxygenase (Alox5) and cathepsin G (Ctsg) were selected from the inflammation category, lipocalin 2 (Lcn2) from the antimicrobial grouping, coagulation factor V (Fv) from the coagulation/thrombosis subset, and killer cell lectin-like receptor α 18 (Klra18) from the leukocyte recruitment category. D site albumin promoter binding protein (Dbp) was selected as a control in that it was expressed higher in the WT compared to the CD21/35−/− samples. Gene-specific oligonucleotide sequences were derived, optimized and used for RT-PCR analysis with mRNA obtained from total splenocytes isolated from individual WT and CD21/35−/− mice not used in the original Affymetrix screen. This analysis confirmed the differential expression of these genes between the splenic samples obtained from the WT and CD21/35−/− animals.

**Altered gene expression in the CD21/35−/− spleen is due to inflammatory cell types**

The microarray analysis was conducted using RNA obtained from total splenocytes. The lack of the signaling potential from the CD21/CD35 proteins on B cells and FDCs could implicate these cells as responsible for the changes in gene expression. Alternatively, the lack of receptors for C3 and C4 breakdown products on B cells and FDCs could alter total C3b/C4b binding within the spleen or influence the decay of complement convertases. The latter could allow for greater complement consumption and enhanced release of anaphylatoxins. Other cells types expressing complement receptor 3 (Cr3), complement receptor 4 (Cr4), or the receptors for C3a and C5a within the spleen might be responsible for the altered gene products.

To begin to address this question, we determined the cell types responsible for the differential expression of a number of inflammatory genes within the CD21/35−/− naive spleen. As seen in Table II, the differential expression of these genes could be traced to a B220/CD11b+ sorted cell population. Alox5 expression,

![Figure 1](http://www.jimmunol.org/DownloadedFromImage.png)
which had a 20-fold increase in expression in the CD21/35\(^{−/−}\) spleen compared with WT, was further targeted to CD11b\(^{+}\)/GR1\(^{high}\)-expressing cells, indicative of neutrophils. Interestingly, CD11b\(^{+}\)/GR1\(^{high}\) cells sorted from WT spleens had undetectable transcript levels for the majority of the genes analyzed suggesting that the subset of neutrophils within the CD21/35\(^{−/−}\) spleen is more transcriptionally active (immature) than the WT counterparts (see below).

**Klra18**, a NK cell-related gene, was paradoxically expressed higher in both B220\(^{+}\) and B220\(^{−}\) cells of the CD21/35\(^{−/−}\) strain compared the WT animal (Table II). The elevated level of expression of inflammatory and other genes in the CD21/35\(^{−/−}\) spleen could be due to increased expression of the genes in the existing population of CD11b\(^{+}\) cells, or the preferential recruitment/retention of such cells in that spleen (compared with WT).

To determine whether splenic cell populations were skewed in these

**FIGURE 2.** RT-PCR confirmation of genes differentially expressed between CD21/35-deficient and WT splenocytes. Gene transcripts, relative to \(β\)-actin, were quantified from total spleen samples harvested from naive WT and CD21/35\(^{−/−}\) mice. Data are fold changes observed in CD21/35\(^{−/−}\) compared with WT (\(n = 5\)).

### Table I. Expression levels of immune response-related\(^a\) gene transcripts differentially expressed in CD21/35\(^{−/−}\) splenocytes relative to BALB/c WT splenocytes

<table>
<thead>
<tr>
<th>Symbol; Gene Title</th>
<th>Probe Set ID</th>
<th>Fold Difference(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>i. Inflammation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alox5; arachidonate 5-lipoxygenase</td>
<td>1441962_at</td>
<td>20.3</td>
</tr>
<tr>
<td>Ear1; eosinophil-associated RNase</td>
<td>1421802_at</td>
<td>9.8</td>
</tr>
<tr>
<td>Csg; cathepsin G</td>
<td>1419594_at</td>
<td>4.3</td>
</tr>
<tr>
<td>Prm3; proteinase 3</td>
<td>1419669_at</td>
<td>4.0</td>
</tr>
<tr>
<td>Slpi; secretory leukocyte peptidase inhibitor</td>
<td>1448377_at</td>
<td>3.5</td>
</tr>
<tr>
<td>Mpo; myeloperoxidase</td>
<td>1415960_at</td>
<td>3.3</td>
</tr>
<tr>
<td>NE; neutrophil elastase</td>
<td>1422928_at</td>
<td>2.7</td>
</tr>
<tr>
<td>Ngp; neutrophilic granule protein</td>
<td>1418722_at</td>
<td>2.4</td>
</tr>
<tr>
<td>Cd177; CD177 Ag</td>
<td>1424509_at</td>
<td>2.2</td>
</tr>
<tr>
<td>Reg2; regenerating islet-derived 2</td>
<td>1416139_at</td>
<td>2.1</td>
</tr>
<tr>
<td>Cd163; CD163 Ag</td>
<td>1419444_at</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>ii. Antimicrobial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Camp; cationic antimicrobial peptide</td>
<td>1419691_at</td>
<td>3.7</td>
</tr>
<tr>
<td>Chi3l3; chitinase 3-like 3</td>
<td>1419764_at</td>
<td>3.3</td>
</tr>
<tr>
<td>Lcn2; lipocalin 2</td>
<td>1427747_at</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>iii. Complement pathways</strong></td>
<td></td>
<td></td>
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<tr>
<td>CD59a; CD59a Ag</td>
<td>1429830_at</td>
<td>2.0</td>
</tr>
<tr>
<td>Adn; adipin</td>
<td>1417868_at</td>
<td>2.4</td>
</tr>
<tr>
<td>Cr2; CR2</td>
<td>1425289_at</td>
<td>6.2</td>
</tr>
<tr>
<td><strong>iv. Coagulation/thrombosis</strong></td>
<td></td>
<td></td>
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<tr>
<td>Cdk5rap1; CDK5 r.a.p. 1</td>
<td>1448626_at</td>
<td>13.9</td>
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<tr>
<td>Fv; coagulation factor V</td>
<td>1418907_at</td>
<td>2.9</td>
</tr>
<tr>
<td>Gp1ba; glycoprotein 1b, Expressed in CD21/35(^{−/−}) splenocytes relative to BALB/c WT splenocytes.-polypeptide</td>
<td>1422316_at</td>
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<tr>
<td>F10; coagulation factor X</td>
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<td>F13a1; coagulation factor XIII, A1 subunit</td>
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<td>F3; coagulation factor III</td>
<td>1417408_at</td>
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</tr>
<tr>
<td>Pafah1b1; p.a.f. acetylhydrolase</td>
<td>1456947_at</td>
<td>2.5</td>
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<td><strong>v. Leukocyte recruitment/immune cell activation</strong></td>
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<tr>
<td>Klra18; killer cell lectin-like receptor</td>
<td>1426127_x_at</td>
<td>4.9</td>
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<tr>
<td>Ly6d; lymphocyte Ag 6 complex</td>
<td>1416930_at</td>
<td>3.5</td>
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<tr>
<td>S100a9; S100 calcium-binding protein A9</td>
<td>1448756_at</td>
<td>3.2</td>
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<td>S100a8; S100 calcium-binding protein A8</td>
<td>1413949_at</td>
<td>2.9</td>
</tr>
<tr>
<td>Cxcr4; chemokines receptor 4</td>
<td>1448710_at</td>
<td>2.0</td>
</tr>
<tr>
<td>Cd3g; CD3 Ag</td>
<td>1419178_at</td>
<td>2.0</td>
</tr>
<tr>
<td>Fcgr2b; FcR, IgG low-affinity IIb</td>
<td>1451941_at</td>
<td>2.1</td>
</tr>
<tr>
<td>Itf7r; IL-7R</td>
<td>1448576_at</td>
<td>2.1</td>
</tr>
<tr>
<td>Cd5; CD5 Ag</td>
<td>1418353_at</td>
<td>2.1</td>
</tr>
<tr>
<td>Cd28; CD28 Ag</td>
<td>1437025_at</td>
<td>2.1</td>
</tr>
<tr>
<td>Tera; TCRα</td>
<td>1439595_at</td>
<td>2.2</td>
</tr>
<tr>
<td>Ifnkr2; IFN-γR2</td>
<td>1423557_at</td>
<td>2.2</td>
</tr>
<tr>
<td>Ilk; IL-2-inducible T cell kinase</td>
<td>1456836_x_at</td>
<td>2.3</td>
</tr>
<tr>
<td>Fcgr3a; Fc fragment of IgG, low affinity III</td>
<td>1425225_at</td>
<td>2.4</td>
</tr>
<tr>
<td>Cxcl1; chemokines receptor C</td>
<td>1416382_at</td>
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<tr>
<td>Alcam; activated leukocyte cell adhesion</td>
<td>1447040_at</td>
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<tr>
<td>Cd96; CD69 Ag</td>
<td>1428735_at</td>
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<td>Klra3; killer cell lectin-like receptor</td>
<td>1425436_x_at</td>
<td>4.8</td>
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<td>Ifi203; IFN-activated gene 203</td>
<td>1452348_s_at</td>
<td>6.1</td>
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\(^{a}\) Gene function inferred determined with Gene Ontology annotations according to Mouse Genome. Expressed in CD21/35\(^{−/−}\) splenocytes relative to BALB/c WT splenocytes. Informatics and from literature searches with public databases such as PubMed.

\(^{b}\) Numbers indicate fold difference. Transcripts with changes of 2-fold or greater were considered differentially expressed.
animals, splenic populations from naive WT and CD21/35−/− mice were analyzed by FACS. There were no differences observed in populations of B cells, T cells, mast cells, neutrophils, or macrophages as demonstrated by cell surface staining of B220, CD3e, c-Kit, CD11b/GR1, and CD11b/F4/80, respectively (Table III). Thus, the increased gene expression observed within the CD21/35−/− spleen is not due to substantial inflammatory cell infiltrate, but likely due to increased transcription of the resident cells.

Altered complement activity and immune complex processing due to a deficiency in CD21/35 proteins on B cells and FDCs could affect the activation state of splenic phagocytes either by allowing for the increased production of the C3a and C5a anaphylatoxins and/or by increasing the number of complement-bound immune complexes. We analyzed the activation state of inflammatory cell types within the CD21/35−/− spleen by FACS analysis to determine whether the level of CD62 ligand (CD62L; L-selectin) was increased (compared with WT cells) (31), whether the level of CD11b was increased (compared with WT cells) (32), and whether the expression of the C5aR was altered. As seen in Fig. 3A, CD11b+sorted splenocytes from CD21/35−/− animals had similar levels of C5aR on the cell surface (Fig. 3B). However, CD11b+ splenocytes, and to a lesser extent B220+ cells, from CD21/35−/− animals had increased mRNA levels for the C5a receptor (C5ar) compared with WT (Fig. 3C). The increase in C5ar transcript did not translate into increased C5ar on the cell surface of splenocytes from CD21/35−/− mice (data not shown). Together, these data indicate that the activation state of inflammatory cell types within the spleens of CD21/35−/− animals is not substantially altered compared with that of WT as might be expected if significant numbers of complement-bound immune complexes were being phagocytized by cells in the CD21/35−/− spleens.

**GR1-expressing cells contribute to the altered gene expression in CD21/35−/− splenocytes**

A number of the differentially expressed genes could be traced to a CD11b/GR1high subset, suggestive of neutrophils. To determine whether these cells were indeed responsible for the altered expression of these genes, we depleted mice of neutrophils with anti-GR1 (24). As shown in Fig. 4A, this treatment resulted in the loss of the CD11b+GR1+ population in the spleen. Analysis of transcripts obtained from total splenocytes from GR1-depleted and isotype-treated animals demonstrated that the absence of the GR1+ cells reduced the expression of Alox5, Fv, KlrA18, and Len2 to WT levels, whereas those for Ctg and Ddb were unaffected (Fig. 4B).

These data thus indicate that a significant subset of the inflammatory gene products identified in the primary gene grid array analysis that were increased in the CD21/35−/− splenocytes are the products of activated, resident GR1-expressing neutrophils in the spleen.

The apparent role for GR1+ cells in increased gene expression in CD21/35−/− splenocytes relative to the WT splenocytes led us to hypothesize that subsets of these cell types were altered in the CD21/35−/− spleen. One subset of GR1-expressing cells has been identified as immature myeloid cells with suppressor activity (33). These cells typically express GR1 at intermediate levels along with CD31 (Pecam1) (34). Analysis of splenocytes from WT and CD21/35−/− mice revealed a significant increase in a GR1low/CD31+ population within the CD21/35-deficient spleen compared with the WT mouse (Fig. 5; 26.1% vs 17.6%, 6 < 0.005). These cells have the potential role of altering the splenic microenvironment of the CD21/35−/− animal.

**Complement depletion alleviates increased gene expression in CD21/35−/− splenocytes**

The preceding data demonstrated that the environment of the naive CD21/35−/− spleen is substantially different from the WT counterpart. The elevated expression of the gene products (such as Alox5) in the deficient animal is indicative of a heightened state of inflammation in the naive CD21/35−/− spleen vs the WT control. Because CD35 serves as a cofactor for factor I, which aids in the inactivation of C3b, we hypothesized that the absence of CD35 on B cells and FDCs within the spleen might result in an increased half-life of naturally occurring C3 and C5 convertases. Such an increase in convertases would result in the
increased generation of the inflammatory anaphylatoxins C3a and C5a. C3a and C5a are potent mediators of inflammatory effector functions (3, 35). Most cells of the immune system (neutrophils and monocytes) have C3a and C5a receptors, and signaling through the C5a receptor and other similar G protein-coupled receptors has been implicated in increased Alox5 expression (36–38). Thus, we hypothesized that cell activation by complement peptides C3a and C5a was responsible for the differential gene expression observed in the CD21/35−/− spleen.

To assess the role of serum C3 in inducing gene expression in vivo, we depleted complement in WT and CD21/35−/− mice by injecting CVF (20) 48 h before spleen RNA collection. We predicted that the CD21/35−/− mice lacking serum C3 should lose the enhanced expression of the inflammatory gene products in the splenic population. Mice treated with CVF had significantly decreased serum levels of C3 as seen by ELISA (Fig. 6A). Expression levels of Alox5, Klra18, Lcn2, and FV in the CD21/35−/− spleen returned to WT levels after 48 h of CVF treatment, compared with PBS-injected controls, whereas expression levels of Cstg and Dbp remained unchanged (Fig. 6B). One caveat in depleting complement before the gene expression analysis is that the ligands for CD21/35 are depleted. Therefore, to distinguish between increased convertase activity in the CD21/35 knockout and active suppression of gene expression downstream of CD21/35 signaling, we analyzed gene expression in the spleens of CVF-treated WT mice. Expression of inflammatory genes was not significantly altered in splenocyte populations derived from the CVF-treated WT animals (compared with mock-depleted controls; data not shown) except in the samples obtained 4 h after CVF treatment. CVF treatment did not alter cell populations or germinal center development in the CD21/35−/− spleens (compared with mock-treated controls; data not shown).

The preceding findings suggested that increased complement convertase activity in the naive CD21/35−/− spleen resulted in the enhanced expression of the inflammatory gene products. One of the primary inflammatory mediators of the complement cascade is C3a. To determine whether this peptide was responsible for the heightened state of activation of cells in the CD21/35−/− spleen, we treated CD21/35−/− mice with the C3aRA SB290157, which blocks C3aR signaling (39). Analysis of CD21/35−/− splenocyte populations treated with C3aRA (vs mock treated) demonstrated decreased splenic expression of Alox5, Klra18, and FV but not Cstg,
Dbp, or Lcn2 (Fig. 6C). These data confirm the role for complement proteins, specifically C3a, in the altered expression of select inflammatory genes in the CD21/35\(^{-/-}\) spleen.

**Complement depletion increases Ag-specific Ab production in CD21/35\(^{-/-}\) mice**

Many, if not all, of the altered phenotypes associated with the CD21/35\(^{-/-}\) mice have been linked to the absence of B cell signaling via the molecularly linked CD19 signal transduction complex. Our findings described above, however, suggest that many of these phenotypic changes could be due to the altered, inflammatory environment of the CD21/35\(^{-/-}\) spleen. This could be particularly important for responses such as isotype switching for which the correct cytokine environment is critical (40). We hypothesized that the enhanced complement-dependent inflammatory responses in the CD21/35\(^{-/-}\) spleen inhibited the ability of B cells to produce IgG3 in response to immunization. Thus, would the removal of complement activation products allow for a reversal of the low IgG3 phenotype? To test for this possibility, we devised a strategy in which complement was depleted (via CVF) before and during an immunization scheme. The work of Pepys (20) and others led us to expect a depression in total Ab response in treated WT animals; however, we anticipated that such treatment of CD21/35\(^{-/-}\) mice might allow for a more robust IgG3 responses compared with mock-depleted CD21/35\(^{-/-}\) mice. To test this hypothesis, WT and CD21/35\(^{-/-}\) mice were immunized with TNP-LPS in the presence or absence of circulating C3. Sera were collected at day 7 and analyzed for Ag specific total IgG and for Ag specific IgG3. As shown in Fig. 7, immunization of the WT animal in the absence of C3 resulted in a significant decrease in total TNP-specific IgG as well as TNP-specific IgG3. Conversely, the depletion of C3 in the CD21/35\(^{-/-}\) mice gave a statistically significant increase in total TNP-specific IgG as

**FIGURE 6.** C3 depletion alleviates increased gene expression in CD21/35-deficient splenocytes. A, Serum C3 levels in CVF- or PBS-treated WT and CD21/35\(^{-/-}\) (knockout; KO) mice. B, Splenocyte gene expression levels of CD21/35\(^{-/-}\) mice injected with CVF or PBS control. C, Splenocyte gene expression analysis 24 h after inhibition of C3aR signaling. Gene expression depicted as fold change expression compared with control WT (n = 5). *, p ≤ 0.05.

**FIGURE 7.** Complement depletion increases Ag-specific Ab production in CD21/35\(^{-/-}\) mice. TNP-specific IgG (A) or IgG3 (B) Ab responses to 30 \(\mu\)g of TNP-LPS-immunized C3-sufficient and -deficient (CVF) BALB/c WT and CD21/35\(^{-/-}\) mice. Results show combined data from two independent experiments.
well as TNF-specific IgG3. Thus, the removal of C3 from the serum of the CD21/35−/− mouse allowed for enhanced Ag-specific Ab production, a finding opposite of that expected if CD21-dependent B cell signaling were required for IgG3 production.

Discussion

Secondary lymphoid organs, such as the spleen, play host to a variety of immune cell and protein interactions. In this report, we have detailed a comparison in steady-state levels of gene expression between spleens obtained from WT mice and those lacking the CD21/CD35 proteins. The knockout mice used in these analyses are true nulls for these gene products, lacking both the CD21/CR2 protein and the CD35/CR1 peptide (12). Of the other two strains of CD21/35−/− mice, one is a true null (13) whereas the other is a hypermorph (11), expressing lower levels of truncated CR1 and CR2 proteins (25). These experiments were initiated because of concerns that a true phenotype for the loss of the CR1-mediated coactivator activity for the degradation of C3-containing convertases was not included within the generally accepted list of deficiencies of such mice. Mice and other lower animals are different from the higher primates such as humans in that the mouse CD21 gene encodes both the CR1 and CR2 proteins via alternative splicing (5) while the human CD21 gene has lost the ability to generate the CR1-like product (41). Instead of producing a CD21-derived CR1 protein, humans use a recently evolved CD35/CR1 gene (derived from components found within the mouse Crty gene) (42). The human CR1 protein recapitulates many of the features of mouse CR1 including N-terminal coactivator activity and size (~200,000 Da). Thus, the function of this protein must be unique, important, and distinguishable from that of the CD21/CR2 product.

The formation and stability of the C3 and C5 complement convertases are regulated by a number of cell surface and soluble proteins. Preventing formation of convertases on the surface of host cells is primarily accomplished by small membrane bound proteins such as decay acceleration factor and membrane cofactor protein which destabilizes the convertases and contributes to factor I-dependent coactivator activity, respectively (43). A large protein such as mouse/human CR1, made up of rigid, repetitive domains, is likely to be more effective at providing complement convertase control activity on immune complexes away from the cell surface, such as those found decorating FDC and Ag-specific B cells via complement and FcRs. A local increased state of complement activation would be expected to be detrimental to that site by the steady production of the inflammatory mediators C3a and C5a, which could in turn serve to chronically activate resident cells of the lymphatic tissue.

The central finding of this work was that the naive CD21/35−/− spleen is fundamentally different from a WT spleen due to an elevated inflammatory state. Marker genes for this inflamed state were traced back to neutrophils and macrophages, and the induced expression was linked to enhanced complement activation and release of the biologically active C3a peptide. Splenic cell populations between WT and CD21/35−/− mice were similar with the exception of the CD31/GR1low myeloid cell population, which has recently been implicated in immunosuppression within the spleen (44, 45) and the modulation of B cell responses (46). Inflammatory gene expression within the CD21/35−/− spleen was further identified to CD11b+ cells or GR1low-expressing cells and confirmed by depletion of GR1+ (including the CD31/GR1low myeloid cell population) cells. The modest, albeit consistent, increase of these suppressor cell types within the CD21/35−/− spleen is by itself of interest. Further studies should confirm their morphology and influences on immune responses, as well as how they are preferentially recruited or expanded in a spleen lacking the CR1 and CR2 proteins.

The role for CD35 as a complement regulatory protein led us to hypothesize that the increased gene expression from inflammatory cell types in the CD21/35−/− spleen was the result of altered complement regulation, such as increased convertase activity and local production of C3a and C5a. The loss of the CD35 coactivator activity could also be expected to increase the ligands (C3b and iC3b) for the CR3 and CR4 receptors expressed by phagocytes. Analysis of activation markers (CD11b/CR3 and CD62L) for the splenic phagocytes obtained from the CD21/35−/− mice, however, did not indicate that such cells were internalizing significantly more C3b-bound complexes than their WT controls. C5aR expression, however, was significantly increased in CD21/35−/− CD11b+ splenocytes compared with WT cells. Others have shown that C5a binding can directly induce the transcription of the C5aR gene (47).

CVF essentially functions as a stable C3 convertase, generating robust amounts of C3a and C5a (48). Analysis of gene expression of WT splenocytes at different time points post-CVF treatment demonstrated that 4 h post-CVF the expression of Alas5, Lcn2, Kat18, and Ctsg were increased, compared with WT mock treated, although this expression profile was not maintained 24 or 48 h post-CVF (data not shown). These data suggest that the anaphylatoxins released via CVF treatment are quickly absorbed and neutralized and that the differential gene expression seen in the naïve CD21/35−/− spleen may be due to chronic C3a/C5a release.

We hypothesized that the altered gene expression in the spleen of the CD21/35−/− mouse is an indication of an altered microenvironment. Depletion of C3, in part, corrected this inflammatory transcriptional signature. Along with reversing this inflamed state, depleting C3 allowed for a reversal of one of the hallmarks of the CD21/35−/− mouse, the selective depression in IgG3 production. Although CD21/CD19 complexes are clearly effective at activating naive B cells under suboptimal activation conditions, the CR1 protein of the mouse (and by extension the human) must be involved in neutralizing complement convertases forming on immune complexes bound to the surface of B cells and FDCs, thus inhibiting the generation of an inflammatory state in the spleen/germinal centers.

Isotype switching involves a complex mechanism that depends not only on the state and Ag specificity of the B cells but also on soluble factors within the microenvironment acting on the B cell. C3a has been demonstrated to act directly on B cells and mediate suppression of polyclonal Ab responses as well as to suppress Ab responses indirectly through other cell types (21, 49). Our studies have led us to the hypothesis that an increase in convertase activity within the CD21/35−/− spleen enhances anaphylatoxin production, resulting in downstream effects such as inhibiting isotype switching to IgG3. However, mice injected with the C3aR antagonist, SB290517, before immunization did not demonstrate enhanced or depressed Ag-specific Ab production (data not shown). These results are not conclusive given that the receptor antagonist potentially does not saturate the C3aR in the spleen and also has a short half-life in vivo, making it undesirable for an immunization study. In vitro studies directly analyzing the effects of C3a and C5a on LPS-induced IgG3 production did not demonstrate an effect of the anaphylatoxins on IgG3 production or I-Cy3 transcript (data not shown). However, taking the cells of the architecture of the spleen may alter their response, and it is difficult to determine relevant in vitro doses of C3a and/or C5a to counteract LPS activation. In this study, we have not determined whether complement activation products directly suppress B cell functions, or whether
the effect is indirect through other cell types such as neutrophils, or perhaps the enhanced immature CD31/GR1<sup>low</sup> myeloid population. Mice depleted of neutrophils and other GR1-expressing cells before immunization did not have altered IgG3 Ab responses (data not shown), suggesting that these cells are not directly responsible for the decreased Ab responses in the CD21/35<sup>−/−</sup> animal. The effects of non-GR1-expressing cells on Ab production cannot be ruled out.

If the IgG3 phenotype of the CD21/35<sup>−/−</sup> mouse was reversible, might other phenotypes associated with the animal be as well? One hallmark of the CD21/35<sup>−/−</sup> mouse is the small and disorganized germinal centers of such mice. Although this observation has been interpreted as a lack of B cell CD21/CD19 coreceptor signaling, it is possible that excess production of C3a/C5a within those sites could depress the formation of germinal centers. The same question can be raised about the vitality of CD21/35<sup>−/−</sup> cells in germinal centers. It has previously been shown that Ag-specific CD21/35<sup>−/−</sup> cells adoptively transferred into a CD21/35 WT host were selectively lost and that this loss was due to the absence of CD21/CD19 signaling (18, 19). An alternative interpretation of these data would be that the formation of membrane attack complexes on the surface of the CD21/35<sup>−/−</sup> cells (via run away complement convertase activity) could lead to their preferential loss in germinal centers compared with the WT counterparts. It has been shown that inhibition of human CR1 activity on the surface of B cells does lead to enhanced formation of membrane attack complexes on the surface of treated cells and their subsequent death (50).

In summary, the data presented in this report suggest that the CR1 and CR2 proteins play a greater role in the biology of the animal than simply serving as coaccessory signaling proteins for CD19-dependent BCR activation. The phenotypes associated with CD21/CD35 loss may well represent blended responses including the lack of intrinsic B cell signaling as well as the elevated inflammatory responses due to excess complement consumption. Controlling immune complex membrane convertase activity on the surface of B cells and FDCs by a CR1-like protein (via either alternative splicing of the CD21 products in the mouse or convergent evolution of the human CR1 protein) must be beneficial to the splenic/germinal center environment to allow for optimal cytokine and receptor coordination between resident T and B cells, and other cells of the lymphatic tissue.

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Disclosures

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