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CUTTING EDGE

Cutting Edge: C3d Functions as a Molecular Adjuvant in the Absence of CD21/35 Expression[1]

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Complement component C3 covalently attaches to Ags following activation, where the C3d cleavage fragment can function as a molecular adjuvant to augment humoral immune responses. C3d is proposed to exert its adjuvant-like activities by targeting Ags to the C3d receptor (CD21/35) expressed by B cells and follicular dendritic cells. To directly assess the importance of CD21/35 in mediating the immunostimulatory effects of C3d, CD21/35-deficient (CD21/35$^{-/-}$) mice were immunized with streptavidin (SA), SA-C3dg tetramers, recombinant HIV gp120 (gp120), or gp120 fused with linear multimers of C3d. Remarkably, SA- and gp120-specific Ab responses were significantly augmented in CD21/35$^{-/-}$ mice when these Ags were complexed with C3d in comparison to Ag alone. In fact, primary and secondary Ab responses and Ab-forming cell responses of CD21/35$^{-/-}$ mice approached those of wild-type mice immunized with SA-C3dg and gp120-C3d. Thus, C3d can function as a molecular adjuvant in the absence of CD21/35 expression. The Journal of Immunology,* 2004, 172: 5833–5837.

Complement is a key component of the innate immune system that can also influence humoral immune responses. Upon activation, C3 cleavage products form covalent bonds with foreign Ags, thereby generating ligands such as C3dg and C3d (a proteolytic fragment of C3dg) that engage CD21/35 complement receptors expressed by mature B cells and follicular dendritic cells. Deficiencies in either C3 or the common gene that generates leukocyte complement receptors 1 (CD35) and 2 (CD21) result in impaired Ab responses in mice (1–7). Moreover, covalently linking C3d fragments to Ags results in a potent adjuvant effect. In the first demonstration of this, fusing multiple copies of C3d to hen egg lysozyme (HEL)[4] lowered the dose of Ag required for Ab responses by at least 1000-fold in transgenic mice expressing B cell Ag receptors (BCR) specific for HEL (8). Likewise, immunization of wild-type mice with DNA-based vaccines encoding HIV gp120 fused to multiple copies of C3d results in higher Ab responses with enhanced avidity maturation when compared with gp120 immunization alone (9, 10). Mice immunized with either influenza or measles virus hemagglutinin fused to multiple copies of C3d also generate more rapid Ab responses and higher neutralizing titers (as high as 8-fold greater) than mice immunized with either Ag alone (11–13). Finally, Ab titers and isotype switching in response to pneumococcal capsular polysaccharide type 14 are enhanced when pneumococcal capsular polysaccharide type 14 is conjugated to C3d (14). C3d is therefore an effective molecular adjuvant that appears safe and acceptable for use in vaccines.

C3d is proposed to function as a molecular adjuvant by efficiently targeting Ags to CD21/35, which interacts with CD19 to regulate transmembrane signals during B cell activation (15, 16). Because a direct role for CD21/35 in this process has never been demonstrated, the importance of CD21/35 receptor engagement in mediating the immunostimulatory effects of C3d was assessed in mice completely deficient in CD21/35 (CD21/35$^{-/-}$) expression (3, 17). Notably, B cells from CD21/35$^{-/-}$ mice do not bind streptavidin (SA)-C3dg tetramers, which are formed by the attachment of four mono-biotinylated C3dg molecules to SA (3, 18). However, C3dg tetramers effectively reveal CD21 ligand binding in wild-type mice and exhibit functional activity on normal, but not CD21$^{-/-}$ B cells, including augmentation of anti-IgM-mediated intracellular Ca^{2+} flux and activation of p38 mitogen-activated protein kinase (3, 18). To investigate the role that C3d-CD21/35 interactions play in humoral responses to C3d-Ag conjugates, CD21/35$^{-/-}$ mice were immunized with SA and recombinant HIV-1 envelope glycoprotein $gp120_{IIIB}$ (gp120), either alone or complexed to C3d. Unexpectedly, Ab responses to SA and gp120 were significantly augmented in CD21/35$^{-/-}$ mice when these proteins were complexed with C3d in comparison to Ag alone. Thus,

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[4] Abbreviations used in this paper: HEL, hen egg lysozyme; BCR, B cell Ag receptor; SA, streptavidin; CGG, chicken γ-globulin.

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C3d can function as a molecular adjuvant through CD21/35 receptor-independent pathways.

Materials and Methods

SA-C3dg and SA-chicken γ-globulin (CGG) formation

Biotinylated C3dg was produced and purified as described (18). Purified C3dg was treated with polymyxin B-agarose (Sigma-Aldrich, St. Louis, MO). Endotoxin contamination was determined to be <0.028 endotoxin U/μg of C3dg (Pyrogent Plus; BioWhittaker, Walkersville, MD; Lineberger Comprehensive Cancer Center Cell Culture Facility, Chapel Hill, NC). To form SA-C3dg tetramers and SA-CGG complexes for injections, 40 μg of biotinylated C3dg or CGG (Sigma-Aldrich) was incubated with 10 μg of SA (Sigma-Aldrich) in 200 μl of PBS for 45 min at room temperature.

gp120 DNA constructs, protein expression, and purification

DNA plasmids encoding soluble gp120 and gp120 fused to three copies of murine C3d (gp120-$C3d_3$) were expressed in 293T cells as described (10). Recombinant gp120 proteins were purified using a HiTrap chelating nickel column using the N-terminal (6×) histidine tag (Amersham Biosciences, Piscataway, NJ). DNA vaccine plasmids encoding soluble gp120 alone or fused to two or three copies of murine C3d (gp120-$C3d_{2-3}$) for use in DNA immunizations were as described (10).

Mice

$CD21/35^{-/-}$ mice were as described (3). Eight- to 10-wk-old $CD21/35^{-/-}$ and wild-type littermates on a mixed B6/129 background were used in SA immunizations. C57BL/6 wild-type mice and $CD21/35^{-/-}$ mice backcrossed six to seven times onto the C57BL/6J background were used in gp120 protein and DNA immunizations. Mice were housed under specific pathogen-free conditions. All procedures conformed to Duke University Animal Care and Use Committee guidelines.

Immunizations

SA (10 μg) was administered alone or complexed with either biotinylated C3dg (18) or biotinylated CGG i.v. in 200 μl of PBS. For gp120 protein immunizations, recombinant gp120 (50 μg) was administered alone or fused with murine $C3d_3$ i.v. in 200 μl of PBS. DNA immunizations were performed on shaved abdominal skin using the hand-held Bio-Rad (Hercules, CA) Gene Delivery System as described (10). Mice received two immunizations at each time point, each containing 1 μg of DNA plasmid encoding soluble gp120, gp120-$C3d_2$, or gp120-$C3d_3$ per 0.5 mg of 1-μm gold beads (Bio-Rad) at a helium pressure setting of 400 psi.

ELISAs

SA or gp120-specific Abs were quantified by coating 96-well plates with SA (5 μg/ml; 100 μl/well) or recombinant gp120 (0.3 μg/ml; 100 μl/well) in 0.1 M borate buffered saline overnight at 4°C. Plates were washed in TBS and blocked with TBS containing 1% gelatin/2% BSA for 90 min at 37°C. Sera were diluted 1/250 in TBS containing 1% BSA and incubated in duplicate wells at room temperature for 90 min. Plates were washed using TBST and incubated with alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, or IgM Abs (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. Plates were developed using *p*-nitrophenyl phosphate substrate (Southern Biotechnology Associates) with OD_{405} values determined.

End-point titers of anti-SA IgG Abs were determined using 3-fold serial dilutions of serum samples. End-point titers were determined as the reciprocal dilution of sera yielding an OD_{405} value that was 3-fold higher than background OD values where sera was omitted. End-point anti-gp120 IgG titers were assessed using recombinant gp120 as described (9, 10). Briefly, plates were coated with gp120 (0.3 μg/ml) overnight at 4°C, blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20 for 1 h at 25°C, and washed with PBS containing 0.05% Tween 20. Plates were incubated with 2-fold serial dilutions of sera for 1 h, washed, and incubated with biotinylated anti-mouse IgG Abs followed by SA-conjugated HRP (Southern Biotechnology Associates). Plates were developed using tetramethylbenzidine substrate (Sigma-Aldrich). End-point titers were determined as the reciprocal dilution of sera yielding an OD value that was 2-fold higher than OD values measured for serum samples from control mice immunized with vector alone.

ELISPOT assays

Immobilon-P Multiscreen 96-well plates (Millipore, Bedford, MA) were precoated with SA (5 μg/ml). Bone marrow and spleen cells were plated at 10^4, 10^5, or 10^6 cells per well in 100 μl of culture medium (RPMI 1640 containing 10% FCS, 10 mM glutamine, 100 U/ml penicillin/streptomycin, and 55 μM 2-ME) for 18 h at 37°C in a CO_2 incubator. The plates were washed three times with TBST, incubated with polyclonal alkaline phosphatase-conjugated goat anti-mouse IgG Abs for 2 h at room temperature, washed, and developed for 30 min using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Sigma-Aldrich).

Statistical analysis

Data are shown as means ± SEM. Student's *t* test was used to identify significant differences between sample means.

Results

C3d augments primary and secondary Ab responses to SA in $CD21/35^{-/-}$ mice

$CD21/35^{-/-}$ and wild-type littermates were immunized with 10 μg of SA protein, either alone or complexed with biotinylated C3dg. SA immunization resulted in modest IgM and IgG responses in both $CD21/35^{-/-}$ and wild-type littermates (Fig. 1*A*). By comparison, SA-C3dg induced significant IgM and IgG anti-SA Ab responses in both $CD21/35^{-/-}$ and wild-type littermates (Fig. 1*A*), without inducing anti-C3dg Ab responses

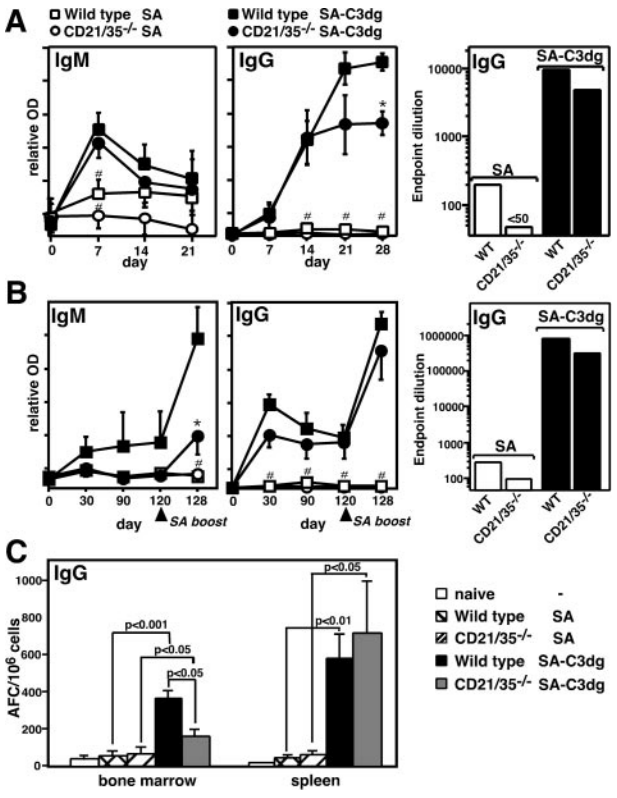

FIGURE 1. C3d enhances humoral responses in $CD21/35^{-/-}$ and wild-type mice. *A*, $CD21/35^{-/-}$ and wild-type (WT) littermates were immunized with SA protein (10 μg) either alone or complexed with biotinylated C3dg (SA-C3dg) on day 0, with serum SA-specific IgM and IgG Ab levels quantified by ELISA. In the *right panel*, SA-specific IgG titers are shown for pooled sera harvested 21 days postimmunization. *B*, Secondary SA-specific Ab responses by $CD21/35^{-/-}$ and wild-type littermates. Mice immunized with SA alone or SA-C3dg on day 0 were boosted at day 120 with 10 μg of SA alone. SA-specific IgG titers are shown for pooled serum samples harvested 7 days after the SA boost. *A* and *B* values represent the mean OD (±SEM) from four to five mice per group. *, Significant differences ($p < 0.05$) between $CD21/35^{-/-}$ and wild-type littermates immunized with SA-C3dg. #, Significant differences ($p < 0.05$) between mice of the same genotype immunized with SA-C3dg compared with SA alone. *C*, Ab-forming cell (AFC) frequencies in naive and immunized $CD21/35^{-/-}$ and wild-type littermates 7 days after a SA boost on day 120.

(data not shown). Seven days after SA-C3dg immunization, SA-specific IgM responses were significantly higher in both CD21/35$^{-/-}$ and wild-type mice ($p < 0.05$). IgG responses were also significantly higher in both CD21/35$^{-/-}$ and wild-type littermates at days 14–28 following SA-C3dg immunization relative to SA-immunized mice ($p < 0.05$). An analysis of serum Ab titers generated similar conclusions: SA-specific IgG responses were ≥50-fold higher in CD21/35$^{-/-}$ and wild-type littermates receiving SA-C3dg compared with SA alone (Fig. 1*A*). Although CD21/35$^{-/-}$ mice responded well to SA-C3dg immunization, they had lower mean SA-specific IgM and IgG titers than wild-type littermates (Fig. 1*A*). Thus, CD21/35 deficiency impairs humoral immune responses to soluble protein Ags as described (1–3). Despite this, C3dg functioned as a molecular adjuvant in the absence of CD21/35 expression.

Whether SA-C3dg immunization augmented secondary anti-SA Ab responses was assessed in CD21/35$^{-/-}$ and wild-type littermates that had been immunized with SA alone or SA-C3dg on day 0. Mice immunized and boosted with SA on days 0 and 120, respectively, did not generate significant IgM or IgG responses (Fig. 1*B*). By contrast, both CD21/35$^{-/-}$ and wild-type littermates first immunized with SA-C3dg generated significantly higher secondary IgM and IgG responses following the SA boost ($p < 0.05$) than mice first immunized with SA alone. IgM Ab responses following SA boosting were lower ($p < 0.05$) in CD21/35$^{-/-}$ mice compared with their wild-type littermates, although IgG responses were similar. In fact, CD21/35$^{-/-}$ and wild-type littermates immunized with SA-C3dg at day 0 had IgG titers on day 128 that were >2500-fold higher than those of mice immunized and boosted with SA alone (Fig. 1*B*). Likewise, the frequencies of SA-specific IgG-secreting cells were 10-fold higher in spleens of CD21/35$^{-/-}$ and wild-type littermates immunized with SA-C3dg compared with mice receiving SA alone (Fig. 1*C*). SA-specific IgG-secreting cell frequencies were also 3- to 6-fold higher in the bone marrow of SA-C3dg-immunized CD21/35$^{-/-}$ and wild-type littermates, respectively. In summary, administration of C3dg-Ag complexes during primary immunization elicited long-lasting Ag-specific IgG production during the primary response and significantly enhanced the secondary Ab response to Ag alone. However, C3d enhancement of the Ab response to SA occurred through a pathway that was largely independent of CD21/35 expression.

C3d augments humoral responses to gp120 in CD21/35$^{-/-}$ mice

CD21/35$^{-/-}$ and wild-type mice were immunized with 50 μg of gp120 protein, either alone or fused with three copies of murine C3d in tandem at the C terminus (9, 10). Wild-type mice immunized with either gp120-C3d$_3$ or gp120 generated similar primary and secondary IgM and IgG responses (Fig. 2, *A* and *B*). Because the effectiveness of C3d as a molecular adjuvant is dependent on the nature of the Ag itself, the dose of Ag, and the route of immunization (11, 13), it was not surprising that gp120-specific responses were similar in wild-type mice immunized with gp120-C3d$_3$ and gp120 proteins. By contrast, gp120 immunization generated low primary IgM and IgG responses in CD21/35$^{-/-}$ mice, although gp120-C3d$_3$ immunization generated elevated titers of gp120-specific IgG in CD21/35$^{-/-}$ mice by day 28 (Fig. 2*B*). However, secondary gp120-specific IgG responses were near wild-type levels in CD21/35$^{-/-}$ mice immunized with gp120-C3d$_3$ (Fig. 2*A*). In fact,

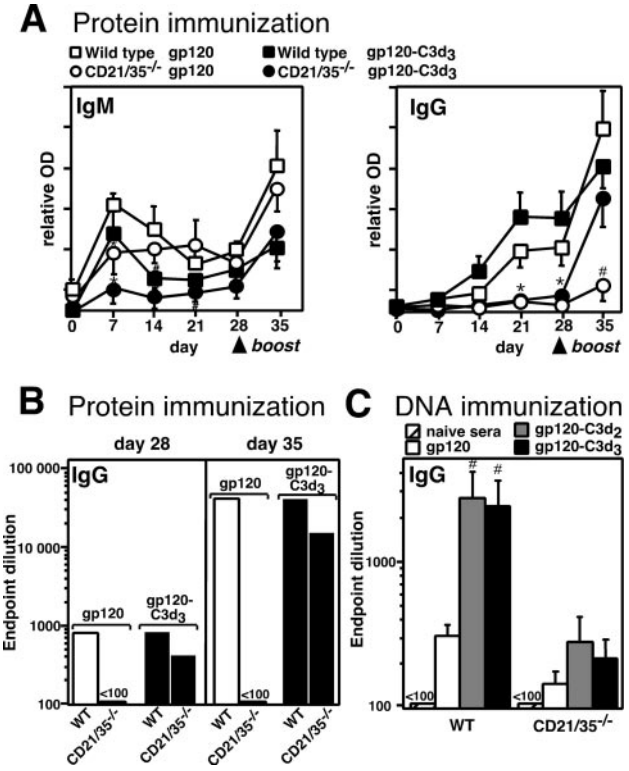

FIGURE 2. HIV gp120-specific Ab responses of CD21/35$^{-/-}$ and wild-type mice following immunization with gp120 alone or gp120-C3d$_{2-3}$. *A*, Mice were immunized on days 0 and 28 with gp120 protein (50 μg) either alone or with three attached copies of C3d. Serum gp120-specific IgM and IgG Abs were quantified by ELISA. Values represent the mean OD (±SEM) from four to five mice per group. *B*, IgG titers of serum from the CD21/35$^{-/-}$ and wild-type mice shown in *A*. *C*, HIV gp120-specific Ab responses of CD21/35$^{-/-}$ and wild-type mice following immunization with DNA encoding gp120 alone, gp120-C3d$_2$, or gp120-C3d$_3$. Mice were immunized with plasmid DNA at wk 0, 4, and 8. Values represent mean IgG titers (±SEM) obtained for four to five mice per group at wk 10. Titers determined to be at or below a dilution of 1/100 are indicated as <100. *, Significant differences ($p < 0.05$) between means of CD21/35$^{-/-}$ and wild-type mice immunized with gp120-C3d$_3$. #, Significant differences ($p < 0.05$) between means for mice of the same genotype immunized with gp120-C3d$_{2-3}$ compared with gp120 alone.

secondary gp120-specific IgG end-point titers were at least 150-fold higher in CD21/35$^{-/-}$ mice immunized with gp120-C3d$_3$ compared with gp120 alone (Fig. 2*B*). Anti-C3d Ab responses were not detected in gp120-C3d$_3$-immunized mice (data not shown). Immunization of mice with DNA-based vaccines encoding gp120-C3d$_2$ or gp120-C3d$_3$ also resulted in increased gp120-specific Ab responses compared with immunization with gp120 alone in both wild-type (by ~8-fold; $p < 0.05$) and CD21/35$^{-/-}$ (by ~1.5- to 2-fold; NS) mice (Fig. 2*C*). However, gp120-specific responses were much weaker than those obtained with direct protein immunizations (Fig. 2, *B* vs *C*). Nonetheless, C3d enhanced gp120-specific IgG responses in CD21/35$^{-/-}$ mice despite their impaired primary and secondary Ab responses to gp120.

CGG augments humoral responses to SA in CD21/35$^{-/-}$ mice

To compare the effect of C3dg on the immune response to SA to that resulting from complexing SA to a well-characterized immunogenic carrier protein, CD21/35$^{-/-}$ and wild-type mice were immunized with SA-CGG complexes. SA-C3d tetramers significantly augmented SA-specific IgM and IgG Ab

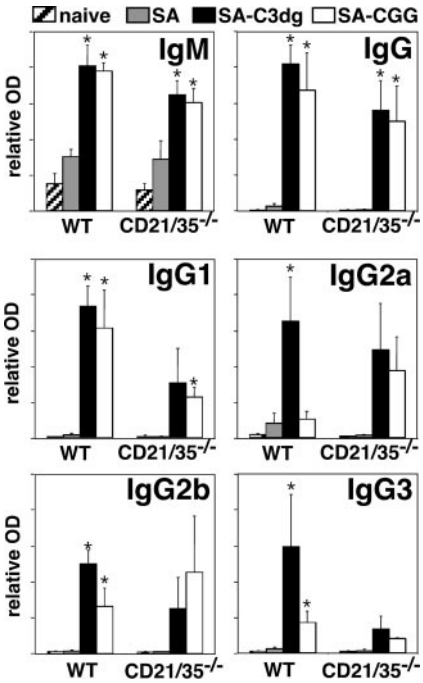

FIGURE 3. C3d and CGG enhance humoral responses in CD21/35$^{-/-}$ and wild-type mice. CD21/35$^{-/-}$ and wild-type littermates were immunized with SA protein (10 μg) either alone or as SA-C3dg or SA-CGG, with serum SA-specific IgM (day 7) and IgG or IgG isotype (day 21) Ab levels quantified by ELISA. Values represent the mean OD (±SEM) from three to five mice per group. *, Significant differences ($p < 0.05$) between mice of the same genotype immunized with SA-C3dg or SA-CGG compared with SA alone.

responses in both wild-type and CD21/35$^{-/-}$ mice to levels similar to those elicited by SA-CGG complexes (Fig. 3), although differences in anti-SA-specific Ab isotypes were observed. Thus, C3dg and CGG similarly augment the overall magnitude of Ab responses in both the presence and absence of CD21/35 expression.

Discussion

This study confirms that C3d can function as a molecular adjuvant during humoral immune responses to Ags administered either directly as proteins (SA and gp120) or as DNA vaccines (gp120). Unexpectedly, C3d could also function as an effective adjuvant in the absence of CD21/35 expression. Ab responses to SA and gp120 were significantly impaired in CD21/35$^{-/-}$ mice, confirming the importance of CD21/35 expression in Ab responses to Ags administered in the absence of adjuvants (1–3). However, IgG Ab responses to SA-C3dg and gp120-C3d were significantly augmented in CD21/35$^{-/-}$ mice in comparison to these Ags given without C3d. These effects were also reflected in the enhanced frequencies of SA-specific Ab-producing cells in CD21/35$^{-/-}$ mice immunized with SA-C3dg tetramers compared with mice immunized with SA alone (Fig. 1*C*). Thus, C3d can function as an adjuvant through pathways that are independent of CD21/35 receptor expression.

C3d can function as a natural adjuvant for a number of physiologically important immunogens, including HIV gp120, viral hemagglutinin, and pneumococcal polysaccharide (9–14). In all cases, C3d has been postulated to augment humoral responses by targeting Ag complexes to B cells and follicular dendritic cells that express CD21/35. On B cells, coligation of the BCR and the CD19/CD21 complex by C3d-Ag complexes is proposed to lower the signaling threshold required for B cell activation and expansion (19–21). Although Dempsey et al. (8) originally proposed that the adjuvant effect of C3d bound to Ag was mediated through coligation of the CD19/CD21 complex with a HEL-specific BCR, the only direct evidence supporting this conclusion was that pretreatment of mice with an Ab against CD21 suppressed the effect elicited by C3d. However, although anti-CD21 mAb treatment is known to inhibit humoral immune responses to a variety of Ags (22–24), anti-CD21 mAb treatment may have effects on B cell function beyond blocking C3d binding (25). In the current study, the importance of CD21 expression in mediating C3d effects was examined using CD21/35$^{-/-}$ mice (3), whereas the study by Dempsey et al. (8) used transgenic mice where all B cells expressed high-affinity Ag-specific BCRs. Thus, differences in conclusions between our current study and those of Dempsey et al. may be explained by differences in experimental approach. Moreover, our data do not discount the model first proposed by van Noesel et al. (26) and evoked by Dempsey et al. (8) for CD21/35 function, because CD21 expression is important for optimal humoral immune responses, and immunization with C3d-conjugated Ags did not always restore immune responses of CD21/35$^{-/-}$ mice to wild-type levels (Figs. 1 and 2). Thus, there may be CD21/35-dependent pathways through which C3d functions, in addition to the CD21/35-independent pathways revealed in the current study.

Although the precise mechanisms through which C3d functions as a molecular adjuvant remain to be elucidated, several hypotheses can be offered. First, C3d interacts with numerous serum proteins, cell surface receptors, and membrane-associated regulatory proteins (27–31). Thus, C3d aggregates may bind Ag complexes to proteins that are distinct from CD21/35 to enhance humoral responses. Alternatively, attachment of C3d to Ags could prolong the in vivo half-life of Ag, perhaps by forming molecular aggregates or facilitating molecular interactions. Finally, C3d could function as a simple protein carrier. In support of this, OVA functions as an adjuvant for pneumococcal polysaccharide in a manner similar to C3d (14). Similarly, SA-C3d tetramers or SA-CGG complexes significantly augmented anti-SA Ab responses in both wild-type and CD21/35$^{-/-}$ mice to similar levels (Fig. 3). However, C3d was not immunogenic and did not elicit anti-C3d Ab production, unlike CGG. Thus, although both C3dg and CGG were effective adjuvants in CD21/35$^{-/-}$ and wild-type mice, they may function through distinct pathways. Given the unexpected finding that C3d augments humoral immune responses through CD21/35-independent pathways, understanding the mechanisms of C3d action may provide important insight into the identity of other molecules with adjuvant activity that will allow the design of even more potent vaccines.

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