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


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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 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 Ca2+ flux and activation of p38 mitogen-activated protein kinase (3, 18). To investigate the role that C3d-CD21 interactions play in humoral responses to C3d-Ag conjugates, CD21/35−/− mice were immunized with SA and recombinant HIV-1 envelope glycoprotein gp120WIIIB (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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2 K.M.H. and F.R.T. contributed equally to this work.
3 Address correspondence and reprint requests to Dr. Thomas F. Tedder, Department of Immunology, Duke University Medical Center, Box 3010, Durham, NC 27710. E-mail address: thomas.tedder@duke.edu
4 Abbreviations used in this paper: HEL, hen egg lysozyme; BCR, B cell Ag receptor; SA, streptavidin; C3d, C3 complement component; C3dg, C3d fragment; gp120, HIV-1 envelope glycoprotein.
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 (Pyrogen Plus; BioWhitaker, 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.

gp1.20 DNA constructs, protein expression, and purification
DNA plasmids encoding soluble gp1.20 and gp1.20 fused to three copies of murine C3d (gp1.20-C3d3) were expressed in 293T cells as described (18). Recombinant gp1.20 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 gp1.20 alone or fused to two or three copies of murine C3d (gp1.20-C3d2,3) for use in DNA immunizations were as described (18).

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 gp1.20 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 gp1.20 protein immunizations, recombinant gp1.20 (50 µg) was administered alone or fused with murine C3d, 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 gp1.20, gp1.20-C3d2, or gp1.20-C3d3, per 0.5 mg of 1-µm gold beads (Bio-Rad) at a helium pressure setting of 400 psi.

ELISAs
SA or gp1.20-specific Abs were quantified by coating 96-well plates with SA (5 µg/ml; 100 µl/well) or recombinant gp1.20 (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. Serum 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 for 90 min. Plates were washed in TBS and blocked with TBS 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 (Southern Biotechnology Associates) for 1 h at room temperature, and the absorbance was measured at 450 nm. 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 value that was 2-fold higher than OD values measured for serum samples from control mice immunized with vector alone.

ELISPOT assays
Immuno-P MultiScreen 96-well plates (Millipore, Bedford, MA) were pre-coated with SA (5 µg/ml). Bone marrow and spleen cells were plated at 104, 105, or 106 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 CO2 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. 1A). By comparison, SA-C3dg induced significant IgM and IgG anti-SA Abs responses in both CD21/35−/− and wild-type littermates (Fig. 1A), without inducing anti-C3dg Ab responses.
Seven days after SA-C3dg immunization, SA-specific IgM responses were significantly higher in both CD21/35<sup>-/-</sup> and wild-type mice (p < 0.05). IgG responses were also significantly higher in both CD21/35<sup>-/-</sup> 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<sup>-/-</sup> and wild-type littermates receiving SA-C3dg compared with SA alone (Fig. 1A). Although CD21/35<sup>-/-</sup> mice responded well to SA-C3dg immunization, they had lower mean SA-specific IgM and IgG titers than wild-type littermates (Fig. 1A). 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<sup>-/-</sup> 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. 1B). By contrast, both CD21/35<sup>-/-</sup> 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<sup>-/-</sup> mice compared with their wild-type littermates, although IgG responses were similar. In fact, CD21/35<sup>-/-</sup> 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. 1B). Likewise, the frequencies of SA-specific IgG-secreting cells were 10-fold higher in spleens of CD21/35<sup>-/-</sup> and wild-type littermates immunized with SA-C3dg compared with mice receiving SA alone (Fig. 1C). SA-specific IgG-secreting cell frequencies were also 3- to 6-fold higher in the bone marrow of SA-C3dg-immunized CD21/35<sup>-/-</sup> 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<sup>-/-</sup> mice**

CD21/35<sup>-/-</sup> 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<sub>3</sub> 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<sub>3</sub> and gp120 proteins. By contrast, gp120 immunization generated low primary IgM and IgG responses in CD21/35<sup>-/-</sup> mice, although gp120-C3d<sub>3</sub> immunization generated elevated titers of gp120-specific IgG in CD21/35<sup>-/-</sup> mice by day 28 (Fig. 2B). However, secondary gp120-specific IgG responses were near wild-type levels in CD21/35<sup>-/-</sup> mice immunized with gp120-C3d<sub>3</sub> (Fig. 2A). In fact, secondary gp120-specific IgG end-point titers were at least 150-fold higher in CD21/35<sup>-/-</sup> mice immunized with gp120-C3d<sub>3</sub> compared with gp120 alone (Fig. 2B). Anti-C3d Ab responses were not detected in gp120-C3d<sub>3</sub>-immunized mice (data not shown). Immunization of mice with DNA-based vaccines encoding gp120-C3d<sub>3</sub> or gp120-C3d<sub>3</sub> also resulted in increased gp120-specific Ab responses compared with immunization with gp120 alone in both wild-type (by ∼2-fold; p < 0.05) and CD21/35<sup>-/-</sup> (by ∼1.5- to 2-fold; NS) mice (Fig. 2C). Nonetheless, 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<sup>-/-</sup> mice despite their impaired primary and secondary Ab responses to gp120.

**C3d augments humoral responses to SA in CD21/35<sup>-/-</sup> 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<sup>-/-</sup> and wild-type mice were immunized with SA-CGG complexes. SA-C3d tetramers significantly augmented SA-specific IgM and IgG Ab responses in CD21/35<sup>-/-</sup> and wild-type mice.
immunized with SA-C3dg or SA-CGG compared with SA alone. These effects were also reflected in the enhanced frequencies of SA-specific Ab production to these Ags given without C3d. These effects were also 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<sup>−/−</sup> mice, confirming the importance of CD21/35 expression in Ab responses to Ags administered in the absence of Ags (1–3). However, IgG Ab responses to SA-C3dg and gp120-C3d were significantly augmented in CD21/35<sup>−/−</sup> 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<sup>−/−</sup> mice immunized with SA-C3dg tetramers compared with mice immunized with SA alone (Fig. 1C). 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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.

**References**


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