Complement Factor H Antibodies from Lung Cancer Patients Induce Complement-Dependent Lysis of Tumor Cells, Suggesting a Novel Immunotherapeutic Strategy

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Abstract

Characterization of the humoral immune response in selected patients with cancer who uniformly do well may lead to the development of novel therapeutic strategies. We have previously shown an association between patients with early-stage non-metastatic lung cancer and autoantibodies to complement factor H (CFH). CFH protects normal and tumor cells from destruction by the alternative complement pathway by inactivating C3b, a protein that is essential for formation of a lytic complex on the cell surface. Here, we show that CFH autoantibodies in lung cancer patients recognize a conformationally distinct form of CFH in vitro, are IgG3 subclass, and epitope map to a crucial functional domain of CFH known to interact with C3b. Purified CFH autoantibodies inhibited binding of CFH to A549 lung tumor cells, increased C3b deposition, and caused complement-dependent tumor cell lysis. This work demonstrates that CFH autoantibodies isolated from patients with lung cancer can kill tumor cells in vitro, suggesting that they may perform this function in vivo as well. Development of specific antibodies to the conformationally distinct epitope of CFH may lead to a useful biologic therapy for lung cancer. Cancer Immunol Res. 3(12): 1325–32. ©2015 AACR.

Introduction

Lung cancer remains a significant public health issue. The majority of tumors are detected at an advanced stage when treatment options are limited. There is a clear need for a greater number and wider variety of effective therapies. This work explores the possibility of using patients’ humoral immune response as a starting point for the development of novel therapeutic agents against lung cancer.

Although activation of the humoral response against malignant cells is well documented in the literature (1), humoral immunity per se has not been very well exploited for cancer therapy. Although circulating antibodies against over 100 different tumor-associated antigens (TAA) have been described, very few are associated with tumor stage or outcome. Yet, it remains possible that certain host antibodies have the potential for anti-tumor activity, but this ability is not fully realized for a number of possible reasons, including low concentration or low affinity of antibodies, or ineffective activation of B lymphocytes.

We propose that clinically relevant and novel therapeutic targets may be discovered by taking cues from the immune response to tumors, particularly in patients who have early-stage disease and never develop recurrence. In a previous report, we demonstrated an association of autoantibodies to complement factor H (CFH) in patients with early-stage, nonmetastatic, non–small cell lung cancer (NSCLC; ref. 2). CFH is one of a class of complement inhibitory factors that protect both normal and tumor cells from attack and destruction by the complement system (3, 4). The primary function of CFH is to inhibit the alternative pathway of complement-mediated lysis. CFH prevents the deposition of complement protein C3b on the cell surface by (i) acting as a cofactor for complement factor I (CFI), a protease that cleaves C3b, and (ii) preventing the formation of and accelerating the decay of the enzyme that forms C3b from its precursor, C3. Deposition of C3b initiates the formation of the cell-lytic membrane attack complex, leading to cell lysis; thus, control of the deposition of C3b on the cell surface by CFH protects against cell lysis. CFH engages with C3b (or degraded C3b, named C3d) on mammalian cell surfaces that contain glycosaminoglycans and sialic acid, as opposed to bacterial surfaces lacking these groups, thus mediating target discrimination (4–6).

Besides protecting normal host cells, CFH has been shown to be expressed by tumor cells, including those from NSCLC, glioblastoma, and colon cancer, and protect them from complement attack (7–10). Neutralizing monoclonal antibodies to CFH and other complement inhibitory proteins increases C3b deposition on cell lines that express and bind these proteins (7, 8, 10). In addition, siRNA knockdown of CFH in the lung tumor cell line A549 slows tumor growth in mice, demonstrating that the tumor cell can take advantage of this protective mechanism to evade one arm of the host immune system (7). However, it has not been clear...
how to specifically target this protein in cancer without having off-target effects. Here, we take cues from the native immune response in patients with a distinct nonmetastatic phenotype and without obvious side effects to carry out a functional analysis and characterization of autoantibodies to CFH. The ultimate goal was to assess their mechanism of action and potential for development into a targeted therapeutic that will cause tumor cell–specific killing.

Materials and Methods

**Dot blot for domain mapping**

Dot blots, kindly supplied by Dr. Michael Pangburn (University of Texas Health Science Center, Tyler, TX), contained cloned, expressed, and purified protein domain subsets of CFH. These proteins were reduced before spotting on nitrocellulose. The blots were probed with human NSCLC serum (1:2,000) and anti-IgG gamma chain–horseradish peroxidase (HRP) conjugate (Millipore; 1:5,000), and then treated with a chemiluminescent substrate, exposed to film, and developed.

**Purification of SCR19–20**

A *Pichia pastoris* clone encoding human CFH short consensus repeat (SCR) domains 19 and 20 (termed SCR19–20) in an integrated *P. pastoris* expression vector was obtained from Dr. Michael Pangburn. The 129 amino acid–long protein was purified from the *P. pastoris* culture medium by sequential differential filtration using Vivacell 70 centrifugal units with 50,000 and 5,000 MW cutoffs (Sartorius), followed by HiTrap SP FF cation exchange chromatography (GE Healthcare Life Sciences).

**Purification of human CFH autoantibodies**

An antibody purification strategy was developed in order to carry out functional studies on CFH autoantibodies. First, bulk immunoglobulin was purified from serum by Protein G chromatography. The CFH-specific antibody was then purified by affinity chromatography over *N*-hydroxysuccinimide ester-activated Sepharose 4 FF (GE Healthcare Life Sciences) conjugated according to the manufacturer's instructions with reduced SCR19–20. Immunoglobulin was loaded onto the SCR19–20 column, the column was washed, and bound anti-CFH antibody was recovered by elution with 3 mol/L sodium thiosulfate, 20 mmol/L Tris–HCl, pH 6.8. Elution buffer was exchanged by sequential steps of dilution with PBS plus 10% glycerol, followed by concentration in an Amicon Ultra 4 spin device (30K MW cutoff), the initial buffer was diluted approximately ×6,000. Recovery of anti-CFH antibody was confirmed by both ELISA and immunoblot.

**CFH autoantibody ELISA**

Wells of a MaxiSorp immunoplate (Nunc International) were coated with 500 ng native, reduced, denatured, or reduced and denatured CFH (Complement Technology, Inc.). Reduction was carried out by incubating 1 mg/mL CFH with 10 mmol/L Tris(2-carboxyethyl) phosphate (TCEP) for 30 minutes, then diluting the protein to 5 µg/mL with phosphate-buffered saline (PBS) and dispensing 100 µL/well into the immunoplate. Denaturation was carried out with 7 mol/L urea followed by dilution with PBS as above. Human NSCLC serum, CFH antibody–positive as assessed by immunoblot, was used as the primary antibody, and anti-IgG gamma chain–HRP (Millipore) was the secondary antibody–enzyme conjugate (1:2,000). Plates were developed using 2,2'-azino-bis[3-ethylbenzothiazoulone-6-sulfonic acid] (ABTS) and hydrogen peroxide and absorbance read at 405 nm in a microplate reader (Tecan).

**Epitope mapping of human CFH autoantibodies**

Epitope mapping was conducted by Pepscan (11). Briefly, 15-mer peptides covering the complete amino acid sequence of SCR19–20 were synthesized with an overlap of 14 amino acids. Peptides were arrayed on a proprietary minicard and screened in an ELISA format using a purified human antibody as the primary antibody and an anti-human peroxidase conjugate as the secondary antibody. The minicards were developed using ABTS and hydrogen peroxide. The color development was quantified with a charge-coupled device (CCD) camera and an image processing system. The values obtained from the CCD camera range from 0 to 3,000 mAU.

**Subclass analysis of CFH autoantibodies**

We determined the subclass of CFH autoantibodies in patient sera using a sandwich ELISA. NeutrAvidin (Pierce) was immobilized in the wells of a 96-well plate, the plate was blocked with BSA, and biotinylated antigen peptide or biotin was added. The sequence of the peptide, synthesized by GenScript, was GPPPDPDNGDIYSG(SGGK)–biotin, where the underscored residues comprise the epitope, amino terminal flanking residues comprise additional SCR19 residues, and the residues in parentheses comprise a linker. After a 30-minute incubation, followed by washing the plate with PBS, 0.1% (v/v) Tween-20 (PBST), serum (diluted 1:250 in PBST) was added and allowed to incubate for 1.5 hours. The plate was washed, and bound IgG was detected with HRP-conjugated secondary antibodies specific for each of the IgG subclasses. The secondary antibodies were purchased from Abcam [cat. nos. 99774 (IgG1), 99784 (IgG2), 99829 (IgG3), 99817 (IgG4)] and used at a 1:2,000 dilution. Plates were developed with ABTS and hydrogen peroxide and read at 405 nm.

**Peptide competition immunoblots**

Affinity-purified autoantibodies (66.7 µg/mL) were incubated overnight at 4°C with the peptide-containing peptide PIDNGDITP(GGGK)–biotin (1.67 mg/mL) in PBS, or in PBS alone (final volume, 6 µL). The next day, the autoantibodies with or without peptide were diluted to a final concentration of 2 µg/mL with PBS containing 0.1% (v/v) Tween-20 and 5% (w/v) nonfat dry milk and used to probe a blot containing full-length CFH and SCR19–20. Bound antibody was detected with anti-IgG gamma chain–HRP conjugate (1:5,000) and a chemiluminescent substrate, followed by film exposure. Peptide competition of antibodies in cell-based assays is described below.

**CFH binding to lung cancer cells**

A549 lung adenocarcinoma cells were obtained from the ATCC and maintained in RPMI-1640 + 1-glutamine medium with 10% fetal bovine serum. CFH was fluorescently labeled using an Alexa Fluor 488 Microscale Protein Labeling kit (Life Technologies). Fluorescently labeled CFH was reduced at 1 mg/mL with 20 mmol/L TCEP for 30 minutes. Reduced or native (nonreduced), fluorescently labeled CFH was added at 0.1 mg/mL to CFH-depleted serum (Complement Technology, Inc.) at a 1:8 final concentration.
dilution. Fluorescently labeled CFH and serum mixtures were then preincubated with affinity-purified human CFH autoantibody (0.2 mg/mL) for 30 minute at 4°C. A549 cells were detached from culture dishes and resuspended in veronal buffer. Cells (2.5 x 10⁶) were added to the CFH, serum, and antibody mixtures and incubated for 30 minutes at 37°C. Human IgG (Jackson ImmunoResearch Laboratories Inc.; 0.2 mg/mL) was substituted for CFH antibody as a negative control. Following two washes of cells in 1% (w/v) BSA in Dulbecco’s PBS (BSA-DPBS), flow cytometry was carried out using a FACSCanto II flow cytometer. Mean Alexa Fluor 488 fluorescence intensity on A549 cells, corresponding to CFH binding to the tumor cell surface, was determined using FlowJo software.

C3 deposition on lung cancer cells

Normal human serum (NHS; Complement Technology, Inc.) at a 1:8 final dilution was used as a source of complement proteins. NHS was preincubated with affinity-purified CFH autoantibody (0.2 mg/mL) for 30 minutes at 4°C. A549 cells were detached from culture dishes using Versene (Life Technologies), washed in DPBS, and resuspended in veronal buffer containing Mg²⁺ and EGTA (Boston Bioproducts), conditions that favor the alternative complement pathway. Cells (2.5 x 10⁶) were added to the NHS–antibody mixtures and incubated for 30 minutes at 37°C. Cells were also incubated with heat-inactivated NHS (HI-NHS; prepared by heating NHS at 56°C for 30 minutes) or NHS preincubated with human IgG (0.2 mg/mL) for 30 minutes at 4°C as negative controls. After two washes in BSA-DPBS, cells were incubated for 30 minutes at 4°C with 0.5 μg of a fluorescein isothiocyanate (FITC)–conjugated mouse anti-human C3 antibody (Lifespan Biosciences). Following the C3–FITC antibody incubation, cells were washed three additional times in BSA-DPBS to remove excess C3 antibody. Flow cytometry was carried out using a FACSCanto II flow cytometer (BD Biosciences) at the Duke Cancer Center Core Facility. Mean fluorescence intensity on A549 cells, corresponding to C3 deposition on the cell surface, was determined using FlowJo software (TreeStar Inc.).

For peptide competition, affinity-purified autoantibodies (0.7 mg/mL) were preincubated overnight at room temperature with the PIDNGDIT(GGGK)-biotin peptide (1.2 mg/mL). After addition to cells, the autoantibody concentration was 0.2 mg/mL, and the peptide concentration was 0.34 mg/mL.

Complement-dependent cytotoxicity of lung cancer cells

The effect of CFH antibodies on complement-mediated cytotoxicity of A549 cells was determined using assay conditions essentially identical to those used to determine C3 deposition as described above. After the incubation of cells, serum, and antibodies, cells were washed three times with BSA-DPBS and then resuspended in 1 μg/mL propidium iodide (Biosource International) in DPBS. Flow cytometry was carried out, and the number of propidium iodide–positive cells was determined. Peptide competition was performed as described for the C3 deposition assay.

Statistical analysis

Data obtained from complement-mediated cytotoxicity and C3 deposition experiments were analyzed using the Student t test. All experiments were completed in triplicate, and cytotoxicity and C3 deposition data are represented as mean ± SD.

Results

CFH Antibodies in NSCLC Patients Are Specific for Reduced CFH

CFH is a 150-kDa protein that is composed of 20 SCRs, also called complement control protein (CCP) modules. Some SCRs function in cell attachment, while others function to eliminate C3b from the cell surface (4, 12). The 20 SCRs that comprise CFH are each approximately 60 amino acids long, are arranged head to tail, and contain four cysteine residues forming two disulfide bonds per module (13). We originally detected antibodies to CFH in the sera of NSCLC patients by immunoblot, for which the CFH was reduced and denatured (2). As shown in Fig. 1, serum antibody recognition of CFH in an ELISA format was dependent on prior treatment of the CFH with a reducing agent, in this case TCEP. Reactivity was not dependent on prior denaturation of CFH. As CFH is a ubiquitous and abundant serum protein, it was surprising to find antibodies directed against it at all. However, the antibodies present in the sera of NSCLC patients all have a distinct preference for the reduced form of CFH, leading us to hypothesize that reduction reveals a cryptic epitope, and that this epitope may be revealed only on the surface of tumor cells. This hypothesis remains to be explored further.

Given that NSCLC patient antibodies recognize a reduced form of the protein, we wanted to examine the possibility that some patients with the antibody may have a mutation in the CFH gene. Such a mutation might create a structural mimic of the reduced form of the protein (such as a Cys to Ser mutation) or expose an epitope obscured in the wild-type form, so that the altered protein was now antigenic. We performed RT-PCR targeting the SCR19–20 domain using RNA isolated from tumor samples from 10 patients who were positive for the CFH antibody. All of the tested samples contained wild-type sequence in this domain.

CFH Autoantibodies Bind to an SCR19–20 Fragment of CFH

Initial domain-mapping experiments were performed by incubating patient sera with “dot blots” containing cloned and
puriﬁed subsets of SCR modules. CFH antibody–positive sera from 3 patients reacted with a fragment containing SCR19 and SCR20 (Supplementary Fig. S1). SCR19 and SCR20 are key domains in CFH function, containing binding sites for C3b/C3d and polyanions typical of self-surfaces (12, 14, 15).

**CFH autoantibodies epitope map to SCR19**

CFH antibodies from 8 patients were puriﬁed using sequential Protein G and SCR19–20 afﬁnity column chromatography steps. Three of the antibodies were epitope mapped on a library of overlapping peptides synthesized from the complete sequence of SCR19–20. The peptide binding data for one antibody are shown in Fig. 2. All three antibodies recognized the epitope PIDNGDIT. This amino acid sequence corresponds to CFH 1114–1121, residues that are located in the binding interface with the C3d portion of C3b in the cryocystal structures of C3d–SCR19–20 (5, 6). C3d is a cleavage product of C3b that remains attached to the cell surface via a thioester domain. CFH D1119 was shown to be critical to the stability of the interface with C3b/d because a D1119G mutant of SCR19–20 abolished binding to C3b/d (6, 16).

A peptide was synthesized with the PIDNGDIT(GGGK)–biotin sequence and was used as a competitor in immunoblots of CFH and SCR19–20 probed with each of the eight puriﬁed human CFH antibodies. Seven of the eight antibodies were competed by the peptide; competition is shown for three antibodies in Fig. 3.

**Subclass of lung cancer patient CFH autoantibodies is IgG3**

Possible functions for CFH autoantibodies fall into two (not mutually exclusive) categories: (i) The antibodies could have an effector function when bound to their target, for example, triggering immune cells expressing Fcγ receptors, leading to phagocytosis, or activating complement. (ii) The antibodies could serve to block a protein–protein interaction and thereby inhibit a speciﬁc function of CFH. We were interested in determining the subclass of the antibodies, as each subclass has a distinct proﬁle of effector function, as well as half-life (17). Subclasses were determined using a sandwich ELISA, incubating patient sera with bound peptide containing the epitope, and detecting bound antibody with a set of secondary antibodies speciﬁc for IgG1–4. The autoantibodies of all 22 lung cancer patients whose sera was tested were IgG3; data for 6 of these patients are shown in Fig. 4.

**Puriﬁed CFH antibodies inhibit binding of CFH to the surface of A549 lung carcinoma cells**

Interaction of both SCR19 and SCR20 to covalently bound C3b and polyanions is necessary for full binding of CFH to the mammalian cell surface (5). Once CFH is bound to the cell surface, the amino terminal regulatory domains of CFH prevent the formation of more C3b. Because lung cancer patient CFH autoantibodies recognize the C3b-binding domain in SCR19, we hypothesized that these antibodies would block the interaction of CFH with the cell surface, presumably by preventing the interaction with cell-bound C3b. Furthermore, because the antibodies recognize the reduced form of CFH with greater speciﬁcity than the native form, inhibition of CFH binding to the cell surface should be greater when CFH is reduced. We tested these hypotheses by incubating ﬂuorescently labeled CFH, native or reduced, with each of several autoantibodies or human IgG (negative control), then adding the mixtures to A549 lung adenocarcinoma cells and measuring CFH binding by ﬂow cytometry. All autoantibodies tested gave similar results, and results for “Antibody G” are shown in Fig. 5. Although Antibody G caused a decrease in the binding of both native and reduced CFH, only the decrease seen with the reduced form was signiﬁcant (0.009 vs. 0.089).

![Image](image-url)
Discussion

The majority of lung cancer patients present with advanced-stage disease and require systemic therapy. Even patients with resectable, early-stage lung cancer have an almost 50% chance of developing recurrence and at some point need adjuvant treatment. Over the past several years, new therapies targeting specific pathways have been introduced and, in selected individuals, these agents produce an initial response. However, almost all patients develop resistance, which is most likely due to tumor heterogeneity and clonal evolution.

We propose that characterization of the immune response to cancer in patients with early-stage disease who uniformly have good outcomes and never experience a recurrence could potentially reveal relevant molecular targets and guide development of an effective therapy. We have previously reported on the association of autoantibodies to CFH and early-stage lung cancer (2). This study is an extension of our earlier effort. Here, we characterize CFH antibodies from patients with a distinct nonmetastatic phenotype and begin to explore potential antibody mechanisms of action in the effort to recapitulate and, eventually, enhance the native immune response in destroying tumor cells.

We showed that the CFH antibodies from NSCLC patients recognize a conformationally distinct form of CFH, which can be revealed by chemical reduction in vitro. CFH is present at a high level in the circulation (0.1–0.8 mg/mL; ref. 18) and would not be expected to be antigenic except when it presents a cryptic epitope or is mutated, as can occur in the kidney disease atypical hemolytic uremic syndrome (19, 20). We saw no evidence that SCR19–20 was mutated in the CFH genes from 10 tumor samples, and none of the NSCLC patients who had CFH autoantibodies had evidence for renal disease. The absence of deleterious effects in normal tissues suggests that the epitope of the CFH antibody is revealed only in the tumor microenvironment.

It has been demonstrated that NSCLC tumors exhibit elevated levels of thioredoxin, the disulfide reductase macrophage migration inhibitory factor (21), and nonprotein thiols such as reduced...
cysteine and glutathione (22). These factors contribute to the production of a more reducing environment in the tumor than in normal tissues. Thus, the anti-CFH epitope(s) may be hidden and only exposed upon reduction of the protein in the intratumoral space. Alternatively, once the soluble form of CFH binds to the tumor cell, the protein may unfold and bind in a tumor cell–specific conformation so that it becomes antigenic; reduction in vitro may simply put CFH in a conformation that mimics this state. However, these hypotheses remain to be investigated.

Identifying the epitope recognized by CFH autoantibodies in NSCLC was of primary interest in order to understand the mechanism by which the autoantibody affects tumor cells and to elucidate the specific region within CFH that is targeted. The NSCLC patient sera we tested that are positive for CFH autoantibody by immunoblot bind to a fragment containing SCR19–20. Three sera we have tested by dot blot of cloned purified SCR domains did not bind to other parts of the protein. The epitope of three affinity-purified CFH autoantibodies was directly shown to be PIDNGDIT, which resides in SCR19. This epitope was also shown indirectly to be the target of four other purified CFH antibodies in an immunoblot/peptide competition assay. Thus, it is likely that this part of the CFH protein is immunogenic in patients with NSCLC who have CFH autoantibodies. SCR19 is a domain that is central to the host cell–protective function of CFH, being involved in binding to the C3d portion of C3b (6).

We determined that the autoantibodies in lung cancer patients were of the IgG3 subclass. This subclass represents only 4% of all immunoglobulins (17) but is the most effective in activating complement via the classical pathway: Using hapten-conjugated cells, Michaelsen and colleagues (23) showed that IgG3 activated CDC better than the other three subtypes at the lowest level of hapten. The CFH autoantibodies bind to a region of CFH known to interact with C3b, prevent C3 binding to tumor cells, and increase CDC of tumor cells, and thus seem to block the function of CFH in vitro under experimental conditions favoring the alternative pathway. However, in vivo, we do not know if these antibodies can bring about CDC due to classical pathway activation by C1q binding to the IgG3 CFH autoantibody. IgG3 is also the most effective subtype of immunoglobulin in activation of Fcγ-mediated effector functions (17). IgG3 has a relatively short half-life (1 week as compared with 3 weeks for the other three subtypes), which may limit excessive inflammatory responses (17, 24). In considering the development of a therapeutic CFH antibody, the properties of the IgG3 subtype of the natural human antibodies must be kept in mind.

Once the domain, epitope, and class of the CFH autoantibodies were elucidated, we focused further on the potential for these antibodies to activate the complement pathway on tumor cells. Purified CFH antibodies (i) inhibited CFH binding to A549 lung adenocarcinoma cells, (ii) increased C3 deposition on A549 cells, and (iii) increased cytotoxicity of A549 cells by the alternative

![Figure 5. CFH binding to A549 lung cancer cells. Antibodies tested are human IgG and human anti-CFH autoantibody from patient G (AbG). Experiments were performed in the presence of CFH-depleted serum. Fluorescently labeled CFH was reduced using TCEP. CFH binding is reported as fluorescence intensity after subtraction of the baseline fluorescence intensity observed using CFH-depleted serum without fluorescently labeled CFH added to the reaction.](image1)

![Figure 6. Deposition of C3-related fragments on A549 lung cancer cells. Antibodies are human IgG and human anti-CFH autoantibody from patient E (AbE), tested with or without blocking peptide (pep), in the presence of NHS or HI-NHS. Fold increase in C3 deposition is reported relative to the baseline observed in the absence of serum.](image2)
CFH Autoantibodies Induce CDC of Tumor Cells

Antibodies possess the inherent ability to kill tumor cells, but their levels or affinities are too low to be effective. Hence, the antibody target is relevant, but the B-cell response is insufficient to drive and sustain high-affinity antibody titers. The same situation may hold true for many antibodies against TAAs. Once an epitope has been identified and validated as a therapeutic target, humanized mouse monoclonal antibodies against it can be developed for potential therapeutic applications. Alternatively, methods have been devised to produce large quantities of high-affinity human antibodies for therapeutic use in order to overcome the limitations of the natural host response (28).

This study suggests that cues from the native humoral response in patients who have limited nonrecurrent disease may be useful in developing novel cancer immunotherapy. As we begin to unravel the complex host response to cancer, more effective systemic treatments derived from the native immune system may be possible. Future studies with recombinant CFH antibodies directed against the specific epitope seen in cancer patients will be investigated as a novel immunotherapy strategy.

Disclosure of Potential Conflicts of Interest
M.J. Campa, E.B. Gottlin, and E.F. Patz have ownership interest (including patents) in CIJE Biologics. No potential conflicts of interest were disclosed by the author.

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Figure 7. Complement-dependent cytotoxicity of A549 lung cancer cells by the alternative pathway. Antibodies tested are human IgG and human anti-CFH autoantibody from patient E (AbE), with or without blocking peptide (pep). Fold increase in cytotoxicity is reported relative to the baseline observed in the presence of NHS.
Campa et al.


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