

# Impaired intestinal tolerance in the absence of a functional complement system

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**Background:** Cells of the innate immune system regulate both adaptive immune responses and the maintenance of tolerance, especially in the gut. However, relatively little is known about the effects of complement on lymphocyte homeostasis.

**Objective:** This study explored complement C3 deficiency in mice and human subjects for its effect on intestinal tolerance.

**Methods:** C3-deficient mice and control C57BL/6 mice were fed ovalbumin (OVA) by means of gavage, and subsequent response to immunization with OVA in Freund's adjuvant was monitored. Serum antibodies against commensal microbes were measured, and the activation status of peripheral blood lymphocytes bearing mucosal homing markers was determined from 2 rare cases of C3-deficient patients.

**Results:** We show in C3-deficient mice and human patients that intestinal tolerance fails in the absence of functional complement. In contrast to wild-type control animals, in which oral tolerance was induced, intragastric administration of OVA did not result in a significantly decreased response to subsequent subcutaneous OVA challenge in C3-deficient mice. In the jejunum of C3-deficient mice the cytokine ratio between IL-10 and IFN- $\gamma$  or IL-17 levels was decreased, indicating a shift in favor of proinflammatory cytokines. In 2 C3-deficient children the frequency of gut-homing T cells expressing activation markers was increased, and the patients had increased serum IgG levels against gut commensal microbes.

The data also suggest that the impaired oral tolerance was at least partly caused by the absence of signaling through C3-binding complement regulators in T cells.

**Conclusions:** Taken together, our results identify complement as an important and nonredundant regulator of intestinal tolerance. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

**Key words:** Mucosal immunity, peripheral tolerance, complement C3, T lymphocytes, humoral immunity

Reflecting the importance of first-line defense, intestinal mucosa-associated lymphoid tissues represent the largest lymphocyte accumulation in the body, accounting for an estimated 70% of all lymphoid cells.<sup>1</sup> At the same time, the gut is a particularly challenging environment for the immunologic decision process. In no other organ does the immune system come regularly into such close contact with microorganisms and foreign antigens, a few of which are pathogenic, although most are not. Thus the local mechanisms have to choose between initiating an immune response, ignoring the foreign antigen, or actively inducing tolerance to it.

The pathogens using mucosal surfaces as a route of entry possess virulence factors that allow them to penetrate the epithelium and to avoid destruction by phagocytes, complement, and other innate mechanisms.<sup>2,3</sup> Proliferation of the pathogens leads to increased signaling through pattern-recognition receptors and, ultimately, to dendritic cell activation and induction of an adaptive immune response in the gut-associated lymphoid tissues.<sup>4,5</sup> The activated effector cells express mucosal homing receptors, which allow them to migrate to the gut epithelium, with subsequent clearing of infection.<sup>5</sup>

However, the default mode of response in the gut favors tolerance.<sup>4,6,7</sup> The interaction of the immune system and normal microbial flora is characterized by limited local IgA responses and systemic ignorance.<sup>3</sup> Commensal microbes lack the virulence factors necessary for penetration of the epithelium and are rapidly cleared by innate immune cells if they reach the lamina propria. Therefore antigens derived from commensal organisms are scarce outside the gut lumen, and the activation of both innate and adaptive immunity is limited.<sup>8,9</sup>

Another potential outcome of intestinal antigen exposure is systemic unresponsiveness, which is often termed oral tolerance. Dendritic cells presenting antigens in the absence of proinflammatory signals induce regulatory T cells in the mesenteric lymph nodes, which then mediate systemic antigen-specific tolerance.<sup>7</sup> In addition to forkhead box P3 (FOXP3)-positive regulatory T cells, especially the IL-10-producing T<sub>R</sub>1 cells, have been implicated in the maintenance of intestinal homeostasis.<sup>10</sup>

Although ultimately mediated by adaptive immunity, the decision between a response and tolerance depends on the

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**Abbreviations used**

|                      |                                                          |
|----------------------|----------------------------------------------------------|
| C3 <sub>ctrl</sub> : | C3 knockout mice, saline feeding, ovalbumin immunized    |
| C3 <sub>OVA</sub> :  | C3 knockout mice, ovalbumin feeding, ovalbumin immunized |
| FOXP3:               | Forkhead box P3                                          |
| HRP:                 | Horse radish peroxidase                                  |
| KO:                  | Knockout                                                 |
| OVA:                 | Ovalbumin                                                |
| PE:                  | Phycoerythrin                                            |
| TCR:                 | T-cell receptor                                          |
| WT:                  | Wild-type                                                |
| WT <sub>ctrl</sub> : | Wild-type mice, saline feeding, ovalbumin immunized      |
| WT <sub>OVA</sub> :  | Wild-type mice, ovalbumin feeding, ovalbumin immunized   |

particular combination of microbe-associated molecular signals sensed by innate immunity.<sup>9</sup> Thus the function of innate immune cells and cellular receptors has been studied in increasing detail and elucidation. In contrast, very little is known about the role of complement in gut-associated adaptive immune responses. Yet complement is a potent modulator of the adaptive immune system on several levels.<sup>11</sup> Complement-mediated opsonization enhances phagocytosis and helps follicular dendritic cells to bind antigens to their surface, thus affecting antigen presentation to lymphocytes.<sup>12-14</sup> The soluble complement cleavage products C3a and C5a participate in regulating cellular traffic.<sup>15</sup> More directly, complement C3d receptor CR2 on B cells decreases their activation threshold and enhances proliferation and survival in the germinal centers.<sup>16,17</sup> T-cell function is also modulated by complement. T-cell responses against viruses are impaired and rejection of allografts is delayed in C3-deficient mice.<sup>18,19</sup> This is at least partly due to disrupted T<sub>H</sub>1 differentiation in the C3-deficient animals, as reported in several studies.<sup>20-22</sup>

Here we have examined the role of complement in the establishment of oral tolerance. Our data, obtained from both C3-deficient mice and 2 rare cases of total C3 deficiency in human subjects, identify complement as an important and nonredundant regulator of oral tolerance.

**METHODS****Study subjects**

PBMCs were isolated from patients 1 and 2 when they were 8.5 and 7 years old, respectively, and sera at the age of 2.5 years. Control samples were collected from children (n = 19; age, 3 days to 3 years) and adults (n = 3; age, 21-38 years). PBMCs were isolated with Ficoll-Paque PLUS (GE Healthcare, Waukesha, Wis) density gradient centrifugation. All subjects were free of acute infections at the time of sampling. Written informed consent was obtained from the study subjects or their parents. The study plan was accepted by the ethics committee of the Joint Municipal Authority of the Pirkanmaa Hospital District and by the ethics committee for pediatrics at the Helsinki University Central Hospital.

**Mice and immunizations**

The mice used were C3 knockout (KO) mice on the C57BL/6 background, with sex- and age-matched wild-type (WT) C57BL/6 mice used as control animals (kindly provided by Professor Marcela Pekna, Gothenburg University, Gothenburg, Sweden). The mice were given an intragastric dose of 1 mg of ovalbumin (OVA) in 100  $\mu$ L of saline or saline only twice a week for 4 weeks. After gavage, the mice were immunized subcutaneously in the neck once with 50  $\mu$ g of OVA (grade V; Sigma-Aldrich, St Louis, Mo) in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich) and reimmunized twice with 50  $\mu$ g of OVA in Freund's incomplete adjuvant (Difco Laboratories)

at intervals of 2 weeks. Animals were killed 1 to 2 weeks after the last subcutaneous boost. The experiment was repeated 3 times independently, each time with 6 mice in each group. The study was approved by the Laboratory Animal Board of the Southern Finland Regional State Administrative Agency.

**Western blotting and complement measurements**

Serum samples were run in a 10% SDS-PAGE gel under reducing conditions. Proteins were transferred to a nitrocellulose membrane, and nonspecific binding sites were blocked with 5% milk in PBS/0.05% Tween 20. Polyclonal rabbit anti-human C3c antibody (1:10,000; Dako, Glostrup, Denmark) was added and incubated at +4°C overnight. The membrane was washed with PBS/0.05% Tween, incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, Pa) for 1 hour at room temperature, and again washed. Goat anti-mouse C3 antibody (1:2000; Bethyl Laboratories, Montgomery, Tex) and HRP donkey anti-goat (1:5000; Jackson ImmunoResearch Laboratories) were used to measure mouse C3 levels in undiluted cell-culture supernatants. The bound antibodies were detected by means of electrochemiluminescence.

Complement total hemolytic activity of the classical pathway (CH100Cl) was measured according to standard diagnostic procedures with the enzyme immunoassay, and the serum concentration of C3 was measured with immunoturbidimetry.

**ELISA**

Mouse OVA-specific immunoglobulin levels were measured as described previously and are presented as OD at 405 nm.<sup>21</sup> Human immunoglobulin levels and vaccine responses were measured by using standard diagnostic ELISA procedures (HUSLAB and National Institute for Health and Welfare, Helsinki, Finland).

Heat-inactivated serum samples from human subjects were used to determine antibodies against commensal microbes. Strains of commensal organisms were isolated from clinical samples or healthy volunteers and grown in suitable broth until the mid-log phase. The microbes were washed twice with PBS, and the concentration was adjusted to 0.6 OD at 600 nm. MaxiSorp microtitre plates (Nunc, Roskilde, Denmark) were coated with 100  $\mu$ L per well of the microbe suspension and allowed to dry overnight at +37°C. The wells were washed with PBS/0.05% Tween, and serum samples were incubated in the wells for 1 hour at room temperature. Bound IgG and IgA were detected by using HRP-conjugated anti-human IgG or IgA (1:5000, Jackson ImmunoResearch Laboratories), followed by OPD substrate solution (Dako). The amount of bound antibody was measured as OD at 492 nm.

Mouse IL-10 levels were measured from cell-culture supernatants with a commercial ELISA reagent set (Mouse IL-10 ELISA Ready-SET-Go; eBioscience, San Diego, Calif), according to the manufacturer's instructions. The supernatants were stored at -70°C until used undiluted for ELISA. Plates were read at 450 nm, and absorbance values of 560 nm and background OD from blank wells without added samples were subtracted.

**Cell stimulation assays**

Mouse spleens were homogenized mechanically, and erythrocytes were lysed with RBC lysing buffer (Sigma-Aldrich). Splenocytes were cultured at  $2 \times 10^5$  cells per well on 96-well plates, as described previously.<sup>21,23</sup> The cells were stimulated in triplicate with OVA (10  $\mu$ g/mL) for 5 days and then pulsed for 6 hours with tritiated thymidine ( $3.7 \times 10^4$  Bq per well; GE Healthcare, Buckinghamshire, United Kingdom). The cells were harvested with a Skatron harvester (Newington, NH) and analyzed with a Microbeta liquid scintillation counter (Wallac, Turku, Finland) by using OptiScint HiSafe scintillation fluid (PerkinElmer, Waltham, Mass). The data are presented as the stimulation index, which was calculated by dividing the average cpm value measured in stimulated wells by the average cpm in control wells.

For Crry/CD3 stimulation, splenocytes from WT C57BL/6 mice (2 individual experiments, n = 4 in each) were stimulated with plate-bound anti-mouse CD3 (clone 145-2C11, 0.3  $\mu$ g/mL) and Crry (clone 1F2, 2  $\mu$ g/mL)

antibodies, both from BD Biosciences (San Jose, Calif). Antibody solutions were incubated in wells overnight at +4°C, and the wells then rinsed twice with PBS. Stimulation was performed in serum-free conditions in CTL-wash medium (CTL, Shaker Heights, Ohio) supplemented with 2 mmol/L L-glutamine, 50  $\mu$ mol/L 2-mercaptoethanol, 100  $\mu$ g/mL streptomycin, and 100 U/mL penicillin (all from Sigma-Aldrich).

## Flow cytometry

Monoclonal anti-mouse CD4–phycoerythrin (PE)/Cy5, CD8-PE, and CD19-PE (BD Biosciences) were used to stain mouse splenocytes. Human PBMCs were first stained for surface markers with anti-human CD4–allophycocyanin/Cy7, CD8-PE/Cy7, integrin  $\alpha$ 4-PE/Cy5 (BD Biosciences), integrin  $\beta$ 7-PE (eBioscience), CD69–fluorescein isothiocyanate (ImmunoTools, Friesoythe, Germany), CD45RO-biotin (BioLegend, San Diego, Calif), and streptavidin-PE/TexasRed (BD Biosciences). The cells were then fixed and permeabilized by using the FOXP3 Fix&Perm kit (eBioscience) and stained with anti-FOXP3–allophycocyanin (eBioscience), according to the manufacturer's instructions. The samples were analyzed by using the FACSaria instrument (BD Biosciences).

## Quantitative RT-PCR

Splenocytes and tissue samples were lysed by using TriPure Isolation Reagent (Roche, Basel, Switzerland) and mechanical homogenization. Total RNA was then isolated with RNeasy MiniKit columns (Qiagen, Crawley, United Kingdom). First-strand cDNA was synthesized by using AMV-reverse transcriptase (Finnzymes, Helsinki, Finland) and oligo-dT-primer (Sigma-Aldrich). Quantitative real-time PCR analysis was performed with the iCycler-IQ instrument (Bio-Rad Laboratories, Hercules, Calif). All assays were intro-spanning primer probe assays purchased from Applied Biosystems (Foster City, Calif); the mouse T-cell receptor (TCR) C $\alpha$  assay has been previously described.<sup>21</sup> Relative expression levels were calculated by subtracting IL-10 cycles from IFN- $\gamma$  or IL-17A cycles or by subtracting the cytokine cycles from TCR C $\alpha$  cycles.

## Immunohistochemical staining of mouse jejunum

From the pylorus to the cecum, the exact middle segment of 1 cm in length was excised and embedded in Tissue Tek OCT-compound (Sakura Finetek, Alphen aan den Rijn, Netherlands). Samples were frozen in liquid nitrogen and cut into 7- $\mu$ m sections. The slides were fixed in –20°C acetone and blocked in 1% rabbit serum for 30 minutes. For the detection of CD3<sup>+</sup> cells, a monoclonal rat anti-mouse antibody was used (RM3400; Caltag, Burlingame, Calif), with a negative isotype-matched control (R2a00, Caltag). Biotinylated anti-rat antibody (BA-4001) and the peroxidase-based detection system were used, according to the manufacturer's instructions (Vectastain ABC Elite; Vector Laboratories, Burlingame, Calif). The number of positively stained cells in the lamina propria was counted through a calibrated graticule at  $\times 60$  magnification by using an Olympus BX50 light microscope (Olympus, Center Valley, Pa). The cell density was expressed as cells per square millimeter.

## Statistical analysis

Data are presented as means  $\pm$  SDs. Statistical analysis was performed with SPSS software, version 19 (SPSS, Chicago, Ill). The statistical significance of differences among the 4 groups of experimental animals were assessed by using 1-way ANOVA, with the Tukey HSD *post hoc* test for comparison of pairs of groups. For Crry stimulation data, the paired samples *t* test was used. The limit for statistical significance was a *P* value of less than .05. Patients' data are shown as individual values.

## RESULTS

### T-cell responses in C3-KO mice after oral tolerization

To elucidate the role of complement in determining the outcome of intestinal antigen exposure, we compared the

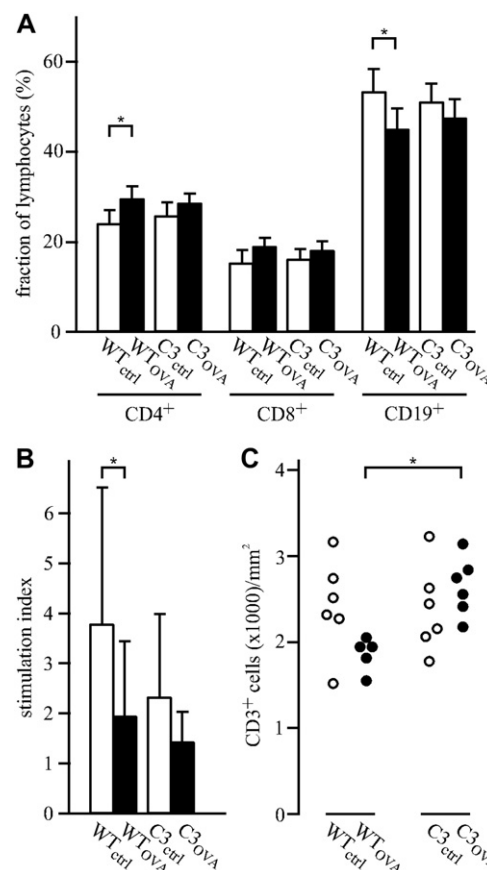


**FIG 1.** Lymphocyte populations in WT<sub>ctrl</sub>, WT<sub>OVA</sub>, C3<sub>ctrl</sub>, and C3<sub>OVA</sub> mice. **A**, Flow cytometric analysis of splenocyte populations at the end of the experiment (*n* = 6). **B**, Proliferative response of splenocytes to OVA stimulation *in vitro*. Data are shown as the stimulation index (*n* = 18). **C**, Immunohistologic quantitation of CD3<sup>+</sup> cells in the jejunum wall (*n* = 6, each symbol represents an individual mouse). Experimental groups are indicated in the figure. \**P* < .05.

induction of oral tolerance in C3-KO and syngeneic WT mice. The mice were given repeated intragastric doses of OVA or physiologic saline and then immunized with OVA. After death, flow cytometric analysis of splenocytes showed a slight increase in the frequency of CD4<sup>+</sup> T cells in the WT mice fed OVA, with a concomitant decrease in the frequency of B cells (Fig 1, A).

We then tested the induction of tolerance in WT mice by stimulating the isolated splenocytes with OVA and measuring the proliferative response in saline-fed and OVA-fed, subsequently immunized animals (hereafter referred to as the WT<sub>ctrl</sub> and WT<sub>OVA</sub> groups, respectively). The data showed a significantly decreased stimulation index in the WT<sub>OVA</sub> group (Fig 1, B), which is consistent with systemic tolerance induced by the mucosal antigen. In line with our previous findings,<sup>21</sup> the OVA-immunized C3-KO mice mounted a poor proliferative response. The stimulation indices in the saline-fed and then OVA-immunized C3-KO animals (hereafter referred to as the C3<sub>ctrl</sub> group) barely reached 2, and therefore no definite conclusions regarding the OVA-fed and immunized C3-KO group (hereafter referred to as the C3<sub>OVA</sub> group) could be drawn (Fig 1, B).

The intragastric administration of OVA should also dampen the subsequent local gut-associated response to OVA immunization. Analysis of the gut-associated response to OVA, which was

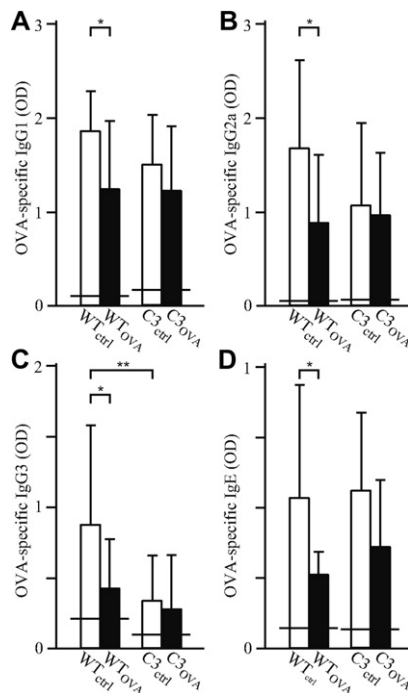


**FIG 2.** OVA-specific serum immunoglobulin response after oral tolerization and OVA immunization in mice. **A**, IgG1. **B**, IgG2a. **C**, IgG3. **D**, IgE. ELISA data are presented as OD values ( $n = 18$ ). Background levels of OVA-binding antibodies in the sera of nonmanipulated mice of each strain ( $n = 3$ ) are indicated with a horizontal line in each panel. Experimental groups are indicated in the figure. \* $P < .05$  and \*\* $P < .01$ .

measured as the density of CD3<sup>+</sup> cells in the gut wall, showed similar numbers in the jejunum of WT<sub>ctrl</sub> and C3<sub>ctrl</sub> mice (Fig 1, C). The OVA-fed and immunized groups, in contrast, showed a significant difference between the WT<sub>OVA</sub> and C3<sub>OVA</sub> groups, with no decrease in response in the latter group, suggesting that in the absence of functional complement, the preceding mucosal exposure to OVA did not prevent a local T-cell response to immunization.

### Analysis of antibody responses after oral tolerization

Next we measured the OVA-specific antibody response. Consistent with complement's direct enhancing effects on B cells,<sup>16</sup> OVA-specific IgG levels were lower in the C3-KO mice (Fig 2). The most pronounced difference was found in T<sub>H</sub>1-associated IgG<sub>3</sub> levels, which is in agreement with previous studies reporting impaired T<sub>H</sub>1 responses in C3-KO mice.<sup>20,21</sup> Nevertheless, all measured classes of OVA-specific antibodies were significantly higher in all experimental groups compared with those seen in nonimmunized control animals ( $n = 3$ ) of the same mouse strain (Fig 2), indicating a broad OVA-specific B-cell response to parenteral immunization.

In WT mice the effect of mucosal OVA administration on subsequent B-cell responses after parenteral immunization was clear. In all the antibody classes tested, the WT<sub>OVA</sub> group had significantly lower levels of OVA-specific antibodies than the WT<sub>ctrl</sub> group (Fig 2). In contrast, there was no significant difference in OVA-specific immunoglobulin levels between the C3<sub>ctrl</sub> and C3<sub>OVA</sub> groups. This was also true of T<sub>H</sub>2-dependent IgE levels, in which the response was similar in the saline-fed groups, but

OVA feeding resulted in a significant decrease only in the WT mice. Taken together, the data confirmed the negative effects of complement dysfunction to T- and B-cell responses but also revealed a perturbation of oral tolerance in the absence of C3.

### Identification and clinical characteristics of 2 C3-deficient patients

Total absence of functional C3 in human subjects is very rare, most likely because of the importance of this central complement factor, with approximately 20 families with inherited C3 deficiency described worldwide. The clinical picture is dominated by recurrent invasive infections, but autoimmune conditions, including immune complex disorders resembling systemic lupus erythematosus, glomerulonephritis, and IgA nephropathy, have also been reported.<sup>24</sup>

We report the identification of 2 male siblings with permanent total lack of C3 (Fig 3, A). The elder brother (patient 1) was referred to specialist consultation at the age of 18 months because of a suspected primary immunodeficiency. He had prolonged pneumonia, which had, despite appropriate antimicrobial treatment, led to pleural effusion and ultimately to pneumococcal sepsis (serogroup 6B). Examination of his immune status revealed undetectable complement hemolytic activity and total absence of C3 (Table I). Serum immunoglobulin levels and leukocyte counts were normal.

Subsequently, the younger brother (patient 2) and the parents were also examined. The younger brother, by the age of 11 months, had no clinical symptoms or infectious complications suggestive of immunodeficiency. However, he was found to lack C3 also. His leukocyte counts and immunoglobulin levels were normal. The nonconsanguineous parents both showed decreased levels of C3 in sera (0.5 and 0.6 g/L in the mother and father, respectively) but normal total hemolytic complement activity. They had no history of autoimmune disease or atypical infections.

At the age of 22 months, the older brother had another episode of pneumococcal sepsis (serogroup 35F). The brothers were then started on prophylactic amoxicillin, and the elder brother has had no further infectious complications. The younger brother has had 2 episodes of fever without focal symptoms. He also had atopic dermatitis in infancy and transient nocturnal wheezing at the age of 4 years, which eased without specific treatment. Skin prick test responses for cat and dog dander were positive.

Both siblings have been vaccinated repeatedly, and the antibody responses have, for the most part, been within normal values. However, the older patient displayed protective antibody response to only 1 of the tested 7 serotypes of *Streptococcus pneumoniae*, despite repeated vaccinations with 7-valent conjugate vaccine (Prevenar; Pfizer, New York, NY) and a previous history of pneumococcal sepsis. Both patients also displayed poor long-term antibody response to tetanus vaccination, and interestingly, the T-cell proliferation response to tetanus toxoid was impaired despite normal response to anti-CD3 stimulation *in vitro* (data not shown).

### Analysis of mucosal immunity in the C3-deficient siblings

Because our analysis of C3-KO mice suggested perturbation of oral tolerance in the absence of functional complement, we analyzed whether the C3-deficient siblings had signs of abnormal mucosal immunity. No other samples than blood were available or feasible. We therefore used the combined

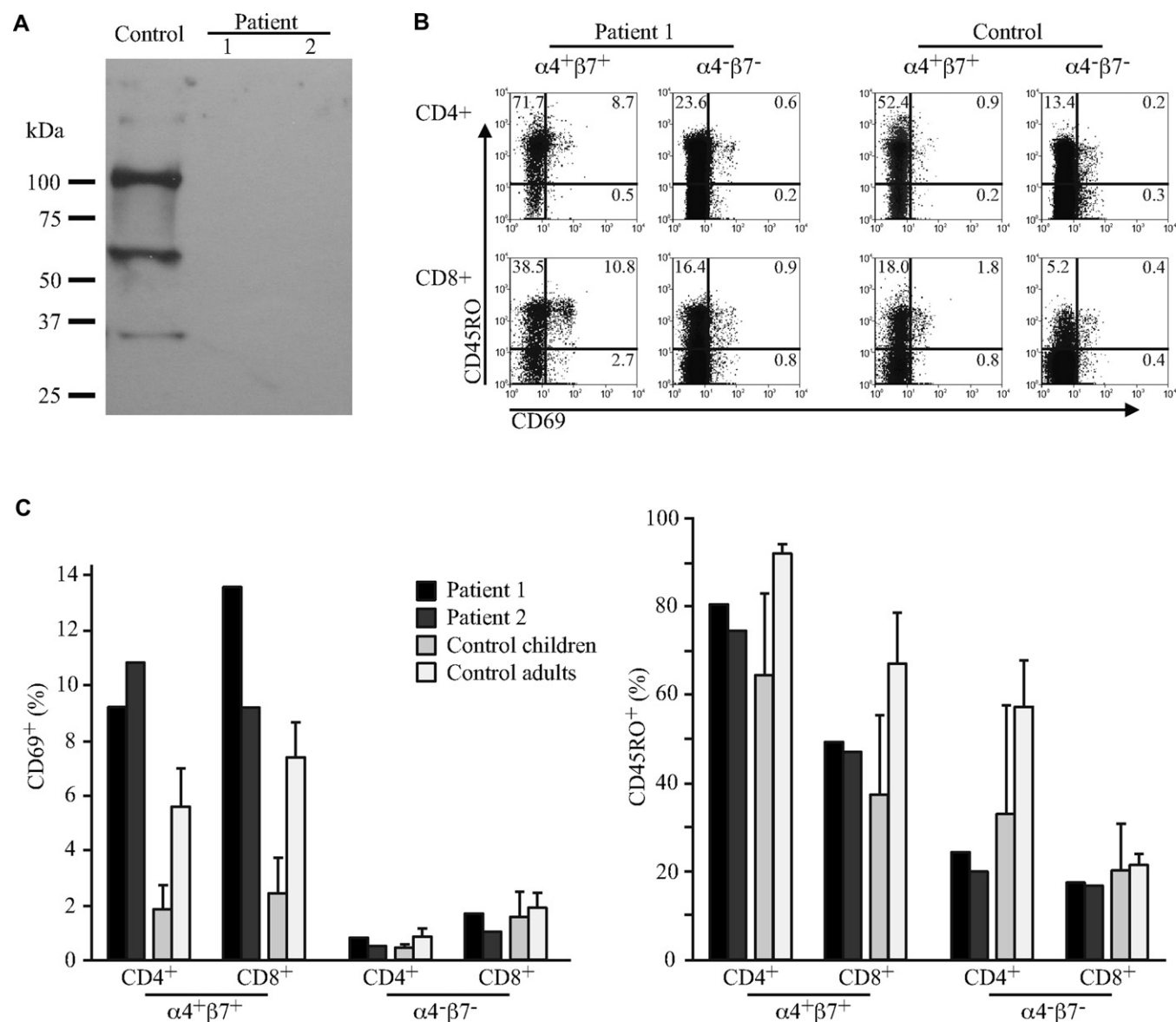


**FIG 3.** Activation markers in gut-homing T cells in C3-deficient patients. **A**, Western blot of serum C3 in the patients and a healthy control subject. **B** and **C**, Flow cytometric analysis of activation and memory markers in gut-homing integrin  $\alpha 4^+ \beta 7^+$  double-positive T cells and nonmucosal  $\alpha 4^- \beta 7^-$  double-negative T cells. The cells were gated to CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes, as indicated. Representative patient and control (child) data are shown in Fig 3, B. Control children, n = 5; control adults, n = 3.

expression of  $\alpha 4$  and  $\beta 7$  integrin on lymphocytes to identify cells en route to or from the gut-associated lymphoid tissues and analyzed the expression of the early activation marker CD69 by using flow cytometry. In both CD4<sup>+</sup> and CD8<sup>+</sup> cells, the integrin  $\alpha 4^+ \beta 7^+$  cells of the C3-deficient boys had a much higher frequency of CD69<sup>+</sup> cells than the normal control cells (Fig 3, B and C). This was most pronounced when compared with samples from healthy children because adults tended to have a higher frequency of CD69 expression. The patients also had a higher than average expression of CD45RO in the  $\alpha 4^+ \beta 7^+$  population, although most of the gut-homing cells were CD45RO<sup>+</sup> in the control subjects as well. In  $\alpha 4^- \beta 7^-$  cells no similar differences were found, indicating that the changes were specific to the gut-associated T cells.

Experiments with oral antigen exposure and immunization were not feasible, and the patients had not received any oral vaccines that would have provided defined antigens for *in vitro* analysis. However, defects in mucosal tolerance have been linked to mishandling of and abnormal immune responses to commensal microbes of the gut.<sup>25,26</sup> We therefore measured the boys' serum IgA and IgG levels against several species of commensal microbes, especially those residing in the gut. The serum samples were taken from both patients at the age of 2.5 years.

Compared with levels seen in age-matched control subjects, the patients showed increased levels of IgG specific to most of the tested species of commensal gut flora (Fig 4), including the fungal commensal organism *Saccharomyces cerevisiae*. Notably, *S. cerevisiae*-specific IgG has been used as a biomarker

**TABLE I.** Serologic characteristics of C3-deficient patients

|                        | Patient 1    | Patient 2    | Reference value |
|------------------------|--------------|--------------|-----------------|
| IgG (g/L)              | 6.1          | 6.14         | 2.3-9.8         |
| IgA (g/L)              | 0.57         | 0.323        | 0.1-1.11        |
| IgM (g/L)              | 1.01         | 0.704        | 0.27-1.86       |
| IgG <sub>1</sub> (g/L) | 4.77         | 4.45         | 3.2-9.0         |
| IgG <sub>2</sub> (g/L) | 0.51         | 1.00         | 0.52-2.8        |
| IgG <sub>3</sub> (g/L) | 0.198        | 0.258        | 0.14-1.20       |
| IgG <sub>4</sub> (g/L) | 0.007        | 0.022        | <0.003-1.06     |
| CH100Cl (IU/mL)        | Undetectable | Undetectable | 22-69           |
| C3 (g/L)               | Undetectable | Undetectable | 0.71-1.41       |
| Pnc (4) (μg/mL)        | 0.48         | 5.03         | 0.34            |
| Pnc (6B) (μg/mL)       | 0.48         | 3.41         | 0.61            |
| Pnc (9V) (μg/mL)       | 0.46         | 0.60         | 0.66            |
| Pnc (14) (μg/mL)       | 0.24         | 5.20         | 0.59            |
| Pnc (18C) (μg/mL)      | 1.17         | 1.34         | 0.31            |
| Pnc (19F) (μg/mL)      | 1.13         | 12.63        | 1.41            |
| Pnc (23F) (μg/mL)      | 0.54         | 2.58         | 0.34            |
| TT (IU/mL)             | 0.34         | 0.80         | >0.1            |

Sera were collected at the age of 2.5 years. Reference values for serogroups of *S pneumoniae* represent average values in healthy, unvaccinated, 4- to 5-year-old subjects.

CH100Cl, Total complement hemolytic activity (classical pathway); Pnc (n), serum IgG against *S pneumoniae* (serogroup); TT, serum IgG against tetanus toxoid.

for disease activity in patients with inflammatory bowel disease.<sup>25,27</sup> Levels of IgG specific to *Staphylococcus epidermidis* and *Streptococcus viridans*, which are mainly associated with the skin and oral cavity, respectively, showed no increase. Serum IgA levels specific to the commensal microbes were generally similar or slightly decreased when compared with those observed in the age-matched control subjects. These findings are consistent with a shift in the mucosal immune homeostasis from tolerance to productive immune responses directed against commensal microbes.

### Shift of intestinal cytokine balance to the inflammatory direction in the absence of C3

Complement activation is mainly an immunostimulatory phenomenon, leading to leukocyte recruitment, augmented activation, and enhanced function.<sup>15,16,20</sup> However, complement also has a crucial role in removal of cellular debris, a physiologic event that should not cause inflammation. The membrane-bound C3 cleavage products are directly involved in this process through recognition by C3 receptors on phagocytes.<sup>13</sup> The noninflammatory state is bolstered by the ligation of C3 to complement regulators on the host cells, which can induce a suppressive phenotype. Thus the ligation of human CD46, a widely expressed complement regulator, induces in T cells a T<sub>H</sub>1-like phenotype that is associated with IL-10 production, suppression, and, interestingly, mucosal homing.<sup>28-30</sup> In C3-deficient patients the CD46-dependent induction of T<sub>H</sub>1 cells has been shown to be impaired.<sup>31</sup> Moreover, IL-10 is a crucial mediator of intestinal tolerance and also regulates anticommensal responses.<sup>10</sup> We therefore examined whether the lack of C3 would have similar consequences for immunoregulation in the C3-KO mice and whether this might be linked to the impaired oral tolerance.

The expression of murine CD46 is limited to the testis. The membrane cofactor protein function of human CD46 is carried out in mice by Crry, a complement regulator expressed also on T cells.<sup>32</sup> Crry has been shown to provide costimulatory signals to

cells,<sup>33,34</sup> but its possible role in immunoregulation is poorly known, with only 2 reports linking it to IL-10 and regulatory T cells in mouse models of arthritis.<sup>35,36</sup>

To test whether a direct interaction between the C3 receptor membrane cofactor protein and C3 could promote intestinal tolerance, we first analyzed the costimulatory and immunoregulatory effects of Crry ligation on mouse splenocytes. To exclude the confounding effects of serum-derived C3, which could be caused, for example, by anti-Crry mAb-mediated inhibition of complement regulation,<sup>32</sup> the experiments were performed with a serum-free culture medium. We also performed a sensitive Western blot analysis of the culture supernatants to ascertain that the cultured cells themselves did not produce C3; none was detected (Fig 5, A).

When used in conjunction with mitogenic anti-CD3 mAb, the anti-Crry mAb had a clear costimulatory effect, increasing the stimulation index from 11 to 23 (n = 4). This showed that ligation of Crry by the mAb was functional, with clear effects on intracellular signaling. We then analyzed the effects of Crry ligation in the absence of immunostimulatory signals by incubating the splenocytes with the anti-Crry mAb alone. As expected, this had no mitogenic effects, but cytokine mRNA levels in the splenocytes showed a shift, with significantly increased expression of IL-10 and decreased expression of IFN-γ (Fig 5, B and C). We also tested the expression of IL-17, but in most cultures no signal was detected.

IL-10 protein levels in the culture supernatants were slightly higher after Crry ligation than in the nonstimulated control wells (80 ± 20 vs 72 ± 20 pg/mL), but the levels in some samples decreased to less than the detection threshold, and the difference was not significant. However, when anti-CD3-stimulated cells were analyzed, costimulation with anti-Crry significantly increased the production of IL-10, from 148 ± 64 pg/mL with anti-CD3 alone to 211 ± 102 pg/mL (P < .02).

We then analyzed the local balance between IL-10 and IFN-γ levels in the jejunum in the 4 experimental mouse groups. The balance between IL-10 and IFN-γ expression was significantly shifted toward the suppressive IL-10 in WT<sub>OVA</sub> mice, whereas no difference was seen between the C3<sub>ctrl</sub> and C3<sub>OVA</sub> groups (Fig 5, D). We also tested the balance between IL-10 and IL-17, an important proinflammatory cytokine expressed by T<sub>H</sub>17 cells. The cytokine balance in the jejunum was shifted toward proinflammatory IL-17 expression in C3<sub>OVA</sub> mice compared with that seen in WT<sub>OVA</sub> mice (Fig 5, E).

We did not see changes in FOXP3 expression in the gut wall (data not shown), despite the fact that FOXP3-expressing regulatory T cells have been shown to be important for oral tolerance in earlier studies.<sup>7</sup> Nevertheless, our data indicate that in the absence of functional C3, the OVA feeding did not shift the subsequent local response to OVA immunization to a protolerance cytokine balance.

### DISCUSSION

It has been previously shown that C3 deficiency affects CD4<sup>+</sup> T-cell functional differentiation and is associated with impaired T<sub>H</sub>1 and enhanced T<sub>H</sub>2 responses.<sup>20,21</sup> Our data showed also that those aspects of the response dependent on T<sub>H</sub>1 function were lower in C3-KO mice. A significant proliferative T-cell response to OVA was not observed, and the T<sub>H</sub>1-dependent IgG<sub>3</sub> response was particularly low in the C3-KO mice. Nevertheless, in all antibody classes the OVA-specific response was significantly

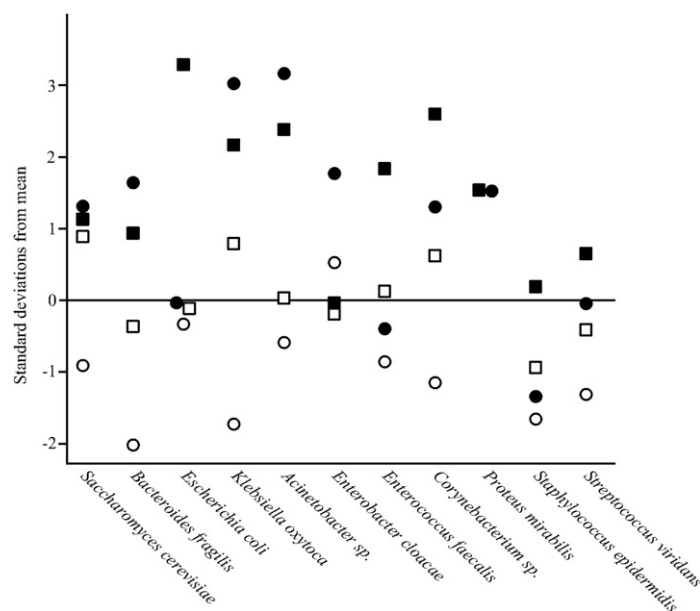


**FIG 4.** Serum IgG and IgA levels against selected microbes in C3-deficient patients compared with those seen in age-matched healthy control subjects. The normal distribution of immunoglobulin levels in control subjects ( $n = 14$ ) was determined, and the data from the C3-deficient patients are presented as SDs from the mean of control samples. Solid symbols, IgG; open symbols, IgA; squares, patient 1; circles, patient 2.

higher than in nonimmunized control animals, indicating successful immunization and induction of a systemic OVA response. Prior oral exposure to OVA did not suppress the response to immunization in C3-KO mice, whereas in the WT control animals a significant decrease in specific antibody levels was readily induced. Thus despite the decreased  $T_H1$ -type responses seen in the C3-KO animals, the failure of oral antigen administration to induce tolerance was clear.

A rare opportunity to extend our studies to human subjects was offered by the diagnosis of 2 brothers with total C3 deficiency. Our analysis of intestinal immunity in these boys was necessarily limited. No other samples than blood were available, and the rarity of the condition precluded comparison with other similar patients. Nevertheless, our findings clearly identified an abnormally high frequency of activated lymphocytes bearing gut-associated homing markers and increased levels of IgG specific to commensal flora. A similar shift from local IgA production to IgG-dominated responses against commensal microbes has been reported in patients with inflammatory bowel diseases, and in these patients the increased IgG levels themselves might be a factor driving inflammation.<sup>25</sup> Gastrointestinal symptoms were not observed in our C3-deficient siblings, and they have not been prominent in other reported cases either. However, C3 deficiency is associated with increased susceptibility to autoimmune manifestations, especially in later age.<sup>24</sup> Chronic stimulation by mishandled commensal antigens might contribute to the immune dysregulation and loss of tolerance.

Our results also suggest that C3 deficiency is associated with a local shift of cytokine balance from tolerogenic IL-10 production to stimulatory IFN- $\gamma$  and IL-17 production. IL-10 is a crucial mediator of intestinal tolerance,<sup>10</sup> and CD46 ligation by C3 has been previously shown to regulate  $T_R1$  differentiation and IL-10 production by  $CD4^+$  T cells.<sup>31</sup> Our data, together with earlier

reports,<sup>35,36</sup> suggest that Crry has an analogous role in promoting IL-10 production in mice, at least in conjunction with TCR/CD3-mediated signaling. It is thus likely that one important mechanism for the disrupted intestinal tolerance is the lack of CD46/Crry signaling and resultant local decrease in IL-10-producing cells. The consequences of this  $T_R1$  defect might be particularly pronounced at interfaces with constant exposure to persisting antigens, especially gut.

Nevertheless, given the central role of C3 in the complement cascade, it is probable that other mechanisms also contribute to the dysregulated responses to commensal organisms. C3 deficiency has a clear negative effect on phagocytes, affecting antigen uptake and dendritic cell and macrophage maturation and function.<sup>31</sup> Moreover, the block at the level of C3 activation also prevents C5 cleavage, and thus anaphylatoxins C3a and C5a are not produced. This is likely to alter the kinetics of cell migration and perhaps also the composition of local cell populations. Defective complement activation might therefore lead to less effective commensal clearance with prolonged stimulatory presence combined with a weakening of tolerogenic signals and IL-10 production in particular. The failure of tolerance induction is then reflected on both B- and T-cell responses at the systemic level. Our findings thus show in both mice and human subjects that complement function is essential to the induction and maintenance of intestinal tolerance.

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**Clinical implications: Recognizing the role of complement as a regulator of intestinal tolerance affects the treatment strategies for inflammatory bowel diseases and food allergies.**

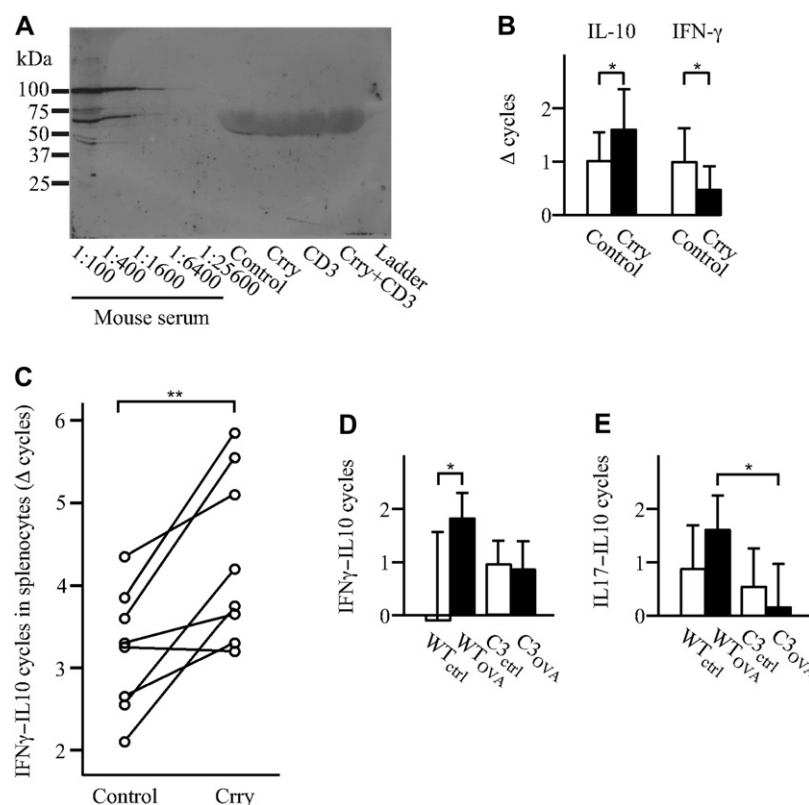


**FIG 5.** Cytokine balance in C3 deficiency in mice. **A**, Western blot analysis of C3 in cell-culture media, with a dilution series of normal mouse serum as a control. **B** and **C**, Relative expression of IL-10 and IFN- $\gamma$  mRNA normalized against TCR C $\alpha$  mRNA expression (Fig 5, **B**) and relative expression of IL-10 mRNA compared with IFN- $\gamma$  mRNA (Fig 5, **C**) in WT splenocytes stimulated with anti-Cry mAb for 3 days. In Fig 5, **C**, each pair of connected circles represents an individual mouse ( $n = 8$ ). **D** and **E**, Relative expression of IL-10 mRNA compared with IFN- $\gamma$  mRNA (Fig 5, **D**) and IL-17A mRNA (Fig 5, **E**) in the mRNA in the jejunum wall ( $n = 6$ ). The samples were analyzed by using quantitative PCR. The data are shown as relative expression ( $\Delta$  cycle) by subtracting the IL-10 cycles from IFN- $\gamma$  or IL-17A cycles. Experimental groups are indicated in the figure. \* $P < .05$  and \*\* $P < .01$ .

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