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THE ROLE OF SPECIFIC IgG AND COMPLEMENT IN COMBATING A PRIMARY MUCOSAL INFECTION OF THE GUT EPITHELIUM

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The role of complement and complement-fixing IgG isotypes at mucosal surfaces is ill defined. Previous data have demonstrated that survival of an infection with the attaching and effacing pathogen *Citrobacter rodentium* requires production of systemic and CD4+ T cell-dependent IgG. We have found that both complement and complement-fixing IgG isotypes are needed to survive a *C. rodentium* infection. Our results indicate that both IgG and complement C3b enter the gut lumen and bind epithelially adherent, and fecally shed *C. rodentium.* Furthermore, C3-deficient mice demonstrate a profound survival defect, though means to replenish C3 in systemic or mucosal sites restores the protective capacity of complement in the host. Our data provide evidence that both IgG and complement interact constructively on both sides of the epithelium to fight colonizing mucosal infections.

Keywords: *C. rodentium,* complement, mucosal infection

Introduction

Citrobacter rodentium causes an attaching and effacing (AE) infection of the distal colon in mice [1–3]. Mouse infections with *C. rodentium* are used as a model system for human gastrointestinal infections with the enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) that cause of approximately 2 million deaths every year [4]. These organisms use a type III secretion system to introduce translocated intimin receptor (Tir), and extracellularly secreted proteins (esp), into target epithelial cells [5–7]. Membrane-associated Tir is tethered to the actin cytoskeleton and binds the bacterially associated intimin, to form attaching and effacing lesions [8, 9]. To get more insight in the human defense mechanism against pathogenic pedestal forming *E. coli,* the mouse *C. rodentium* colitis mode is used in this study

Resolution of infection relies on a variety of innate and adaptive responses. Pro-inflammatory cascades resulting in local secretion of IFNg, IL-8/KC, IL6, and TNFa-member cytokines are believed to mediate protection through recruitment of inflammatory infiltrates, and stimulation of anti-microbial peptides, from infected epithelium [10–13]. Surprisingly, mucosal IgA has negligible impact in *C. rodentium* infection, where the primary infection occurs over the luminal surface of the gut [14, 15]. Prior studies have demonstrated that systemic pathogen-specific IgG and CD4+ T cell responses are required for survival and resolution of this colonizing infection of the gut epithelium [15–17].

IgG's mechanism(s) of action during a colonizing infection of the luminal epithelial surface remains ill defined. However, this question is critical not only for the study of attaching and effacing pathogens, but for the development of systemic/IgG-based vaccines that serve to protect the host prior to, or during early phases of infections involving mucosal contact. Studies by Masuda et al. [18] have previously shown the importance of Fc-receptor-bearing cells in facilitating survival and clearance of *C. rodentium,* suggesting that opsonization and uptake of the pathogen, or pathogen antigens by macrophages and DCs, contribute to host defense.

The protective host IgG response to a *C. rodentium* infection consists largely of complement-fixing isotypes, namely IgG2c and IgG2b [15]. IgG's entry into the gut lumen during this infection likely occurs through a variety of mechanisms, including active uptake and release, and more passive entry through damaged mucosa. Active uptake and release of IgG by gut epithelium, particularly within the small bowel, is mediated by the neonatal Fc receptor [19, 20]. The receptor also transports IgG into the gut lumen before, and during, *C. rodentium* infection [21]. FcRn-deficient mice demonstrated decreased survival with *C. rodentium* infection. Absence of the receptor results in poor uptake of antigen-antibody complexes from the gut lumen during infection, a defect that can be circumvented by expressing the receptor solely in gut epithelium in FcRn–/– mice [21]. While intestinal expression may promote local immune responses, extra-intestinal expression of the receptor likely

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also contributes to host defense by prolonging the half-life of pathogen-specific IgG during infection. Infection also impacts normal barrier function of the epithelium, allowing passive leakage of not only macromolecules such as IgG, but other serum proteins including those of the complement cascade as well as cellular component [22].

IgG commonly combats pathogens through a combination of complement-dependent, opsonization, and phagocytic mechanisms, as well as direct inhibitory effects on microbial growth or neutralization of adhesins [23–25]. Binding of IgG to microbial adhesins can prevent epithelial adherence of enteric pathogens, as has been shown with polyclonal antibody against EPEC intimin [26]. Subsequent antimicrobial effects include lysis through activation of the classical arm of the complement cascade, and opsonization of bacteria with IgG and/or C3, which allows efficient uptake and killing by macrophages and neutrophils.

In addition to hepatic and myeloid sources of complement, other tissues, including gut epithelium, express components of each pathway. C3, C4, factor B and MBL have been demonstrated in luminal secretions from healthy stomach, small intestine, pancreatic, and biliary secretions [27–31]. As an acute-phase reactant, local inflammation up-regulates complement expression, particularly in response to IL-1, IL-6, IFNg, and TNF-a in intestinal epithelial cell lines Caco2 and T84 [27]. While components of the membrane attack complex (C7–C9) have been shown to be produced in myocardium, renal tubular epithelium, and other sites under inflammatory conditions, or after ischemia/reperfusion [31, 32]; this type of expression has not been demonstrated to date in gut epithelium.

While effector functions of IgG and complement at locations basolateral to mucosal surfaces are well defined, mechanisms of action across infected epithelium and within the gut lumen are not. The presented experiments indicating complement binds to, and acts upon luminal *C. rodentium,* support the hypothesis that IgG interacts constructively with complement on both sides of the epithelium to combat infection.

Results

IgG2b and complement C3b bind luminally adherent C. rodentium

To determine if both IgG and complement were interacting at the gut surface, we stained for IgG2b and cleaved C3b in sections of infected distal colon from wildtype mice *(Fig. 1).* The immunofluorescent staining indicates the binding capability of secreted IgG and C3b to *C. rodentium* at the epithelial surface. Fecal FACS of GFP-tagged *C. rodentium,* shed in feces, was also used to evaluate the isotypes binding to bacteria. Bacteria were first analyzed by flow cytometry by gating on GFP+ events and analyzing signal in FL-2 for C3b, C4 and MBL-C bound to the bacterial cell surface. *Figure 2* shows C3-opsonization of bacteria shed in the feces at day 15 of infection.

Cobra venom factor (CVF) rapidly depletes circulating C3 when administered IP or IV but does not enter the gut lumen to degrade mucosally secreted C3. CVF treatment of wildtype mice was thus used to assess effects of systemic depletion of C3. Mice treated with CVF *(Fig. 2D–F)* and C3-deficient mice systemically reconstituted with C3-sufficient serum *(Fig. 2G–I)* show similar patterns to the wildtype mice. This result indicates the presence and importance of luminal-secreted complement and its binding capability there.

Serum antibody is also capable of entering the gut lumen to bind apically adherent *C. rodentium* during acute infection *(Fig. 3).* The amount of specific IgG2b isotype increased in the feces 15 days after infection. *Figure 3* shows antibody levels in serum and on bacteria shed in the feces. Serum levels of IgM, IgG2c, and IgG2b all rice during infection. Increased levels of IgA, IgG2b, and IgG3 are detected in the feces during acute infection. This is suggestive of sufficient deterioration of epithelial barrier function to enable diffusion through the mucosal barrier.

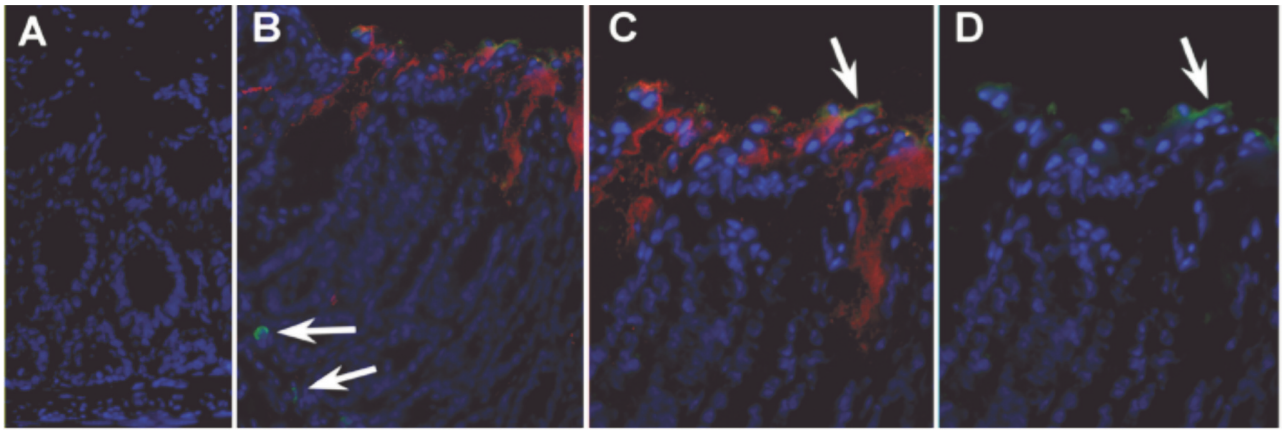

Fig. 1. *In vivo* association of IgG and C3 with apically adherent *C. rodentium:* (A) Section of mouse colon (×200) stained with Hoescht dye donkey anti-rabbit-CY3 and rat-anti-mouse IgG-FITC. (B) Section from infected mouse (×200) showing host nuclei in blue, prominent C3 staining of apically adherent *C. rodentium* (red), faint IgG straining at the luminal surface (green), as well as IgG+ B cells in the lamina propria (arrows). (C) ×400 magnification of previous image showing distribution of C3+ *C. rodentium* and co-localization of IgG at the luminal surface. (D) Composite of previous image showing only IgG and Hoescht staining

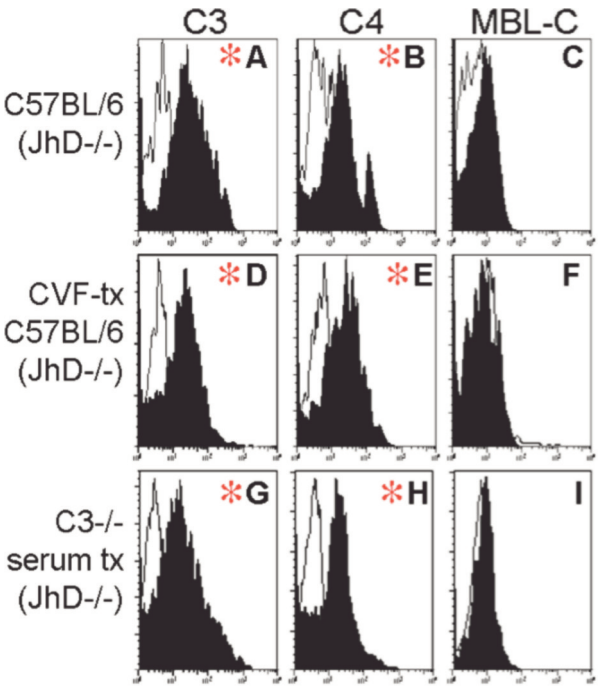

Fig. 2. Fixation of C3 on fecally shed *C. rodentium:* Representative histograms from flow cytometry of GFP+ events in fecal bacterial isolated from mice at 15 days of infection with *C. rodentium. X*-axis indicates fluorescence intensity in FL-2 ($\log_{10}$ scale); *Y*-axis indicates number of events. Panels A–C demonstrate staining in a representative wildtype mouse. *(A)* C3 and *(B)* C4, but not *(C)* MBL-C to shed GFP+CR (black plots). Staining in control antibody-deficient JhD–/– mice (white tracings) are shown. Red asterisk indicates populations where the mean fluorescence intensity between wildtype and JhD–/– mice was significant. Similar plots are shown from a representative CVF-treated mouse showing *(D)* C3, *(E)* C4, and *(F)* MBL-C. The bottom row of plots shows representative histograms from a C3–/– mouse reconstituted intravenously with wildtype serum containing C3. *(G)* C3, *(H)* C4, and *(I)* MBL-C

Complement-dependent lysis of C. rodentium

Our data suggest that both reactive IgG and C3 enter the gut lumen and bind apically adherent *C. rodentium.* Next the ability of these immune molecules to mediate complement-dependent lyses was analysed. Combinations of pre-immune or specific serum IgG against *C. rodentium* with or without the presence of serum complement were added to 10^6 CFU of mid-log phase *C. rodentium* cultured *in vitro*. Bacteria were plated after 2 hours of incubation to assess effect on growth. Growth inhibition of *C. rodentium* occurred only in the presence of immune IgG and complement. Pre-immune serum, lacking specific IgG and immune serum treated with CVF failed to inhibit growth *(Fig. 4)*. These findings demonstrate that the polyclonal serum antibody response, and monoclonal antibodies against *C. rodentium* O-antigen, can inhibit actively dividing bacteria in the presence of serum complement.

Complement is required for effective host defense against C. rodentium

Complement has key protective functions in a variety of infections. To evaluate if complement is important in the defense against the intestinal pathogen, *C. rodentium,* we first performed mouse survival studies with complement C3-defficiant mice. Infection demonstrated a significant survival defect as 40% of C3–/– mice survived while >90% of wildtype mice survived *(Fig. 5A)*. These studies demonstrate the need for complement in surviving *C. rodentium* infection. Subsequent experiments dissected the functions of mucosal vs. systemic stores of C3 in infection. CVF treatment of wildtype mice was thus used to assess effects of systemic depletion of C3, as shown in *Fig. 5*. To evaluate effects of systemic C3, in the absence of mucosally secreted C3, we reconstituted C3–/– mice with wildtype or CVF-treated serum administered intravenously. Systemic reconstitution with C3-sufficient serum was 100% protective, while 50% of mice receiving CVF-depleted serum failed to survive infection *(Fig. 5C)*. Treatment of wildtype mouse with CVF does not affect their survival *(Fig. 5B)*. Two CVF-treated mice succumbed to infection while wildtype controls survived, though differences failed to attain significance ($p = 0.2605$). These data suggest that systemic complement, as well as complement released from mucosal compartments, can provide protection.

Impact of C3-deficiency on the development of pathogen-specific antibody responses

C3-deficiency has previously been shown to impact the development of antigen-specific IgG responses in the host [33–36]. Prior studies by our group have shown that mice surviving *C. rodentium* infection produce detectable pathogen-specific titers of IgG2b and IgG2c by 15 days of infection, whereas mice succumbing to infection do not. Adoptive transfer of purified IgG from day 15 serum of infected mice can rescue CD4-deficient mice that otherwise succumb to infection, demonstrating the protective capacity of IgG.

We evaluated the profile of pathogen-specific IgG isotypes in wildtype and C3-deficient mice prior to infection and at 15 days post-challenge to determine if lack of C3 prevented development of specific IgG. IgG2b and IgG2c IgM were assayed by ELISA to quantify titers against the following *C. rodentium antigens:* (1) purified LPS which contains the carbohydrate O-antigen, (2) his-tagged intimin, a primary virulence factor and protein antigen, and (3) sonicates of cultured *C. rodentium* which contain a mix of antigens and have served as capture material in prior studies [15, 16].

In C3–/– mice, IgM and IgG2b titers against all antigens are comparable to those of the wildtype. However, the IgG2c antibody response was reduced in C3–/– mice both against intimin (–1628 mean relative endpoint titer) and whole cells (–388 mean relative endpoint titer) at day 15 during the infection *(Fig. 6)*.

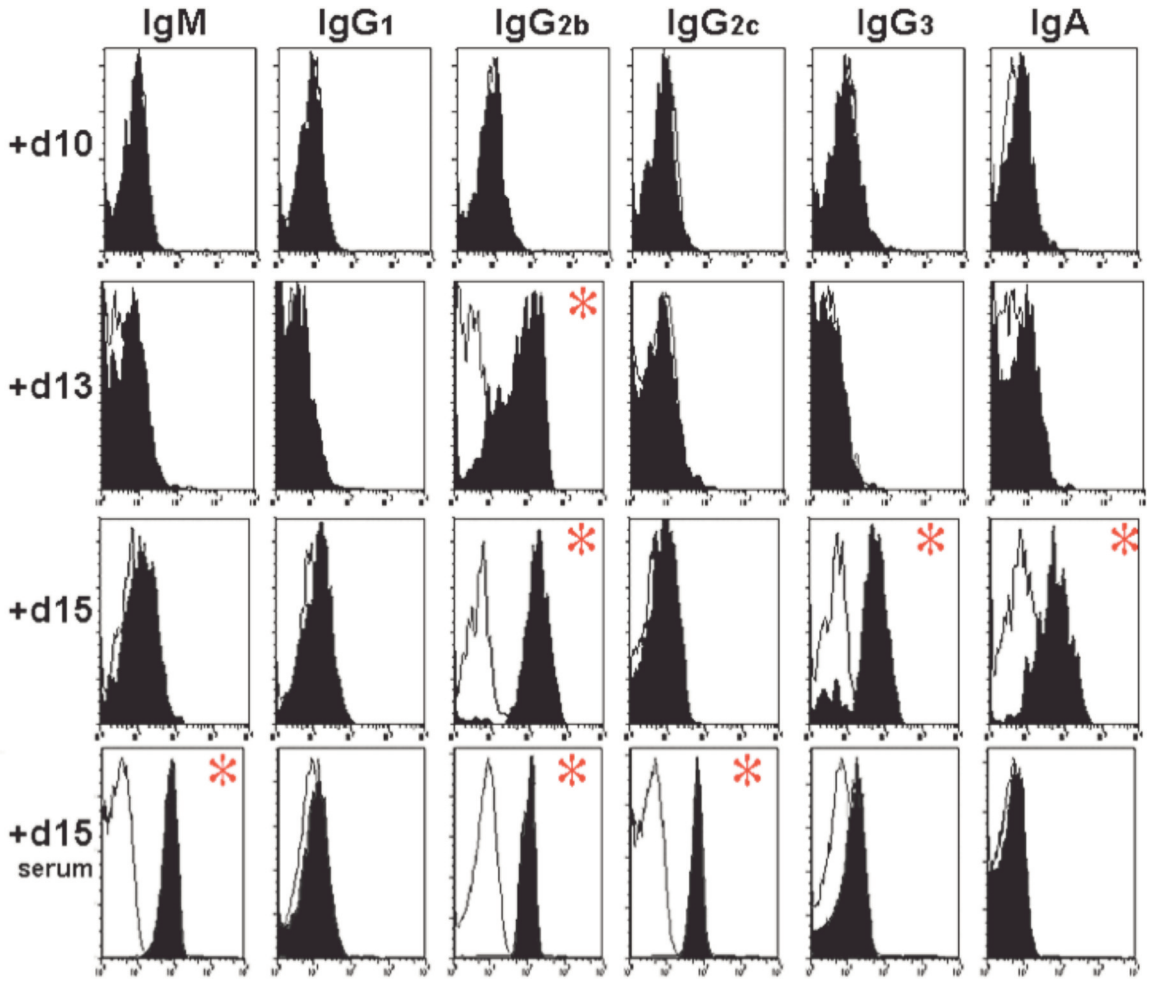

Fig. 3. Ig Binding to fecal bacteria during infection: Representative histograms from flow cytometry of GFP+ events in fecal bacteria isolated from mice at 10, 13, and 15 days of infection with *C. rodentium. X*-axis indicates fluorescence intensity in FL-2 (log_{10} scale); *Y*-axis indicates number of events. Panels: Staining in control antibody-deficient JhD–/– mice mice (white tracings) are shown. From left to right, the different antibodies are shown (IgM, IgG1, IgG2b, IgG2c, IgG3, and IgA). The three top rows indicate levels in the fecal pellets 10, 13, and 15 days after infection. The bottom row shows antibody levels in serum 15 days after infection. Asterisks indicate a significant difference between wildtype and immune compromised mice

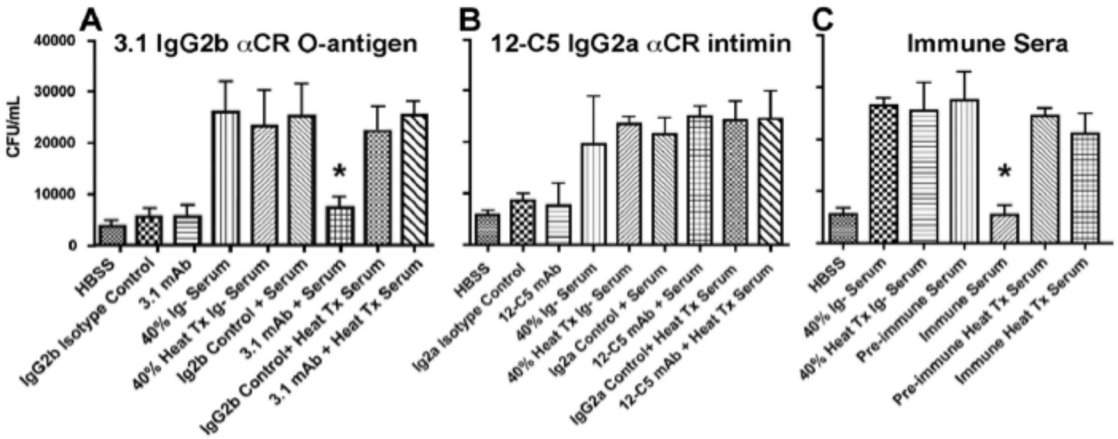

Fig. 4. Anti-bacterial activities of mAbs with complement-sufficient serum: Assays for complement-dependent lysis after 2-h incubation with (A) 3.1 mAb against *C. rodentium* O-antigen; asterisk indicates significant growth inhibition of bacteria in the presence of serum and 5 μg of 3.1 mAb, (B) 12-C5 mAb against bacterial intimin, or (C) effects of immune and pre-immune serum on bacterial growth inhibition. Asterisk indicates growth inhibition in the presence immune serum, as compared with adjacent peaks measuring growth in pre-immune serum or in heat-treated sera

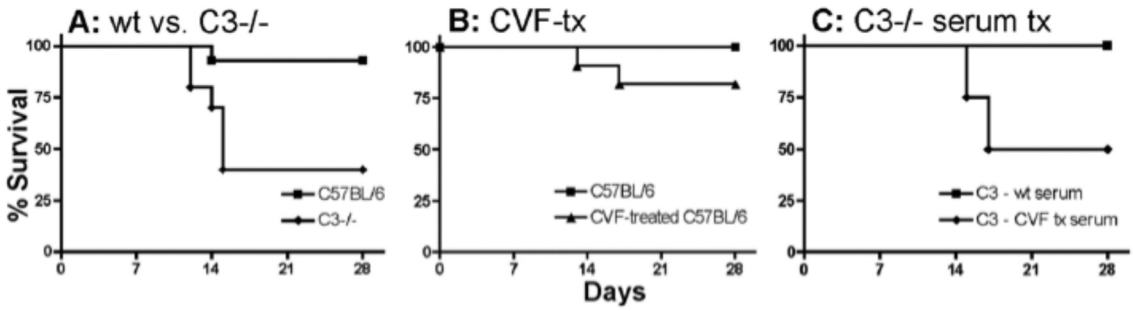

Fig. 5. Survival studies in C3–/– and CVF-treated mice infected with *C. rodentium:* (A) Comparison of survival in wildtype (11 of 12 mice survived) and C3–/– mice (4 of 10 mice survived) infected with *C. rodentium* at 21 days of age. (B) Wildtype CVF ($n = 10$) or saline ($n = 10$) mice. (C) Infection in C3–/– mice treated with wildtype serum ($n = 5$) or CVF-treated serum ($n = 4$ mice)

Discussion

The *C. rodentium* mouse model has helped our understanding of innate and adaptive immunity against A/E *pathogens pathogens,* which are the cause of worldwide morbidity and mortality [4]. The adaptive immune response involves CD4+ T cells and B cells via IgG secretion [14, 16]. Here we demonstrate that mucosal IgG and complement can act in the gut lumen and impact the course of *C. rodentium* infection in means of antimicrobial activity and stimulation of protective adaptive responses. Our data support the hypothesis that the secreted IgG together with complement acts across infected epithelium, and within the gut lumen.

As demonstrated in sepsis models, complement affects the development of Th1 IgG responses against protein antigens [34]. Herein, we have demonstrated that complement

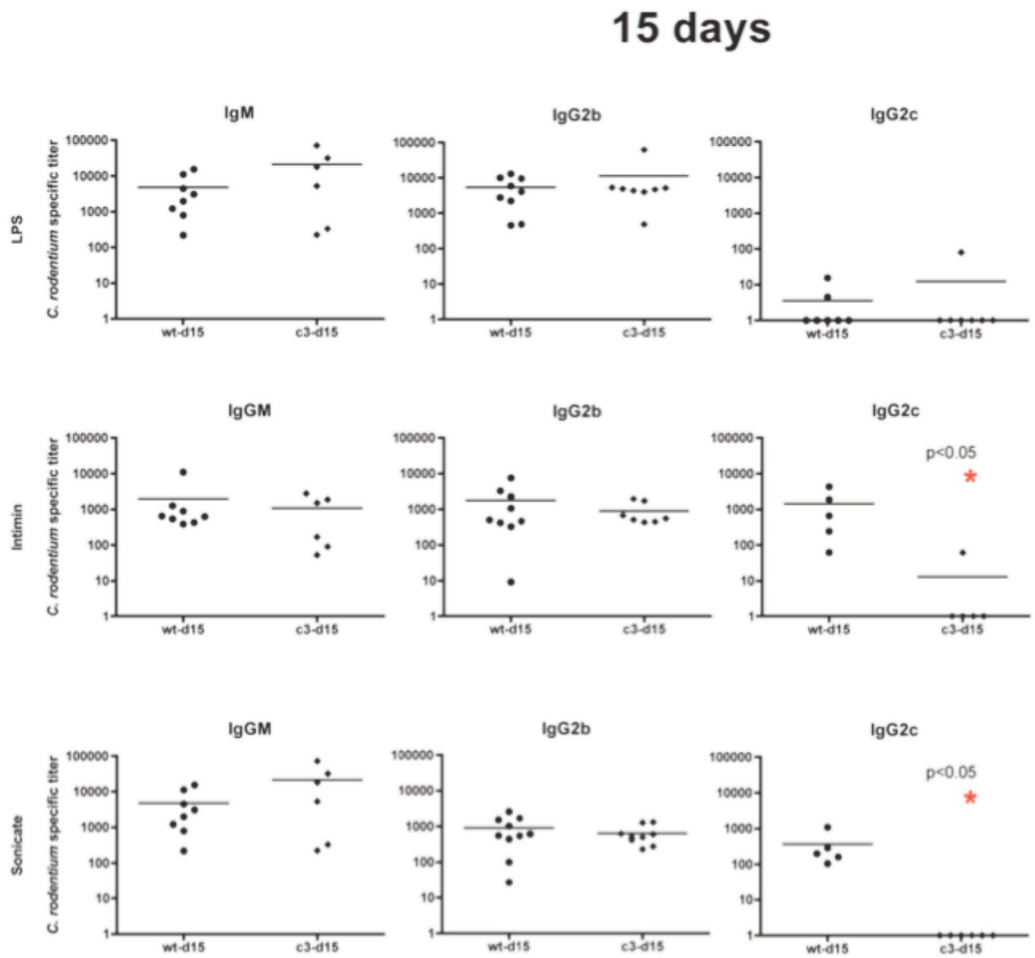

Fig. 6. Complement and antibody responses: Demonstrating antibody titer against *C. rodentium* Intimin, LPS and sonicate in wildtype (circles) and C3–/– (diamonds) 14 days post-inoculation with *C. rodentium.* From left to right: the different antibodies that were tested (IgM, IgG2b and IgG2c). From top to bottom: the three antigens (Intimin, LPS and sonicate). Asterisk indicates a significant difference between wildtype and C3–/–

also affects development of Th1-depdendent antibody responses against a protein antigen from a mucosal pathogen. C3–/– mice demonstrated significantly lower titers of IgG2c against bacterial intimin, while deficiency had no effect on development of responses against LPS. The fact that C3–/– mice have delayed IgG2c responses against protein antigens is supported by other studies [37] demonstrating delay or absence of antibody responses against protein-antigen, but not carbohydrate antigens such as LPS O-antigen. Our results clearly demonstrate the importance of complement in development of responses against a known vaccine target for A/E pathogens. Further study is needed to determine whether complement-dependent IgG2c responses occur in the organized GALT, draining mesenteric lymph nodes or more systemic sites.

The importance of specific IgG in the resolution of *C. rodentium* infection is strengthened by a recent study using a using *Lactobacillus casei*-Int$_{cv}$ vaccine against *C. rodentium* infection. It was demonstrated that subcutaneous immunization with purified int$_{cv}$ induced concentration of ant-Int$_{cv}$ IgG1 ans IgG2a in sera, indicating a balanced Th1:Th2 profile [38]. In contrast, protection elicited by *L. casei*-Intcv vaccines was observed in conditions where no significant induction of anti-Intcv IgG in sera was detected [38]. These data implicate that other mechanisms are involved in the protection elicited by *L. casei*-Int$_{cv}$ oral vaccines as compared to subcutaneous immunization.

The overall importance of complement coming from mice bearing a deficiency in complement C3, C4, or CD21/CD35 is described and reviewed [36]. Our data are complementary and show that C3 complement is essential for mice surviving a *C. rodentium* infection. C3–/– mice could be rescued with C3-sufficient serum from uninfected mice, but not CVF-treated serum. As wildtype mice that were systemically treated with CVF show no survival defect during the infection, these findings suggest that mucosal stores of C3 may be protective in the absence of systemic protein. Alternatively, CVF-treatment may not be 100% effective at depleting systemically released C3 that had circulated into mucosal locations, or whether locally produced C3 from epithelial cells or myeloid cells, mediated protection.

Our data clearly demonstrate the protective role of complement, specifically C3, in the survival and resolution of mucosal infection with *C. rodentium*. Interestingly, we have demonstrated that both cleaved C3b and IgG isotypes are present on shed bacteria from a wildtype murine host, demonstrating that these factors enter the gut lumen in sufficient quantities to interact with luminal bacteria. This opsonization lead to growth inhibition of *C. rodentium,* and should be efficient for stimulation of cells of the adaptive immune system [39, 40].

In conclusion, the occurrence of multiple antibiotic resistance strains like EPEC favors the vaccine approach rather than conventional antibiotics treatment. Our data strengthen the finding that complement is important in humoral immunity and, thus, support the potential of development of specific vaccine against the emerging antibiotic resistant A/E pathogens.

Experimental procedures

GFP-tagged C. rodentium

GFP-CR was created by electroporating ClonTech's eGFP plasmid into *C. rodentium*. Plasmid selection was on 100 μg/ml carbenicillin to overcome any induced expression of *C. rodentium's* chromosomal ß-lactamase [41]. Mice were then infected with 5X108 CFU of CR-GFP. *In vivo* stability of GFP expression was first determined by plating fecal homogenates to MacConkey agar and replica plating colonies to LB agar to assess GFP in isolates. The plasmid GFP is stably expressed for a period of 2 weeks *in vivo* without antibiotic selection. Beyond 2 weeks, <90% of isolated *C. rodentium* maintain the tag. In the presence of ß-lactam selection, *C. rodentium* can de-repress its chromosomal lactamase, rendering useless *in vivo* antibiotic selection of the plasmid-expressed ß-lactamase (data not shown).

Survival studies

C3–/– mice were infected with *C. rodentium* and followed to define roles of complement in infection. Twenty-one-day-old wildtype and C3–/– C57BL/6 mice were orally infected with 5×10^8 CFU of *C. rodentium* and followed for 28 days. All studies performed under an IACUC protocol.

Cobra venom factor (CVF) treatment of wildtype mice

Wildtype mice received 2 doses of CVF IP, one day before infection, and maintenance IP doses on days 4, 8, 12, 16, and 20 after infection with *C. rodentium*. Control mice received saline only. To test CVF depletion of C3, serum from CVF, or saline-treated mice were collected at d15 of infection and incubated with 10 μl of rabbit RBCs (Pelfreez) for 30 min at 37 °C. After centrifugation, supernatants were analyzed at OD 405 to measure hemolysis. Mouse serum lyses rabbit RBCs through alternative pathway activation, while CVF-treated serum does not.

Reconstitution of C3–/– mice with systemic C3

Mice received 250 μl of serum on the day of infection with *C. rodentium* and on days 5, 10, 15, and 20 post-inoculation.

Fecal FACS

Fecal bacteria were stained for surface-bound C3 in mice in the following manner: (1) wildtype, (2) CVF-treated C57BL/6, or (3) control antibody-deficient JhD–/– Balb/c, which lack B cells, were infected with GFP-CR as described. Fecal pellets were collected on days 3, 7, 10, 13, and 15 post-inoculation and vortexed in 1ml PBS to obtain homogenates. A 3-min low-speed spin pelleted larger material.

Supernatants, enriched for fecal bacteria, were blocked at 4 °C with 5% heat-treated donkey serum in PBS, followed by staining with rat anti-mouse C3, sheep anti-mouse C4 (Cell Sciences) or isotype control. After washing, bacterial cells were stained with donkey anti-rat-PE or donkey anti-sheep-PE antibody, washed and fixed in 2% paraformaldehyde. Bacteria were analyzed by flow cytometry by gating on GFP+ events and analyzing signal in FL-2 for C3, C4, and MBL-C bound to the bacterial cell surface. Lack of available mouse reagents for alternative pathway components prevents analysis of alternative activation with this method.

Localization of C3 and IgG in infected colon

Frozen sections of acutely infected mouse colon stained with Hoescht nuclear dye (blue), C3 antibody (red) and IgG (green).

Complement-dependent lysis

Mid-log phase *C. rodentium* (5×10^3) were added to 100 µl of Hanks buffer salt solution (HBSS) + 2 mM $CaCl_2$ + 2 mM $MgCl_2$. Aliquots were incubated alone, with 5 µg specific or isotype control antibody, or with normal or heat-treated antibody-deficient mouse serum as a source of complement (40% final volume of serum in assays). Serum heated at 56 °C for 1 h inactivated complement proteins. Bacteria were also incubated in immune or pre-immune serum from C57BL/6 mice *(Fig. 5C)*. Bacteria were incubated for 30 min at 37 °C to measure lytic effects in the absence of bacterial growth, and for two hours to assess effects on dividing cultures in 40% serum. After incubation, reactions were serially diluted and plated to LB agar to quantify remaining CFU. Experiments were repeated in triplicate and findings subjected to Kruskal-Wallis non-parametric test to identify significant differences among groups.

Purified his-tagged beta-intimin of C. rodentium

A vector expressing the his-tagged C-terminus of *C. rodentium* beta-intimin was kindly provided by Dr. Jim Sinclair (USAMRIID) [42]. Intimin's C-terminus is surface exposed and facilitates bacterial binding to TIr. Expression in *E. coli* was induced by incubating cells in 2 mM IPTG at 37 °C for 6 h, binding of his-tagged protein to nickel columns (Qiagen), subsequent dialysis of eluate against PBS to obtain purified his-intimin for use in ELISAs.

Sonicates from C. rodentium

Stationary phase *C. rodentium* were washed with PBS + 5 mM EDTA, pelleted, and resuspended in PBS, sonicated and centrifuged pellets were filter sterilized over 0.22-µM filters prior to use in ELISAs.

LPS purification of C. rodentium

LPS was isolated by the hot phenol method. A 100 ml of *C. rodentium* stationary phase were washed and resuspended in 10 ml 2:1 Ccl3: MetOH for overnight incubation. Hereafter, cells were pelleted and incubated for 20 min with 68 C phenol. Hereafter, the aquatious phase was re-extracted with hot phenol twice and thereafter dialyzed against water, followed by RNAse, DNAse, and proteinase treatment. LPS was detected using a Limulus amebocyte lysate (LAL) assay (Sigma-Aldrich) and visualized by silver staining after acrylamide gel electrophoreses.

ELISA

Polystyrene microtiter plates (Immunoplate Maxisorb; Nunc) were coated o.n. with either *C. rodentium* LPS, *C. rodentium* Intimin (1 mg/ml) or *C. rodentium* sonicate (1 mg/ml protein) as antigen. The next day plates were washed twice with PBS + 0.5% Tween (PBST). Serum was added to the plates in serial dilutions and incubated for 2 h. Hereafter the plates were washed five times with PBST and the specific immunoglobuline conjungated to AP (Zymed) was added to the plates in a 1:1000 dilution and incubated for another hour. Plates were washed five more times with PBST and the reaction was developed with Alkaline Phosphatase Yellow (pNPP) (Sigma).

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