Efficacy of an experimental gonococcal lipooligosaccharide mimitope vaccine requires terminal complement

Lisa A. Lewis[1], Sunita Gulati[1], Wioleta M. Zelek[2], B. Paul Morgan[2], Wen-Chao Song[3], Bo Zheng[1], Nancy Nowak[1], Rosane B. DeOliveira[1], Bryan Sanchez[1], Leandro DeSouza Silva[1], Janine Schuurman[4], Frank Beurskens[4], Sanjay Ram[1] and Peter A. Rice[1]

[1]Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Worcester, MA 01605, [2]Systems Immunity Research Institute and Dementia Research Institute, Henry Wellcome Building for Biomedical Research, Cardiff University, Heath Park, Cardiff, CF14 4XN, [3]Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104 and [4]Genmab, Utrecht, The Netherlands

Running title: Gonococcal vaccine efficacy requires MAC

Word Count Abstract: 99

Word Count Text: 1392

Main point: An experimental gonococcal peptide vaccine that mimics a gonococcal glycan epitope on lipooligosaccharide requires activation of the terminal complement pathway for its efficacy in the mouse vaginal colonization model of gonorrhea.

Footnotes:

Conflicts of interest: Peter Rice, Sunita Gulati and Sanjay Ram are listed as inventors on patents related to the TMCP2 vaccine (Assignee: University of Massachusetts Medical School). Sanjay Ram serves a consultant for Apellis Pharmaceuticals and Ionis Pharmaceuticals. Frank Beurskens and Janine Schuurman have financial interests in Genmab (stocks and/or warrants). B. Paul Moran serves as a consultant for UCB and Kira Pharmaceuticals.

Financial support: This work was supported by National Institutes of Health grants R01 AI141181 and R21 146621.

Address correspondence to: Lisa A. Lewis, Division of Infectious Diseases and Immunology, University of Massachusetts Medical School, Lazare Research Building, Room 214, 364 Plantation Street, Worcester MA 01605, USA. Tel: +1-508-856-8587. Fax: +1-508-856-8447. E-mail: lisa.lewis@umassmed.edu

Abstract

A safe and effective vaccine against multidrug-resistant gonorrhea is urgently needed. An experimental peptide vaccine called TMCP2 that mimics an oligosaccharide epitope in gonococcal lipooligosaccharide, when adjuvanted with glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE), elicits bactericidal IgG and hastens clearance of gonococci in the mouse vaginal colonization model. Here, we show that efficacy of TMCP2 requires an intact terminal complement pathway, evidenced by loss of activity in *C9*$^{-/-}$ mice or when C7 function was blocked. In conclusion, TMCP2 vaccine efficacy in the mouse vagina requires membrane attack complex. Serum bactericidal activity may serve as a correlate of protection for TMCP2.

Key words: *Neisseria gonorrhoeae*; gonorrhea; vaccine; lipooligosaccharide; complement; terminal complement pathway

Background

The emergence of multidrug-resistant *Neisseria gonorrhoeae* constitutes a global public health problem. A safe and effective vaccine against gonorrhea is urgently needed. Several gonococcal vaccine candidates are being evaluated in pre-clinical studies (reviewed in [1]). We previously identified a peptide mimic (mimitope) of the lipooligosaccharide (LOS) epitope recognized by mAb 2C7, which when configured as a tetramer (called TMCP2) and adjuvanted with GLA-SE, attenuated colonization of mice by gonococci [2]. One impediment to the development of gonococcal vaccines is the lack of a correlate of protection. Here, we elucidate the mechanism of action of TMCP2 in mice and define a corelate of protection that will facilitate further pre-clinical development.

Methods

Bacterial strain. *N. gonorrhoeae* strain FA1090 has been described previously [2].

Mouse strains. C57BL/6 and BALB/c mice were from Jackson Laboratories. *C9*$^{-/-}$ mice in a C57BL/6 background have been described previously [3].

Antibodies. Function-blocking anti-mouse C7 mAb (IgG2κ) was produced as recently described [4]. A chimeric human IgG1 derivative of mAb 2C7 with a complement-enhancing Fc mutation (E430G) has been described previously [5].

Immunization of mice. Six week-old C57BL/6 and *C9*$^{-/-}$ mice were immunized with three doses of 50 µg TMCP2 plus 5 µg GLA-SE adjuvant at weeks 0, 3 and 6. BALB/c mice used in experiments with anti-C7 were given a fourth dose of vaccine at 9 weeks. Mice were challenged with *N. gonorrhoeae* strain FA1090 10-14 d after the last dose of vaccine.

LOS ELISA. Antibody elicited against the 2C7 LOS epitope was measured by ELISA using LOS purified from 2C7-positive *N. gonorrhoeae* strain 15253 as described previously [2].

Opsonophagocytosis. Mouse PMNs were elicited via intraperitoneal injection of thioglycolate broth and killing of FA1090 opsonized with normal mouse sera was performed as described [6].

Murine model of gonococcal vaginal colonization. Use of animals was performed in strict accordance with recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol (protocol number A-1717) was approved by the IACUC at the University of Massachusetts Medical School. Immunized female mice (10-14 d after the last vaccine dose) in the diestrus phase of the estrous cycle were treated with Premarin (Pfizer) and antibiotics (vancomycin and streptomycin) as described previously [2, 5]. Mice were infected intravaginally with *N. gonorrhoeae* FA1090 (CFU specified for each experiment). Daily bacterial burdens were measured by enumerating CFU by rinsing vaginal swabs in 100 µl of normal saline and then plating serial 10-fold dilutions onto chocolate agar plates containing vancomycin, colistin, nystatin, and trimethoprim sulfate) supplement (Becton Dickinson, Cockeysville, MD, US) plus 100 mg of streptomycin sulfate (Sigma, St. Louis, MO, US) per ml of media [2].

Statistical analysis. Clearance of *N. gonorrhoeae* across groups was compared using three characteristics of the data, as described previously [2, 5]: Time to clearance, longitudinal trends in mean $\log_{10}$ CFU and the cumulative CFU as area under the curve (AUC). Median time to clearance was estimated using Kaplan-Meier survival curves; the times to clearance were compared between groups using a log-rank test. Mean $\log_{10}$ CFU trends over time were compared between groups using 2-way ANOVA and Dunnett's multiple comparisons test. The

mean AUC (log_{10}CFU vs. time) was computed for each mouse to estimate the bacterial burden over time (cumulative infection); the means under the curves were compared between groups using 1-way ANOVA (Kruskal Wallis test) because distributions were skewed or kurtotic; pairwise comparisons between groups was carried out using Dunn's post-hoc test. Comparisons of anti-LOS IgG titers across vaccine immunized groups were made by Mann-Whitneys non-parametric test.

Results

Antibody responses to TMCP2 are unimpaired in C9$^{-/-}$ mice. We compared 2C7 epitope-specific anti-LOS IgG responses in *C9$^{-/-}$* and wild-type C57BL/6 mice 2 weeks after dose 2 and dose 3 (i.e., at weeks 5 and 8, respectively). As shown in Fig. 1A, both strains of mice showed similar anti-LOS IgG responses following immunization with TMCP2/GLA-SE.

TMCP2 loses efficacy in mice without functional terminal complement. The terminal pathway comprises five components; C5b, C6, C7, C8 and C9, plasma proteins that when combined together form the lytic membrane attack complex (MAC), penetrating membranes to kill microbes. Complement C9 is the last step in assembly of the terminal complement pathway (membrane attack complex). The role of the early stages of the complement pathway in bacterial killing is well defined; Fc-Fc receptor (FcR) and C3 fragment-complement receptor 3 (CR3) interactions, as well as signaling through the C5a receptor (C5aR) all contribute to opsonophagocytic uptake and killing of Neisseriae [7, 8]. We confirmed that opsonophagocytosis remained unaffected in *C9$^{-/-}$* mice (Supplemental Figure S1). As shown in

Fig. 1B, TMCP2 lost all activity in *C9*$^{-/-}$ mice, suggesting that MAC formation was essential for TMCP2-mediated protection.

A second independent line of evidence for the role of the terminal pathway in the efficacy of TMCP2 was provided by a function-blocking anti-C7 mAb [4]. This mAb, at the dose used in this study, completely blocks terminal pathway in mice for at least 48 hours after administration [4]. To confirm that C7 function was blocked in the current study, we tested the efficacy of a human IgG1 chimeric mAb 2C7 with the E430G Fc mutation that promotes the formation of Fc hexamers on the bacterial surface, increases C1 complex engagement and enhances classical complement pathway activation; activity of mAb 2C7-E430G requires the terminal pathway for its efficacy [5]. Administering anti-C7 rendered the chimeric mAb 2C7 ineffective (Fig. 2A). Similarly, TMCP2 immunization also failed to hasten the clearance of gonococcal colonization in mice given anti-C7 (Fig. 2B), confirming results obtained with *C9*$^{-/-}$ mice.

Discussion

Serum bactericidal activity is widely accepted as a correlate of protection against meningococcal disease [9]. A major obstacle in the development of gonococcal vaccines is the lack of an established correlate of protection. A group B meningococcal vaccine (4CMenB) showed 31% efficacy in retrospective epidemiologic study [10], however its mechanism of action remains unclear. Here, we show that activity of a candidate gonococcal vaccine in the mouse vaginal colonization model relies on a functional terminal complement pathway (Fig. 1 and Fig. 2). These results mirror prior data with passively administered chimeric mAb 2C7 [5], which targets the same LOS epitope that is mimicked by TMCP2 [11]. We also show that

antibody responses to TMCP2 in *C9*$^{-/-}$ mice are intact, consistent with normal antibody responses in humans with terminal complement deficiencies given meningococcal vaccines [12, 13].

C9-deficient human serum can also kill *N. gonorrhoeae* strains that are susceptible to killing by complement-sufficient human serum, but at rates far slower than seen in normal serum [14]. By contrast, C8-depleted human serum did not kill gonococci even at later time points [14]. Our data demonstrate that MAC formation is essential for activity of anti-gonococcal LOS antibodies in mice; either absence of C9 or inhibition of C7 ablates activity. Delayed killing reported *in vitro* with C9-depleted/C9-deficient serum [14] may not suffice for vaccine efficacy *in vivo,* although we acknowledge that differences in gonococcal strains and sources of complement may preclude extrapolation of our data to humans. The presence of active complement in the female mouse genital tract that can support gonococcal killing are consistent with previous studies showing that human cervical secretions contains hemolytically active complement [15]. These data and our previous results with mAb 2C7 [5] suggest that serum bactericidal assay may serve as a correlate of protection for the TMCP2 vaccine. *N. gonorrhoeae* have evolved numerous strategies to evade killing by neutrophils [7], therefore it is not surprising that opsonophagocytosis may not contribute significantly to clearance of gonococci. Accordingly, depletion of PMNs did not have any negative impact on the efficacy of mAb 2C7 [5].

A recent study showed that *C6*$^{-/-}$ mice (derived from the Peru-Coppock strain) have impaired innate immune responses, including defective expression of surface adhesion molecules, generation of superoxide anion, and appearance of reactive oxygen species and histone release after activation of PMNs, along with defective phagocytosis [16]. Loss of C6,

C7, or C8 activity does not impair opsonophagocytic killing of meningococci by neutrophils derived from normal individuals [8, 12]. To minimize the possibility of impaired neutrophil function in mice genetically deficient of C9, we confirmed the role of terminal complement using a function-blocking anti-C7 mAb in wildtype mice.

In conclusion, terminal complement is necessary for efficacy of a peptide vaccine that targets gonococcal LOS. The serum bactericidal assay may serve as a correlate of protection for the TMCP2 vaccine.

References

1. Rice PA, Shafer WM, Ram S, Jerse AE. Neisseria gonorrhoeae: Drug Resistance, Mouse Models, and Vaccine Development. Annu Rev Microbiol **2017**; 71:665-86.

2. Gulati S, Pennington MW, Czerwinski A, et al. Preclinical Efficacy of a Lipooligosaccharide Peptide Mimic Candidate Gonococcal Vaccine. MBio **2019**; 10.

3. Ueda Y, Miwa T, Ito D, et al. Differential contribution of C5aR and C5b-9 pathways to renal thrombic microangiopathy and macrovascular thrombosis in mice carrying an atypical hemolytic syndrome-related factor H mutation. Kidney Int **2019**.

4. Zelek WM, Morgan BP. Monoclonal Antibodies Capable of Inhibiting Complement Downstream of C5 in Multiple Species. Front Immunol **2020**; 11:612402.

5. Gulati S, Beurskens FJ, de Kreuk BJ, et al. Complement alone drives efficacy of a chimeric antigonococcal monoclonal antibody. PLoS Biol **2019**; 17:e3000323.

6. Wu H, Jerse AE. Alpha-2,3-sialyltransferase enhances Neisseria gonorrhoeae survival during experimental murine genital tract infection. Infect Immun **2006**; 74:4094-103.

7. Palmer A, Criss AK. Gonococcal Defenses against Antimicrobial Activities of Neutrophils. Trends Microbiol **2018**.

8. Konar M, Granoff DM. Eculizumab treatment and impaired opsonophagocytic killing of meningococci by whole blood from immunized adults. Blood **2017**; 130:891-9.

9. Goldschneider I, Gotschlich EC, Artenstein MS. Human immunity to the meningococcus. I. The role of humoral antibodies. J Exp Med **1969**; 129:1307-26.

10. Petousis-Harris H, Paynter J, Morgan J, et al. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. Lancet **2017**; 390:1603-10.

11. Ngampasutadol J, Rice PA, Walsh MT, Gulati S. Characterization of a peptide vaccine candidate mimicking an oligosaccharide epitope of Neisseria gonorrhoeae and resultant immune responses and function. Vaccine **2006**; 24:157-70.

12. van den Broek B, van Els C, Kuipers B, et al. Multi-component meningococcal serogroup B (MenB)-4C vaccine induces effective opsonophagocytic killing in children with a complement deficiency. Clin Exp Immunol **2019**; 198:381-9.

13. Fijen CA, Kuijper EJ, Drogari-Apiranthitou M, Van Leeuwen Y, Daha MR, Dankert J. Protection against meningococcal serogroup ACYW disease in complement- deficient individuals vaccinated with the tetravalent meningococcal capsular polysaccharide vaccine [see comments]. Clin Exp Immunol **1998**; 114:362-9.

14. Harriman GR, Esser AF, Podack ER, et al. The role of C9 in complement-mediated killing of Neisseria. J Immunol **1981**; 127:2386-90.

15. Price RJ, Boettcher B. The presence of complement in human cervical mucus and its possible relevance to infertility in women with complement-dependent sperm- immobilizing antibodies. Fertil Steril **1979**; 32:61-6.

16. Fattahi F, Grailer JJ, Parlett M, et al. Requirement of Complement C6 for Intact Innate Immune Responses in Mice. J Immunol **2020**.

Figures / Figure Legends

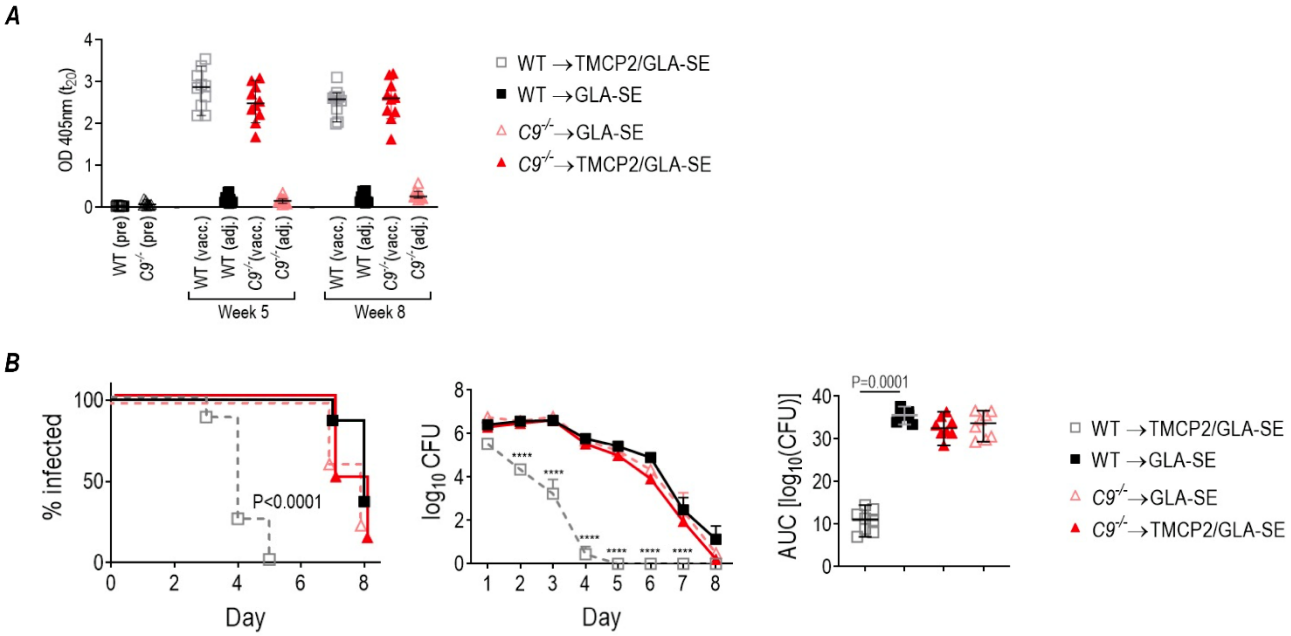


Fig. 1. An intact terminal complement pathway is required for TMCP2 vaccine efficacy. Wildtype C57BL/6 or *C9*$^{-/-}$ mice were immunized with either TMCP2 (50 µg) plus GLA-SE adjuvant (5 µg), or GLA-SE alone intramuscularly at 0, 3 and 6 weeks. ***A***. *C9*$^{-/-}$ mice immunized with TMCP2/GLA-SE mount normal IgG responses. Anti-LOS IgG in sera (n=10/group) collected at week 0 (pre-immune sera, labeled 'pre') and at weeks 5 and 8 (two weeks after doses 2 and 3, respectively) were measured by LOS ELISA. Mice that received TMCP2/GLA-SE and GLA-SE alone are indicated as 'vacc' and 'adj' respectively. Horizontal bars represent the median and error bars the 95% confidence interval. The differences between the immunized groups were not significant. ***B***. TMCP2 is ineffective in the absence of C9. Wild-type C57BL/6 mice or *C9*$^{-/-}$ mice immunized according to the schedule above were challenged with *N. gonorrhoeae* strain FA1090 (3 x 10^7 CFU) intravaginally (n=8 mice/group). Vaginas were swabbed daily to obtain CFUs. *Left graph*: Kaplan-Meier curves for time to

clearance of infection (analyzed by Mantel-Cox Log Rank test). *Middle graph*: $\log_{10}$ CFU vs time (comparisons by 2-way ANOVA; ****, P<0.0001). *Right graph*: Area Under Curve (AUC) analysis (groups compared by one-way ANOVA using Kruskal Wallis' non-parametric test and pairwise comparisons were made with Dunn's test).

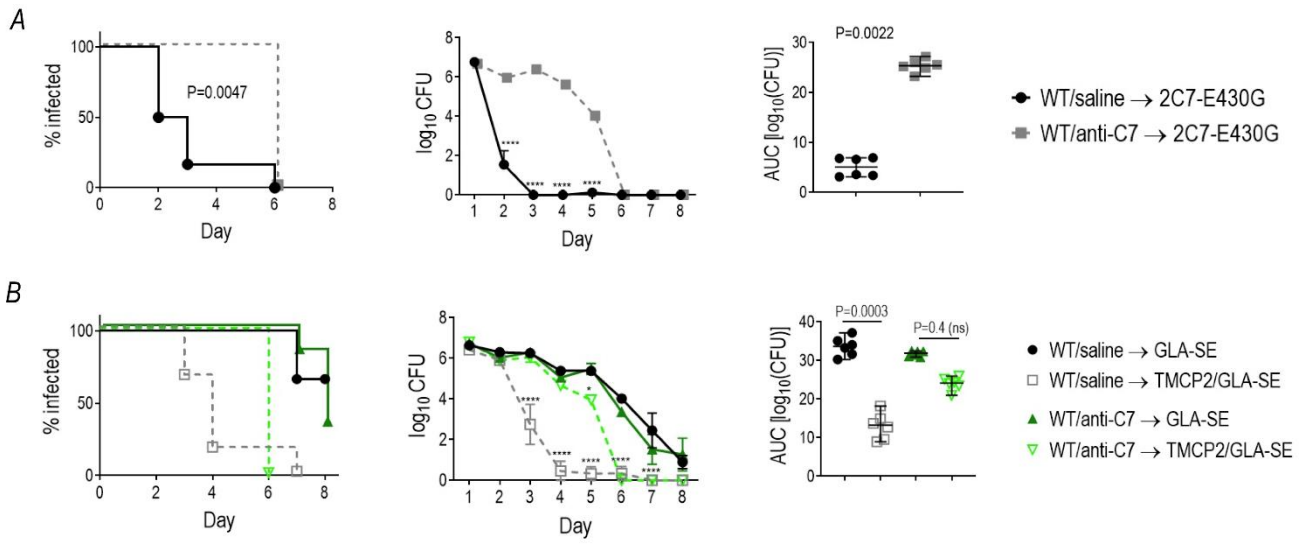
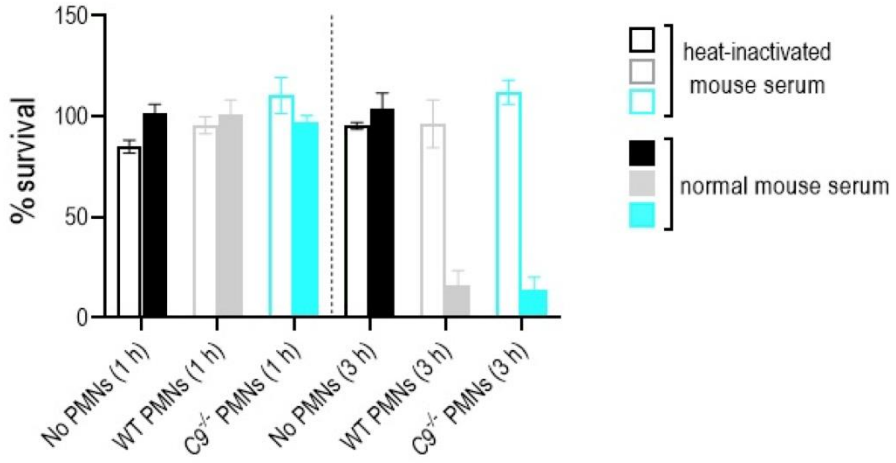


Fig. 2. C7 is required for activity of the TMCP2 vaccine. ***A.*** Verification of the function of anti-mouse C7 function blocking mAb. Wild-type BALB/c mice (n=6/group) were treated with either saline or with anti-mouse C7 mAb (1 mg) intravenously on day -1 and infected with *N. gonorrhoeae* FA1090 (2.6 x 10^7 CFU) on day 0. Anti-C7 or saline (control) was administered again on days 2 and 5. Vaginas were swabbed daily to enumerate CFUs. *Left graph*: Kaplan-Meier curves for time to clearance of infection (analyzed by Mantel-Cox Log Rank test). *Middle graph*: $\log_{10}$ CFU vs time (comparisons by 2-way ANOVA; ****, P<0.0001). *Right graph*: Area Under Curve (AUC) analysis (groups compared by Mann-Whitney's test). ***B***. Blocking C7 function decreases efficacy of TMCP2. Wild-type BALB/c mice were infected with FA1090 and treated with anti-C7 (or saline) as described in panel B and CFUs monitored daily. *Left graph*: Kaplan-Meier curves for time to clearance of infection. *Middle graph*: $\log_{10}$ CFU vs time (comparisons by 2-way ANOVA; ****, P<0.0001). *Right graph*: Area Under Curve (AUC) analysis (groups compared by one-way ANOVA using Kruskal Wallis' non-parametric test and pairwise comparisons were made with Dunn's test).



Supplemental Figure S1. Opsonophagocytic killing of *N. gonorrhoeae* FA1090 by PMNs isolated from WT and *C9*$^{-/-}$ C57BL/6J mice. Gonococci (10^5 to 10^6) opsonized with 10% normal mouse sera (NMS, filled bars) or 10% heat-inactivated mouse sera (HI-NMS, open bars) were incubated with 10^6 thioglycolate-elicited murine PMNs (>94% viability) for 3 hours at 37°C. Gonococci were diluted in GC broth with 0.05% saponin and enumerated by plating on Chocolate agar at 0, 1 and 3 hours (1h, 3h). Results are expressed as percent survival at 1 hour and 3 hours relative to CFU at time 0 ([CFU at 1 or 3 hours/CFU at time 0] x 100). Reactions without PMNs were included as controls. Each experiment used PMNs pooled from 3 WT and 3 *C9*$^{-/-}$ mice. Bars represents the mean survival from 3 independent experiments and error bars are SEM.