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Enterococcus faecalis capsular polysaccharide and mechanisms of host innate immune evasion

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Abstract

It has become increasingly difficult to treat infections caused by *Enterococcus faecalis* due to the high levels of intrinsic and acquired antibiotic resistances. However, few studies have explored the mechanisms that *E. faecalis* employs to circumvent the host innate immune response and establish infection. Capsule polysaccharides are important virulence factors that are associated with innate immune evasion. We demonstrate that capsule producing *E. faecalis* strains of either serotype C or D are more resistant to complement-mediated opsonophagocytosis compared to un-encapsulated strains using cultured macrophages (RAW 264.7). We show that differences in opsonophagocytosis are not due to variation in C3 deposition, but due to the ability of capsule to mask bound C3 from detection on the surface of *E. faecalis*. Similarly, *E. faecalis* capsule masks detection of lipoteichoic acid which correlates with decreased TNF-α production by cultured macrophages in the presence of encapsulated strains compared to unencapsulated strains. Our studies confirm the important role of the capsule as a virulence factor of *E. faecalis*, and provide several mechanisms by which the presence of the capsule influences evasion of the innate immune response, and suggest that the capsule could be a potential target for developing alternative therapies to treat *E. faecalis* infections.

Introduction

Enterocoocus faecalis is an important nosocomial pathogen associated with many types of infections including surgical site infections, bacteremia, urinary tract infections, and endocarditis (32). Many infections caused by *E. faecalis* are difficult to treat due to increasing resistance to conventional antibiotic therapies including vancomycin (11-13). Apart from studies on the roles of gelatinase and cytolysin (6, 23, 28), relatively little is known about the mechanisms employed by *E. faecalis* to circumvent host innate immune responses.

In other bacterial pathogens, the production of capsular polysaccharide is a known virulence factor as it aids in avoidance of the host innate immune response (26, 31, 37). *E. faecalis* is known to produce two capsular polysaccharide serotypes (C and D)(11, 13, 15, 41) that contribute to pathogenesis and evasion of the host innate immune response (11). Hufnagel *et al*. reported decreased neutrophilic killing of encapsulated serotype C and D strains compared to the un-encapsulated A and B strains (16). In addition, a recent comprehensive analysis of clinical *E. faecalis* isolates indicated that most pathogenic strains of *E. faecalis* belonged to serotypes C (20). Despite a link between capsule and virulence, little is known about the specific mechanism(s) of how capsule enhances pathogenesis.

The complement system plays a central role in the activation of the immune system and in the clearance of pathogens. Cleavage of C3 to C3b provides a highly effective opsonin in the absence of antibodies. Several reports have shown that capsule producing species of bacteria are more resistant to opsonophagocytosis by inhibiting the deposition and/or detection of C3b on the

surface of the organism (29, 33, 43). Encapsulated bacteria employ numerous mechanisms to resist C3 opsonization and subsequent phagocytosis, including overall reduction in C3 deposition (7). The abundance of C3 deposition is known to differ between capsule producing serotypes of *Streptococcus pneumoniae* (21). In *Staphylococcus aureus,* C3 is buried beneath the surface of the capsule rendering C3 less accessible to complement receptors on the surfaces of macrophages and neutrophils (42).

Bacterial capsular polysaccharides are also known to aid in the avoidance of innate immune responses including immune surveillance. Immune surveillance relies on pathogen recognition receptors (PRRs), including Toll-like receptors, to sense pathogen associated molecular patterns (PAMPs). Two common PAMPs associated with Gram-positive microorganisms are lipoteichoic acid (LTA) and peptidoglycan (PGN). Detection of these PAMPs by Toll-like receptor 2 in conjunction with Toll-like receptors 1 and 6 induces the production of cytokines. In other instances, capsule prevents the detection of PAMPs by PRRs which leads to decreased or altered cytokine production (10). The altered cytokine response to encapsulated pathogens appears to contribute to pathogenicity and virulence.

Our data indicate that the *E. faecalis* capsular polysaccharides from serotypes C and D attenuate C3 opsonized phagocytosis, and that this attenuated response is likely due to decreased recognition of bound C3 on the bacterial surface. Similarly, capsule inhibits detection of *E. faecalis* LTA on the surface and the absence of recognition of this molecule and/or other surface PAMPs in the presence of capsule results in decreased TNF-α production by macrophages.

Material and Methods

Bacterial Strains, plasmids, and growth conditions. All relevant bacterial strains are listed in table 1. *E. faecalis* strains were cultivated in Todd-Hewitt broth supplied with the appropriate antibiotics when needed (THB; Becton, Dickinson and Company, Sparks, Maryland).

Culture of Macrophages.

The macrophage like RAW 264.7 (ATCC TIB-71) cells were cultured in DMEM (Invitrogen, Grand Island, N.Y.) supplemented with 100 U penicillin per mL, 100 µg streptomycin per mL, 2 µg L-glutamine per mL, and 5% heat inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA).

Complement C3 Deposition

Overnight cultures of *E. faecalis* were diluted 1:100 in fresh media. The cultures were allowed to reach mid-log phase (O.D. 600 of 0.6), and were washed 3X in sterile phosphate buffered saline (PBS) pH 7.4. Approximately $2X10^7$ cells of each strain were re-suspended in 10% normal CD1 mouse serum containing complement (Innovative Research, Southfield, MI) diluted in PBS. Serum for negative controls was heat inactivated prior to the addition of bacteria by incubating at 56ºC for 30 minutes. Bacteria were incubated in 10% serum for 30 minutes at 37ºC with agitation. Complement deposition was stopped by addition of EDTA to a final concentration of 10mM followed by incubation on ice for 5 minutes. The bacteria were pelleted at 4ºC, washed 3 times with sterile PBS to remove unbound complement, and finally re-suspended in 30 µL of 1X SDS-PAGE loading buffer. Whole bacteria were boiled vigorously for five minutes, and the cell debris was removed by centrifugation. The remaining supernatants were loaded on an SDS-PAGE gel and electrophoresed. Proteins in the gel were transferred to nylon membranes, and detection of C3 was carried out by western blot analysis using goat anti-

mouse C3 polyclonal antibodies (Bethyl Laboratories, Montgomery, TX) and rabbit anti-goat conjugated with horse radish peroxidase (HRP) as secondary antibody (Bethyl Laboratories, Montgomery, TX) followed by development with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL).

ELISA

The concentration of naturally occuring anti-enterococcal antibodies present in the CD1 (Innovative Research, Southfield, MI) mouse serum (used for subsequent phagocytosis assays) was analyzed by ELISA. In addition, ELISA was performed to investigate the serotype specificity conferred by the presence of CpsF among *E. faecalis* isolates using serotype C-specific antibodies. Briefly, log phase *E. faecalis* strains were washed 3 times in PBS and aliquoted (50 μL) into high binding 96 well Costar plates (Corning). The washed cells were allowed to adhere overnight at 4°C. Bound cells were then incubated with either CD1 mouse serum or rabbit anti-serotype C serum (19) followed by incubation with either goat anti-mouse IgG HRP conjugate (Sigma, Saint Louis, MO) or goat anti-rabbit IgG HRP conjugate (Jackson ImmunoResearch, West Grove, PA). ELISAs were developed using o-phenylenediamine dihydrochloride (OPD, Sigma) as the HRP substrate, and the results were read at O.D. 490 on a Bio-Tek PowerWave XS 96 well plate reader.

Opsonophagocytosis assay

E. faecalis strains V583, LT02 (V583 Δ*cpsF*), and LT06 (V583 Δ*cpsC*) were transformed by electroporation with the plasmid pMV158GFP (25) giving rise to LT12, LT13, and LT14 respectively (Table 1). Strains LT12, LT13, and LT14 constitutively express GFP allowing fluorescent detection during the opsonophagocytosis assay.

Log phase bacteria were washed three times in PBS prior to re-suspending in HBSS (Invitrogen) media. Harvested RAW 264.7 cells were also re-suspended HBSS media. A concentration of $2X10^6$ CFU/mL bacteria were added to $2X10^5$ RAW 264.7 cells/mL followed by the addition of complement containing CD1 mouse serum to a concentration of 10% to give a final volume of 500 µL and a bacteria to macrophage ratio of 10:1. The samples were incubated at 37ºC for 20 minutes to allow uptake of bacteria by macrophages. Trypsin was then added at 0.25% final concentration and incubated for 10 minutes to remove any bacteria bound to the external surfaces of the RAW 264.7 cells. The free bacteria were removed by three PBS washes with low speed centrifugation (750Xg) (8, 9). The washed cells were fixed to glass slides by cyto-centrifugation. The samples were viewed under 100X oil immersion using a Ziess Axioplan 2 fluorescent microscope to visualize the GFP expressing bacteria inside the RAW 264.7 cells. The intracellular bacteria of at least 100 RAW 264.7 cells were counted for each experimental replicate. The phagocytic index was calculated by dividing the number of phagocytic cells (cells that had consumed bacteria) by the total number of macrophages counted and multiplying that number by the number of bacteria per phagocytic macrophage ($\frac{\#\ phagocytic\ cells}{total\ cells\ counted}$ X $\frac{bacteria}{\#\ phagocytic\ cells}$) as previously described (22, 30). Data are presented as percent phagocytic index with the phagocytic index of LT14 (V583Δ*cpsC*, capsule -) set to 100%. Data were compiled from three separate experiments and the standard error of the mean and statistical significance were calculated with Graphpad Prism software.

Slide Agglutination

Un-encapsulated and encapsulated strains were tested for their reactivity to serotype A antiserum, previously reported to be specific for enterococcal lipoteichoic acid (LTA) (40).

Slide agglutination assays were preformed as previously described (19, 41). Briefly, log phase bacteria were washed three times with PBS. Following the PBS washes, 5.0 μL of LTA antiserum or pre-immune serum was added to 15.0 μL of test cells on a glass slide, and gently rotated for one minute. Agglutination was determined by visual clumping of the cells. Sterile PBS and pre-immune serum were used as negative controls.

Flow Cytometry

Flow cytometry was used to determine if C3 or LTA accessibility to antibodies was altered by the presence of capsule. Log phased bacteria were washed three times in PBS, diluted 1:2, and blocked in 5% donkey serum (Jackson ImmunoResearch). Bacteria used for analyzing C3 accessibility were incubated in 50 μL of CD1 mouse serum for 20 minutes at 37ºC to allow for C3 deposition and washed three times in PBS prior to blocking with donkey serum. Blocked cells were incubated for 15 minutes on ice with 2.0% goat anti-C3 antibodies followed by three washes in PBS. Similarly diluted goat serum was used as an isotype control. The bacteria were then incubated with FITC conjugated donkey anti-goat antibody (1:1000) (Jackson ImmunoResearch) for 15 minutes on ice in the dark. The bacteria were again washed three times with PBS and analyzed by flow cytometry. For detection of LTA accessibility, washed and blocked bacterial cells were incubated on ice for 15 minutes with 2.0% anti-LTA rabbit serum (40). Similarly diluted pre-immune rabbit serum was used as an isotype control Cells were then washed three times in PBS and incubated for 15 minutes on ice in the dark with FITC conjugated donkey anti-rabbit antibody (1:100) (Jackson ImmunoResearch). Bacteria were washed three times in PBS and analyzed by flow cytometry. For both the C3 and LTA experiments, flow cytometry analysis of 50,000 bacteria was performed using a FACSCalibur flow cytometer

(Becton and Dickinson, San Jose, CA) at a flow rate of ~2000 cells per second. Data were analyzed using the WinList software program (VerityHouse, Topsham, ME).

TNF-α production

Log phase bacteria were washed three times in PBS and heat killed by incubation at 80ºC for 30 minutes. RAW 264.7 cells were harvested and re-suspended in fresh DMEM culture media to a concentration of $1X10^6$ cells per mL. RAW cells at a concentration of $1X10^6$ cells/mL in a total volume of 2.0 mL were seeded in 24 well plates. The cells were allowed to adhere to the plate surface for two hours prior to induction. Bacteria were added to each well at a concentration of $1X10^7$ Cfu. Lipopolysaccharide (LPS) from *Salmonell enterica* serotype typhimurium (Sigma) was used as a positive control for TNF-α production at a concentration of 10 ng per mL. Clarified supernatants were collected from each well at four hours after the bacterial inoculation. The amount of TNF-α present in the supernatants was determined by ELISA (eBioscience, San Diego, CA) following the manufacturer instructions. One way ANOVA in correlation with a Newman-Kuels post hoc test were used to evaluate statistical significance (GraphPad Prism).

Results

Protective effects of capsule on opsonophagocytosis

The capsular polysaccharides of many bacterial species confer resistance to complement mediated opsonphagocytosis. We examined whether *E. faecalis* capsule conferred resistance to C3 opsonophagocytosis mediated by macrophages. We used ELISA to confirm that our complement source (CD-1 mouse serum) was free of detectable *E. faecalis* antibodies (Data not shown).

Previous studies have shown that *E. faecalis* opsonizing antibodies exist in normal human serum; however, these antibodies are only directed towards the un-encapsulated serotype A and B strains of *E. faecalis* (2, 15). In view of these studies, we determined if *E. faecalis* capsule serotypes C or D conferred resistance to complement mediated opsonophagocytosis compared to an isogenic acapsular mutant. The encapsulated *E. faecalis* strains LT12 (serotype C), LT13, an isogenic *cpsF* deletion mutant which results in the production of a serotype D capsular polysaccharide (40) and LT14, an isogenic *cpsC* deletion mutant which is un-encapsulated (40) were compared. For this assay, we followed the method of Drevets et al. (8, 9) which calls for trypsin treatment and subsequent washes to remove externally bound bacteria as opposed to antibiotic treatment with gentamicin which has been shown to be internalized by macrophages leading to antibiotic killing affects independent of macrophage activity (9). Our data shows a 50% reduction in the opsonophagocytosis of capsule producing strains by macrophages in the presence of complement compared to unencapsulated strains (Fig. 1). These data also show that there is no statistical difference in opsonophagocytosis between isogenic serotype C (LT12) and serotype D (LT13) strains (Fig. 1), suggesting that the mere presence of capsule regardless of serotype provides protection against bacterial uptake by macrophages .

Complement C3 deposition and surface accessibility

Bacterial resistance to complement mediated opsonophagocytosis has been attributed to decreased amounts of C3 deposition on the surface of encapsulated strains (7). We used western blot analysis to assess the abundance of complement C3 deposited on both encapsulated and un-encapsulated *E. faecalis* strains. Two encapsulated strains FA2-2 and LT01(FA2-2Δ*cpsF*) and two un-encapsulated LT05 (FA2-2Δ*cpsC*) and OG1RF strains were used in this experiment.

Complement C3 is composed of an α and a β chain (35). The 75 kDa C3 β chain is left intact through the processing events of C3, and was used to determine differences in overall C3 deposition. Figure 2 shows the deposition of the 75 kDa β chain of C3 on different strains of *E. faecalis*. There is no difference in the amount of C3 deposited on the surfaces of the un-encapsulated strains OG1RF and LT05 when compared to the encapsulated V583 and LT01 strains (Fig. 2). The other detected fragments in this blot are known breakdown products of C3 and C3b.

The amount of complement deposition does not vary between strains, but the presence of complement on the encapsulated strains could be masked from detection by complement receptors leading to decreased phagocytosis. We used complement opsonized strains of V583 (serotype C), LT02(V583Δ*cpsF*, serotype D) and LT06 (V583Δ*cpsC*, capsule -) in conjunction with flow cytometry to determine C3 surface accessibility to antibodies. Our data show that C3 deposited on the surface of LT06 is more detectable than C3 deposited on the surface of encapsulated strains V583 and LT02 (Fig. 3). Statistical analysis using one-way ANOVA in conjunction with a Newman-Keuls post hoc test show a significant statistical difference (p-values < 0.05) between V583 and LT06, and also between LT02 and LT06 (Fig. 3). There was also a statistically significant difference between V583 and LT02 even though they appear to be equally resistant to complement mediated opsonophagocytosis (Fig. 1). The basis for this difference is not known at the present time, but may relate to structural differences in the capsular polysaccharides between these two serotypes.

Lipoteichoic acid and capsule

Lipoteichoic acid (LTA) and peptidoglycan are PAMPs present on *E. faecalis* that are known to stimulate the immune system through pathogen recognition receptors including TLR-2 (36). The capsules produced by other bacteria shield PAMPs resulting in altered cytokine production (31). We examined differences in LTA accessibility between encapsulated and un-encapsulated strains by slide agglutination assays. *E. faecalis* serotype A anti-serum is directed against enterococcal LTA (40). We tested the ability of these antibodies to agglutinate either encapsulated or un-encapsulated *E. faecalis* strains. The encapsulated strains V583 (serotype C) and LT02(Δ*cpsF*, serotype D) were not agglutinated by the anti-serum, whereas the un-encapsulated strains LT06(Δ*cpsC*) and 12030 (serotype A reference strain) were both agglutinated (data not shown).

As agglutinating antibodies are generally of the IgM class, we also used flow cytometry to quantify the differences of LTA availability to the IgG class. Strains V583, LT02, and LT06 were incubated with Serotype A antiserum followed by a FITC conjugated secondary antibody. Figure 4A and B show the percentage of the cells that were positive for FITC labeling. One-way ANOVA followed by a Newman-Keuls post hoc test showed significant statistical differences (p-values < 0.05) in the amount LTA detected between V583 (serotype C) and LT06 (capsule -), and also between LT02 (serotype D) and LT06. However, there was no significant statistical difference when the encapsulated strains V583 and LT02 were compared. These data indicate that capsule produced by either serotype C or D strains masks LTA from antibody detection.

TNF-α production in response to capsule

The presence of a capsule is known to alter the macrophage cytokine response in other microorganisms (10). To examine this possibility in *E. faecalis*, we used ELISA to assess the ability of capsule producing and non-producing strains to induce TNF-α production by RAW 264.7 cells. We predicted that the ability of capsule to inhibit detection of LTA (Fig. 4) would translate to less TNF-α production by RAW 264.7 cells. The capsule producing strains T-5 (serotype D), V583 (serotype C), LT02 (serotype D) along with the un-encapsulated strains (LT06, 12030, OG1RF) were heat-killed and incubated with RAW 264.7 cells. Clarified supernatants were collected at 4 hours post inoculation, and were analyzed for TNF-α production. The TNF-α produced in response to the un-encapsulated strains is significantly higher than that produced in response to encapsulated strains with p-values < 0.05 using one-way ANOVA and a Newman-Keuls post hoc test analysis (Fig. 5). However, there is no statistically significant difference when comparing the encapsulated strains with each other or when comparing the un-encapsulated strains with each other. Strikingly, there is no statistically significant difference in the amount of TNF-α produced by RAW cells when comparing the strains T-5, V583, and LT02 to the un-induced RAW control cells.

Discussion

Capsular polysaccharides contribute to the virulence of microorganisms through multiple mechanisms including resistance to opsonophagocytosis, and by masking bacterial surface antigens from detection by the host immune system (1, 10). Several Gram-positive cocci including *S. aureus* (26), *S. pneumoniae* (1), and group-B streptococci (4) produce capsular polysaccharides that are known to contribute to virulence. Previous reports have indicated that *E. faecalis* strains can be classified by the presence or absence of capsular polysaccharide (11, 15, 16, 40). Hancock and Gilmore (11) showed that the presence of capsule enhances persistence at infectious sites using a murine infection model, and subsequently showed that encapsulation protects the bacteria from killing by neutrophils, whereas an unencapsulated isogenic mutant was readily killed by neutrophils. The killing of the unencapsulated mutant by neutrophils was dependent on the opsonic activity of complement.

Here, we demonstrate that *E. faecalis* capsular polysaccharide serotypes C and D provide resistance to complement opsonized phagocytosis by macrophages. In good agreement with previously reported work on the role of the *E. faecalis* capsule in affecting resistance to opsonic killing by neutrophils (11, 16), we observed a 50% reduction in phagocytic killing in encapsulated strains compared to the isogenic acapsular mutant. An additional cell wall polysaccharide in *E. faecalis* termed Epa has also been shown to contribute to resistance to phagocytic killing (38), and may account for why the protective affect of the capsule is not more substantial in *E. faecalis*. Unlike the capsule, the Epa polymer and its genetic locus appear to be highly conserved in *E. faecalis* (11, 38). A direct comparison on the relative contribution of Cps and Epa in the same strain background has not possible to date, because the OG1RF strain in

which Epa mutants were created lacks the capsule locus (15, 41), and in our hands we have been unable to generate Epa mutants in encapsulated strain backgrounds (L.T., unpublished data). A recent report by Teng et al. (39) demonstrated gross changes in the bacterial cell shape of Epa mutants in the OG1RF background and this may account for our inability to generate such mutants in our encapsulated strains and may partially explain the pleiotropic affects ascribed to the Epa locus in virulence studies (39, 44).

An additional benefit of the macrophage system is the use of cultured cells that are less likely to vary from experiment to experiment compared to the neutrophil assay, which requires fresh isolation of neutrophils from human blood donors. Furthermore, because the strains used in this comparative study were isogenic derivatives we can make a direct assessment on the role of capsule and serotype differences in host immune evasion as has been observed in other microbial pathogens (29, 33, 43) 43). Our findings show that *E. faecalis* capsular polysaccharides alter the detection of C3 and LTA by antibodies (Figs. 3-4). Paralleling these findings, we also demonstrate that the presence of capsule also abrogates TNF-α production by macrophages (Fig. 5). Together these data provide a mechanism by which the presence of capsule alters complement-mediated opsonophagocytosis by altering accessibility of the bound C3b opsonin, as well as limiting the recruitment of phagocytes to sites of infection by altering the production of TNF-α in response to encapsulated *E. faecalis*. It is noteworthy that capsule serotype differences in an isogenic background did not result in significant changes in resistance to opsonin-mediated phagocytosis, or in altered TNF-α response. McBride et al. (20) recently showed that clinical isolates of *E. faecalis* possessing multiple virulence factors, as well as multi-drug resistance were more likely to be identified as capsule serotype C. Our findings suggest

that either of the encapsulated serotypes (C or D) benefit the bacterium in evasion of the host innate response. We did however observe a significant difference in the amount of bound C3 detectable on the surface of isogenic serotype C compared with serotype D capsule, but this difference did not correlate with changes in the phagocytic index of these strains, leaving open the question as to why the more pathogenic and drug-resistant clinical isolates are more frequently identified as serotype C as opposed to D. In *S. aureus*, comparison of the contribution of type 5 and type 8 capsule in the same strain background revealed that the presence of N-acetylation on the type 5 capsule structure conferred a fitness advantage *in vivo* (42). Whether a similar affect will also be observed in the comparison of *E. faecalis* serotype C and D strains *in vivo* will be the focus of future studies.

The mechanism by which capsule alters the phagocytic response in the presence of complement opsonization differs depending on the microorganism. The capsule of group-B streptococci contains a sialic acid side chain that inactivates C3b, whereas the capsule serotypes 5 and 8 of *S. aureus* shield C3 from detection by the cognate receptors (4, 42). In contrast, the presence of capsule in *S. pneumoniae* decreases the amount of complement deposited on the cell surface (21). Our results suggest that the mechanism of evading opsonophagocytosis by encapsulated *E. faecalis* is dependent on the ability of the capsule to mask the bound C3b to prevent it from being recognized by host effector cell receptors targeted to bound complement.

Aside from anti-phagocytic properties, bacterial capsules also act as barriers that limit detection of PAMPs by PRRs (1, 10). A common PAMP shared by all strains of enterococci is LTA. The LTA of *E. faecalis* is known to stimulate TNF-α production via TLR-2 and TNF-α is thought to

play a key role in *E. faecalis*-mediated inflammatory responses (3), though the full role of TNF-α in *E. faecalis* infections is not fully understood (27). A study involving *Enterococcus faecium*, which produces serologically identical LTA to *E. faecalis*, showed that TLR-2 mediated signaling was critical for early immune response and clearance of *E. faecium* (18). Based on these studies, recognition of enterococcal LTA and/or peptidoglycan by TLR-2 would appear critical for an efficient host immune response, and the masking of these integral wall components by capsule could result in increased pathogenesis by limiting the host response to the organism.. Interestingly, a study by Kau et al. (17) demonstrated that the response to *E. faecalis* in a urinary tract infection model is not TLR-2 dependent. The capsule phenotype of the clinical isolate used in this study is not known, and based on our finding that the presence of the capsule alters recognition of an important PAMP (LTA) known to be recognized by TLR-2, it would suggest that TLR-2 signaling might only be of benefit against *E. faecalis* strains that lack capsule.

Our goal was to understand the mechanism of how encapsulation enhances the resistance of *E. faecalis* to innate immunity. Taken together, our results show that the two capsule serotypes produced by *E. faecalis* can subvert host innate immune responses by conferring resistance to complement-mediated phagocytosis, as well as altering the innate response to the pathogen. This study provides mechanistic evidence demonstrating that the *E. faecalis* capsule alters the accessibility of bound C3 supporting the observation that the most pathogenic lineages of *E. faecalis* are encapsulated (20, 40). By masking PAMPs on the surface of *E. faecalis*, the capsule also alters the host response to infection by encapsulated strains. It is our contention that the capsule produced by *E. faecalis* serotypes C and D is an important virulence determinant that

plays multi-faceted roles in evasion of host innate immune responses. Because of this, we believe that the *E. faecalis* capsule could serve as a target for developing future therapeutics.

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References

1. **Abeyta, M., G. G. Hardy, and J. Yother.** 2003. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of Streptococcus pneumoniae. Infect Immun **71:**218-225.
2. **Arduino, R. C., B. E. Murray, and R. M. Rakita.** 1994. Roles of antibodies and complement in phagocytic killing of enterococci. Infection and Immunity **62:**987-993.
3. **Baik, J. E., Y. H. Ryu, J. Y. Han, J. Im, K. Y. Kum, C. H. Yun, K. Lee, and S. H. Han.** 2008. Lipoteichoic acid partially contributes to the inflammatory responses to Enterococcus faecalis. J Endod **34:**975-982.
4. **Campbell, J. R., C. J. Baker, and M. S. Edwards.** 1991. Deposition and degradation of C3 on type III group B streptococci. Infect Immun **59:**1978-1983.

5. **Clewell, D. B., P. K. Tomich, M. C. Gawron-Burke, A. E. Franke, Y. Yagi, and F. Y. An.** 1982. Mapping of Streptococcus faecalis plasmids pAD1 and pAD2 and studies relating to transposition of Tn917. J Bacteriol **152:**1220-1230.
6. **Coburn, P. S., C. M. Pillar, B. D. Jett, W. Haas, and M. S. Gilmore.** 2004. Enterococcus faecalis senses target cells and in response expresses cytolysin. Science **306:**2270-2272.
7. **Cunnion, K. M., H. M. Zhang, and M. M. Frank.** 2003. Availability of complement bound to Staphylococcus aureus to interact with membrane complement receptors influences efficiency of phagocytosis. Infect Immun **71:**656-662.
8. **Drevets, D. A., and P. A. Campbell.** 1991. Macrophage phagocytosis: use of fluorescence microscopy to distinguish between extracellular and intracellular bacteria. J Immunol Methods **142:**31-38.
9. **Drevets, D. A., B. P. Canono, P. J. Leenen, and P. A. Campbell.** 1994. Gentamicin kills intracellular Listeria monocytogenes. Infect Immun **62:**2222-2228.
10. **Graveline, R., M. Segura, D. Radzioch, and M. Gottschalk.** 2007. TLR2-dependent recognition of Streptococcus suis is modulated by the presence of capsular polysaccharide which modifies macrophage responsiveness. Int Immunol **19:**375-389.
11. **Hancock, L. E., and M. S. Gilmore.** 2002. The capsular polysaccharide of Enterococcus faecalis and its relationship to other polysaccharides in the cell wall. Proc Natl Acad Sci U S A **99:**1574-1579.
12. **Hancock, L. E., and M. S. Gilmore.** 2000. Pathogenicity of Enterococci, p. 251-258. *In* V. Fischetti, R. Novick, J. Ferretti, D. Portnoy, and J. Rood (ed.), Gram-Positive Pathogens. American Society of Microbiology, Washington D.C.
13. **Hancock, L. E., B. D. Shepard, and M. S. Gilmore.** 2003. Molecular analysis of the Enterococcus faecalis serotype 2 polysaccharide determinant. J Bacteriol **185:**4393-4401.
14. **Huebner, J., Y. Wang, W. A. Krueger, L. C. Madoff, G. Martirosian, S. Boisot, D. A. Goldmann, D. L. Kasper, A. O. Tzianabos, and G. B. Pier.** 1999. Isolation and chemical characterization of a capsular polysaccharide antigen shared by clinical isolates of Enterococcus faecalis and vancomycin-resistant Enterococcus faecium. Infect Immun **67:**1213-1219.
15. **Hufnagel, M., L. E. Hancock, S. Koch, C. Theilacker, M. S. Gilmore, and J. Huebner.** 2004. Serological and genetic diversity of capsular polysaccharides in Enterococcus faecalis. J Clin Microbiol **42:**2548-2557.
16. **Hufnagel, M., A. Kropec, C. Theilacker, and J. Huebner.** 2005. Naturally acquired antibodies against four Enterococcus faecalis capsular polysaccharides in healthy human sera. Clin Diagn Lab Immunol **12:**930-934.
17. **Kau, A. L., S. M. Martin, W. Lyon, E. Hayes, M. G. Caparon, and S. J. Hultgren.** 2005. Enterococcus faecalis tropism for the kidneys in the urinary tract of C57BL/6J mice. Infect Immun **73:**2461-2468.
18. **Leendertse, M., R. J. Willems, I. A. Giebelen, P. S. van den Pangaart, W. J. Wiersinga, A. F. de Vos, S. Florquin, M. J. Bonten, and T. van der Poll.** 2008. TLR2-dependent MyD88 signaling contributes to early host defense in murine Enterococcus faecium peritonitis. J Immunol **180:**4865-4874.
19. **Maekawa, S., M. Yoshioka, and Y. Kumamoto.** 1992. Proposal of a new scheme for the serological typing of *Enterococcus faecalis* strains. Microbiology and Immunology **36:**671-681.

20. **McBride, S. M., V. A. Fischetti, D. J. Leblanc, R. C. Moellering, Jr., and M. S. Gilmore.** 2007. Genetic diversity among Enterococcus faecalis. PLoS ONE **2:**e582.
21. **Melin, M., H. Jarva, L. Siira, S. Meri, H. Kayhty, and M. Vakevainen.** 2009. Streptococcus pneumoniae capsular serotype 19F is more resistant to C3 deposition and less sensitive to opsonophagocytosis than serotype 6B. Infect Immun **77:**676-684.
22. **Mikerov, A. N., T. M. Umstead, X. Gan, W. Huang, X. Guo, G. Wang, D. S. Phelps, and J. Floros.** 2008. Impact of ozone exposure on the phagocytic activity of human surfactant protein A (SP-A) and SP-A variants. Am J Physiol Lung Cell Mol Physiol **294:**L121-130.
23. **Miyazaki, S., A. Ohno, I. Kobayashi, T. Uji, K. Yamaguchi, and S. Goto.** 1993. Cytotoxic effect of hemolytic culture supernatant from Enterococcus faecalis on mouse polymorphonuclear neutrophils and macrophages. Microbiol Immunol **37:**265-270.
24. **Murray, B. E., K. V. Singh, R. P. Ross, J. D. Heath, G. M. Dunny, and G. M. Weinstock.** 1993. Generation of restriction map of Enterococcus faecalis OG1 and investigation of growth requirements and regions encoding biosynthetic function. J Bacteriol **175:**5216-5223.
25. **Nieto, C., and M. Espinosa.** 2003. Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid **49:**281-285.
26. **O'Riordan, K., and J. C. Lee.** 2004. Staphylococcus aureus capsular polysaccharides. Clin Microbiol Rev **17:**218-234.
27. **Papasian, C. J., R. Silverstein, J. J. Gao, D. M. Bamberger, and D. C. Morrison.** 2002. Anomalous role of tumor necrosis factor alpha in experimental enterococcal infection. Infect Immun **70:**6628-6637.
28. **Park, S. Y., K. M. Kim, J. H. Lee, S. J. Seo, and I. H. Lee.** 2007. Extracellular gelatinase of Enterococcus faecalis destroys a defense system in insect hemolymph and human serum. Infect Immun **75:**1861-1869.
29. **Peterson, P. K., B. J. Wilkinson, Y. Kim, D. Schmeling, and P. G. Quie.** 1978. Influence of encapsulation on staphylococcal opsonization and phagocytosis by human polymorphonuclear leukocytes. Infect Immun **19:**943-949.
30. **Popi, A. F., J. D. Lopes, and M. Mariano.** 2004. Interleukin-10 secreted by B-1 cells modulates the phagocytic activity of murine macrophages in vitro. Immunology **113:**348-354.
31. **Raffatellu, M., D. Chessa, R. P. Wilson, R. Dusold, S. Rubino, and A. J. Baumler.** 2005. The Vi capsular antigen of Salmonella enterica serotype Typhi reduces Toll-like receptor-dependent interleukin-8 expression in the intestinal mucosa. Infect Immun **73:**3367-3374.
32. **Richards, M. J., J. R. Edwards, D. H. Culver, and R. P. Gaynes.** 2000. Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol **21:**510-515.
33. **Rubens, C. E., L. M. Heggen, R. F. Haft, and M. R. Wessels.** 1993. Identification of cpsD, a gene essential for type III capsule expression in group B streptococci. Mol Microbiol **8:**843-855.
34. **Sahm, D. F., J. Kissinger, M. S. Gilmore, P. R. Murray, R. Mulder, J. Solliday, and B. Clarke.** 1989. In vitro susceptibility studies of vancomycin-resistant Enterococcus faecalis. Antimicrob Agents Chemother **33:**1588-1591.

35. **Sahu, A., and J. D. Lambris.** 2001. Structure and biology of complement protein C3, a connecting link between innate and acquired immunity. Immunol Rev **180:**35-48.
36. **Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning.** 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. J Biol Chem **274:**17406-17409.
37. **Stollerman, G. H., and J. B. Dale.** 2008. The importance of the group a streptococcus capsule in the pathogenesis of human infections: a historical perspective. Clin Infect Dis **46:**1038-1045.
38. **Teng, F., K. D. Jacques-Palaz, G. M. Weinstock, and B. E. Murray.** 2002. Evidence that the enterococcal polysaccharide antigen gene (epa) cluster is widespread in Enterococcus faecalis and influences resistance to phagocytic killing of E. faecalis. Infect Immun **70:**2010-2015.
39. **Teng, F., K. V. Singh, A. Bourgogne, J. Zeng, and B. E. Murray.** 2009. Further Characterization of the epa Gene Cluster and Epa Polysaccharides of Enterococcus faecalis. Infect Immun.
40. **Theilacker, C., Z. Kaczynski, A. Kropec, F. Fabretti, T. Sange, O. Holst, and J. Huebner.** 2006. Opsonic antibodies to Enterococcus faecalis strain 12030 are directed against lipoteichoic acid. Infect Immun **74:**5703-5712.
41. **Thurlow, L., V.C. Thomas, L.E. Hancock** 2009. Capsular polysaccharide production in Enterococcus faecalis and the contribution of cpsF to capsule serospecificity J Bacteriol **In Press**.
42. **Watts, A., D. Ke, Q. Wang, A. Pillay, A. Nicholson-Weller, and J. C. Lee.** 2005. Staphylococcus aureus strains that express serotype 5 or serotype 8 capsular polysaccharides differ in virulence. Infect Immun **73:**3502-3511.
43. **Wessels, M. R., A. E. Moses, J. B. Goldberg, and T. J. DiCesare.** 1991. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. Proc Natl Acad Sci U S A **88:**8317-8321.
44. **Zeng, J., F. Teng, G. M. Weinstock, and B. E. Murray.** 2004. Translocation of Enterococcus faecalis strains across a monolayer of polarized human enterocyte-like T84 cells. J Clin Microbiol **42:**1149-1154.

List of Figures

Figure 1. Capsule serotypes C and D are resistant to opsonophagocytosis in the presence of complement. **A.** Representative micrographs depicting from left to right LT12 (V583 expressing Gfp), LT13 (Δ*cpsF* expressing Gfp), and LT14 (Δ*cpsC* expressing Gfp) incubated with RAW 264.7 macrophage like cells. **B.** Quantification of phagocytic index expressed as the percentage of the un-encapsulated LT14 strain (see Materials and Methods for calculating Phagocytic index). The light gray bar (LT12: serotype C) and the dark gray bar (LT13: serotype D) both show a significant reduction in phagocytic index when compared to LT14 (black bar). Error bars represent SE of three replicates.

Figure 2. The amount of C3 deposition does not differ between strains. Western blot analysis was employed to examine the amount of the C3 deposited on the cell surface of serotype C (FA2-2), serotype D (LT01), and un-encapsulated (LT05 and OG1RF) strains. The blot shows the 75 kDa β chain of C3 for FA2-2 (A)**,** LT02 (B), LT05 (C), OG1RF (D) ,and the negative control, FA2-2 incubated with heat inactivated serum (E).

Figure 3. Complement C3 is masked from detection by capsule. Flow cytometry was used in conjunction with anti-C3 antibodies and FITC conjugated secondary antibodies to evaluate the availability of C3 to detection. **A.** Representative histograms depicting (from left to right) flow cytometry results for serotype C (V583), seroypte D (LT02) and un-encapsulated (LT06) *E. faecalis* strains. The isotype controls are light gray and the C3 antibody treated cells are dark gray. **B.** Quantification of the C3 positive cells. Using one-way ANOVA in conjunction with a Newman-Keuls post test, statistical analysis for three replicates showed statistically significant differences (p-value < 0.05) in the amount of positively labeled bacteria when V583 (light gray

bar) and LT06 (black bar) were compared, and when LT02 (dark gray bar) and LT06 were compared. Statistical analysis also revealed a significant difference in C3 detection between V583 and LT02 ($P < 0.05$). Error bars represent SE for three replicate. Approximately 50,000 bacteria were analyzed for each replicate.

Figure 4. The presence of capsule masks LTA from detection by antibodies. Flow cytometry was used in conjunction with LTA antiserum and FITC conjugated secondary antibodies to evaluate the levels of LTA accessibility. **A.** Representative histograms depicting (from left to right) flow cytometry results for serotype C (V583), seroypte D (LT02) and un-encapsulated (LT06) *E. faecalis* strains. The isotype controls are in light gray and the C3 antibody treated cells are dark gray. **B.** Quantification of LTA detection by flow cytometry. Statistical analysis for three replicates using a one-way ANOVA in conjunction with a Newman-Keuls post test showed significant differences ($P < 0.05$) in the amount of LTA detected between V583 (light gray bar) and LT06 (black bar), and between LT02 (dark gray bar) and LT06 with p-values less than 0.05. However, there is no statistical difference in LTA detection when LT02 is compared V583. Error bars represent SE for three replicate. Approximately 50,000 bacteria were analyzed for each replicate.

Figure 5. *E. faecalis* capsule reduces TNF-α production by RAW 264.7 cells. Macrophage like RAW 264.7 cells were incubated with serotype C (V583), serotype D (T-5 and LT02), and un-encapsulated (LT06, 12030, and OG1RF) *E. faecalis* strains. Supernatants were collected and analyzed by ELISA for TNF-α content. Results show pg/mL of TNF-α production by RAW 264.7 cells in the presence of each strain. Statistical analysis of three replicates using one way ANOVA and a Newman-Kuels post hoc test shows significant differences between the amount of TNF-α produced in response to T-5, V583 and LT02 when compared to LT06, 12030, and

OG1RF. Interestingly, there is no statistically significant difference between the amount of TNF-α produced by un-induced RAW cells when compared to the three encapsulated strains. Error bars represent SE for three replicate experiments.

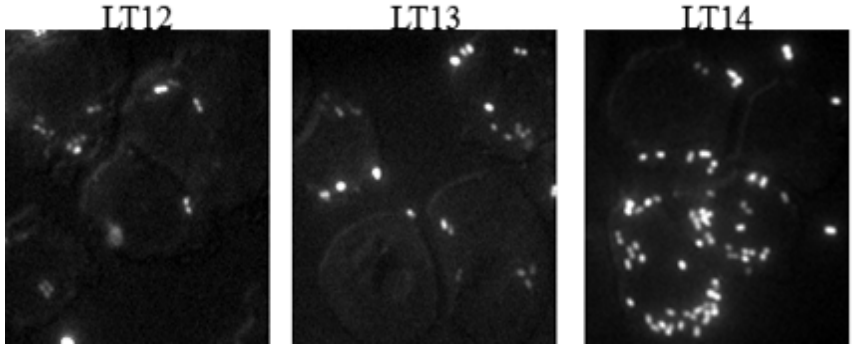
Table 1

Table 1: *E. faecalis* Strains used in this study

Strain	Description	Reference
FA2-2	Capsule + (Serotype C)	(5)
V583	Capsule + (Serotype C)	(34)
OG1RF	Capsule -	(24)
12030	Capsule -	(14)
LT01	FA2-2 Δ*cpsF* Capsule + (Serotype D)	(41)
LT02	V583 Δ*cpsF* Capsule + (Serotype D)	(41)
LT05	FA2-2 Δ*cpsC* Capsule -	(41)
LT06	V583 Δ*cpsC* Capsule -	(41)
LT12	V583 + pMV158gfp	This Study
LT13	LT02 + pMV158gfp	This Study
LT14	LT06 + pMV158gfp	This Study

Figure 1

A.



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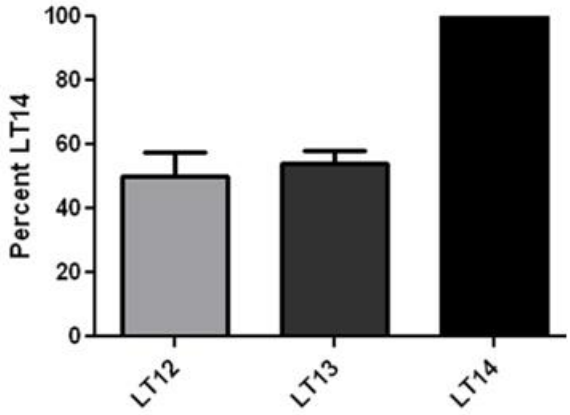


Figure 2

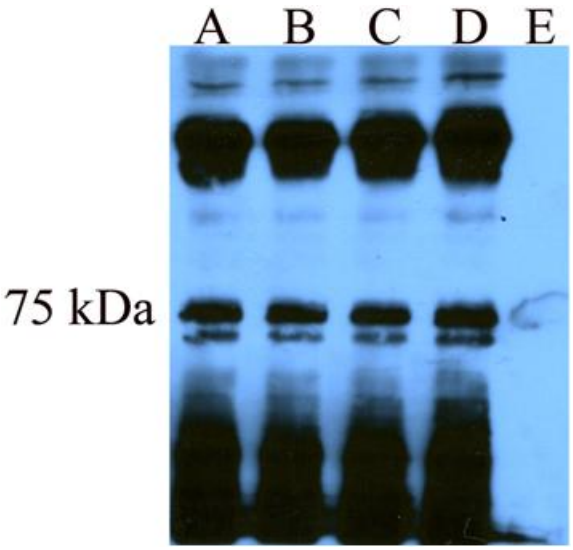
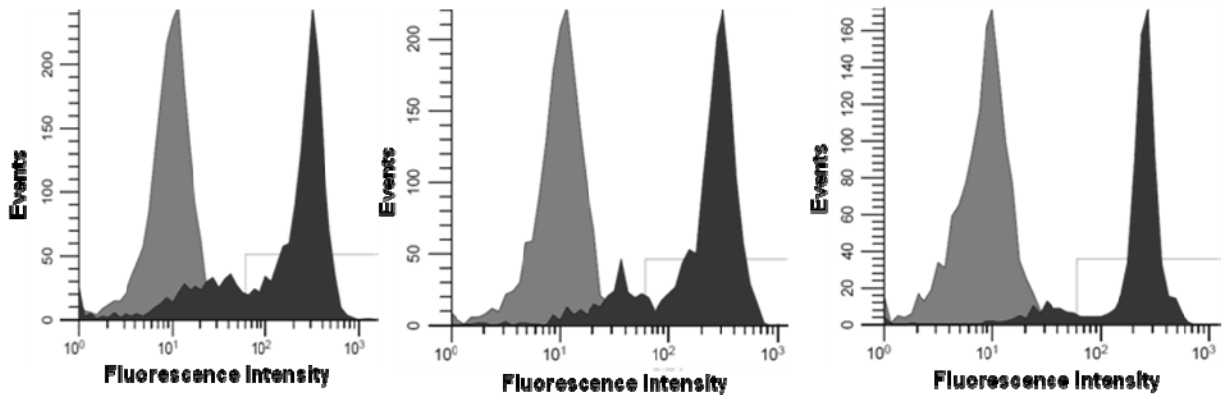


Figure 3:

A.


Events
Fluorescence Intensity
Events
Fluorescence Intensity
Events
Fluorescence Intensity

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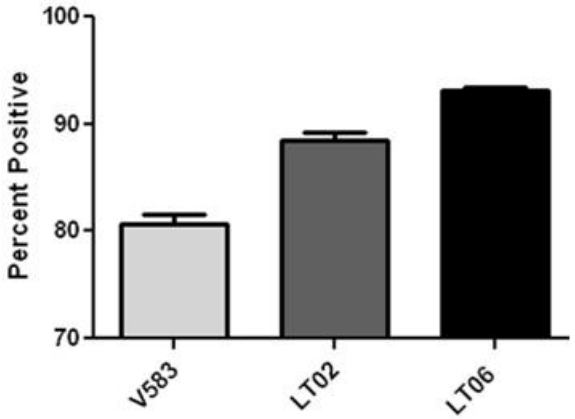
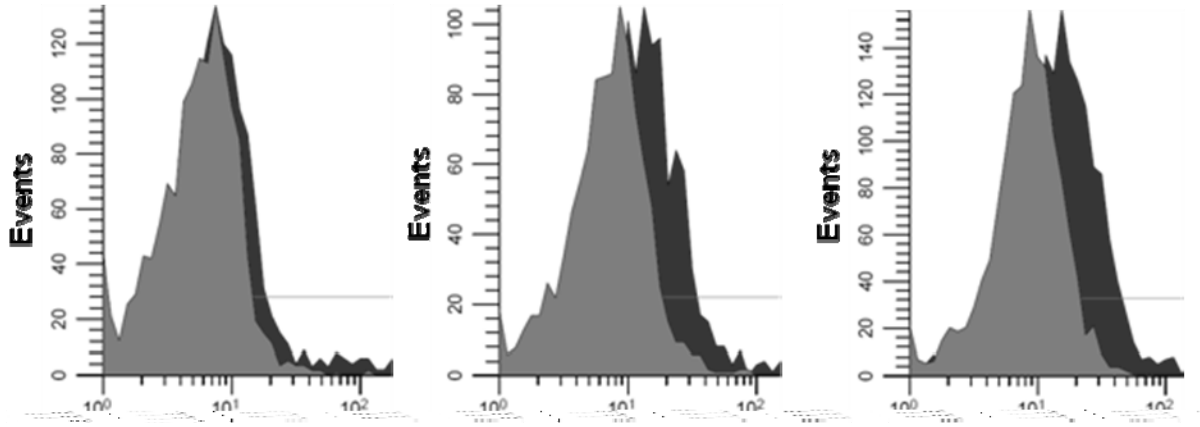

Percent Positive
100
90
80
70
V583
LT02
LT06

Figure 4:

A.


Events
Events
Events

B.

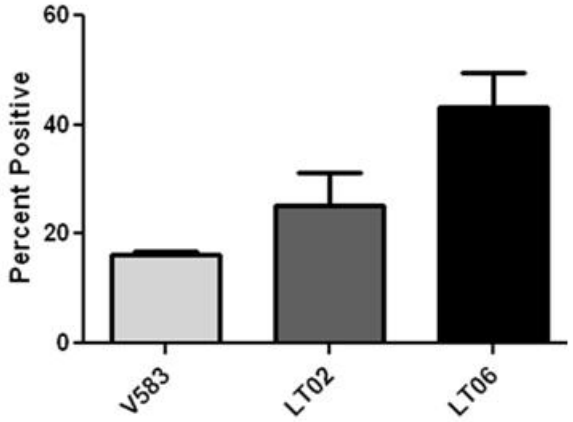
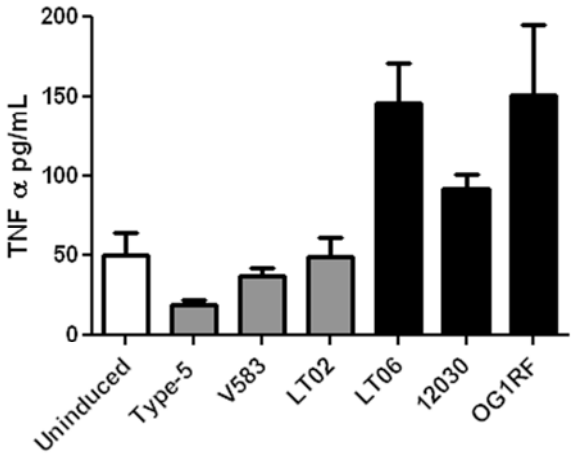

Percent Positive
60
40
20
0
V583
LT02
LT06

Figure 5:


TNF α pg/mL
200
150
100
50
0
Uninduced
Type-5
V583
LT02
LT06
12030
OG1RF