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Improvement of a Surrogate Assay for Protective Antibodies to Pneumococcal Surface Protein A

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IMPROVEMENT OF A SURROGATE ASSAY FOR ANTIBODIES TO PNEUMPCOCCAL SUEFACE PROTEIN A

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BIOLOGY

ABSTRACT

Despite the availability of vaccines for pneumococcal infections, their protective efficacy is not ideal. This problem is due to the replacement of the strains that are not covered by the current vaccines. One potential resolution of this problem is to develop a pneumococcal vaccine that is not limited to serotypes of *Streptococcus pneumoniae*.

On the surface of pneumococci, there are several virulence factors in addition to capsule. Pneumococcal surface protein A (PspA) is a major virulence factor that presents on the surface of virtually all pneumococcal strains. PspA is also known to elicit protective immune responses and as a result PspA is a strong candidate for a protein-based vaccine.

However, an *in vitro* assay to test the protective capacity of immunity PspA is not available. Therefore, developing an *in vitro* assay that can measure the killing of pneumococci by anti-PspA antibody is necessary. The modified surface killing assay (MSKA) is a complement and antibody-dependent assay that measures the killing of pneumococci by phagocytic cells. We project that the MSKA will serve as a guide during phase 2 trials to identify the best vaccine dose for use in a phase 3 efficacy trial of a PspA-containing vaccine.

Keywords: *Streptococcus pneumoniae*, pneumococcal surface protein A, modified surface killing assay, Neutrophils

DEDICATION

I dedicate this thesis to my parents. They have taught me the importance of learning and being respectful to others. Without their trust and support, I would not be able to pursue my education and dreams.

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I would like to thank my mentor, Dr. David Briles, for his support, guidance, and encouragement, which made me become more confident with science throughout the years. I would also like to thank his lab staff for continued support and help. In particular, I would like to thank Kristopher Genschmer for his instruction, advice, and answers to my endless questions; Janice King for helping me obtain human blood for neutrophils; Yvette Hale for collecting mouse serum for complement sources; and Flora Gathof for helping me navigate the ocean of paperwork. I would like to thank Dr. Vithal Ghanta for her truthful advice and encouragement. Also, Dr. William Benjamin for his sincere insight and advice, and for the opportunity to explore infectious diseases in the clinical field. I would like to thank the Nahm lab, specifically Rob Burton and Juan Calix, for providing me with HL-60 cells and helping me to use these cells in my assay. Thank you to Dr. Robert Fischer and Dr. Stephen Watts for their help and encouragement. I would like to thank my parents and siblings for their endless support and love. Thank you to my first host parent, Holly Smith, for accepting me as a part of her family and helping me adapt to a new environment when I first came to the United States. I would like to thank my other host parents, Millie and Henry Huff, for helping me have a close relationship with God and patiently correcting my pronunciation. I would like to thank my best friend Gaëlle Gourmelon for always being there and being sincerely supportive. Finally, I would like to thank my friends, specifically, Christina Croney, Evida Dennis, and Reshmi Mukerj for their support and encouragement.

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INTRODUCTION

Background of *Streptococcus pneumoniae*

Streptococcus pneumoniae (*S. pneumoniae*, pneumococcus) is a Gram-positive bacterium and a human pathogen that causes invasive diseases such as bacteremia, pneumonia, sepsis, and meningitis. These diseases frequently occur in young children, elderly people, and immunocompromised patients (35). A high morbidity and mortality rate caused by this organism is observed worldwide (23).

Current Pneumococcal Vaccines

An important treatment of pneumococcal diseases is the use of antibiotics. Due to the development of resistance by the pneumococci to several antibiotics, treatment of these infections has become difficult (5). Often severe morbidity and mortality can occur before diagnosis, thus an effective vaccine would be very useful. Two main types of polysaccharide vaccines are currently available. One of these vaccines contains 23 different purified capsular polysaccharide antigens that cover 85-90% of disease-causing bacteria in adults (1). The availability of polysaccharide vaccines have greatly reduced the incidence of the invasive pneumococcal diseases in adults but not in children under age of 2 (5). This is because adults elicit antibody to polysaccharide antigens whereas young children do not (21, 36). To increase the efficacy of a polysaccharide vaccine in

young children, polysaccharides are conjugated to a protein, detoxified diphtheria toxin (4). The first successfully developed polysaccharide-protein pneumococcal conjugate vaccine (PCV7) was introduced in 2000 and contained 7 capsular serotypes that caused 80 – 85% of invasive pneumococcal disease in children at that time (5, 44). PCV7 has effectively decreased the incidence of infections (45) because the immune system of young children responds well when protein antigen is used (33). However, non-vaccine serotypes have filled in the niche left behind by these vaccine types (18). This phenomenon is known as "serotype replacement" (25). Because there are more than 90 different known serotypes (35) it is difficult to develop a polysaccharide vaccine that will protect against all strains. Thus, vaccines containing pneumococcal surface proteins need to be investigated (2, 3, 33).

Pneumococcal surface protein A (PspA)

Numerous surface proteins of *S. pneumoniae* have been identified (40). Among these surface proteins, PspA is a good protein vaccine candidate for the following reasons: 1) PspA is present on clinically relevant strains and 2) it elicits protective antibodies (11, 13, 22). PspA induces protective antibodies in human and mice (10, 11, 33, 39). Monoclonal antibodies to PspA protect mice from fatal pneumococcal infections by diverse challenge strains (30-32). PspA is a choline-binding protein and has proline-rich and choline-binding domains (12). It is divided into 2 major families and 5 clades by amino acid sequence (12, 26). Although PspA families from different strains are variable, they all share common antigenic epitopes, which allows cross-reactive protection against

PspA (22, 33, 41). In most cases, immunity to one PspA family will protect against all pneumococci of the same family (41, 43) and often protects against some strains of the other family.

PspA interferes with complement deposition on the surface of pneumococci, slowing clearance in the blood (16, 32, 39).

However binding of antibodies to PspA enhances complement deposition. Therefore, one mechanism to kill bacteria is by opsonization, which involves complement and antibodies, and phagocytosis.

Modified Surface Killing Assay (MSKA)

Although an *in vitro* surrogate assay is available for polysaccharide vaccines (34), there is no reliable *in vitro* surrogate assay that measures and identifies functional and nonfunctional antibodies. An *in vitro* assay is necessary to efficiently develop a protein-based vaccine. The *in vitro* assay for polysaccharide vaccines, the opsonophagocytosis killing assay (OPKA), distinguishes between protective and non-protective antibodies to the capsular polysaccharides and measures antibody function (27). Antibodies to PspA protect against pneumococcal sepsis and enhance blood-clearance of pneumococci in the presence of complement (15). This has led to the expectation that antibody to PspA could opsonize pneumococci for killing by phagocytes (8). However, in suspension, antibody to PspA did not result in the killing of pneumococci by phagocytes (8). We hypothesized that this meant the solution killing assay was too stringent to detect protection mediated by antibody to antigens that are not expressed as densely on the

surface as the repeating epitopes of capsular polysaccharides. Prior studies by Wood and Smith in 1958 revealed that phagocytes were much more efficient at phagocytosis of pneumococci on surfaces than in solution. Thus, to mimic what happens *in vivo*, we developed a modified surface killing assay (MSKA) based on a recently described assay conducted on the surface of a blood agar plate (29, 44).

HL-60 cells

The HL-60 cell-line was derived from a patient with acute promyelocytic leukemia (19). This cell-line can be passaged, maintained, and differentiated into different effector cells such as monocytes, granulocytes, and eosinophils (19, 20). The state of differentiation depends on the chemicals used, the environment the cells are grown in, and the time of exposure to the chemical inducer used and environmental conditions (19, 20). For example, when HL-60 cells are exposed to dimethyl formamide (DMF) the cells differentiate into granulocytes (20). The opsonophagocytosis assay (OPA) showed phagocytosis of antigen-antibody-complement complexes by HL-60 cells (24). Differentiatied-HL-60 cells have been used in the OPA and have become a phagocytic cell source for the standardized OPA (38). The standard OPA did not detect the protective effects of antibody to PspA. In an effort to develop a surrogate assay to detect the protective effects of antibody to PspA, I have adapted HL-60 cells as an alternative phagocytes in the MSKA. (38).

Aims of these studies

For the development of pneumococcal vaccines, it is necessary to have assays to measure the immunity produced. For pneumococci, the vaccine-mediated protection against invasive disease is almost exclusively mediated by antibody. Thus, vaccine induced immunity can be evaluated by measuring antibody levels. However, all antibodies are not equally protective and such measures are generally not considered adequate to guide a multimillion-dollar vaccine trail. A biologically relevant surrogate assay is needed. In the case of PspA, the antibody and complement deposit on the pneumococcal surface and increase the rate of clearance of bacteria from the blood. It is assumed that this occurs by phagocytosis of the opsonized bacteria. The standard opsonization and killing assay used for antibody to polysaccharides is not predictive of the protective capacity of antibodies to PspA. In the studies presented here, I have strengthened our group's earlier findings that the MSKA may be a useful surrogate assay for detecting antibody to PspA. This assay allows the neutrophils to phagocytose the bacteria on an agar surface rather than in suspension.

My studies sought to: **1)** extend earlier findings to a second pneumococcal challenge strain, **2)** extend findings to strains with family 2 PspA, **3)** determine whether a transformed line of phagocytic cells (HL-60) can be used in place of fresh human neutrophils, **4)** optimize the conditions for use of HL-60 cells, and **5)** compare different complement sources.

METERIALS AND METHODS

Pneumococcal strains

Strains and monoclonal antibodies (mAbs) used in the MSKA are provided in Tables 1 and 2. Serotype 3 pneumococcal strain WU2 (PspA+) and its PspA- mutant, BR260.1, were grown in Todd-Hewitt Broth with 0.5% yeast extract (THY) (Becton Dickinson, Sparks, MD) at 37°C until the OD_{600}=0.45 was reached. The bacterial stocks were then frozen at -80°C in 10% glycerol.

Table 1: Pneumococcal strains used in the MSKA

Strain	**Capsular Serotype**	**PspA family**
WU2	3	1
BR260.1	3	No PspA present
BR93.1	3	2

Table 2: Anti-PspA mAbs used in the MSKA

anti-PspA mAb	PspA Family
1b2.21	1
8b2.19	1
5c6.1	2
6e5.5	2

Isolation of phagocytic cells

Human neutrophils were isolated using the Polymorph prep system (Axis-Shield, Oslo, Norway) following manufacturer's instructions. Neutrophils were washed, resuspended in Hanks' Balanced Salt Solution (HBSS) (with Ca^{2+} and Mg^{2+}) (Invitrogen, Auckland, NZ) with 1% bovine serum albumin (BSA) (ICN Biomedicals, Aurora, OH) and diluted to $2x10^6$ cells/ml.

HL-60 cells were obtained from ATCC (catalog no. CCL-240). The cells were maintained, passaged, and differentiated with DMF into phagocytic cells as described by Romero-Steiner *et al*. (38). HL-60 cells on the 4^{th} and 5^{th} day of differentiation were used. The cells used in the MSKA were prepared as previously described (37). The differentiated cells were washed with 1x HBSS (without Ca^{2+} and Mg^{2+}), suspended in 1x HBSS (+Ca^{2+} and Mg^{2+}), and diluted to $4x10^6$ cells/ml in HBSS supplemented with 1% gelatin and 5% fetal bovine serum.

Modified Surface Killing Assay

The MSKA was based on an assay developed by Weinberger *et al*. (44) with several modifications. Monoclonal antibodies to capsule type 3 (mAb 16.3), PspA, and phosphocholine were serially diluted as indicated in HBSS with 1% BSA. WU2, BR260.1, and BR93.1 strains were diluted to $5x10^3$ CFU/ml, $1.5x10^4$ CFU/ml, and $5x10^3$ CFU/ml in HBSS with 1% BSA, respectively (about 75 CFUs per spot). 200 µl of each strain was added to 80 µl of mAb in HBSS with 1% BSA. A control tube had bacteria and 80 µl HBSS with 1% BSA only. Each tube was incubated at 37°C for 30 minutes

with shaking at 200 rpm or as indicated. Normal mouse serum (NMS) was obtained from female BALB/cByJ mice or CBA/N mice (Jackson Laboratories, Bar Harbor, ME) as indicated and was used as a complement source (final concentration 6.7%). Heat-inactivated NMS (6.7%) was added as a negative control. After the complement was added, the tubes were incubated at 37°C for another 30 minutes with shaking at 200 rpm. Six spots of 15 μl of the incubated mixtures were plated onto dried sheep blood agar plates (THY II) (Becton Dickinson). After the 6 spots were absorbed into the agar, 20 μl of prepared neutrophil suspension was overlaid on top of 3 of the spots. When the neutrophil suspension had absorbed into the agar, the plates were incubated in a candle jar at 37°C with increased CO_2 for 3 hours in candle jar. The plates were then placed at 4°C for 20 minutes to stop the phagocytic reaction. After 20 minutes, the plates were placed at 37°C with increased CO_2 to allow the bacteria to grow. CFUs in each spot were counted. Percent killing was determined by comparing spots with neutrophils to spots without neutrophils and normalizing to the NMS count. In other studies, we compared groups with complement to groups where the complement had been heat inactivated (HI).

Dot Blot

WU2 and BR260.1 strains were diluted to $1x10^8$ CFU/ml and $1x10^8$ CFU/ml, respectively, in PBS and 1 μl of each bacterial dilution? was placed on a nitrocellulose membrane (Millipore, Billerica, MA), allowed to dry, and blocked in 1x Dulbecco's Phosphate Buffered Saline (PBS) (Mediatech, Manassas, VA) with 1% BSA. The membrane was incubated with mAb 16.3, mAb 1b2.21, mAb 5c6.1, or mAb 8b2.19 as

the primary mAb for 1 hour at room temperature then washed 5 times for 5 minutes each in wash buffer (PBS plus 0.1% Tween20). The membrane was then incubated with a solution containing biotin-conjugated goat anti-mouse antibodies (Southern Biotech, Birmingham, AL) and strepavidin (Southern Biotech) for 1 hour at room temperature and washed 5 times for 5 minutes each time. The dot blot was developed with a BCIP+NBT tablet (Sigma-Aldrich, St. Louis, MO) per manufacturer's instructions.

Statistical analysis

Percent killing from the MSKA was evaluated with unpaired, two-tailed student t-test using Graphpad Prism version 5.0 for Windows (GraphPad Software, San Diego, California). *P* values of significance are $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

RESULTS

Experiment 1: Modified surface killing assay as a potential *in vitro* surrogate assay using human neutrophils.

WU2 and BR260.1 have similar growth curves.

Growth curves of WU2 and its PspA –mutant, BR260.1, were measured (Figure 1). Although the *pspA* mutant appeared to grow measurably slower, the difference was small enough that it was not considered necessary to incubate plates bearing the bacteria for different time periods prior to counting the CFU in the MSKA.

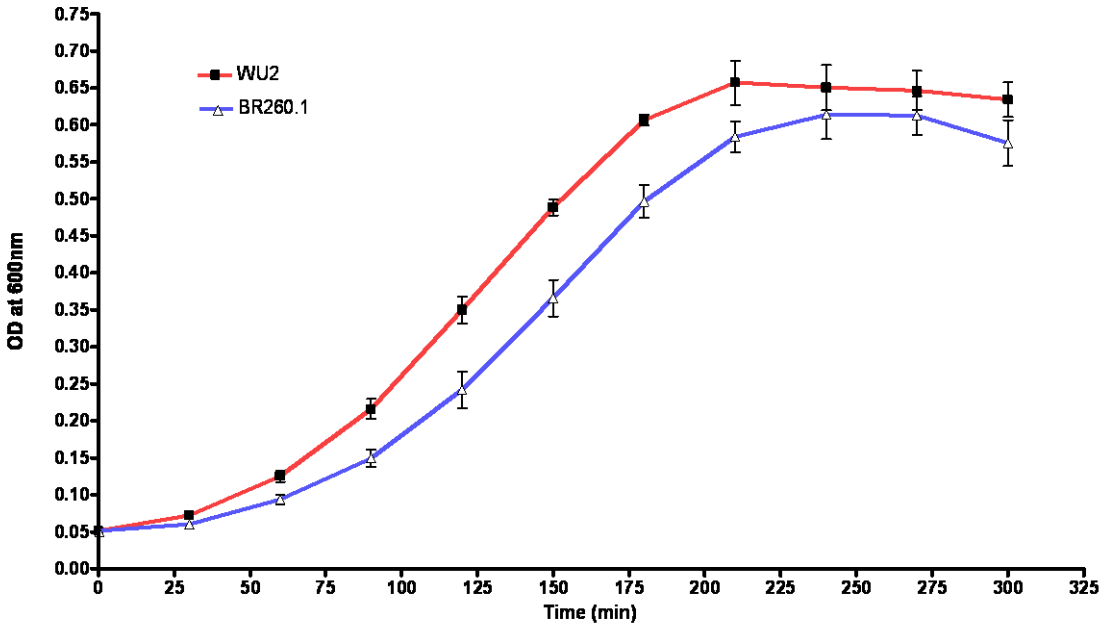


Figure 1: Growth curve of WU2 and its PspA$^-$ mutant, BR260.1. OD of each strain, WU2 and BR260.1, was measured at intervals of 30 minutes. Both strains reached plateau phase about 210 minutes.

WU2 and BR260.1both bind to mAb 16.3, whereas only WU2 binds to anti-PspA mAB 1b2.21.

The reactivity of pneumococcal strains WU2 and BR260.1 with anti-capsule type 3 mAb 16.3 and anti-PspA mAb 1b2.21 was tested prior to their use in the MSKA (Figure 2). Each strain was reciprocally diluted (1:3) from $1x10^8$ CFUs/ml to $4.6x10^4$CFUs/ml. The concentration of Anti-PspA mAbs used was 0.14mg/ml. Anti-capsule type 3 mAb 16.3 bound strongly to both WU2 and BR260.1 and the binding weakened as each bacterial solution was diluted. mAb 1b2.21 bound to WU2 but not BR260.1 due to absence of PspA on the surface of BR260.1.

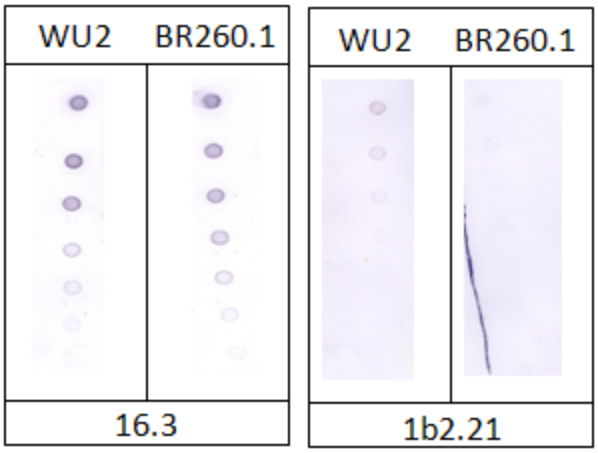


Figure 2: Dot blot of WU2 and BR260.1 with mAb 16.3 and mAb 1b2.21. Dot blot was done with WU2 and its isogenic PspA-mutant BR260.1 with anti-capsule type 3 mAb (16.3) and anti-PspA mAb (1b2.21). mAb 16.3 was used as a positive control.

Anti-Capsule Type 3 mAb 16.3 mediates killing of WU2 and BR260.1 in the MSKA.

Differences in the susceptibility of WU2 and BR260.1 to phagocytosis by human neutrophils were observed due to differential complement fixation (Figure 3). The percent killing of BR260.1, the $PspA^-$ mutant, was significantly higher (p <0.0001) than WU2 in the presence of only NMS. The mean percent killing of BR260.1 and WU2 with NMS were 17.98±2.1 and 1.74±3.8, respectively. The larger amount of killing of

BR260.1 is likely due to lack of PspA on its surface, which allows greater complement deposition on the bacteria compared to its wild type, WU2 (28), and thus greater phagocytosis and killing by PMNs, which is also known as neutrophils.

Anti-capsule type 3 mAb 16.3 mediated killing of both WU2 and BR260.1 by human neutrophils compared to NMS control with each strain (Figures 4 and 5). The mean percent killing of BR260.1 and WU2 at the highest concentration of mAb 16.3 were 12.1±1.6 and 39.7±5.4, respectively. This mAb-mediated killing is consistent with the fact that both strains express serotype 3 polysaccharide. BR260.1 had a lower killing effect (Figure 5) compared to WU2 (Figure 4) ($p<0.001$). This observation is consistent with the fact that BR260.1 was already efficiently killed in the presence of complement alone (Figure 3).

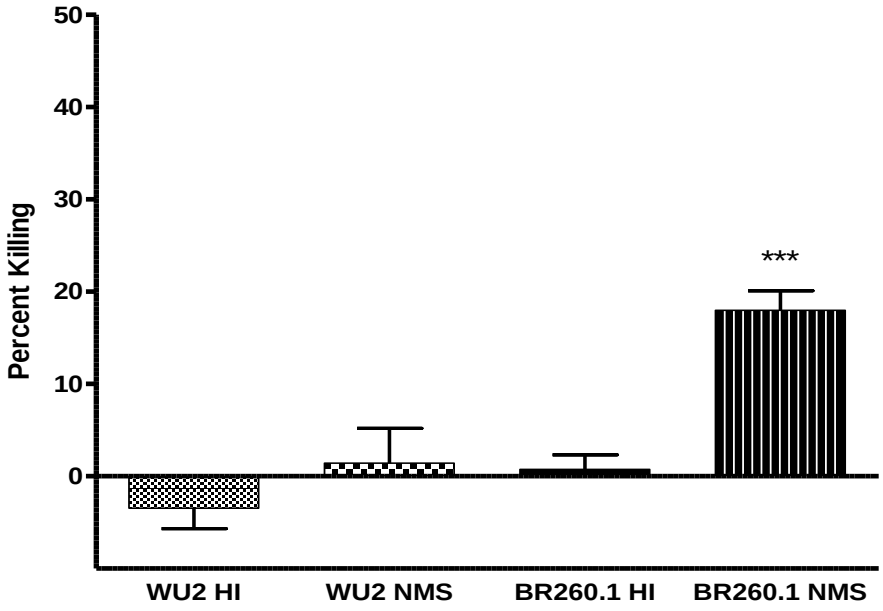


Figure 3: Killing effect of WU2 and BR260.1 by complement. Percent killing of WU2 and BR260.1 with heat inactivated mouse serum (HI) and normal mouse serum (NMS) with human PMNs was tested. Significantly higher percent killing (*P=0.0004)* of BR260.1 in presence of NMS than WU2 with NMS was seen. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

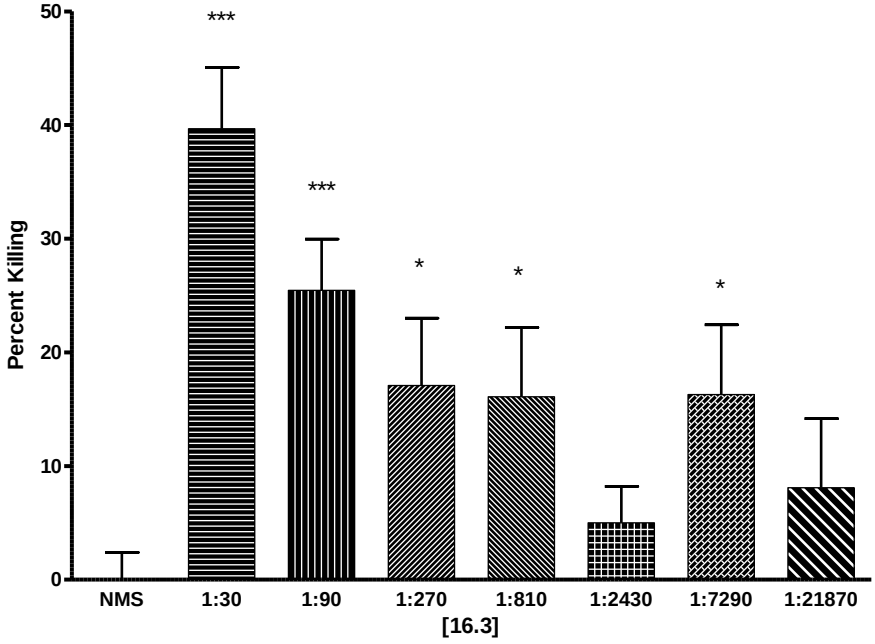
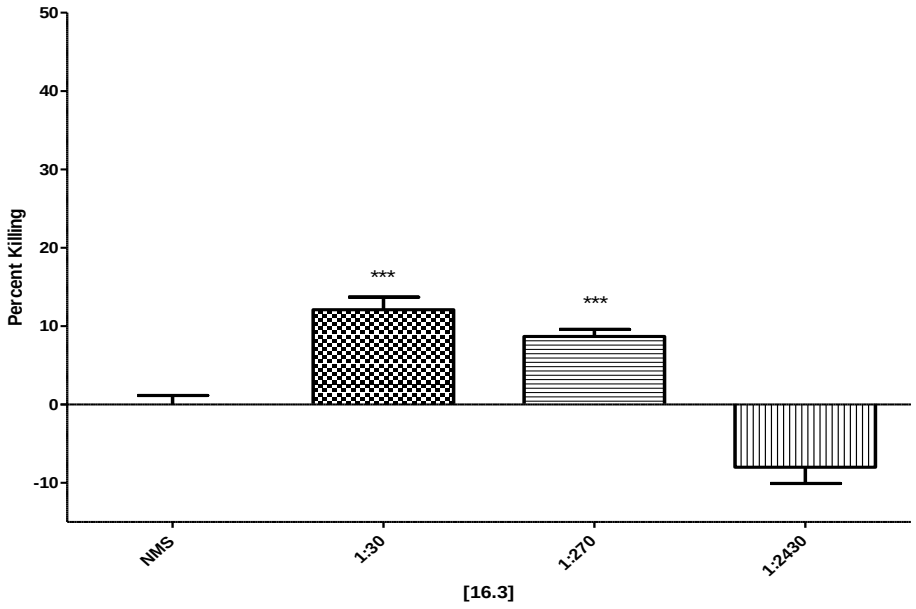


Figure 4: MSKA of WU2 with mAb 16.3 by human neutrophils. Percent killing of serial dilutions of mAb 16.3 with WU2. NMS used as a control.* p value < 0.05, ** p value < 0.01, *** p value < 0.001.

A)



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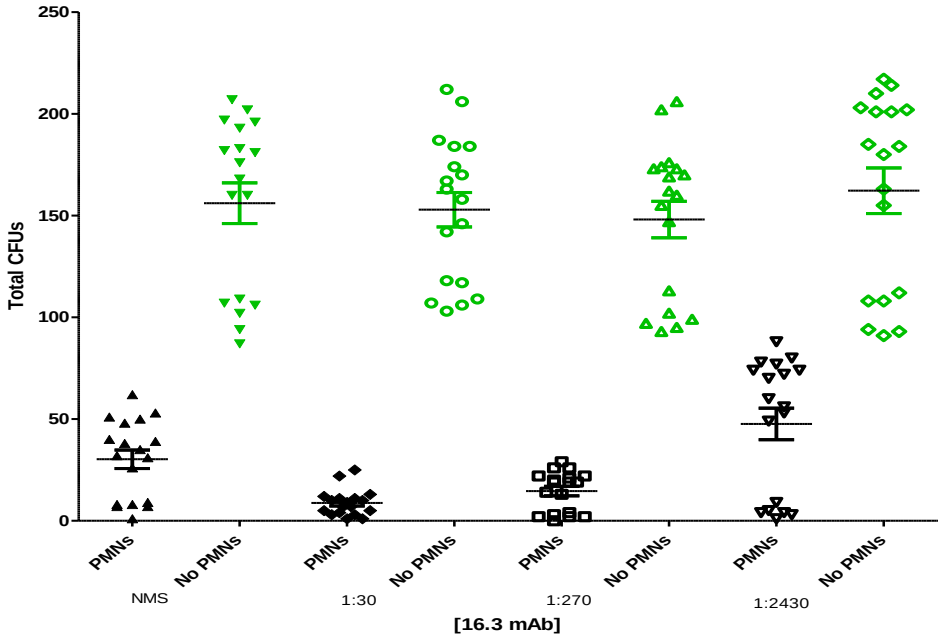


Figure 5: MSKA of BR260.1 with mAb 16.3 by human neutrophils. Percent killing of BR260.1 with serial dilutions of mAb 16.3 is compared to its NMS control. A)Percent killing of BR260.1 with 16.3 mAb. B) Survival of BR260.1 (Total CFUs) with 16.3 mAb. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

The MSKA can distinguish mAbs that are protective against pneumococcal challenge from those that are non-protective.

Two different anti-PspA family 1 mAbs, 1b2.21 and 8b2.19, significantly increased killing of WU2 by neutrophils in the MSKA compared to its NMS control (Figure 6 and 7). 20 to 40 ng of each mAb was able to mediate significant killing in the MSKA. Thus, the PspA appears to be sufficiently surface exposed to permit anti-PspA mAb to mediate killing of bacteria. This also shows the potential of this assay to serve as an *in vitro* surrogate assay to identify protective antibody responses to PspA.

As expected, anti-PspA mAb 1b2.21 did not have a significant effect on killing of BR260.1 by neutrophils in the presence of complement in the MSKA compared to its NMS control (data not shown). This shows that the lack of PspA makes BR260.1 more susceptible to killing due to higher complement deposition on its surface (28).

Anti-PspA family 2 mAb 5c6.1, an anti-PspA family 2 mAb, did not show binding on dot blot to WU2, which expresses family 1 PspA (data not shown). mAb 5c6.1 did not mediate a significant effect on killing of WU2 by neutrophils in the MSKA compared to its NMS control (Figure 8). The failure of mAb 5c6.1 to facilitate killing of WU2 is consistent with its inability to bind the PspA of WU2. This illustrates that the binding of anti-PspA mAb is specific. The finding also supports our assumption that the MSKA can function as an *in vitro* surrogate assay that will be able to discriminate between protective and non-protective mAb to PspA.

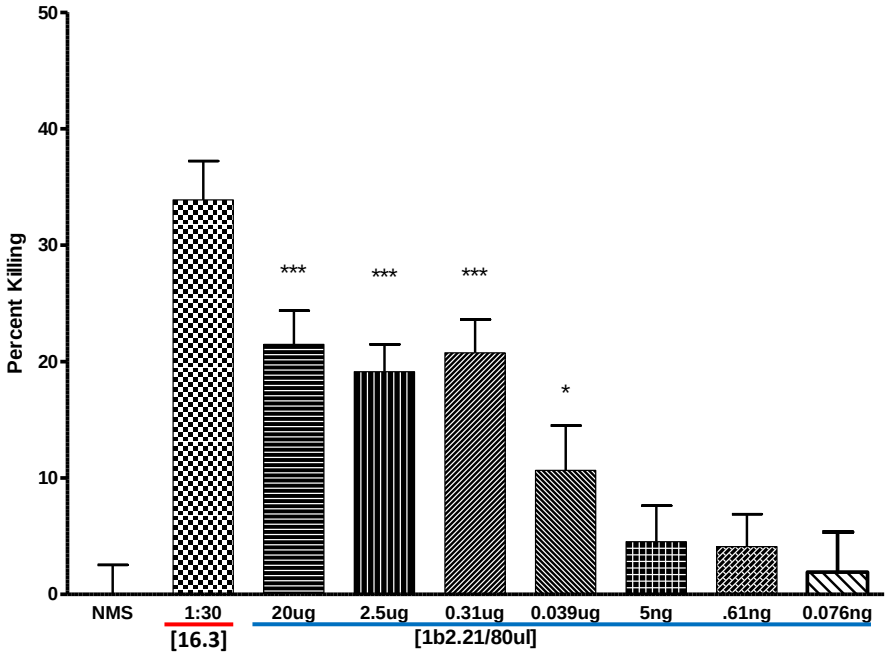
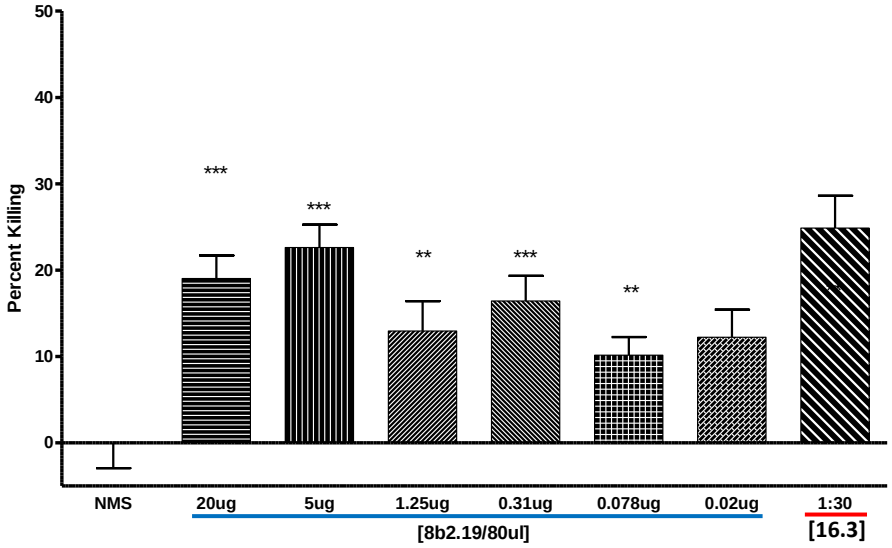


Figure 6: MSKA of WU2 with mAb 1b2.21 using human neutrophils. Percent killing of WU2 colonies by mAb 16.3 (positive control) and mAb 1b2.21 compared to NMS is shown. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

A)



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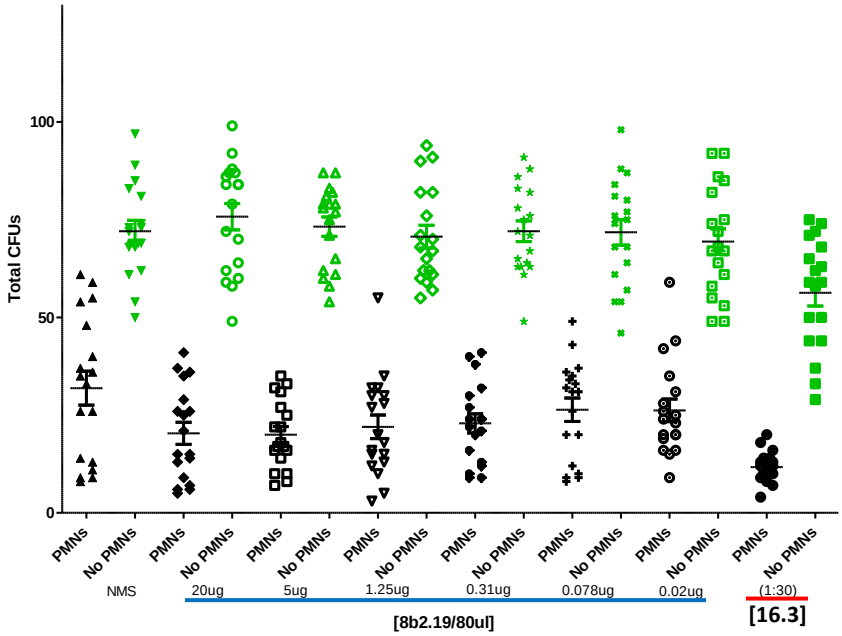


Figure 7: MSKA of WU2 with mAb 8b2.19 using human neutrophils. Percent killing of WU2 by different concentrations of mAb 8b2.19 compared to NMS. A) mAb 8b2.219 mediated killing of WU2. B) mAb 8b2.19 mediated killing of WU2 as a decrease in total CFUs. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

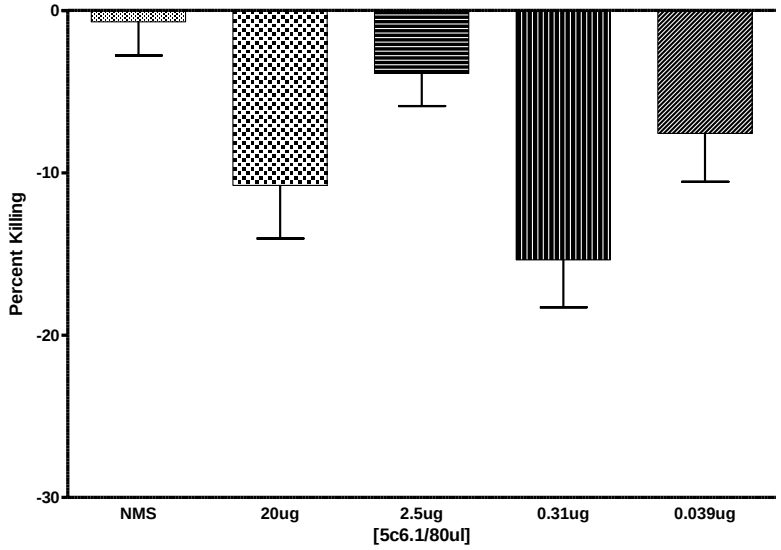


Figure 8: MSKA of WU2 with mAb 5c6.5 using human neutrophils. Percent killing of WU2 by different concentrations of mAb 5c6.1 compared to. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

Experiment 2: The MSKA as a potential *in vitro* surrogate assay using an immortal cell-line, HL-60 cells.

An immortalized line of phagocytic cells, such as HL-60, would have a great advantage over fresh human PMNs because they provide a source of homogenous cells that can be produced in large quantities for use in large-scale assays of pre- and post-immune sera from immunized subjects. This could have a clear advantage over fresh human PMN, which are obtained in relatively small numbers from the blood of each donor, and the activity of the PMN is variable from different donors, or even the same donor on different days.

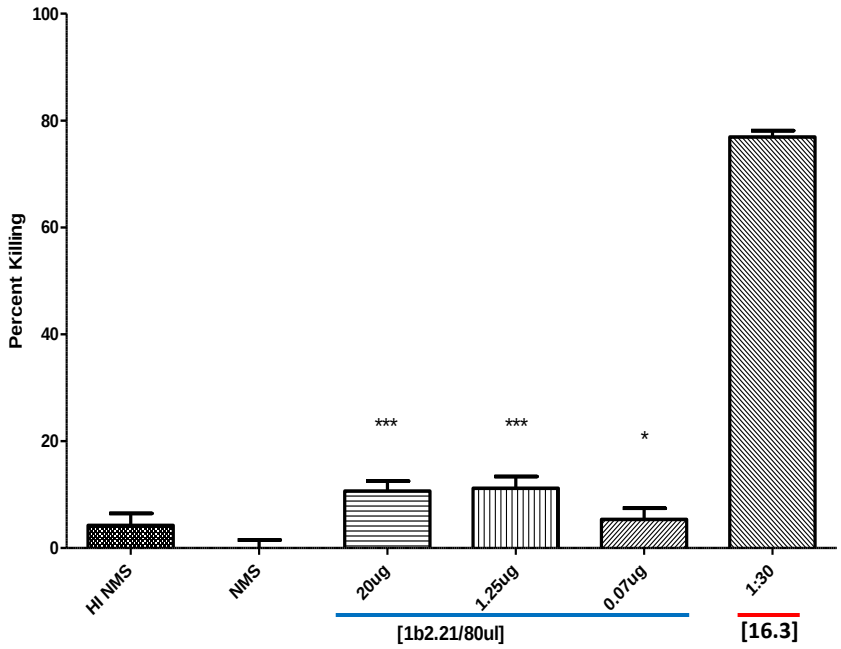
The optimal conditions for use of HL-60 cells in the MSKA are $4x10^6$ cells/ml in a sequential method for a 3 hour incubation time.

The optimal cell number, incubation method, and incubation time were determined for use of HL-60 cells in the MSKA (Figures 9, 10, and 11). HL-60 cells were tested at $1x10^7$ cells/ml and $4x10^6$ cells/ml with samples that were incubated separately (Figure 9). Both mAb 16.3 (positive control) and mAb 1b2.21 mediated significant killing at both HL-60 concentrations ($p < 0.001$). However, in comparison with our previous results with PMNs, the difference in killing mediated by anti-capsule type 3 versus anti-PspA mAb was magnified with the use of the two different concentrations of HL-60 cells. With mAb to capsule type 3, we observed about 80% killing of the WU2 bacteria with HL-60 cells (Figure 9) versus ~30% killing by PMNs (Figure 7). With mAb to PspA, however, the percent killing was slightly less with HL-60 cells than with PMNs under similar conditions. When the two HL-60 cell densities were compared, the lower cell concentration of $4x10^6$ cells/ml appeared to work better than $1x10^7$ cells/ml; specifically, a greater killing was observed using $4x10^6$ cells/ml at each of the concentrations of mAb to PspA that was tested.

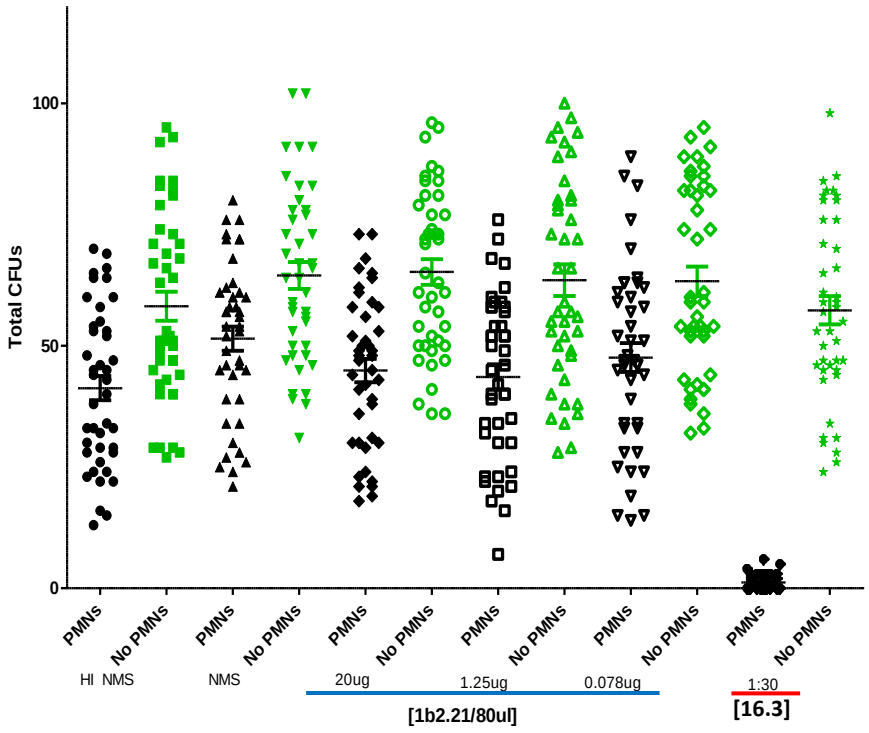
The killing effects from different incubation methods in the MSKA were also measured (Figure 10). Sequentially incubating WU2 with mAb to PspA and complement resulted in greater killing than when WU2, anti-PspA mAb, and complement were added all at once. Significant killing was shown with both mAb 16.3 and mAb 8b2.19. mAb 8b2.19 was serially diluted 1:16. The percent killing of mAb 16.3 with the sequential method was 1.18-fold greater. Also, the percent killing of mAb 8b2.19 with sequential method was 2.6-fold, 5.38-fold, and 0.84-fold greater in the concentrations of 20μg/80μl, 1.25μg/80μl, and 0.078μg/80μl respectively.

Next, the effect of different incubation times at 37ºC after the HL-60 cells were overlaid on the mixture of opsonized bacteria was evaluated (Figure 11). An incubation time of 3 hr had the most significant killing (~27%) with 20 µg mAb 1b2.21 compared to both 2 hr (~17%) and 7 hr (~26%) in the MSKA. At 3 and 7 hr of incubation, there was less killing with an antibody concentration of 0.078µg versus 20 µg, however, this was not observed at 2 hrs of incubation. The fact that the 3 and 7 hr incubations had essentially the same results raise the possibility that the plates could be placed at 37ºC until the bacteria are ready to be counted.

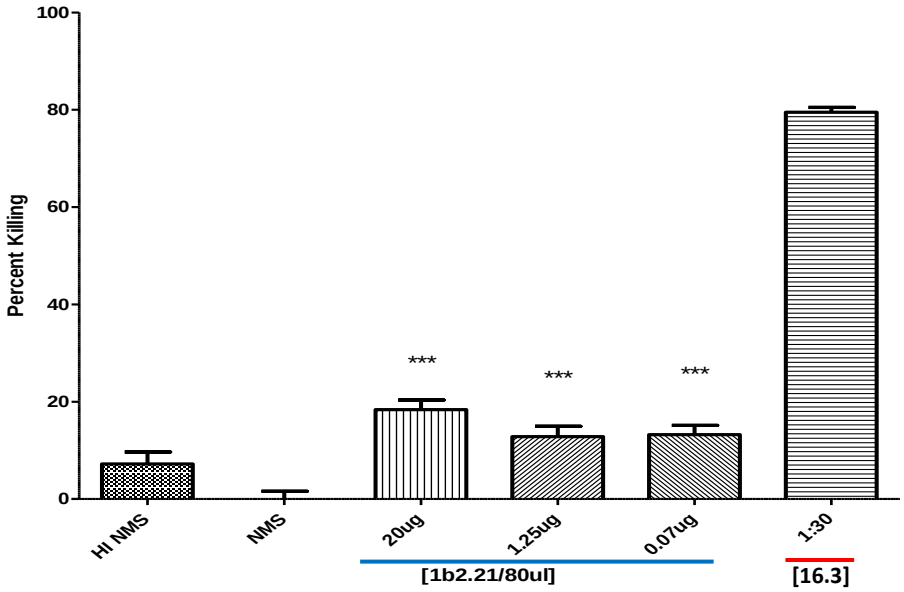
A)


Percent Killing
100
80
60
40
20
0
***
***
*
HI NMS
NMS
20ug
1.25ug
0.07ug
1:30
[1b2.21/80ul]
[16.3]

B)


Total CFUs
100
50
0
PMNS
No PMNS
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[1b2.21/80ul]
[16.3]

C)



D)

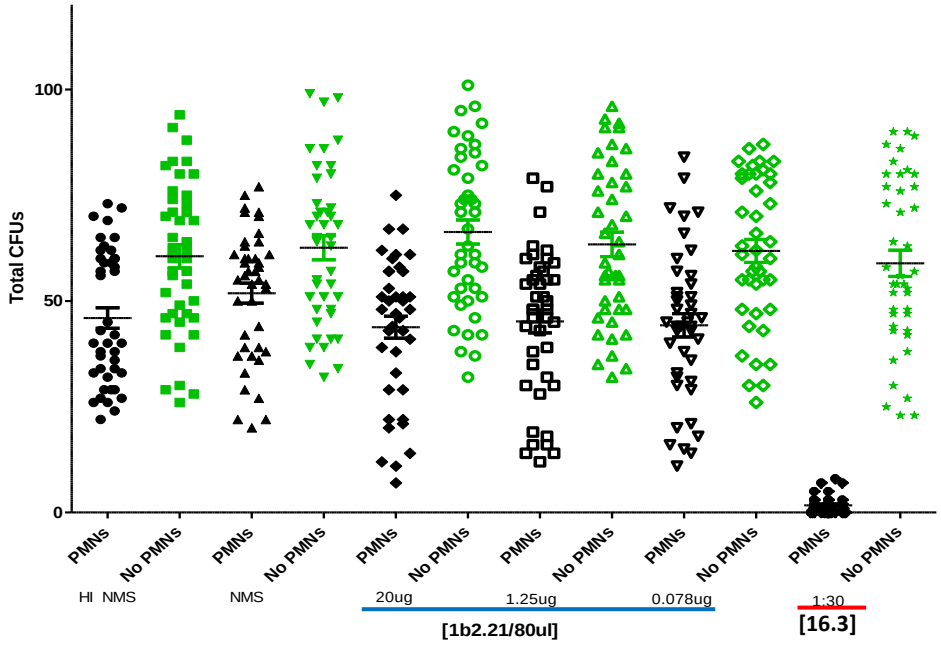
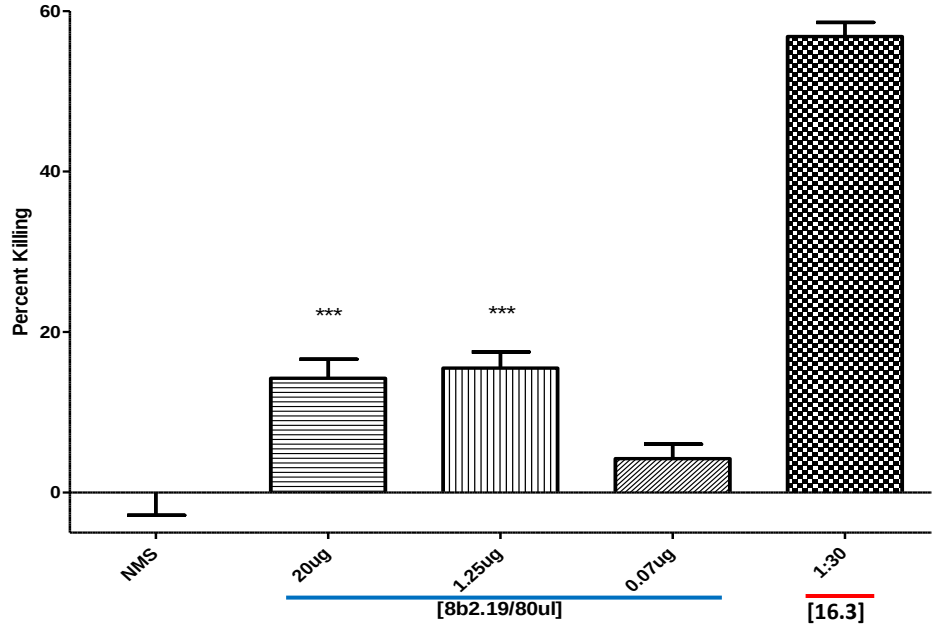
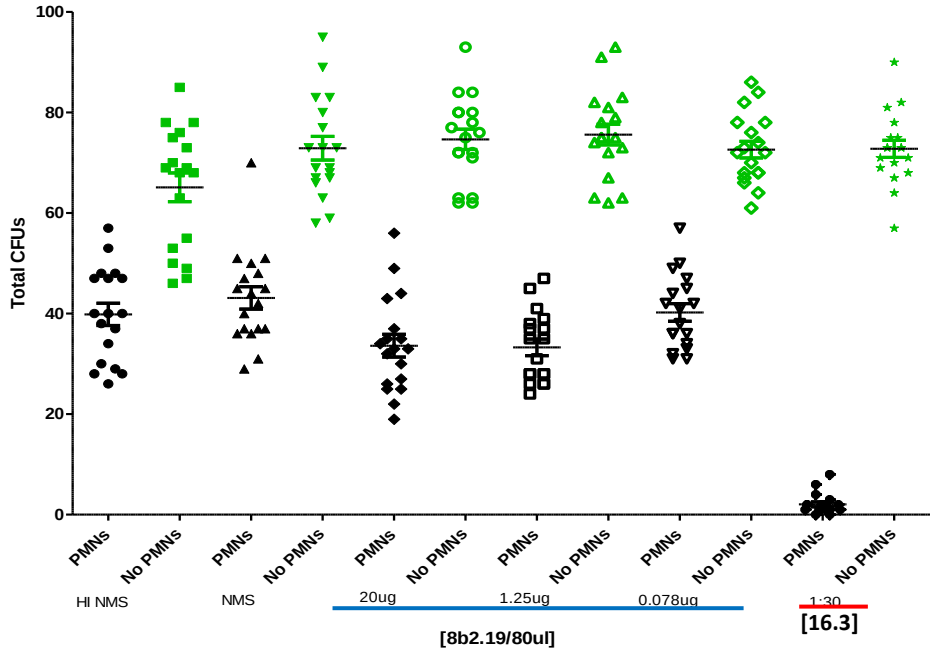


Figure 9: MSKA of WU2 with different concentration of HL-60 cells. Percent killing of different concentrations of HL-60 cells with mAb 16.3 (positive control) and mAb 1b2.21 compared to NMS. A) $1x10^7$ cells/ml, B) Total CFUs of $1x10^7$ cells/ml , C) $4x10^6$ cells/ml, and D) Total CFUs of $4x10^6$ cells/ml. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

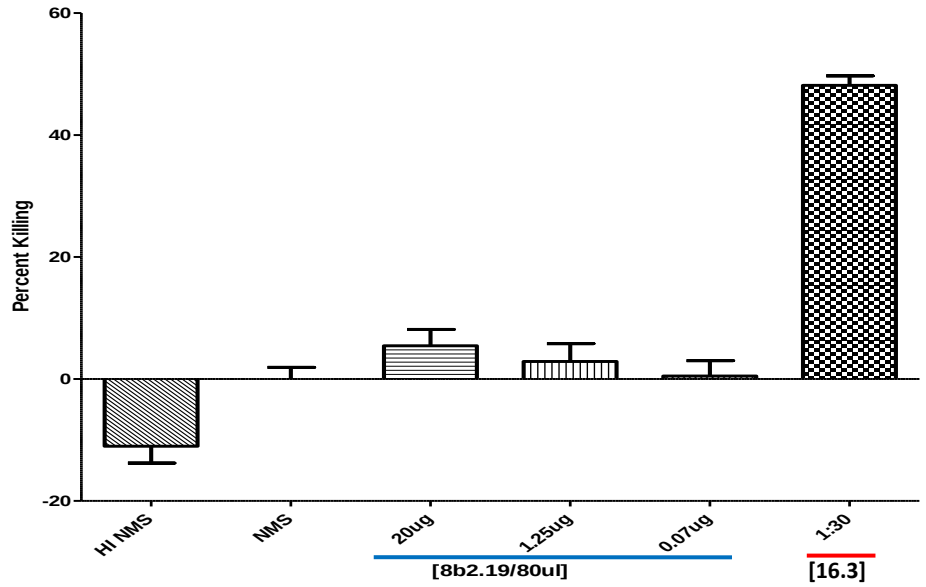
A)


60
40
20
0
Percent Killing
***
***
NMS
20ug
1.25ug
0.07ug
1:30
[8b2.19/80ul]
[16.3]

B)


100
80
60
40
20
0
Total CFUs
PMNs
No PMNs
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[8b2.19/80ul]
[16.3]

C)



D)

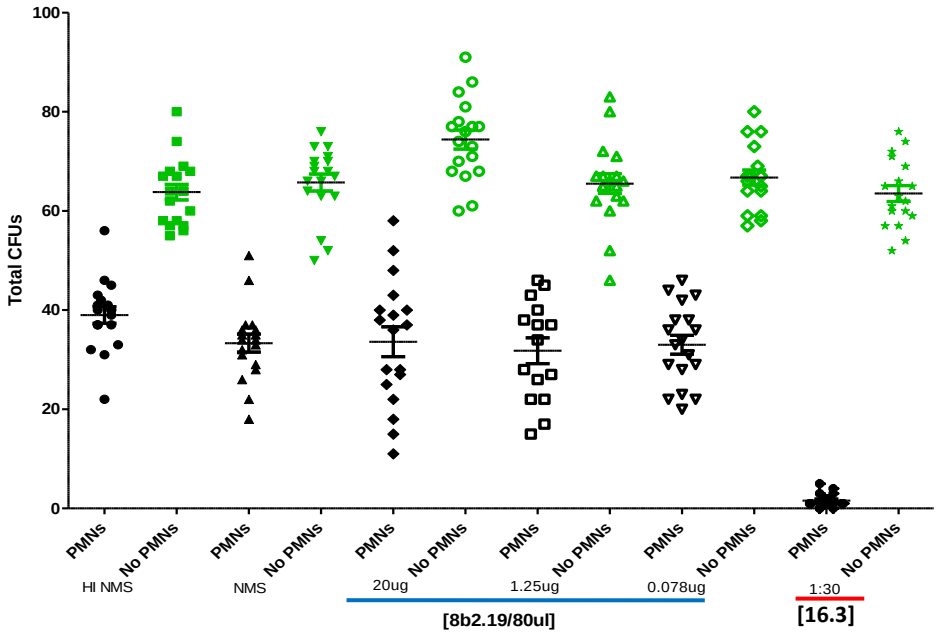
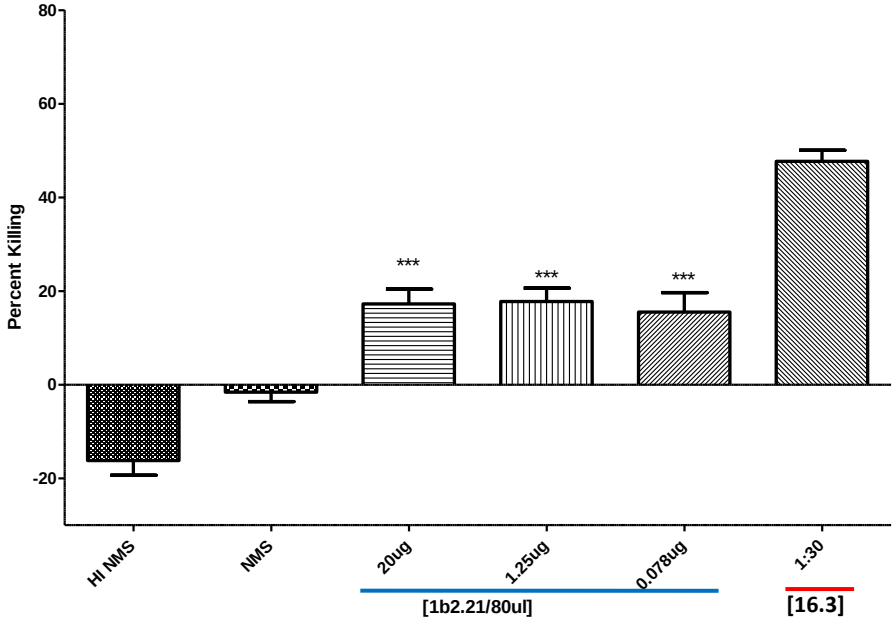
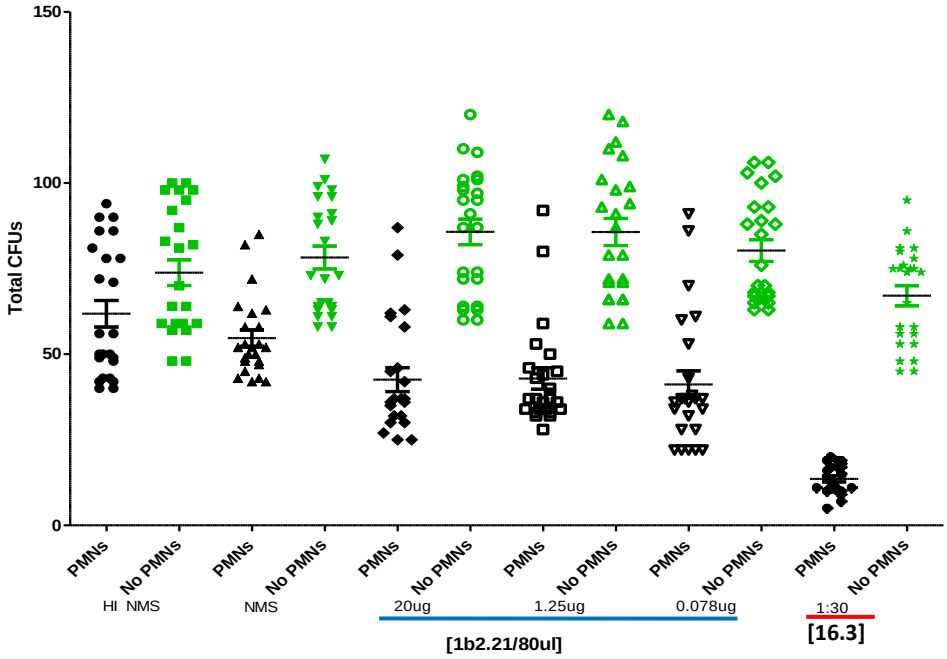


Figure 10: MSKA of WU2 with different incubation steps. The effect of different incubation methods using WU2 strain with serial dilutions of mAb 8b2.19 is shown. A) First WU2 was incubated with mAb for 30 min, then complement was added and the mixture was incubated for an addition 30 min, B) Separated incubations with WU2 in total CFUs, C) One hour incubation of WU2 with mAb, and D) One hour incubation of WU2 with complement in total CFUs. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

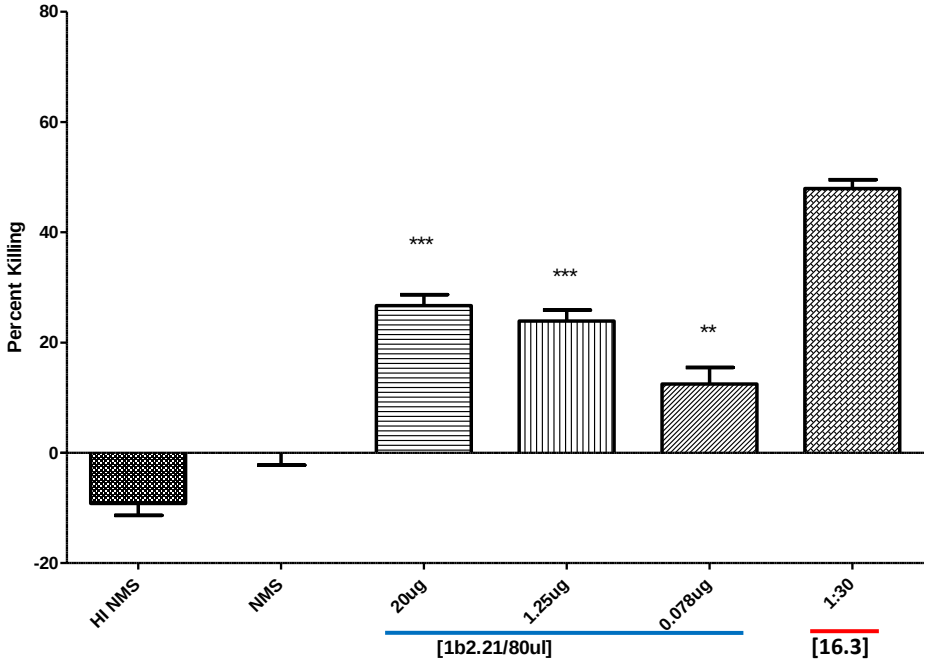
A)


Percent Killing
80
60
40
20
0
-20
***
***
***
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[1b2.21/80ul]
[16.3]

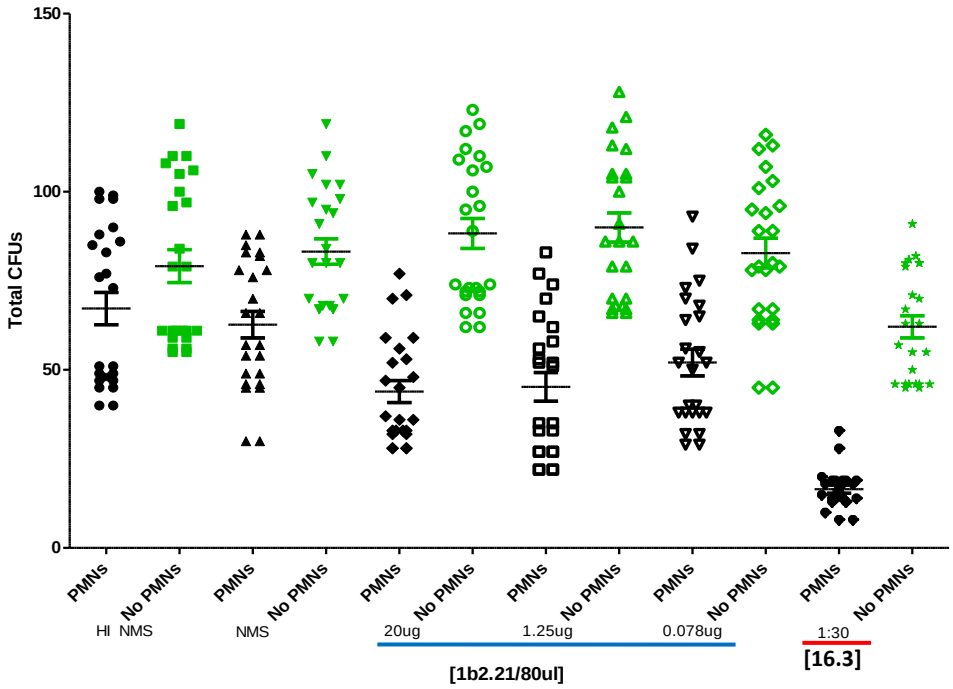
B)


Total CFUs
150
100
50
0
PMNs
No PMNs
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[1b2.21/80ul]
[16.3]

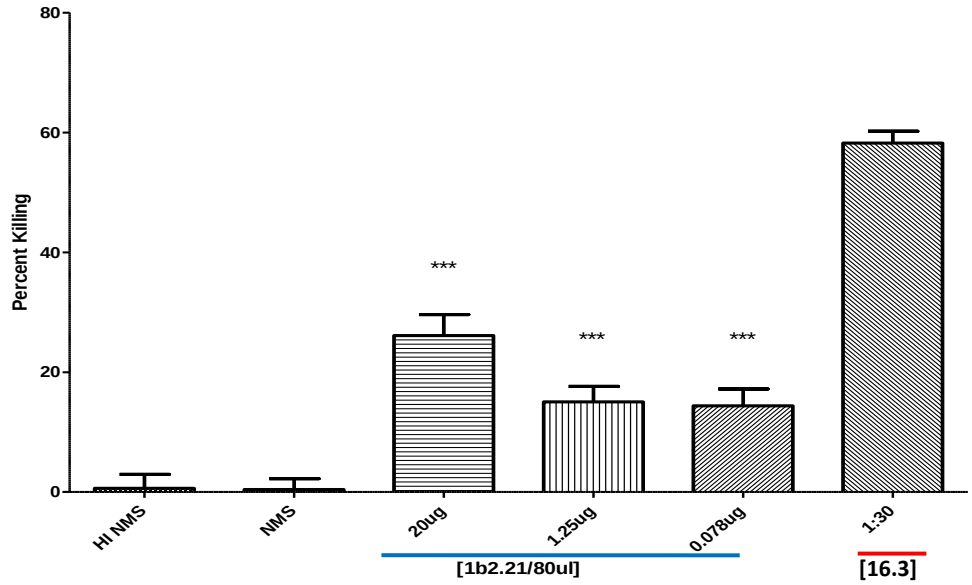
C)


Percent Killing
80
60
40
20
0
-20
***
***
**
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[1b2.21/80ul]
[16.3]

D)


Total CFUs
150
100
50
0
PMNs
No PMNs
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[1b2.21/80ul]
[16.3]

E)



F)

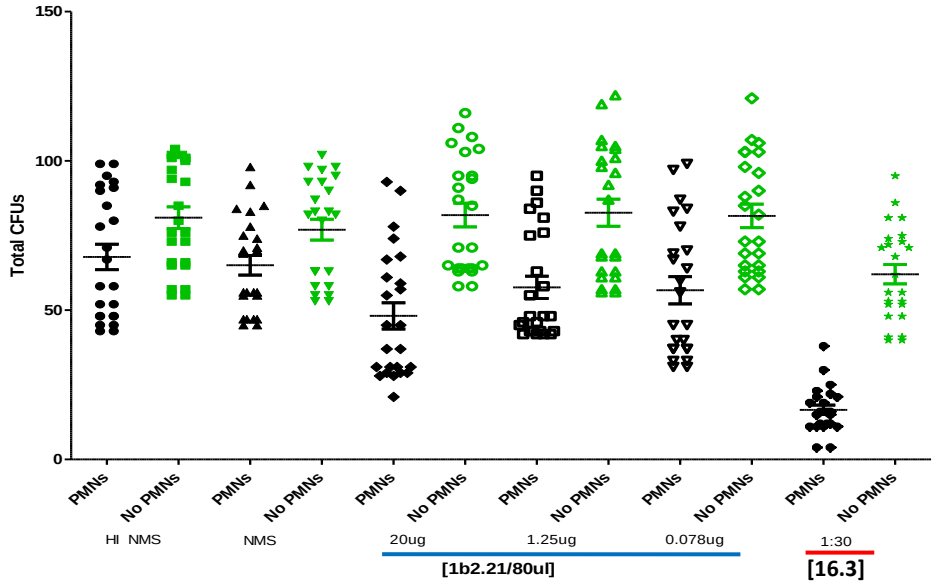


Figure 11: MSKA of WU2 with different incubation (phagycytosis phase) times. The killing effect of different incubation times at 37ºC of the bacteria and phagocytesis shown. WU2 was opsonized with mAb 1b2.21 at various concentrations while all other variables were kept constant. mAb 16.3 was used as a positive control. A) 2 hr incubation time in percent killed, B) 2 hr incubation time in total CFUs, C) 3 hr incubation time in percent killed, D) 3 hr incubation time in total CFUs, E) 7 hr incubation time in percent killed, and F) 7 hr incubation time in total CFUs. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

HL-60 cells can be a replacement for fresh human PMNs.

Differentiated HL-60 cells were used in place of human neutrophils in the MSKA with mAb 16.3, mAb 1b2.21, and mAb 8b2.19 (Figures 12, 13, and 14). With the differentiated HL-60 cells, the dose response killing effect was steeper than when human neutrophils were used (Figures 4 and 12). When HL-60 cells were used with mAb 16.3, a greater killing effect (1.3-fold) with the 1st dilution (1:30) on WU2 compared to human neutrophils was observed. However, it was not statistically greater when the 2 were compared. In the case of mAb to PspA, the dose-response curve was just as shallow with the HL-60 cells as with human neutrophils (Figures 13 and 14). When mAb 1b2.21 was used, both human neutrophils and HL-60 cells had similar killing effect on WU2 (Figures 6 and 13). Percent killing by both phagocytes at the highest concentration of mAb 1b2.21 was ~ 20%. Similar results were observed with mAb 8b2.19 (Figures 7 and 14). However, an extended and greater killing effect was observed with HL-60 cells compared to human neutrophils.

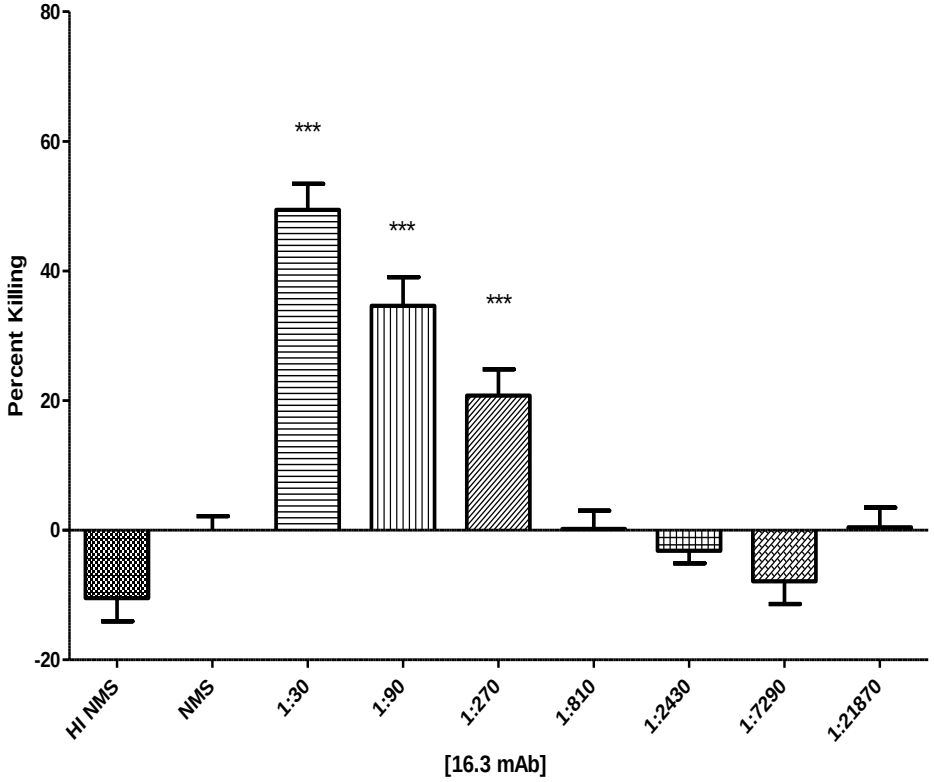
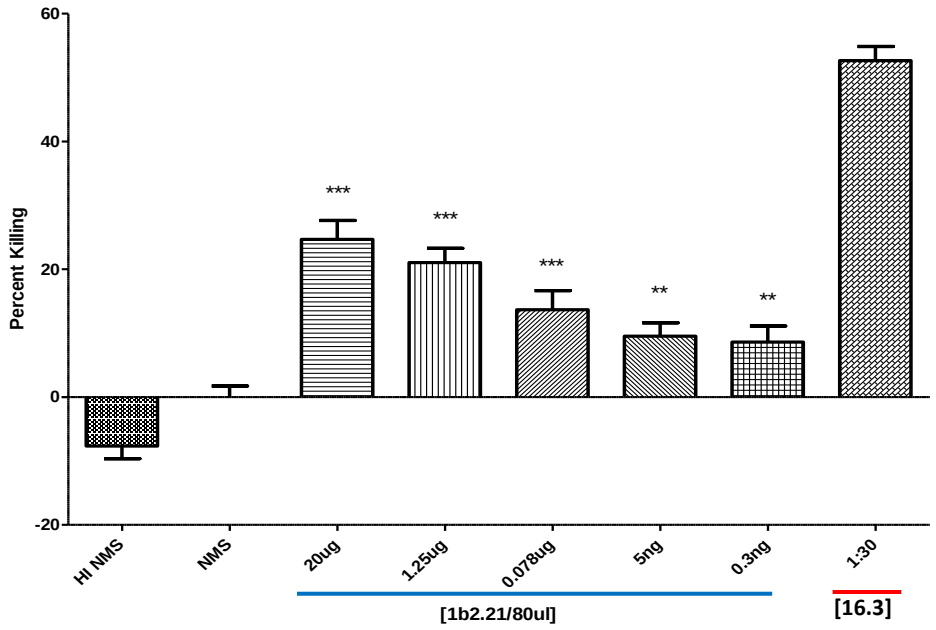


Figure 12: MSKA of WU2 with mAb 16.3 by HL-60 cells. Effect of serial dilutions of mAb 16.3 on WU2 and killing effect of neutrophils vs. differentiated HL-60 cells are shown compared to its control * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

A)



B)

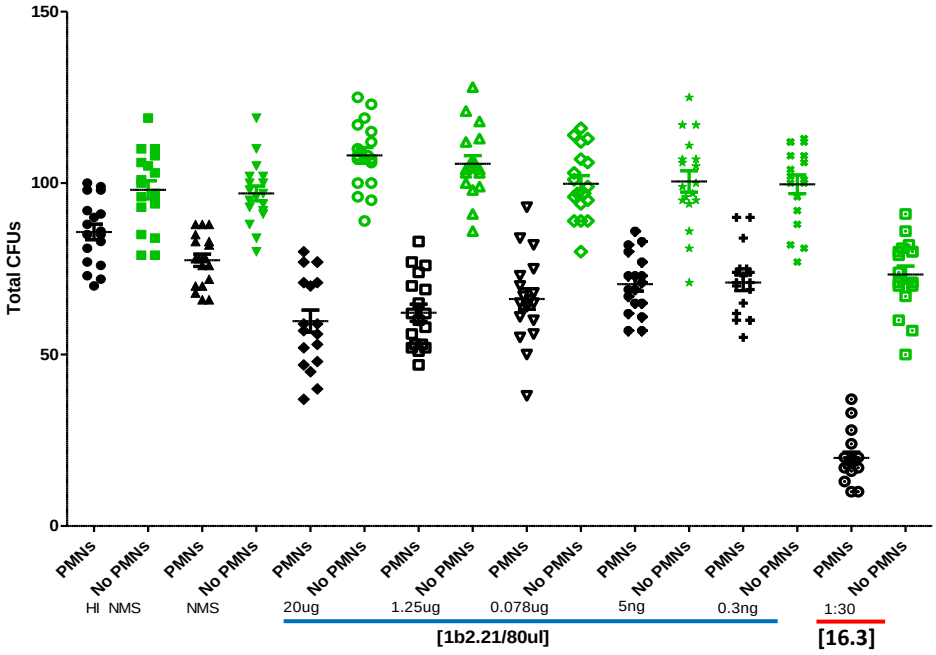
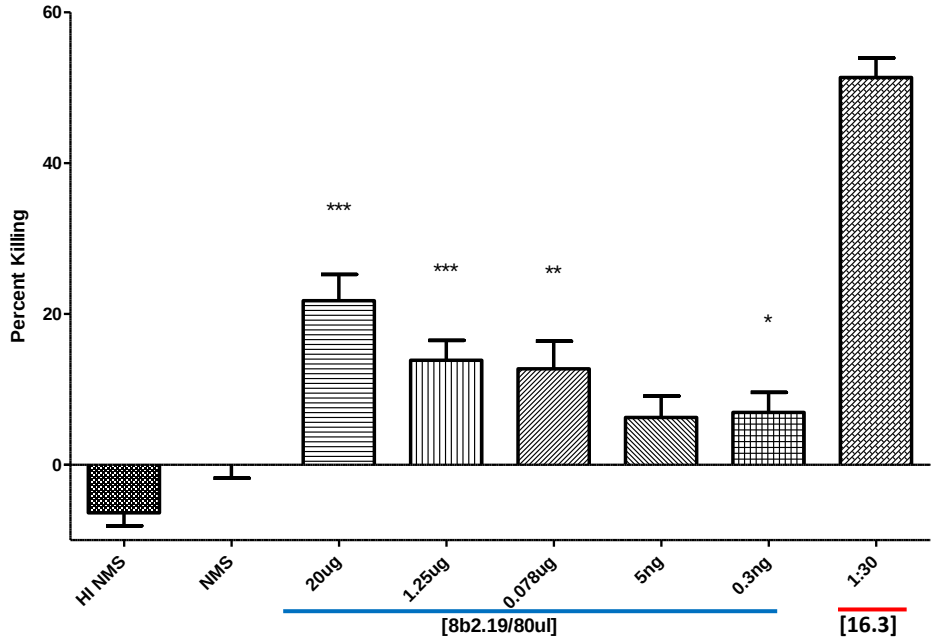


Figure 13: MSKA of WU2 with mAb 1b2.21 by HL-60 cells. Effect of serial dilution of anti-PspA mAb 1b2.21 on WU2 and killing effect of neutrophils vs. differentiated HL-60 cells are shown compared to its control. A) Killing effect of 1b2.21 in killing percent B) Killing effect mediated by 1b2.21 in total CFUs. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

A)



B)

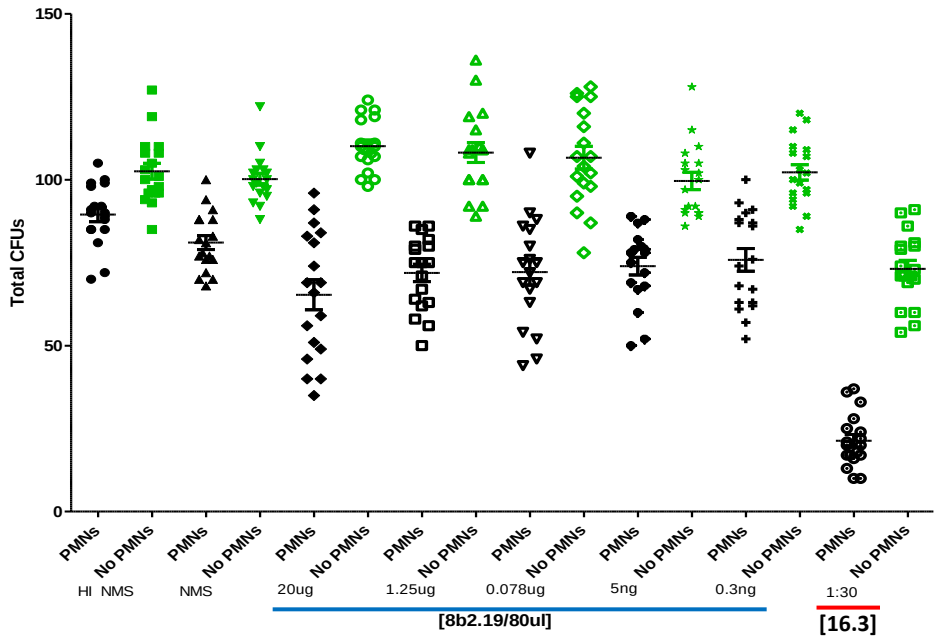


Figure 14: MSKA of WU2 with mAb 8b2.19 by HL-60 cells. Change in WU2 colonies with mAbs 16.3 and 8b2.19 when compared to NMS and killing effect of different phagocytic cells are shown. Both mAbs 16.3 and 8b2.19 greatly increased percent killing. A) Killing effect mediated by 8b2.19 in MSKA and B) Killing effect mediated by mAb 8b2.19 in total CFUs. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

Anti-PspA mAb mediated killing is a PspA family specific reaction.

BR93.1, capsule type 3 with family 2 PspA, was used as the challenge strain. Monoclonal antibodies to family 2 PspA, 5c6.1 and 6e5.5, were used (Figure 15 and 16). For this assay, differentiated HL-60 cells were used instead of human neutrophils. The mean highest percent killing with 5c6.1 mAb and 6e5.5 mAb were 22.4±3.36 and 19.5±4.8, respectively. The highest killing percent of mAb to both family 1 and family 2 ranged from 19.5 to 24.7% when homologous mAbs were used. The dose response curve was shallow as seen with mAb to family 1 PspA (Figures 13 and 14).

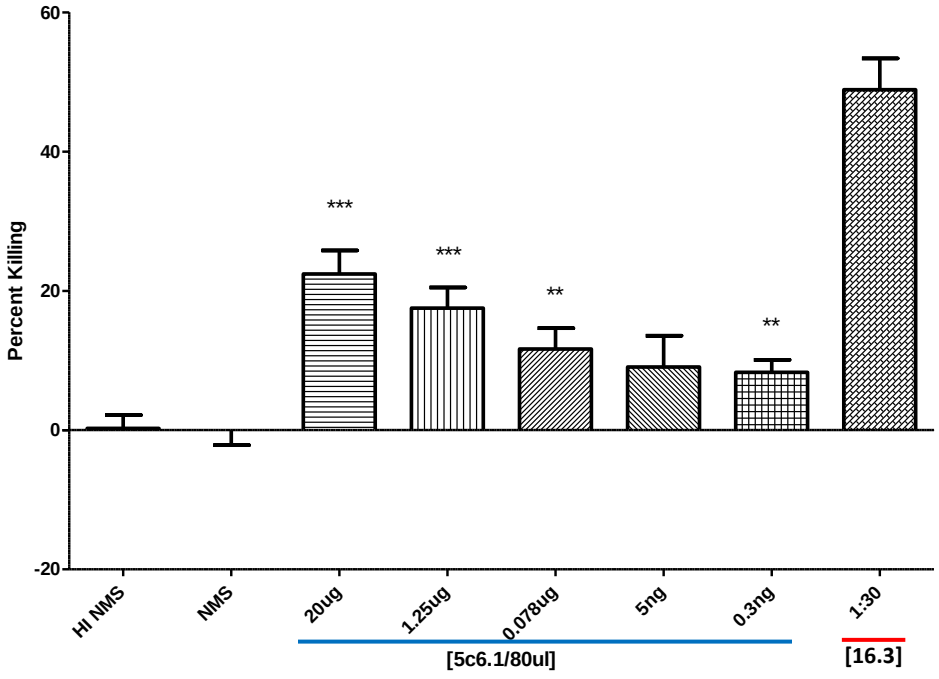


Figure 15: MSKA of BR93.1 with mAb 5c6.1 by HL-60 cells. Percent of BR93.1 (PspA family 2) killed by mAbs 16.3 and 5c6.1 compared to NMS and killing effect of differentiated HL-60 cells are shown. Both 16.3 and 5c6.1 monoclonal antibodies greatly increased percent killing. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

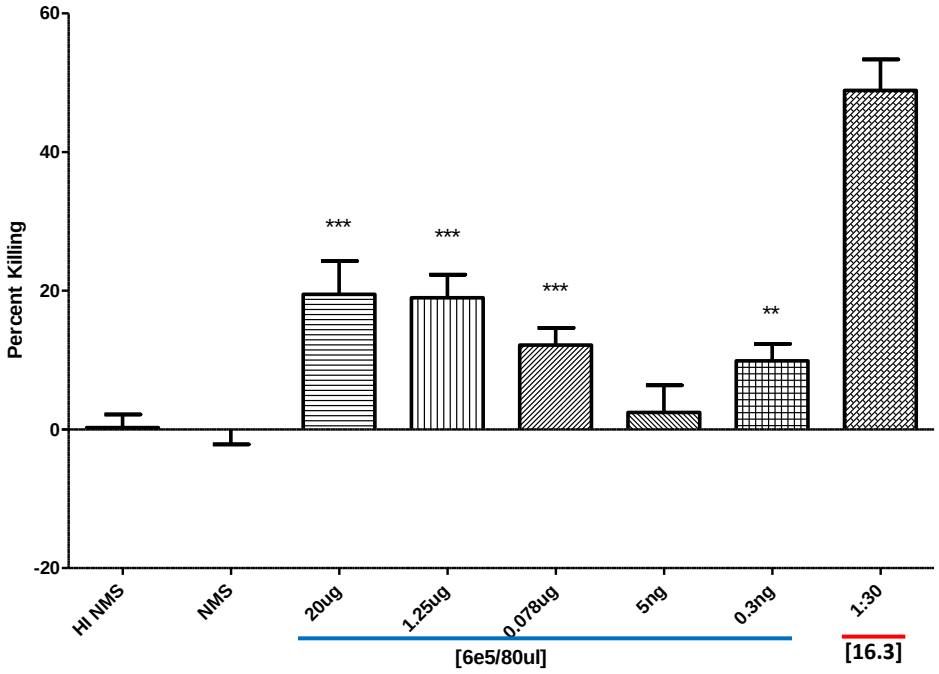


Figure 16: MSKA of BR93.1 with mAb 6e5.5 by HL-60 cells. Percent killing of BR93.1 PspA family 2 strain killed by mAbs 16.3 and 6e5.5 compared to NMS is shown. Both 16.3 and 6e5.5 mAbs greatly increased percent killing. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

Experiment 3: Effect of different complement sources.

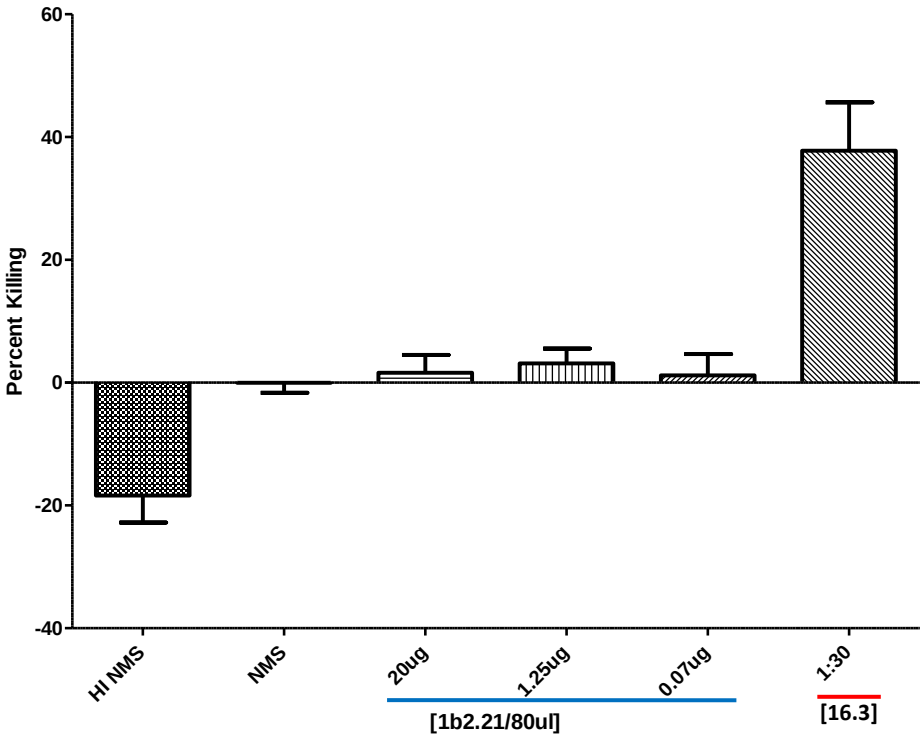
The ability of mAb to PspA to mediate detectable killing in the MSKA is a strong indication that this assay can detect killing mediated by anti-PspA antibody directly. However, it must be remembered that complement is actually serum and serum contains antibodies to many different antigens, some of which may be able to synergize with mAb to PspA. All of the data presented above were obtained with normal BALB/ cByJ mice serum because it is readily available in our laboratory. The only known anti-pneumococcal antibody in this serum is a naturally occurring antibody that reacts with the phosphorylcholine (PC) epitope on the teichoic acids and lipoteichoic acids of the

pneumococcal cell wall and cell membrane(11, 14, 17, 42)**.** Because CBA/N mice are unable to make antibodies reactive with most epitopes on polysaccharides including PC, these mice are highly susceptible to pneumococcal invasive. The absence of naturally occurring anti-PC antibodies in the serum of CBA/N mice makes them highly susceptible to pneumococcal invasive diseases (6, 7, 9, 11, 14, 30, 46).

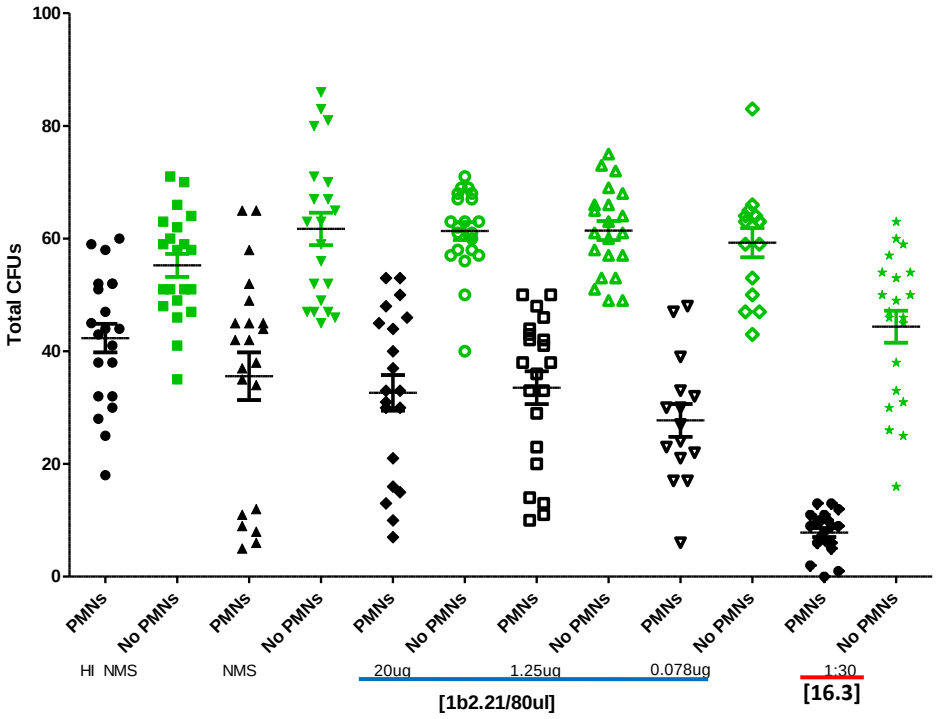
These considerations raise the possibility that anti-PC antibody in the normal serum of BALB/ cByJ mice might be synergizing with the mAb to PspA, and that mAb to PspA may not be protective on its own in the MSKA. This is an important issue because if anti-PC antibody plays an important role, then it would be important to include it in the assay at a standardized concentration.

To address this issue, I have compared the sera from CBA/N and BALB/cByJ mice in the MSKA. Large differences in percent killing with the two complement sources were observed (Figure 17). Complement from BALB/cByJ mice had significantly greater killing activity than complement from CBA/N mice ($p<0.001$) With mAb 16.3, the percent killing with CBA/N and BALB/cByJ NMS were about 38% and 48%, respectively. For anti-PspA mAb 1b2.21, the percent killing with complement from CBA/N and BALB/cByJ were 2% and 18%, respectively. So while the use of the CBA/N mouse serum in place of BALB/c mouse serum had resulted in a slightly lower killing with anti-type 3 mAb, it virtually eliminated detectable killing mediated by mAb to PspA.

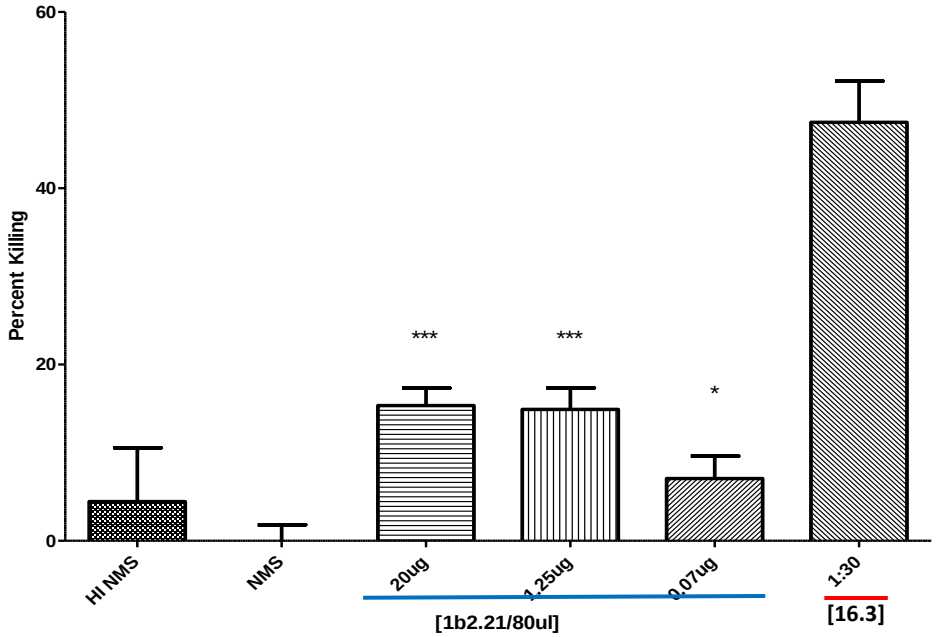
A)


Percent Killing
60
40
20
0
-20
-40
HI NMS
NMS
20ug
1.25ug
0.07ug
1:30
[1b2.21/80ul]
[16.3]

B)


Total CFUs
100
80
60
40
20
0
PMNs
No PMNs
HI NMS
NMS
20ug
1.25ug
0.078ug
1:30
[1b2.21/80ul]
[16.3]

C)



D)

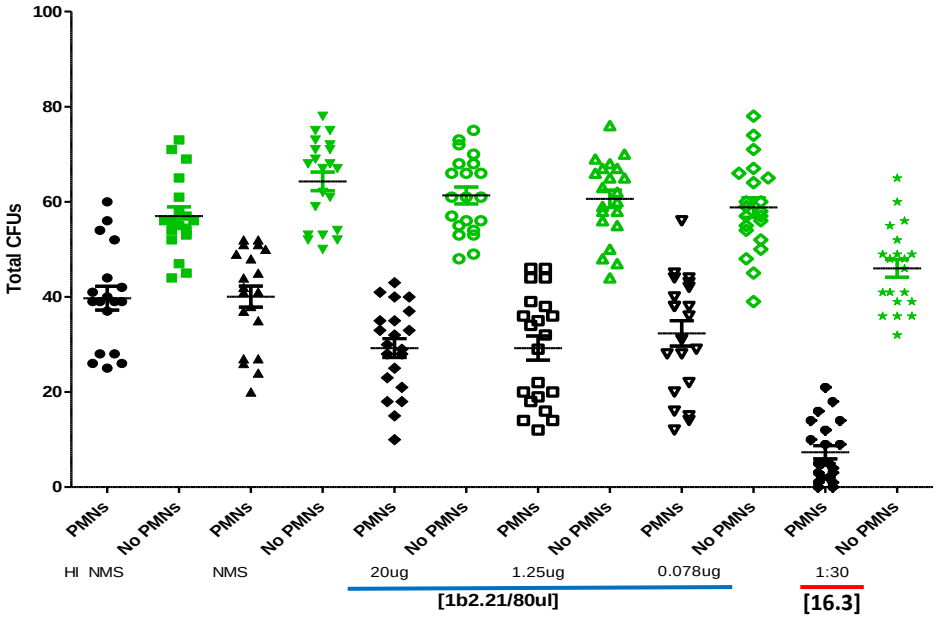


Figure 17: MSKA of WU2 with different complement sources by HL-60 cells. Percent killing and changes in CFU of WU2 strain bacteria with different complement sources. A) Complement from CBA/N mice, B) Complement from CBA/N mice in total CFUs, C) Complement from BALB/cByJ female mice, and D) Complement from BALB/cByJ female mice in total CFU assay. * p value < 0.05, ** p value < 0.01, *** p value < 0.001.

These findings raise the need for three follow-up studies, although they may not be completed during this thesis project. One study is to compare the level of complement activity in the BALB/cByJ and CBA/N NMS. This will allow us to determine if there is less functional complement in the CBA/N versus the BALB/cByJ NMS. The next study will be to use CBA/N and BALB/cByJ sera as complement sources and measure their ability to deposit complement component C3 onto pneumococci in the presence and absence of mAb to PspA. The third study will be to observe if mAb to PC can synergize with mAb to PspA in the MSKA.

DISCUSSION

Immunization of mice and humans with PspA yields antibodies that have been shown to be able to protect mice from infection with pneumococci (10, 30, 32). To use PspA in phase 2 immunogenicity studies in humans, it will be necessary to not just measure antibody to PspA but also have an assay that can measure the protective capacity of the antibodies produced. The only useful surrogate assay for immunity to PspA is passive protection of mice with pre-and post-immune sera (11). That assay is very time consuming and when done properly requires over 100 CBA/N mice ($80 each) to examine the pre- and post-immune sera from a single individual. With this labor intensive technique the mouse assay costs about $10,000 per immunized subject (11).

The data presented here are part of an effort in our laboratory to develop an *in vitro* surrogate assay that can be used in clinical settings. One prior study in our laboratory demonstrated that the MSKA using fresh human PMN could detect a protective effect of antibody to PspA using a single strain of pneumococci (Genshemer and Briles, unpublished data). The present studies have extended those findings by providing several important results: **1)** a second challenge strain (WU2) of pneumococci also worked in the MSKA, **2)** opsonization of bacteria was PspA family-specific, **3)** it was possible to conduct the MSKA with HL-60 cells, **4)** the conditions for use of HL-60 cells in the MSKA were optimized, **5)** an appropriate complement source is critical, and **6)** for anti-PspA antibody to show protection in the MSKA, sub-protective levels of other antibodies may need to be present to synergize with the antibodies to PspA. The latter

finding may mean that the MSKA will be an especially sensitive assay for detecting synergy between immunity to PspA and immunity to other surface proteins on pneumococci.

My observations that HL-60 cells can be used in place of fresh human PMNs is a major breakthrough because it will enable the MSKA to be used on a large-scale to measure the protective levels of antibody in serum from hundreds of patients immunized with different doses of a PspA-containing vaccine. With capsule type 3, we observed that the use of HL-60 cells gave a higher percent killing and a better dose response when the antibody is diluted. With antibody to PspA, we saw little change in the percent killing, but the dose response curve was very shallow. One implication of this is that the assay will be very sensitive to antibody to PspA, but the other implication is that it will require an improvement in reproducibility of the percent killing for it to become quantitative.

The finding that antibody to PspA might require synergy with antibody to PC (or other pneumococcal antibodies) to be effective in the MSKA gives us insight into ways to improve the assay. Although it is known that immunity to PspA can synergize with other antibodies in mice (10), it is also known that passive antibody to PspA is highly protective in CBA/N mice in the absences of any other antibody to pneumococci (32). This may suggest that antibodies to PspA are more effective *in vivo* than in the MSKA and might work by different mechanisms. If this is the case, it would suggest that the MSKA may not be a perfect mimic of the protective activity of antibody to PspA *in vivo*. Thus, it could turn out that the assay is not a perfect *in vivo* surrogate.

Further studies will be required to resolve these issues. At present, our laboratory is comparing the ability of antibodies to protect in the MSKA with their ability to protect in passive transfer studies. A strong correlation would be a good argument that the MSKA will in fact be a valid *in vitro* surrogate assay to measure the protective efficacy of immunity to PspA in humans.

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APPENDIX [IRB APPROVAL FORM]

Project Revision/Amendment Form

Form version: October 28, 2010

In MS Word, click in the white boxes and type your text; double-click checkboxes to check/uncheck.

- **Federal regulations require IRB approval before implementing proposed changes. See Section 14 of the IRB Guidebook for Investigators for additional information.**
- **Change means any change, in content or form, to the protocol, consent form, or any supportive materials (such as the Investigator's Brochure, questionnaires, surveys, advertisements, etc.). See Item 4 for more examples.**

1. Today's Date	5/19/2011

2. Principal Investigator (PI)

Name (with degree)	David E. Briles, Ph.D.	**Blazer ID**	Dbriles
Department	Microbiology	**Division (if applicable)**	
Office Address	BBRB 658 2170	**Office Phone**	934-6595
E-mail	dbriles@uab.edu	**Fax Number**	N/A

Contact person who should receive copies of IRB correspondence (Optional)

Name	Flora Gathof	**E-Mail**	florag@uab.edu
Phone	934-1924	**Fax Number**	N/A
Office Address (if different from PI)			

3. UAB IRB Protocol Identification

3.a. Protocol Number	X080428011
3.b. Protocol Title	PspA: A Potential Pneumococcal Vaccine Component

3.c. Current Status of Protocol—Check ONE box at left; provide numbers and dates where applicable

- ☐ **Study has not yet begun** — **No participants, data, or specimens have been entered.**
- X **In progress, open to accrual** — **Number of participants, data, or specimens entered:** 26
- ☐ **Enrollment temporarily suspended by sponsor**
- ☐ **Closed to accrual, but procedures continue as defined in the protocol (therapy, intervention, follow-up visits, etc.)**
  - **Date closed:**
  - **Number of participants receiving interventions:**
  - **Number of participants in long-term follow-up only:**
- ☐ **Closed to accrual, and only data analysis continues**
  - **Date closed:**
  - **Total number of participants entered:**

4. Types of Change

Check all types of change that apply, and describe the changes in Item 5.c. or 5.d. as applicable. To help avoid delay in IRB review, please ensure that you provide the required materials and/or information for each type of change checked.

- ☐ **Protocol revision (change in the IRB-approved protocol)**
  In Item 5.c., if applicable, provide sponsor's protocol version number, amendment number, update number, etc.
- ☐ **Protocol amendment (addition to the IRB-approved protocol)**
  In Item 5.c., if applicable, provide funding application document from sponsor, as well as sponsor's protocol version number, amendment number, update number, etc.
- X **Add or remove personnel**
  In Item 5.c., include name, title/degree, department/division, institutional affiliation, and role(s) in research, and address whether new personnel have any conflict of interest. See "Change in Principal Investigator" in the IRB Guidebook if the principal investigator is being changed.
  - X **Add graduate student(s) or postdoctoral fellow(s) working toward thesis, dissertation, or publication**
    In Item 5.c., (a) identify these individuals by name; (b) provide the working title of the thesis, dissertation, or publication; and (c) indicate whether or not the student's analysis differs in any way from the purpose of the research described in the IRB-approved HSP (e.g., a secondary analysis of data obtained under this HSP).
- ☐ **Change in source of funding; change or add funding**
  In Item 5.c., describe the change or addition in detail, include the applicable OGCA tracking number(s), and provide a copy of the application as funded (or as submitted to the sponsor if pending). Note that some changes in funding may require a new IRB application.
- ☐ **Add or remove performance sites**
  In Item 5.c., identify the site and location, and describe the research-related procedures performed there. If adding site(s), attach notification of permission or IRB approval to perform research there. Also include copy of subcontract, if applicable. If this protocol includes acting as the Coordinating Center for a study, attach IRB approval from any non-UAB site added.

☐ **Add or change a genetic component or storage of samples and/or data component—this could include data submissions for Genome-Wide Association Studies (GWAS)**
To assist you in revising or preparing your submission, please see the IRB Guidebook for Investigators or call the IRB office at 934-3789.

☐ **Suspend, re-open, or permanently close protocol to accrual of individuals, data, or samples (IRB approval to remain active)**
In Item 5.c., indicate the action, provide applicable dates and reasons for action; attach supporting documentation.

☐ **Report being forwarded to IRB (e.g., DSMB, sponsor or other monitor)**
In Item 5.c., include date and source of report, summarize findings, and indicate any recommendations.

☐ **Revise or amend consent, assent form(s)**
Complete Item 5.d.

☐ **Addendum (new) consent form**
Complete Item 5.d.

☐ **Add or revise recruitment materials**
Complete Item 5.d.

☐ **Other (e.g., investigator brochure)**
Indicate the type of change in the space below, and provide details in Item 5.c. or 5.d. as applicable.
Include a copy of all affected documents, with revisions highlighted as applicable.

5. Description and Rationale

In Item 5.a. and 5.b, check Yes or No and see instructions for Yes responses.
In Item 5.c. and 5.d, describe—and explain the reason for—the change(s) noted in Item 4.

☐Yes ☐No **5.a. Are any of the participants enrolled as normal, healthy controls?**
If yes, describe in detail in Item 5.c. how this change will affect those participants.

☐Yes ☐No **5.b. Does the change affect subject participation, such as procedures, risks, costs, location of services, etc.?**
If yes, FAP-designated units complete a FAP submission and send to fap@uab.edu. Identify the FAP-designated unit in Item 5.c.
For more details on the UAB FAP, see www.uab.edu/cto.

5.c. Protocol Changes: In the space below, briefly describe—and explain the reason for—all change(s) to the protocol.

Addition of Master Degree Student to protocol.

(A) Eunlim Kim ✓ Added in SIRB.
(B) Thesis title "Modified Surface Killing Assay to Measure the Ability of Monoclonal Antibodies to PspA to Mediate Killing of Streptococcus pneumoniae"
(C) This student's work and analysis does not differ in any way from the purpose of the research described in the IRB-approved HSP.

Description of studies for Eunlim Kim:

Eumlim Kim is a Masters Graduate Student who will be working on the purification of human neutrophils from whole blood received from human donors. These purified neutrophils will be utilized in an in vitro surrogate assay that is being developed to predict the ability of monoclonal antibodies to passively protect mice. The assay entails the opsonization of the target bacteria with the antibody in question and normal mouse serum as the source of complement. The bacteria are then spotted on a blood agar plate and the purified human neutrophils are then overlayed on top of half of the spots and incubated over night. The assay will detect how the presence of the monoclonal modifies the ability of the human neutrophils to "kill" the bacteria on the plate.

There is no conflict of interest for this student.

This student is being added to the protocol because she is using cells from the blood samples that will be obtained from volunteers.

5.d. Consent and Recruitment Changes: In the space below,
(a) describe all changes to IRB-approved forms or recruitment materials and the reasons for them;
(b) describe the reasons for the addition of any materials (e.g., addendum consent, recruitment); and
(c) indicate either how and when you will reconsent enrolled participants or why reconsenting is not necessary (not applicable for recruitment materials).

Also, indicate the number of forms changed or added. For new forms, provide 1 copy. For revised documents, provide 3 copies:
- **a copy of the currently approved document (showing the IRB approval stamp, if applicable)**
- **a revised copy highlighting all proposed changes with "tracked" changes**
- **a revised copy for the IRB approval stamp.**

▶

Signature of Principal Investigator David L Brilliant Date 7/12/11

FOR IRB USE ONLY

☐ Received & Noted ☑ Approved Expedited* ☐ To Convened IRB

Marilyn Davis 7-13-11
Signature (Chair, Vice-Chair, Designee) Date

DOLA 4-13-11

Change to Expedited Category **Y / N / NA**

*No change to IRB's previous determination of approval criteria at 45 CFR 46.111 or 21 CFR 56.111

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