PLATELET AND ERYTHROCYTE PHAGOCYTOSIS KINETICS ARE DIFFERENTIALLY CONTROLLED BY PHOSPHATASE ACTIVITY WITHIN MONONUCLEAR CELLS.[a]

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Running Title: Kinetics of platelet and RBC phagocytosis.

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Background: **Anti-D treatment is effective in raising platelet counts in patients with autoimmune thrombocytopenic purpura (AITP), however, the exact mechanism of action is unknown. Previous results have suggested that anti-D coated erythrocytes affect RES phagocytosis by stimulating agents (e.g. reactive oxygen species) that alter signaling pathways within the phagocyte. To address this, we used a flow cytometric assay to compare the kinetics and signaling pathways responsible for opsonized platelet and RBC phagocytosis.**

Study Design and Methods: **Human platelets or RBC were labeled with the fluorescent dye CM-Green, opsonized with Rh immune globulin or anti-MHC respectively and incubated with THP-1 monocytes with or without signal transduction inhibitors and intracellular fluorescence was analyzed.**

Results: **Compared with opsonized platelets, phagocytosis of opsonized RBC was significantly slower ($p<0.0001$) and within 2h, induced a state of phagocytic refractoriness; resting the mononuclear cells for up to 24h did not rescue their ability to further mediate platelet phagocytosis. Inhibitors of phosphatidylinositol 3-kinase (wortmannin, LY294002, myricetin and quercetin), protein kinase C (staurosporine) and Syk kinase (piceatannol) inhibited both opsonized RBC and platelet phagocytosis. In contrast, opsonized RBC phagocytosis was significantly ($p<0.0001$) enhanced by the tyrosine phosphatase inhibitor phenyl arsine oxide (PAO) whereas platelet phagocytosis was significantly reduced ($p<0.0001$). Of interest, phosphatase inhibition during opsonized RBC phagocytosis induced a longer (48h) phagocytic refractoriness period in the mononuclear cells.**

Conclusion: **These results suggest that the early kinetics and signaling events related to phosphatase activity regulate how mononuclear phagocytes engulf opsonized RBC and induce phagocytic refractoriness for further platelet phagocytosis.**

INTRODUCTION

Autoimmune thrombocytopenic purpura (AITP) is an immune-mediated bleeding disorder in which platelets are opsonized by autoantibodies and prematurely destroyed by Fc receptor (R)-mediated phagocytosis by the reticuloendothelial system (RES) within the spleen.[1,2] Treatment options for chronic AITP include corticosteroids, intravenous gammaglobulin and splenectomy, and if these fail to raise platelet counts, more potent immunosuppressive compounds such as azathioprine may be used.[1,2] Anti-D has also been shown to raise the platelet counts of RhD positive patients with autoimmune thrombocytopenic purpura (AITP),[3,4] however, little is known about the mechanisms of action of anti-D, although it has been suggested that it opsonizes D+ red blood cells (RBC) and blocks platelet destruction by adhering to the Fc receptors (FcR) of leukocytes.[3,4] Alternatively, it has been suggested that anti-D may cause immunosuppression by interacting with Fcγ receptors (R) on B lymphocytes and perhaps stimulating production of immunosuppressive cytokines.[5] Related to the latter hypothesis, three groups have demonstrated that within hours after anti-D administration to patients with chronic AITP, there was a significant, but transient increase in the plasma levels of several cytokines including IL1 receptor antagonist (ra), IL-6 and TNF-α.[6-9] These in vivo data were subsequently confirmed in vitro where it was shown that anti-D-opsonized RBC incubated with cells of the RES stimulated an early respiratory burst followed by the production of IL1ra that halted opsonized-RBC phagocytosis.[10] It was suggested that the early RBC-induced reactive oxygen species production may have played a role in modifying intracellular signaling related to RBC phagocytosis.[10]

Phagocytosis of antibody opsonized particles is primarily mediated by the actin-based cytoskeleton and is a dynamic process involving a complex mix of proteins including actin, the Arp2/3 complex, Rho-family GTPases, filament-capping proteins, tropomyosin, Rho kinase and myosin II.[11-12] For example, engagement of particle-bound IgG by Fc receptors (R) causes FcR aggregation and recruitment of cytosolic tyrosine kinases, particularly Syk.[13] The onset of uptake is

accompanied by protein phosphorylation and the concentration of several proteins at the phagocytic cup and correlated with the accumulation of actin filaments.[13,14] These events are counteracted by the inhibition of phosphorylation by, for example, phosphatase activity leading to a complete halt of phagocytosis.[15] Subsequently, during pseudopod extension around the opsonized particle, phosphatidylinositol 3-kinase (PI3K) is recruited to the plasma membrane, triggering the exocytosis of internal stores of membranes for the growing pseudopod extensions.[15-17] Ultimately, psuedopod extension leads to engulfment of the particle and formation of the phagosome for particle destruction.[17] With regards to the phagocytosis of opsonized RBC, some of the above processes have been confirmed.[18-20] On the other hand, it is not known if these processes are also responsible for the phagocytosis of platelets although a previous report suggested that platelet phagocytosis by macrophages within atherosclerotic lesions was associated with novel processing and signaling pathways related to β-amyloid precursor protein.[21]

The current study was designed to compare the kinetics and intracellular signaling mechanisms associated with opsonized platelet and RBC phagocytosis. Our results demonstrate that phagocytosis of opsonized RBC and platelets by mononuclear cells differ in kinetics and early signaling events related to phosphatase activity. More importantly, anti-D opsonized RBC induced a state of phagocytic refractoriness in mononuclear cells against further platelet phagocytosis. The results suggest a potential mechanism of action of how anti-D opsonized RBC may affect opsonized platelet phagocytosis and may form a basis of designing novel therapies to prevent platelet phagocytosis in diseases such as AITP.

MATERIALS AND METHODS

Preparation of platelets and RBC: Peripheral blood was obtained by venipuncture (Citrated Venutubes, BD) from Rh positive healthy laboratory volunteers under approval of the St. Michael's Hospital REB. Platelets were prepared from the blood using the PRP method. Briefly, blood was centrifuged at 120g for 15 minutes and the PRP aspirated off. Platelets were washed once in 1.0 % (vol/vol) EDTA/phosphate-buffered saline (PBS) containing 5 uM prostaglandin E1 (160 ng/ml final) to minimize activation. Enriched RBC were obtained by overlaying blood on a 1.091 g/ml Percoll cushion (Amersham Biosciences, Sweden) and centrifuging it at 1660xg for 30 minutes at room temperature. The sedimented erythrocytes were washed six times in PBS.

Cell Line: THP-1 is a human cell line obtained from the American Tissue Culture Collection (ATCC# TIB-202) and maintained in RPMI-1640 with 5% fetal calf serum (FCS), 100 µg/mL Penicillin/ Streptomycin/Fungizone, 100 mM L-glutamine and 5 x 10^{-5} M 2-mercaptoethanol (cRPMI). THP-1 was derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia. They express FcR and C3b receptors, lack surface and cytoplasmic immunoglobulins and stain positive for alpha-naphthyl butyrate esterase.[22] They produce lysozyme, are phagocytic (for both latex beads and sensitized erythrocytes) and can restore the response of purified T lymphocytes to Con A.[22]

Antibodies and Reagents: Rh immune globulin (Anti-D; WinRho SDF, Cangene Corporation, Winnipeg, MB) was used to opsonize RBC. High titred human pooled sera (N=20) that was polyspecific in a 30 panel LCT assay (anti-HLA) was used to opsonize platelets and was obtained from Dr. B Hannach (Canadian Blood Services, Toronto Center). The selective PIP3K inhibitors Wortmannin and LY294002 and the broad-spectrum PKC inhibitors Myricetin, Quercetin dihydrate and Staurosporine were obtained from the Sigma Chemical Co. (St. Louis, MO). Phenyl arsine oxide

(PAO), an inhibitor of tyrosine phosphatases and the Syk kinase inhibitor piceatannol were also from Sigma.

IgG and F(ab')$_2$ preparation: F(ab')$_2$ fragments were prepared from the anti-D and anti-HLA as previously described.[23] Briefly, IgG was precipitated with 50% saturated ammonium sulphate, dialysed against 50 mM Tris-saline, pH 8.0, and adsorbed on QAE-Sephadex A-50 to remove albumin. The IgG (1-3% w/v) was dialysed against 0.2 M sodium acetate, pH 4.5 and digested with 2% (w/w) pepsin (Sigma, St. Louis, MO) for 24 h at 37^{o}C. F(ab')$_2$ fragments were then purified by Sephadex G150 gel filtration and Protein G-Sepharose adsorption. F(ab')$_2$ purity was determined by HPLC analysis and typically >96%. Binding of the anti-D and anti-HLA F(ab')$_2$ fragments was tested by flow cytometry using a FITC-labeled goat anti-human IgG (H+L chain specific, #GTX72737, GeneTex, San Antonio, TX); both fragments bound to their respective targets in an equivalent manner to their whole immunoglobulin counterparts.

Fluorescent labeling and opsonization of platelets and RBC: RBC ($5x10^{10}$ in 5 ml) were incubated with an equivalent volume of 20 uM CellTracker Green CMFDA (CM-Green; Molecular Probes, Eugene, OR.) for 2 h at 37°C. The RBC were centrifuged at 120xg and the CM-Green-containing solution was replaced with 10 ml PBS and further incubated for 1 h to allow attachment of CM-Green to thiol-containing proteins and conversion to its fluorescent conjugate. Labeled RBC were washed six times in PBS. To fluorescently label platelets, the same procedure was used except that 5 uM prostaglandin E1 was added to the buffer to reduce platelet activation. In an attempt to standardize antibody opsonization, both antibody preparations (anti-D and anti-HLA) were titred on RBC or platelets respectively and equivalent fluorescence was determined by flow cytometry using a FITC-labeled goat anti-human IgG (H+L chain specific, GeneTex); the amounts of IgG that showed equivalent fluorescence were used. Based on the titration studies, opsonization of CM-Green labeled RBC was performed by incubating $3x10^{10}$ RBC in 3 ml PBS with 5 μg/ml (final concentration) of

anti-D for 1 hour at room temperature. Opsonization of 10^9 platelets in 1ml was performed with a 1:100 dilution of anti-HLA sera. A similar protocol was used to coat RBC with F(ab)'$_2$ fragments of anti-D or anti-HLA.

Phagocytosis Assay: Phagocytosis of platelets and RBC by THP-1 cells was measured by flow cytometry as previous described.[24] Briefly, 10^6 THP-1 cells were first incubated with 1.62 x 10^{-5} uM PMA (Sigma) for 15 minutes at room temperature. Non-opsonized- or opsonized-CM-Green-labeled platelets or RBC were then added to 5 x 10^5 THP cells (final ratios were 20:1 RBC:THP; 5:1 Platelet:THP based on a previous method[25]) in 0.1 ml PBS for 2 hours. One duplicate tube was incubated on ice (0^{o}C) while the other was incubated at 37^{o}C to allow phagocytosis to proceed. The cells were washed in PBS, quenched with 100 ul 0.1% trypan blue in PBS for 20 min and washed once. The cells were resuspended in DNA stain LDS-751 and were acquired by a FACSort flow cytometer (Becton Dickinson, San Jose, CA) equipped with an Argon laser operating at 15mW; 10,000 events were acquired through a "live" gate drawn on the FL3 nucleated cells. Platelet or RBC phagocytosis was determined by comparing the intracellular FL1 fluorescence between the tubes incubated at 0^{o}C and 37^{o}C. Phagocytic index was calculated by the formula: median fluorescence at 37^{o}C / median fluorescence at 0^{o}C. Where indicated, titrations of the inhibitors were added to the THP-1 cells 60 min prior to the phagocytosis assay. This type of flow cytometric assay has been validated for both opsonized platelets and RBC by several groups and accurately assesses true intracellular engulfment.[26-29]

Testing phagocytic refractoriness: To determine whether exposure of THP-1 cells to either opsonized platelets or RBC caused long-term effects on opsonized platelet phagocytosis, 5 x 10^7 THP-1 cells were first exposed to non-labelled but opsonized platelets (2.5x10^8) or RBC (1x10^9) in the presence or absence of the indicated signaling inhibitors for two hours as above. The opsonized platelets or RBC were then removed by washing twice or adding acid-citrate-dextrose (ACD) lysis

buffer respectively and then washing twice. The lysis step removed 99.9% of the initial RBC as determined by flow cytometry. These levels of residual RBC have been determine to not inhibit opsonized platelet phagocytosis.[25] The THP-1 cells were then harvested and used in the phagocytosis assay to determine their ability to mediate opsonized platelet phagocytosis.

RESULTS

When fluorescently-labeled opsonized platelets or RBC were incubated with THP-1 cells for 2 hours, THP-1 cell intracellular fluorescence for both anti-HLA opsonized platelets and anti-D opsonized RBC was observed (Figures 1A and 1B). The phagocytosis was Fc dependent since $F(ab')_2$-bound RBC or platelets were not engulfed (Figure 1C). Compared with opsonized platelets, however, the kinetics of phagocytosis of opsonized RBC were significantly slower ($p<0.0001$); opsonized platelets induced higher levels of intracellular THP-1 fluorescence within 30 minutes whereas RBC phagocytosis was not observed until 2 hours (Figure 2).

To determine whether opsonized platelets or RBC affected THP-1-mediated phagocytosis over time, THP-1 cells were first incubated with non-labelled but opsonized platelets or RBC and then rested for various times to determine if they could still mediate the phagocytosis of opsonized platelets. Opsonized platelet exposure for 2h before resting did not change the ability of THP-1 cells to subsequently mediate phagocytosis of opsonized platelets after 24h or 48 h (Figure 3). In contrast, exposure of the THP-1 cells to opsonized RBC significantly ($p<0.0002$) inhibited the mononuclear cell's ability to subsequently mediate platelet phagocytosis (Figure 3). The effect was transient, however, because by 48 hours rest, THP-1 cells could mediate opsonized platelet phagocytosis (Figure 3).

To address the intracellular signaling events related to opsonized platelet and RBC phagocytosis, THP-1 cells were incubated with various inhibitors of signaling. The selective (Wortmannin and LY294002) and non-selective (Quercetin and Myricetin) inhibitors of PI3K reduced both opsonized platelet and RBC phagocytosis (Figure 4A-D). Similar results were also observed when Staurosporine, a potent inhibitor of PKC, or Piceatannol, and inhibitor of Syk kinases, were added to the phagocytosis assay (Figure 4E-F). On the other hand, PAO, an inhibitor of tyrosine phosphatases significantly ($P<0.0001$) enhanced opsonized RBC phagocytosis while significantly ($p<0.0001$)

inhibiting opsonized platelet phagocytosis (Figure 5). To determine if the enhanced RBC effect affected phagocytic refractoriness, opsonized RBC or platelets were added to the THP-1 cells in the presence of PAO during the initial 2h incubation and the THP-1 cells were rested. Compared with opsonized platelets, PAO and opsonized RBC significantly ($p<0.0001$) prolonged the anti-platelet phagocytic refractoriness period to 48h (Figure 6).

DISCUSSION

Although anti-D is effective in raising the platelet counts in patients with AITP, little is known about its exact mechanism of action. Previous results have demonstrated that anti-D-opsonized RBC stimulated an early (within 10 min) respiratory burst in mononuclear phagocytes followed by a reduction in RBC phagocytosis due to inhibitory cytokines (e.g. IL1-receptor antagonist) suggesting that the ROS may have modified intracellular signaling pathways.[8] This study was designed to compare the ability of THP-1 cells to mediate opsonized platelet and RBC phagocytosis. Our data suggests that opsonized RBC phagocytosis occurs at a slower rate than opsonized platelet phagocytosis and the opsonized RBC can induce a transient phagocytic refractoriness in mononuclear cells against further platelet phagocytosis. These events appear related to intracellular phosphatase activity.

The greater ability of THP-1 cells to mediate phagocytosis of opsonized platelets compared with RBC was of interest since it is thought that anti-D's mechanism of action in raising platelet counts in patients with AITP is due to interference with platelet phagocytosis.[1] We observed that the rate of opsonized RBC phagocytosis was significantly lower than opsonized platelet phagocytosis (Figures 1 and 2). The reasons for this may be related to multiple factors. For example, the larger size of the RBC may be at least one determining factor for the lower ability of the THP-1 cells to engulf opsonized RBC since several reports have demonstrated that FcR-mediated signaling and membrane movement patterns vary with the size of the IgG particle being ingested.[17] In addition, the anti-HLA used to opsonized the platelets may have had a higher density than anti-D on RBC (even though we attempted to equilibrate the binding of the two polyclonal antibodies and used lower ratios of platelets in the assay) and this may have lead to more platelet-associated IgG to interact with Fc receptors on the THP-1 cells. Furthermore, the mobility of the D antigen on RBC compared with adsorbed HLA on platelets may have influenced the kinetics. Nonetheless, these reasons do not

totally account for the increased opsonized platelet phagocytosis because we observed platelet phagocytosis at lower platelet:THP-1 ratios or lower anti-HLA antibody titres (ratios and titres where RBC engulfment was not observed, not shown). What was more striking was that opsonized RBC, in contrast to platelets, appeared to induce a transient refractory state in THP-1's ability for further phagocytosis of opsonized platelets (Figure 3). This result may be related to recent observations that anti-D opsonized RBC can directly inhibit opsonized platelet phagocytosis when the two are mixed in vitro with THP-1 cells.[25] The current data further suggest that anti-D opsonized RBC may actively inhibit THP-1 cells from further phagocytosis even after they are no longer present. The mechanisms related to this induction of refractoriness are unknown, however, there are studies to suggest that RBC and platelets contain several surface molecules that can affect macrophage function. For example, Oldenborg et al[30] have demonstrated that RBC express CD47 that binds a molecule on the macrophage surface called signal regulatory protein α (SIRPα). This interaction can potently inhibit macrophage activation and phagocytosis via phosphorylation of SIRPα's intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) and recruitment of tyrosine phosphatases such as SHP-1 and SHP-2.[30-32] SHP-1 and SHP-2 in turn, interrupt signaling from tyrosine kinase–dependent receptors.[30-32] On the other hand, platelets also express CD47,[16] but in addition, express counteracting molecules that have been shown to significantly activate macrophages and dendritic cells.[33-36] Perhaps the balance of these types of molecular interactions determine the rate of phagocytosis for opsonized platelets and RBC. We are currently studying the effects of these molecules on opsonized RBC and platelet phagocytosis.

The transient nature of opsonized RBC inhibition of monocyte phagocytosis led us to examine how RBC and platelet phagocytosis was affected by signaling pathways. Various inhibitors of PI3K, PKC and phosphatases were utilized. The results suggest for both opsonized platelet and RBC phagocytosis, THP-1 cells utilize similar phagocytic signaling pathways with respect to late PI3K and

PKC events (Figure 4). In contrast, however, the most striking difference between platelet and RBC phagocytosis was the differential effect of PAO, an inhibitor of tyrosine phosphatases (Figure 5). Phosphatases remove phosphate groups from amino acids and this process has generally been shown to be associated with an inhibition of phagocytosis via reduction in phosphorylation of FcR signaling molecules.[15-17] In addition, several reports have demonstrated that the balance of kinase and phosphatase activity within the macrophage and neutrophils is critical for differentially regulating the cells ability to mediate phagocytosis.[37-41] PAO significantly increased the rate of RBC phagocytosis by THP-1 cells and this was associated with a prolonged platelet phagocytic refractory period in the cells (Figure 6) The reasons for these observations are not known but suggest that early tyrosine phosphatase activity during RBC phagocytosis is associated with an induction of mononuclear cell refractoriness against opsonized platelet phagocytosis. We are currently studying whether opsonized RBC can enhance the activity of these types of phosphatases within the macrophage and affect opsonized platelet phagocytosis. If this is true, it may, in part, explain how anti-D-opsonized RBC affect platelet phagocytosis in AITP.

In summary, our results suggest that opsonized platelet and RBC phagocytosis by mononuclear cells significantly differ in their kinetics of uptake and early phosphatase utilization and this be related to the ability of anti-D opsonized RBC to induce a state of mononuclear cell refractoriness against further platelet phagocytosis. Thus, targeting early FcR-related phosphorylation/dephosphorylation reactions within mononuclear phagocytes may be a therapeutic modality for reducing platelet phagocytosis in AITP.

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FIGURE LEGENDS

Figure 1: Flow cytometric histogram analysis of THP-1 intracellular fluorescence of **(A)** anti-HLA-opsonized platelets at 0°C (——) and 37°C (▬▬), **(B)** anti-D-opsonized RBC at 4°C (——) and 37°C, (▬▬) and **(C)** anti-HLA $F(ab')_2$ bound platelets (——) and anti-D $F(ab')_2$ bound RBC (▬▬). Typical histograms are shown. A histogram shift to the right indicates intracellular fluorescence within THP-1 cells. The assay was performed at 0°C to distinguish opsonized platelet and RBC adherence from internalization (phagocytosis).

Figure 2: Kinetics of opsonized platelet (●) and RBC (■) phagocytosis and non-opsonized platelets (□) or RBC (○) by THP-1 mononuclear cells. Labelled and opsonized platelets or RBC were added to the phagocytosis assay for the indicated times and intracellular fluorescence was analyzed. The data are expressed as the mean fluorescence intensity ($\pm$95% confidence interval) from 10 independent experiments. The confidence interval was calculated by the formula: 2.36xSD/sq.root of N. Statistical significance between platelet and RBC phagocytosis was calculated by a one way analysis of variance (ANOVA) and was $p<0.0001$.

Figure 3: Induction of refractoriness to phagocytosis of opsonized platelets. THP-1 cells were first pre-incubated with either non-opsonized RBC (□), anti-D opsonized RBC (■), non-opsonized platelets (○) or anti-HLA opsonized platelet (●) for 2h at 37°C and then platelets were washed out or RBC were lysed. The THP-1 cells were then rested for the indicated time periods and were then used to test their ability to mediated opsonized platelet phagocytosis. The results are expressed as mean phagocytic index ($\pm$95% confidence interval) calculated by the formula: median fluorescence at 37°C / median fluorescence at 0°C from 8 independent experiments. Statistical significance (p values) between THP-1 cells pre-incubated with either opsonized platelets (●) or RBC (■) at 24h was calculated by an unpaired t test and was $p=0.0002$. There was no significance after the 48h rest period.

Figure 4: Summary of the effects of various concentrations of (A) wortmannin, (B) LY294002, (C) Quercetin, (D) Myricetin, (E) staurosporine and (F) piceatannol on THP-1 phagocytosis of opsonized platelets (open bars) or RBC (solid bars). Data is expressed as Percent Control Phagocytosis (±SD) and was calculated by the following formula: median channel fluorescence with inhibitor / median channel fluorescence without inhibitor x 100 from 10 independent experiments.

Figure 5: Summary of the effects of various concentrations of PAO on THP-1 mononuclear cell phagocytosis of opsonized platelets (open bars) or RBC (solid bars). Data is expressed as Percent Control Phagocytosis (±SD) and was calculated by the following formula: median channel fluorescence with inhibitor / median channel fluorescence without inhibitor x 100 from 7 independent experiments. Statistical significance between the 0 columns and columns with inhibitor present for both platelet and RBC phagocytosis was calculated by a one way analysis of variance (ANOVA) and was found to be $p<0.0001$ for both RBC and platelets.

Figure 6: Effect of the tyrosine phosphatase inhibitor PAO on the ability of anti-D opsonized RBC (□) or platelets (○) to induce refractoriness to further phagocytosis of opsonised platelets. THP-1 cells were incubated with anti-D opsonized RBC or anti-HLA opsonized platelets with 10 μM PAO for 2h and then rested for the indicated times and compared for their ability to mediated opsonized platelet phagocytosis. The data are expressed as mean phagocytic index (±95% confidence interval) as calculated in Figure 2 from 5 independent experiments. Statistical significance (p values) between the two groups was calculated by an unpaired t test and found to be $p=.0007$for 24h and $p=0.0001$ for 48h.

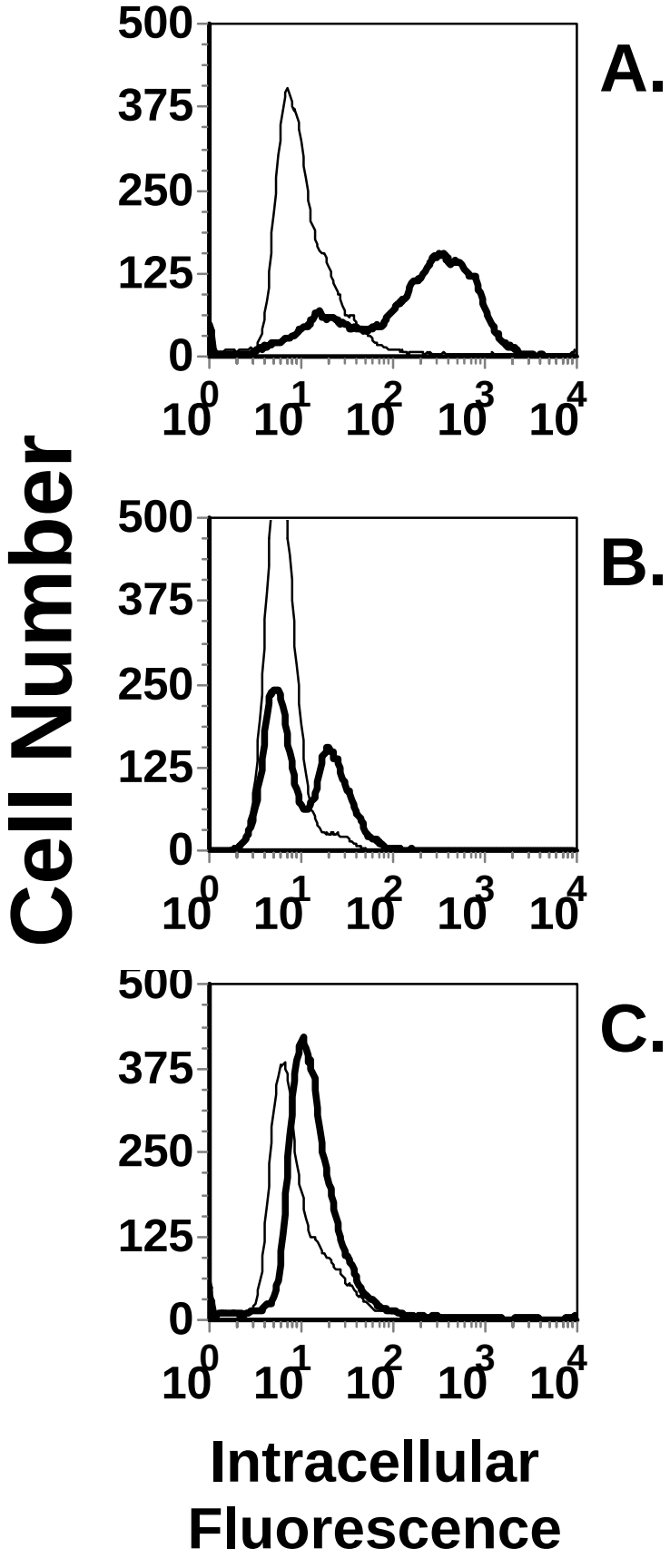
A.
B.
C.
Cell Number
500
375
250
125
0
10^0 10^1 10^2 10^3 10^4
Intracellular
Fluorescence

Figure 1

FI

Figure 2

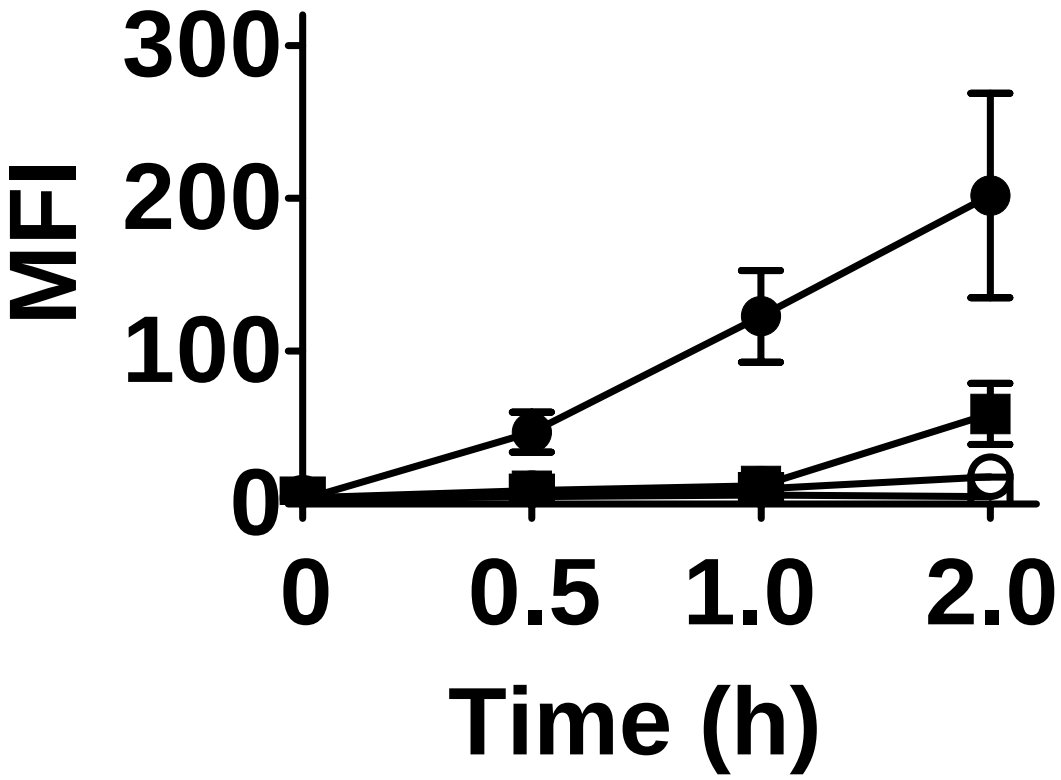

Figure 3

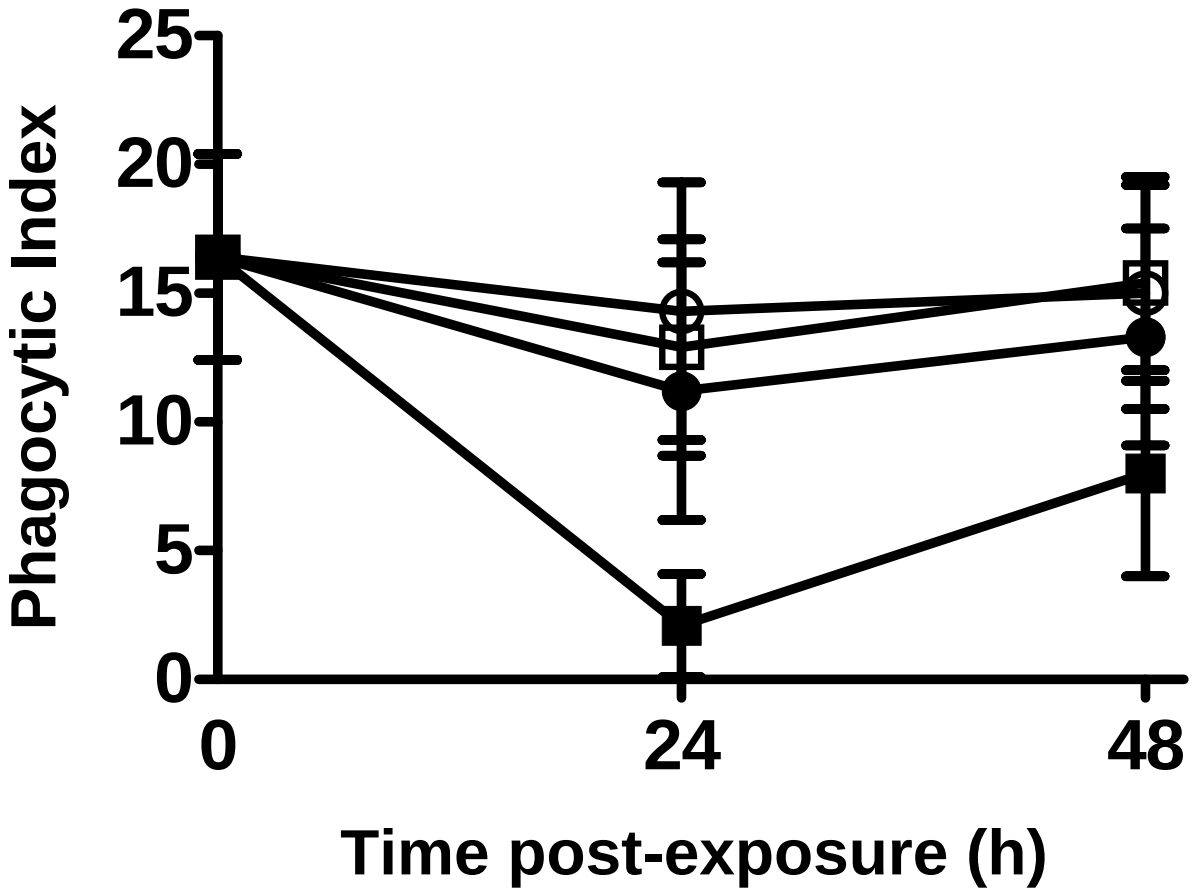

Figure 4

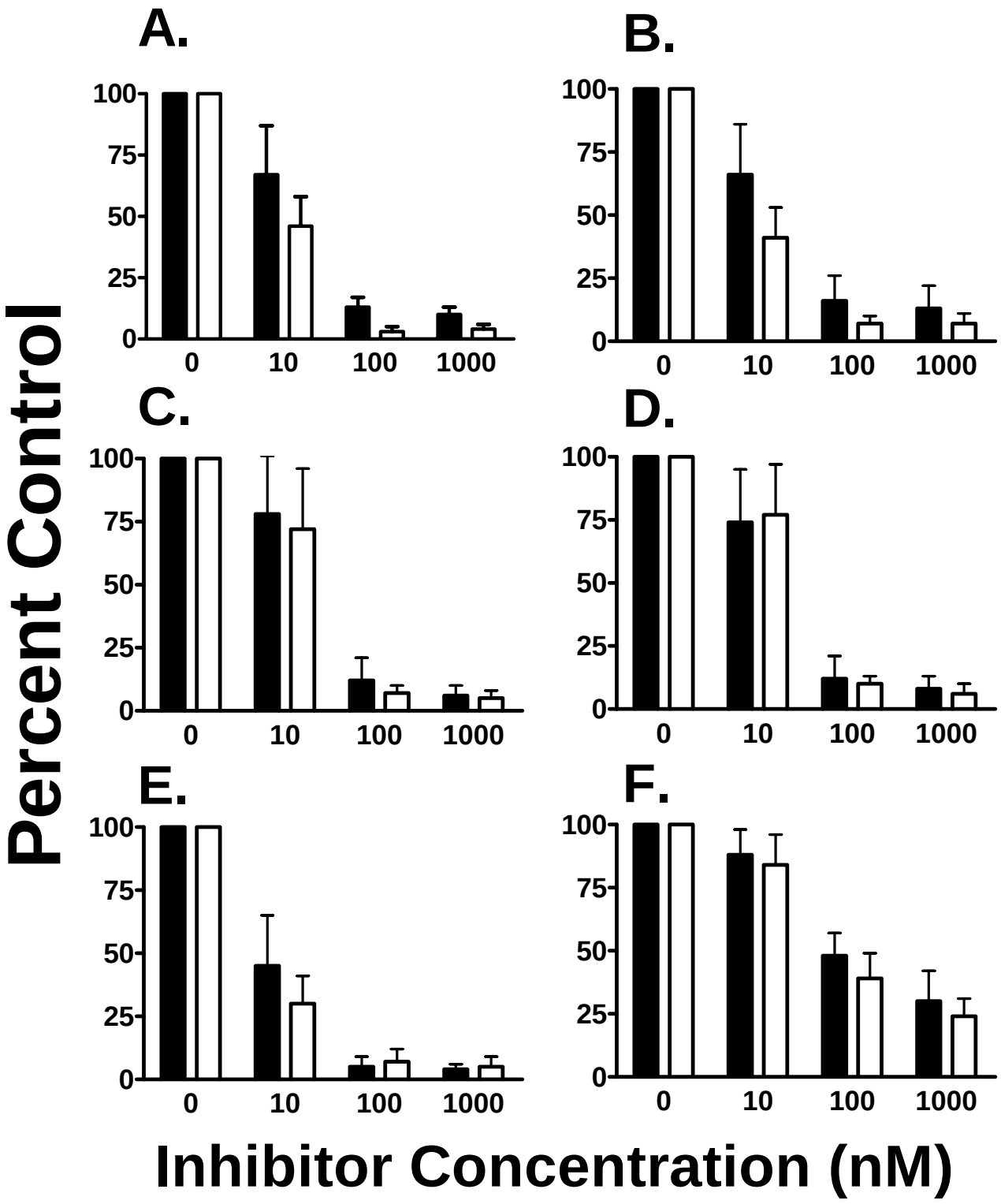
A.
B.
C.
D.
E.
F.
100
75
50
25
0
0
10
100
1000
Percent Control
Inhibitor Concentration (nM)

Figure 5

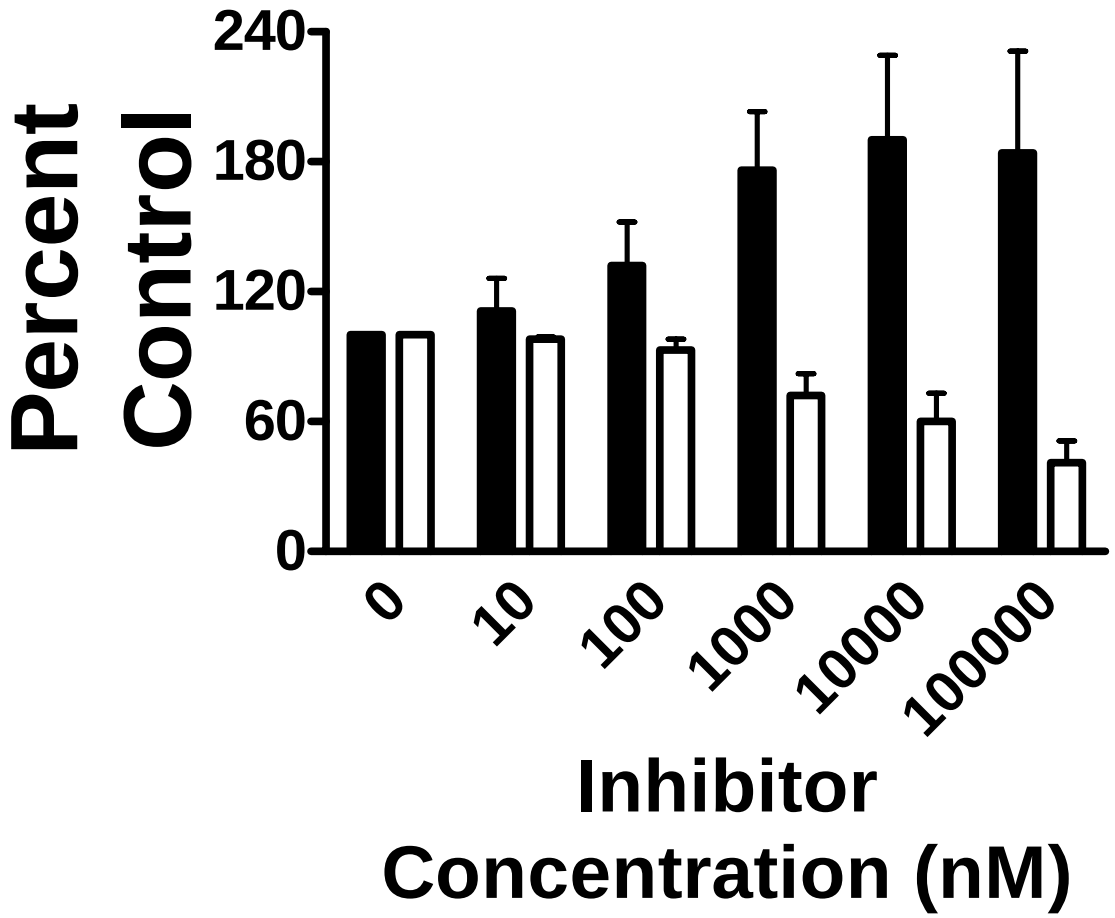
Percent Control
240
180
120
60
0
0
10
100
1000
10000
100000
Inhibitor Concentration (nM)

Figure 6