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TITLE PAGE

PECAM targeted oxidant-resistant mutant thrombomodulin fusion protein with enhanced potency *in vitro* and *vivo*

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RUNNING TITLE PAGE

Running title: Targeted TM generates APC under oxidative conditions

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Non-Standard Abbreviations: APC, Activated Protein C; APTT, Activated Partial Thromboplastin Time; BSA, Bovine Serum Albumin; DMEM, Dulbecco's Modified Eagle Medium; ELISA, Enzyme Linked Immunosorbent Assay; FBS, Fetal Bovine Serum; HRP, Horse Radish Peroxidase; MPO, Myeloperoxidase; MS1, Mouse Pancreatic Islet Endothelial Cells; NaI, Sodium Iodide; OPD, Ortho-phenilen diamine; PBS, Phosphate Buffered Saline; PC, Protein C; PECAM, Platelet Endothelial Cell Adhesion Molecule; P/S, Penicillin and Streptomycin; REN, Human Mesothelioma cell line; scFv, single chain variable fragment; sTM, soluble thrombomodulin; Thrombomodulin, TM; WT, Wild-type.

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ABSTRACT

Thrombomodulin (TM) is a glycoprotein normally present in the membrane of endothelial cells that binds thrombin and changes its substrate specificity to produce activated protein C (APC) that has anti-thrombotic and anti-inflammatory features. To compensate for loss of endogenous TM in pathology, we have fused recombinant TM with scFv fragment of an antibody to mouse platelet endothelial cell adhesion molecule-1 (PECAM). This fusion, anti-PECAM scFv/TM, anchors on the endothelium, stimulates APC production and provides therapeutic benefits superior to sTM in animal models of acute thrombosis and inflammation. However, in conditions of oxidative stress typical of vascular inflammation, TM is inactivated via oxidation of the methionine 388 (M388) residue. Capitalizing on the reports that M388L mutation renders TM resistant to oxidative inactivation, in this study we designed a mutant anti-PECAM scFv/TM M388L. This mutant has the same APC-producing capacity and binding to target cells, yet, in contrast to wild type fusion, it retains APC-producing activity in an oxidizing environment *in vitro* and *in vivo*. Therefore, oxidant resistant mutant anti-PECAM scFv/TM M388L is a preferable targeted biotherapeutic to compensate for loss of anti-thrombotic and anti-inflammatory TM functions in the context of vascular oxidative stress.

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INTRODUCTION

Thrombomodulin (TM) is a glycoprotein present in the membrane of endothelial cells that regulates the coagulation pathway by modifying the action of thrombin (Esmon CT 2005; Weiler H et al., 2003; Ding BS et al., 2009; Glaser CB et al., 1992 and Ware LB et al., 2003). As a cofactor for thrombin-catalyzed activation of protein C, TM enhances the reaction rate by > 1,000-fold. The resultant serine protease, activated protein C (APC) inactivates factors Va and VIIIa, thus inhibiting coagulation by blocking further thrombin generation and exerts anti-inflammatory features (Esmon CT 2005; Weiler H et al., 2003 and Mosnier LO et al., 2007).

Endothelial surface density and activity of endogenous TM are suppressed under many pathological conditions (Aird WC 2003 and Lentz SR et al., 2003). In order to alleviate the negative consequences of this abnormality, recombinant soluble TM (sTM) is being proposed and clinically tested as a replacement therapy (van Irsel T et al., 2011 and Ikezoe T et al., 2012). However, the therapeutic effects and utility of sTM are limited by elimination from plasma (Zaitsev S et al., 2012 and Kumada T et al., 1988). Further, protective activities of endogenous TM are amplified by cofactors expressed on the endothelial plasmalemma (Weiler H et al., 2003 and Aird WC 2003). In order to optimize retention and microenvironment of recombinant TM in the vascular lumen, we have fused sTM with a scFv fragment of an antibody to endothelial surface glycoprotein, PECAM. Endothelial cell adhesion molecules including PECAM are good molecular anchors for targeted delivery of drugs to endothelium (Ding BS et al., 2005; Ding BS et al., 2008; Charoenphol P et al., 2011; Kuldo JM et al., 2012 and Koren E et al., 2011).

It has been previously demonstrated by our lab that after intravenous injection in mice, anti-PECAM scFv/TM, but not sTM, binds to vascular endothelium (Ding BS et al., 2009). As result, it accumulates preferentially in the pulmonary vasculature that represents ~25% of the endothelial surface and has a privileged perfusion of more than

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50% of total cardiac blood output; approximately 35% of the injected dose of scFv/TM accumulated per gram of lung, which is similar to the accumulation of other anti–PECAM-1 conjugates (Ding BS et al., 2005 and Ding BS et al., 2008). However, endothelial binding of PECAM-targeted scFv-TM depletes the circulating pool, which precludes fair comparative study of the pharmacokinetics of sTM vs scFv/TM fusions.

Moreover, we demonstrated that scFv/TM stimulates APC production and provides therapeutic benefits superior to sTM in animal models of acute thrombosis and inflammation (Ding BS et al., 2009).

The pathogenesis of thrombosis and especially inflammation is intertwined with vascular oxidative stress (Molema G 2010 and Ng K et al., 2002), a condition in which excessive oxidant molecules damage endothelial cells and directly inactivate endothelial surface proteins (Eiserich JP et al., 1998). Oxidative inactivation of proteins can have physiological importance (Abrams WR et al., 1981). The biological properties of a number of proteins and peptides, including α I-protease inhibitor (α_1–PI) (Johnson D et al., 1978; Johnson D et al., 1979 and Carp H et al., 1982), plasminogen activator inhibitor 1 (Lawrence DA et al., 1986), α2-antiplasmin, antithrombin III (Stief TW et al., 1988), and N-formylmethionyl-leucyl-phenylalanine (Harvath L et al., 1984 and Cochrane CG et al., 1983), are altered as a consequence of methionine oxidation. The inactivation of α_1–PI by oxidation of a methionine to methionine sulfoxide is well characterized and occurs in patients with acute respiratory distress syndrome (Cochrane CG et al., 1983) and in smokers' lungs (Carp H et al., 1982). It has been previously demonstrated that like α_1–PI, TM is sensitive to physiological oxidants (Glaser CB et al., 1992), suggesting that inactivation of TM occurs during inflammation. Previous studies identified that oxidation of M388 methionine residue inhibits TM activities and M388L mutation renders TM resistant to oxidative inactivation (Glaser CB et al., 1992). In order to maximize effect of targeted scFv/TM in conditions involving oxidative stress, in this study we designed

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mutant anti-PECAM scFv/TM M388L and characterized its salient functional features *in vitro* and *in vivo*.

MATERIALS AND METHODS

Unless otherwise indicated, cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). Chloramine-T, mouse anti-flag M2 HRP mAb, heparin from porcine intestinal mucosa from Sigma-Aldrich (St. Louis, MO, USA), Human protein C was kindly supplied by Dr. Sriram Krishnaswamy at the University of Pennsylvania, Human APC was purchased from Haemotologic Technologies (Essex Junction, VT, USA), S-2366 from Diapharma (Columbus, OH, USA), Bovine thrombin from GE Amersham Biosciences (Pittsburgh, PA, USA), Recombinant Hirudin from EMD Chemicals (Billerica, MA, USA), Benzamidine HCl hydrate, Myeloperoxidase, Sodium iodide, Hydrogen peroxide, Bovine serum albumin type V, 96-well Costar (cat# 3595) plates for *in vitro* experiments and 8-well EIA/RIA Corning strips (cat# 2590) for *in vivo* experiments and Activated Partial Thromboplastin Time (APTT) clotting reagent from Fisher Scientific (Pittsburgh, PA, USA). C57BL6J 6-8 week old male mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). Mouse monoclonal antibodies against human APC (1555) were kindly provided by Charles Esmon and colleagues (Liaw PC et al., 2003)**.**

Production and Characterization of mouse PECAM (i.e. mPECAM) targeted and non-targeted TMs:

Generation of soluble thrombomodulin (sTM) and PECAM targeted fusion proteins: cDNA encoding the extracellular domain of TM (Leu^{17}-Ser^{517}) was amplified from whole mouse lung cDNA (Ding BS et al., 2009). sTM was constructed with a triple-FLAG tag at the 3' end and purified by anti-FLAG affinity. The scFv fusion proteins were generated as previously described (Ding BS et al., 2009), containing a triple-FLAG at the 3’ end like its non-targeted counterpart described above. The M388L substitution was performed using

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site-directed mutagenesis primers, Forward 5'-CCG CAC AAG TGC GAA CTC TTC TGC AAT GAA ACT TCG-3'; Reverse – 5'-CGA AGT TTC ATT GCA GAA GAG TTC GCA CTT GTG CGG-3'. The resulting product was sequenced and analyzed using the Penn Sequencing Center to verify that there were not any undesirable mutations. The dna constructs were transfected into drosophila S2 cells and proteins were expressed and isolated as previously described (Ding BS et al., 2009). Resulting protein fractions were concentrated using a 10kDa MW cut-off. sTM, anti-PECAM scFv/TM and anti-PECAM scFv/TM M388L protein concentrations were quantitated using Nanodrop (ThermoFisher), using MWs of 56.7kDa and 83.84kDa and extinction coefficients of 1.09 and 1.39, respectively.

Cell lines: Mouse pancreatic islet endothelial cells (MS1) cells endogenously expressing native PECAM-1 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in complete media (DMEM with 10% (v/v) FBS and 1% (v/v) P/S). Human non-endothelial cell line (REN cells), either stably transfected to express mouse PECAM-1, or naïve PECAM-negative REN cells used as a negative control (Chacko AM et al., 2012) were maintained in RPMI-Glutamax supplemented with 10% (v/v) FBS, 1% (v/v) P/S, and G418, [250 µg/mL].

ELISA: Cells were grown to confluence in 1% gelatin-coated 96-well plates (BD Biosciences) and ELISA performed as previously reported (Chacko AM et al., 2012). Briefly, indicated dilutions of sTM or scFv/TM or mutant scFv/TM in complete media were added in triplicate to the wells and incubated at 4°C for 2 h. Cells were washed with 3% BSA/PBS (100µL/well) two times and incubated at 4°C for 1 h with a monoclonal anti-flag M2-peroxidase (HRP) antibody at a dilution of 1:20,000 in complete media. Wells were subsequently washed with 3% BSA/PBS and ortho-phenilen diamine (OPD) substrate with H_2O_2 added. The reaction quenched with 100µL of 50% H_2SO_4. The plates were read at

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A490 nm (OD 490nm) on a Multiskan FC Microplate reader (Thermofisher). Experiments were performed three independent times, with each experiment performed in triplicate.

Analysis of TM-mediated stimulation of conversion protein C into APC by thrombin:

To compare APC-producing activity of different formulations of recombinant TM, human protein C [300nM] was incubated for 20 min in a 1.7mL microcentrifuge tube with bovine thrombin [5nM] pre-incubated with 4nM sTM, scFv/TM, scFv/TM M388L or PBS with Ca^{2+}. A 25µL aliquot was removed and added to wells of a 96-well plate containing 25µL recombinant hirudin [0.25U/µL]. A 50µL solution of chromogenic substrate of S2366 [4mM] Diapharma was added to wells and the OD at 405nm was measured at 60 min (Mean ± SEM, N=3). To test effect of oxidation, scFv/TM or scFv/TM M388L was incubated with a mixture of sodium iodide (NaI) [1.25mM], myeloperoxidase (MPO) [0.5U] and hydrogen peroxide (H_2O_2) [10µM], or chloramine-T [84µM] for 10 min APC production has been tested as above. To test APC production by PECAM-anchored fusions, MS-1 cells, REN-PECAM or REN wild type cells (the latter used as a negative control) were incubated for 30 min at 37°C with scFv/TM fusion proteins in serum free media, washed and treated with chloramine-T in PBS with Ca^{2+}/Mg^{2+} for 10 min at room temperature. After additional washings, cells were incubated with recombinant human protein C [100nM] and bovine thrombin [1nM] for 1 h at 37°C, supernatant was collected and treated with hirudin (3µL of 100nM) to inhibit thrombin and incubated with a chromogenic substrate S-2366 [4mM] (Diapharma) to assay for APC content. Absorbance at 405nm was monitored kinetically and the slope was measured. A standard curve was generated using purified human APC, allowing conversion of slopes in OD/min into nM of APC.

Analysis of APC-producing potency of scFv/TM fusions *in vivo*: Samples of scFv/TM or scFv/TM-M388L were incubated with PBS or chloramine-T [84µM] for 10 min, incubated with bovine thrombin for 10 min and injected into the jugular vein of

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C57BL6J mice. Immediately thereafter, human protein C [64.5nM] was injected into the contralateral jugular vein. Ten minutes later, blood was collected from the inferior vena cava in 3.8% sodium citrate and 1M benzamidine HCl (v/v 2:1) with 100µL of the sodium citrate mix added per 600µL of collected blood. Blood was spun at 1500×g for 5 min. Plasma was collected and flash frozen in liquid nitrogen and stored at -80°C until use. Level of human APC in the samples of murine plasma was analyzed by ELISA as previously described (Carnemolla R et al., 2012).

Activated Partial Thromboplastin Time (APTT) Clotting time assay: Blood was collected from the tail vein in 3.8% sodium citrate (1:10 v/v) from C57BL6J mice. Blood was spun at 1500×g for 5 min. Plasma was collected and flash frozen in liquid nitrogen and stored at -80°C until use. To run the assay, with or without TM [40nM], thrombin [1nM] and human protein C [64.5nM] were incubated in PBS (w/o Ca^{2+}) for 10 min. 25µl of the reaction, plasma, APTT reagent were incubated at 37°C for 170 sec, before being spiked with 25µl 25mM $CaCl_2$. The time to clot was measured by a Diagnostica Stago Start4 Instrument.

RESULTS

Binding and APC-producing properties of anti-PECAM scFv/TM mutant M388L

Figure 1 shows the design of the cDNA encoding sTM, wild type anti-PECAM scFv/TM (indicated as WT scFv/TM unless indicated otherwise) and scFv/TM M388L mutant. Transfected S2 cells secreted these proteins that migrated as a single band with MW ~ 56.7kDa for sTM and ~83.8 kDa for both WT and mutant scFv/TM (not shown). Both WT scFv/TM and M388L mutant bound equally well to mouse endothelial MS1 cells, while non-targeted sTM did not (Figure 2).

Both WT and M388L mutant scFv/TM have almost identical capacity to stimulate APC production by thrombin in liquid phase (Figure 3A). Using a non-endothelial cell line transfected with mouse PECAM we tested APC production by target-bound fusions.

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Unlike endothelial cells that naturally express PECAM, using this model offers a negative control for binding and analysis of activity of cell-bound scFv/TM that is not convoluted by contribution of endogenous TM and cofactor proteins typical of endothelial cells. This test showed that WT and mutant scFv/TM fusions bound to model target cells have similar ability to stimulate APC production by thrombin (Figure 3B).

APC-producing capacity of scFv/TM M388L is resistant to oxidative stress

Oxidation of scFv/TM by chloramine-T caused approximately 60% inhibition of APC producing capacity of the WT scFv/TM, whereas the mutant scFv/TM M388L retained full APC-producing capacity (Figure 4A). To validate this finding with a more physiological oxidative agent, we tested effect of scFv/TM exposure to a mixture that mimics myeloperoxidase (MPO)-mediated oxidative stress caused by activation of neutrophils, which plays a pathophysiological role in vascular pathology (Eiserich JP et al., 2002). In a good correlation with chloramine data, this treatment caused a 60% loss of APC-producing capacity of WT scFv/TM, but not scFv/TM mutant M388L (Figure 4B).

Oxidant-resistant scFv/TM M388L fully restores APC-producing capacity of oxidized endothelial cells

Next, we tested whether scFv/TM M388L resistance to oxidative inactivation provides an advantage in restoring endothelial TM function in conditions of oxidative stress. In the first series, PECAM-expressing cells were incubated with equal doses of WT or mutant fusion, washed and exposed to the oxidative stress. In this series, similarly to fluid phase test described in the previous section, cell-bound WT fusion lost ~60% of APC-producing capacity, whereas cell-bound scFv/TM M388L mutant was not affected (Figure 5A). Next, we exposed mouse endothelial cells to two different concentrations of oxidative stress and found that this causes ~40% reduction of cellular APC-producing capacity (Figure 5B-C). This outcome, reflecting inhibition of endogenous TM, could not be alleviated by delivery of WT scFv/TM followed by

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oxidative stress (closed circles). In a sharp contrast, oxidant resistant mutant fusion completely restored endothelial APC-producing capacity under the same conditions (open circles).

Oxidant resistant scFv/TM M388L mutant has superior APC generating potency *in vivo*

We next tested whether enhanced oxidative resistance of scFv/TM M388L translates into being more protected from oxidative stress production of APC by TM replacement therapy *in vivo*. WT and mutant fusion proteins exposed to oxidative stress injected in mice and the subsequent conversion of human protein C to APC has been analyzed. Oxidized WT scFv/TM produced ~30% less APC *in vivo* than intact counterpart, whereas APC-producing potency of scFv/TM M388L mutant *in vivo* has not been affected by this insult (Fig. 6).

Oxidant resistant scFv/TM M388L mutant prevents clots *in vitro*

We were able to demonstrate that our oxidant resistant mutant generates more APC than wild-type, scFv/TM, but the next question we wanted to ask was what is the effect of that APC? To do this we utilized a classical APTT clotting assay. As expected, under normal conditions, the wild-type scFv/TM and mutant have similar influences on clotting time, >200 sec (machine stops measuring at this point) (Figure 7). However, under oxidative conditions, the scFv/TM fails to prevent the clot from forming, and the time is very similar to plasma without TM. In contrast, scFv/TM M388L does not clot at all, with the instrument stopping measurement at 200 sec.

DISCUSSION

Inflammation, oxidative stress, ischemia-reperfusion and thrombosis represent common interconnected and mutually propagating mechanisms in cardiovascular pathology (Esmon CT 2005 and Weiler H et al., 2003). Unfortunately, under these circumstances, activity of endogenous TM, which exerts protective anti-thrombotic and

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anti-inflammatory effects, is suppressed via mechanisms including suppression of synthesis, enhanced shedding and direct oxidative inactivation (Esmon CT 2005 and Weiler H et al., 2003). In particular, the latter mechanism involves oxidation of the specific methionine residue (M388) that results in loss of TM function (Glaser CB et al., 1992).

In order to alleviate this effect of oxidative stress, a mutant form of recombinant soluble TM, sTM-M388L, has been developed and is currently being evaluated in Phase I clinical studies (van Irsel T et al., 2011). In this study, we have used this rationale to molecularly engineer and improve the functional features of a novel targeted version of TM replacement therapy, namely, TM fused with scFv targeted to endothelial cells (scFv/TM). Stable anchoring of scFv/TM on the luminal vascular surface more closely resembles restoration of endogenous protective mechanisms of TM/APC axis and offers advantages of more sustained and efficient interventions.

The results of *in vitro* and *in vivo* experiments performed in the present study indicate that PECAM-targeted scFv/TM M388L mutant exerts both targeting and APC-producing activities similar to those of WT fusion. However, the mutant fusion retained these activities under oxidative conditions that otherwise inhibit APC production in the WT counterpart. As result, targeted delivery of scFv/TM M388L mutant to endothelial cells in oxidative conditions allows full restoration of APC producing activity, whereas with the WT fusion restoration was incomplete.

Nuclear magnetic resonance (NMR) spectroscopy of the smallest active fragment of TM with EGF domains 4 and 5 (TMEGF45), and a crystal structure of thrombin bound to a larger TM fragment with EGF domains 4, 5 and 6 (TMEGF456) show that M388 is packing within the fifth EGF domain (Wood MJ et al., 2003). Multidimensional NMR reveals differences in 3-dimensional structures of TMEGF45, in which M388 is oxidized (TMEGF45ox) and TMEGF45, in which M388 is mutated to Leu (TMEGF45ML).

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Comparison of the structures shows that the fifth domain has a somewhat different structure depending on the residue at position 388, and several of the thrombin-binding residues are buried into the fifth EGF domain in the oxidized protein whereas they are exposed and free to interact with thrombin in the native structure and the Met-Leu mutant.

This observation is consistent with kinetic measurements showing that the K_m for TMEGF45ox binding to thrombin is 3.3-fold higher than for the native protein. Most importantly, the connection between the two domains, as indicated by NMR-measured interdomain nuclear overhauser effects (NOEs), appears to be essential for activity. In the TMEGF45ox structure, which has a reduced k_{cat} for protein C activation by the thrombin-TMEGF45ox complex, interaction between the two domains is lost. Conversely, a tighter connection is observed between the two domains in TMEGF45ML, which has a higher k_{cat} for protein C activation by the thrombin-TMEGF45ML complex. Therefore, it seems oxidant-resistant scFv/TM fusion is a preferable candidate for therapeutic interventions aimed at compensation for the loss of endogenous TM.

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AUTHORSHIP CONTRIBUTION

Participated in research design: Carnemolla, Chacko, Greineder and Muzykantov.

Conducted experiments: Carnemolla, Chacko, Greineder and Patel.

Contributed new reagents or analytic tools: Esmon

Performed data analysis: Carnemolla, Chacko, Greineder and Muzykantov.

Wrote or contributed to the writing of the manuscript: Carnemolla, Chacko, Greineder, Esmon and Muzykantov.

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LEGENDS FOR FIGURES

Figure 1: Molecular design of recombinant thrombomodulin TM constructs used in the study. Schematic diagram describes the strategy and main protein domains in the non-targeted soluble (sTM) and targeted fusion protein constructs: To generate the scFv fusion protein, anti-PECAM variable domains of the heavy and light chain were linked by a $(Gly_4Ser)_3$ linker and then fused to the N-terminus of thrombomodulin (TM) by a $(Ser_4Gly)_2Ala_3$ linker. sTM or TM and anti-PECAM scFv were ligated and cloned into the SpeI and XhoI sites of the pMT drosophila cell protein expression vector. Protein expression and isolation is described within methods section.

Figure 2: Mutation M388L in scFv/TM does not affect binding of the fusion protein to PECAM-1 expressing endothelial cells. Cell surface binding of fusion proteins to PECAM-1 was determined by ELISA-based method with PECAM-1 expressing REN cells. Proteins were added to confluent cellular monolayer at the indicated dilutions and incubated for 2 h at 4°C. Non-PECAM-1 expressing cells were used as negative control. The results shown are from a representative experiment ± SD. Three independent experiments were performed in triplicate.

Figure 3: Wild-type and mutant fusion proteins have similar ability to stimulate production of activated protein C (APC) by thrombin. (A) Generation of APC by thrombin in presence of sTM (open bar), scFv/TM (black bar) and scFv/TM M388L (grey bar) vs thrombin alone (dotted line). Three independent experiments were performed in triplicate, Mean ± SEM. (B) Activation of thrombin conversion of protein C to APC by PECAM-associated scFv/TM. Model human cell line stably transfected with mouse PECAM (upper curves) or control PECAM-negative naïve cells (lower curves) were incubated with WT or mutant scFv/TM (open or closed symbols, respectively), washed

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and incubated further with thrombin and protein C. APC values for both graphs A-B were extrapolated from a APC linear standard curve (R^2 = 0.99).

Figure 4: scFv/TM M388L fusion protein is resistant to oxidant-induced suppression of APC generation. (A) MPO/NaI/H_2O_2 induced oxidative suppression of APC generation capability. Dotted line represents thrombin with protein C alone (without TM); *, p<0.005 vs scFv/TM M388L w/ MPO/NaI/H_2O_2. (B) Chloramine-T suppression of scFv/TM ability to stimulate APC generation by thrombin. Dottled line represents thrombin with protein C alone. For both A-B, represented is the average of three independent experiments ± SEM; *, p<0.004 vs. scFv/TM-M388L w/ chloramine T.

Figure 5: PECAM-targeted scFv/TM M388L resistant to oxidative inactivation restores thrombomodulin function of oxidized endothelial cells. (A) Functional resistance to chloramine oxidation of WT vs mutant scFv/TM bound to model PECAM-expressing cells. Model human cell line stably transfected with mouse PECAM control PECAM-negative naïve cells (data not shown) were incubated with WT or mutant scFv/TM (left or right bar pairs, respectively), thrombin alone (dotted line), washed, exposed to 250μM chloramine-T, incubated further with thrombin and protein C, and the media was examined for APC. Three independent experiments were performed in triplicate, Mean ± SEM; *, p<0.05 vs oxidized scFv/TM-M388L. (B and C) Restoration of TM activity in oxidized endothelial cells. Mouse endothelial MS1 cells were incubated with PBS, WT or mutant scFv/TM, washed, exposed to chloramine-T [500μM] or [1000μM] and the media was examined for APC generated upon addition of thrombin and protein C as in panel A. Three independent experiments were performed in triplicate, Mean ± SEM; *, p<0.002 for open vs oxidized scFv/TM-M388L.

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Figure 6: Oxidant-exposed scFv/TM M388L provides more effective than WT scFv/TM stimulation of APC generation *in vivo*. WT or mutant scFv/TM (left or right bar pairs, respectively), thrombin alone (dotted line) were exposed to chloramine-T [84μM] and injected in mice intravenously with thrombin and human protein C. Ten min later blood plasma was obtained and level of activated protein C was examined by ELISA for human APC. Three independent experiments were performed in triplicate, Mean ± SEM; *, $p<0.005$ vs scFv/TM M388L.

Figure 7: Oxidant resistant scFv/TM M388L mutant prevents clots *in vitro*. Chloramine-T suppression of scFv/TM (open bars) APC generating function results in clotted plasma. To run the assay, with or without TM [40nM], thrombin [1nM] and human protein C [64.5nM] were incubated in PBS (w/o Ca^{2+}) for 10 min. 25μl of the reaction, plasma, APTT reagent were incubated at 37°C for 170 sec, before being spiked with 25μl 25mM $CaCl_2$. The time to clot was measured by a Diagnostica Stago Start4 Instrument. Three independent experiments were performed in duplicate, Mean ± SEM; *, $p<0.005$ vs scFv/TM M388L (dark bars).

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Figure 1

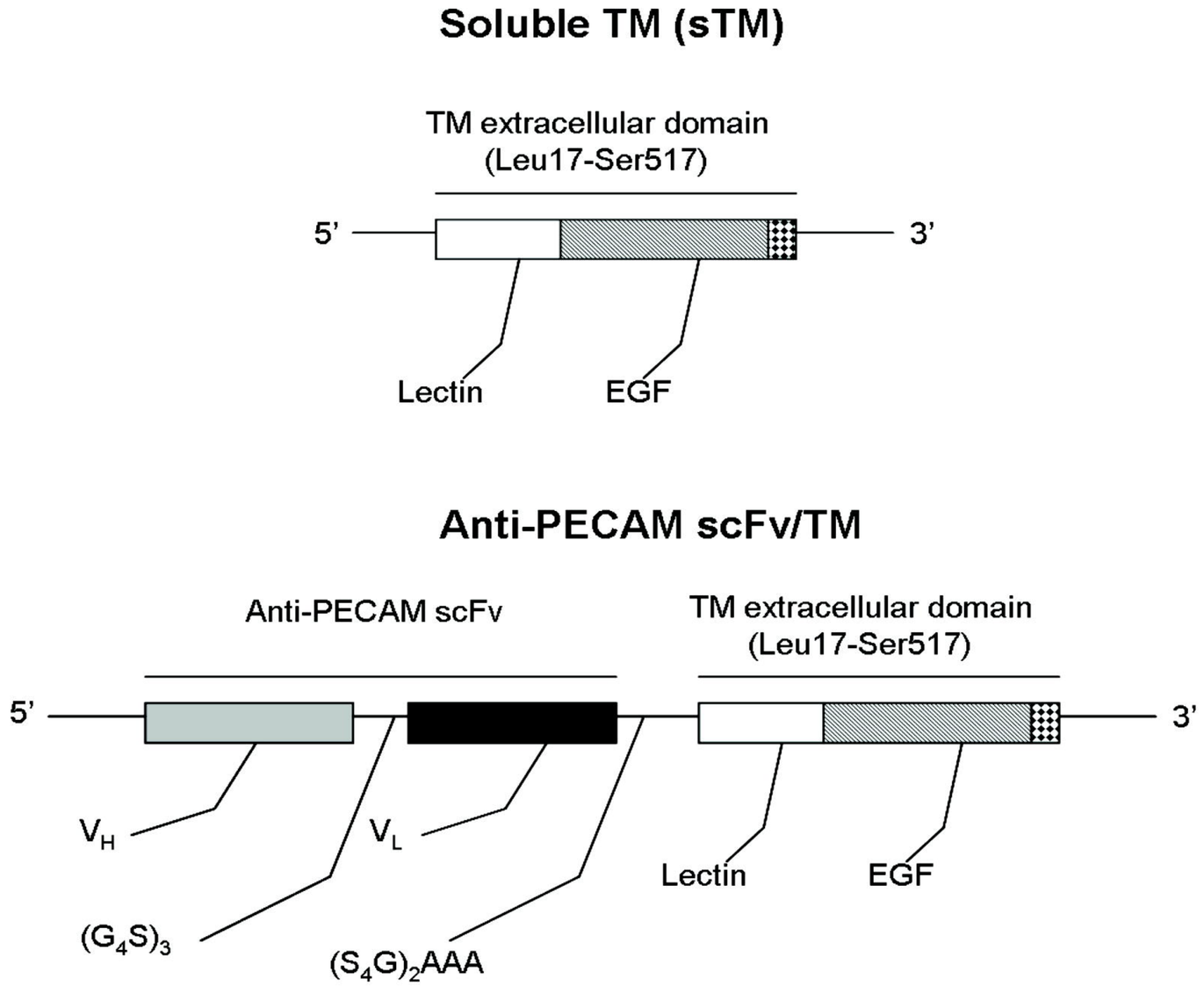

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Figure 2

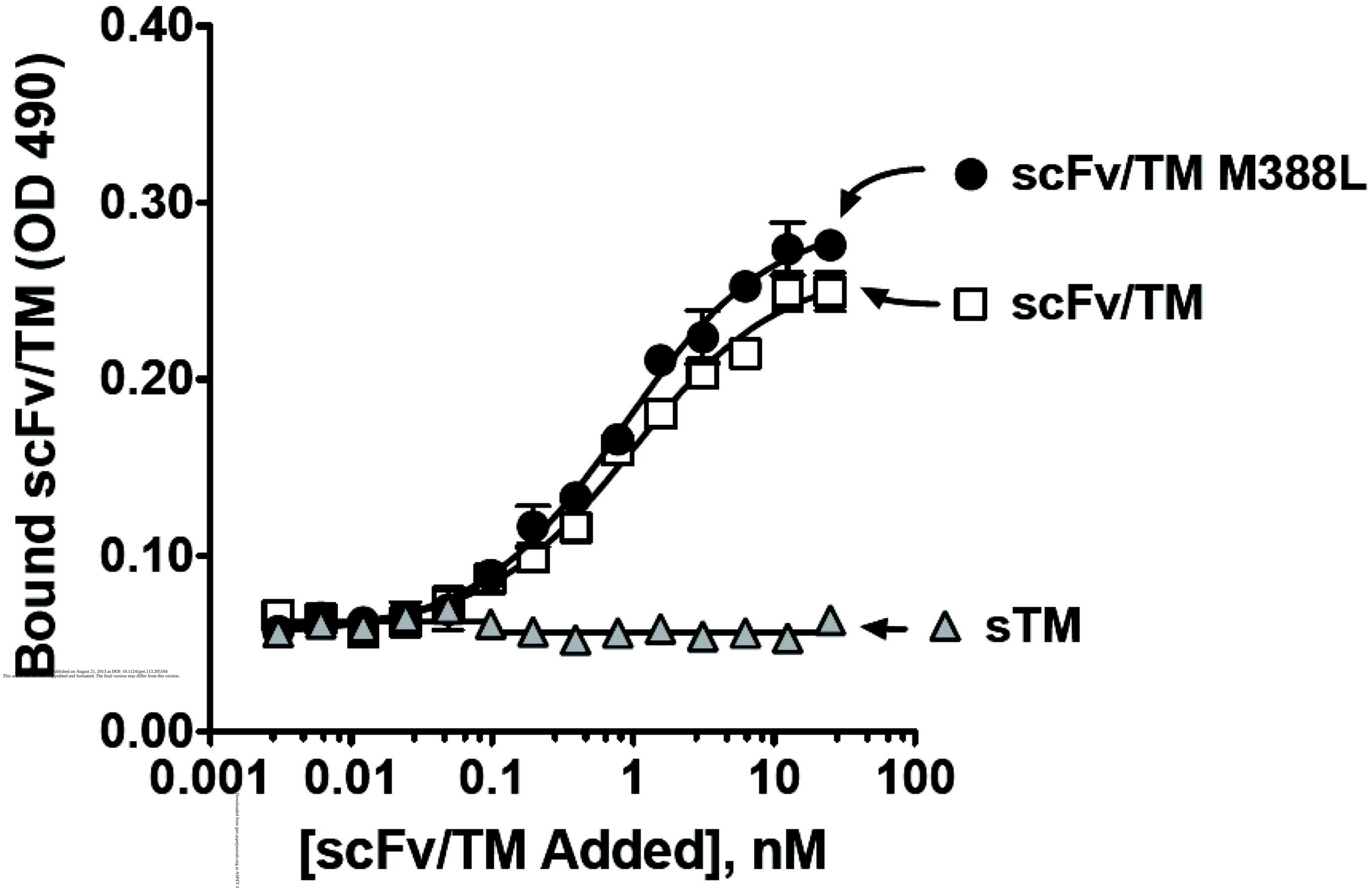

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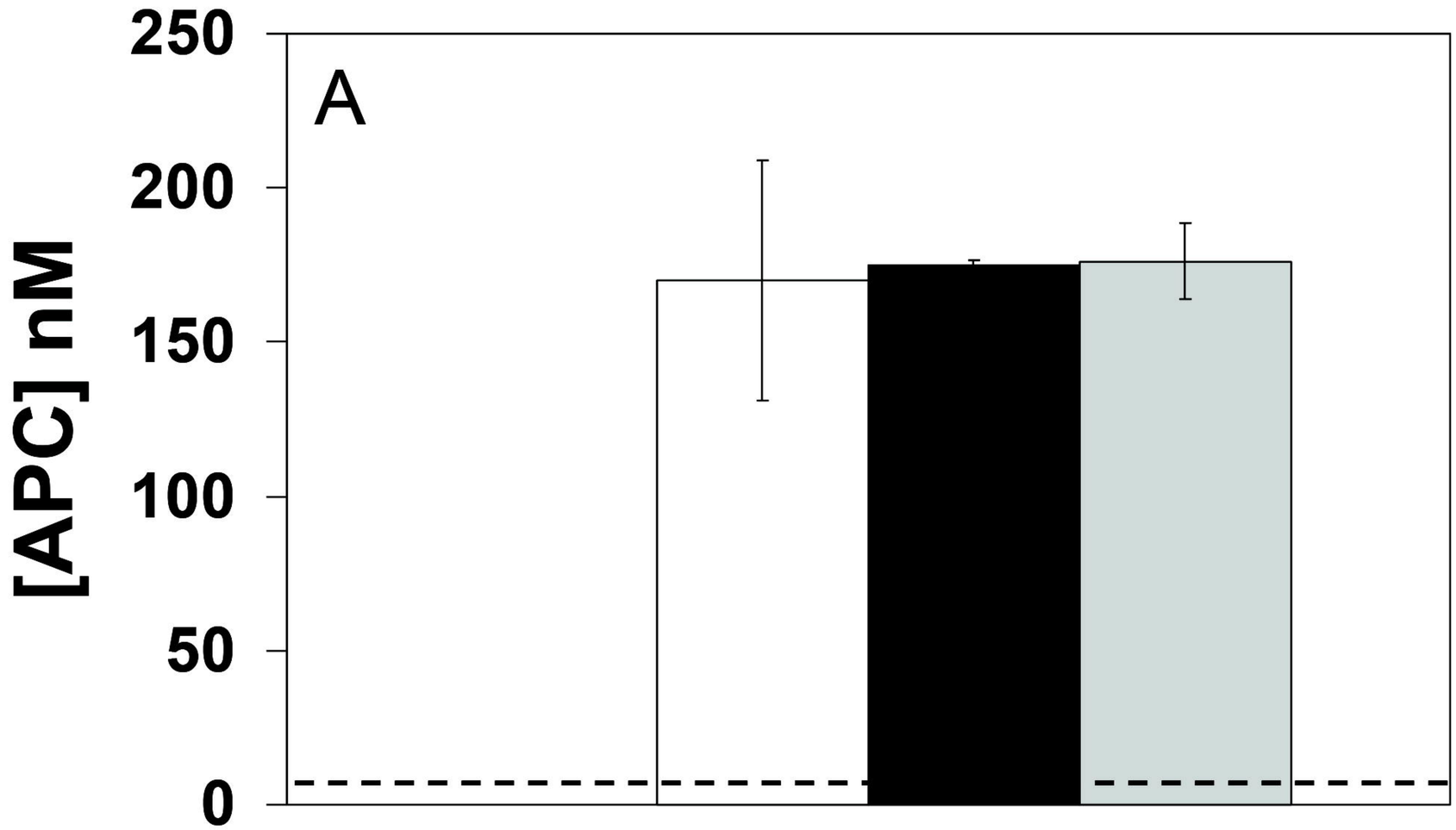
Figure 3



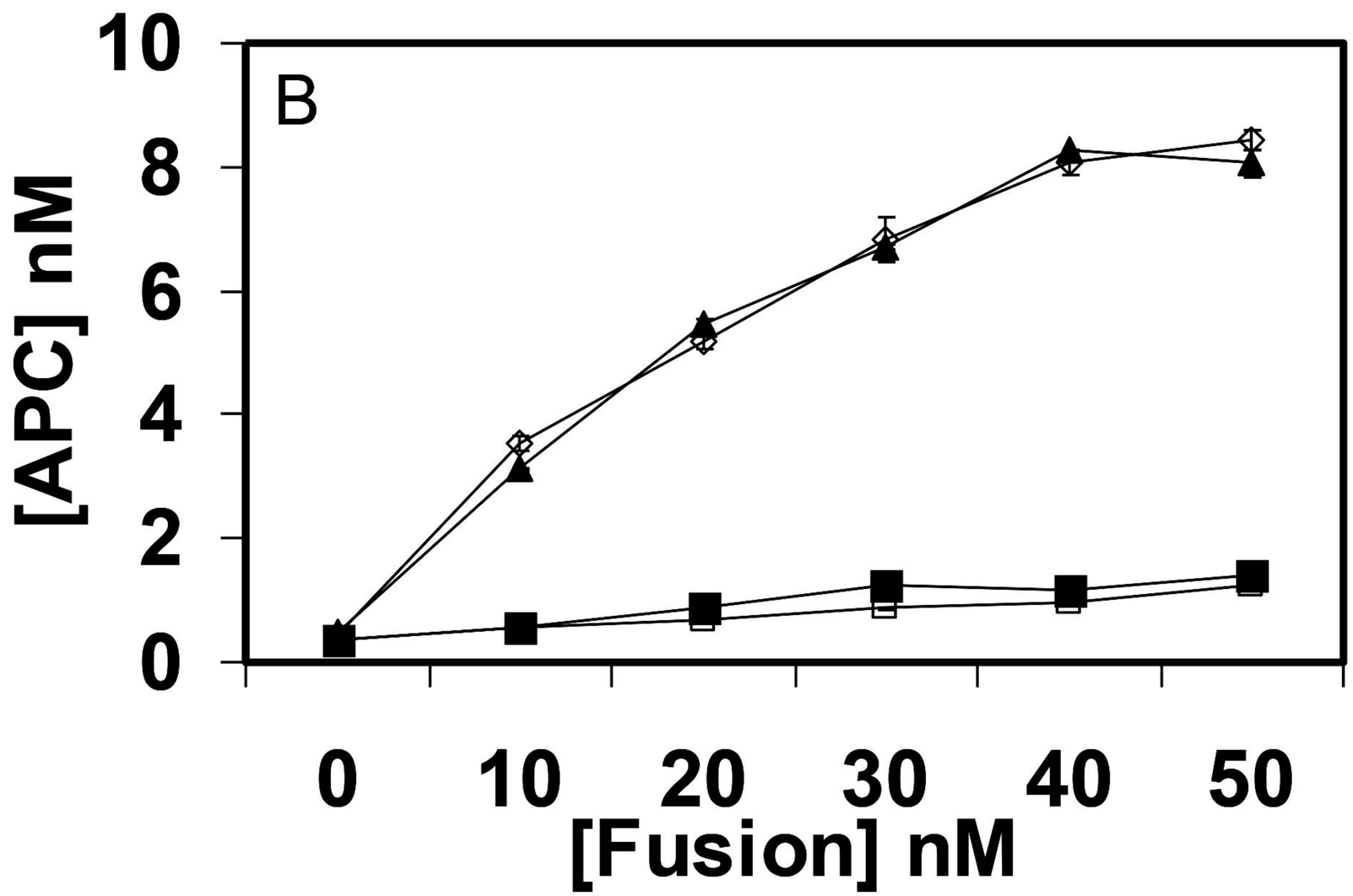


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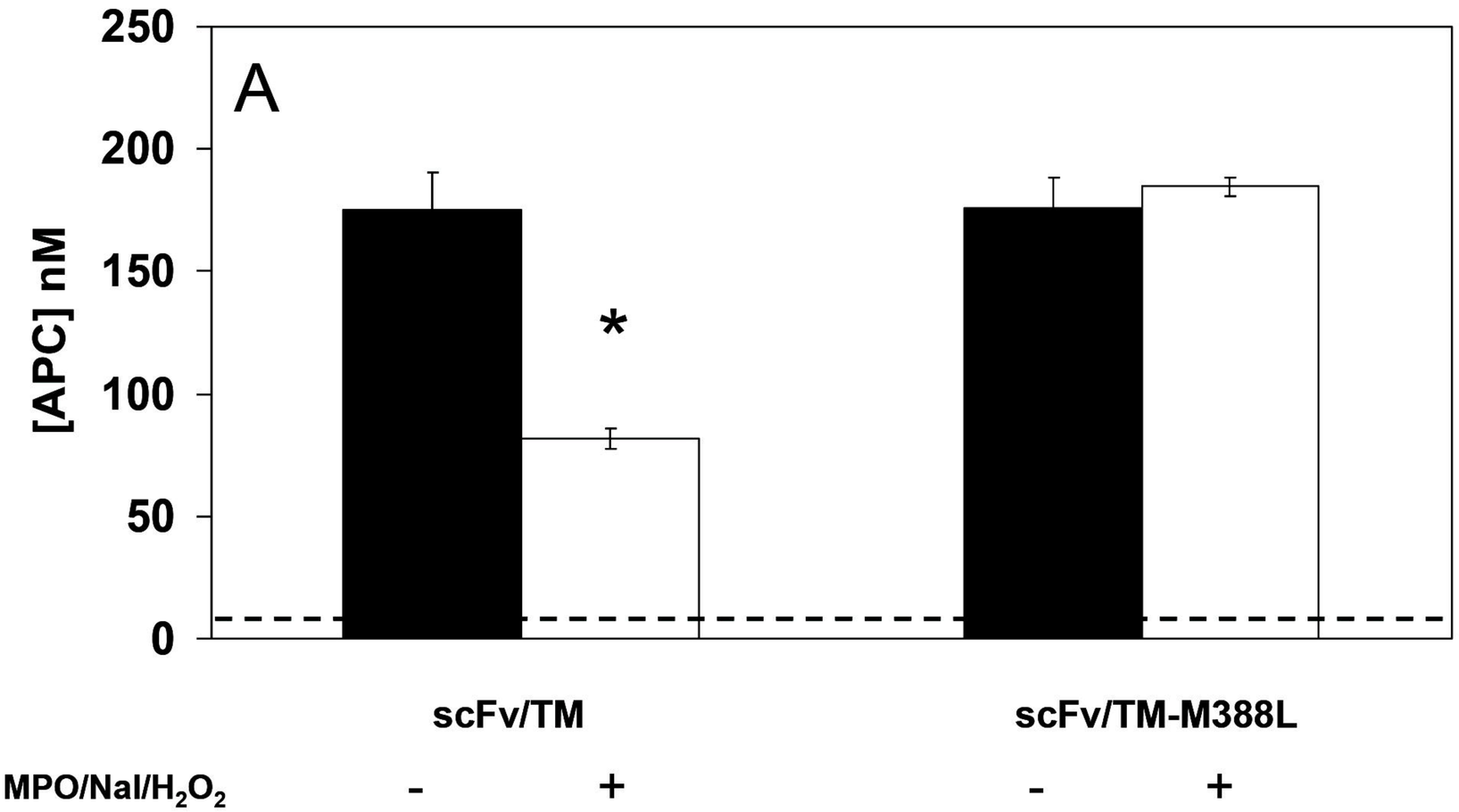
Figure 4



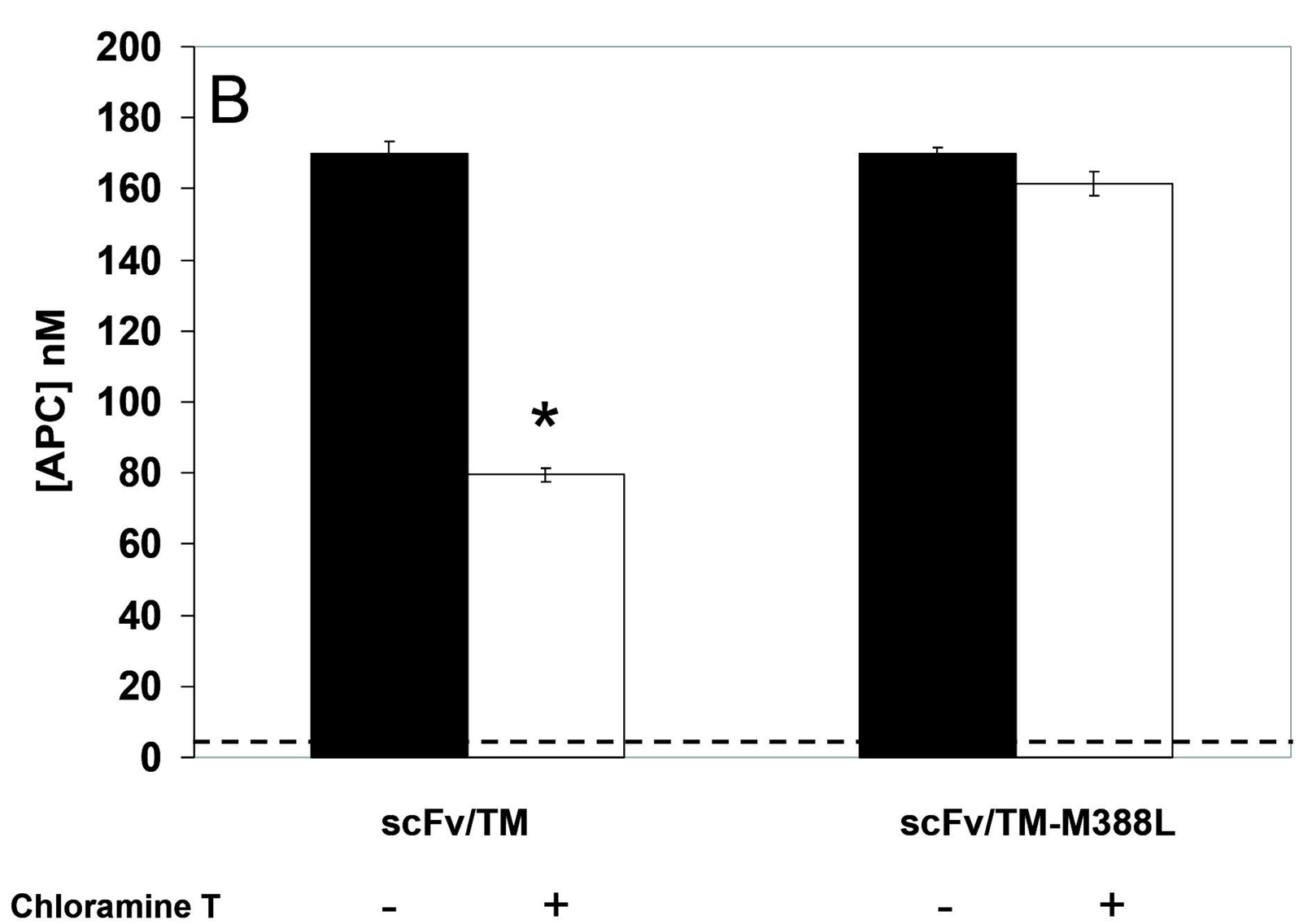


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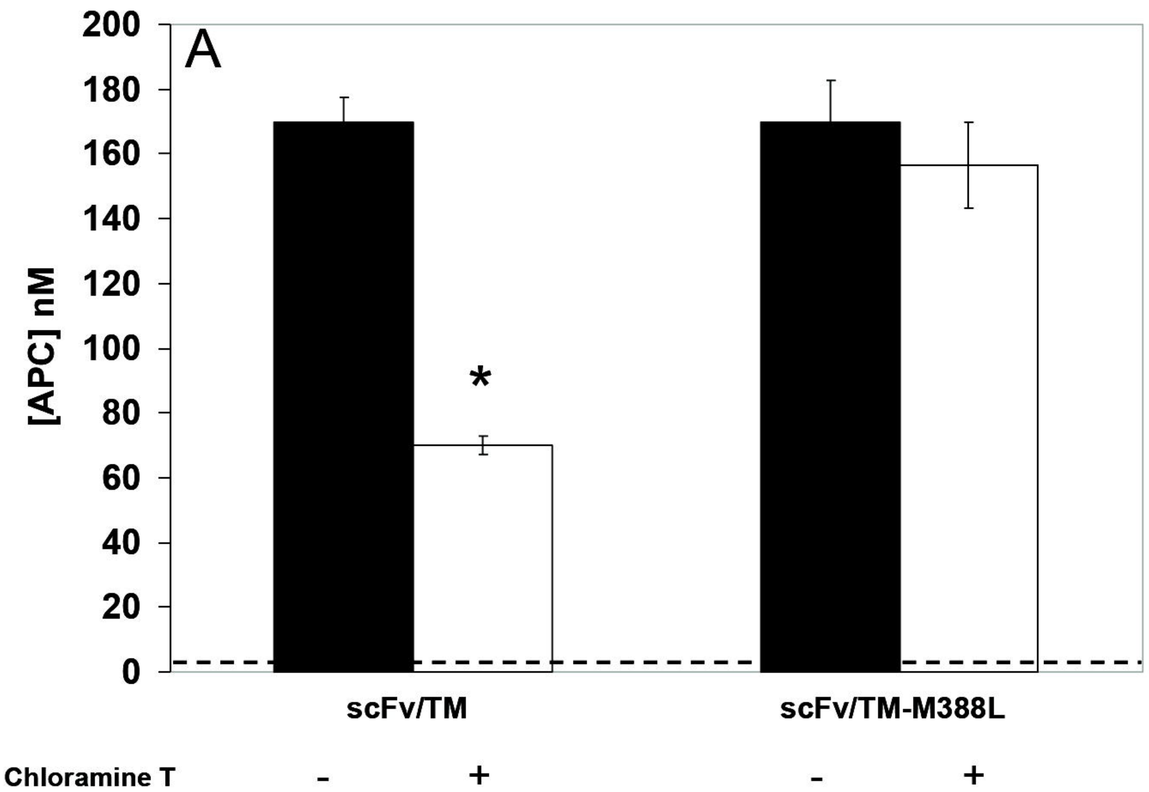
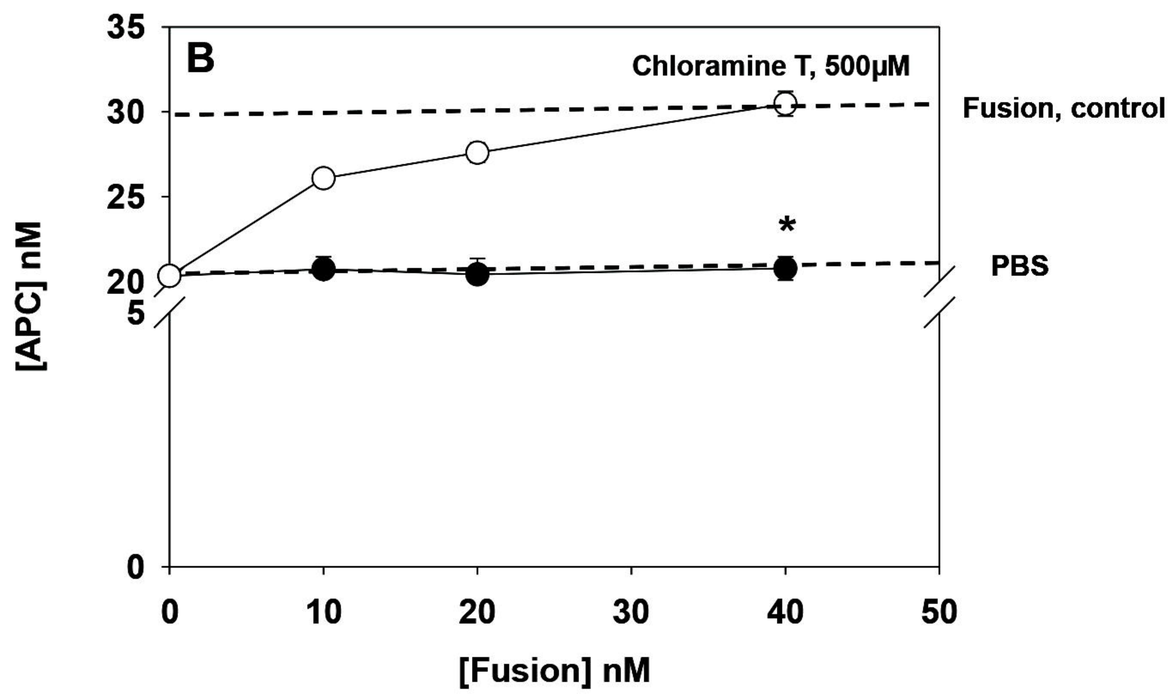
Figure 5





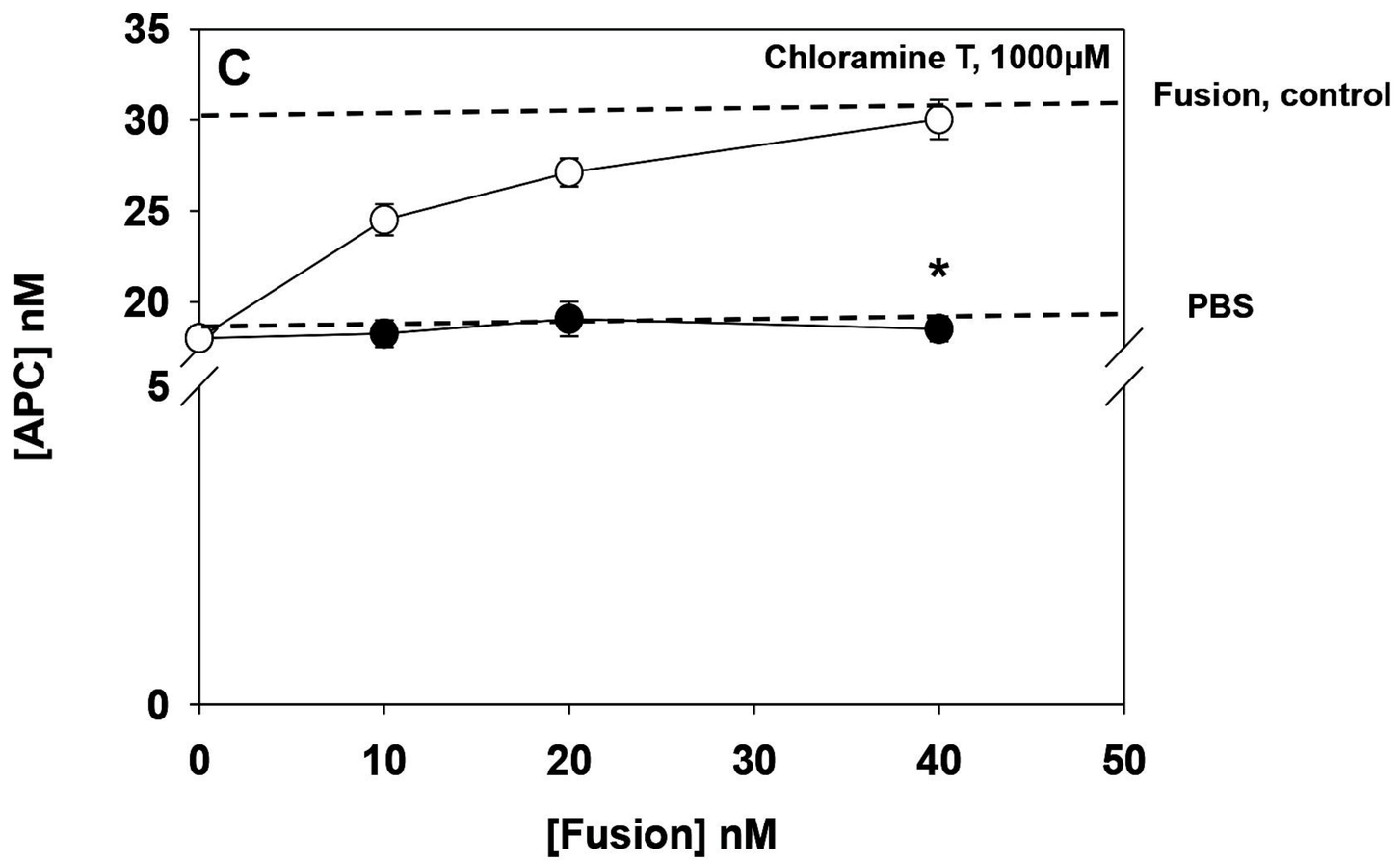


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Figure 6

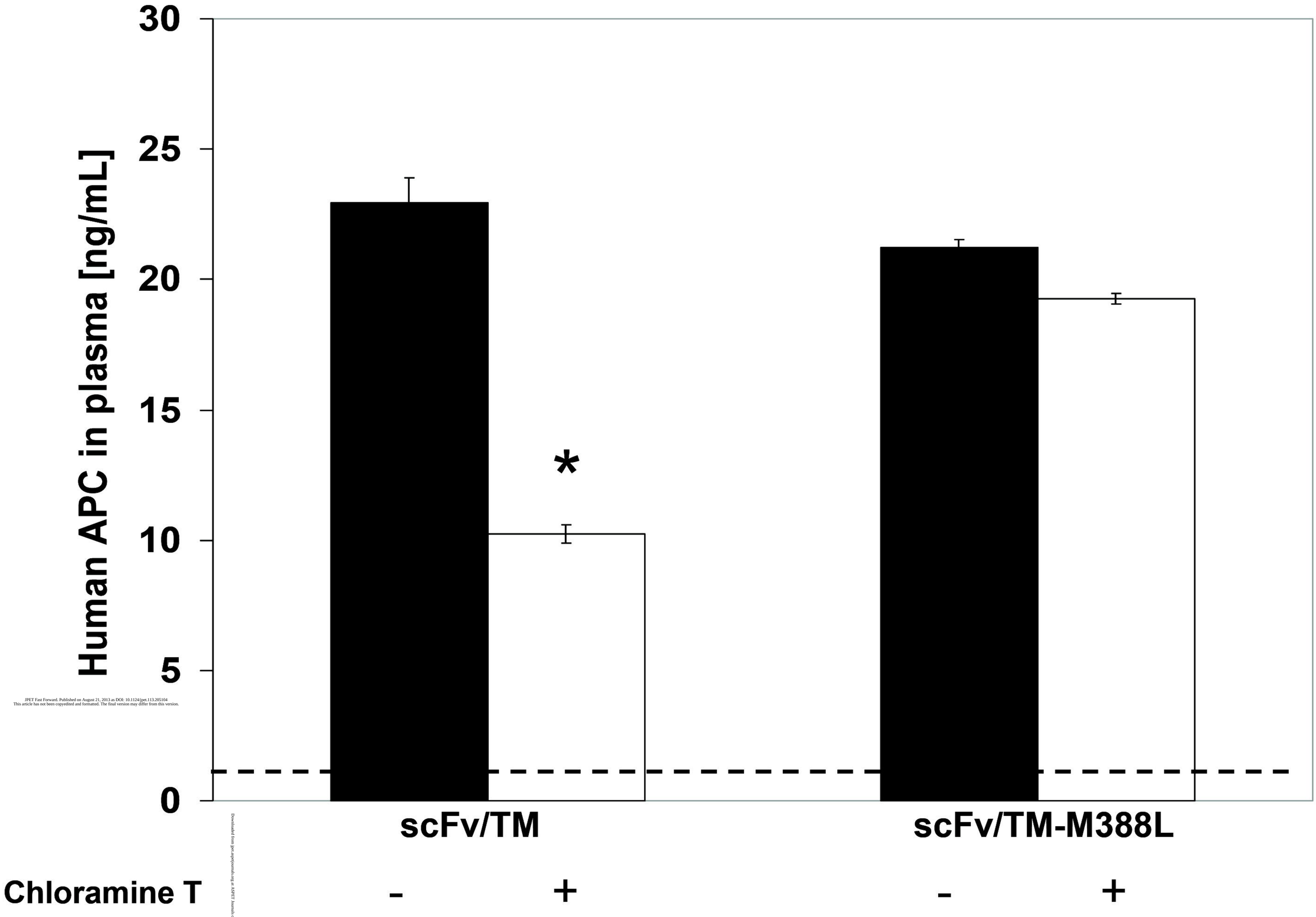

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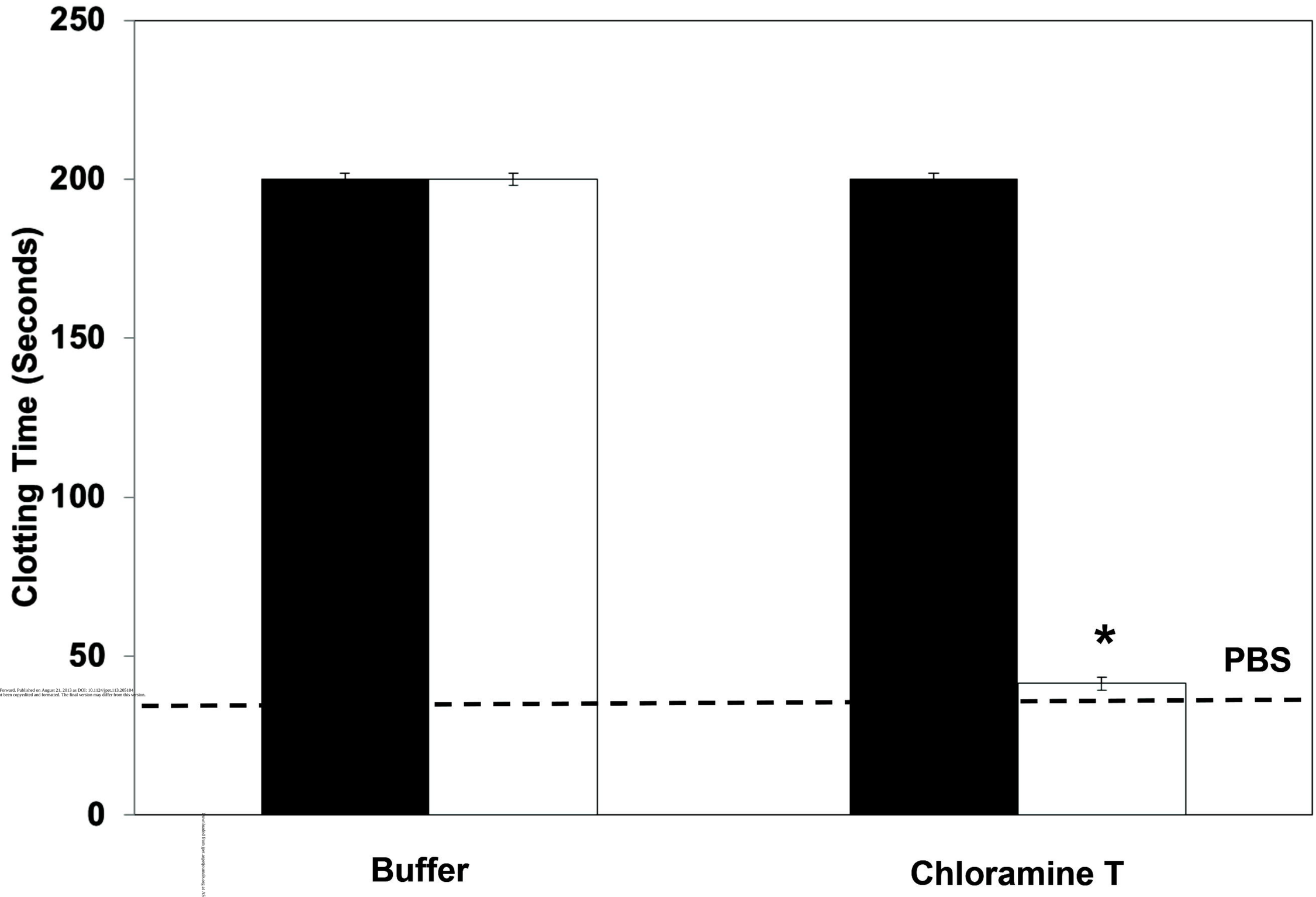
Figure 7



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