

1 **Human blood exposure to *Clostridium perfringens* epsilon toxin may shed light on**
2 **erythrocyte fragility during active multiple sclerosis**

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9 Running Head: *C. perfringens* ETX is hemolytic to human RBCs

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21 **ABSTRACT**

22 During active multiple sclerosis (MS), red blood cells (RBCs) harvested from patients
23 reportedly display increased osmotic fragility and increased cellular volume
24 (macrocytosis). The cause of these abnormalities remains unknown. We have previously
25 proposed that *Clostridium perfringens* epsilon toxin (ETX) may be a blood-borne trigger
26 for newly forming MS lesions based on its tropism for blood-brain barrier vasculature
27 and CNS myelin. Recently, Gao et al. have reported that ETX binds to and damages
28 human RBCs, leading to hemolysis. Moreover, the authors suggest that purinergic
29 nucleotide (P2) receptor activation amplifies the hemolytic process. Here, we confirm
30 that ETX indeed causes human-specific RBC lysis. However, our data suggest that the
31 hemolytic process is mediated by metal-catalyzed oxidation of the swell-induced,
32 nucleotide-sensitive ICln chloride channel. We use spectrophotometry, flow cytometry
33 and Western blotting to show that ETX targets human RBCs and T lymphocytes via their
34 shared expression of Myelin and Lymphocyte protein (MAL); a protein shown to be both
35 necessary and sufficient for ETX binding and toxicity. ETX likely triggers T cells to
36 release redox-active heavy metals, Cu^+ and Fe^{3+} , via the lysosomal exocytosis pathway,
37 while RBCs likely release these heavy metals via ETX pore formation within the RBC
38 membrane. Extracellular Cu^+ and Fe^{3+} may then amplify hemolysis by oxidizing a
39 previously identified heavy metal-binding site within the ICln channel pore, thus
40 deregulating its normal conductance. Elucidating the precise mechanism of ETX-
41 mediated hemolysis may shed light on the underlying etiology of MS, as it would explain
42 why MS RBC abnormalities occur during active disease.

43

44 **IMPORTANCE**

45 During active MS, numerous reports suggest that circulating RBCs are larger than normal
46 and fragment more easily. The exact trigger(s) for these RBC abnormalities and for
47 newly forming MS lesions remains unidentified. We have proposed that ETX, secreted
48 by the gut bacterium *Clostridium perfringens*, may be an environmental trigger for newly
49 forming MS lesions. Indeed, ETX has been shown to breakdown the BBB, enter the
50 brain and damage the myelin sheath. Because ETX is typically spread through the
51 circulatory system, we wished to determine how the toxin affects human blood.
52 Provocatively, there has been a recent report that ETX produces cellular abnormalities in
53 human RBCs, reminiscent of what has been described during active MS. In our study,
54 we sought to elucidate the precise mechanism for how ETX causes RBC damage. In
55 addition to triggering BBB breakdown and CNS demyelination, ETX might also explain
56 why RBCs appear abnormal during MS attacks.

57

58 **INTRODUCTION**

59 Although typically considered a disease confined to the central nervous system (CNS),
60 multiple sclerosis (MS) has frequently been shown to cause hematologic abnormalities.
61 To our knowledge, Waksman provided the lone report of T-lymphocyte abnormalities
62 during active MS, which entailed cellular enlargement and a decline in circulating T cell
63 numbers [1]. However, there have been numerous reports of red blood cell (RBC)
64 abnormalities during, and up to one week prior to the onset of neurologic symptoms [2-
65 7]. These decades-old RBC studies have described increased RBC volume
66 (macrocytosis) and increased osmotic fragility, which makes hemolysis more likely to

67 occur. Interestingly, Lewin et al. have recently shed additional light on MS-related RBC
68 abnormalities by showing that free hemoglobin, presumably released after hemolysis,
69 correlates with iron deposition along CNS blood vessels. Iron deposition may lead to
70 neuronal toxicity, axonal loss and the progression from relapsing-remitting MS (RRMS)
71 to secondary-progressive MS (SPMS) [8]. Despite the possible relevance to how MS
72 fundamentally progresses, these hematologic abnormalities remain unexplained.

73

74 We have previously suggested that newly forming MS lesions may be triggered by a gut-
75 derived neurotoxin, *Clostridium perfringens* epsilon toxin (ETX), which is disseminated
76 via the circulatory system [9-12]. In support of this theory, Wagley et al. have recently
77 identified serological evidence of prior ETX exposure in MS patients from the United
78 Kingdom [13], complementing what we have observed in an American cohort [9].

79

80 *C. perfringens* is an anaerobic, spore-forming, gram-positive bacillus that is sub-
81 classified into seven distinct toxinotypes (A-G) based on differential exotoxin production
82 [14]. *C. perfringens* type A typically colonizes the human gut with a prevalence of 63%
83 among healthy individuals [15], while *C. perfringens* types B and D, the producers of
84 ETX, are commonly found in the intestines of ruminant animals such as sheep, goats, and
85 cattle but rarely in humans [16]. ETX is a potent neurotoxin secreted as a 33 kDa
86 inactive precursor during the logarithmic growth phase of *C. perfringens* in the
87 mammalian intestine. This poorly active precursor is cleaved by gut trypsin,
88 chymotrypsin and several other carboxypeptidases [17]. The ~27 kDa ETX cleavage
89 product permeabilizes the gut epithelium, enters the blood stream and binds to receptors

90 on the luminal surface of brain endothelial cells [9,16]. Once bound to brain
91 microvessels, ETX oligomerizes and forms a heptameric pore in the endothelial cell
92 plasma membrane. Brain endothelial cell damage leads to breakdown of the BBB [16].
93 In addition to its known effects on BBB vasculature, ETX has been found to specifically
94 bind to and damage myelin when incubated with mammalian brain slices [11, 18, 19].
95 This unique ability to interact specifically with the tissues that are damaged in MS, i.e.,
96 the BBB and CNS myelin, makes ETX a promising candidate as an environmental MS
97 trigger.

98

99 ETX binds to and damages cells that express the tetraspan transmembrane myelin and
100 lymphocyte protein (MAL); relevant cell types containing MAL include the myelin-
101 oligodendrocyte unit, blood-brain barrier endothelial cells and circulating CD4+ T-
102 lymphocytes [10]. Intriguingly, Gao et al. have recently reported that ETX directly binds
103 to the human RBC plasma membrane, matures into the ETX pore-complex and triggers
104 dose-dependent hemolysis, while sparing RBCs from other species. Furthermore, they
105 propose that ETX-mediated hemolysis is amplified by purinergic nucleotide (P2) receptor
106 activation [20].

107

108 In this study, we confirm that ETX indeed binds to and damages human RBCs, likely due
109 to their expression of MAL isoform C. However, our data point to a different nucleotide-
110 sensitive system being responsible for amplifying hemolysis. We find that inhibitors of
111 the nucleotide-sensitive, swell-induced chloride channel, ICLn, successfully inhibit the
112 hemolytic process.

113

114 Because ETX has only recently garnered interest in its potential to cause human disease,
115 there is a paucity of studies published on how it affects human blood. Gaining insight
116 into how ETX triggers human RBC fragility may provide ancillary support for the ETX-
117 MS hypothesis in light of the fact that MS-associated RBC abnormalities remain
118 unexplained. Furthermore, if ETX is ultimately shown to be involved in triggering new
119 MS lesions, understanding the precise mechanism of toxin-triggered hemolysis may open
120 avenues for novel biomarkers capable of predicting the onset of neurologic symptoms, as
121 RBC abnormalities have been shown to precede clinical relapse in some instances.
122 Considering the recent report that hemolysis may play a critical role in the transition from
123 RRMS to SPMS [8], elucidating how hemolysis occurs may allow for clinical
124 interventions aimed at preventing disease progression and permanent neurological
125 decline.

126

127 **RESULTS**

128 **Human blood is uniquely sensitive to ETX-mediated hemolysis via MAL expression**

129 We first attempted to repeat the finding by Gao et al. that ETX-mediated hemolysis is
130 specific to human blood [20]. We compared the lytic effect of ETX on human blood vs.
131 blood from ruminant animals such as sheep, goats and cows, as these animals are the
132 natural hosts for ETX-secreting *C. perfringens* strains [16]. We also tested guinea pig
133 blood as a non-human, non-ruminant control. Highly purified epsilon protoxin
134 (protoETX), as determined by SDS-PAGE (Supplemental Fig 1) was trypsin activated,
135 and time-course analysis showed that this activated ETX (15nM) caused hemolysis in

136 PBS-washed whole human blood. However, blood harvested from non-human species
137 was completely refractory (Fig 1a). Importantly, we also determined that trypsin
138 activation was necessary for hemolytic activity, as non-activated protoETX failed to
139 trigger hemolysis (Supplemental Fig 2). Moreover, inhibition of activated ETX with an
140 anti-ETX neutralizing antibody, JL008, inhibited hemolysis in a dose-dependent manner
141 (Supplemental Fig 3). We also confirmed that ETX directly binds human RBCs by
142 incubating cells harvested from human, rhesus macaque, sheep and rat with non-activated
143 protoETX (50nM), which is capable of cellular binding, but is incapable of forming pores
144 within the cell membrane [16]. We visualized human-specific ETX binding by flow
145 cytometry using custom anti-ETX antibody, JL001.2 (Fig 1b).

146

147 Our previous work has shown that lipid raft protein, MAL (17 kDa, predicted), is both
148 necessary and sufficient for ETX binding and toxicity, thus we sought to determine if
149 human RBCs express MAL. Consistent with MAL's proposed role in ETX toxicity and
150 with previously published RBC membrane proteomic analysis [21], we corroborated that
151 human RBCs indeed express MAL; specifically a shortened isoform predicted to be
152 11kDa, MAL isoform C (Fig 1c). RBCs from refractory species, cow and rat, were both
153 negative for MAL expression. However, we cannot exclude the possibility that the
154 recognition antibody used may be specific for human MAL. Interestingly, there seemed
155 to be a trace expression of full length MAL (open arrowhead) in addition to the dominant
156 band appearing for shortened MAL isoform C (closed arrowhead).

157

158

159 **P2 receptor agonists and antagonists both inhibit ETX-mediated hemolysis**

160 While assessing the possible involvement of the P2 purinergic nucleotide receptors in
161 ETX-mediated hemolysis (as suggested by Gao et al.), we first exposed PBS-washed
162 whole human blood to: i) the irreversible P2 receptor antagonist, oxidized ATP (oxATP);
163 ii) P2 receptor agonists ATP and GTP and; iii) UMP, as a poorly interacting nucleotide
164 control, before treatment with ETX [22]. Surprisingly, we found that both P2 agonists
165 and P2 antagonists inhibited ETX-mediated hemolysis (Fig 2a). Furthermore, we found
166 that overnight incubation with oxATP and subsequent washout of this irreversible P2
167 inhibitor prior to ETX treatment largely abolished its anti-hemolytic effect (Fig 2b).
168 These results led us to search for alternative pathways capable of amplifying ETX-
169 mediated hemolysis in a nucleotide-sensitive fashion.

170

171 **O₂ and redox-active heavy metals, Cu⁺ and Fe³⁺, are involved in ETX-mediated**
172 **hemolysis**

173 A serendipitous finding, observed upon exposing ETX-treated blood to different
174 experimental conditions, was that atmospheric oxygen is required for hemolysis. One
175 concern was that anaerobiosis may be blocking a pathway crucial to ETX-mediated
176 hemolysis that is dependent on the electron-transport chain and oxidative
177 phosphorylation. To address this, we compared the hemolytic effect of ETX under
178 aerobic, anaerobic and sodium azide (10mM) conditions. Sodium azide was used as an
179 alternative method to inhibit oxidative phosphorylation in lieu of atmospheric oxygen
180 depletion. The results revealed that ETX-mediated hemolysis was inhibited under

181 anaerobic conditions, but not by oxidative phosphorylation inhibition via sodium azide
182 blockade (Fig 3a).
183
184 Considering the involvement of ambient O₂, we explored various sources of cellular free-
185 radical generation. A potent way for macromolecules to be specifically targeted and
186 damaged in an oxygen-dependent manner is by metal-catalyzed oxidation, where redox-
187 active transition metals coordinate with metal-binding amino acid residues, e.g., cysteine,
188 histidine and methionine, and locally generate reactive oxygen species (ROS) [23].
189 Along these lines, we pre-incubated ETX-treated human blood with the hydrophilic Cu⁺
190 chelator, tris-hydroxypropyltriazolylmethylamine (THPTA) and the hydrophilic Fe³⁺
191 chelator, deferoxamine (DFO), both of which significantly inhibited ETX-mediated
192 hemolysis. In contrast, the Cu²⁺ chelator, penicillamine (PncI), and the non-specific
193 metal chelator, citrate, were not effective at inhibiting hemolysis (Fig 3b). As an
194 alternative to “artificial” metal chelators, we also exposed ETX-treated blood to
195 “endogenous” metal chelators, such as the heavy metal-coordinating amino acids,
196 cysteine, histidine and methionine [24], each of which transiently inhibited ETX-
197 mediated hemolysis, cysteine > histidine > methionine. However, the control amino
198 acids, lysine, glutamate and glycine were significantly less effective (Fig 3c).
199
200 A metal-coordinating protein involved in how the RBC membrane interacts with
201 liberated Cu⁺ and Fe³⁺ is likely to bind more than one metallic species, and with varying
202 affinities. This notion led us to hypothesize that pretreatment of human blood with
203 redox-silent transition metals, such as Ni²⁺ and Mn²⁺, might protect RBCs from ETX-

204 mediated hemolysis by competing for crucial metal-binding sites on the RBC membrane.
205 Indeed, we observed that both Ni^{2+} and Mn^{2+} significantly inhibited ETX-mediated
206 hemolysis, $\text{Ni}^{2+} > \text{Mn}^{2+}$. Ca^{2+} also exhibited inhibitory properties, but to a lesser extent
207 when compared to the redox-silent transition metals (Fig 3d).

208

209 **The heavy metal binding, nucleotide-sensitive ICln chloride channel amplifies ETX-**
210 **mediated hemolysis**

211 The sensitivity of ETX-mediated hemolysis to the presence of nucleotides, ambient
212 oxygen and the extracellular chelation of redox-active transition metals led us to search
213 the literature for a surface molecule, which may be sensitive to these stimuli. We
214 identified the ubiquitously expressed, outwardly-rectifying ICln chloride channel as a
215 lead candidate. Under normal conditions, the ICln protein is found to be closely
216 associated with the actin cytoskeleton abutting the inner leaflet of the plasma membrane
217 [25]. Upon cellular swelling, as would be expected from ETX pore formation, ICln
218 rapidly inserts into the plasma membrane forming a channel that causes intracellular
219 chloride to flow out of the cell, against its diffusion gradient. This outward rectification
220 allows the cell to decrease its volume, thus avoiding osmolysis [26]. Remarkably, a key
221 histidine residue, His64, resides within the ICln pore. His64 coordinates with Ni^{2+} to
222 alter ICln's normal conductance [27]. We hypothesize that this regulatory, heavy metal-
223 binding residue may be the site at which redox-active Cu^+ and Fe^{3+} , previously liberated
224 by initial ETX pore formation, bind to and damage this volume-regulating channel. Of
225 note, ICln also possesses distinct Ca^{2+} binding sites, located near the extracellular
226 opening of the channel that allow for conductance inhibition by extracellular Ca^{2+} [27].

227

228 An alternate name for ICln is chloride channel nucleotide sensitive 1A (CLNS1A). As
229 the name suggests, the presence of extracellular nucleotides also regulates ICln
230 conductance, i.e., ATP and GTP inhibit ICln [28]. To further investigate ICln's potential
231 role in ETX-mediated hemolysis, we tested other distinct classes of ICln inhibitors,
232 namely the chromones (cromolyn and nedocromil) [29], and the cyclamate anion [30];
233 each of which successfully inhibited ETX-mediated hemolysis (Figs 4a and 4b). We
234 tested each class of ICln inhibitor in an aggregate experiment to provide a comparative
235 analysis (Fig 4c). Please note that we omitted the anti-retroviral nucleoside analogue
236 ICln inhibitors, acyclovir and AZT [31] due to poor solubility relative to the other ICln
237 inhibitors. Interestingly, when we assessed RBC ICln expression via Western blot, we
238 observed the occasional appearance of an ICln doublet (open arrowhead), consistent with
239 what has been observed in previous studies [25]. However, the doublet did not correlate
240 with changes in the ETX concentration (Fig 4d).

241

242 Because other pore-forming toxins that form similarly sized pores, e.g., *Staphylococcus*
243 *aureus* alpha toxin, have been reported to cause hemolysis via P2 receptor amplification
244 [32], we explored the possibility that ICln may also be a target for other toxins. Indeed,
245 when we pre-incubated human blood with a large panel of ICln inhibitors and heavy
246 metal chelators, we found that *S. aureus* alpha toxin also employed the ICln channel,
247 similar to what we had observed for ETX (Fig 4e).

248

249

250 **T cell lysosomal exocytosis contributes to ETX-mediated hemolysis**

251 Because human T-lymphocytes also express MAL [33, 34], we also wanted to explore
252 the possibility that T cells might contribute to ETX-mediated hemolysis. To assess
253 possible leukocyte involvement, we compared the rate of ETX-mediated hemolysis in
254 PBS-washed whole blood to that of PBS-washed RBCs that were leukocyte depleted (Fig
255 5a). The slowed rate of ETX-mediated hemolysis in the case of leukocyte depletion
256 prompted to us to confirm that MAL-expressing CD4⁺ T cells do indeed bind ETX (Fig
257 5b). In agreement with the literature, ETX binding suggests that human T cells express
258 MAL, while non-malignant B cells do not [35].

259

260 Blanch et al. have recently determined that ETX forms pores in the membranes of a
261 human T-lymphocyte cell line [36]. Moreover, they demonstrated that T-lymphocyte cell
262 lines are sensitive to ETX toxicity, while B-lymphocyte cell lines are not. We wished to
263 confirm that primary T cells are indeed susceptible to ETX. Therefore, we isolated CD3⁺
264 T cells and exposed them to ETX (50nM) for 1 hour at 37°C and assessed cell death by
265 propidium iodide (PI) uptake. Isolated CD20⁺ B-lymphocytes were used as a control
266 lymphocyte population. Results revealed that a subset of human T cells is sensitive to
267 ETX-mediated damage, while human B cells are completely refractory (Fig 5c).

268

269 One way that nucleated cells can defend against pore-forming toxins is via lysosomal
270 exocytosis [37, 38]. However, this pathway has been described for toxins that form
271 relatively large pores (> 2nm in diameter) in the plasma membrane. For example,
272 streptolysin O allows the influx of Ca²⁺, which then triggers the lysosomal exocytosis

273 pathway and lysosomal hydrolase-mediated membrane internalization and repair [37, 39].
274 Toxins similar to ETX that form much smaller pores, such as *Staphylococcus aureus*
275 alpha toxin, have not previously been reported to trigger lysosomal exocytosis.
276 Nucleated cells exposed to small pore formers are thought to first internalize these toxins
277 and then expel them in a membrane-bound exosome-like form [40]. However, the
278 exocytosis machinery has not been clearly identified. Along these lines, we wished to
279 determine if the lysosomal exocytosis pathway might be involved in how nucleated cells,
280 e.g., human T cells, process small pore-forming toxins such as ETX. Intriguingly, similar
281 to *Staphylococcus aureus* alpha toxin, ETX has been reported to undergo internalization
282 soon after binding the plasma membrane of nucleated cells [41].

283

284 Administration of ETX (20nM) to human T cells caused significant surface expression of
285 lysosome associated membrane protein-1 (LAMP1), a marker for the limiting membrane
286 of the lysosome, and thus a marker for lysosomal exocytosis [39]. To determine if
287 normal lysosomal function is required for ETX-mediated lysosomal exocytosis, we
288 disrupted lysosomal acidification and subsequent function, by exposing ETX-treated T
289 cells to chloroquine, a lysosomotropic agent that strongly suppressed ETX-mediated
290 lysosomal exocytosis (Fig 5d). Chloroquine preferentially accumulates in acidic
291 compartments of the cell and prevents acidification because of its protonated basic amine
292 groups [42, 43].

293

294 Remarkably, lysosomal exocytosis has previously been proposed as a hemolytic
295 mechanism in the case of anti-Rh sensitized RBCs and engaging monocytes [44]. In this

296 previous study, the drugs colchicine (a microtubule depolymerizing agent), and
297 hydrocortisone hemisuccinate (a corticosteroid), suppressed monocyte-mediated
298 hemolysis. The authors suggested that the anti-hemolytic effect of these drugs was the
299 result of suppressing monocyte lysosomal exocytosis [45]. Similarly, we wished to
300 determine if blockade of lysosomal exocytosis by chloroquine or other inhibitory
301 compounds, colchicine and hydrocortisone hemisuccinate, was sufficient to inhibit ETX-
302 mediated hemolysis. Indeed, we found that each of these compounds inhibited ETX-
303 mediated hemolysis, chloroquine > hydrocortisone > colchicine (Fig 5e).

304

305 **DISCUSSION**

306 We have confirmed the findings of Gao et al. that human blood exposure to *C.*
307 *perfringens* epsilon toxin results in hemolysis. However, our data extend these findings
308 and suggest that metal-catalyzed oxidation of the nucleotide-sensitive, volume-regulating
309 ICln channel is likely responsible for amplifying the hemolytic process, rather than P2
310 receptor activation. Moreover, we find that ICln may be involved in hemolytic
311 amplification for pore-forming toxins beyond ETX, e.g., *S. aureus* alpha toxin and
312 perhaps others. An important experimental distinction between this study and that of Gao
313 et al. is the substantial difference in the concentration of ETX used. Our hemolytic
314 assays were conducted at 15nM ETX, while Gao et al. used ≥ 100 nM ETX [20].

315

316 We have also shown that human RBCs express a minor MAL isoform (likely MAL
317 isoform C), which would explain why human RBCs are uniquely susceptible to ETX-
318 mediated damage, as compared to RBCs from other species that do not express MAL.

319 Similarly, ETX also damages MAL-expressing human T cells, causing them to undergo
320 lysosomal exocytosis. Our data also show that lysosomal exocytosis contributes to ETX-
321 mediated hemolysis, as evidenced by hemolytic blockade by lysosomal inhibitors such as
322 chloroquine. Considering chloroquine's ability to increase pH in a general fashion, we
323 suspect that it may have a dual effect on inhibiting ETX-mediated hemolysis, as ICln's
324 ion conductance is pH sensitive [27]. Along these lines, we have observed that
325 conducting hemolysis assays in bicarbonate buffer also results in slowed ETX-mediated
326 hemolysis (data not shown). Therefore, the inhibitory action of hydrocortisone and
327 colchicine might more accurately reflect the true contribution of T cell lysosomal
328 exocytosis.

329

330 To better understand how lysosomal exocytosis might influence ETX-mediated
331 hemolysis, we reviewed the literature and identified that lysosomes serve as storage
332 compartments for redox-active heavy metals such as Cu^+ and Fe^{3+} [46]. For example, the
333 copper transporter ATP7B actively transports Cu^+ into the late endosome, which later
334 fuses with the lysosome [47]. Furthermore, an excess of extracellular copper stimulates
335 lysosomal copper uptake, and the cell can go on to release accumulated copper into the
336 extracellular space via expulsion through the lysosomal exocytosis pathway [48]. For
337 these reasons, we favor the idea that ETX triggers the release of previously stored redox-
338 active heavy metals from the lysosomal compartment of MAL-expressing T cells, which
339 are predominantly of the CD4+ lineage [35]. To help visualize this process, we have
340 composed a diagram to illustrate the proposed mechanism in a step-wise fashion (Fig 6),

341 while figure 7 aims to illustrate a more comprehensive schema for ETX-mediated
342 hemolysis.
343
344 The notion that ICln must first insert into the RBC membrane for metal-catalyzed
345 oxidation to ensue might lead to the prediction that supernatant harvested from ETX-
346 exposed T cells would not be sufficient to trigger ETX-mediated hemolysis, and indeed,
347 we find this to be the case (Supp Fig 4). However, an important caveat to this
348 experiment is that Cu^+ ions are unstable in aqueous solution due to rapid oxidation
349 (oxygen-dependent) and rapid disproportionation (oxygen-independent), and thus must
350 be chelated in the cytosol by chaperones such as glutathione that are largely absent in the
351 extracellular space [49]. This lack of extracellular stability could lead to a proximity
352 requirement for toxin-exposed T cells to have a hemolytic effect on neighboring RBCs.
353 To address this caveat, we transplanted isolated human leukocytes into blood harvested
354 from non-human species, i.e., cow and rhesus macaque; RBCs that have been shown to
355 either lack MAL (cow) or that fail to bind ETX (macaque). ETX-sensitive human
356 leukocytes were unable to trigger hemolysis in non-human RBCs, even when exposed to
357 150nM ETX (Supp Fig 5).
358
359 In its entirety, this study may help to shed light on how RBCs incur damage during an
360 acute MS relapse because ETX, a blood-borne neurotoxin, has previously been indicated
361 as a potential MS trigger due to its remarkable specificity for BBB vasculature and for
362 CNS myelin [9,10]; the two tissues damaged during each MS attack. The finding that
363 ETX also causes RBC abnormalities, reminiscent of what has been observed during

364 active MS, may further support the ETX-MS hypothesis. Of note, even though we
365 assessed ETX-mediated hemolysis in > 70 study participants, we did not observe any
366 ETX-resistant phenotypes, suggesting that all blood types tested were ETX-susceptible
367 and express MAL. To our knowledge, MAL expression by circulatory cells has not been
368 reported in any non-human species to date, thus blood cell sensitivity to ETX may be an
369 exclusively human trait.

370

371 Should ETX be conclusively shown to trigger nascent MS lesion formation, ICIn
372 insertion into the RBC plasma membrane and surface LAMP1 expression on CD4+ T
373 cells may lay the groundwork for novel biomarker development. To date, there are no
374 biomarkers that can predict MS disease activity. The identification of such biomarkers
375 may be useful in predicting the onset of neurological symptoms and/or identifying occult
376 disease activity, as is the case for “silent lesions,” which are detectable on MRI but yield
377 no observable symptoms [50].

378

379 In addition to surface LAMP1 expression, lysosomal exocytosis also causes the release of
380 lysosomal hydrolases into the extracellular milieu, and the enzymatic activity of acid
381 hydrolases such as β -hexosaminidase are commonly used as markers for this cellular
382 process [48]. These enzymes may also be candidate biomarkers for ETX blood exposure
383 and early disease activity in multiple sclerosis.

384

385 Finally, identifying the mechanism by which ETX causes RBC lysis may allow for novel
386 clinical interventions. For example, metal chelation therapy might inhibit hemolysis, thus

387 preventing vascular iron deposition and neuronal toxicity/axonal loss. Although
388 hemolysis and free hemoglobin have recently been shown to correlate with the transition
389 from RRMS to SPMS [8], the root cause of this hemolysis has not yet been identified. *C.*
390 *perfringens* epsilon toxin may adequately explain this phenomenon, in addition to how
391 nascent MS lesions form in the absence of an inflammatory infiltrate [9, 51].

392

393 **MATERIALS AND METHODS**

394 **Ethics Statement**

395 Research protocol RRU-0952 for the collection of samples from individuals with MS and
396 healthy controls was reviewed and approved by the Rockefeller University institutional
397 review board. All participants in the study gave written informed consent.

398

399 **Epsilon Toxin**

400 His-tagged protoxin was procured from BEI Resources, activated by adding an equal
401 volume of 0.25% trypsin (ThermoFisher) and incubating for 1 hour at 37°C. Trypsin was
402 then inactivated by the addition of 1:1 volume Defined Trypsin Inhibitor (ThermoFisher).

403

404 **Blood Sample Collection and Manipulation**

405 Human blood from healthy adult donors was obtained at the Rockefeller University
406 hospital using heparinized tubes. Cow, goat, sheep, rat, guinea pig and rhesus macaque

407 blood and enriched human, cow and rat RBCs were all purchased from Innovative
408 Research, Inc. All whole blood and enriched RBC samples were centrifuged for 5 mins
409 at 600g, the supernatant was aspirated, and the cell pellet was washed with PBS (20x the
410 original volume) prior to re-suspension in PBS so as to match the original starting blood
411 volume. For experiments using transition metals nickel and manganese, human blood was
412 washed as previously described. However, normal saline was used instead of PBS to
413 avoid the formation of insoluble $\text{Ni}_3(\text{PO}_4)_2$ and $\text{Mn}_3(\text{PO}_4)_2$ salts.

414 For the transfer of human leukocytes to the blood of non-human mammals, human blood
415 was centrifuged at 600 x g for 5 mins and the plasma layer removed. Pelleted cells were
416 suspended in 50 volumes of phosphate buffered saline (PBS), centrifuged at 1000 x g,
417 and suspended in RBC lysis buffer (155 mM NH_4Cl , 12 mM NaHCO_3 , 0.1 mM EDTA).
418 RBCs were allowed to lyse for 5 mins, after which, leukocytes were centrifuged at 600 x
419 g for 5 mins. Pelleted leukocytes were washed 3 times in PBS and transferred to washed
420 whole cow or whole macaque blood of a similar original volume.

421

422 **Hemolysis Quantitation**

423 After ETX incubation, cells were pelleted at 600g for 5mins, supernatant was harvested
424 and the degree of hemolysis was determined by light absorbance (OD 540nm) using a
425 SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices).

426

427 **Lymphocyte Isolation**

428 Harvested human blood was incubated with RosetteSep Human T cell enrichment
429 cocktail (STEMCELL Technologies) or RosetteSep Human B cell enrichment cocktail
430 (STEMCELL Technologies) and then isolated with Ficoll-Paque Plus (GE Healthcare),
431 as per the manufacturer's instructions.

432

433 **Flow Cytometry Staining and Analysis**

434 For protoETX binding, isolated cells were washed 3 times with PBS 1% BSA buffer, and
435 incubated for 2 hours at 4°C with 5% donkey serum (PBS 1% BSA) containing either
436 50nM or 0nM protoETX. Cells were washed 3 times with chilled PBS 1% BSA buffer,
437 and then stained for 1 hour at 4°C with primary antibodies: rabbit anti-ETX (JL001.2,
438 1:1000), mouse anti-CD3 Alexa 660 (eBioscience) 1:200 or mouse anti-CD20 APC
439 (eBioscience) 1:200. After 3 washes with chilled PBS 1% BSA, cells were then
440 incubated with Alexa 488-conjugated donkey anti-rabbit (1:1000, Jackson
441 ImmunoResearch) so as to detect bound JL001.2 antibody, and then thoroughly washed
442 prior to Flow Cytometry analysis. For cytotoxicity assays, isolated lymphocytes were
443 stained with mouse anti-CD3 Alexa 660 (1:200) or mouse anti-CD20 APC (1:200), and
444 propidium iodide (ThermoFisher) 1:500. For lysosomal exocytosis assays, isolated T
445 cells were stained with mouse anti-LAMP1 Alexa 488 (ThermoFisher) 1:200. All cells
446 were analyzed by Flow Cytometry with BD AccuriC6 at our core facility.

447

448 **Western blot Analysis of RBC membrane proteins**

449 100 μ L of purified RBCs were lysed in 900 μ L ammonium chloride RBC lysis buffer for
450 10 mins at 37°C. The cell lysate was centrifuged at > 16,000g for 5 mins, and the pellet
451 was washed 3 times in PBS and re-suspended in 50 μ L PBS. An equal volume of 2X
452 Laemmli sample buffer (Bio-Rad) was added to each sample and the dissolved
453 membranes were stored at -20°C. Thawed samples were diluted 2.5 fold in 1X Laemmli
454 sample buffer prior to SDS PAGE. Proteins were transferred to an Immobilon-P
455 membrane (MilliporeSigma) and probed with either mouse-anti MAL 1:1000 (clone E-1,
456 Santa Cruz Biotechnology, Inc.) or rabbit anti-ICln 1:1000 (PA5-13450, ThermoFisher).
457 HRP-conjugated donkey anti-mouse IgG and donkey anti-rabbit IgG secondaries
458 (Jackson ImmunoResearch) were used with the corresponding primary antibody to
459 visualize MAL expression and ICln expression respectively (1:100,000). SuperSignal
460 West Femto Maximum Sensitivity Substrate (ThermoFisher) was used to visualize bound
461 antibody.

462

463 **Anaerobiosis**

464 Anaerobic experiments were performed in an anaerobic hood (BACTRON Anaerobic
465 Chamber).

466

467 **Drugs and Compounds**

468 All drugs and compounds used in this study were purchased from Sigma Aldrich.

469

470 **Statistical Analysis**

471 Results are representative of data obtained from repeated independent experiments. Each
472 value represents the mean \pm *SD* for three replicates. Statistical analysis was performed
473 using the two-tailed Student *t*-test (GraphPad Software, San Diego, CA, USA).

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694

Figure Legends

695

696 **Fig 1. ETX causes human-specific hemolysis via human RBC MAL expression.**

697 **a)** Washed whole human, cow, sheep, goat and guinea pig blood was suspended in PBS
698 and incubated with 15nM ETX at 37°C, and sampled over 24 hours. Data shown are
699 from experiments performed in triplicate. Error bars represent standard deviations, and
700 asterisks indicate that results are statistically significant compared with refractory guinea
701 pig blood (dark blue); Student's *t*-test, **P* < 0.0001. **b)** Human, rhesus macaque, rat and
702 sheep RBCs were incubated with non-toxic protoETX (50nM) for 2 hours at 4°C. Toxin
703 binding was detected via flow cytometry using custom anti-ETX antibody, JL001.2.
704 Data shown are from a single experiment and are representative of 3 independent
705 experiments, using 3 distinct blood donors. **c)** Human, cow and rat RBC membranes were
706 analyzed for MAL expression by Western blot (left). RBC membranes harvested from
707 additional human donors were also analyzed (right). The open arrowhead signifies full
708 length MAL, while the closed arrowhead signifies the shortened MAL isoform.

709

710 **Fig 2. Extracellular nucleotides inhibit ETX-mediated hemolysis.**

711 **a)** Washed whole human blood was pre-incubated with oxidized ATP (oxATP), ATP,
712 GTP and UMP (10mM each) prior to ETX exposure (15nM) at 37°C, and sampled over
713 24 hours. Data shown are from experiments performed in triplicate. Error bars represent
714 standard deviations, and asterisks indicate that results are statistically significant
715 compared with PBS vehicle control (gray); Student's *t*-test, **P* < 0.0002. **b)** Washed
716 whole human blood was exposed to the irreversible P2 receptor inhibitor, oxATP,

717 overnight at 37°C. OxATP was either allowed to remain in the suspension buffer
718 (oxATP 10mM) or washed out. All samples were then incubated with ETX (15nM) at
719 37°C, and sampled over 24 hours. Data shown are from experiments performed in
720 triplicate. Error bars represent standard deviations, and asterisks indicate that results are
721 statistically significant compared with PBS vehicle control (green); Student's *t*-test, **P* <
722 0.0002.

723

724 **Fig 3. Oxygen and redox-active transition metals, Cu⁺ and Fe³⁺, are integral to ETX-**
725 **mediated hemolysis. a)** Washed whole human blood was suspended in PBS and
726 exposed to 15nM ETX at 37°C under anaerobic conditions, aerobic conditions or co-
727 incubated with sodium azide (10mM) and sampled over 24 hours. Data shown are from
728 experiments performed in triplicate. Error bars represent standard deviations, and
729 asterisks indicate that results are statistically significant compared with aerobic control
730 (green); Student's *t*-test, **P* < 0.0001.

731 **b)** Washed whole human blood was exposed to ETX (15nM) at 37°C over 24 hours, and
732 co-incubated with metal chelators (5mg/mL each), deferoxamine (DFO), THPTA,
733 penicillamine (Pncl), citrate or PBS buffer control. Data shown are from experiments
734 performed in triplicate. Error bars represent standard deviations, and asterisks indicate
735 that results are statistically significant compared with PBS vehicle control (gray);
736 Student's *t*-test, **P* < 0.0003. **c)** The inhibitory effects of endogenous metal chelating
737 amino acids (5mg/mL each), cysteine, histidine and methionine were compared to that of
738 non-chelating amino acids (5mg/mL each), lysine, glutamate and glycine. All samples
739 were exposed to ETX (15nM) at 37°C and sampled over 30 mins. Data shown are from

740 experiments performed in triplicate. Error bars represent standard deviations, and
741 asterisks indicate that results are statistically significant compared with glycine (farthest
742 right); Student's *t*-test, $*P < 0.01$. **d)** Washed whole human blood was suspended in
743 isotonic saline with or without divalent cations (15mM each), Ni²⁺ or Mn²⁺ (redox-silent
744 transition metals) or Ca²⁺ (alkaline earth metal), and assessed for inhibition of ETX-
745 mediated hemolysis (15nM ETX at 37°C) over 24 hours. Data shown are from
746 experiments performed in triplicate. Error bars represent standard deviations, and
747 asterisks indicate that results are statistically significant compared with NaCl vehicle
748 control (green); Student's *t*-test, $*P \leq 0.0003$.

749

750 **Fig 4. Inhibitors of the nucleotide-sensitive, heavy metal-binding ICln chloride**
751 **channel block pore forming toxin-mediated hemolysis. a)** Washed whole human blood
752 was pre-incubated the chromone class of ICln inhibitors, cromolyn and nedocromil
753 (5mg/mL) prior to ETX exposure (15nM at 37°C), and sampled over 24 hours. Data
754 shown are from experiments performed in triplicate. Error bars represent standard
755 deviations, and asterisks indicate that results are statistically significant compared with
756 PBS vehicle control (green); Student's *t*-test, $*P < 0.0004$. **b)** Washed whole human
757 blood was pre-incubated with the ICln inhibitor, cyclamate anion (154mM) or acetate as
758 a control organic anion (154mM) prior to ETX exposure (15nM at 37°C), and sampled
759 over 24 hours. Data shown are from experiments performed in triplicate. Error bars
760 represent standard deviations, and asterisks indicate that results are statistically
761 significant compared with the NaCl control (green); Student's *t*-test, $*P < 0.0001$. **c)**
762 Washed whole human blood was pre-incubated with a full panel of known ICln

763 inhibitors, ATP, oxATP, cromolyn (5mg/mL each); sodium cyclamate (154mM); and
764 NiCl₂ (15mM) prior to ETX exposure (15nM at 37°C), and sampled over 24 hours. Data
765 shown are from experiments performed in triplicate. Error bars represent standard
766 deviations, and asterisks indicate that results are statistically significant compared with
767 the NaCl control (blue); Student's *t*-test, **P* < 0.0001. **d)** Human RBC membranes were
768 analyzed for differential ICln expression in the setting of increasing ETX concentrations
769 by Western blot analysis. Arrowheads signify individual components of the ICln
770 monomer (closed) and its doublet (open). Data shown are from a single experiment and
771 are representative of 3 independent experiments, using 3 distinct blood donors. **e)**
772 Washed whole human blood was exposed to P2 receptor inhibition (green asterisks *), a
773 full panel of ICln inhibitors (red asterisks *) and redox-active metal chelators (blue
774 asterisks *) prior to being exposed to a hemolytic dose of *S. aureus* alpha-toxin (525nMα
775 at 37°C) and sampled over 24 hours. Data shown are from experiments performed in
776 triplicate. Error bars represent standard deviations, and black asterisks indicate that
777 results are statistically significant compared with the NaCl as a control for NiCl₂ and
778 NaCyclamate, and PBS as a vehicle control for all nucleotides and metal chelators;
779 Student's *t*-test, **P* < 0.0007.

780

781 **Fig 5. Leukocytes contribute to hemolysis via ETX-triggered T cell lysosomal**
782 **exocytosis.**

783 **a)** Leukocyte-depleted human blood was washed and suspended in PBS, incubated with
784 15nM ETX at 37°C and sampled over 24 hours. Hemolysis was compared to that of
785 washed whole human blood incubated under the same experimental conditions. Data

786 shown are from experiments performed in triplicate. Error bars represent standard
787 deviations, and asterisks indicate that results are statistically significant compared with
788 human blood (blue); Student's *t*-test, $*P < 0.0001$. **b)** Isolated B cells, T cells and
789 human RBCs were incubated with non-toxic protoETX (50nM) for 2 hours at 4°C. Toxin
790 binding was detected via flow cytometry using custom anti-ETX antibody, JL001.2.
791 Data shown are from a single experiment and are representative of 3 independent
792 experiments, using 3 distinct blood donors. **c)** Isolated T and B cells were exposed
793 activated ETX (50nM) for 1 hour at 37°C and assessed for membrane integrity by
794 propidium iodide (PI) uptake. Data shown are from a single experiment and are
795 representative of 3 independent experiments, using 3 distinct blood donors. **d)** Isolated T
796 cells were exposed to activated ETX (20nM) for 1 hour at 37°C with or without the
797 lysosomotropic agent, chloroquine (ChlQ). Data shown are from a single experiment and
798 are representative of 3 independent experiments, using 3 distinct blood donors. **e)**
799 Washed whole human blood was suspended in PBS and incubated with different
800 inhibitors of lysosomal exocytosis, chloroquine, hydrocortisone hemisuccinate and
801 colchicine, 4 hours prior to ETX administration. All samples were then treated with ETX
802 (15nM), incubated at 37°C, and sampled over 24 hours. Data shown are means and SD
803 from a single experiment and are representative of 3 independent triplicate experiments.
804 Asterisks indicate that results are statistically significant compared with PBS vehicle
805 control (purple); Student's *t*-test, $*P < 0.005$.

806

807

808

809 **Fig 6. A schematic illustration of ETX-mediated lysosomal exocytosis**

810 **1)** Epsilon toxin (ϵ) binds to the T cell plasma membrane via its cellular receptor
811 (possibly Myelin And Lymphocyte protein, MAL). **2)** Seven ETX subunits form a pre-
812 pore complex at the cell surface. **3)** The heptameric pre-pore matures into a
813 transmembrane pore (cylinder). **4)** Pore formation triggers membrane invagination and
814 endosome formation. **5)** As the late endosome matures, ETX-containing intraluminal
815 vesicles (ILV) begin to form. Concurrently, cytosolic Cu^+ ions are being transported into
816 the late endosome via the ATP7B copper transporter. **6)** Upon becoming a mature
817 lysosome, fusion occurs between the lysosomal membrane and the plasma membrane,
818 resulting in lysosomal exocytosis. Lysosomal exocytosis releases exosome-like, ETX-
819 containing microparticles (Exo), similar to what has previously been described for toxins
820 that form small pores ($< 2\text{nm}$ in diameter) such as *S. aureus* alpha toxin [40], into the
821 extracellular space. In addition to releasing ETX-containing microparticles, redox-active
822 heavy metals stored in the lysosome such as Cu^+ are also released.

823

824 **Fig 7. A schematic illustration of ETX-mediated RBC lysis**

825 **1)** Epsilon toxin (ϵ) binds to the human RBC membrane via a cellular receptor (possibly
826 MAL isoform C). **2)** Oligomerized toxin forms a pore and inserts into the RBC
827 membrane, allowing unregulated influx of ions and water. **3)** Cellular swelling causes
828 rapid insertion of the ICln chloride channel as a countermeasure to resist osmolysis,
829 resulting in the efflux of ions and water. **4)** The ETX pore facilitates the release of
830 intracellular redox-active heavy metals from both the T cell (Cu^+) and RBC cytosol
831 (Fe^{3+}). **5)** Extracellular heavy metals bind to and damage the ICln pore in an oxygen-

832 dependent fashion. **6)** Metal-catalyzed oxidation of the ICln pore results in a deregulated
833 channel that allows ions and water to flow down their diffusion gradient resulting in
834 eventual osmolysis.

835

836 **Supplemental Fig 1. SDS PAGE analysis of His-tagged protoETX**

837 His-tagged prototoxin (BEI Resources) was diluted in PBS and combined with 2X
838 laemmli sample buffer (1:1 volume), heated at 90°C for 6 minutes and subjected to
839 electrophoresis on a 4-12% Bis-Tris gel. Proteins were stained using a colloidal blue
840 protein staining kit.

841

842 **Supplemental Fig 2. ProtoETX requires trypsin activation to gain hemolytic**

843 **activity.**

844 Washed whole human blood was exposed to trypsin activated ETX (15nM) or non-toxic
845 protoETX (15nM), incubated at 37°C, and sampled over 24 hours. Data shown are means
846 and SD from a single experiment and are representative of 3 independent triplicate
847 experiments. Asterisks indicate that results for protoETX (blue) are significant different
848 when compared to active ETX (red); Student's *t*-test, **P* < 0.0001.

849

850 **Supplemental Fig 3. Neutralizing anti-ETX monoclonal JL008 inhibits ETX-**

851 **mediated hemolysis in a dose-dependent manner. a)** Washed whole human blood was
852 pre-incubated with a neutralizing anti-ETX rabbit monoclonal (JL008) at varying
853 concentrations (10^{-2} , 10^{-3} and 10^{-4}) and compared to a rabbit isotype control antibody (Rb
854 IgG), and PBS vehicle control. All samples were treated with ETX (15nM), incubated at

855 37°C, and sampled over 24 hours. Data shown are means and SD from a single
856 experiment and are representative of 3 independent triplicate experiments. Asterisks
857 indicate that results are statistically significant compared with PBS vehicle control (light
858 blue); Student's *t*-test, **P* < 0.0007.

859

860 **Supplemental Fig 4. Supernatant from ETX-exposed human T cells is not sufficient**
861 **to trigger hemolysis.** Human T cell isolates were treated with ETX (30nM) at 37°C for
862 1 hour in PBS buffer (1% BSA). Supernatant was harvested and unbound ETX was
863 neutralized with anti-ETX rabbit monoclonal, JL008 (10⁻²). Alternatively, the supernatant
864 was incubated with a control rabbit isotype control antibody, Rb IgG (10⁻²). Supernatants
865 were added to washed whole human blood and incubated at 37°C, and sampled over 24
866 hours. Data shown are means and SD from a single experiment and are representative of
867 3 independent triplicate experiments. Asterisks indicate that results are statistically
868 significant compared with the positive control, 30nM ETX (purple); Student's *t*-test, **P* <
869 0.0001.

870

871 **Supplemental Fig 5. Transfer of human leukocytes into non-human, ETX-resistant**
872 **blood, fails to confer ETX hemolytic sensitivity.** Human leukocytes were isolated and
873 transferred into washed whole cow or whole rhesus macaque blood, incubated with
874 150nM ETX at 37°C, and sampled over 24 hours. Data shown are means and SD from a
875 single experiment and are representative of 3 independent triplicate experiments.
876 Asterisks indicate that results are statistically significant compared with ETX (150nM)
877 treated, washed whole human blood (blue); Student's *t*-test, **P* < 0.0001.

Figure 1a

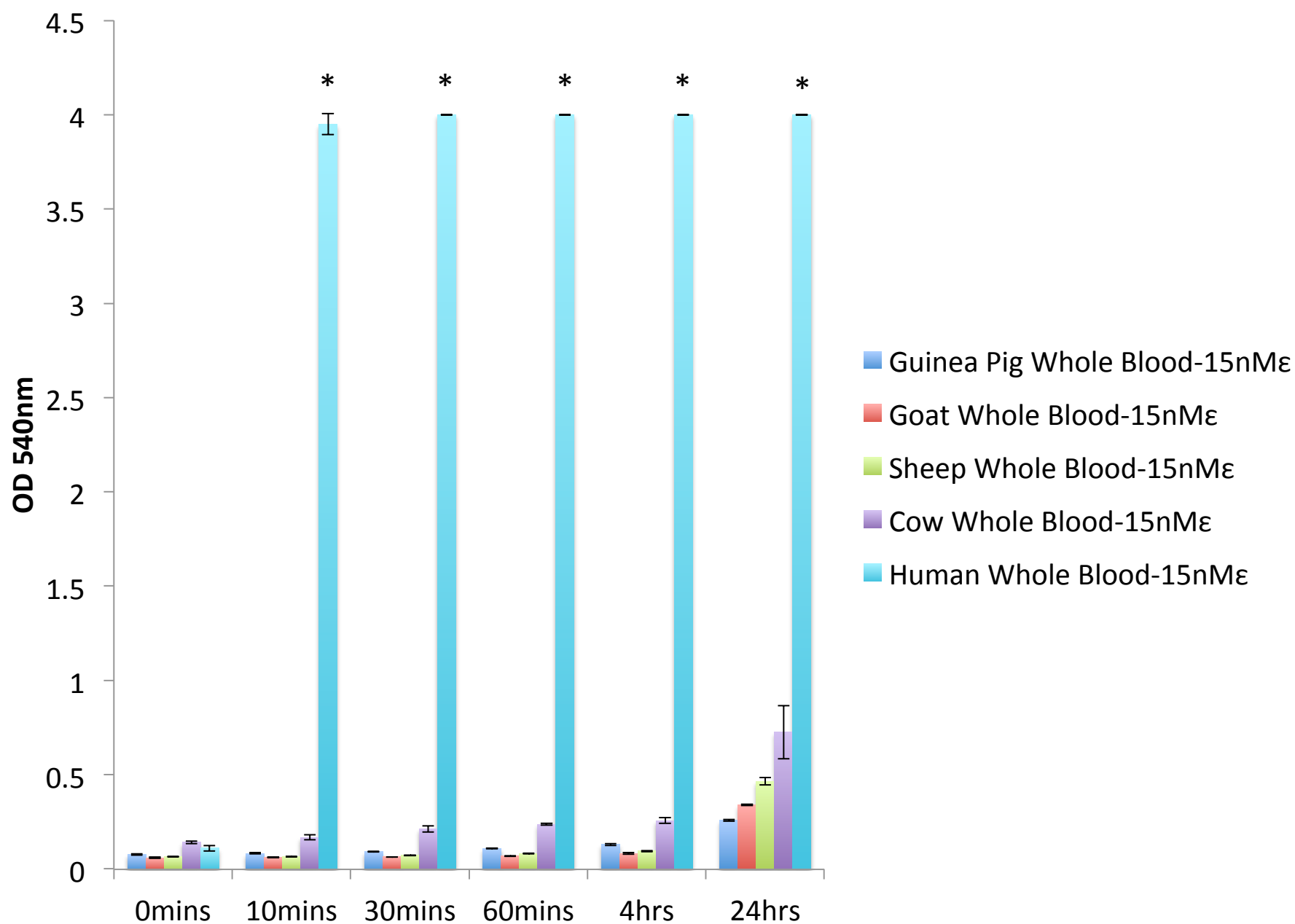


Figure 1b

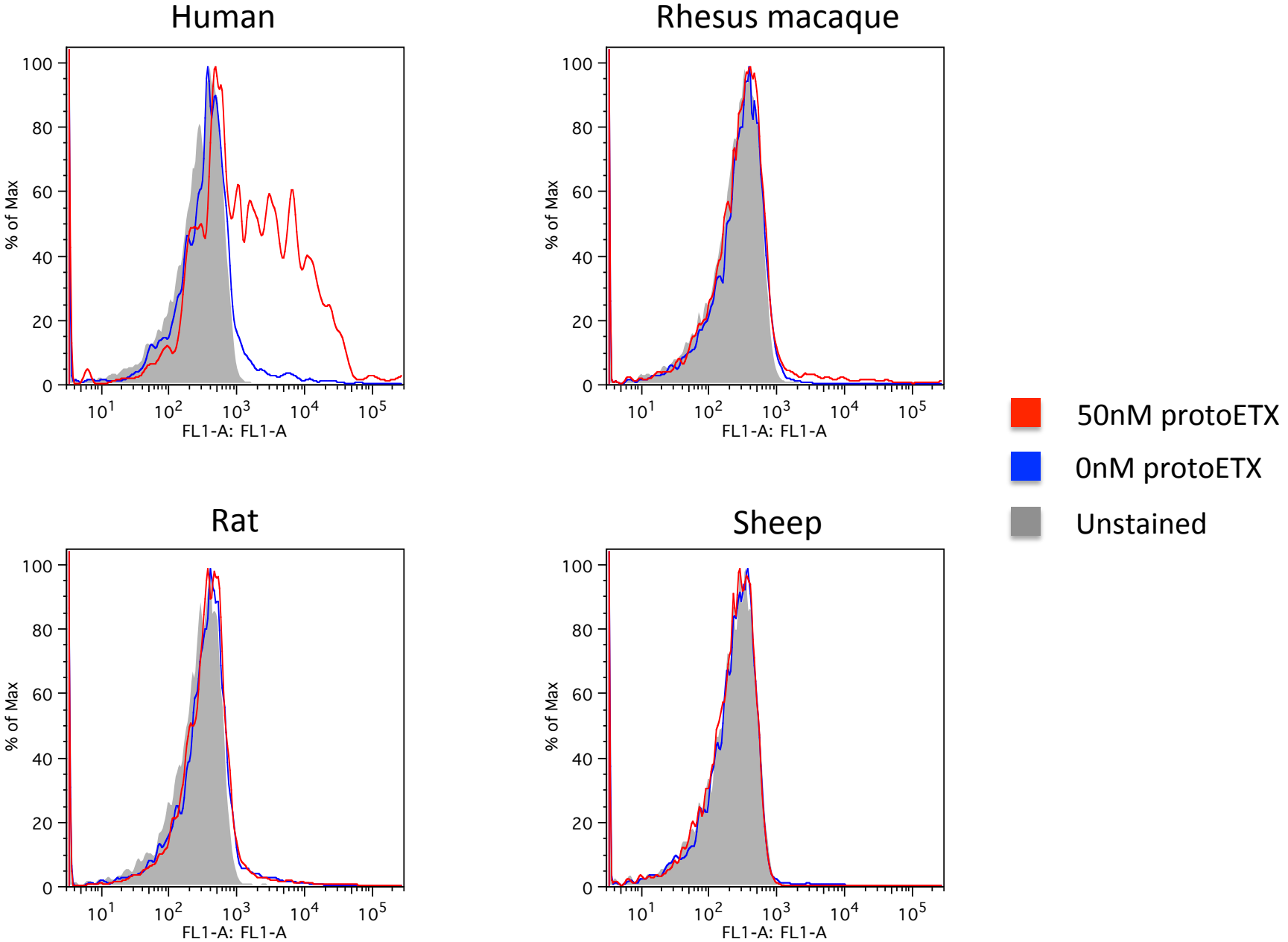


Figure 1c

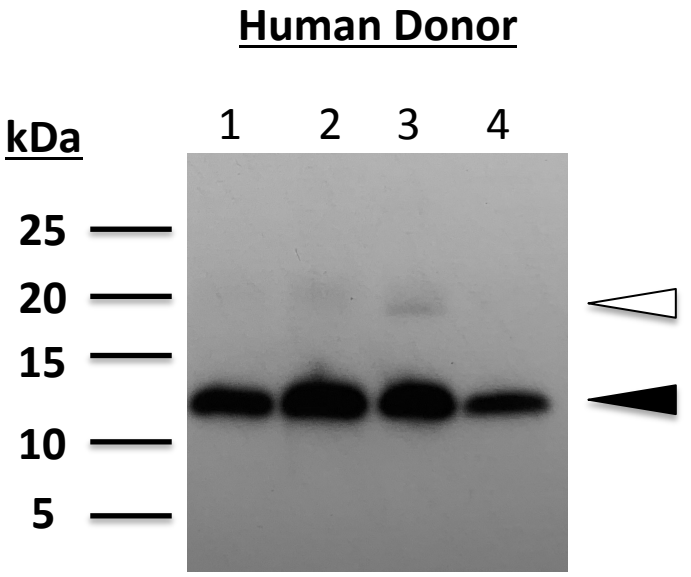
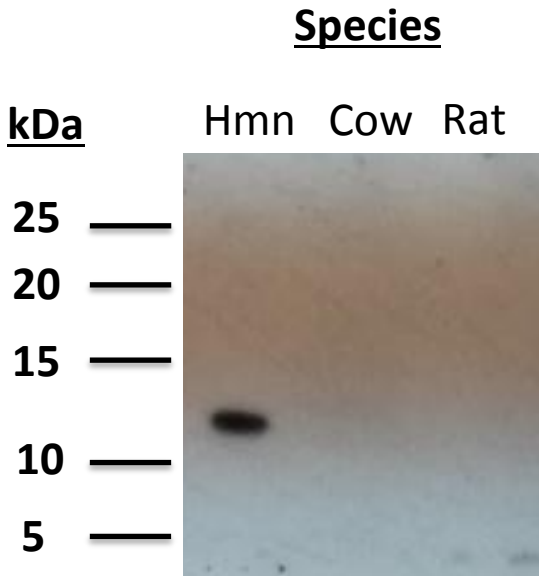


Figure 2a

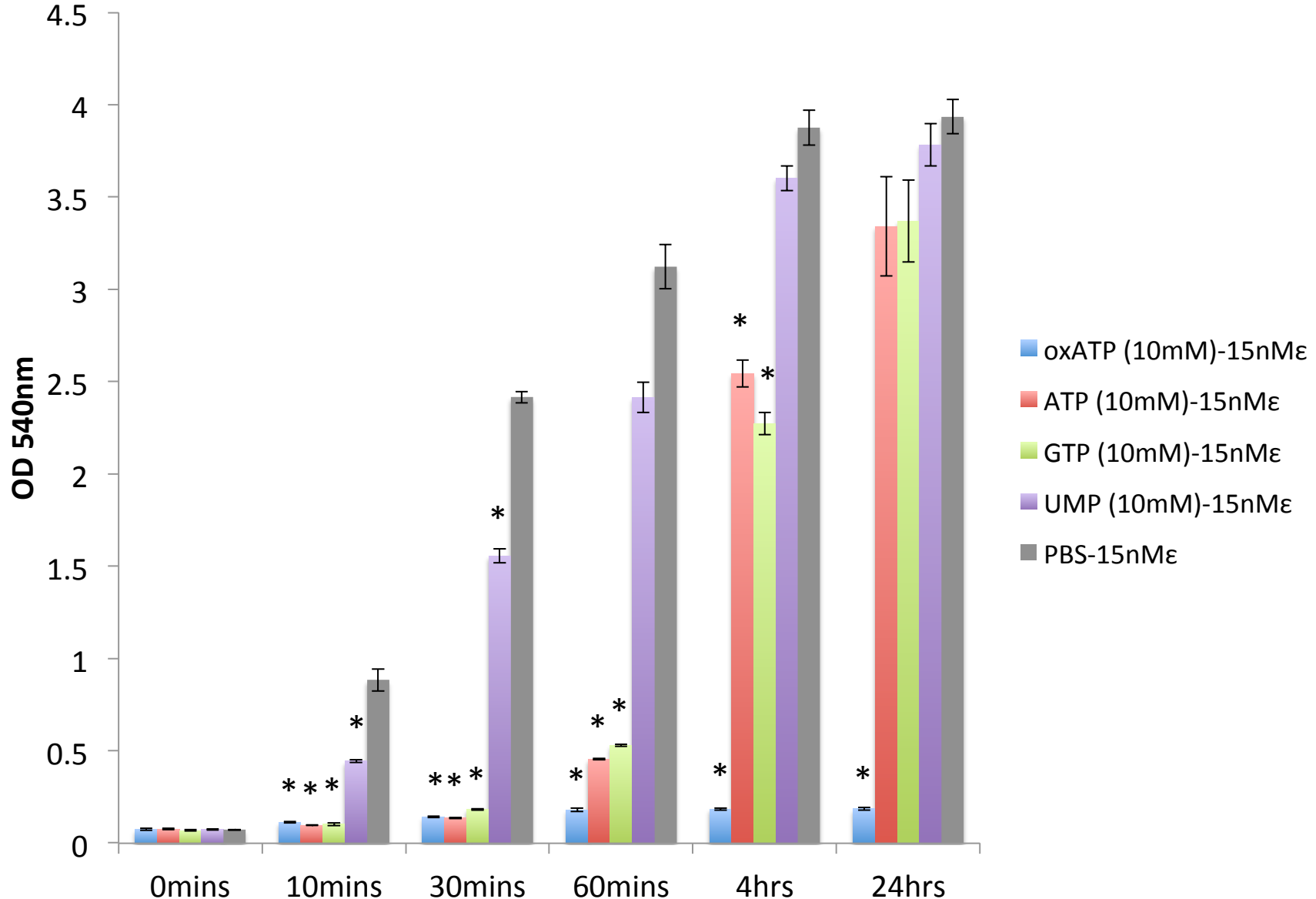


Figure 2b

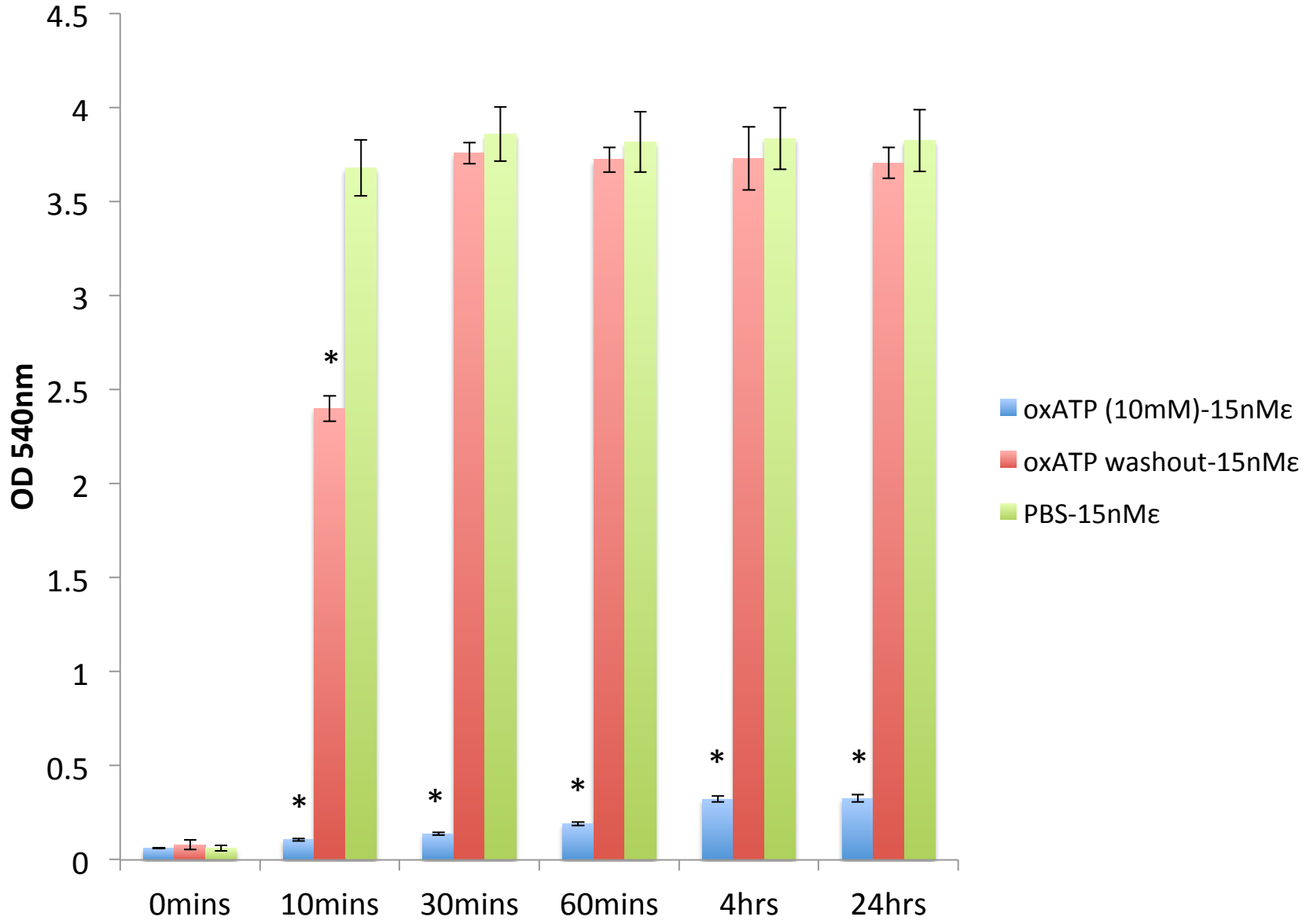


Figure 3a

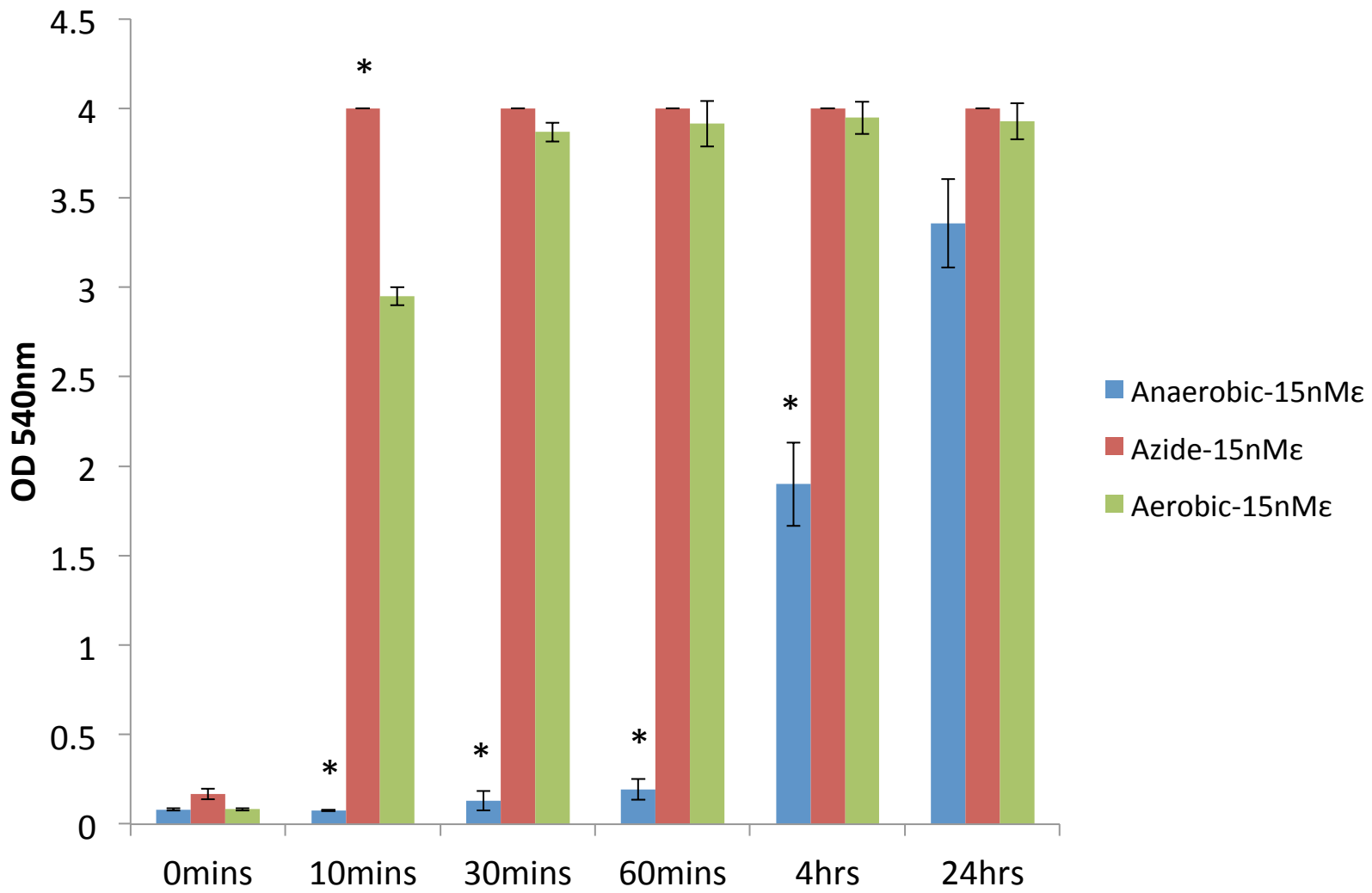


Figure 3b

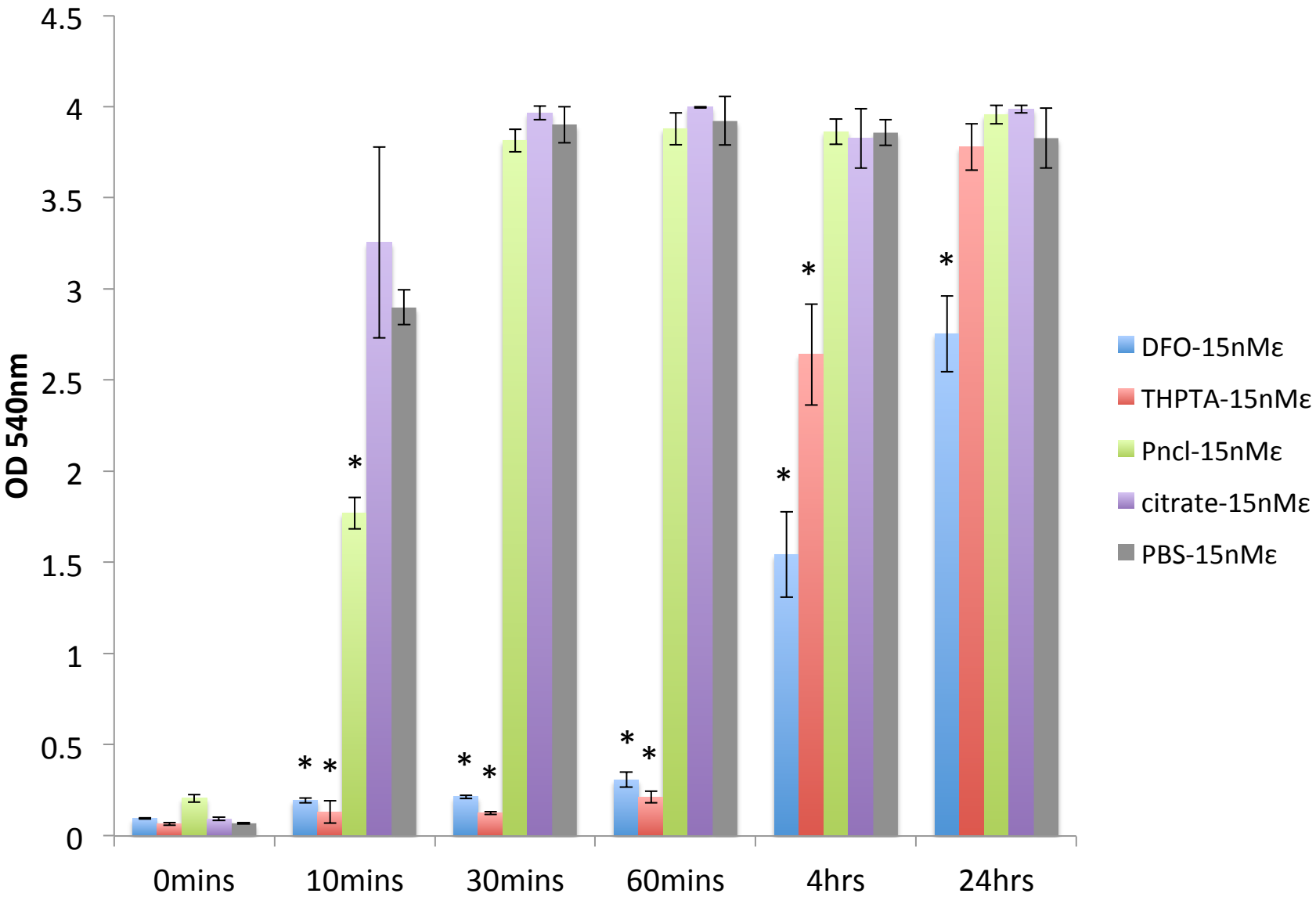


Figure 3c

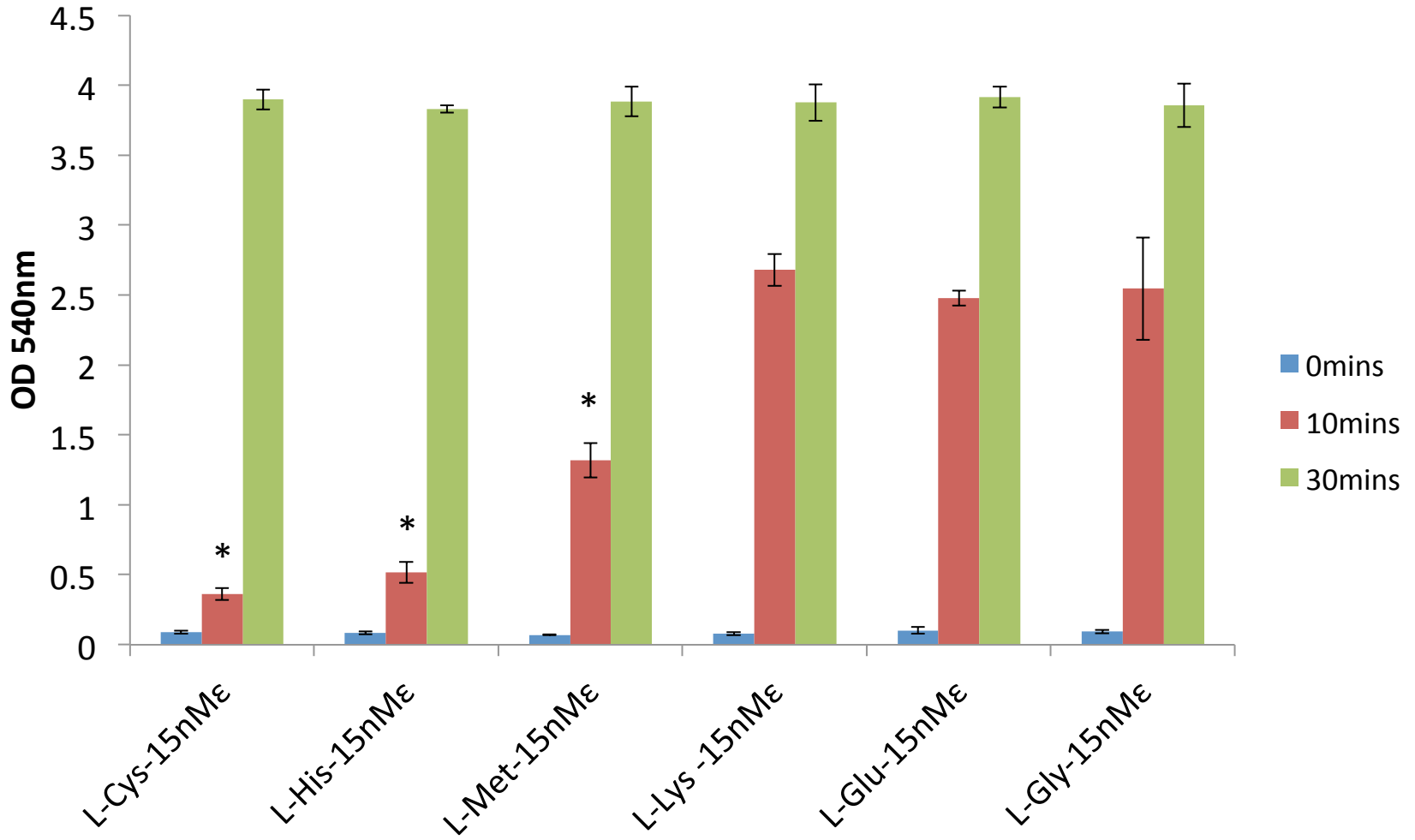


Figure 3d

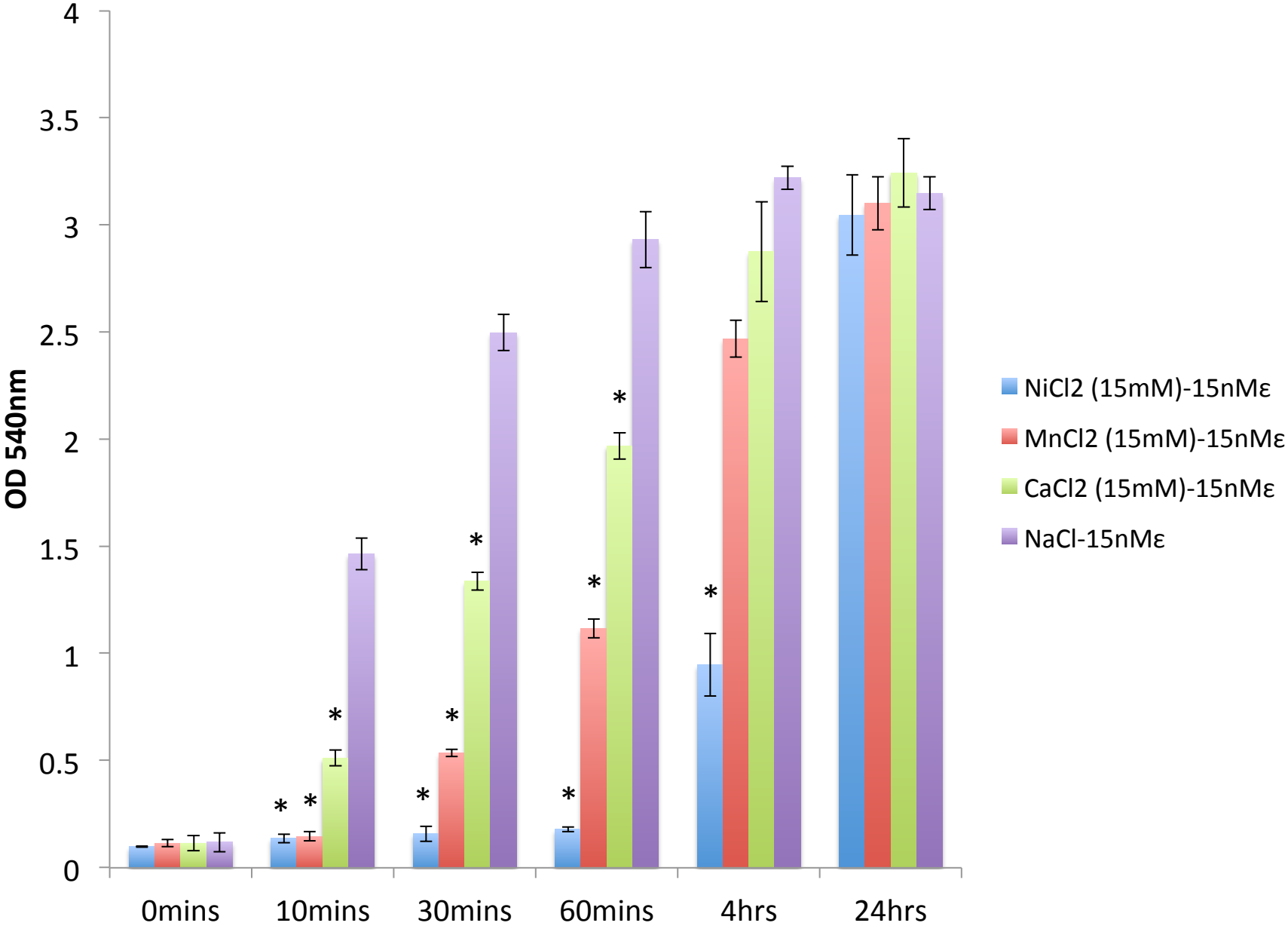


Figure 4a

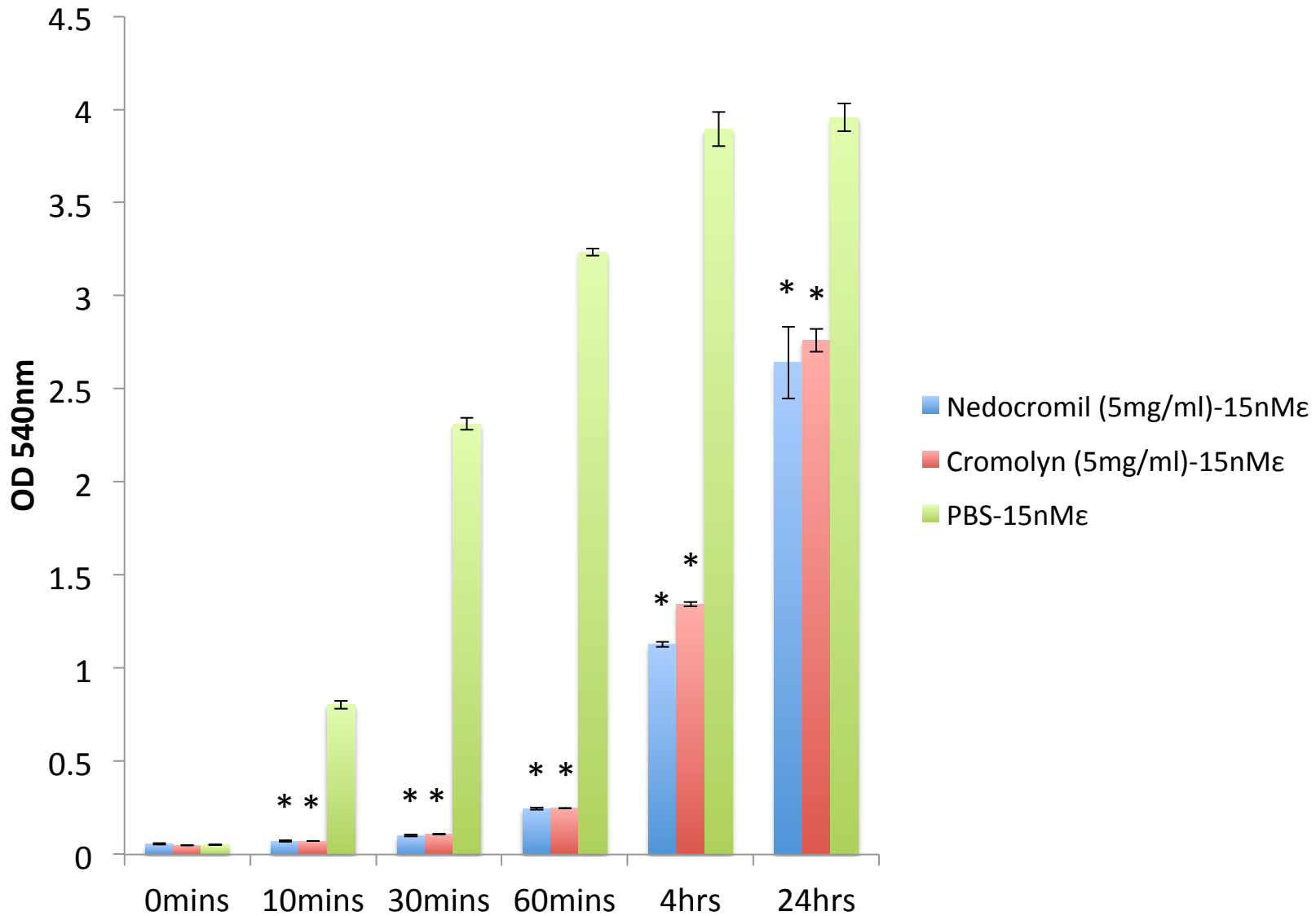


Figure 4b

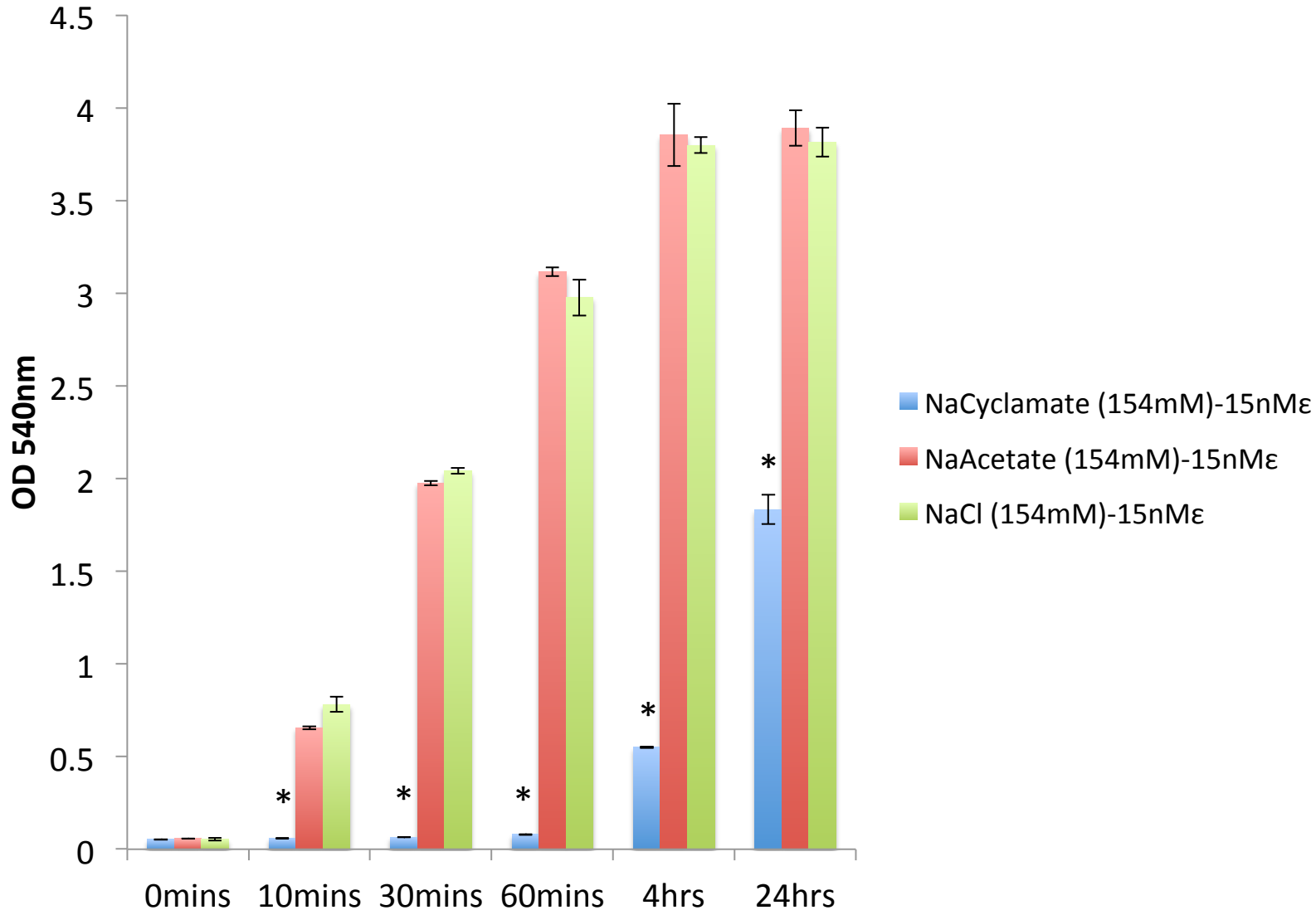


Figure 4c

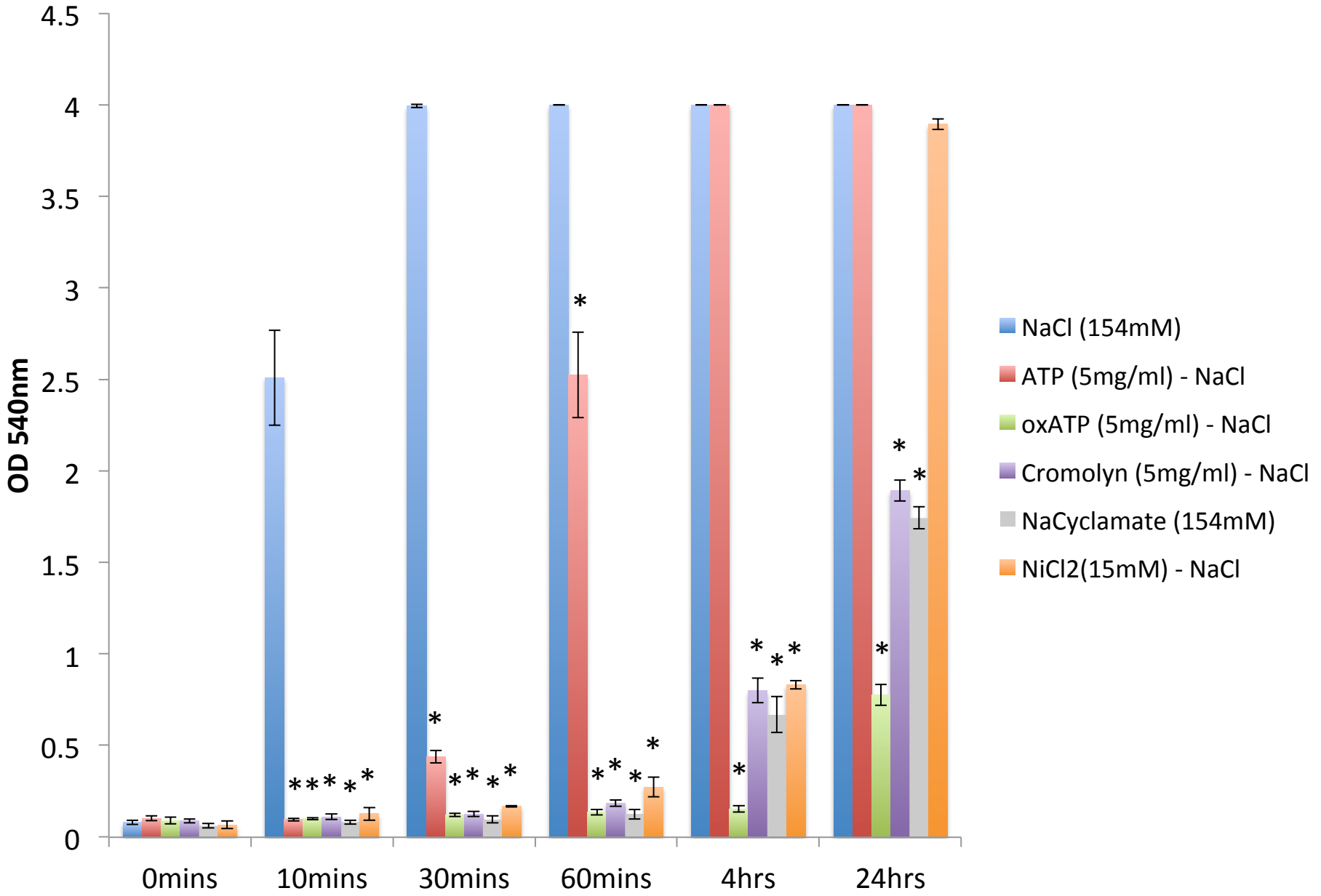


Figure 4d

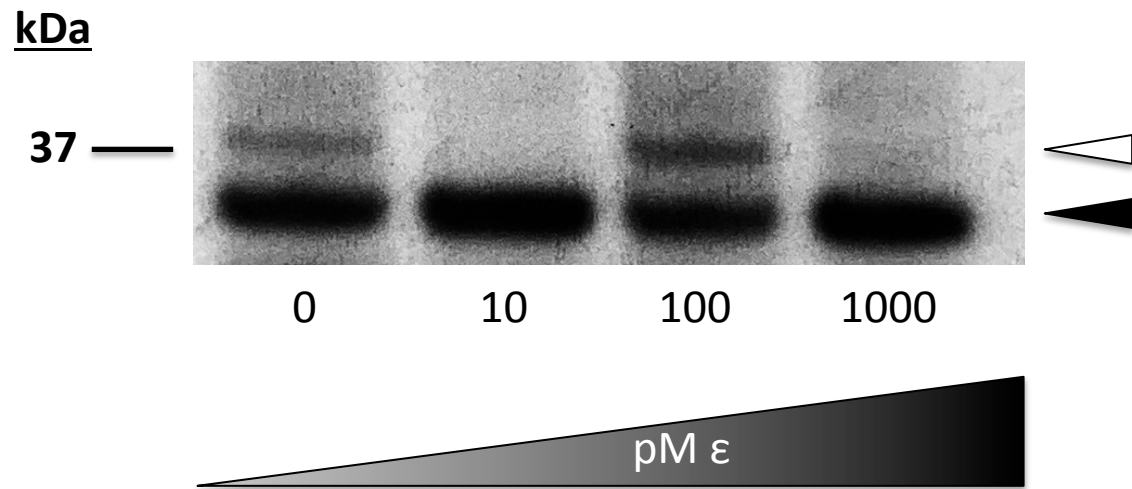


Figure 5a

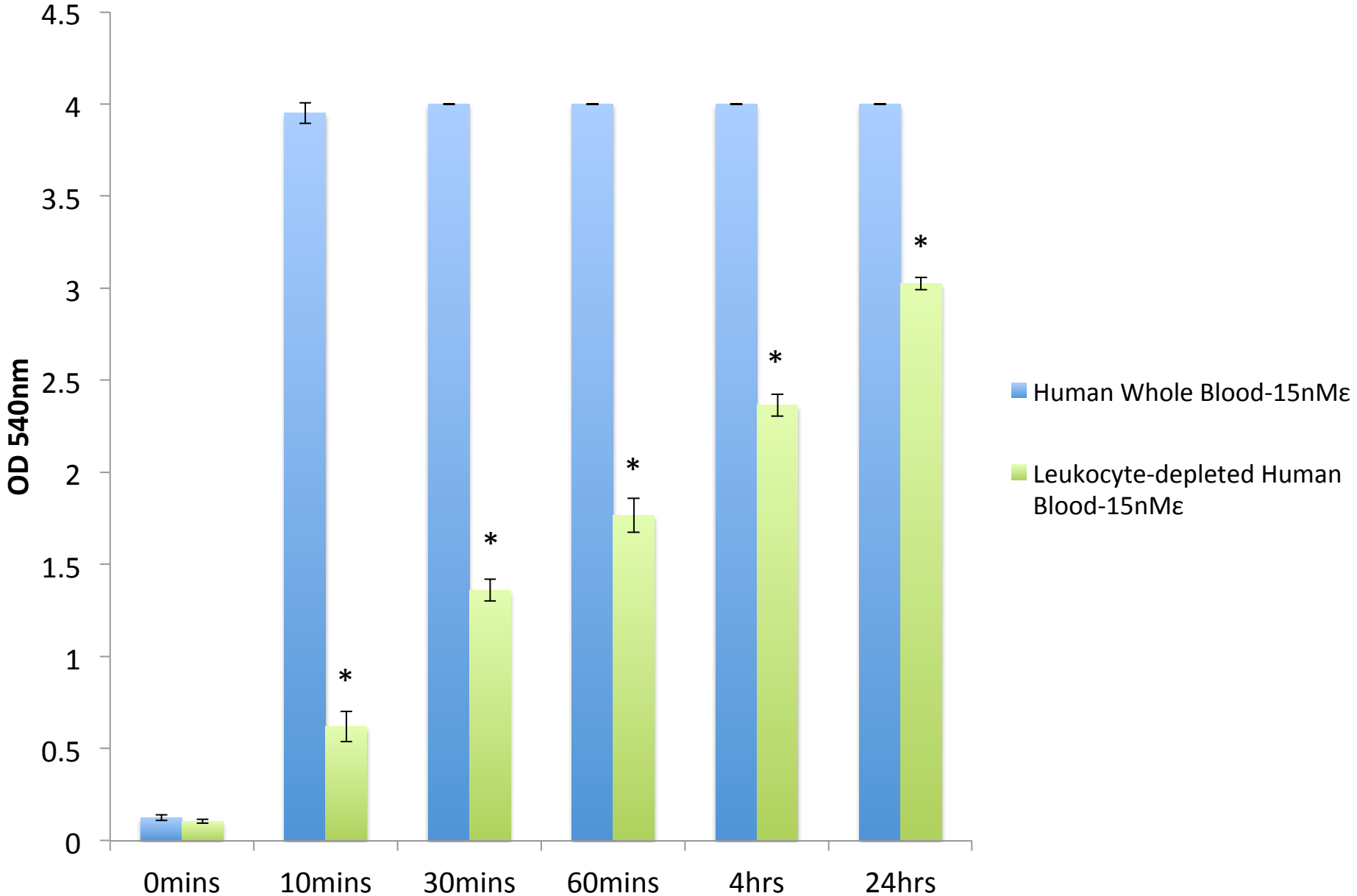
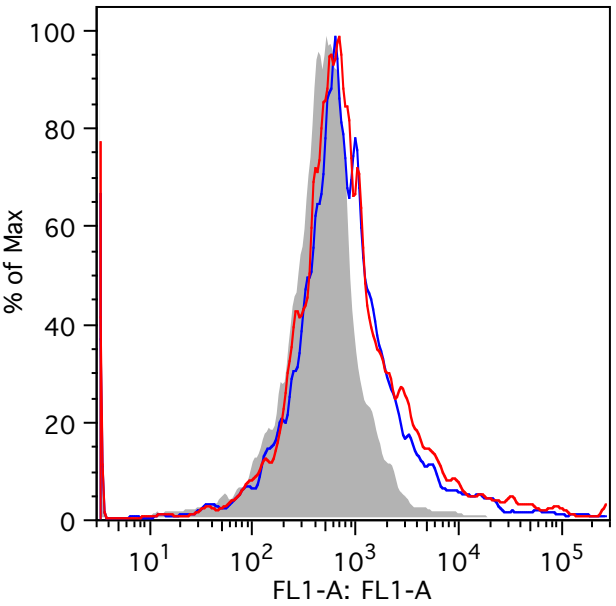
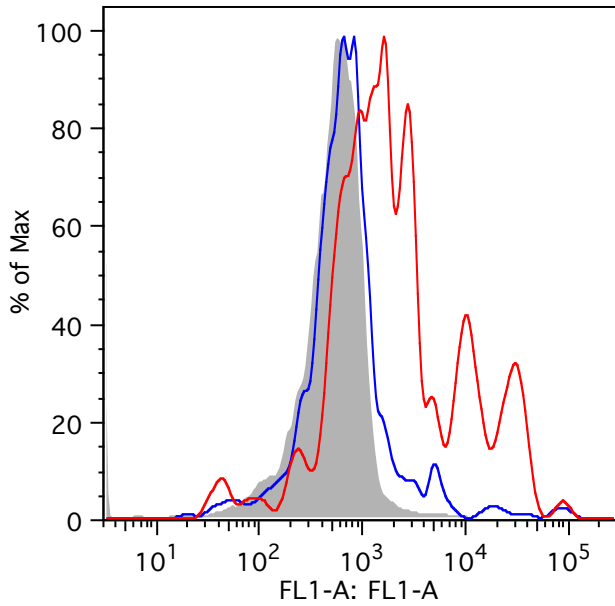


Figure 5b

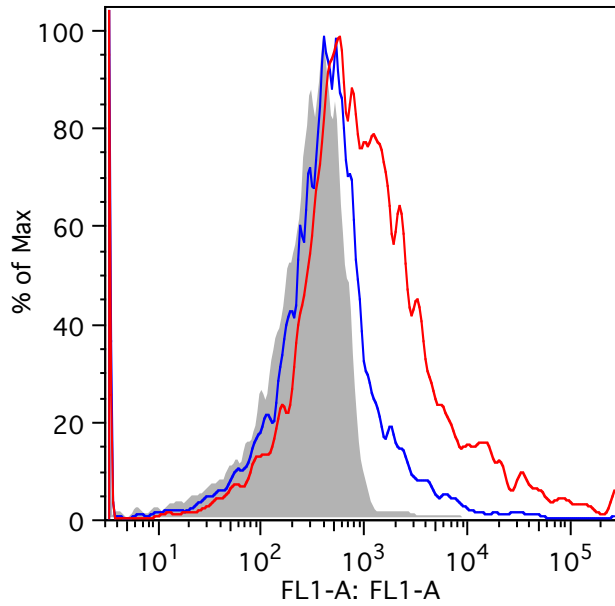
B cells



CD4⁺ T cells



RBCs



- 50nM protoETX
- 0nM protoETX
- Unstained

Figure 5c

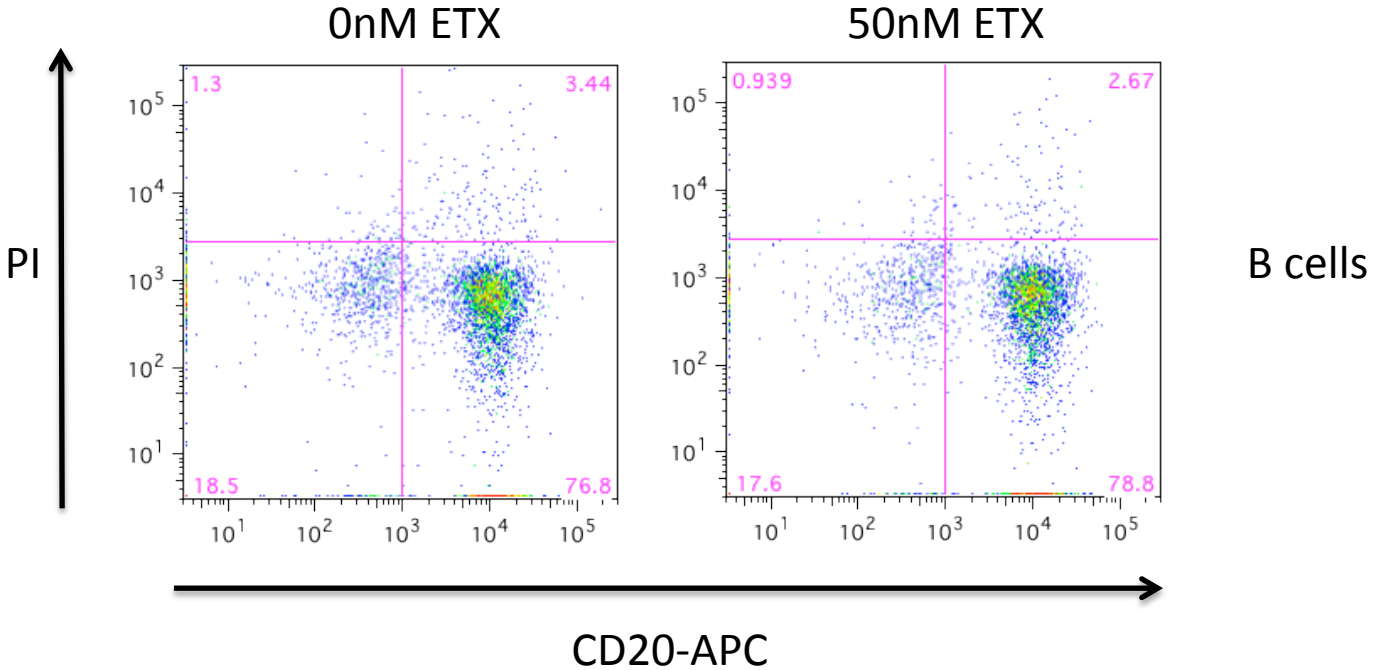
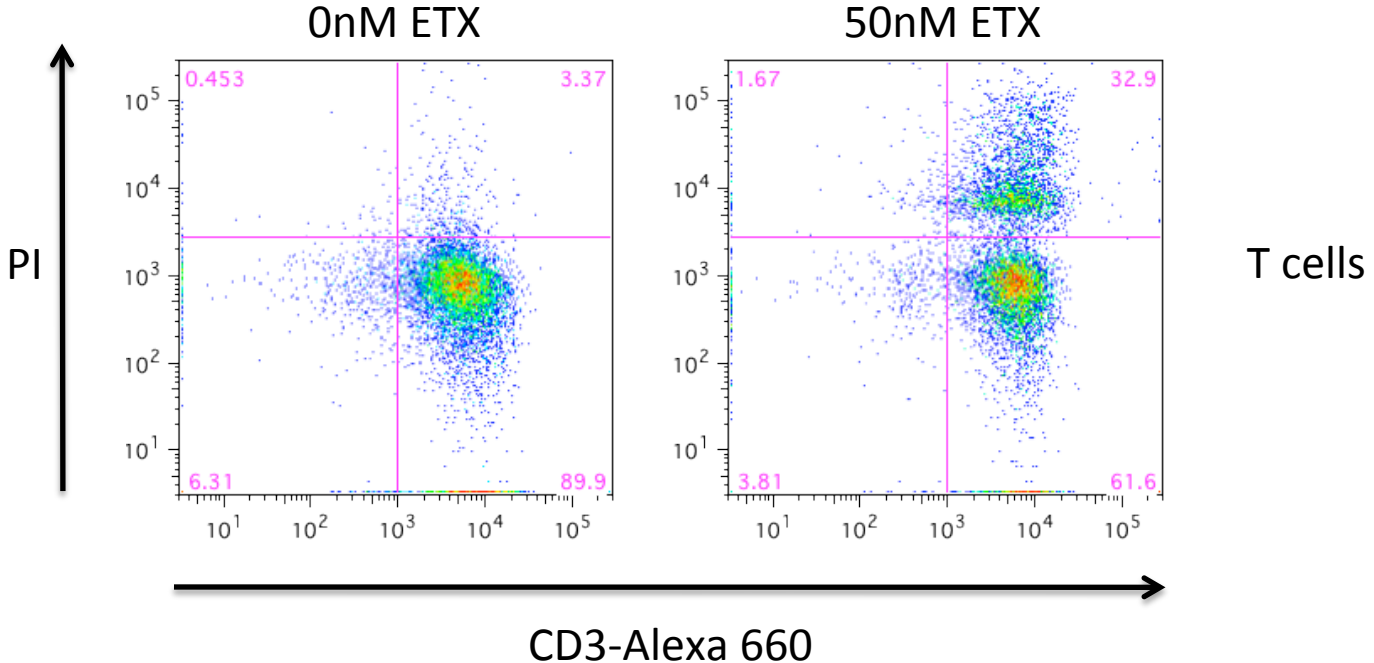


Figure 5d

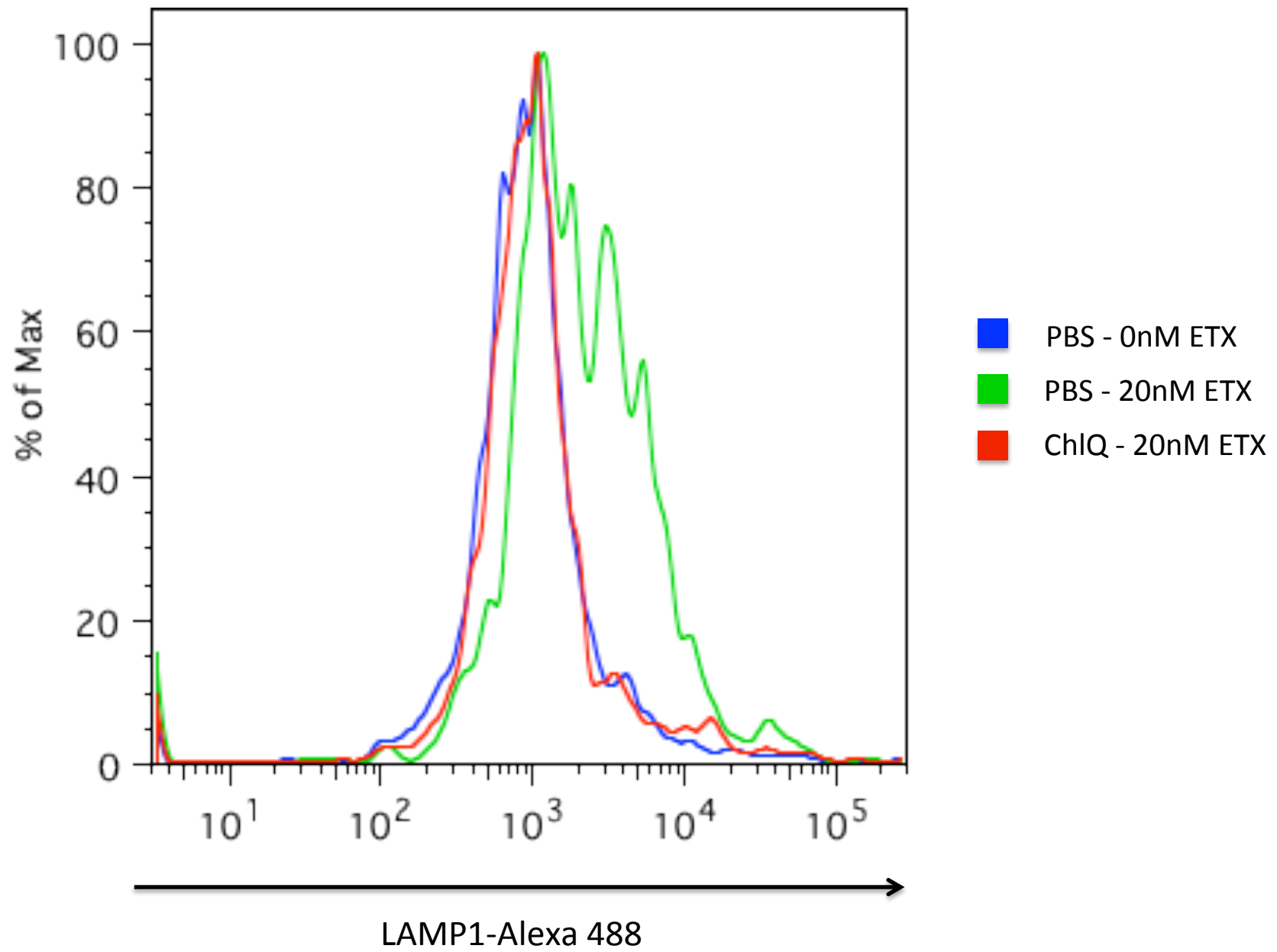
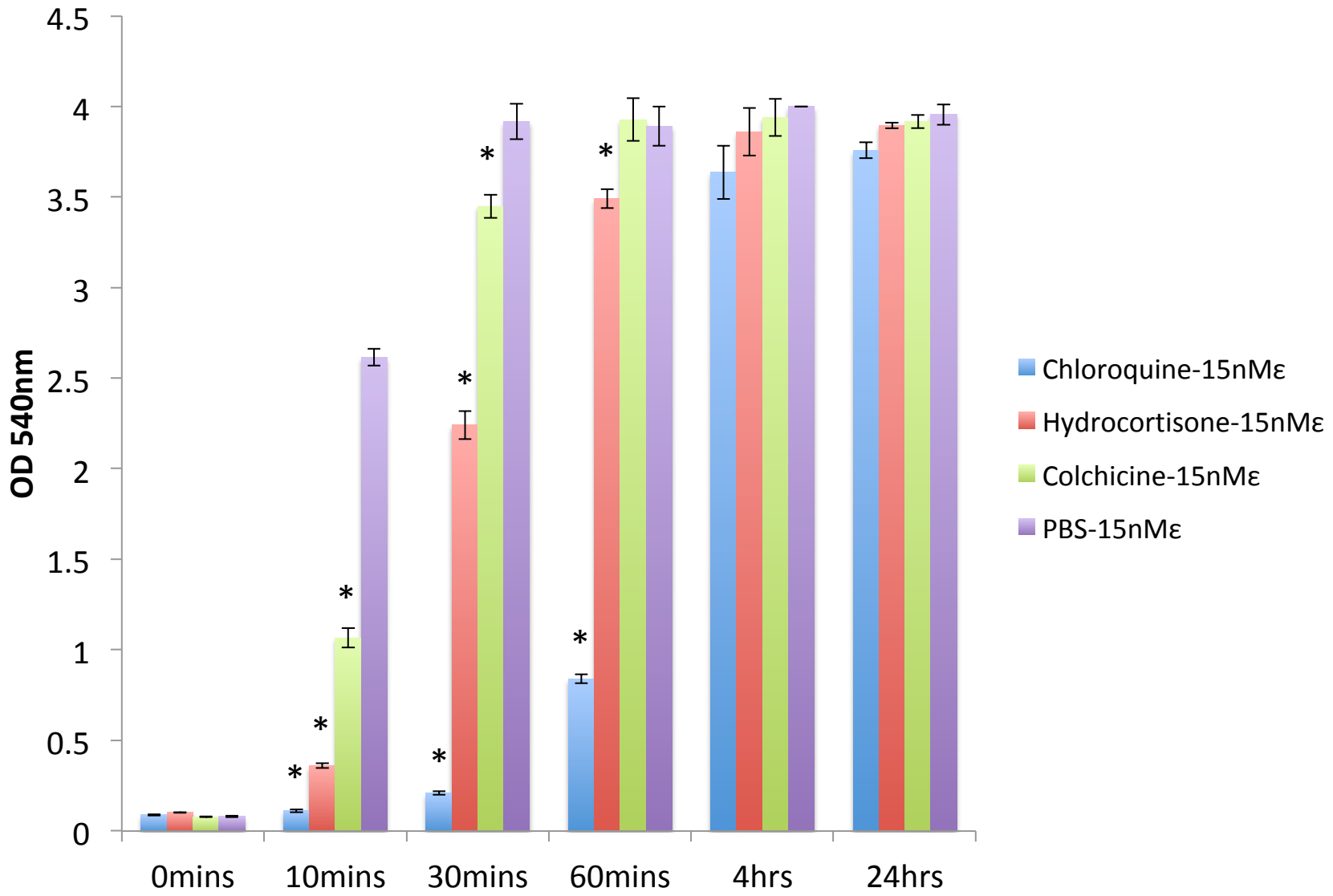


Figure 5e



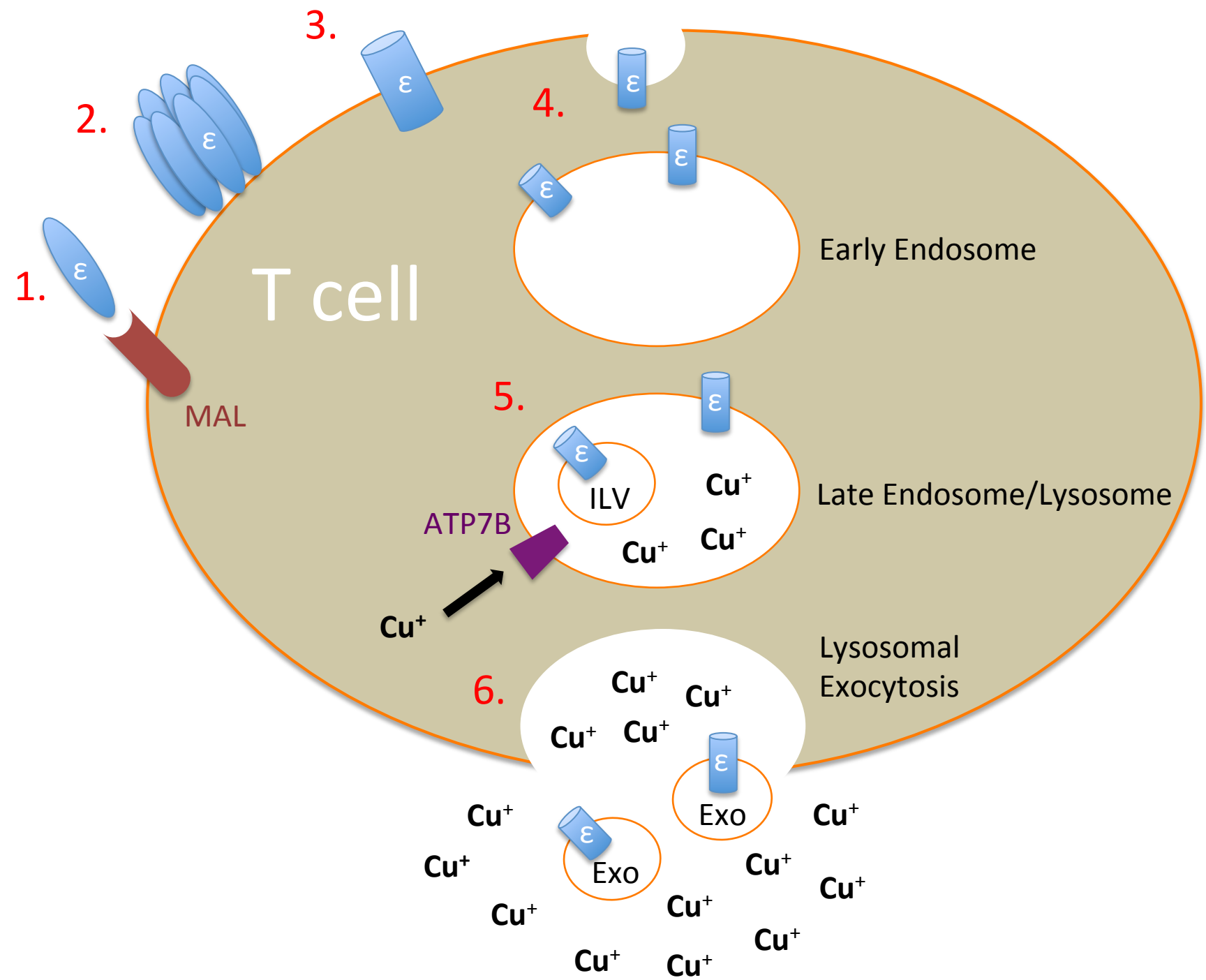


Figure 6

Figure 7

