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Epsilon Toxin from *Clostridium perfringens* Induces the Generation of Extracellular Vesicles in T-Lymphocytes

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INTRODUCTION & AIM

INTRODUCTION

Epsilon toxin (ETX) is a pore-forming toxin (PFT) capable of crossing the blood-brain barrier and binding to myelin structures. *In vitro* assays have demonstrated that ETX impairs oligodendrocytes and induces demyelination. Notably, ETX has been implicated in the pathogenesis of multiple sclerosis (MS), with ETX-specific antibodies detected in sera from MS patients.

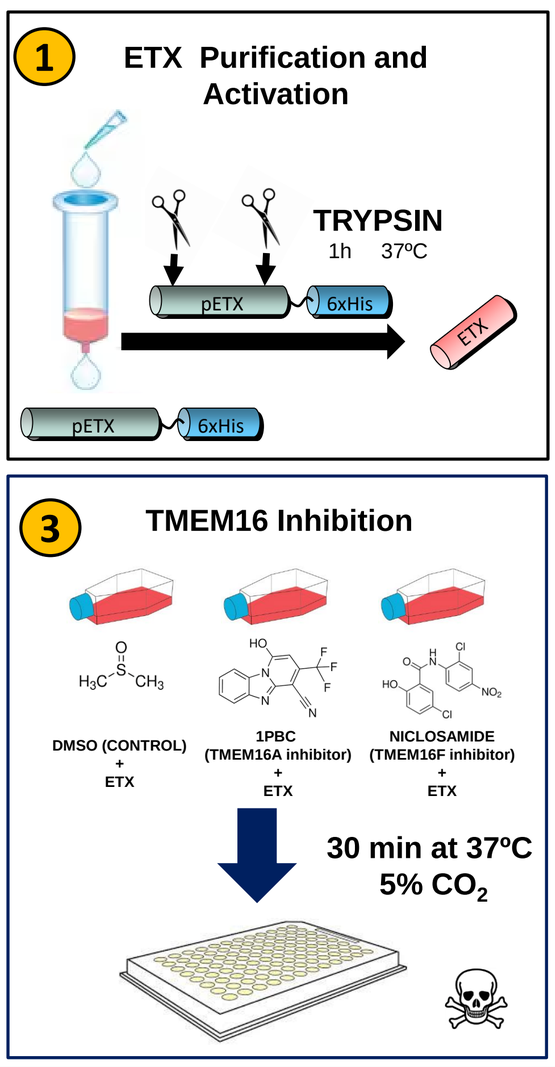
Myelin and lymphocyte protein (MAL) is widely recognized as the receptor for ETX. Its presence is essential for ETX-induced pore formation in the plasma membrane of host cells, ultimately leading to cell death. Recent findings have shown that ETX also binds to and kills primary human lymphocytes, which express elevated levels of MAL. This suggests that ETX may influence immune responses associated with MS, although its precise mechanism of action remains unclear.

To counteract the damage caused by PFTs, some host cells release extracellular vesicles (EVs) to reduce pore insertion into the plasma membrane. ETX has been shown to induce EV production in HeLa cells overexpressing MAL-GFP. Similar effects have been observed in MOLT-4 cells, a T-lymphocyte cell line that endogenously expresses MAL protein. Our observations confirm that ETX also stimulates EV formation in MOLT-4 cells. Moreover, both MAL protein and ETX oligomers are present in these EVs, making them a valuable tool for investigating ETX's mode of action and its interaction with its receptor. We speculate that these EVs may play a role in the induction of MS.

AIM

Study the mechanism of action of ETX in inducing the generation of extracellular vesicles (EVs) in the MOLT-4 T-lymphocyte cell line.

METHOD



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RESULTS & DISCUSSION

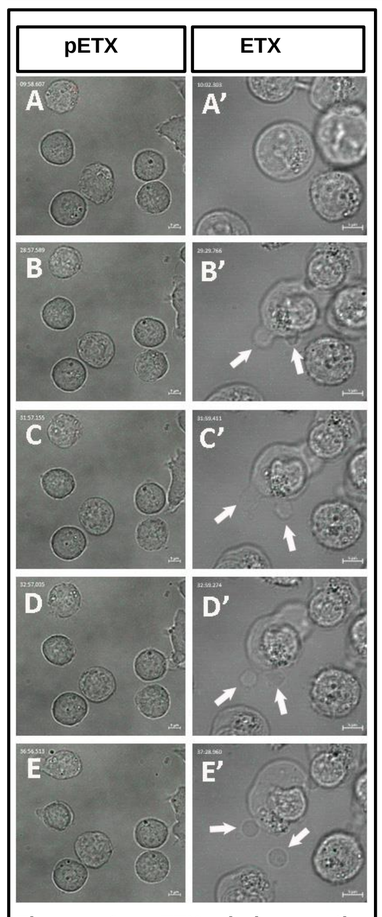

Figure 1.- ETX induces the generation of extracellular vesicles (EVs) in MOLT-4 cells. EVs (indicated by arrows) are observed in ETX-treated cells. In contrast, pETX does not induce EV formation. Scale bars represent 5 µm.

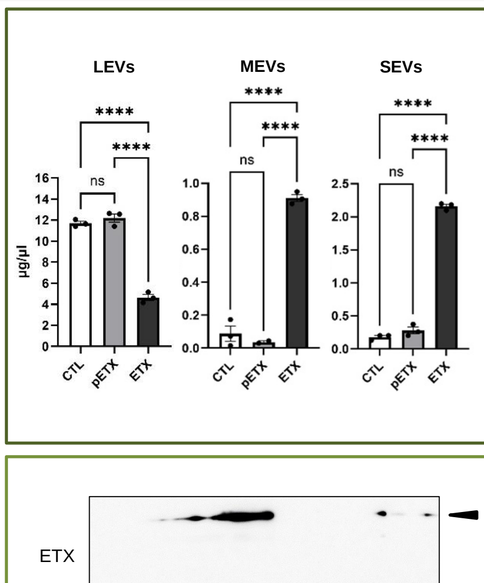

Figure 2.- ETX induces the generation of extracellular vesicles (EVs) from MOLT-4 cells. Quantification of large EVs (LEVs), medium EVs (MEVs), and small EVs (SEVs) generated by ETX (dark gray bars), pETX (gray bars), or PBS (white bars). ETX significantly increased the production of MEVs and SEVs, while interestingly, it significantly reduced the LEV population. (*****p* < *0.0001, ns = not significant*). A one-way ANOVA was performed using GraphPad Prism 10 for each group, comparing CTL, pETX, and ETX across three independent experiments.

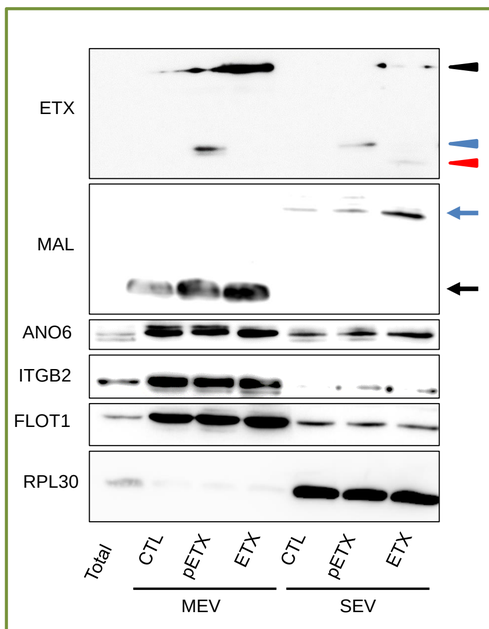

Figure 3.- ETX induces the generation of MEVs and SEVs containing ETX, MAL and TMEM16F proteins. ETX is present as an oligomer (>200 kDa, black arrowhead). Monomeric forms of pETX and ETX appear as bands at 33 kDa and 29 kDa, respectively (blue and red arrowheads). MAL protein is detected as a monomer (~12 kDa) in MEVs (black arrow) and as a trimer (~36 kDa) in SEVs (blue arrow). The calcium-activated phospholipid scramblase TMEM16F (ANO6) is detected at ~100 kDa. ITGB2 (~100 kDa), FLOT1 (~50 kDa), and RPL30 (~10 kDa) were used as EV purity markers. A total of 80 µg of protein was loaded per lane. Cells were treated for 1 h at 37 °C with 50 nM of pETX, ETX, or PBS (CTL), and EVs were isolated (MEVs and SEVs). "Total" refers to the crude fraction from untreated MOLT-4 cells. The Western blot image is representative of three independent experiments.

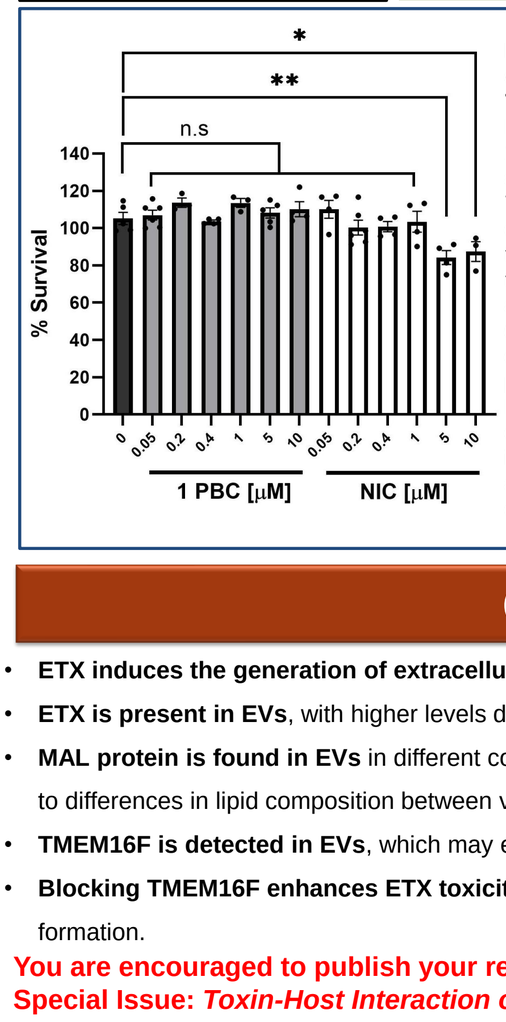

Figure 4.- The optimal concentration for using TMEM16 inhibitors is 1 µM. MOLT-4 cells were treated with 1PBC (a TMEM16A inhibitor) and Niclosamide (NIC, a TMEM16F inhibitor) at concentrations ranging from 0 to 10 µM. Niclosamide induces toxicity at concentrations above 1 µM. (***p < 0.01, *p < 0.05, ns = not significant*). A one-way ANOVA was performed using GraphPad Prism 10, comparing each condition to the control. The image is a representative assay of 3 independent experiments.

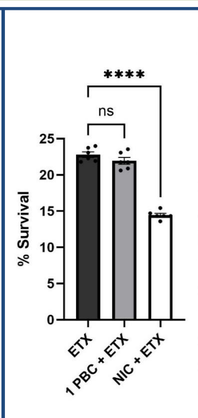

Figure 5.- Blocking TMEM16F increases ETX toxicity. MOLT-4 cells were pre-treated with 1 µM 1PBC and Niclosamide (NIC) for 10 minutes at 37 °C in a 5% CO_2 atmosphere, followed by incubation with 10 nM ETX for 30 minutes under the same conditions. Cells treated with Niclosamide showed increased sensitivity to ETX. (*****p* < *0.0001, ns = not significant*).

A one-way ANOVA was performed using GraphPad Prism 10, comparing each condition. The image is a representative assay of 3 independent experiments.

CONCLUSION

- **ETX induces the generation of extracellular vesicles (EVs)** of different sizes and densities in T lymphocytes.
- **ETX is present in EVs**, with higher levels detected in medium-sized EVs (MEVs) compared to small EVs (SEVs).
- **MAL protein is found in EVs** in different conformations: as a monomer in MEVs and as a trimer in SEVs, likely due to differences in lipid composition between vesicle types.
- **TMEM16F is detected in EVs**, which may explain the scramblase activity induced by ETX.
- **Blocking TMEM16F enhances ETX toxicity**, possibly due to a reduced ability of the cells to counteract ETX pore formation.

You are encouraged to publish your results in the upcoming Special Issue: *Toxin-Host Interaction of Clostridium Toxins*.

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