An in vitro cell based model for testing NETosis inhibitors and proNETotic agents.

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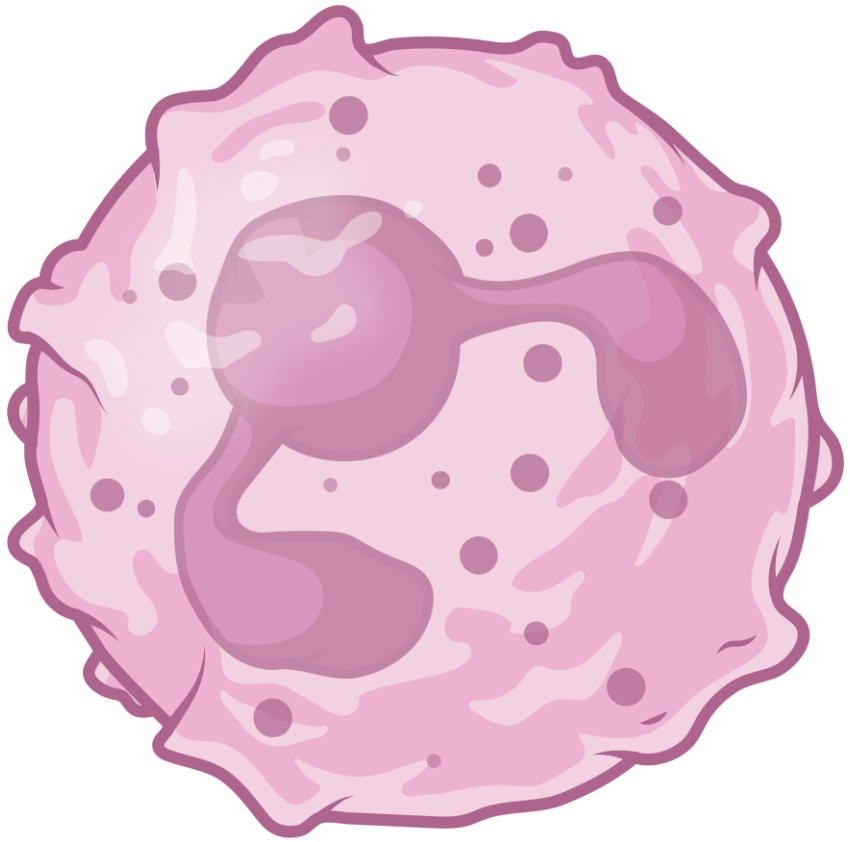
Introduction
From Neutrophils to NETosis



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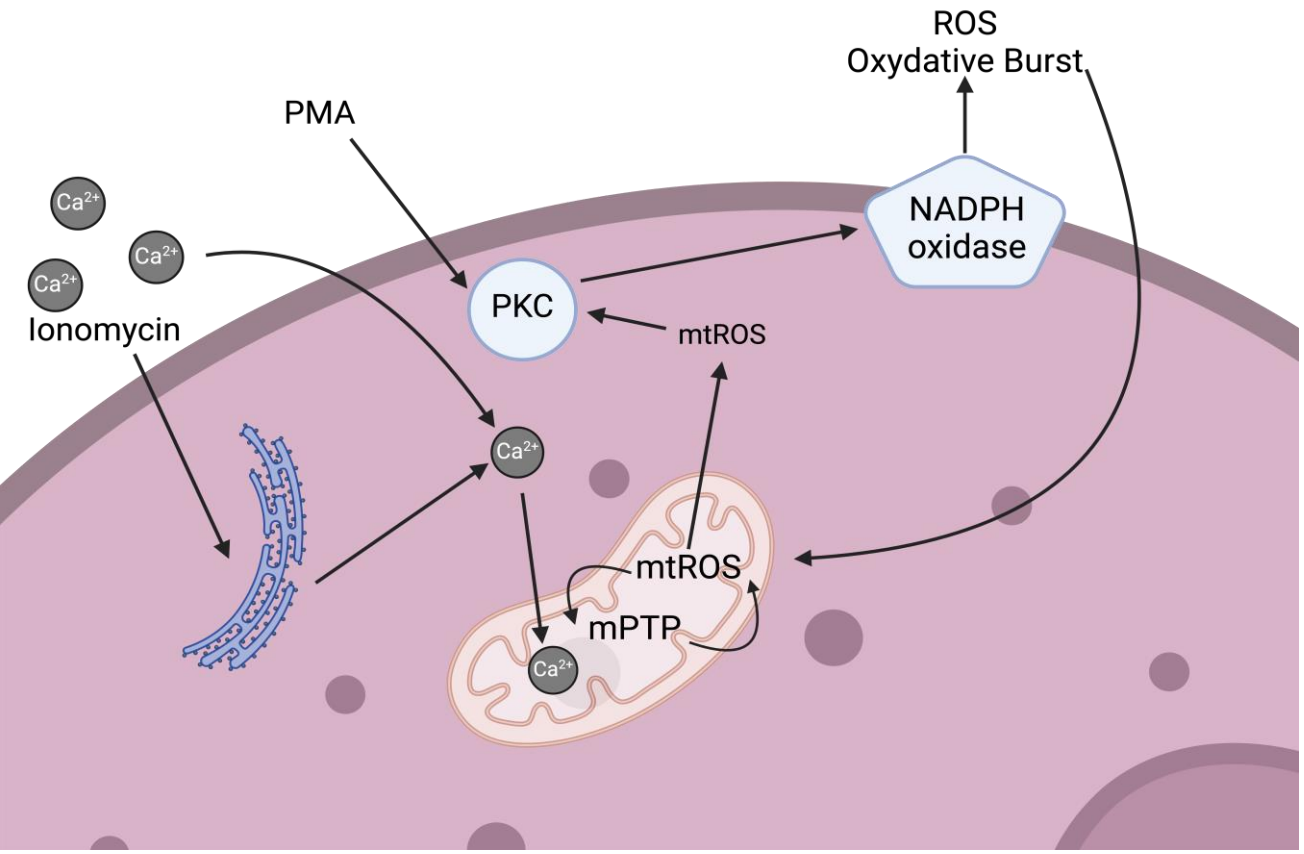
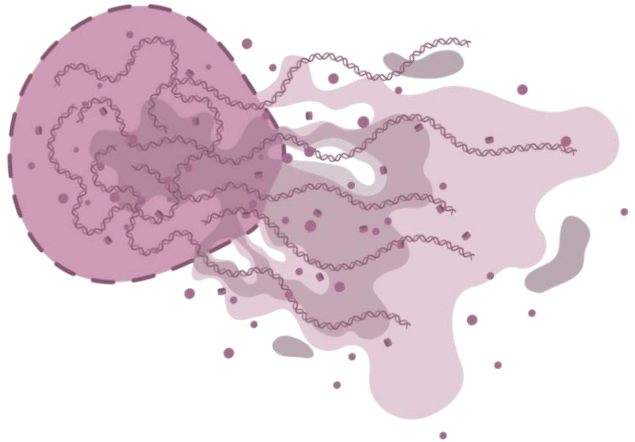
- **Most abundant type of white blood cells** in humans (70%).
- First responders to infection and inflammation.
- Characterized by a multi-lobed nucleus (polymorphonuclear leukocytes).
- Produced in the bone marrow and circulate in the bloodstream.
- **Short-lived cells**, surviving only a few hours to days.
  - Circulate in the bloodstream for approximately 6 to 10 hours
  - After migrating into tissues, they survive for 1 to 2 days
- Eliminate pathogens through multiple mechanisms:
  - Phagocytosis: engulfing and digesting microorganisms
  - Degranulation: releasing antimicrobial substances
  - **NETosis: forming Neutrophil Extracellular Traps (NETs) composed of DNA and proteins to trap and kill pathogens**

Gillot et al. Frontiers in Pharmacology 2021
Amulic B. et al. Annual Review of Immunology 2012

Introduction
From Neutrophils to NETosis





- Cell-free DNA
- Histones (citrullinated)
- Protein such as **myeloperoxidase** (MPO) and **neutrophil elastase** (NE)

- ✓ Different **stimulus** leads to generation of the **neutrophil extracellular traps** (NETs) such as pathogen-associated molecular pattern, toll-like receptor (TLR) 4,7,8, pro-inflammatory cytokines, tumor necrosis factor α (TNF-α), activated platelets,…
- ✓ Two important intracellular signaling pathways regulate NETosis: **protein kinase C** (PKC) and **NADPH oxidase 2** (NOX2)

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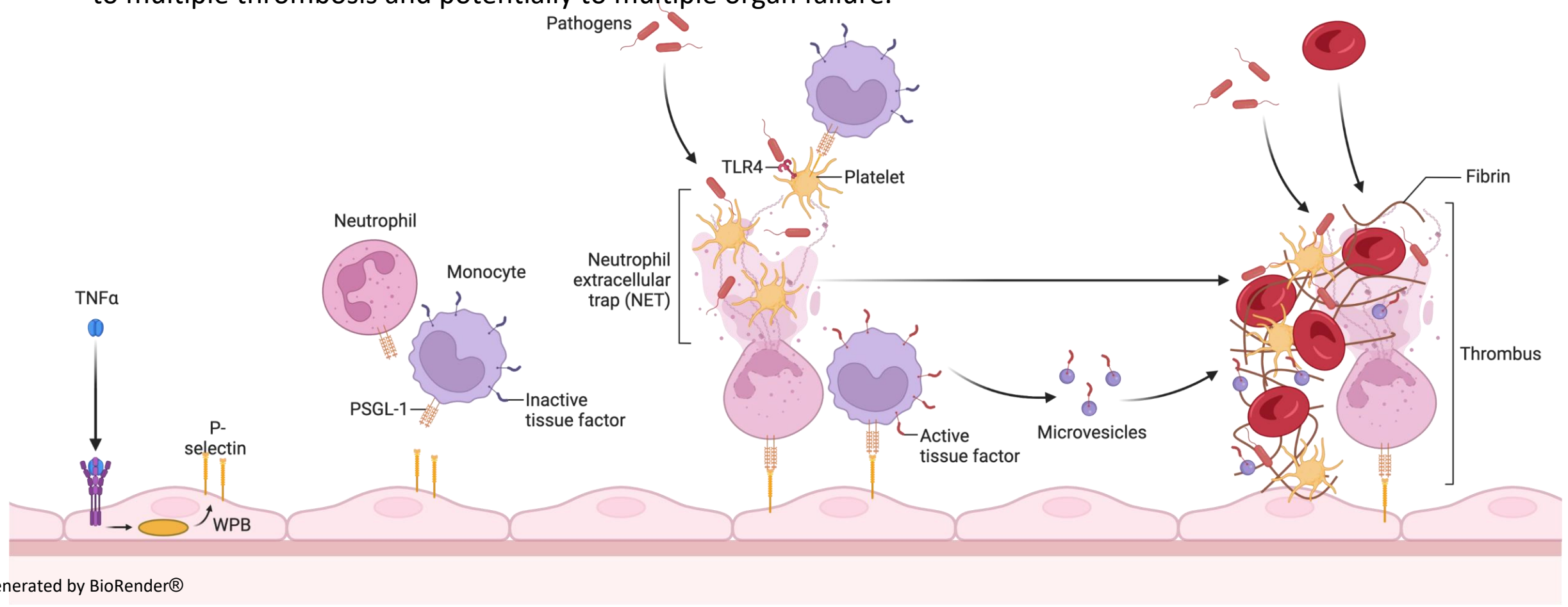
Introduction

From NETosis to Immunothrombosis

- NETs serve as **a scaffold for thrombus formation** which, in the case of uncontrolled activation, can lead to multiple thrombosis and potentially to multiple organ failure.



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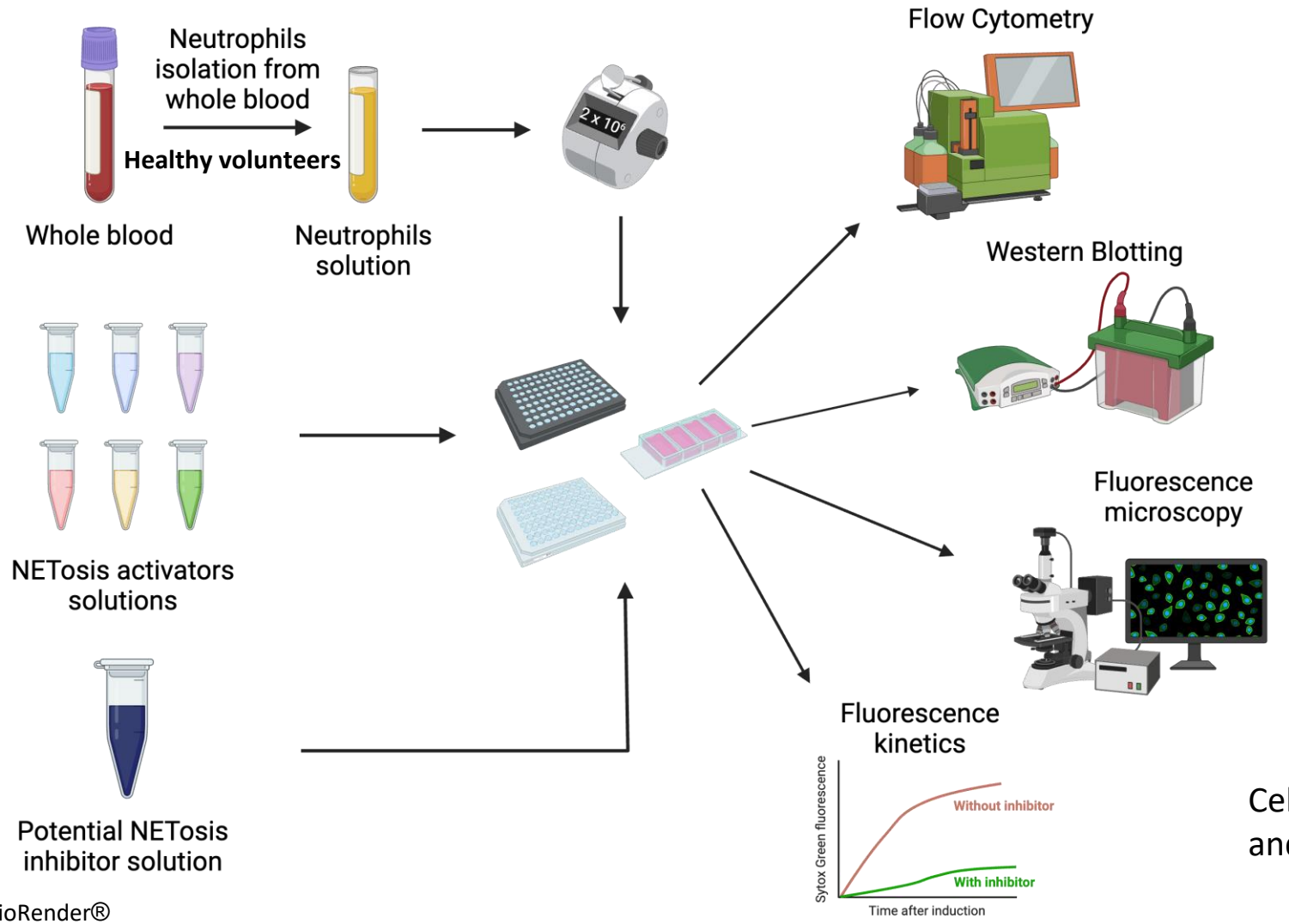
NETosis cell-based model
Protocol Overview



Neutrophil counting and viability assay

Intracellular signaling pathways: PKC δ and NOX2

Visualizing NETs

Cell-free DNA release and NE activity

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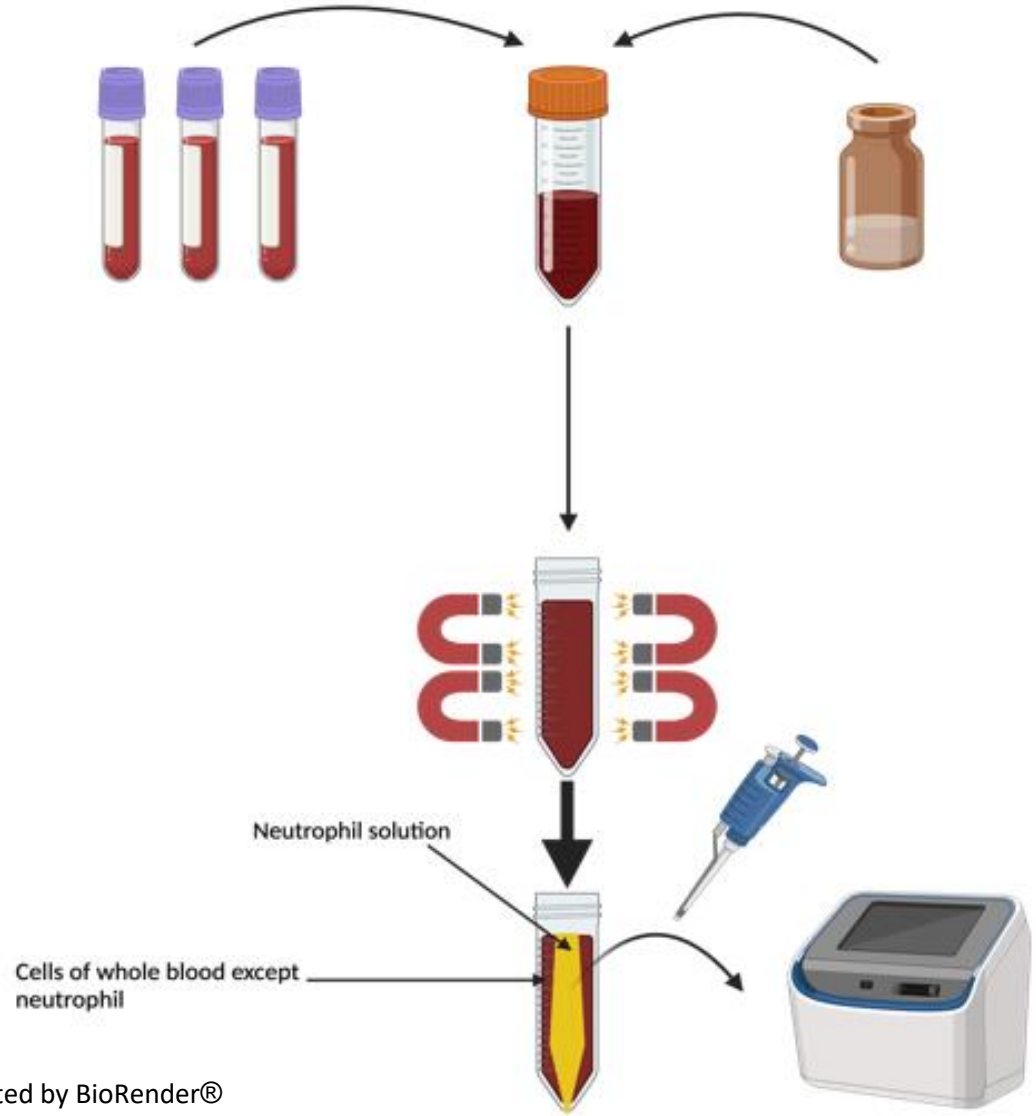
NETosis cell-based model

Neutrophil isolation – Negative selection



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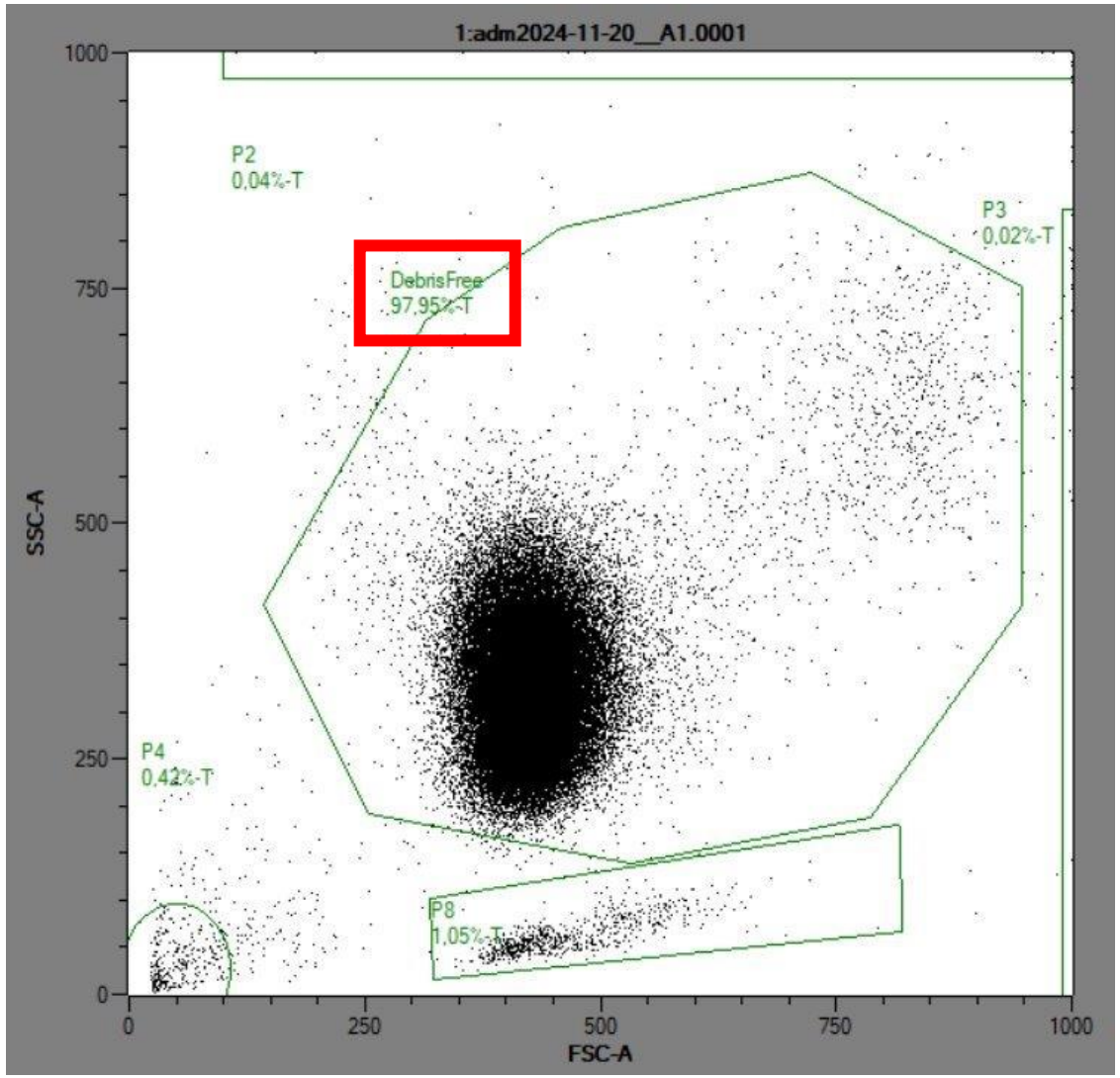
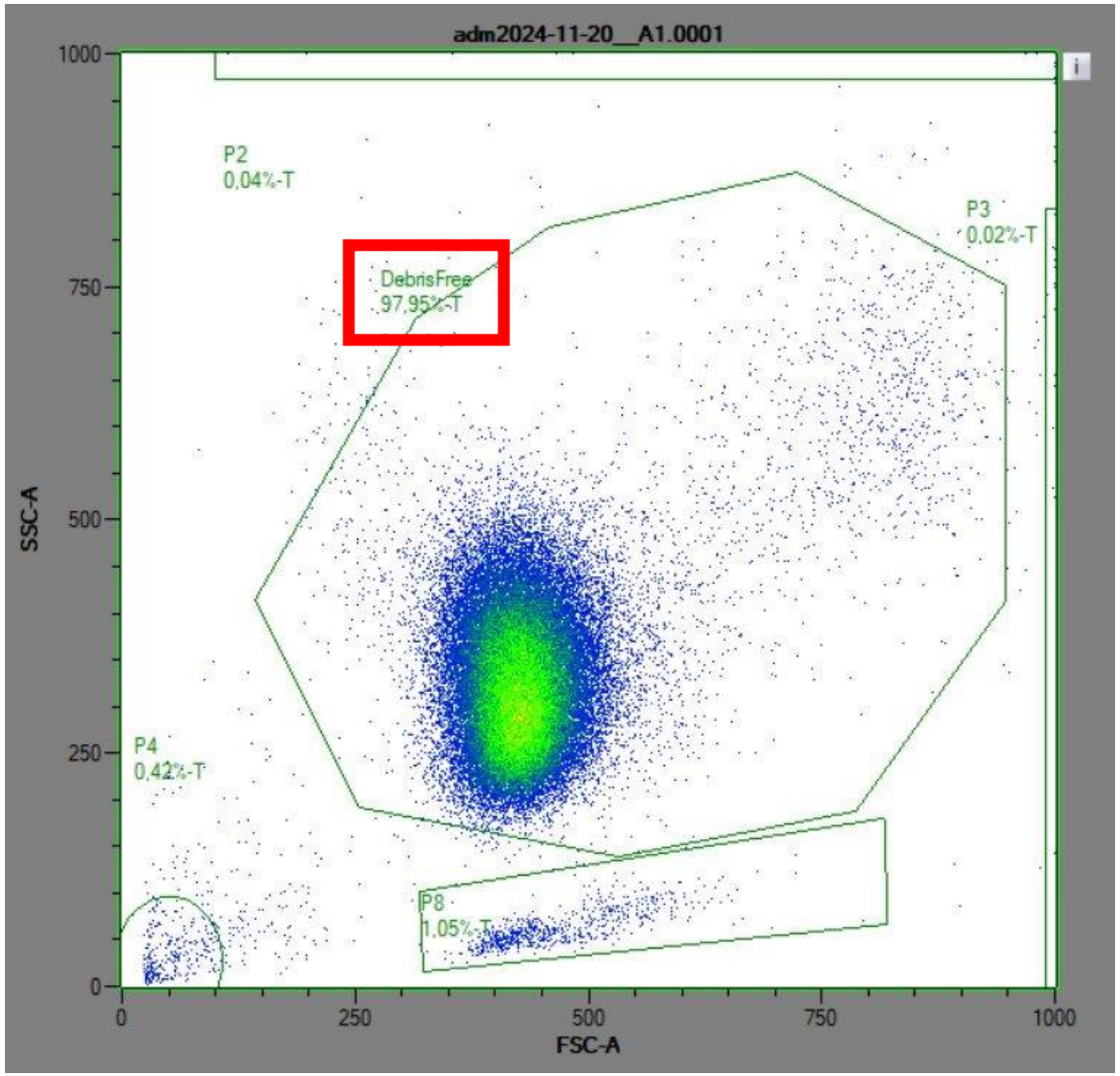
- Neutrophils are extracted from whole blood collected on EDTA tubes.
- The whole blood is mixed with magnetic beads allowing a **negative selection of neutrophils.**
- The mixture is inserted into a magnet to separate the components.
- The neutrophils in the center of the falcon are collected and counted.
- **This is a critical step as rough handling could kill neutrophils or activate NETosis before the test starts.**

NETosis cell-based model

Neutrophil counting and viability assay

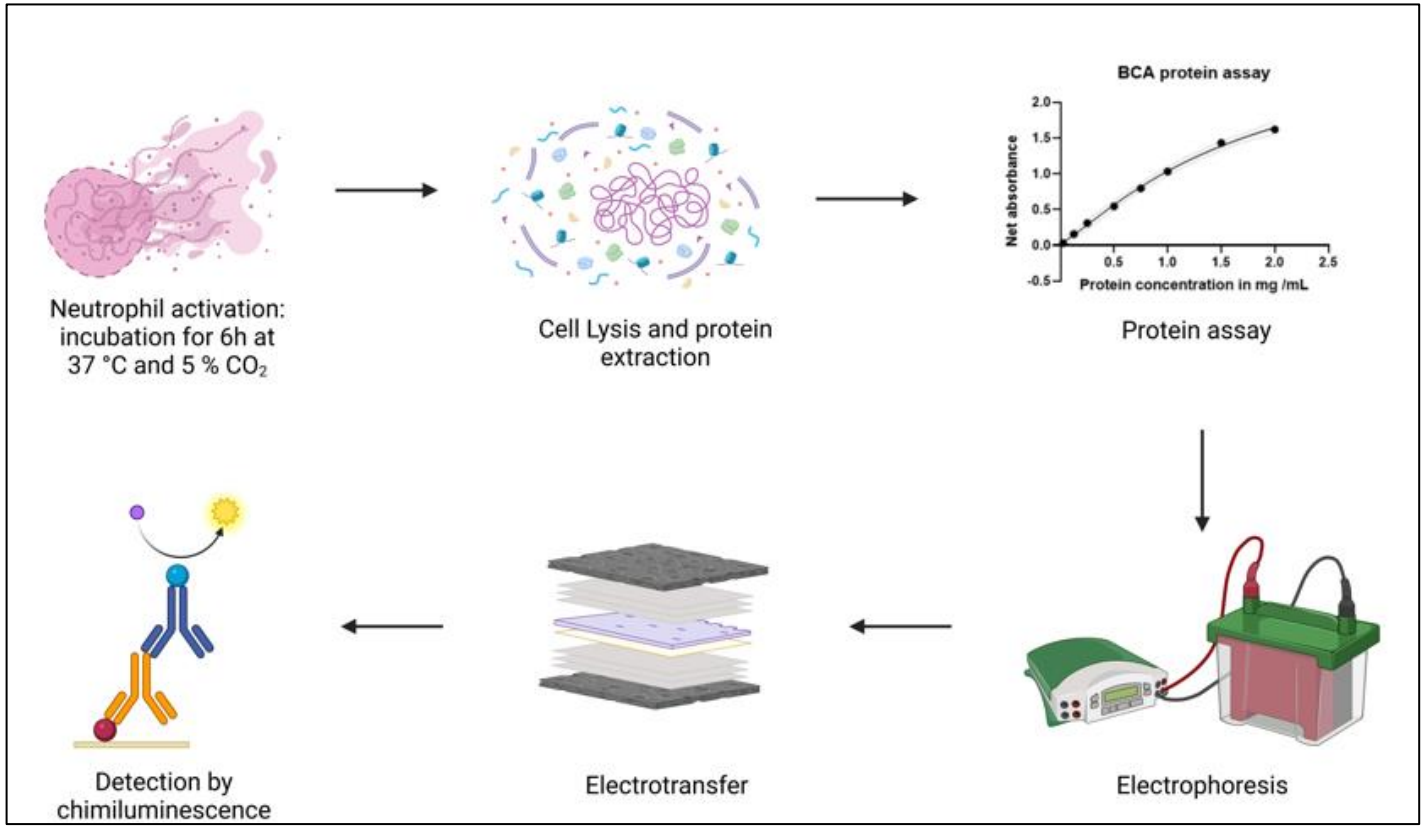




NETosis cell-based model

Intracellular signaling pathways: PKC δ and NOX2

- PKC δ is strongly involved *in vivo* in neutrophil migration, NETosis induction, and neutrophil–platelet aggregate formation.
- PKCδ activates NOX2, leading to ROS generation, oxidative stress, and further amplification of NETosis.



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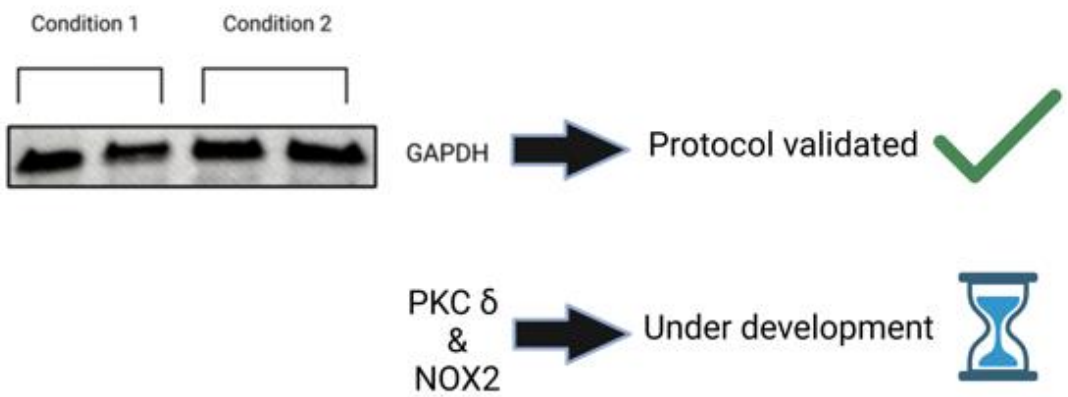

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NETosis cell-based model

Visualizing NETs in ICF

- Immunocytochemistry with fluorescence (**ICF**): visualization of specific cellular structures through fluorescent labeling. Essential for studying NETosis, a process by which neutrophils release extracellular DNA structures.

Objectives:

- ✓ Identify **activated**, **inactive**, or **inhibited** neutrophils
- ✓ Visualize the various structures involved in NETosis

- **Fluorescent markers used:**

- **Nucleus (blue):** DAPI (350 nm)
- **Actin filaments (red):** Phalloidin (647 nm)
- **Extracellular DNA (green):** Sytox Green (500 nm)
- **Citrullinated histone H3 (yellow):** Anti-citH3 antibody (580 nm)

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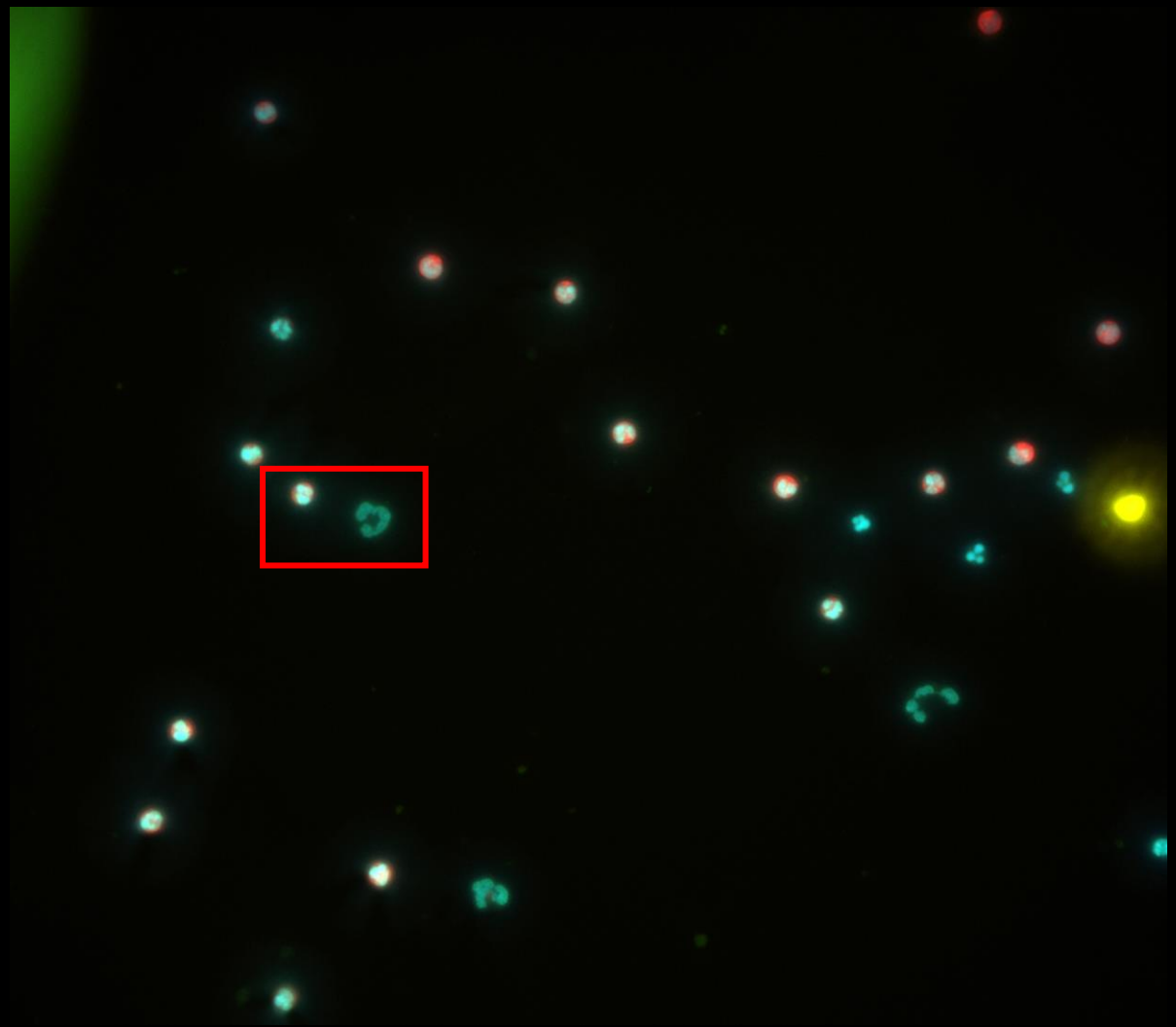
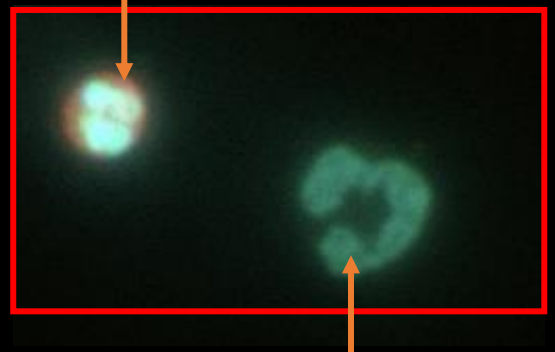
NETosis cell-based model

Visualizing NETs in ICF

Unactivated neutrophils

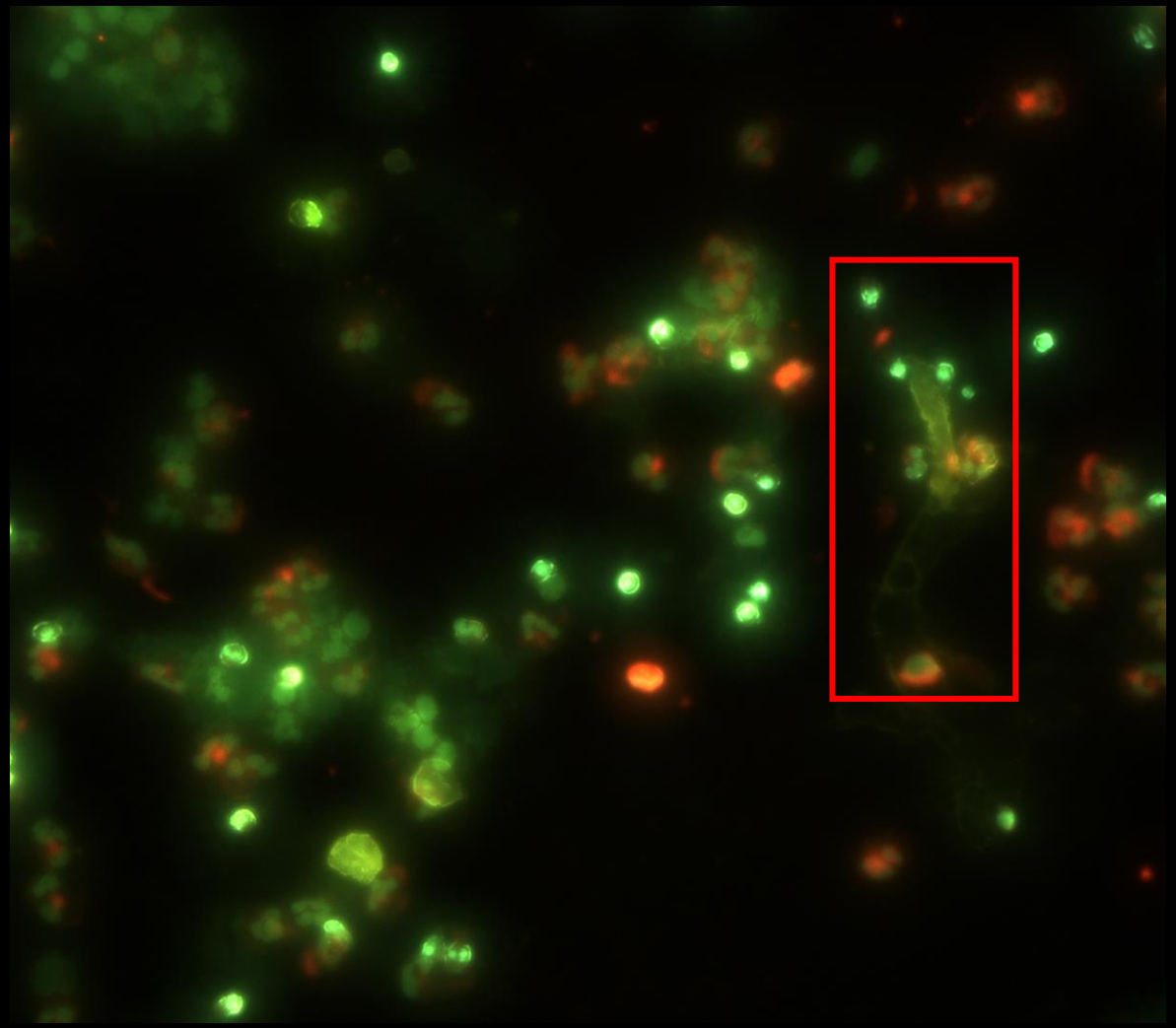


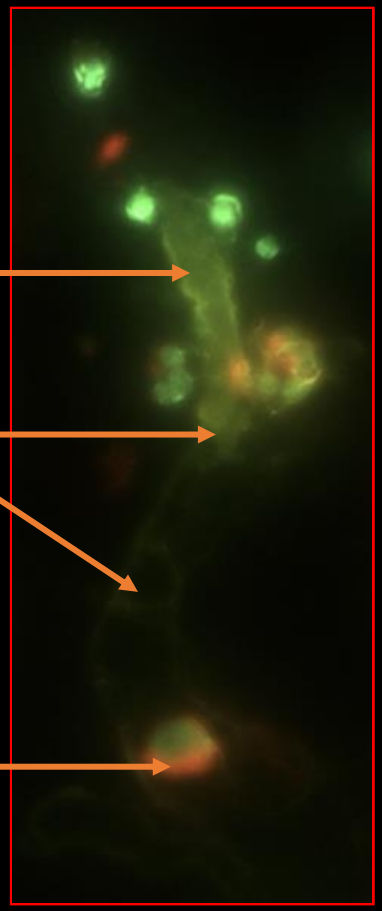


NETosis cell-based model

Visualizing NETs in ICF

Activated neutrophils





Extracellular
DNA 500 nm

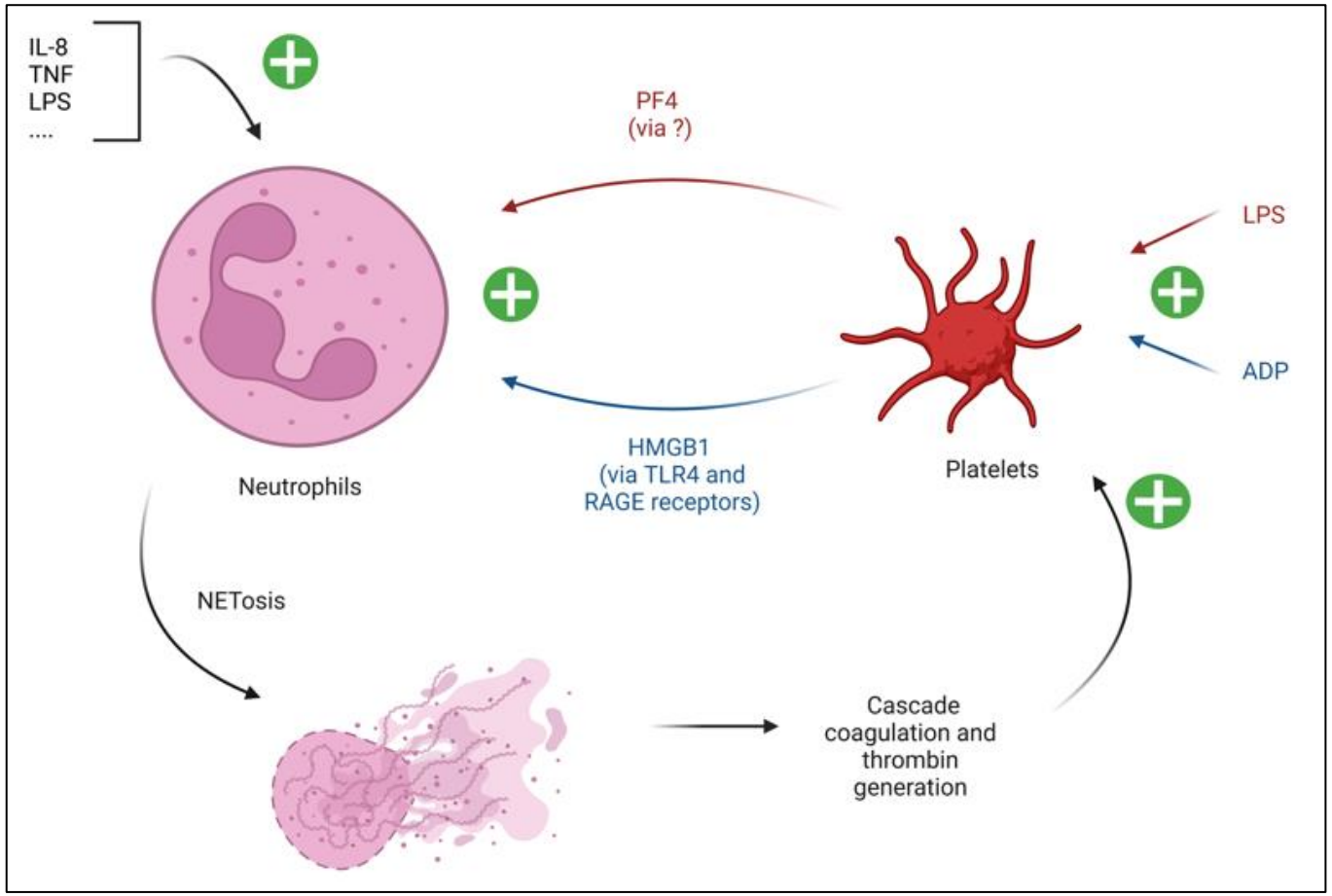
Histone citH3
580 nm

Actine infrared
647 nm

NETosis cell-based model

Cell-free DNA release and NE activity: testing activators (HMGB1 & PF4)

- Real-time fluorescence tracks **cell-free DNA** and **NE activity.**
- Data analyzed through **T50** and **AUC**.
- **DNA quantification** is performed using a calibration curve.
- Allows screening of potential NETosis activators such as: **HMGB1** and **PF4.**



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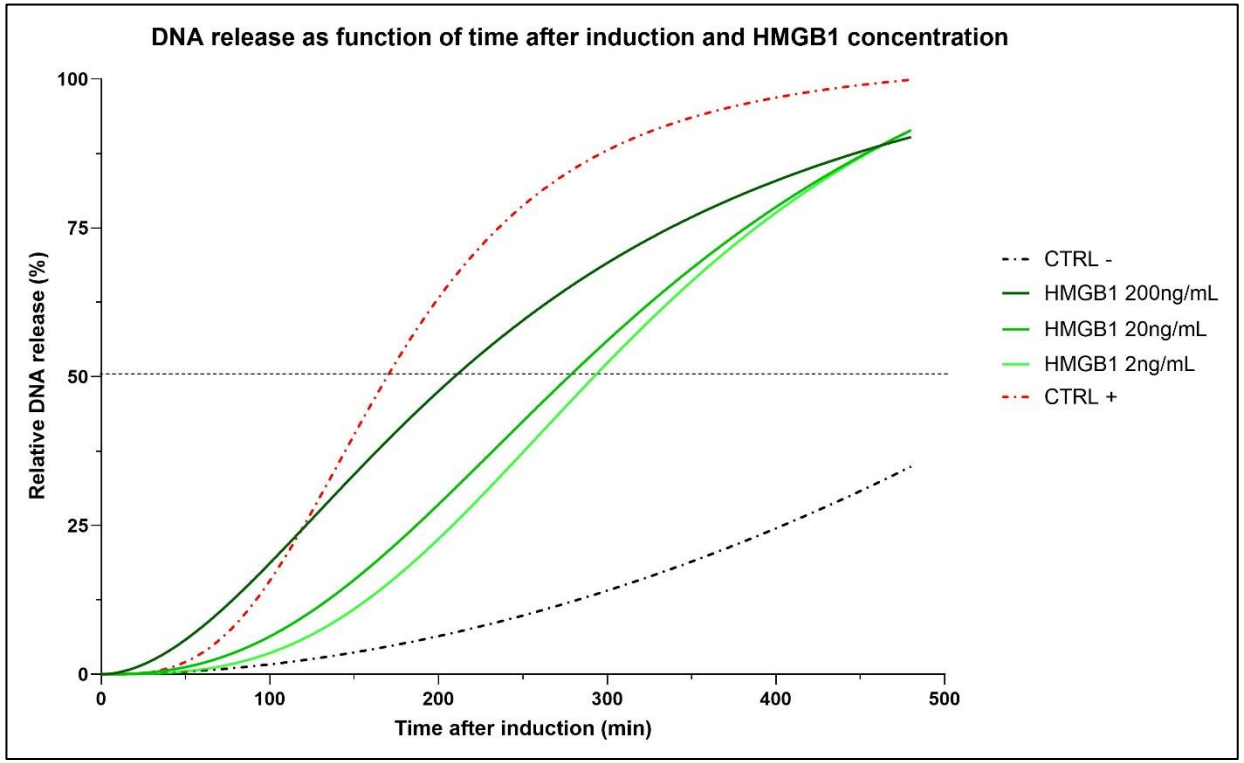
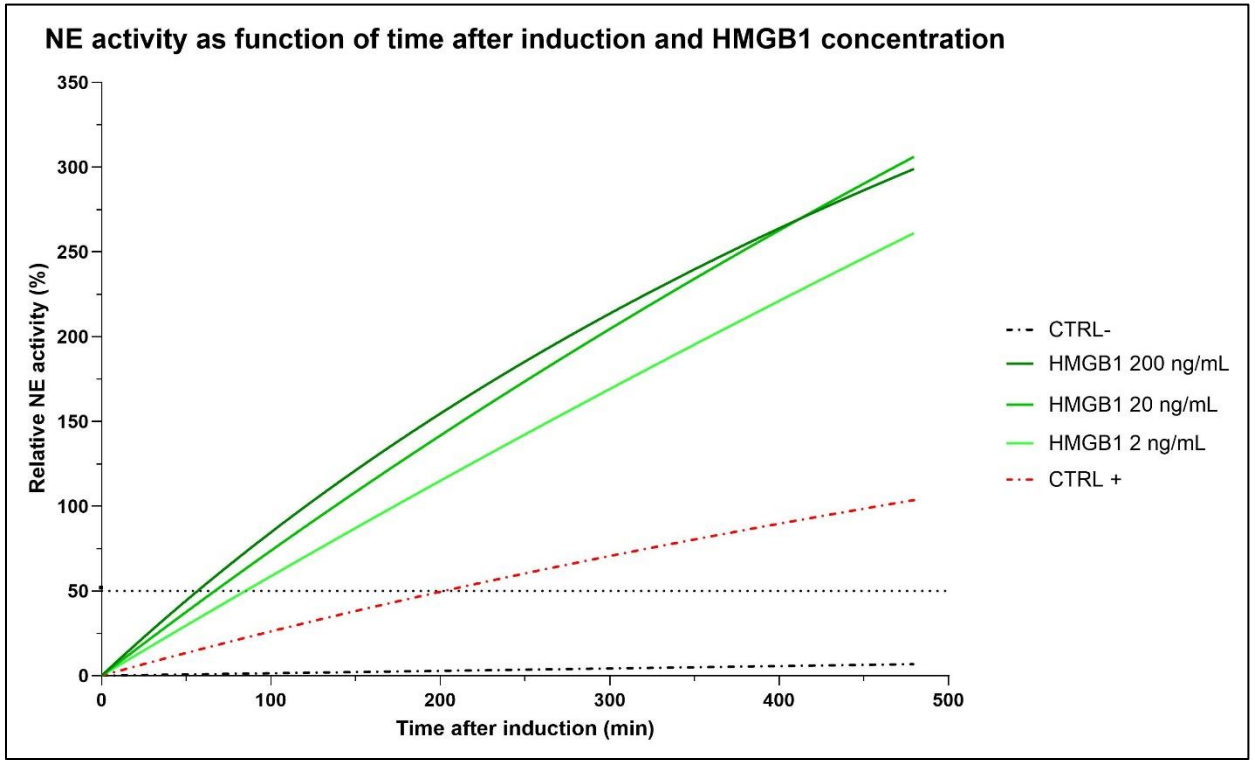
NETosis cell-based model

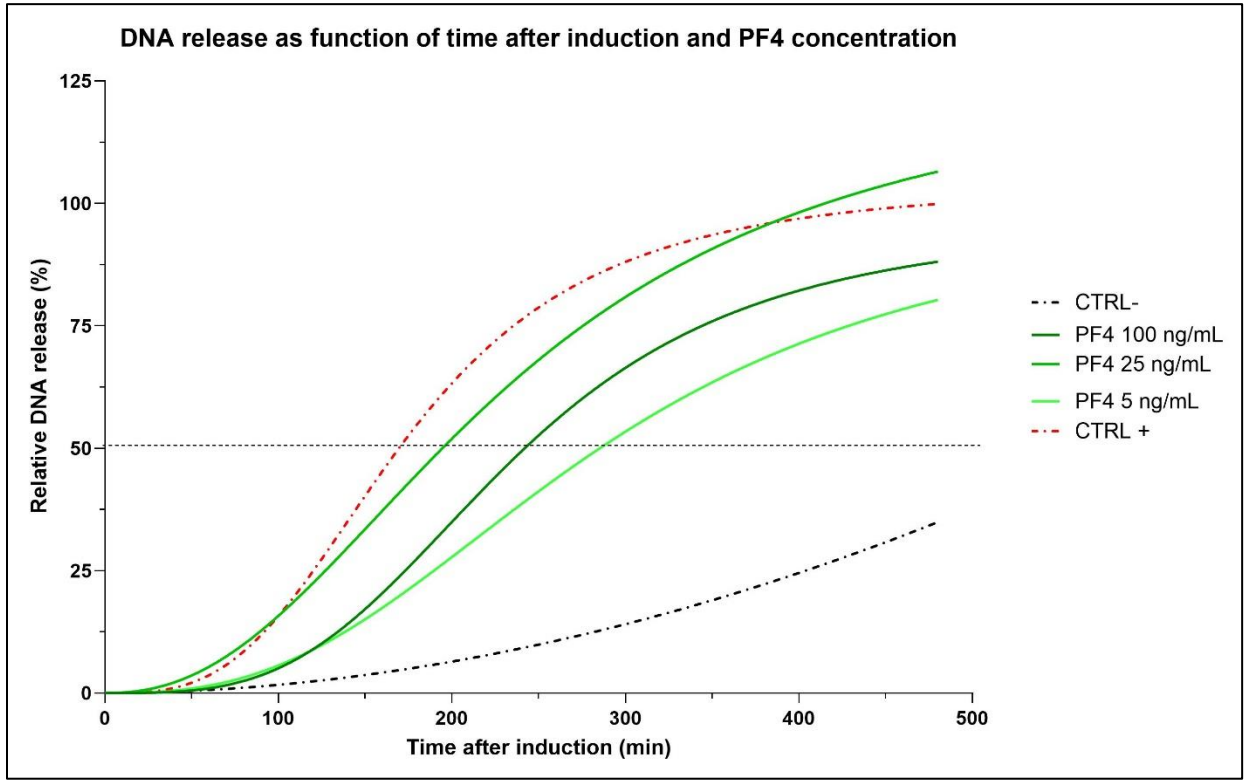
Cell-free DNA release and NE activity: testing activators HMGB1

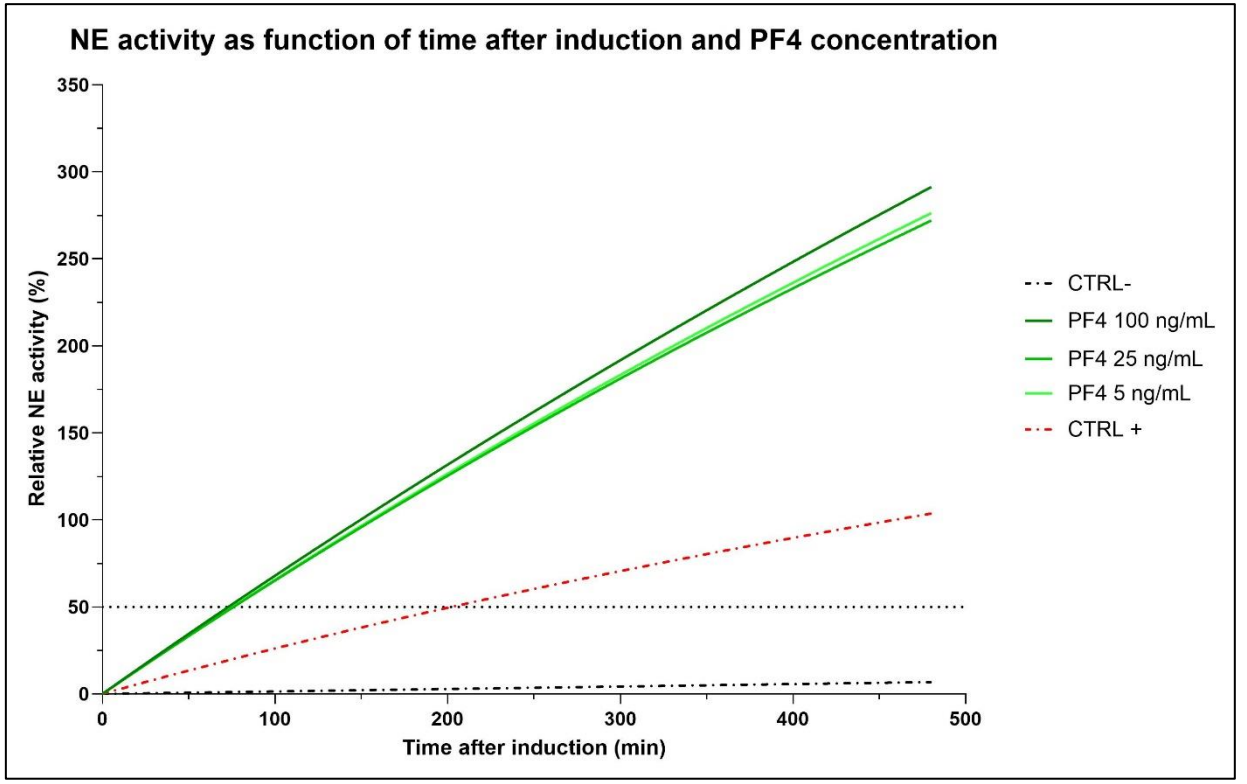




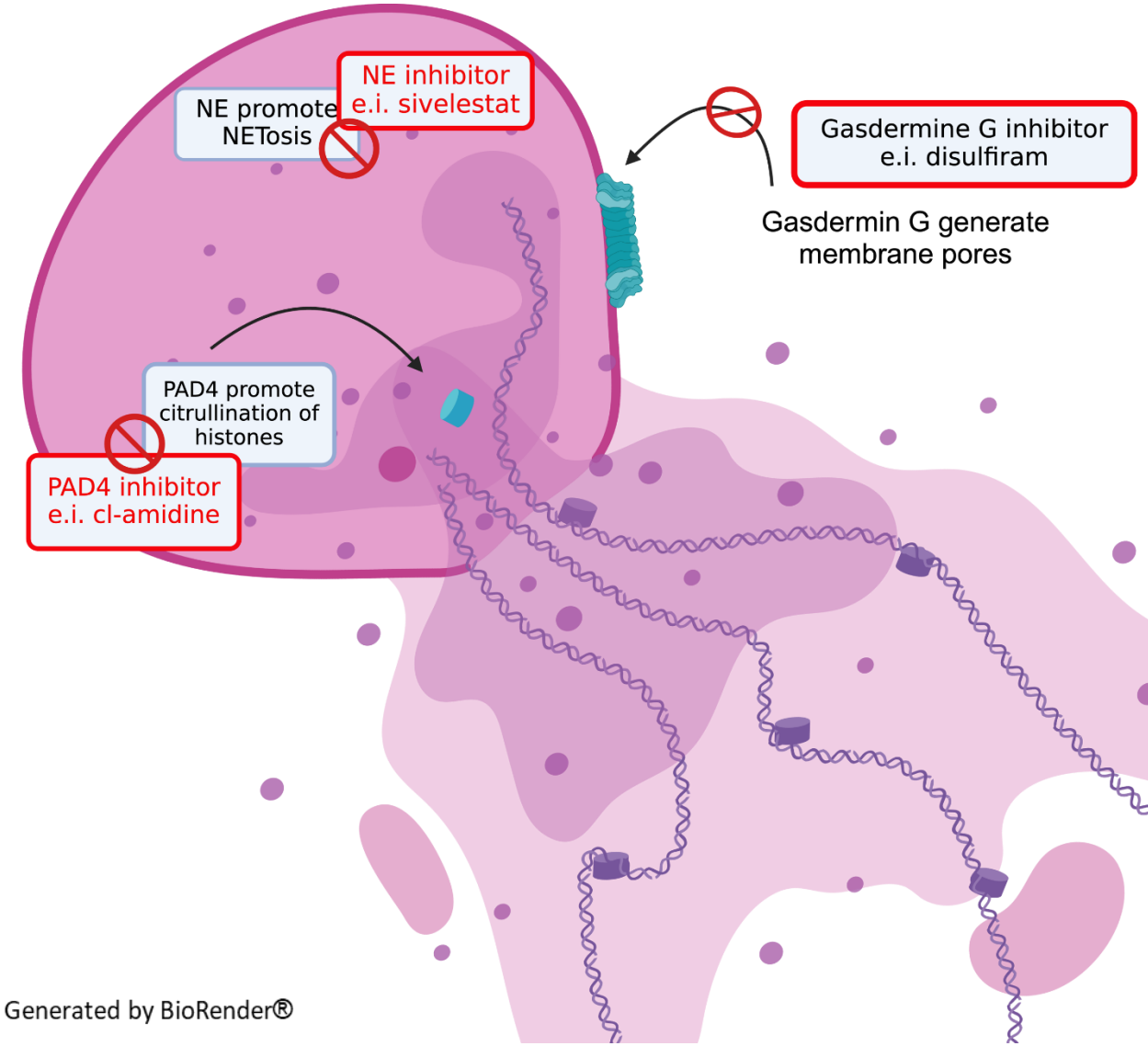
NETosis cell-based model

Cell-free DNA release and NE activity: testing activators PF4





NETosis cell-based model
Modulation of NETs formation



NETs activation

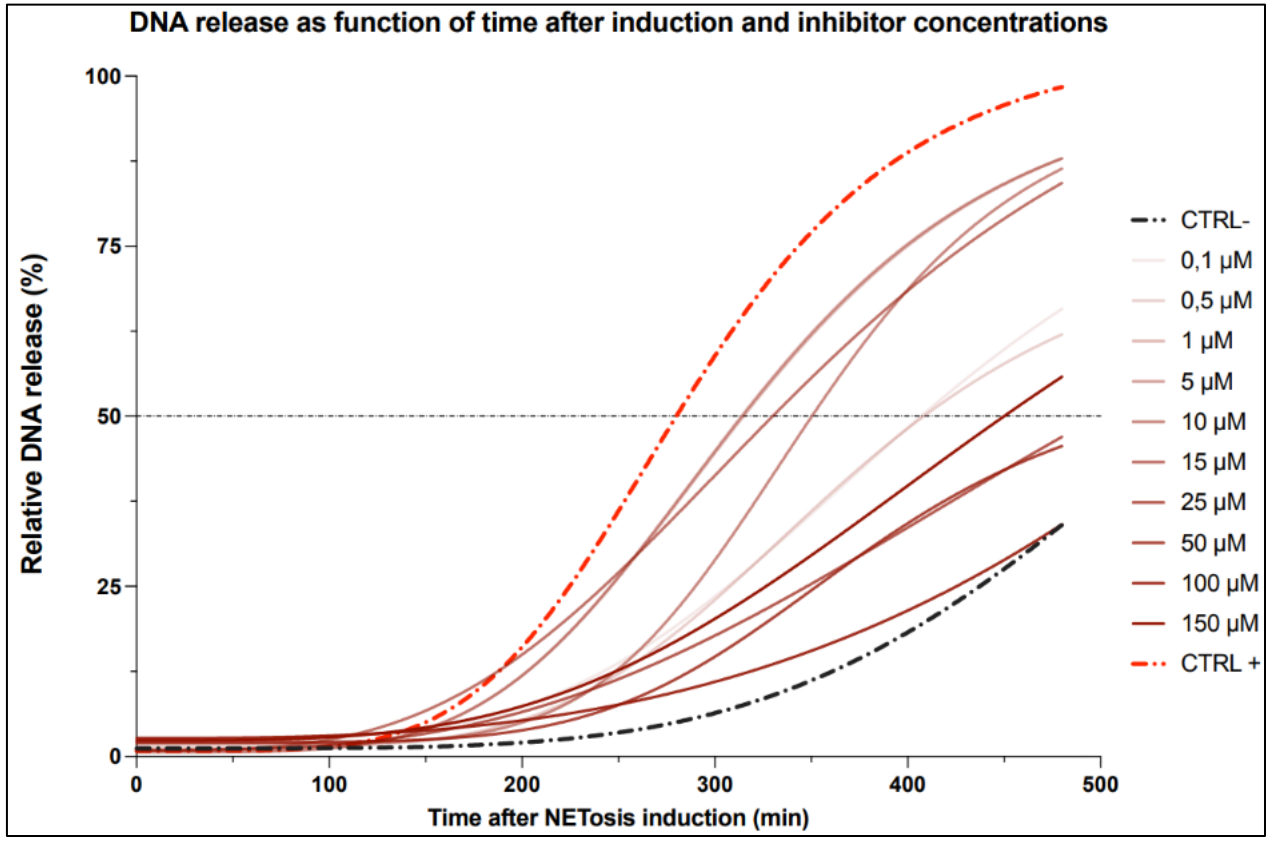
Sivelestat: a neutrophil elastase inhibitor

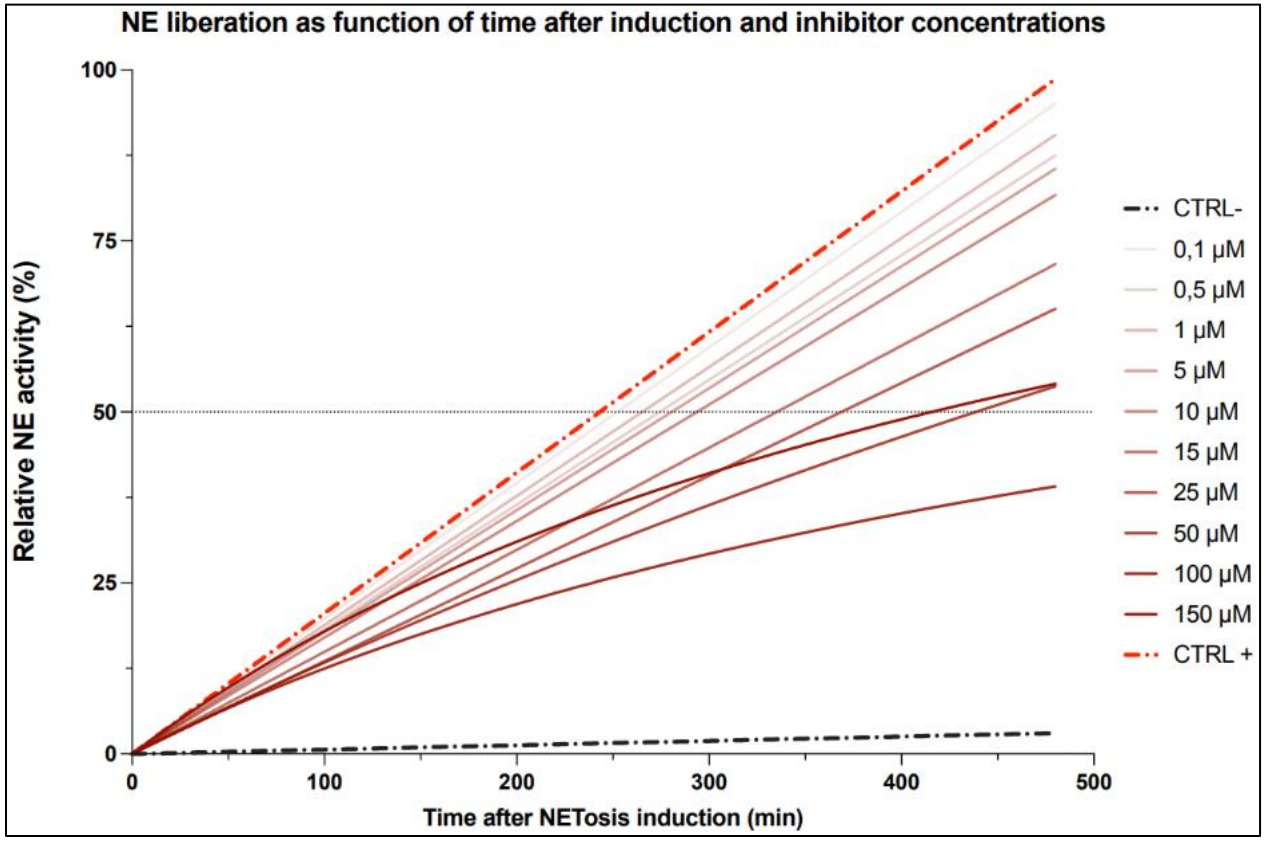
Chlor-amidine: PAD_4 inhibitor which blocks chromatine decondensation

Disulfiram: a gasdermine G inhibitor which reduces the formation of NETs

NETosis cell-based model

Cell-free DNA release and NE activity: testing inhibitors





Perspectives and future directions

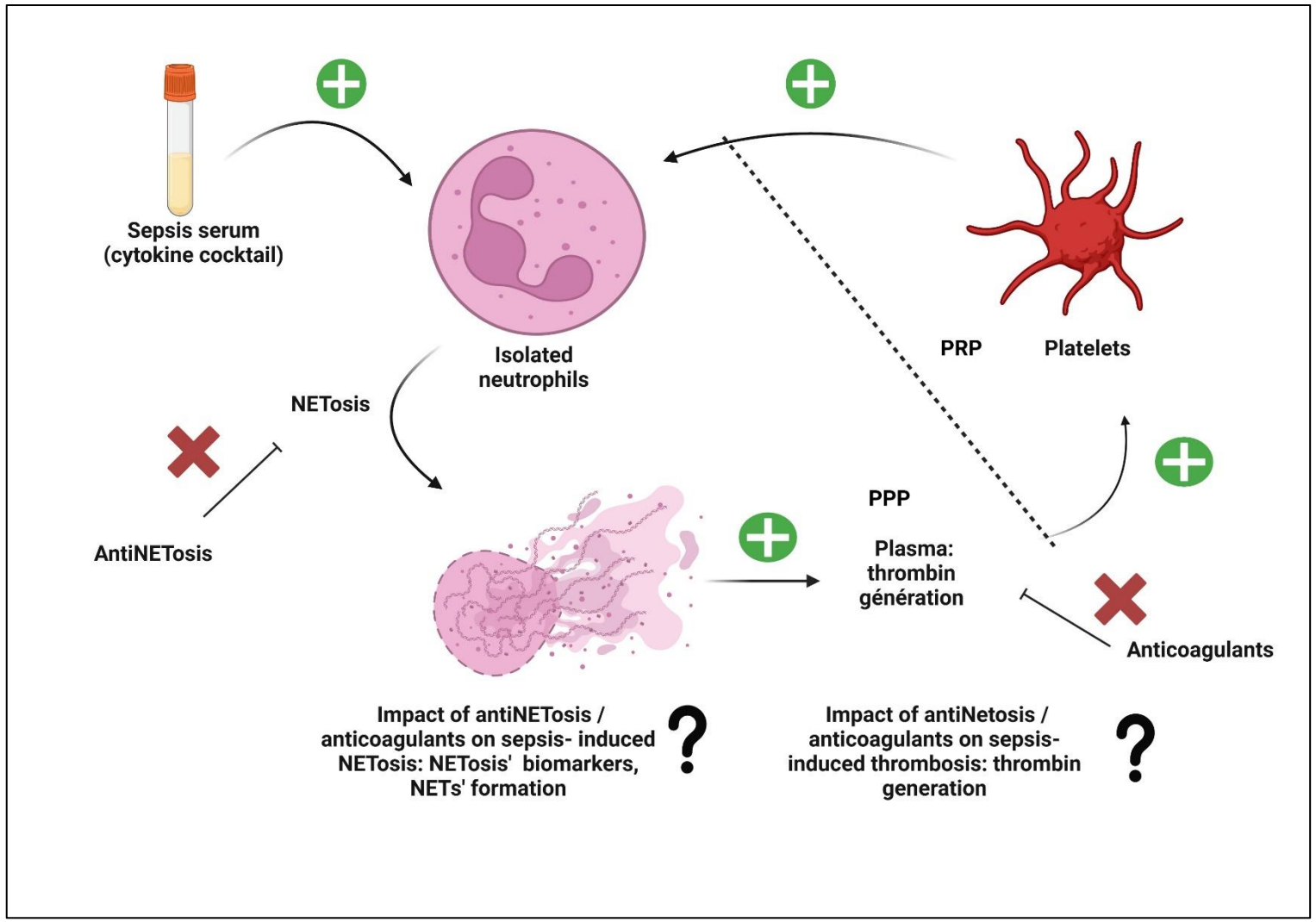
- Currently, the cell-based model allows for the **exploration of NETosis** only by evaluating the effects of **activators and inhibitors**.
- The model does not yet enable the study of the **NETosis-hemostasis relationship.**
- The **objective** is to optimize the model by adding **plasma (PRP** or **PPP**).
- This model will be used in:

1. **Sepsis:** To investigate the role of this interaction in pathophysiology and screen potential drugs for treatment.
2. **In-house development of new AAV viral vectors:** assessing hemocompatibility to validate their use in gene therapy treatments.

Perspectives and future directions

NETosis in sepsis: mechanisms and drug screening

- **Sepsis** is a life-threatening condition caused by a **dysregulated** immune response.
- **NETosis** plays a crucial role in the **pathophysiology** of sepsis and is a key driver of **sepsis-induced thrombosis**.
- **Objectives:** To **develop** a **method** to assess **NETosis-induced thrombin generation** in sepsis, and to **evaluate** the **effect of anti-NETosis agents** and **anticoagulants** on the **NETosis-hemostasis relationship.**



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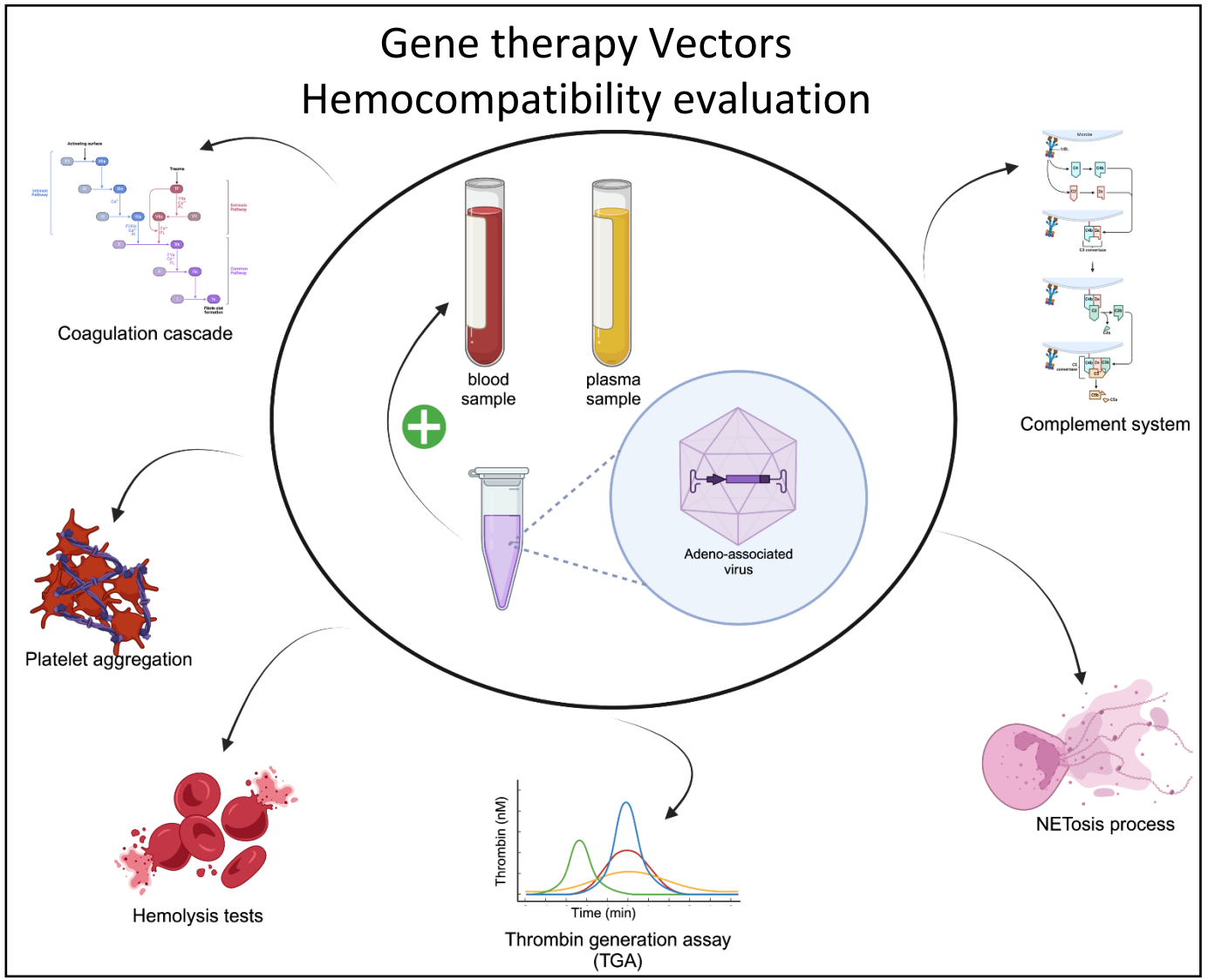
Perspectives and Future Directions

Hemocompatibility of adenoviruses produced In-house

- **Viral** and **non-viral vectors**: to develop innovative personalized therapeutic solutions (hereditary diseases, chronic conditions, cancer).
- **Problem**: AAVs **toxicity** → adverse effects such as **thrombotic microangiopathies**.
- **Studies**: AAVs **can activate** the complement system, leading to platelet activation and thrombotic events.
- **Objective**: evaluation of the impact of viral and non-viral vectors on the hemostatic system, the complement system and NETosis for a **safer** use of these vectors.



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Thank you for your attention !
Any questions ?

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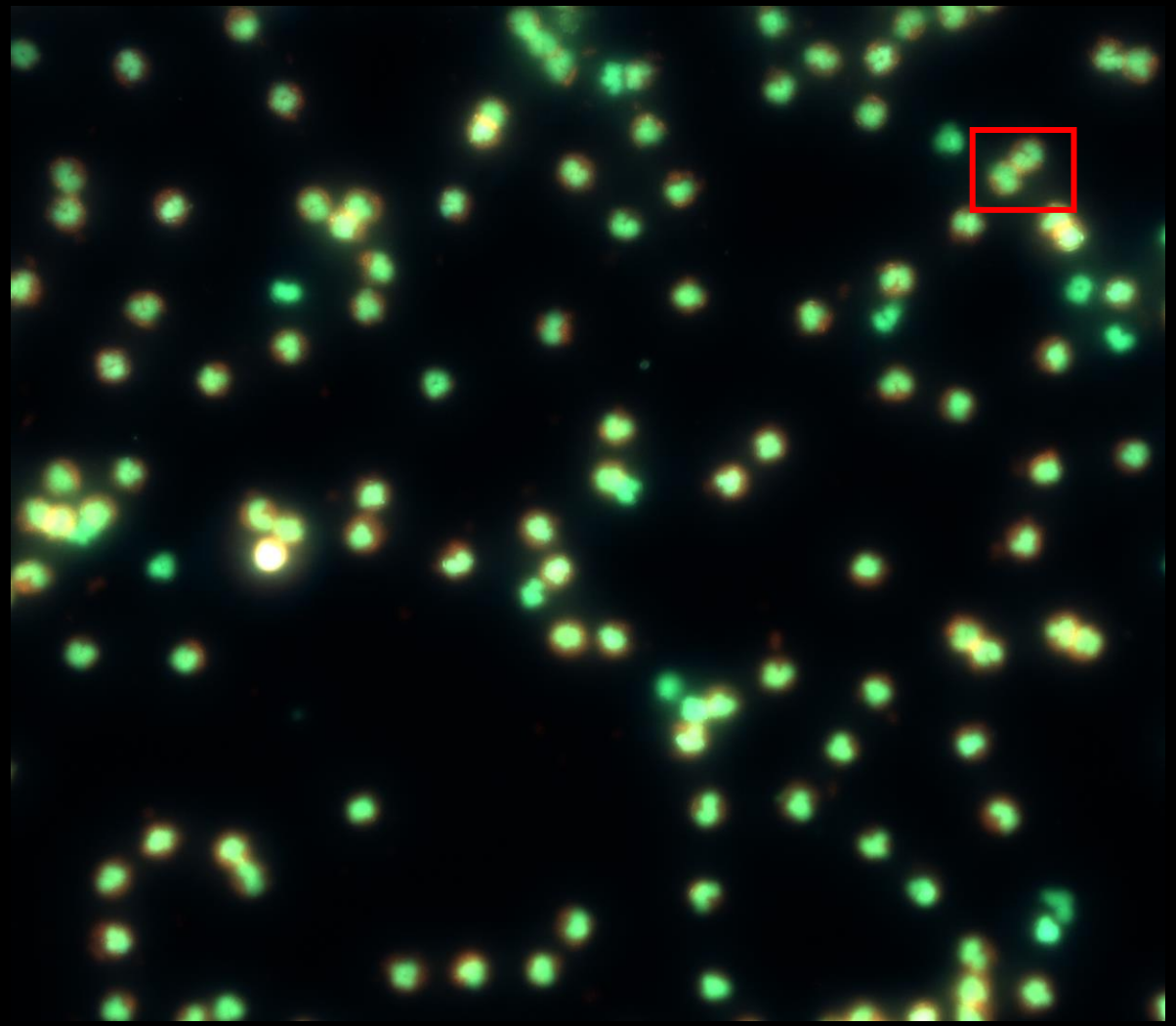
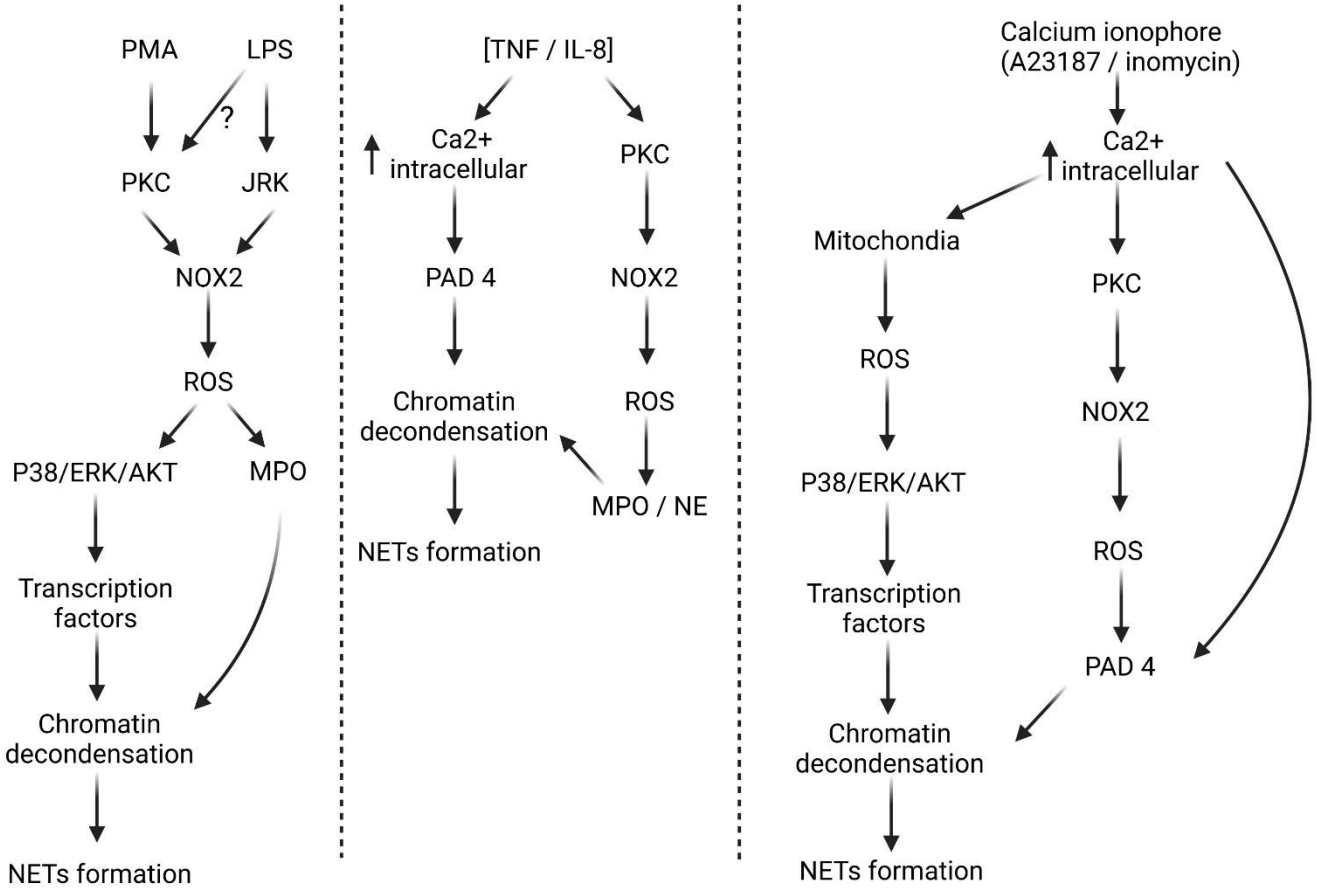
NETosis cell-based model
visualizing NETs

Inhibited neutrophils



Intracellular signaling pathways



Intracellular Signaling Pathway of Different NETosis Activators

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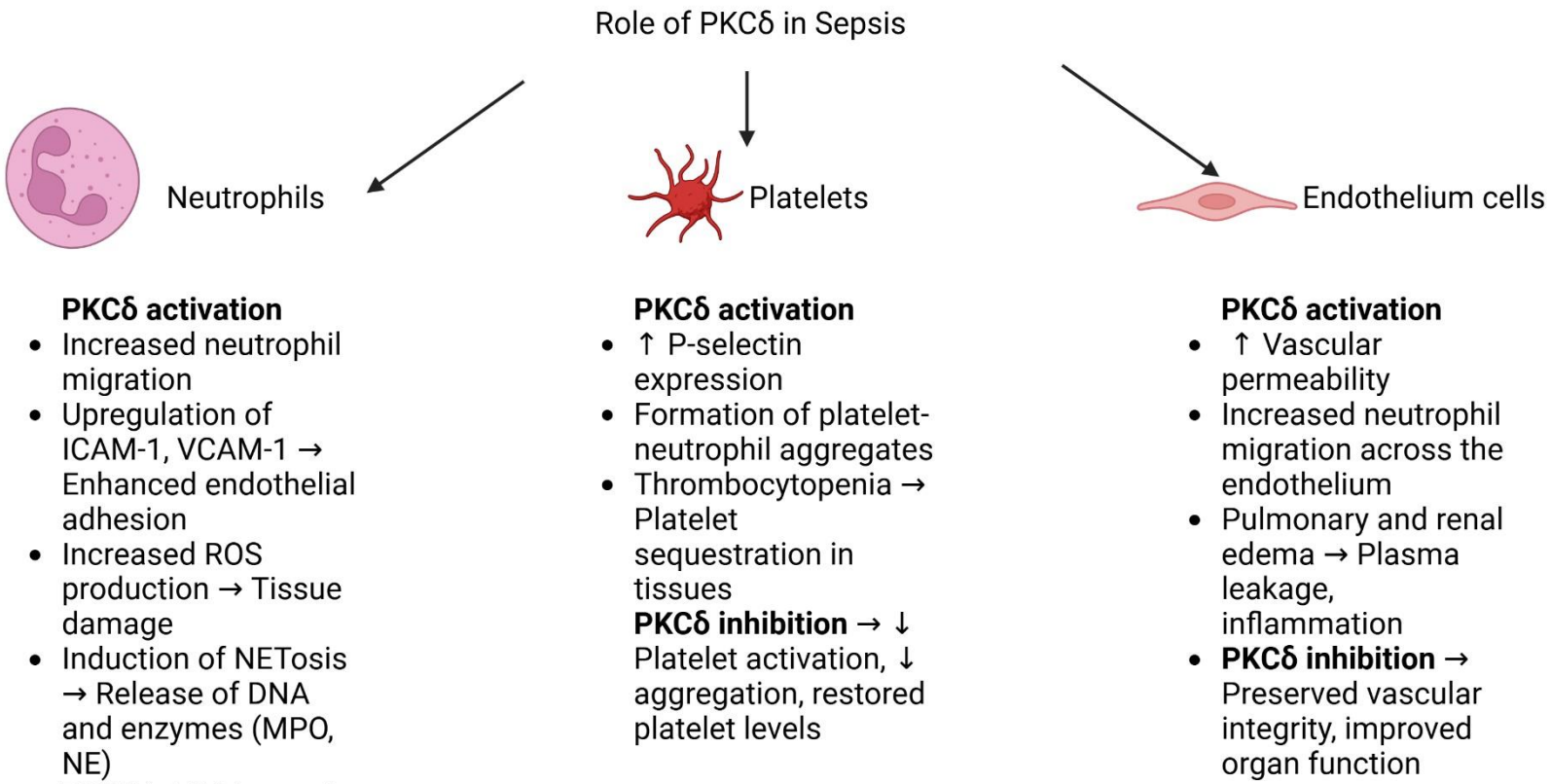
PKC δ in sepsis



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