SCHOOL OF LIFE SCIENCES
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Extracellular Traps: Potential Armamentarium of Neutrophils

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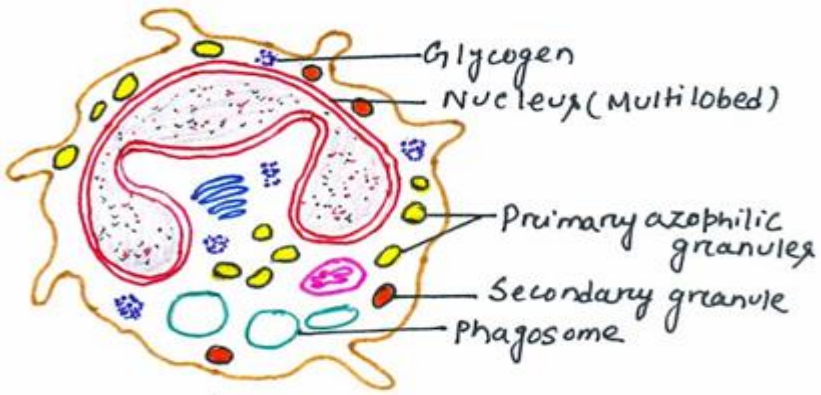
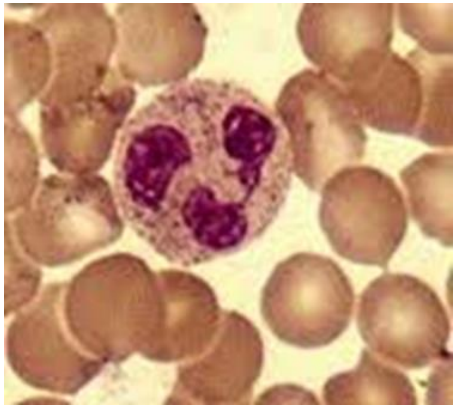
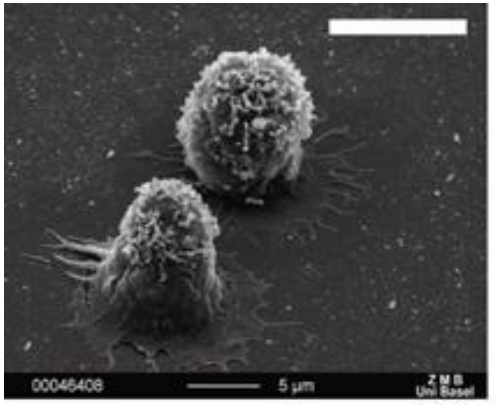
Neutrophils

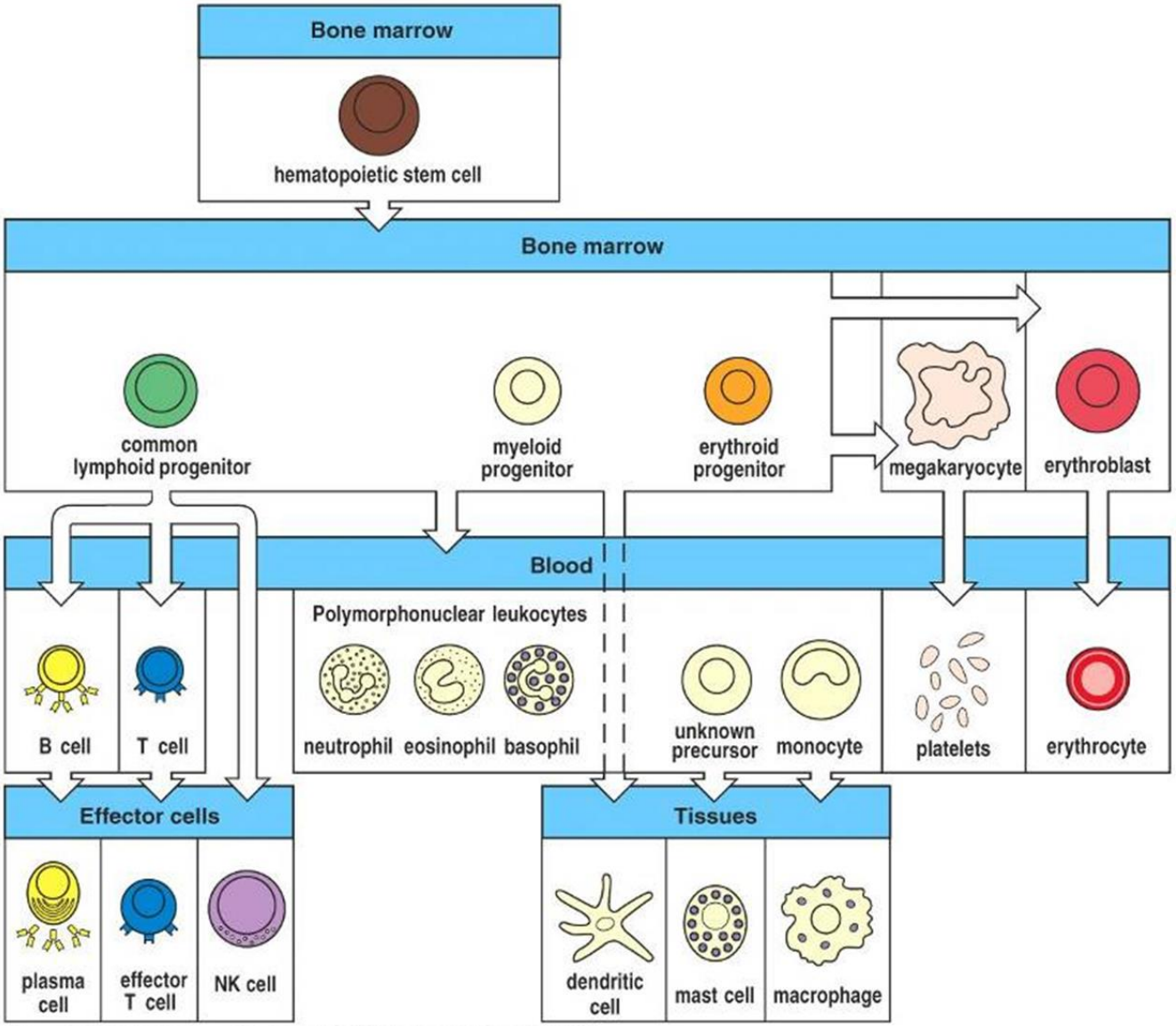
- ✓ Neutrophils play central role in fighting infections
- ✓ Reach area of inflammation within minutes by migrating (diapedesis) from circulatory system to site of infection.
- ✓ Granulocyte
- ✓ Terminally differentiated
- ✓ 55-75 % total blood cells
- ✓ An abundance of granules
- ✓ Multi-lobed nucleus (Polymorpho nuclear cell)
- ✓ Prominent cytoskeleton for locomotion and chemotactic functions



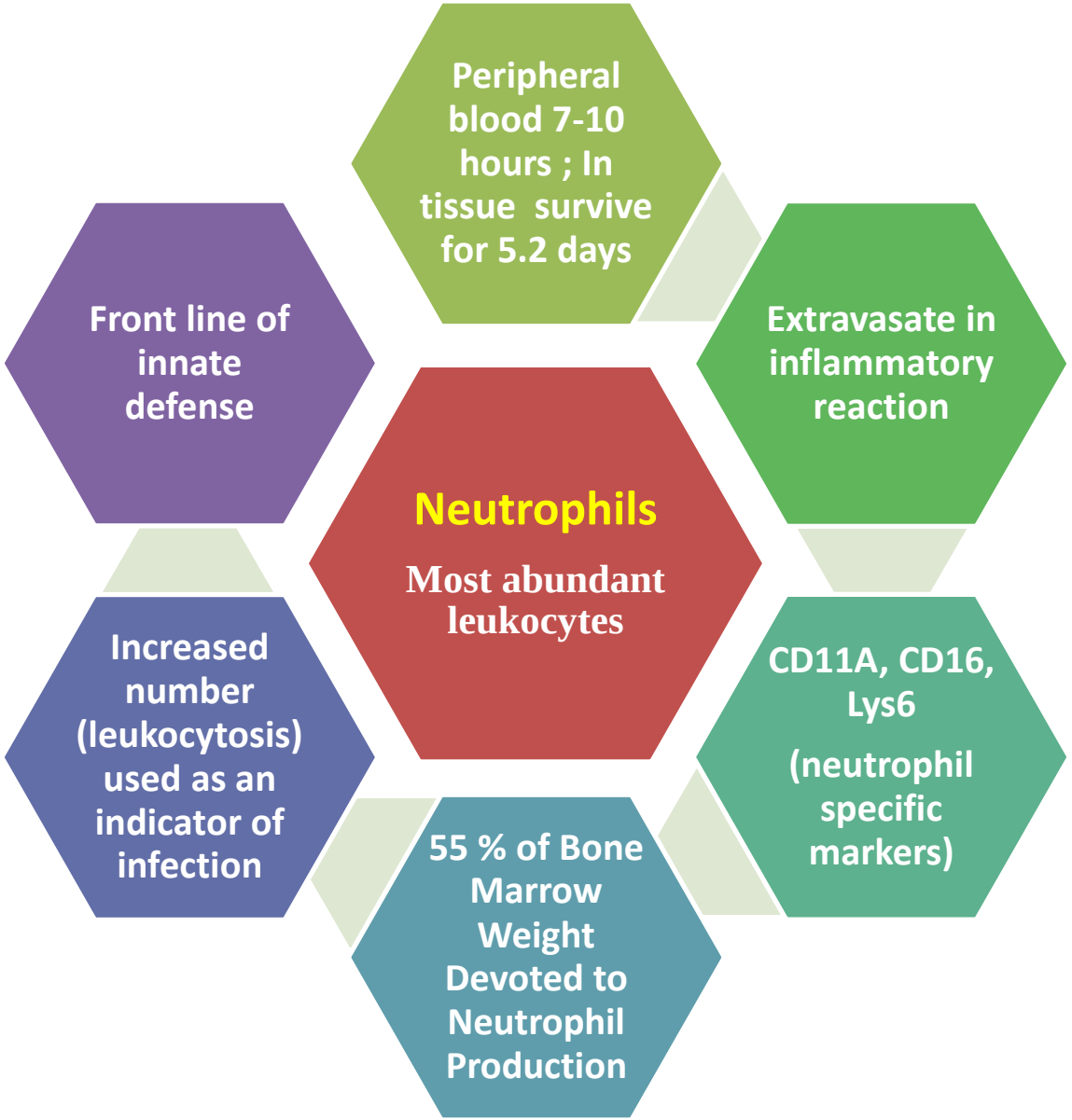




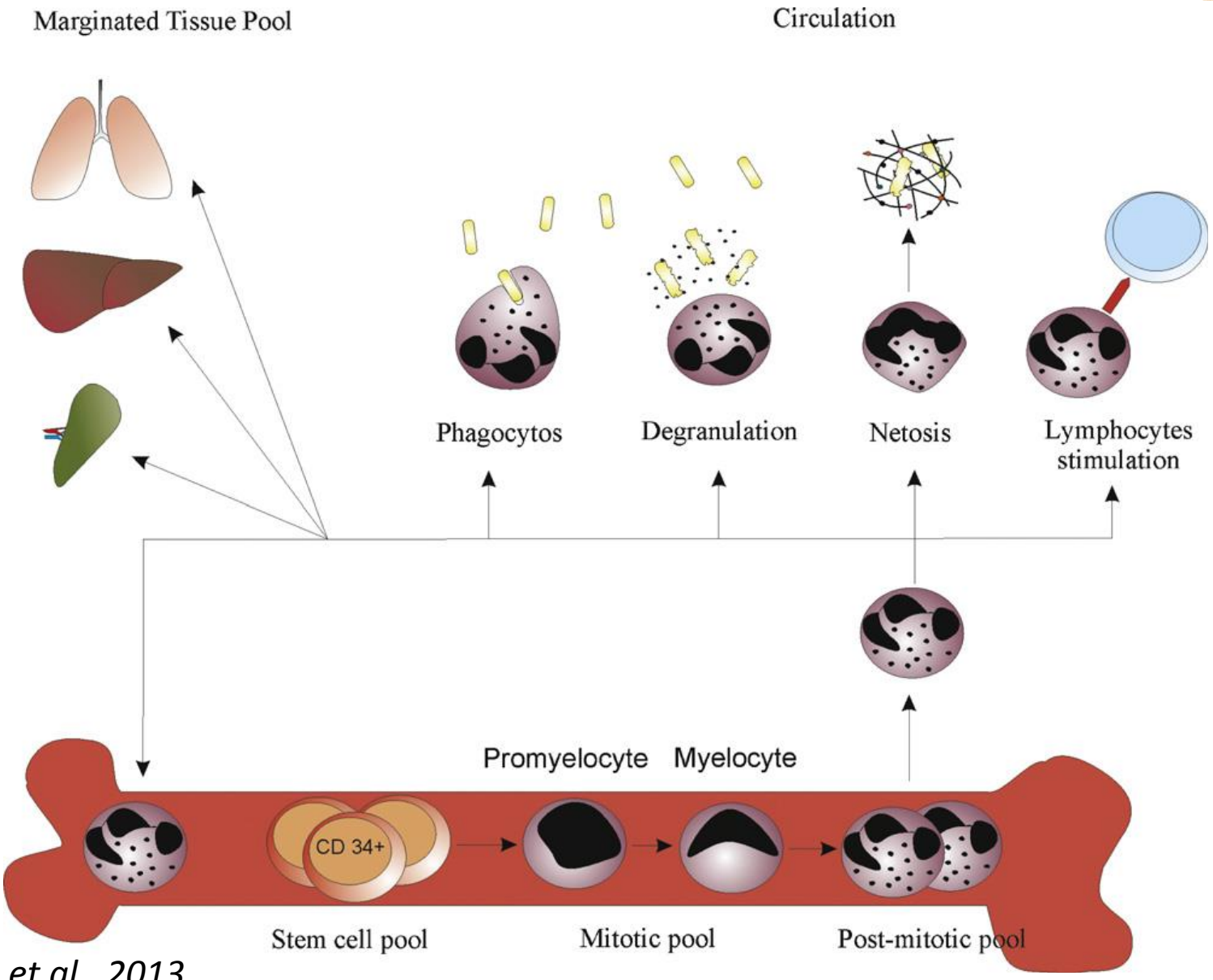
Neutrophils are derived from myeloid lineages



Properties of Neutrophils



Life of Neutrophil



Purchniak et al., 2013

What's there in neutrophil granules?

Azurophilic or Primary : The first granules formed in the developing neutrophil and peak degranulation is 90 minutes.

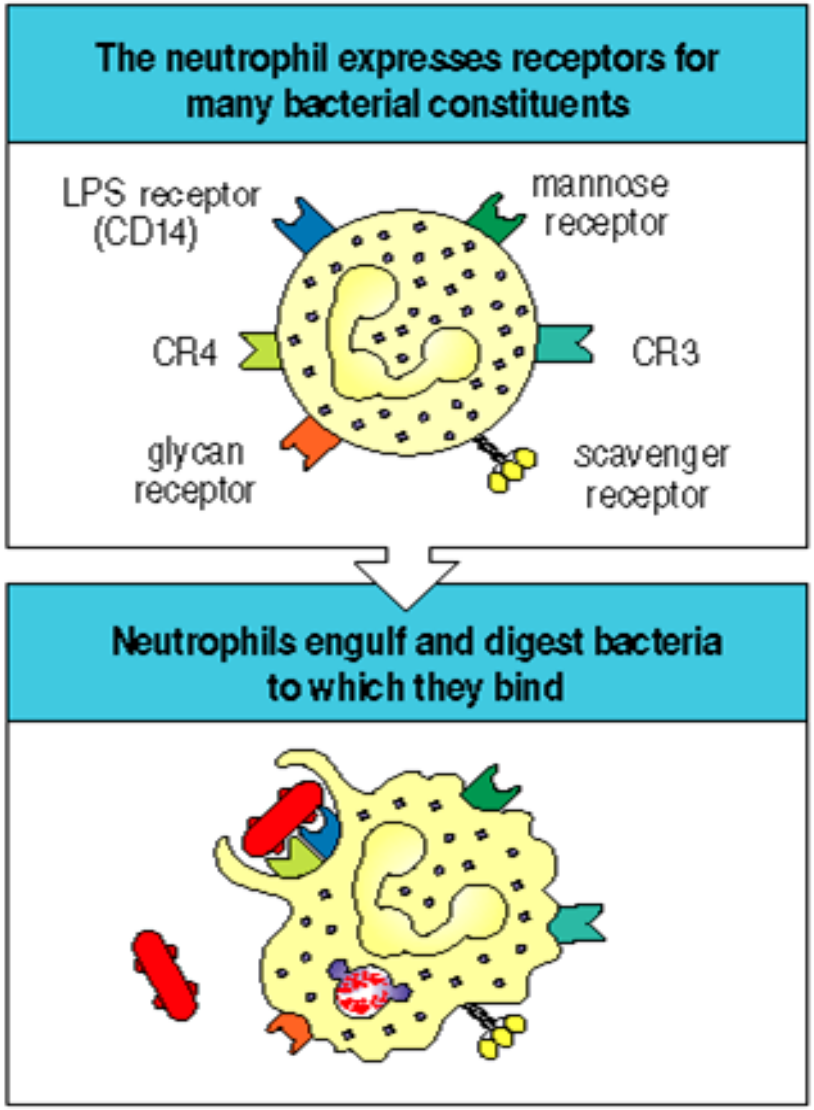
Myeloperoxidase
Defensins
Lysozyme
Elastase
Cathepsin G
Alkaline phosphatase
Proteinase 3
β-glucuronidase
α-fucosidase
Phospholipases A2, C, D
α-mannosidase

Secondary: formed later in the development of the neutrophil. These enzymes are released within 15 seconds after contact with the pathogen.

Lactoferrin
Lysozyme
Collagenase
Gelatinase
Vitamin B12-binding protein
Cytochrome b558
fMLP receptor
CD11b/CD18, CD11c/CD18 (integrins)
Complement receptor 3 (CR3)
Histaminase
Plasminogen activator

Neutrophil Mediated Killing Mechanisms

1. Phagocytosis



- Phagosome - membrane bounded vesicle that becomes acidified
- Lysozome - granules that contain products that damage or kill pathogens
  - Enzymes

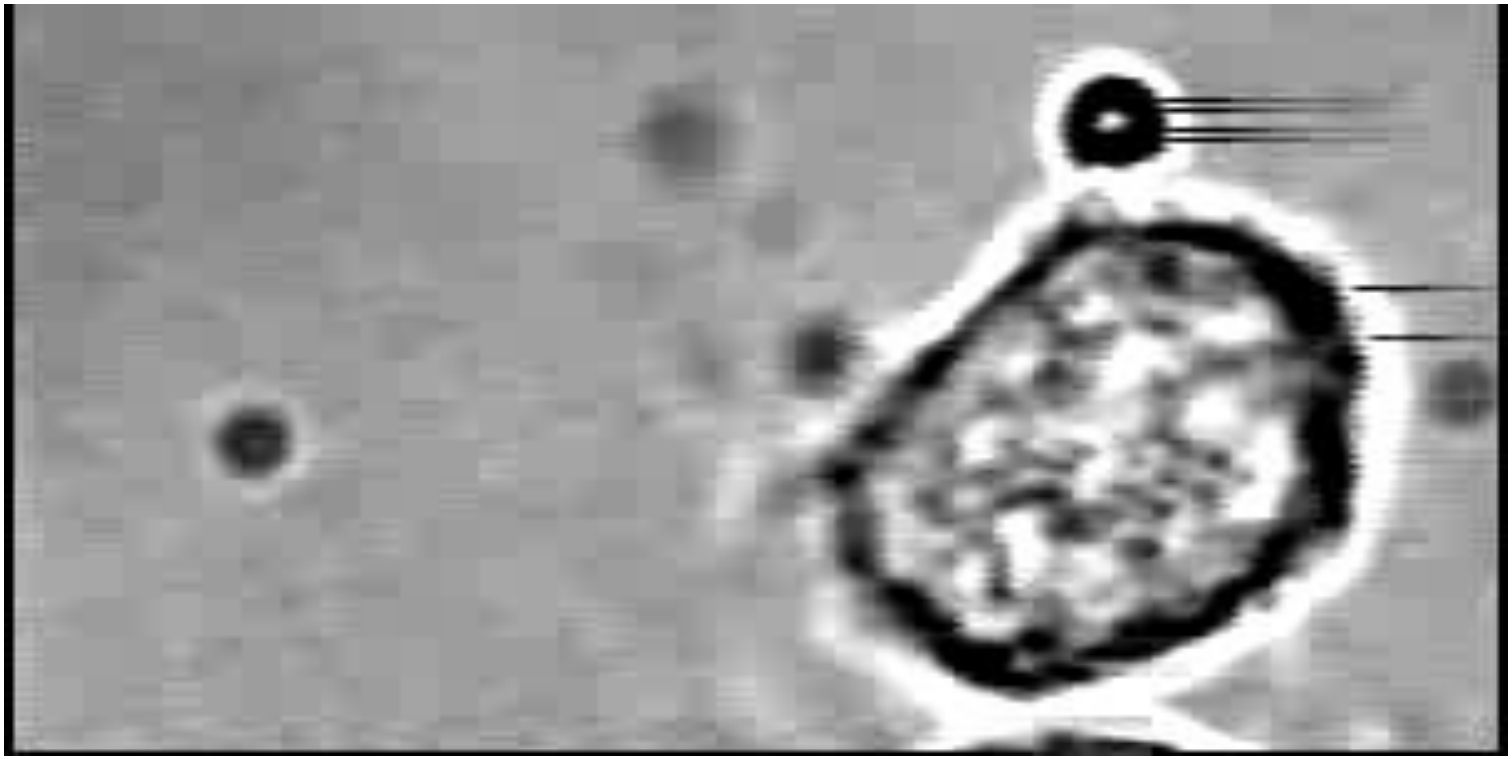
    Lysozyme - dissolves cell walls of some bacteria

    Acid hydrolases - digests bacteria
  - Proteins

    Lactoferrin - binds Fe^{++} needed for bacterial growth
  - Peptides

    Defensins and cationic proteins - direct antimicrobials

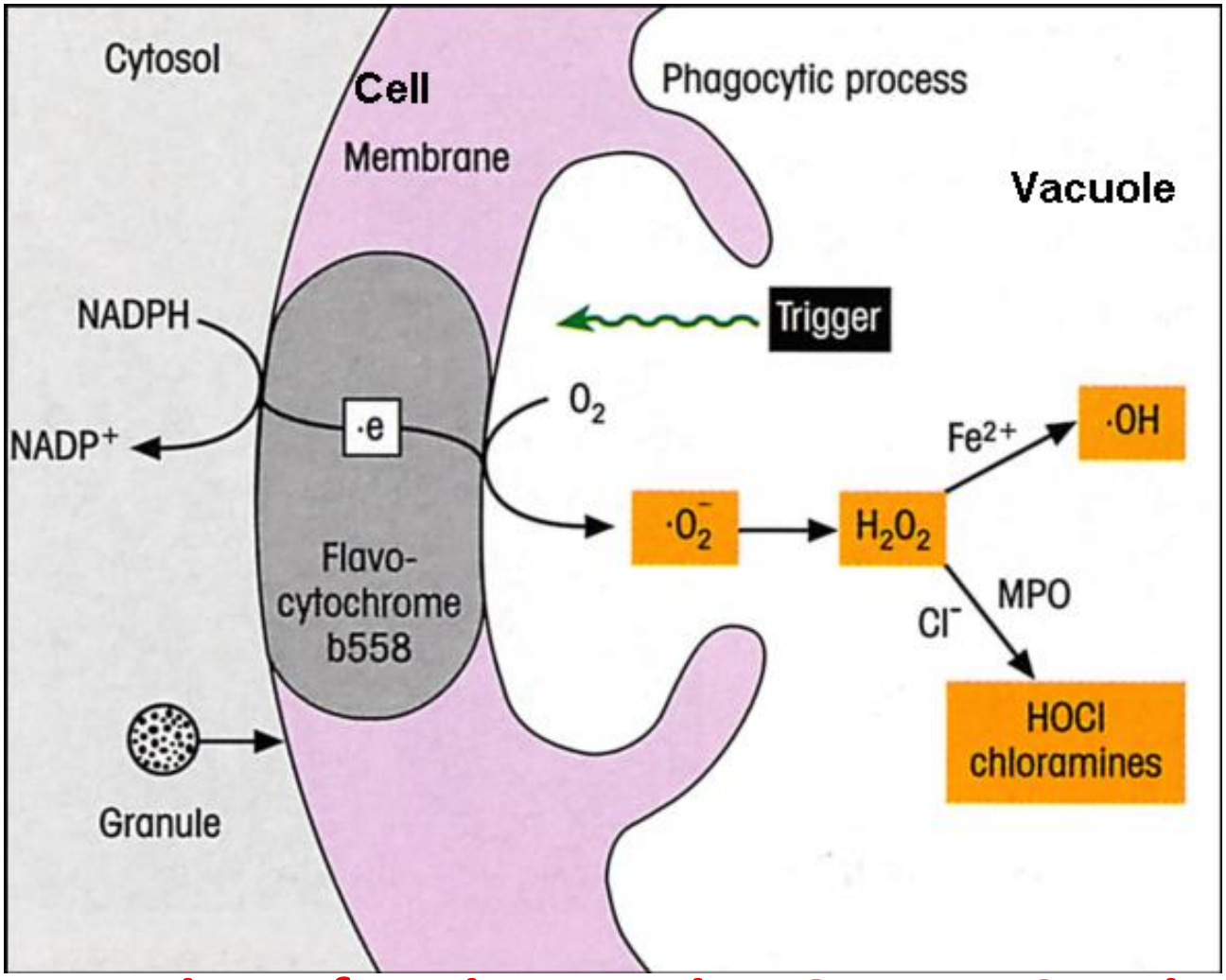
Phagocytosis



www.hopkinsmedicine.org/cellbio/devreotes

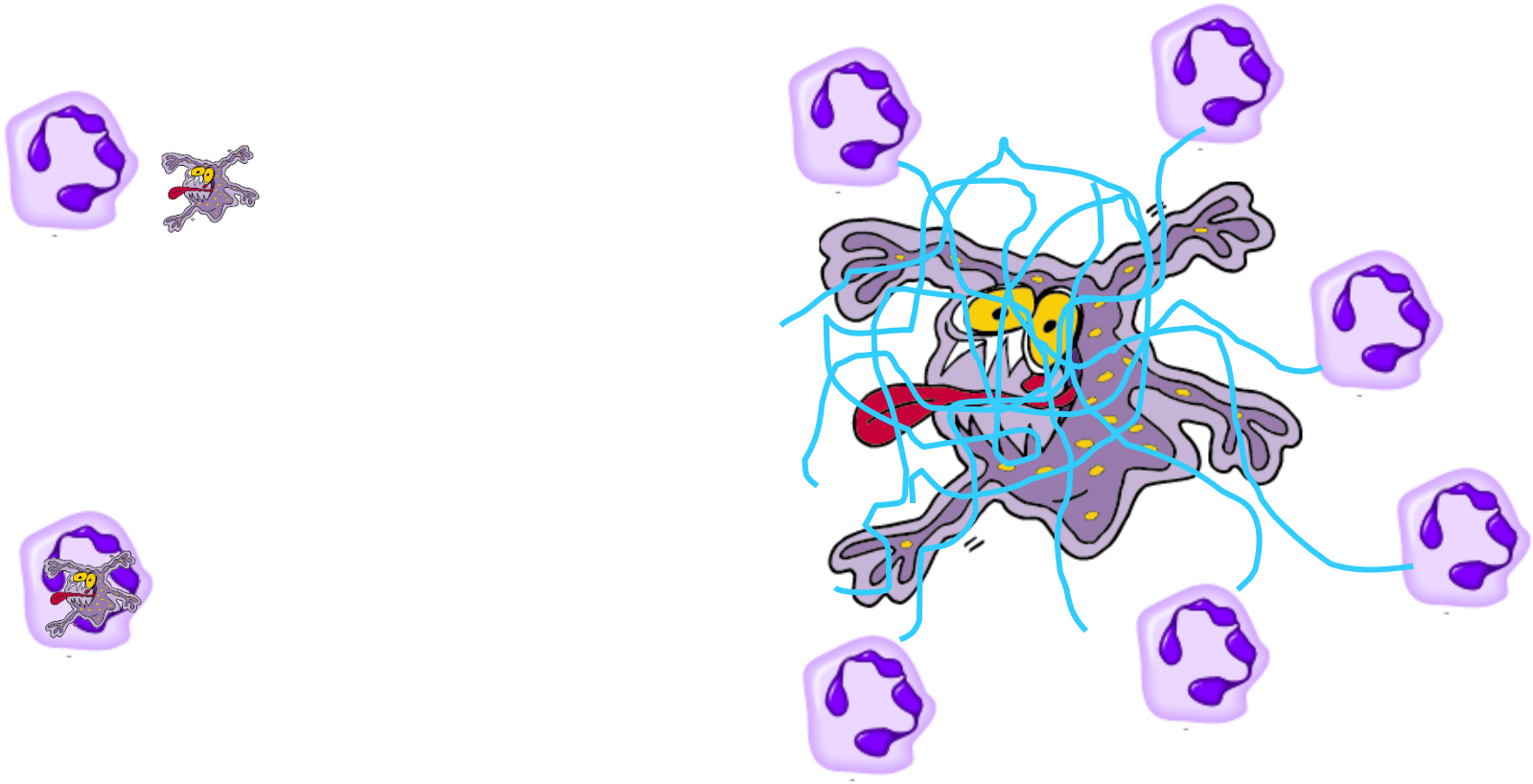
Neutrophil Mediated Killing Mechanisms

2. Oxidative (Respiratory) Burst

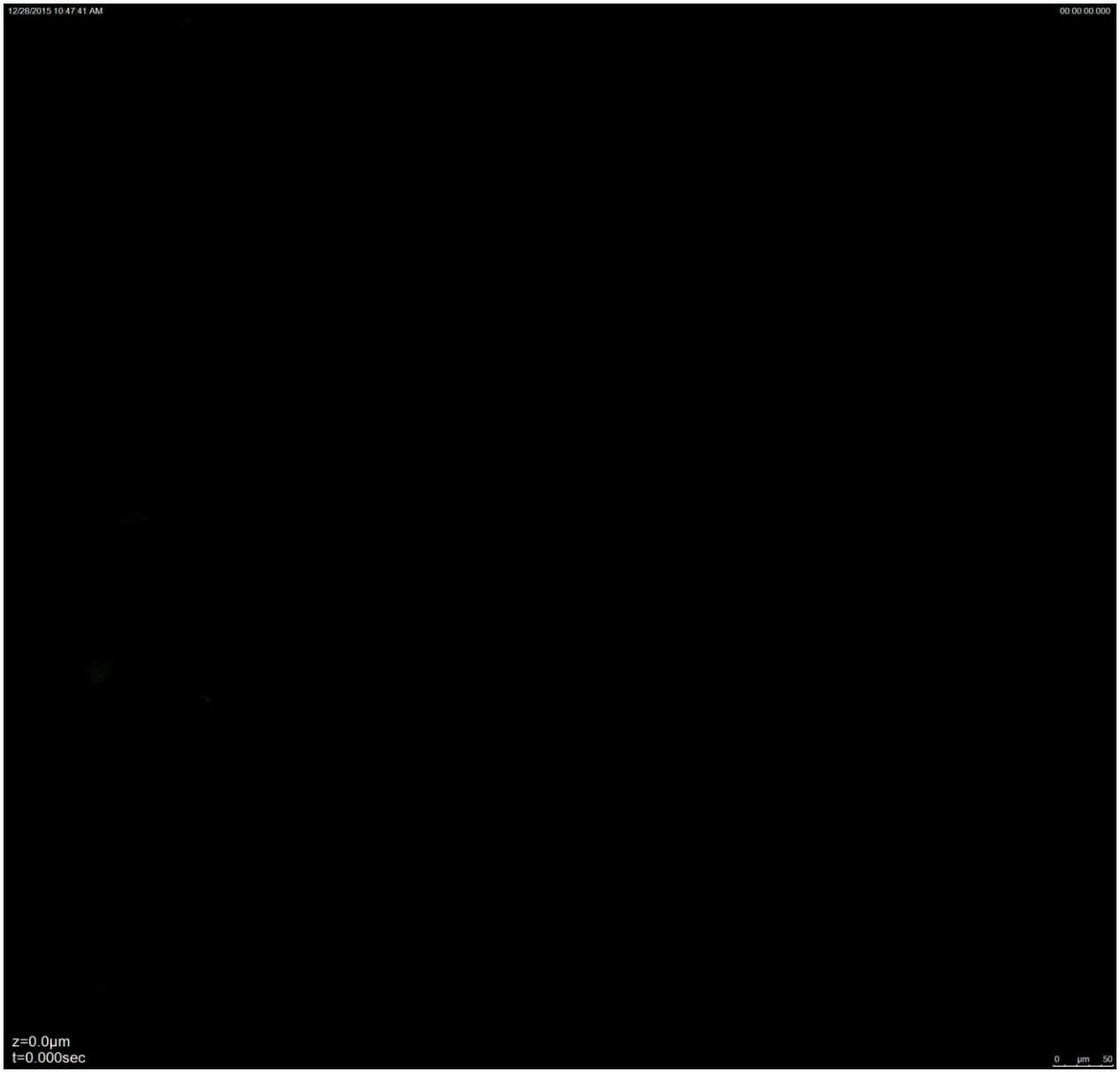


Generation of Toxic Reactive Oxygen Species

Neutrophils: The Spiderman of Our System Kill Pathogens by Neutrophil Extracellular Traps (NETs)



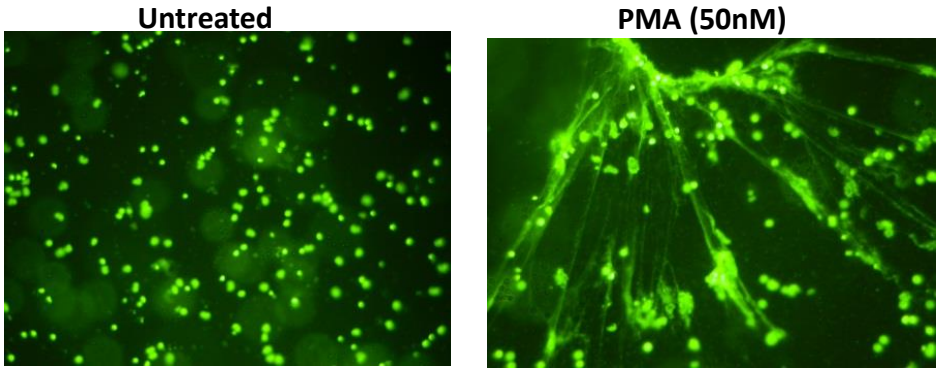
Neutrophils Extracellular Traps (NETs)



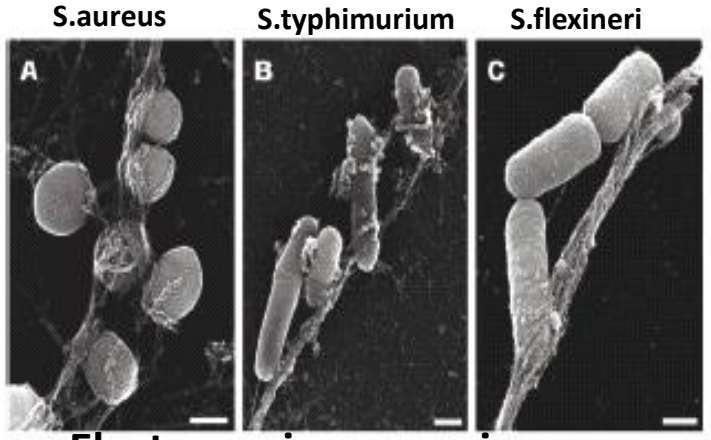
Neutrophils: Smart Cells- Smart Ways to Kill Pathogens

Neutrophil Extracellular Traps (NETs)

- ✓ NETosis: Release of chromatin and granular proteins to form an extracellular fibrillar matrix to trap pathogens *(Brinkman et al, Science, 2004)*
- ✓ Reactive oxygen species (NADPH oxidase) dependent. Mutations in any component of NADPH oxidase gene leads to Chronic Granulomatous Disease; patients fail to produce NETs (*Fuchs et al, J Cell Biol, 2007)*
- ✓ NETs are composed of variety of bactericidal proteins such as elastase, myeloperoxidase, cathelicidin etc, bound to DNA.



NETs stained with DNA binding dye Sytox Green



Electron microscopy images showing bacteria trapped in NETS

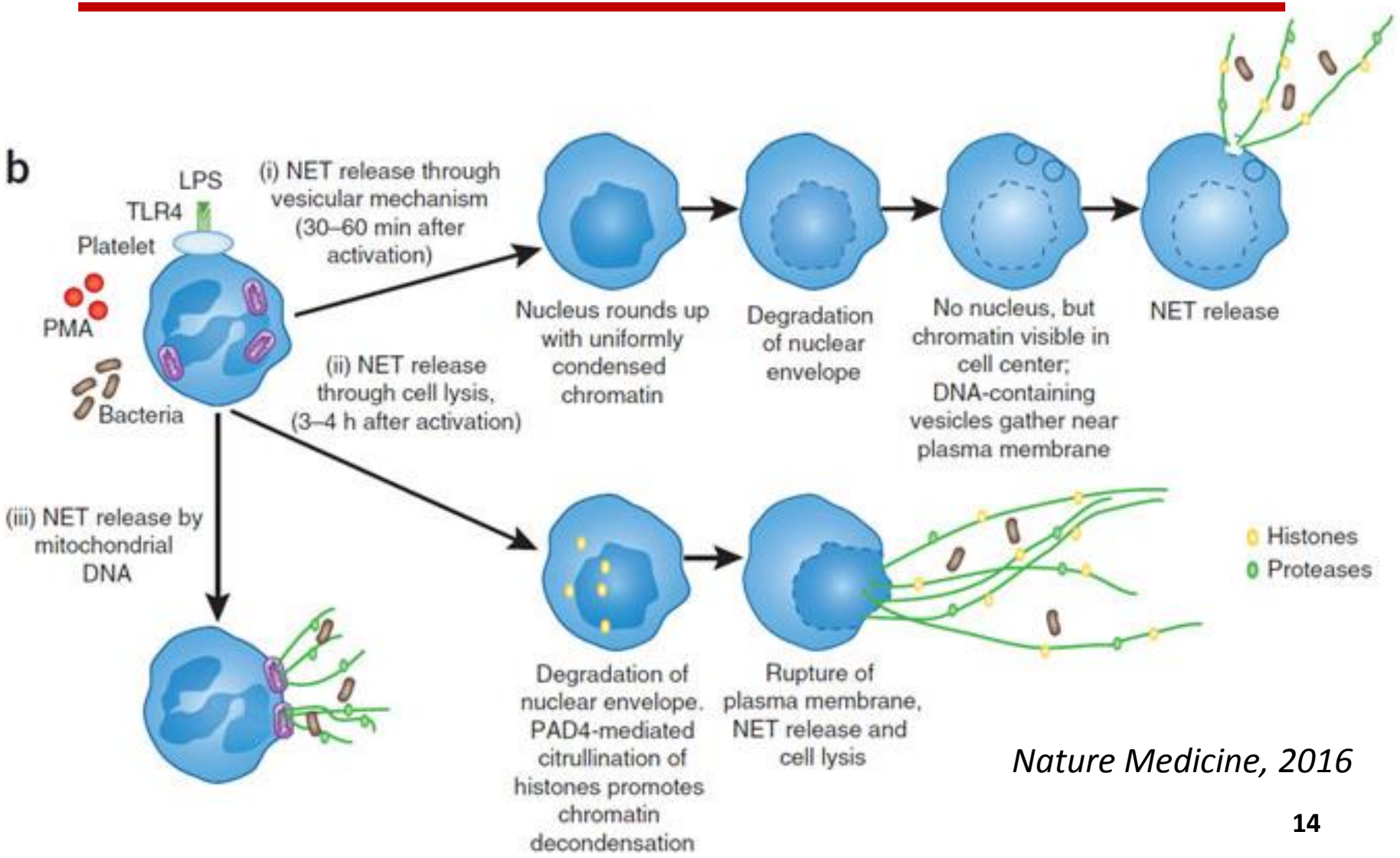
(Brinkman et al, 2004)

Microbes and Chemical Factors Inducing NETs

Aspergillus fumigatus (conidia and hyphae)
Candida albicans (conidia and hyphae)
Cryptococcus gattii
Cryptococcus neoformans
Eimeria bovis
Enterococcus faecalis
Escherichia coli
Haemophilus influenzae
Helicobacter pylori
Klebsiella pneumoniae
Lactococcus lactis
Leishmania amazonensis
L. donovani, L. major, L. chagasi
Listeria monocytogenes
Mannheimia haemolytica and leukotoxin
Mycobacterium tuberculosis, M. canettii
Serratia marcescens
Shigella flexneri
Staphylococcus aureus
Streptococcus (group A – GAS)
Streptococcus dysgalactiae
Streptococcus pneumoniae
Toxoplasma gondii
Yersinia enterocolitica
HIV1

calcium ions
glucose oxidase
GMCSF + C5a
GMCSF + LPS
hydrogen peroxide
IFNα + C5a
IFNγ + C5a
IL8
lipopolysaccharide (LPS)
protein M1/protein M1 + fibrinogen complex
NO
phorbol12myristate13acetate (PMA)
PMA + ionomycin
platelet activation factor
TLR4
TNF
statins
δtoxin from *Staphylococcus epidermidis*
Glucose
Homocystiene
IL-6

Molecular and Cellular Mechanism Involved in NETs Formation



Nature Medicine, 2016

What are NETs Comprised of?

Granules
Neutrophil elastase (NE)
Lactoferrin
Azurocidin
Cathepsin G
Myeloperoxidase (MPO)
Proteinase 3
Lysozyme C
Defensins 1 and 3
BPI

Nucleus
Histone H2A
Histone H2B:
a) histone H2B
b) H2Blike histone
Histone H3
Histone H4
Myeloid nuclear differentiation antigen

Cytoplasm
S100 calciumbinding protein A8
S100 calciumbinding protein A9
S100 calciumbinding protein A12

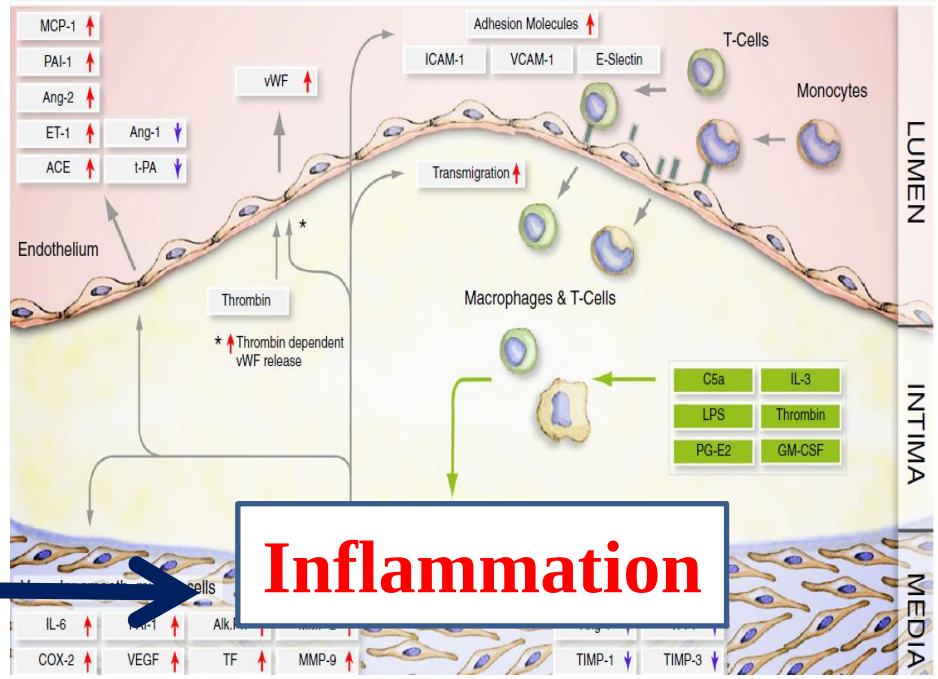
Cytoskeleton
Actin (β and/or γ)
Myosin9
αactinin (1 and/or 4)
Plastin2
Cytokeratin1

Peroxisomes
catalase

Glycolytic enzymes
αenolase
Transketolase

Pro-Inflammatory Milieu in Diabetes

- ✓ Diabetes : associated with chronic and low grade inflammation
- ✓ Increased levels of pro-inflammatory cytokines
- ✓ Diabetic patients are predisposed to recurrent bacterial and viral infections
- ✓ Abnormalities in innate and acquired immunity



Diabetes
Hyperglycemia
Hyperlipidemia
Oxidative/ER stress

Inflammation

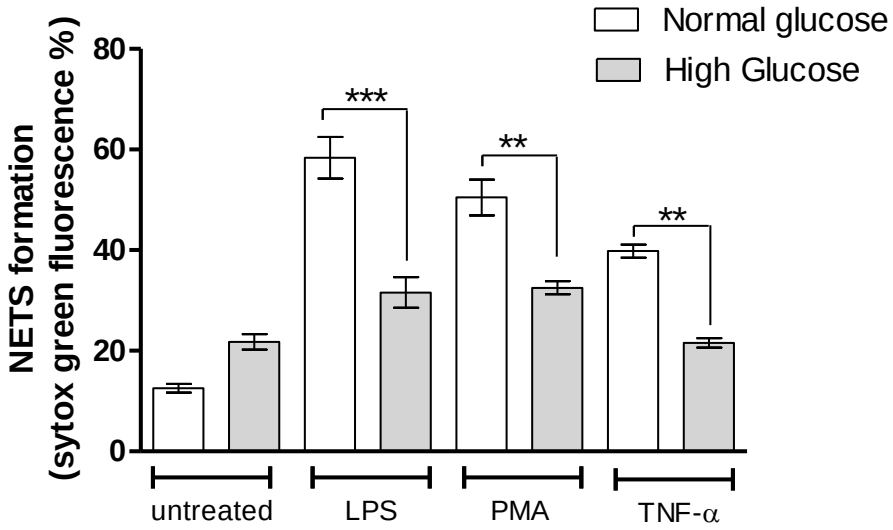
Demyanets et al, 2011

Working Hypothesis & Key Question

- ✓ **Metabolic and Inflammatory pathways are interdependent**
- ✓ **Both metabolism and immune system are deregulated during diabetes**
- ✓ **Diabetic patients are predisposed to recurrent bacterial and viral infections**

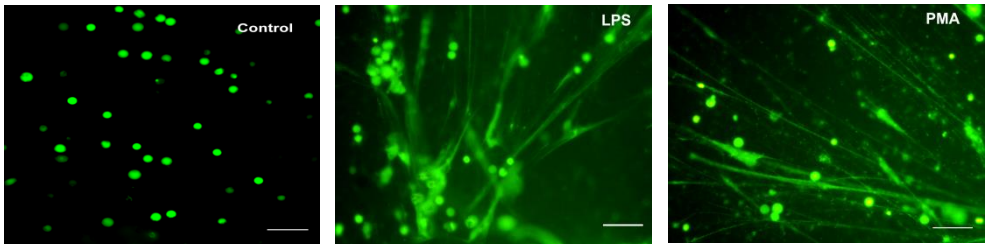
"Consequences of diabetic microenvironment on NETosis?"

High Glucose Inhibits NETs Formation

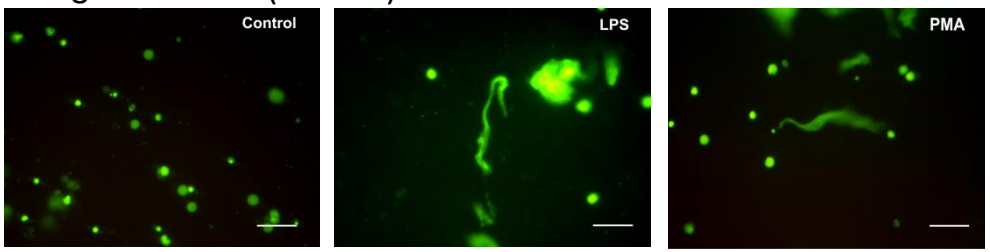


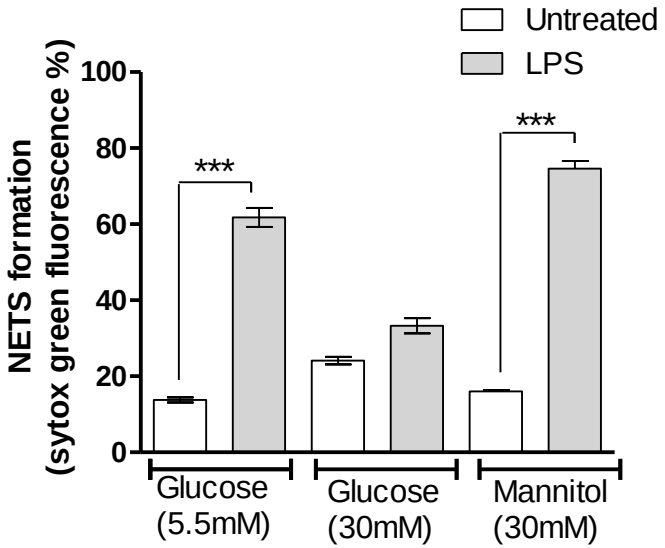
LPS : 2μg/ml
PMA : 25nM
TNF-α: 20 ng/ml
} 3 hours

Normal Glucose (5.5 mM) (24 hours)

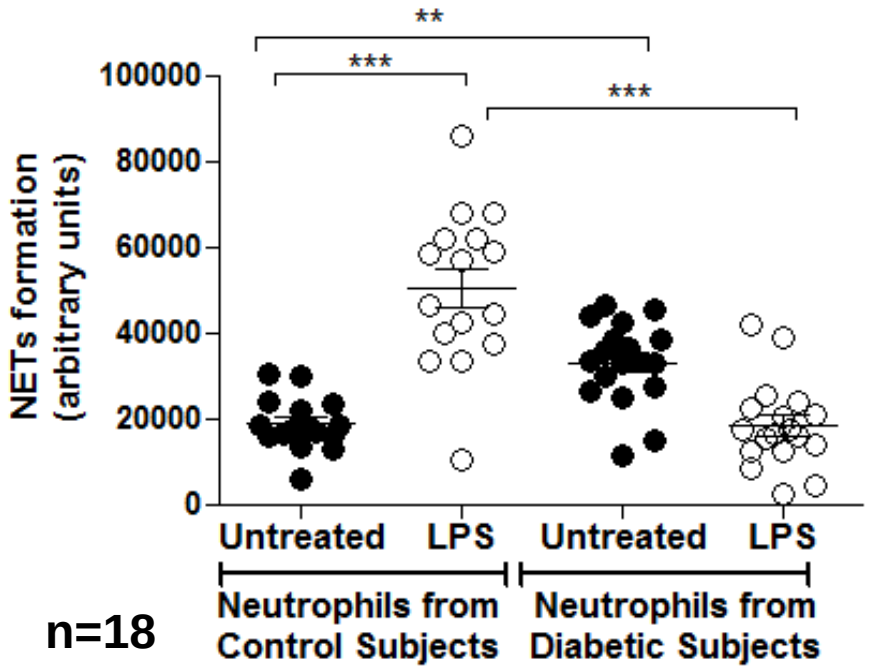


High Glucose (30 mM) (24 hours)





Constitutive NETs Production and Blunted Response to LPS in Diabetic Subjects



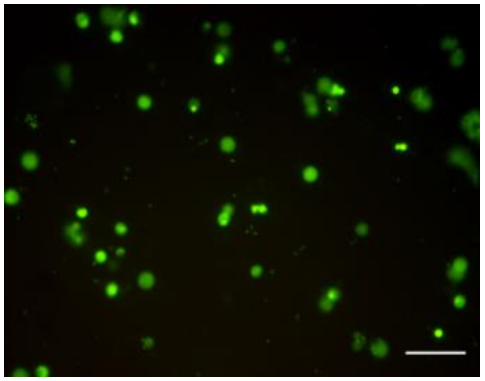
LPS : 2µg/ml for 3 hours

n=18

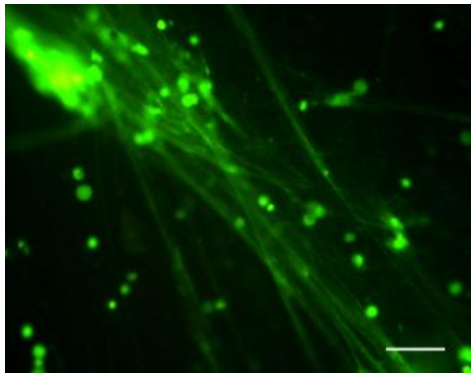
n=18

Control Subject

Untreated

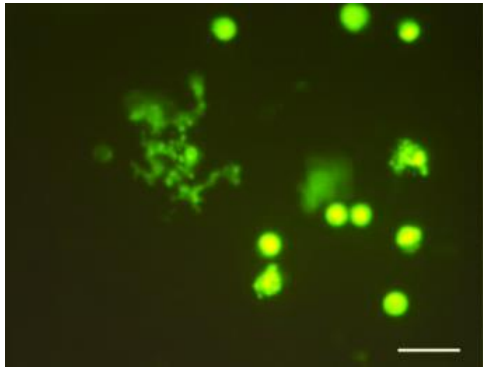


LPS-Treated

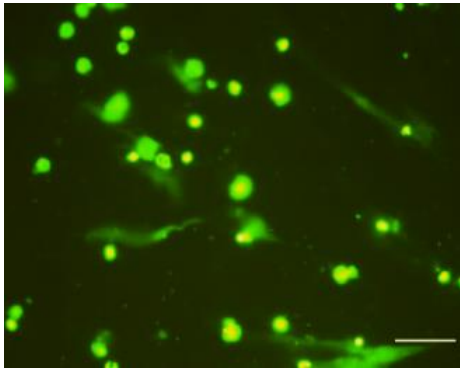


Diabetes Subject

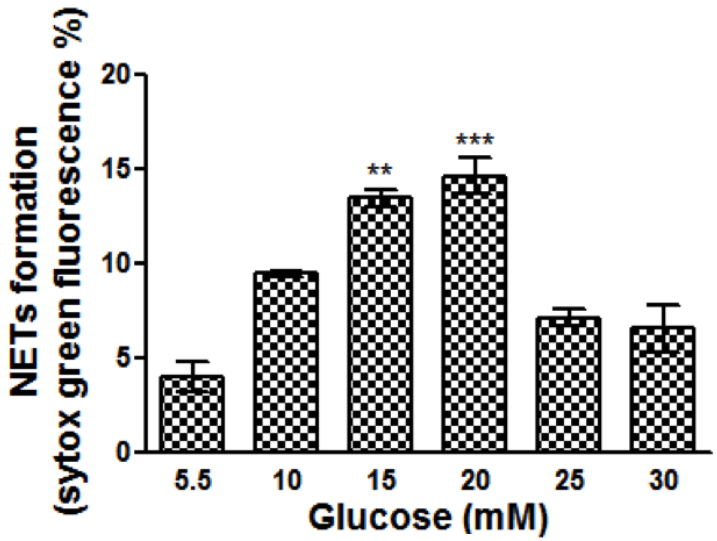
Untreated

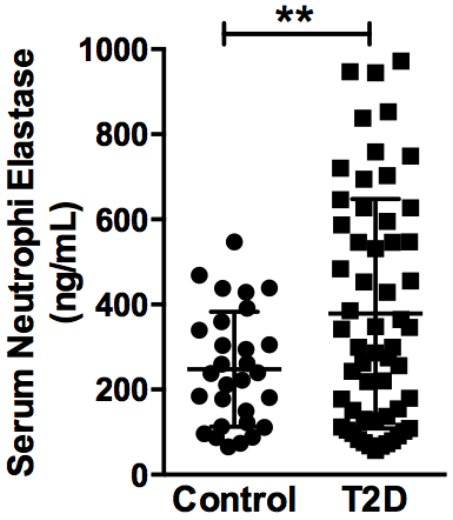


LPS-Treated

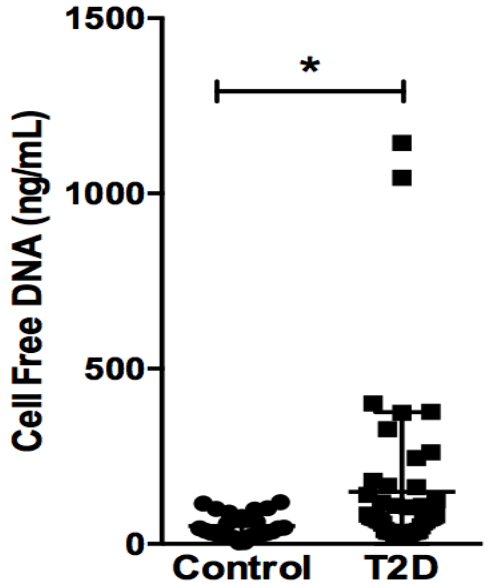


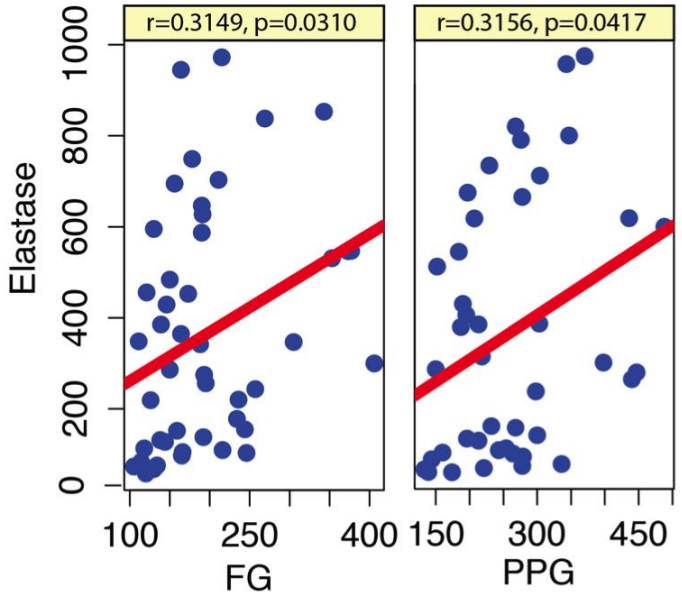
Serum Glucose Levels in T2D Strongly Correlates with NETs Components



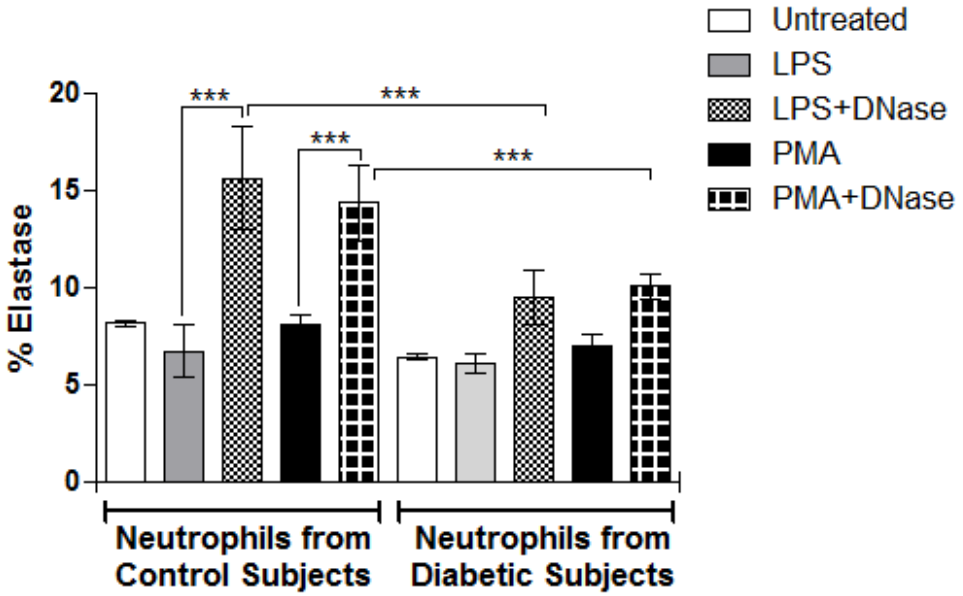


Control (n=31)
T2D (n=57)

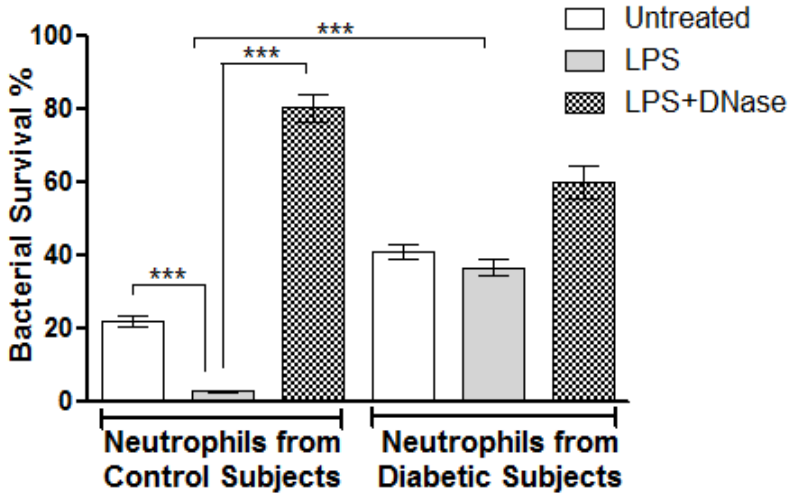




NETs Associated Elastase Activity is Decreased in Diabetic Subjects and Leads to Reduced Anti-Bacterial Activity

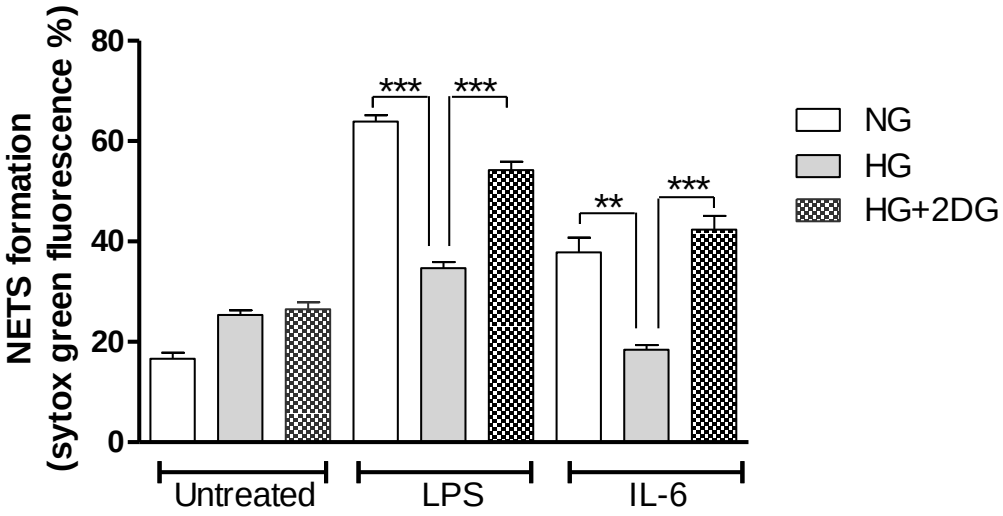


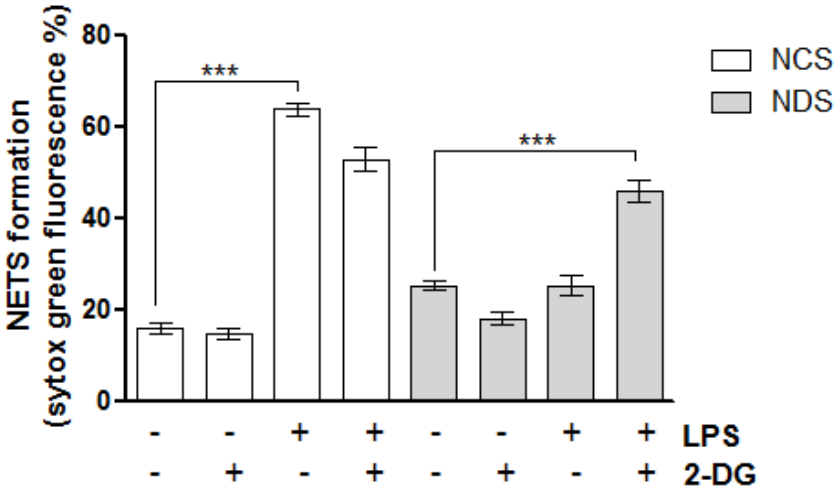
LPS: 2μg/ml
PMA: 25nM
Dnase: 8U/ml
Elastase activity: Flurometric Enz Check elastase assay



LPS: 2μg/ml
Dnase: 8U/ml
E. coli DH5α

Glycolysis Inhibitor 2-Deoxyglucose Restores NETs Formation





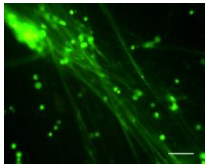
2DG (2- deoxy glucose): glycolysis inhibitor 500μM
NG: Normal Glucose (5.5mM)
HG: High Glucose (30mM)
LPS: 2μg/ml
IL-6: 25ng/ml

NCS: Neutrophils from Control Subjects
NDS: Neutrophils from Diabetic Subjects

Glucose Metabolism in Neutrophils

- Neutrophils are metabolically active and neutrophil functions are energy dependent
- Neutrophils lack gluconeogenesis machinery and hence do not produce glucose on its own

Glycogen formation → Phagocytosis



NETosis

Glucose utilization in Neutrophils

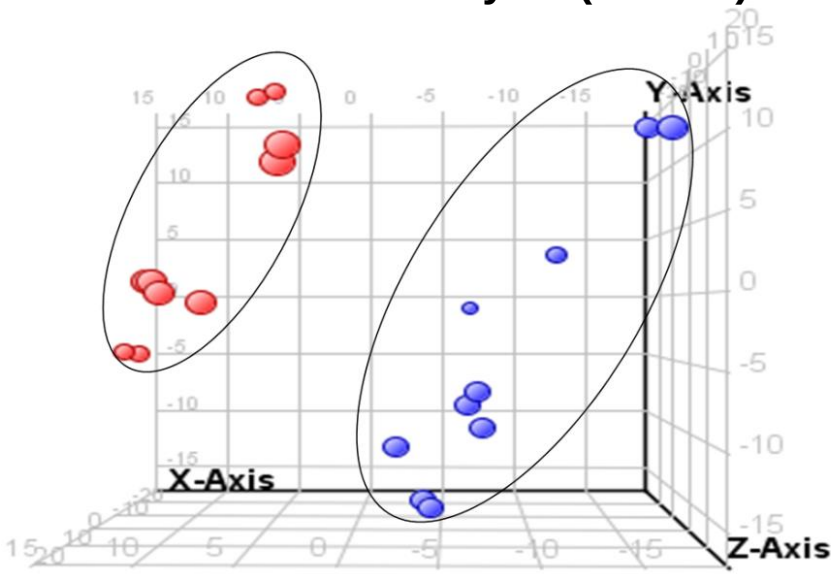
NADPH production ← HMP shunt pathway

Glycolysis → Chemotaxis & Locomotion

Metabolite Profiling in Neutrophils in Response to High Glucose

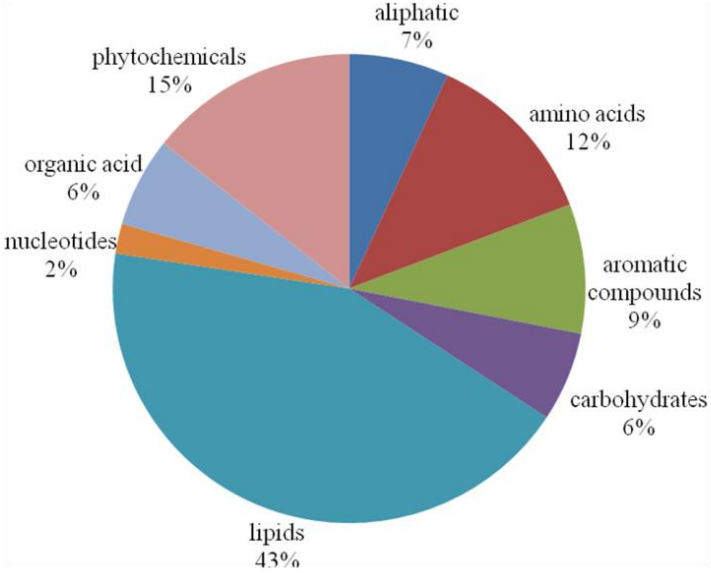
Mass spectrometry based high throughput untargeted metabolomics

Partial Least Square Discrimination Analysis (PLSDA)



Accuracy: 91%

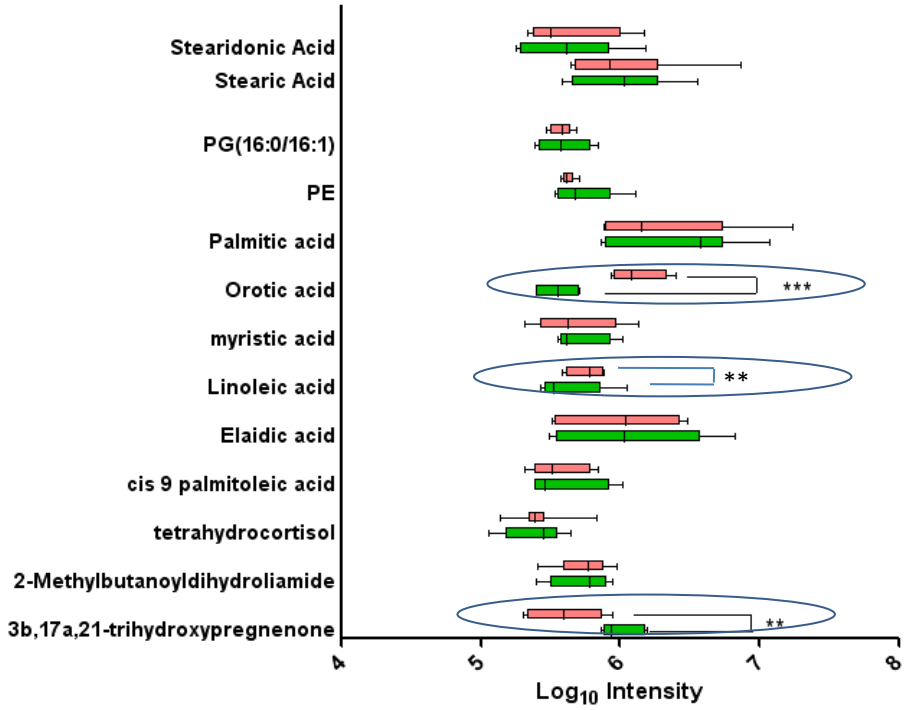
Metabolite classification



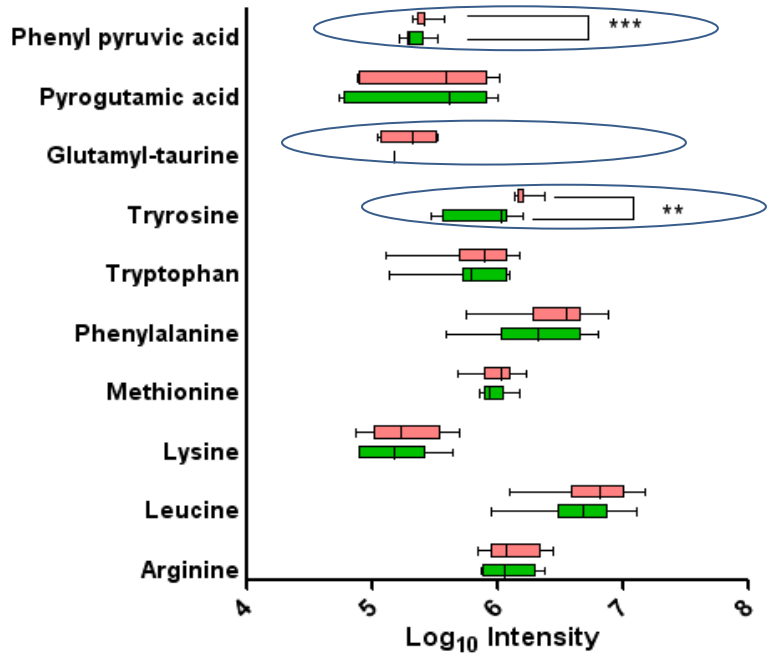
Peripheral Neutrophils from healthy individuals → Cultured in Normo/Glycemic conditions for 24 hrs → Endometabolites extraction (Methanol extraction) → Mass spectrometry analysis (Agilent ESI-QTOF) MS and MS/MS)

High Glucose Perturbs Metabolome of Neutrophils

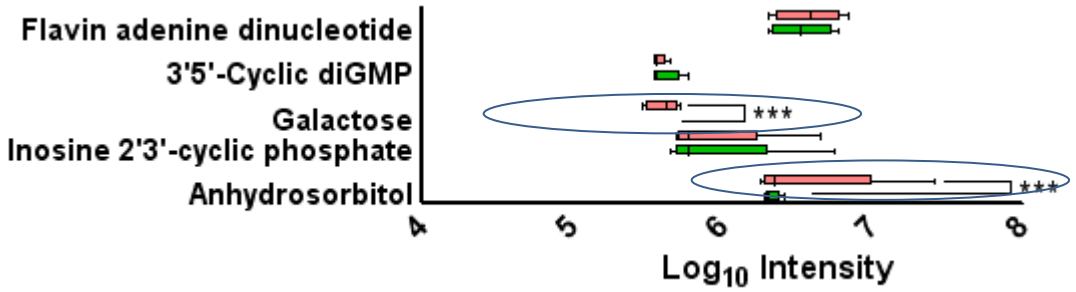
Lipids



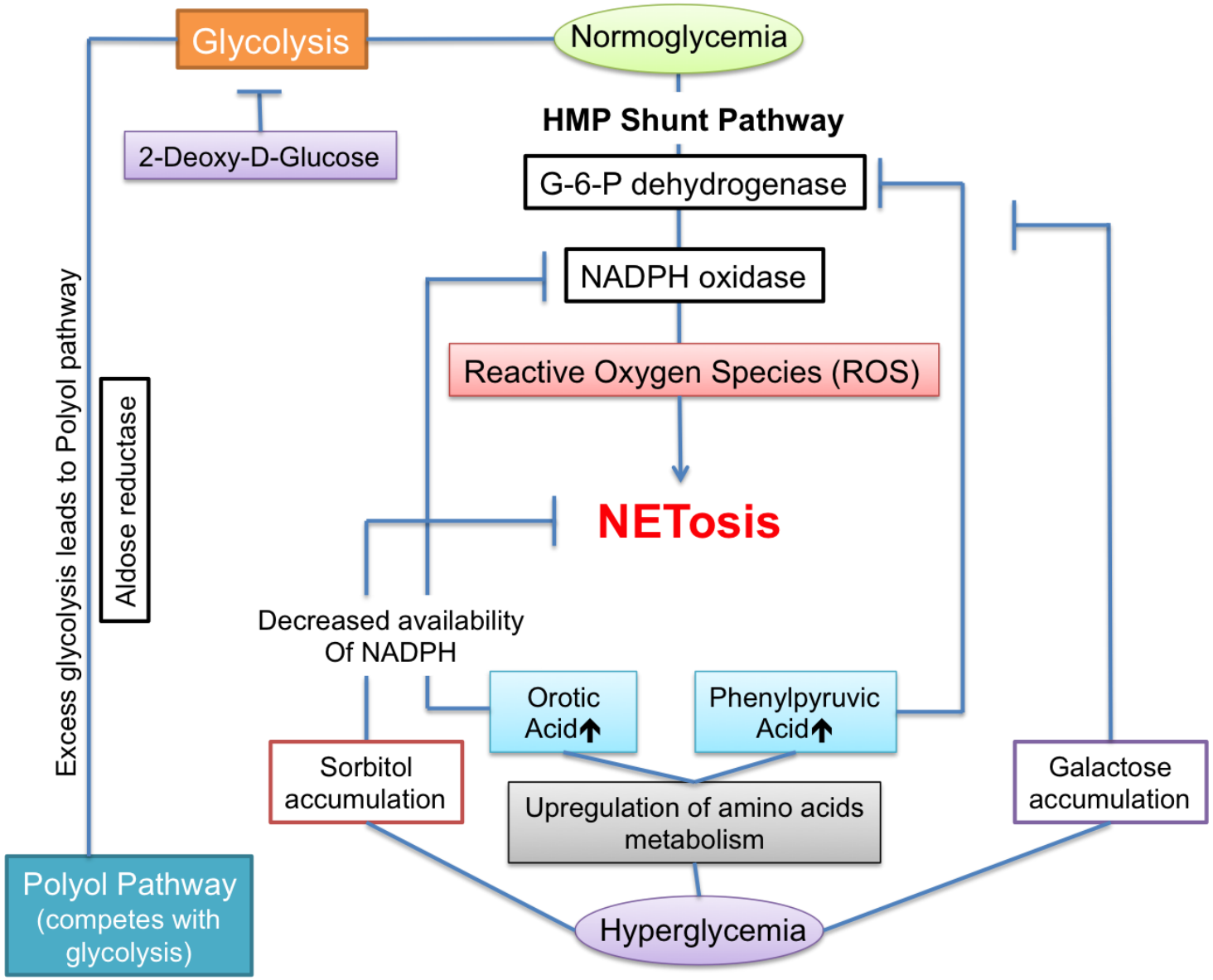
Amino acids & derivatives



Sugars and nucleotides

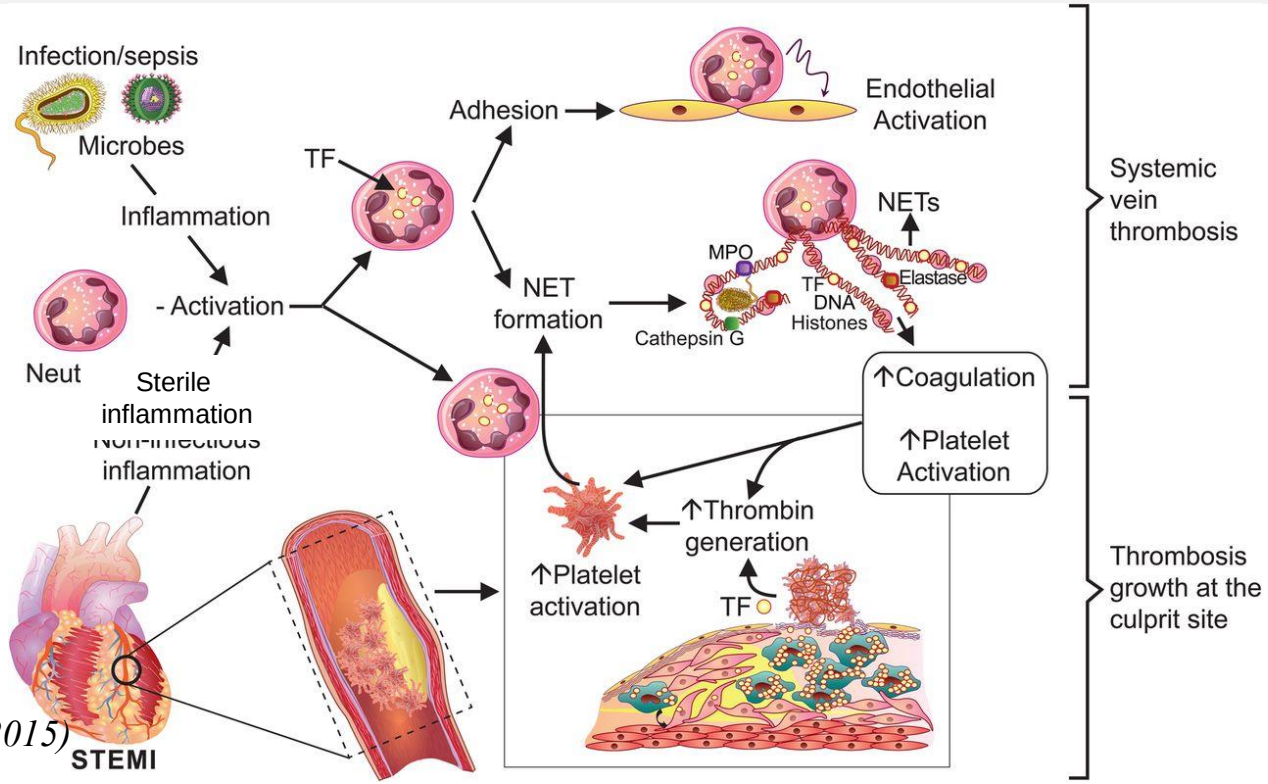


High Glucose Leads to Competition for NADPH Between NADPH oxidase and Polyol Pathway



NETs are Associated with Vascular Diseases

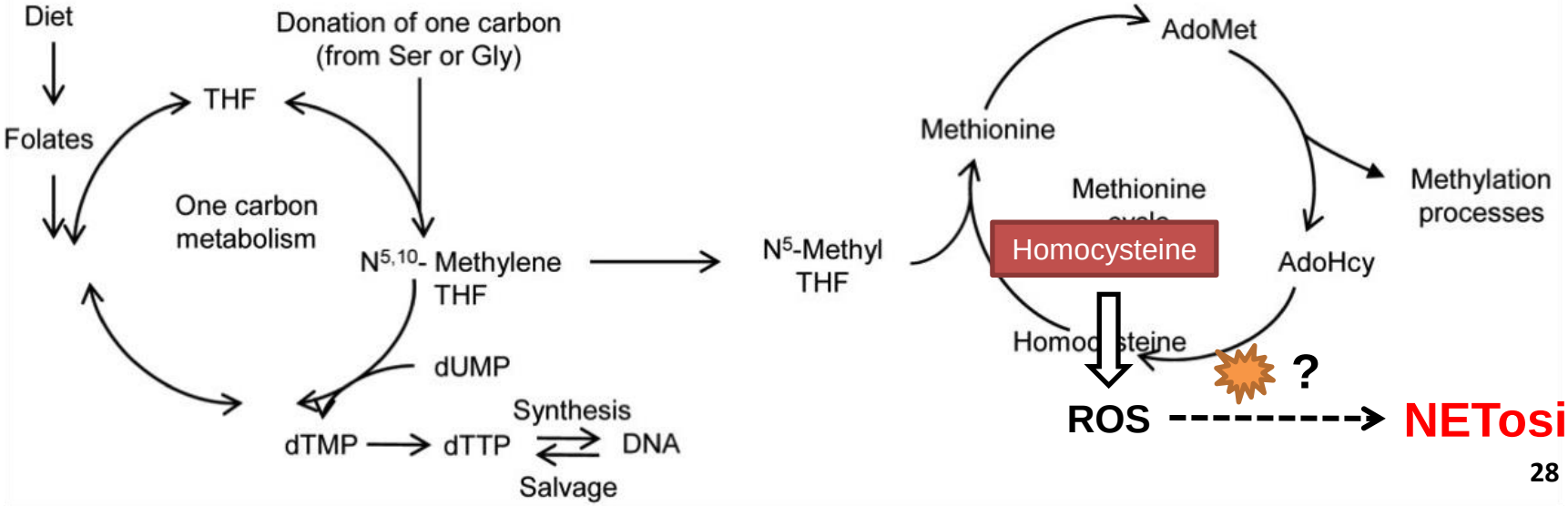
- NETs are associated with vascular diseases such as stroke and atherosclerosis
- Uncontrolled and excess NETs have been implicated in tissue damage and possess pro-thrombotic and atherogenic properties
- Diabetic subjects are prone to cardiovascular diseases



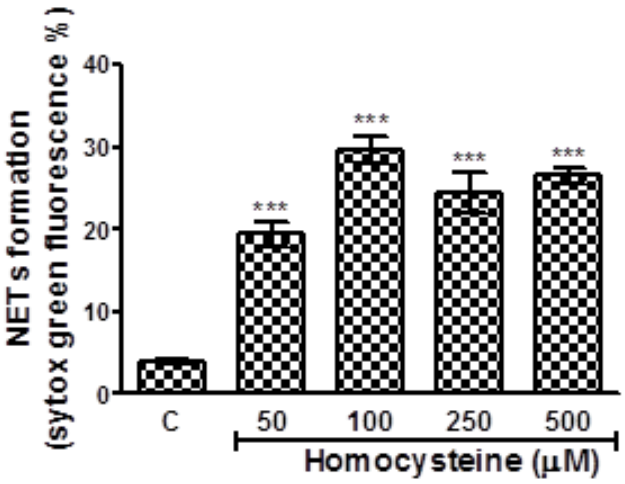
(Badimon & Vilahur, 2015)

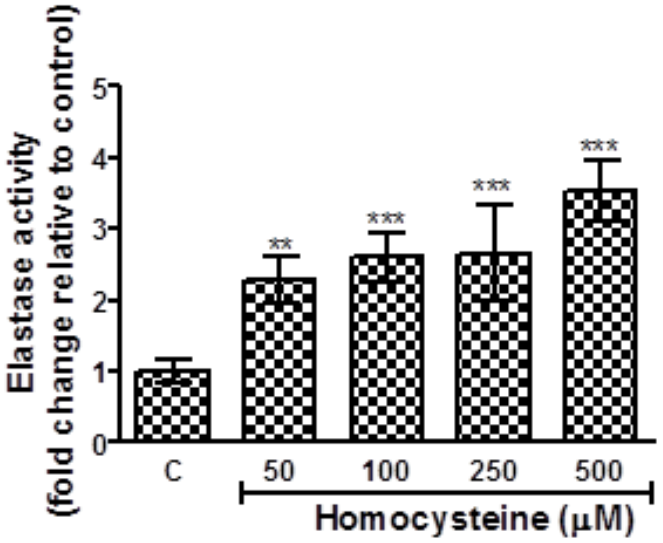
Hyperhomocysteinemia : Known Risk Factor for Vascular Diseases

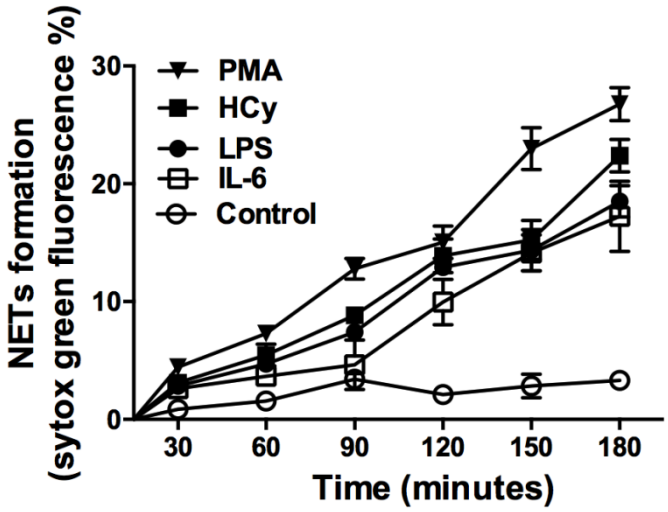
- Elevated homocysteine levels are associated with diabetes & cardiovascular diseases
- Innate immune response and molecules of coagulation pathway are influenced by inherent increase in plasma homocysteine which impact all the tissues associated with thrombosis.
- Homocysteine levels induces oxidative stress and releases ROS



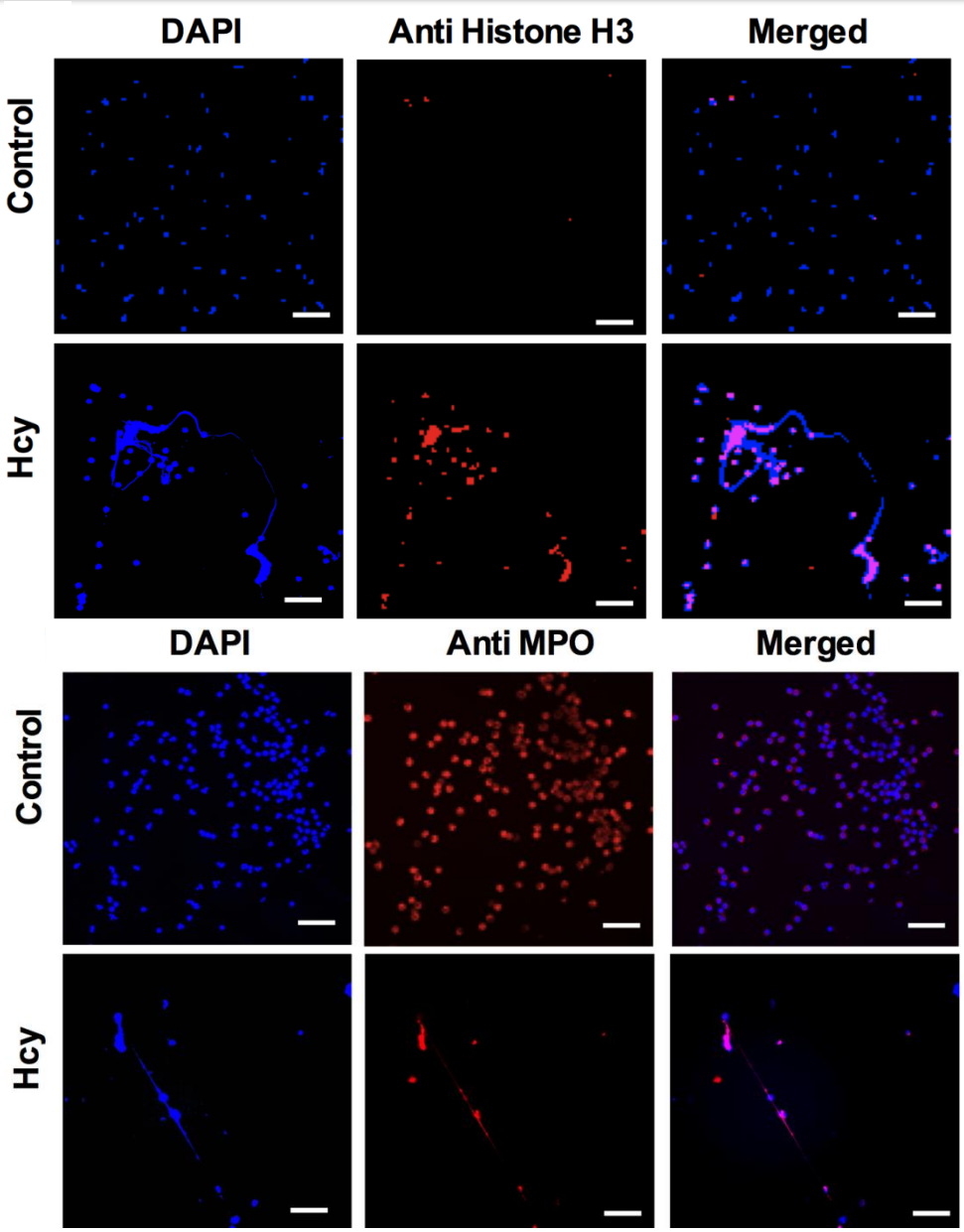
Homocysteine : Potential Inducer of NETs



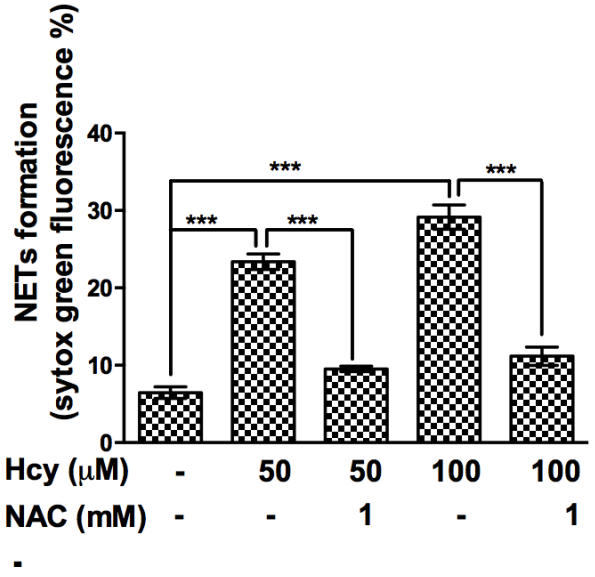


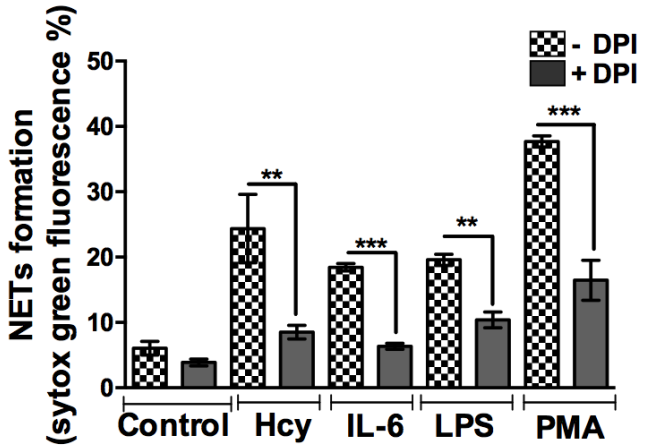


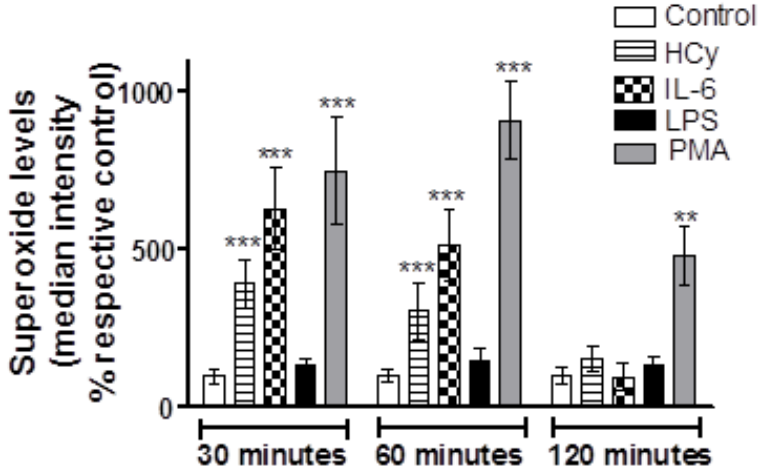
Homocysteine : Potential Inducer of NETs

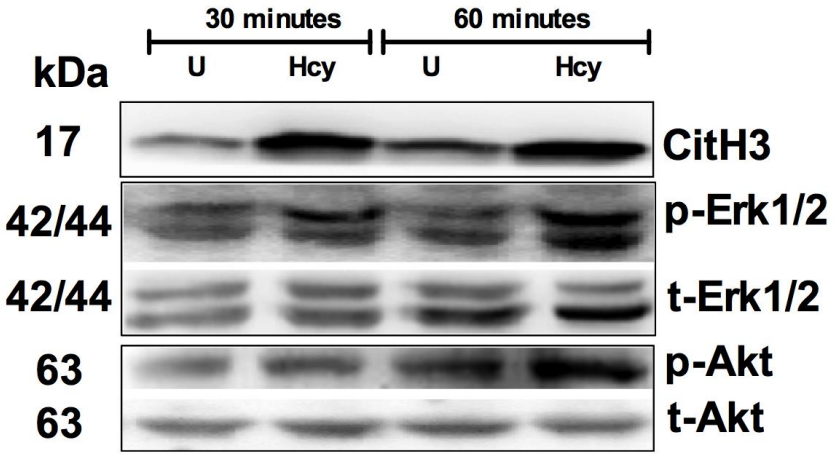


Homocysteine Induce both NADPH Oxidase Dependent and Independent ROS

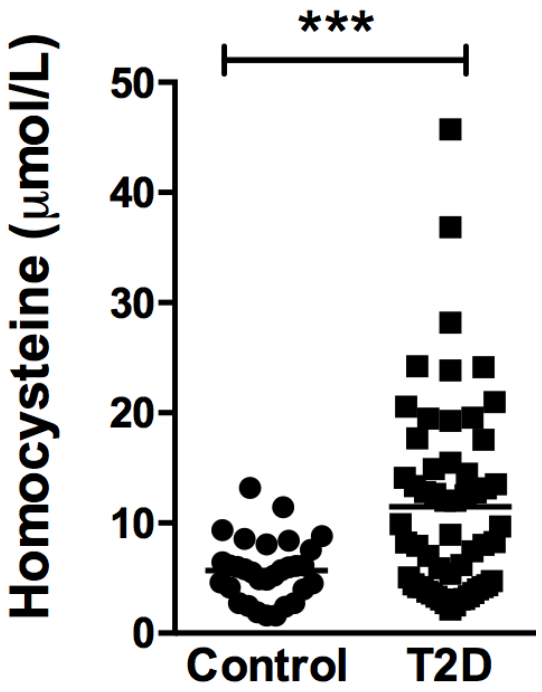


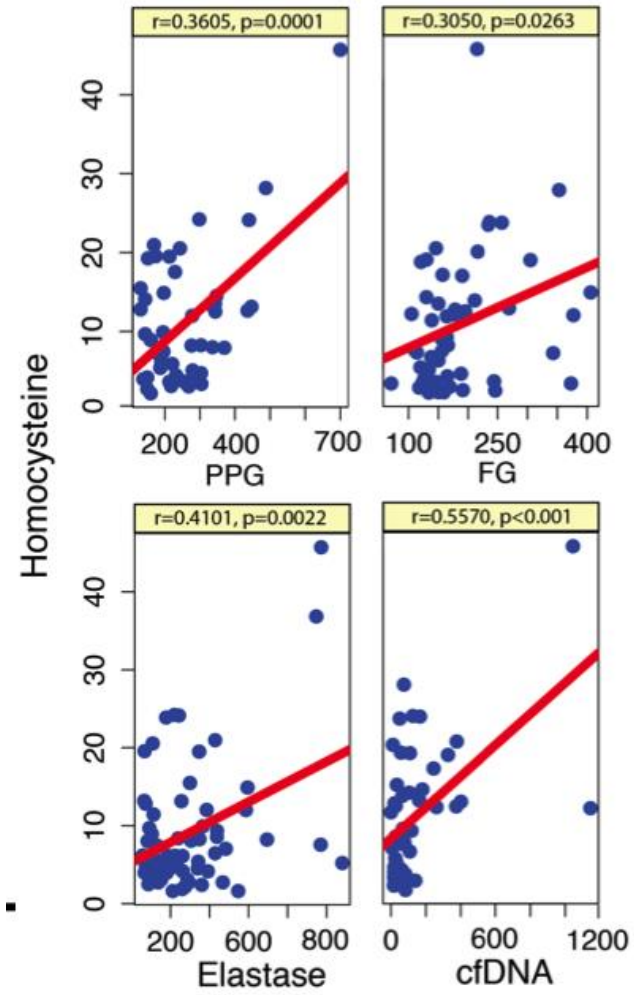




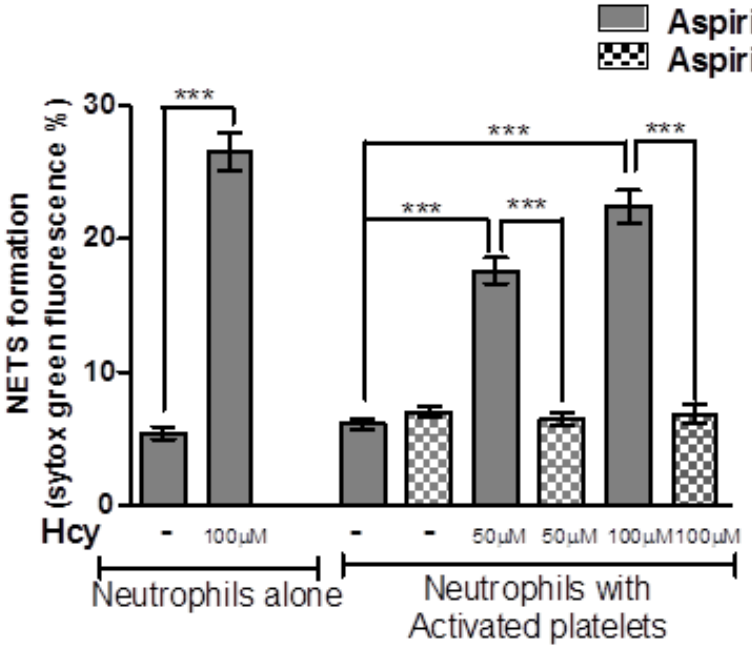


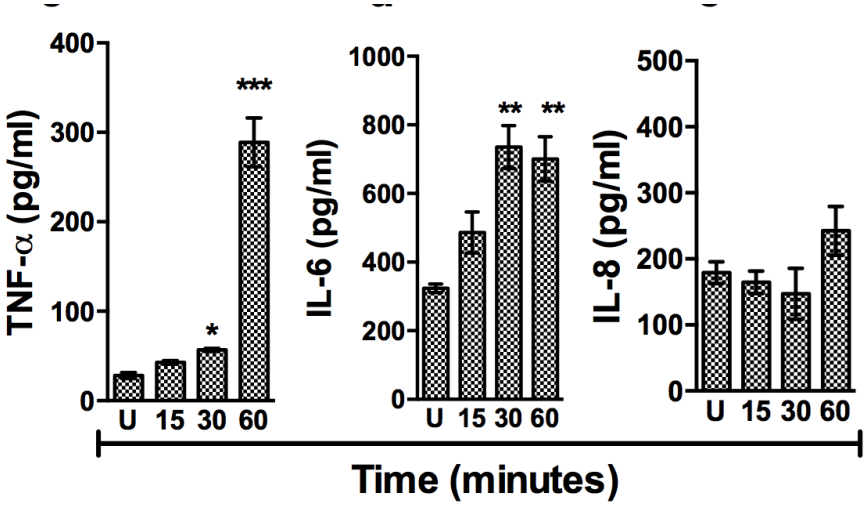
Plasma Homocysteine Levels are Elevated in T2D Subjects and Correlates with NETs Components





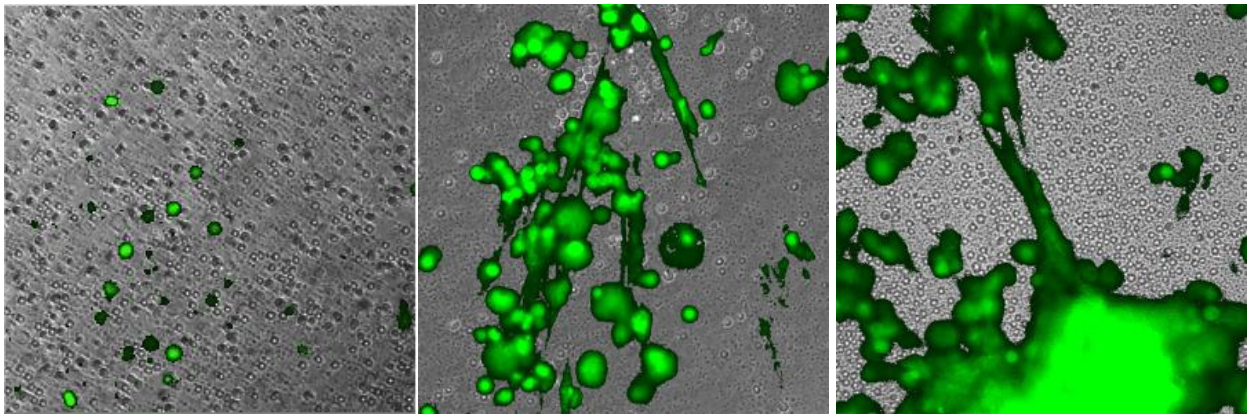
Homocysteine Activated Platelets Release Cytokines and Facilitates NETosis





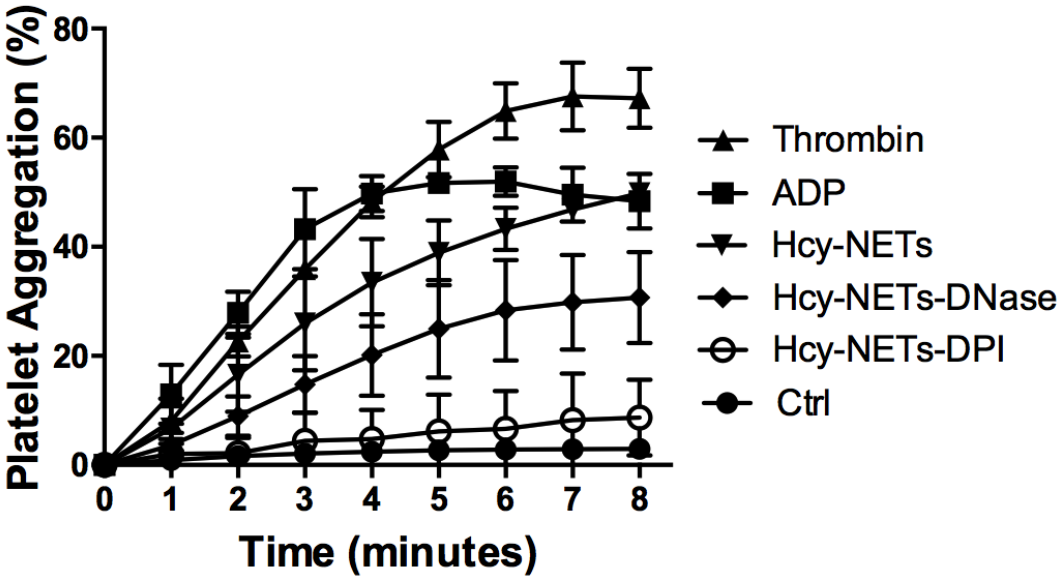
Untreated Platelets+ neutrophils

Platelet treated with homocysteine + neutrophils



Homocysteine Induced NETs Activates Platelet Aggregation

Neutrophil-Platelet Bidirectional Activation



Summary

- ✓ NETs are influenced by glucose homeostasis
- ✓ Hyperglycemia mimics a state of constitutively active pro-inflammatory condition in neutrophils leading to reduced response to external stimuli which might be responsible for diabetic subjects susceptible for infections
- ✓ Hyperglycemia induced polyol pathway and NADPH Oxidase competes for NADPH leading to reduced response to LPS
- ✓ Increased homocysteine levels in T2D induces constitutive NETosis which might be responsible for accelerated vascular disease

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