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Aging Impairs HIV Responses in Neutrophils from Blood and the Female Genital Tract

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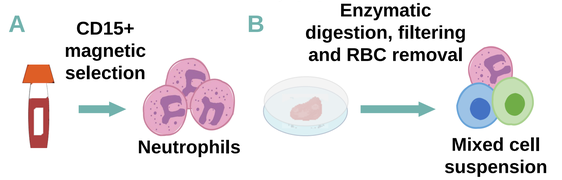
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BACKGROUND

Women acquire HIV through sexual transmission, with HIV infection rates progressively increasing in older women (>50 years) since 2012 (1). However, low transmission rates per sexual act indicate local immune protection against HIV acquisition at the main portal of entry, the female genital tract. We demonstrated that genital neutrophils respond to HIV stimulation with Neutrophil Extracellular Trap (NET) release to inactivate the virus (2). However, the mechanisms involved in HIV recognition, molecular events that lead to NET release, and how aging affects this protective mechanism are unknown.

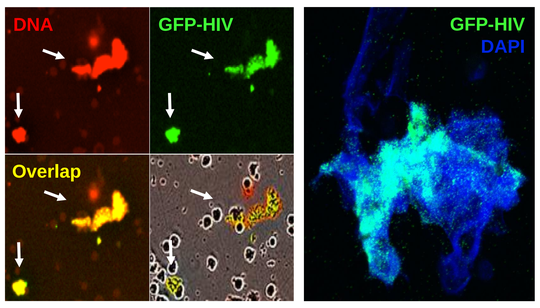
METHODS

Human neutrophils were purified from blood (A) (n=51) and genital tract tissues (n=33) after digestion of hysterectomy samples (endometrium, ectocervix and endocervix) (B).



Neutrophils were stimulated *in vitro* with GFP-labeled HIV viral-like particles (HIV-VLPs) to induce NET release and quantify HIV-NETs by time-lapse imaging (C) and confocal microscopy (D). To study HIV recognition and signaling mechanisms, prior to HIV stimulation, neutrophils were incubated with endosomal TLR7/8 inhibitors; Rhod-3 AM to stain cytosolic calcium; and Annexin V dye to assess cell death.

C D



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RESULTS

1. Early NET-release is impaired with aging after menopause.

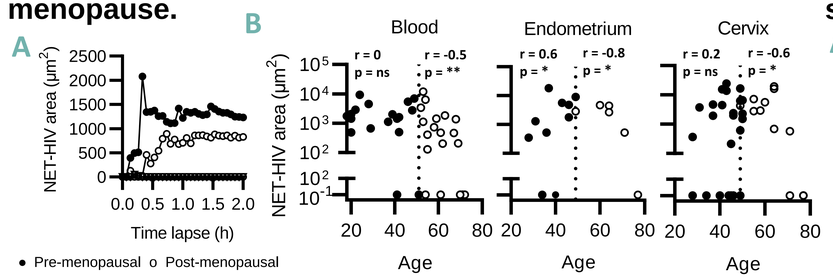


Fig. 1 (A) Representative curve of NET release over time by pre-menopausal (black dots) and post-menopausal women (white dots) after HIV stimulation. Unstimulated neutrophils from pre-menopausal and post-menopausal women overlap at the bottom. **(B)** Correlation of NET release at early times (0-15min) and women's age (pre-menopausal, black dots; post-menopausal, white dots) in blood and genital tissues (endometrium; cervix). Dotted line separates pre- from post-menopausal women. **p<0.01; *p<0.05

2. TLR8 and TLR7 mediate early and late HIV-induced NET release in a temporal sequential manner.

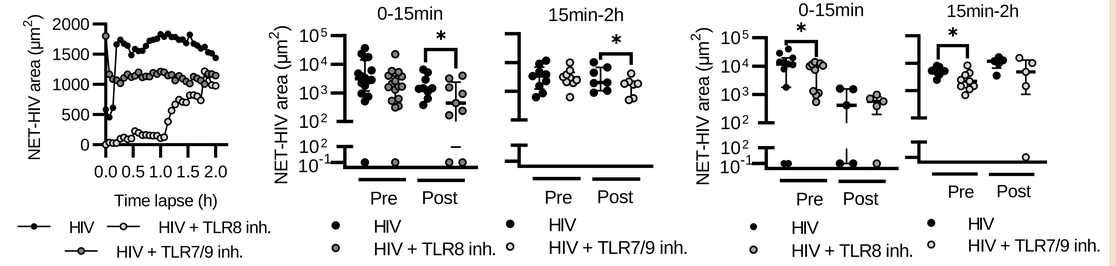


Fig. 2 (A) Representative quantification of NET release over time after HIV stimulation (black), HIV stimulation plus TLR8 inhibitor pretreatment (light grey), and HIV stimulation plus TLR7/9 inhibitor pretreatment (dark grey) in blood neutrophils. **(B)** Comparison of early (left graph) and late (right graph) NET release after HIV stimulation in the presence or absence of TLR8 inhibitor (left graph) and TLR7/9 inhibitor (right graph) in blood neutrophils from pre- and post-menopausal women **(C)** and in genital neutrophils from pre- and post-menopausal women. *p<0.05

3. Neutrophils do not undergo apoptosis following HIV stimulation, but Annexin V identifies a subset of NETs with inflammatory properties.

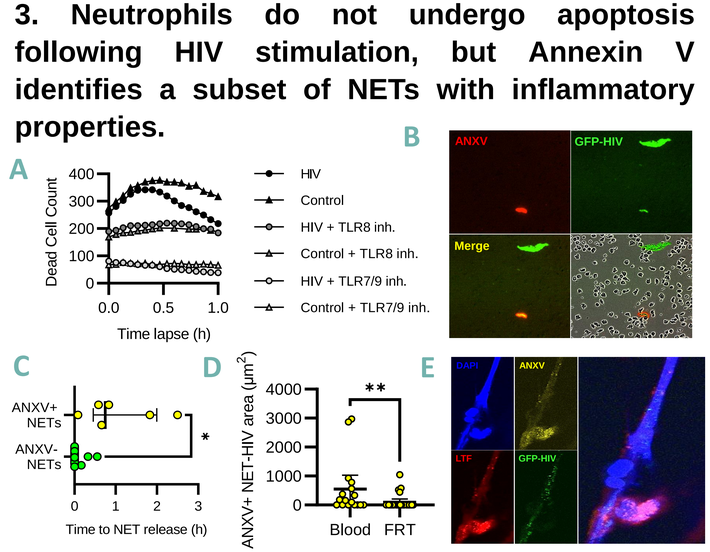


Fig. 3 (A) Representative quantification of dead cell count after HIV stimulation and unstimulated controls with and without pretreatment with TLR8 inhibitor or TLR7/9 inhibitor. **(B)** Representative images of Annexin V+ and Annexin V- NETs after HIV stimulation by blood neutrophils. Images were taken using IncuCyte, where Annexin V is shown in red, HIV-VLPs in green (GFP) and merge is shown as yellow. **(C)** Quantification of time until release of the first Annexin V+ and Annexin V- NET by blood neutrophils after HIV stimulation. **(D)** Blood neutrophils release more Annexin V+ NETs than genital neutrophils after HIV stimulation **(E)** Representative confocal microscopy images of Annexin V+ NETs by blood neutrophils. Annexin V+ NETs specifically incorporate lactoferrin (LTF), a proinflammatory antimicrobial protein. **p<0.01;*p<0.05

4. HIV stimulation induces a rapid increase of cytosolic calcium in blood neutrophils from younger women but not in genital neutrophils.

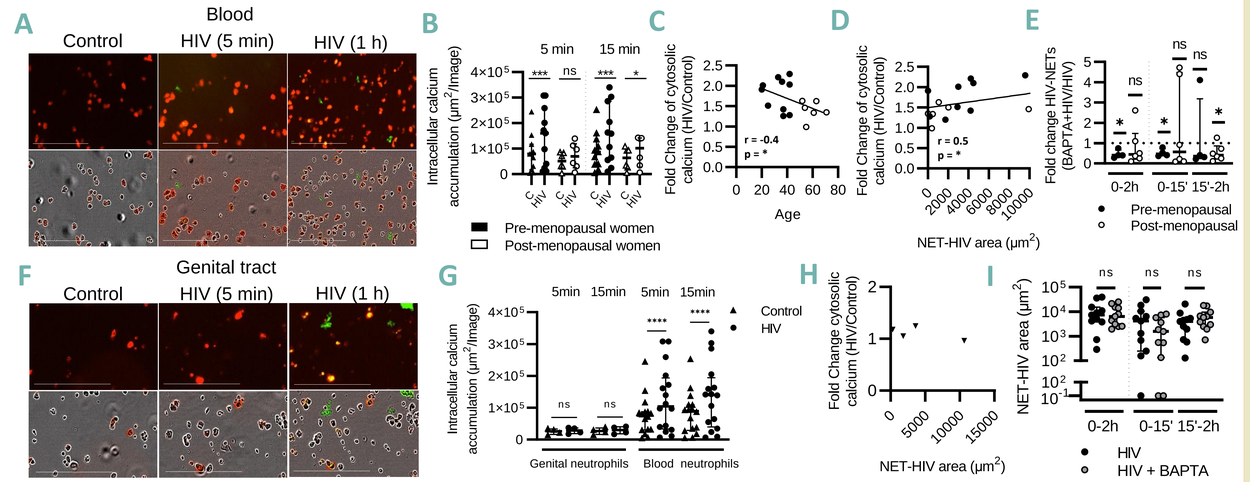


Fig. 4 (A) Representative images of blood neutrophils stained with Rhod-3 AM dye (red) to detect intracellular calcium release after stimulation with HIV-VLPs (GFP, green). **(B)** Intracellular calcium accumulation in blood neutrophils from pre- (black) and post-menopausal women (white) after HIV stimulation for 5 and 15 min or unstimulated neutrophils (C=control). **(C)** Negative correlation between age and the fold-change in intracellular calcium after HIV stimulation for 5 min. **(D)** Positive correlation between early NET release by blood neutrophils and the fold-change in intracellular calcium after HIV stimulation for 5 min. **(E)** Blockade of calcium by BAPTA reduces early NET-release in pre-menopausal (black dots) but not post-menopausal (white dots) women. Dots represent the fold-change in HIV-NET release after HIV stimulation in the presence of BAPTA ((HIV+BAPTA)/HIV). **(F)** Representative images of purified genital neutrophils stained with Rhod-3 AM dye (red) after stimulation with HIV-VLPs (GFP, green). **(G)** Comparison of intracellular calcium accumulation in blood neutrophils after 5 and 15 min of HIV stimulation versus lack of calcium response in genital neutrophils. **(H)** Lack of correlation between early NET release by genital neutrophils and intracellular calcium after HIV stimulation for 5 min. **(I)** Blockade of calcium by BAPTA does not affect early NET-release by genital neutrophils. (ns=non-significant; ****p<0.0001; ***p<0.001;*p<0.05)

CONCLUSIONS

- HIV-induced NET release is delayed and progressively declines with age in post-menopausal neutrophils from blood and genital tissues.
- Endosomal TLR8 and TLR7 HIV recognition is the preferential pathway for NET release in blood neutrophils from older women and in genital neutrophils from younger women, but this pathway is impaired as women age.
- In younger women, HIV triggers rapid cytosolic calcium increase leading to NET release, but this response is compromised with aging, and absent in genital neutrophils.
- Importantly, we identified differential responses in blood and tissue, with blood neutrophils more prone to release proinflammatory NETs, highlighting the need to study genital neutrophils to develop prevention strategies.

REFERENCES

1. CDC. Diagnoses of HIV infection in the United States and dependent areas, 2019. HIV Surveillance Report 2021;32.
2. Fiona D Barr, Christina Ochsenbauer, Charles R Wira, **Marta Rodriguez-Garcia**. Neutrophil extracellular traps prevent HIV infection in the female genital tract. Mucosal Immunol. 2018 Sep;11(5):1420-1428.