# Novel Anti-Viral & Immunomodulatory roles of Neutrophils

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**Introduction:**

Neutrophils:

- most abundant immune cell
- crucial in the response against invading pathogens
- classically eliminate microbes via phagocytosis, reactive oxygen species, degranulation & NETosis

**Pathogen Recognition Receptors (PRRs)**

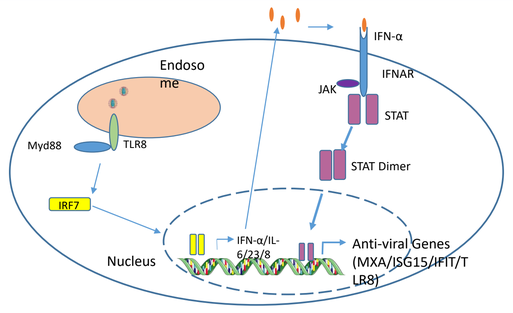
- Found extracellularly and intracellularly
- TLR7/TLR8 responsible for the recognition of ssRNA viruses intracellularly
- Induce the expression of proinflammatory cytokines and type 1 Interferons that in turn are responsible for the induction of Interferon stimulated Genes
- many ISG block viral infection & replication, thus eliminating the pathogen
- Viruses have developed numerous viral immune evasion strategies that target the type 1 IFN JAK/STAT pathway

## Hypothesis

Neutrophil depletion a) increases influenza viral load & b) lethality of hepatitis A Virus in mice.

Therefore, we hypothesise that neutrophils are crucial immune cells in combating viral infection & have developed a redundant anti viral mechanism independent of type 1 Interferons.

To test this hypothesis, we aim to analyse the role of Primary Human Neutrophils in 1) viral RNA sensing & 2) the induction of anti-viral ISGs; while also investigating their immunomodulatory capacity through the induction of cytokines




**Schematic Diagram of Type 1 IFN-α JAK/STAT signalling**

## Results

### IFN-α-mediated pSTAT1 in Primary Human Neutrophils is prolonged

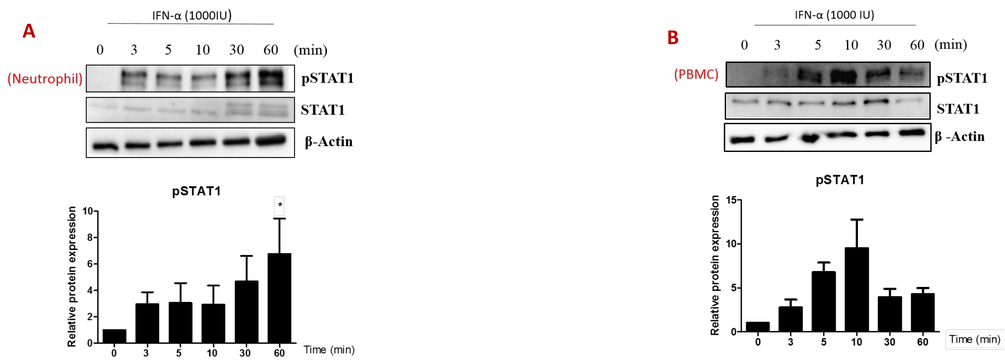



**Fig 1:** pSTAT1 is significantly upregulated after 60min of IFN-α (1000IU) in neutrophils. pSTAT1 is expression peaks at 10 minutes post IFN-α treatment and declines thereafter in human PBMCs . Densitometric analysis was performed using Image Lab software and values for pSTAT1 and STAT1 were measured relative to β-actin; the IFN-stimulated timepoints were made relative to the unstimulated, which was normalised to 1. Graphs (A)are the mean ± SD of three independent experiments and (B) are the mean ± SD of two independent experiments

### TLR8 activation results in the induction of ISG expression

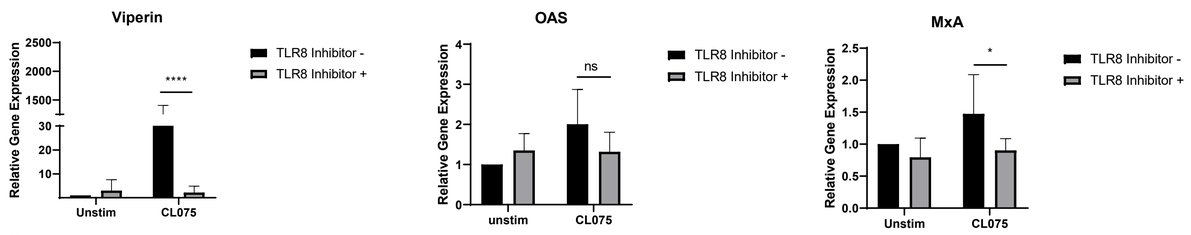



**Fig 2:** TLR8 activation results in the induction of ISG expression. Primary human neutrophils were stimulated the endosomal agonist CL075 for 4 hours either in the presence or absence of a TLR8 inhibitor. Using qRT-PCR ISG15, MxA and Viperin were quantified and calculated relative to the house keeping gene β–aActin and compared with the unstimulated control which was normalised to 1. Graphs are the mean ± SD (n=X). ns, non-significant, * p<0.05 etc. are all significantly induced in a TLR8 dependant manner

### TLR8 is required for mounting an efficient IFN independent antiviral response to HIV-1

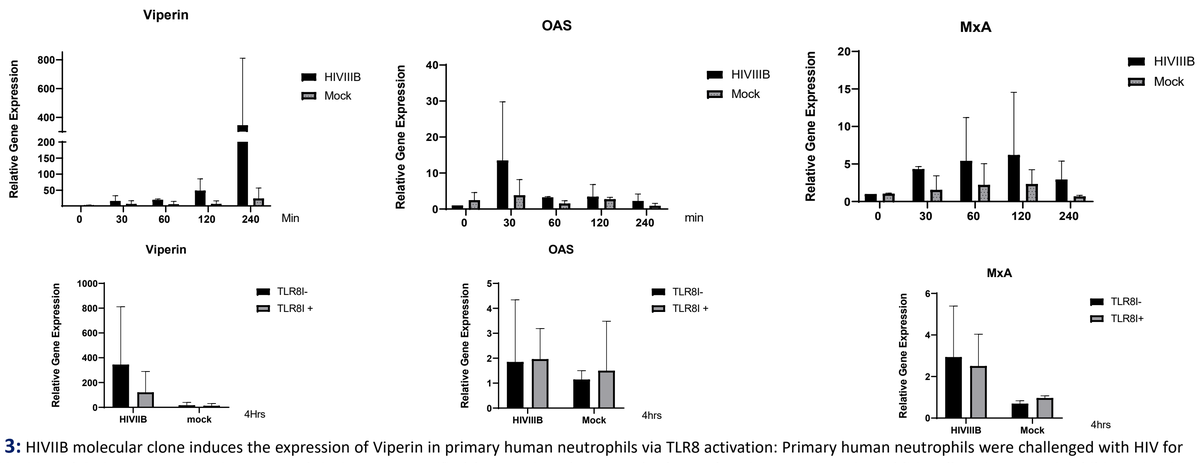



**Fig 3:** HIVIIIB molecular clone induces the expression of Viperin in primary human neutrophils via TLR8 activation: Primary human neutrophils were challenged with HIV for 30,60,120 and 240 minutes. Viperin, MxA and OAS were quantified by RT-PCR relative to the house keeping gene B-actin. Additionally using a TLR8 inhibitor viperin expression was reduced

### TLR8 activation results in an increase in the expression of IRF1

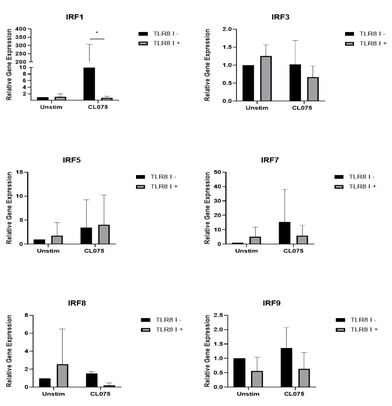



**Figure 4.** TLR8 activation results in an increase in the expression of IRF1 in primary human neutrophils. Primary human neutrophils were treated with CL075 for 24 hours either in the presence or absence of a TLR8 inhibitor. Total RNA was extracted and converted to CDNA which was used as a template to measure levels of IRF1,3,5,7,8,9 induction was measured using qRT-PCR.

### Inhibition of IRF signalling results in the abrogation of an early antiviral response by TLR8

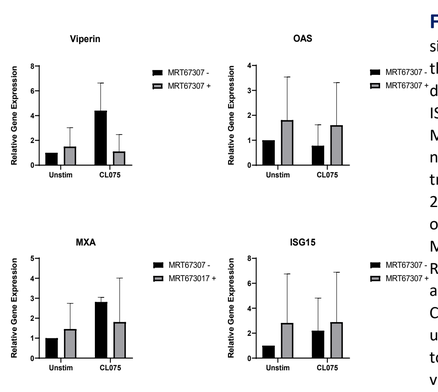



**Fig 5**: TBK1/IRF signalling results in the early downregulation of ISGs Viperin and MxA. Primary human neutrophils were treatd withCL075 for 2 hours either with or without MRT67307. Total RNA was extracted and converted to Cdna which was used as a template to measure levels of viperin, MxA, ISG15 and OAS.

### TLR8 activation results in the expression proinflammatory and chemotactic cytokines

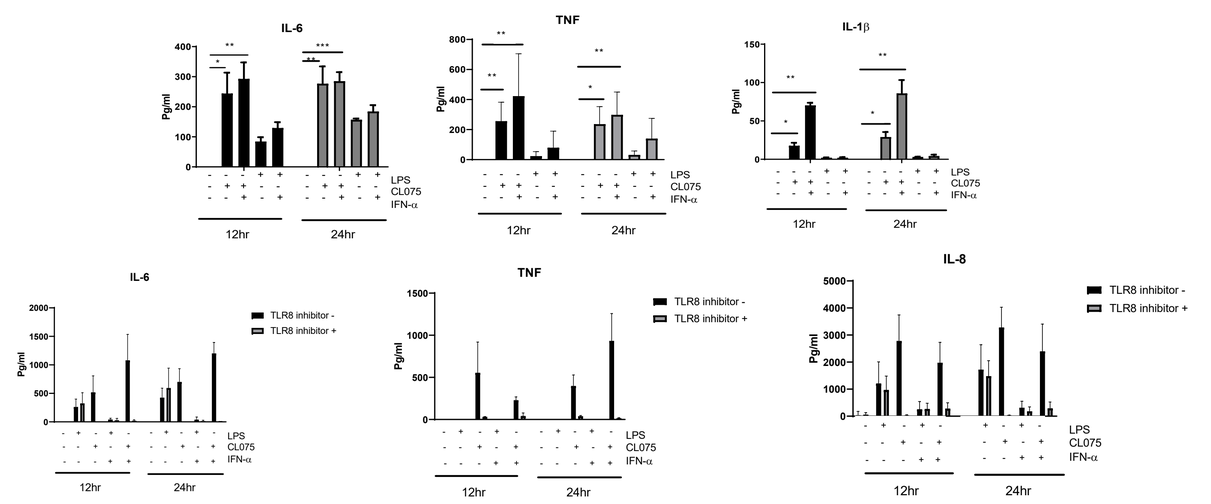



**Fig 6**: **TLR8 agonist CL075 and IFN-α synergistically enhance the expression of proinflammatory cytokines in primary human neutrophils**. neutrophils (1 x10^6) were cultured in the presence of either LPS, (100ng/ml), IFN-α (1000IU) or CL075 (2µg/ml) over the indicated time points. mRNA levels of (A) IL-1β (B) IL-6 and (C) TNF calculated relative to the RPS15 house-keeping gene and stimulated samples were compared to unstimulated . Additionally supernatants were analysed by ELISA for the presence of (D) IL-6, (E) TNF, (F) IL-1β and (G) IL-23. Graphs are the mean ± SD of 4 independent experiments.

### TLR8-activated Neutrophil Supernatant enhances Dendritic Cell (DC) maturation

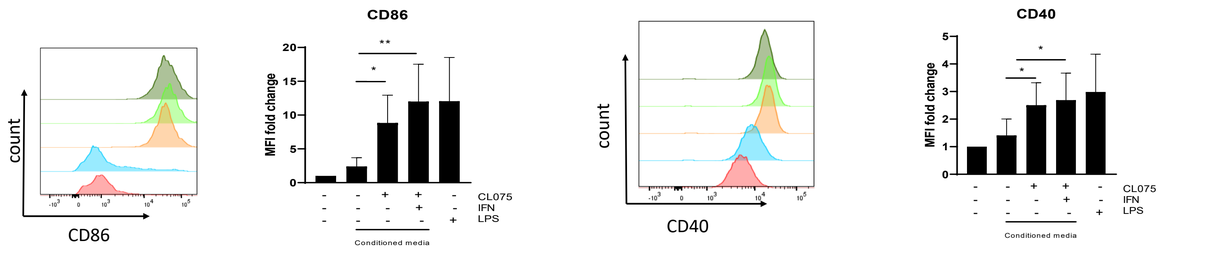



**Fig 7**: **Primary human dendritic cell markers CD86 and CD40 are significantly upregulated when cultured in TLR8 conditioned neutrophil media**. Neutrophils (1 x10^6) we cultured for 24 hours in the presence of IFN-α/CL075/LPS. Immature dendritic cells were then cultured in this neutrophil conditioned media for a further 24 hours and the subsequent maturation markers CD40/CD86 analysed using FACS. Mean fluorescent intensity (MFI) was measured and treated samples were compared to the unstimulated, which was normalised to 1. Graphs are the mean ± SD of 5 independent experiments.

### Neutrophil derived TNF enhances expression of CD86 and CD40

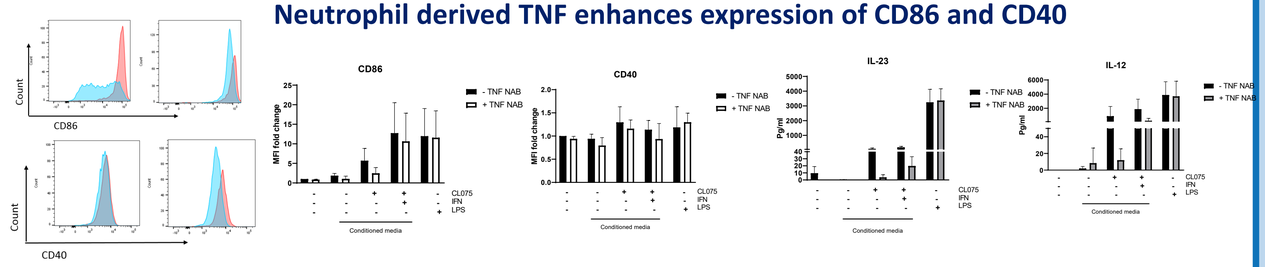



**Fig 8:. TNF inhibition of TLR8 stimulated neutrophils reduces the expression of Dendritic cell maturation markers CD86/CD40** Neutrophils (1 x10^6) we cultured for 24 hours in the presence of IFN-α/CL075/LPS, with or without the addition of a TNF neutralising antibody. Immature dendritic cells were then cultured in this neutrophil conditioned media for a further 24 hours and the subsequent maturation markers CD40/CD86 analysed using FACS. Mean fluorescent intensity (MFI) was measured and treated samples were compared to the unstimulated, which was normalised to 1. Graphs are the mean ± SD of independent experiments.

### DC:Neutrophil co-culture Supernatant polarises CD4 T-cells towards a Th1 phenotype

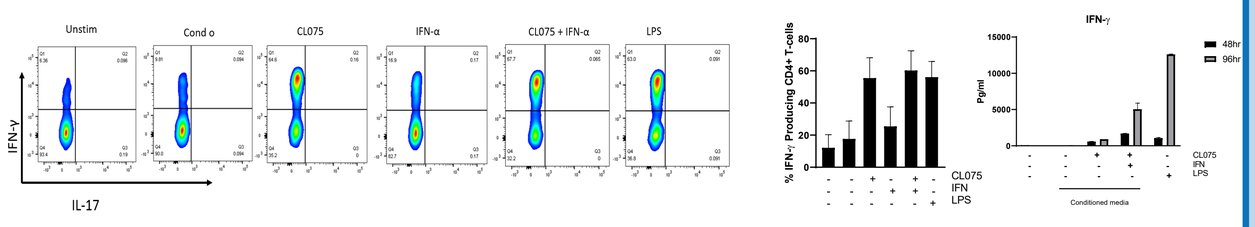
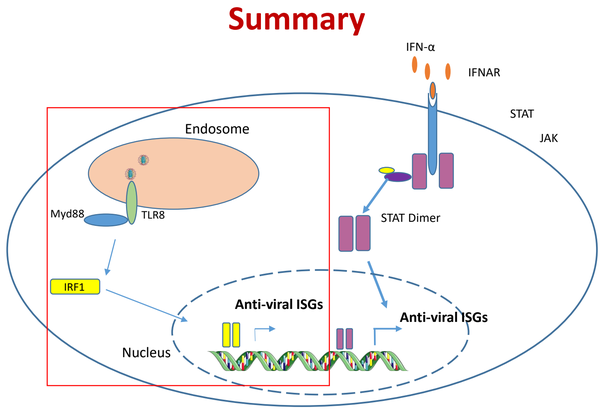
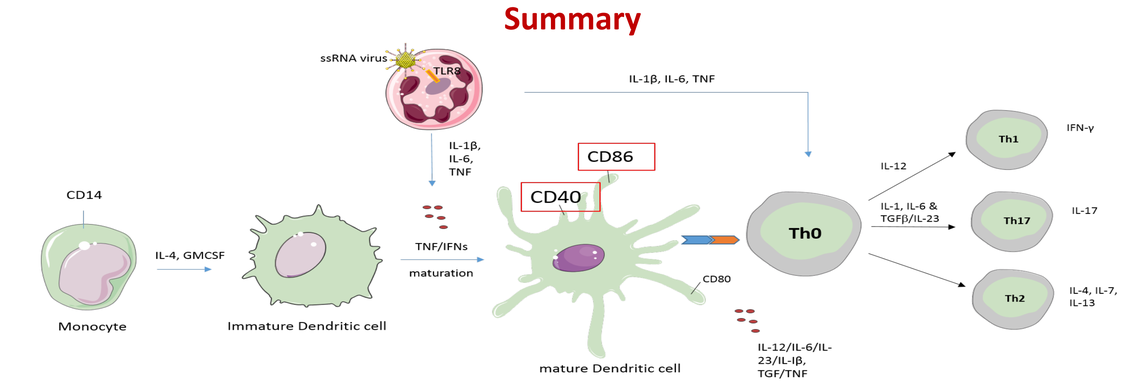



**Fig 9: Dendritic cell/neutrophil coculture supernatant polarises CD4 T-cells towards a Th1 phenotype.** Dendritic cells were cultured in TLR8 conditioned neutrophil media for 24 hours. Isolated peripheral blood CD4+ T-cells were then cultured in this dendritic cell conditioned media for 96 hours in the presence of anti-CD3 and CD28. live singlet CD3+/CD4+/CD8- were gated on their expression of intracellular IL-17/IFN-γ. Additionally T cell culture supernatants were analysed using ELISA for IFN-γ to determine the optimal time point to measure T-cell responses. Graphs are the mean ± SD of 2 independent experiments.

## Summary




## Summary




**Schematic diagram showing how neutrophils modulate the adaptive responses in viral infection**

## Summary/conclusion

- pSTAT1 is significantly expressed in a delayed and sustained fashion in response to IFN-α, with optimal induction observed at 60min in primary human neutrophils
- HIV-1 signals via TLR8 do induce the successful expression of ISGS independent of Type 1 IFNs
- IFN independent induction of ISG Viperin is dependant on the expression of IRF1
- TLR8 activation results in the production of Cytokines IL-8,IL-6, IL-1β and TNF
- TLR8-activated neutrophil-derived supernatant stimulates DC maturation through TNF
- This maturation of DCs may also polarise CD4 T-cells towards a Th1 Phenotype

**Primary Human Neutrophils respond to Viral Agonists & Anti-Viral Cytokine, thereby stimulating ISGs, DC Maturation & Th1 Differentiation; revealing Novel Anti-Viral & Immunomodulatory Roles for Neutrophils**