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Study of neutrophils in a cynomolgus macaque model of HIV infection : how do they impact T cells and inflammation?

Gaëlle Sandillon

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Study of neutrophils in a cynomolgus macaque model of HIV infection: how do they impact T cells and inflammation?

Etude des neutrophiles chez le macaque cynomolgus comme modèle de l'infection VIH : comment impactent-ils les lymphocytes T et l'inflammation ?

Thèse de doctorat de l'Université Paris-Saclay

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Thèse soutenue à Paris-Saclay, le 31 Mars 2025, par

Gaëlle SANDILLON

Composition du Jury

Membres du jury avec voix délibérative

Pierre DELOBEL

Professeur des Universités – Praticien Hospitalier, CHU Purpan, INSERM UMR1291-CNRS UMR5051-Université Toulouse III

Président du jury

Aude REMOT-BRIZION

Chargée de Recherche, UMR1282, Infectiologie et Santé Publique, Centre INRAE Val de Loire

Rapportrice & Examinatrice

Maria NARANJO-GOMEZ

Chargée de Recherche, INSERM U1183, IRMB, Centre Hospitalier Régional Universitaire de Montpellier

Rapportrice & Examinatrice

Lisa CHAKRABARTI

Directrice de Recherche, CNRS UMR 3569, Unité Virus et Immunité, Institut Pasteur, Université Paris Cité

Examinatrice

Lai Guan NG

Directeur de Recherche, Shanghai Immune Therapy Institute, Renji Hospital, Shanghai Jiao Tong University School of Medicine

Examinateur

Titre : Etude des neutrophiles chez le macaque cynomolgus comme modèle de l'infection VIH : quel est leur impact sur les lymphocytes T et l'inflammation ?

Mots clés : Neutrophiles ; Lymphocytes T ; VIH/SIV ; régulation ; inflammation

Résumé : Malgré les traitements antirétroviraux (ARV), les personnes vivant avec le VIH (PVVIH) sont exposées à une inflammation chronique persistante, qui augmente les risques de développer des comorbidités comme les maladies cardiovasculaires ou certains cancers. Alors que de nombreuses études se sont intéressées aux rôles des monocytes et des macrophages dans l'inflammation au cours du VIH, l'impact des neutrophiles et leurs interactions avec les autres cellules de l'immunité sont peu connus. Notre objectif était ainsi de caractériser la dynamique des neutrophiles, leurs capacités modulatrices envers les lymphocytes T et leur rôle dans l'inflammation dans l'infection VIH. Nous avons utilisé le modèle macaque cynomolgus infecté par le Virus d'Immunodéficience Simienne (SIV) comme modèle préclinique du VIH, qui présente un phénotype et des fonctions neutrophiles similaires à l'Homme. Pour cette étude, quinze macaques ont été infectés par le SIV et ont reçu ou non un TARV à 4 semaines post-infection. Nous avons analysé les neutrophiles en combinant de la cytométrie en flux, des co-cultures neutrophiles-lymphocytes T avec détection de cytokines et chimiokines solubles et marquages intracellulaires afin de mesurer l'activation T ; ainsi qu'une analyse protéomique des neutrophiles avant et pendant l'infection SIV.

Durant l'infection aiguë par le SIV, la fréquence de neutrophiles CD101+ CD10- immatures circulants était augmentée par rapport aux animaux non infectés, et l'expression de CD62L était diminuée chez les neutrophiles sanguins totaux. Après six mois d'infection, les neutrophiles CD62_{low} et la fréquence d'immatures étaient toujours plus élevées chez les macaques non traités, alors que les macaques traités par ARV présentaient un phénotype neutrophile similaire à la baseline.

Pendant les phases aiguës et chroniques de l'infection SIV, nous avons montré par des co-cultures de neutrophiles et de cellules sanguines mononucléées

périphériques (PBMCs) que les neutrophiles pouvaient moduler la concentration de molécules solubles en lien avec l'activation T. Ainsi, les neutrophiles ont induit la diminution de cytokines solubles comme l'IL-6, l'IL-1 β ou le TNF α dans les surnageants de co-culture, tandis que la concentration de la chimiokine IP-10 était augmentée. En parallèle, lors des phases aiguës et chroniques, nous avons observé que les neutrophiles induisaient une augmentation de l'activation des lymphocytes T CD4+ et CD8+, avec une augmentation du CD69, des cytokines intracellulaires et de la cytotoxicité CD8. En utilisant des puits Transwell, nous avons vu que les effets modulateurs induits par les neutrophiles dépendaient d'un contact avec les PBMCs. Enfin, nous avons observé que les neutrophiles des macaques infectés par le SIV présentaient des signatures protéomiques antivirales, ainsi que des signatures interféron de type I à 21 jours post-infection et à 6 mois post-infection. En revanche, les animaux sous ARV à 6 mois présentaient des signatures neutrophiles associées au métabolisme, ce qui suggère que le traitement pourrait restaurer les fonctions neutrophiles et diminuer l'inflammation. A travers ces résultats, nous démontrons que l'infection par le SIV chez le macaque cynomolgus induit l'augmentation de neutrophiles immatures et CD62_{low} capables de moduler l'activation T et leur production de cytokines de manière contact-dépendante. De plus, les neutrophiles étaient associés à des signatures protéomiques antivirales et liées aux voies interféron, ainsi qu'une augmentation de la production d'IP-10, un phénotype inflammatoire partiellement résolu par le ARV. Notre travail permet une meilleure appréhension de la modulation induite par les neutrophiles de l'activation T et de l'inflammation au cours du SIV. Comprendre les capacités modulatrices et inflammatoires des neutrophiles dans l'infection SIV/VIH est cruciale dans l'optique de définir de nouvelles cibles thérapeutiques pour diminuer l'inflammation chez les PVVIH.

Title: Study of neutrophils in a cynomolgus macaque model of HIV infection: how do they impact T cells and inflammation?

Keywords: Neutrophils ; T lymphocytes ; HIV/SIV ; regulation ; inflammation

Abstract: Despite antiretroviral therapy (ART), people living with HIV (PLWHIV) are exposed to a persisting chronic inflammation, leading to increased risks of developing comorbidities such as cardiovascular diseases, neurocognitive disorders, or cancer. While many studies have explored the roles of monocytes and macrophages in immune activation and inflammation, less is known about the role of neutrophils and their interactions with other immune cells during HIV infection. In this work, we aimed to characterize neutrophil dynamics, how they modulate T lymphocyte activation, and their impact on inflammation during HIV infection. We used cynomolgus macaques infected by Simian Immunodeficiency Virus (SIV) as a preclinical model of HIV, displaying neutrophil phenotype and functions similar to humans. Fifteen macaques were infected with SIV and received ART or not at four weeks post-infection. We monitored neutrophils by combining whole blood flow cytometry, along with neutrophil-T cells co-culture assays with cytokine and chemokine detection as well as intracellular staining assays to evaluate T cell activation. Additionally, we analyzed the proteome of neutrophils before and during SIV infection.

During primary infection, the frequencies of circulating immature CD101⁺ CD10⁻ neutrophils increased significantly, and neutrophils displayed a CD62L_{low} phenotype. After six months of infection, low-CD62L and immature PMNs remained higher than before infection in the blood of untreated SIV-infected macaques. On the other hand, macaques receiving ART showed a neutrophil phenotype similar to baseline.

In primary and chronic phases of SIV infection, co-cultures of autologous neutrophils with peripheral mononuclear blood cells (PBMCs) revealed that neutrophils could modulate the concentration of molecules produced by PBMCs. In acute infection, neutrophils increased IL-1RA and granzyme B concentrations, while soluble cytokines such as IL-6, IL-1 β and TNF α were decreased. Importantly, the levels of the chemokine IP-10 significantly increased in the presence of neutrophils in the acute and chronic phases of SIV infection.

In parallel, we investigated the direct effects of neutrophils on T cells. We observed that neutrophils increased CD4⁺ T cell and CD8⁺ T cell activation, intracellular cytokine production and CD8 cytotoxicity during early and, at a lower level, late SIV infection. Using Transwell assays, we demonstrated that the immunomodulatory properties of neutrophils relied on contact-dependent mechanisms.

Lastly, in order to decipher the impact of SIV infection on neutrophils, we performed a proteomic analysis of purified neutrophils using mass spectrometry. We demonstrated that neutrophils from SIV-infected macaques displayed antiviral and interferon type I signatures at 21dpi, which persisted in untreated chronic infection. Contrary to viremic animals, neutrophils from ART-treated macaques showed proteome signatures associated with metabolism, which suggests the treatment may restore neutrophil function and lower inflammation.

Overall, we found that SIV infection in cynomolgus macaques induced immature and CD62L_{low} neutrophils capable of modulating T cell activation and cytokine production in a contact-dependent manner. Neutrophils in SIV infection were associated with antiviral and interferon-related proteome signatures, as well as IP-10 production, an inflammatory phenotype that was partially resolved with ART. Our work provides a better understanding of how neutrophils shape T cell activation and inflammation in SIV infection. In the future, deciphering neutrophil modulatory and inflammatory properties in SIV/HIV infection will be crucial in defining new therapeutic targets to lower inflammation in PLWHIV.

Synthèse des travaux en Français

Malgré les traitements antirétroviraux (ARV), les personnes vivant avec le virus d'immunodéficience humaine (PVVIH) sont exposées à une inflammation chronique persistante, qui augmente les risques de développer certaines comorbidités comme les maladies cardiovasculaires, neurocognitives ou encore certains cancers. Alors que les rôles des populations mononucléées inflammatoires comme les macrophages et les cellules dendritiques, ont été explorés au cours du VIH, l'impact des polynucléaires neutrophiles sur l'inflammation est peu connu. Pourtant, les neutrophiles constituent les leucocytes les plus nombreux du sang et présentent de hauts potentiels inflammatoires et anti-infectieux.

Précédemment, Julien Lemaitre et al. ont démontré que l'infection par le Virus d'Immunodéficience Simienne (SIV) SIVmac251 chez le macaque cynomolgus (MCy), modèle pré-clinique du VIH, induisait un phénotype immature et activé des neutrophiles, partiellement résolu lorsque les animaux étaient traités par ARV. De plus, les neutrophiles co-cultivés avec des cellules mononucléées périphériques du sang (PBMCs) induisaient une augmentation des fréquences de lymphocytes T CD4⁺ et T CD8⁺ activés, en particulier en phase aiguë de l'infection. Ces travaux ont montré que les neutrophiles étaient capables de moduler l'activation T au cours de l'infection par le SIVmac251. Cependant, la nature précise des interactions entre neutrophiles et lymphocytes T ainsi que leur impact sur l'inflammation chez les MCys restaient à explorer. Ainsi, pour ce projet, notre objectif était d'investiguer l'impact des neutrophiles sur les lymphocytes T et les conséquences sur l'inflammation au cours de l'infection par le SIVmac251. Plus précisément, nous avons analysé la dynamique des neutrophiles aux échelles cellulaire et protéomique, la nature de leurs capacités modulatrices envers les lymphocytes T et les PBMCs, et leur lien avec l'inflammation au cours de l'infection. Nous avons utilisé le modèle MCy infecté par le SIVmac251, un modèle d'infection par le VIH qui a été caractérisé lors de nombreuses études dans notre unité de recherche IDMIT et qui permet de reproduire une physiopathologie chez le MCy très proche de celle induite par le VIH chez l'Homme. De plus, les MCys présentent des neutrophiles ayant un développement, des phénotypes et des fonctions similaires aux neutrophiles humains. Pour cette étude, quinze MCys ont été infectés par le SIVmac251 et ont reçu ou non un traitement ART à quatre semaines post-infection. A différents temps de l'infection, nous avons analysé la dynamique des neutrophiles en combinant de la cytométrie en flux sur sang total, des co-cultures neutrophiles-PBMCs avec détection de cytokines et chimiokines solubles pro et anti-inflammatoires, ainsi que des marquages extra et intracellulaires afin de mesurer l'activation T. Enfin, nous avons caractérisé les changements de protéome des neutrophiles sanguins par spectrométrie de masse au cours de l'infection.

En premier lieu, nous avons analysé le nombre absolu de neutrophiles sanguins ainsi que leur phénotype au cours de l'infection par le SIVmac251 chez le MCy. A 10 jours post-infection (jpi), correspondant à la phase d'infection aiguë, le nombre absolu de neutrophiles sanguins était significativement diminué chez les MCys par rapport aux

animaux non infectés (baseline). Par ailleurs, la fréquence de neutrophiles CD101+ CD10- immatures circulants était significativement augmentée et l'expression de CD62L était diminuée chez les neutrophiles sanguins totaux par rapport à la baseline, démontrant un phénotype plus activé qu'avant l'infection. A 6 mois post-infection (mpi), alors que le phénotype de neutrophiles CD10_{low} CD62L_{low} était toujours observable chez les CMys infectés sans traitement, les MCys traités par ARV présentaient des neutrophiles phénotypiquement similaires à la baseline. Ces données montrent que l'infection des MCys par le SIVmac251 induit en phase aiguë une déplétion temporaire des neutrophiles, cellules caractérisées par un phénotype immature et plus activé qui persiste en phase chronique en l'absence de traitement.

Dans un second temps, nous avons investigué l'impact des neutrophiles sur la concentration de cytokines et chimiokines pro ou anti-inflammatoires produites par les PBMCs stimulées, cultivées à différents temps de l'infection avec ou sans neutrophiles. A 15 jpi, les concentrations de cytokines solubles comme l'IL-6, l'IL-1 β ou le TNF α étaient diminuées dans les surnageants de culture en présence de neutrophiles, tandis que la concentration d'IL1-RA d'une part, et d'IP-10 d'autre part, étaient augmentées. A 6 mpi, les neutrophiles co-cultivés avec les PBMCs tendaient toujours à diminuer les concentrations de plusieurs cytokines pro-inflammatoires comme l'INF γ , le TNF α ou l'IL-6, indépendamment du traitement. De plus, la concentration d'IP-10 soluble tendait à être augmentée chez tous les animaux en présence de neutrophiles, mais de manière plus forte chez les animaux non traités. Ces données indiquent que lors de l'infection SIVmac251, les neutrophiles induisent la modulation de cytokines en lien avec l'inflammation produites par les PBMCs. En particulier, les neutrophiles diminuent les taux de plusieurs cytokines pro-inflammatoires d'une part, mais augmentent la concentration d'IP-10, une chimiokine impliquée dans l'inflammation.

En parallèle, nous avons étudié si l'impact direct des neutrophiles sur l'activation des lymphocytes T dépendait d'un contact entre ces deux types cellulaires. A 10 jpi, nous avons confirmé que les neutrophiles induisaient une augmentation des fréquences de lymphocytes T CD4+ et CD8+ activées. Cette activation était caractérisée par une expression plus importante du marqueur CD69, de la production de cytokines intracellulaires INF γ et TNF α , ainsi que du marqueur CD107a, associé à la cytotoxicité des T CD8+. En phase chronique, les neutrophiles induisaient toujours une augmentation de l'expression du CD69 sur les lymphocytes T, mais pas des autres marqueurs étudiés, chez les animaux traités et non traités par ART. En utilisant des puits Transwell, nous avons démontré que ces effets modulateurs induits par les neutrophiles dépendaient d'un contact avec les PBMCs, mais nous n'avons pas pu identifier le(s) mécanisme(s) associé(s).

Enfin, nous avons interrogé l'impact de l'infection SIVmac251 sur les neutrophiles de MCys en réalisant une analyse protéomique par spectrométrie de masse de neutrophiles purifiés à différents temps d'infection. A partir de ces données, nous avons observé que les protéomes des neutrophiles des macaques infectés par le SIV à 21 jpi présentaient d'importantes signatures interféron de type I. Ces signatures interféron

étaient liées à l'augmentation de la présence de protéines issues de gènes stimulés par les interférons (ISG), comme les protéines des familles IFIT ou OAS. De plus, ces signatures étaient aussi liées à la présence de protéines de défense antivirales comme RIG-I ou SAMHD1. Alors que ces signatures protéiques étaient toujours fortement présentes chez les animaux non traités à 6 mpi, les animaux traités présentaient davantage de signatures neutrophiles associées au métabolisme cellulaire. Cependant, certaines protéines liées aux ISG étaient élevées chez les deux groupes, comme IFIT1 ou MX2. Ces données suggèrent que le traitement pourrait induire une réduction partielle des signatures interféron chez les neutrophiles sanguins et diminuer l'inflammation.

A travers ces résultats, nous démontrons que l'infection par le SIVmac251 chez le macaque cynomolgus induit l'augmentation de neutrophiles immatures et CD62L_{low} capables de moduler le micro-environnement cytokinique des PBMCs ainsi que l'activation des lymphocytes T de manière contact-dépendante. Au cours de l'infection, les neutrophiles étaient associées à des signatures protéomiques liées aux voies interféron et à la défense antivirale, et induisaient d'autre part une augmentation de la production d'IP-10 soluble, une chimiokine impliquée dans l'inflammation dont la concentration plasmatique est élevée chez les PVVIH. Ces données tendent à suggérer que les neutrophiles pourraient participer à l'inflammation au cours de l'infection par le SIV chez le MCy via les voies interféron, et ce phénotype inflammatoire serait partiellement résolu par le traitement ARV. Notre travail permet une meilleure appréhension des interactions neutrophiles-lymphocytes T, ainsi que du rôle des neutrophiles dans l'inflammation au cours du SIVmac251 chez le MCy. Dans le futur, les neutrophiles pourraient constituer de nouvelles cibles thérapeutiques intéressantes pour réguler l'inflammation chez les PVVIH.

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J'espère sincèrement que ce travail contribuera dans le futur à améliorer le quotidien des personnes vivant avec le VIH, en France et dans le monde.

LIST OF ABBREVIATIONS

AIDS: Acquired immunodeficiency syndrome	ISG: Interferon-stimulated genes
AGM: African green monkey	LDN: Low-density neutrophils
APCs: Antigen-presenting cells	PLS: Lipopolysaccharide
ART: Antiretroviral therapy	MDSC: Myeloid-derived suppressor cells
AZT: Azidothymidine	MHC: Major histocompatibility complex
BM: Bone marrow	MMP: Matrix metalloproteinase
CCR5: C-C chemokine receptor 5	MPO: Myeloperoxidase
CD: Cluster of differentiation	MSM: Men who have sex with men
CDC: Centers for Disease Control	Mtb: Mycobacterium tuberculosis
CXCR4: C-X-C-chemokine receptor 4	NK: Natural killer cells
CMV: Cytomegalovirus	NETs: Neutrophil extracellular traps
CyMs: Cynomolgus macaques	NHP: Non-human primate
DCs: dendritic cells	PLWHIV: People living with HIV
DNA: Deoxyribonucleic acid	PLS-DA: Partial least squares discriminant analysis
FcγR: Fragment crystallizable receptor for immunoglobulin G	PBMC: Peripheral mononuclear cells
fMLP: N-Formylmethionyl-leucyl-phenylalanine	PMA: Phorbol myristate acetate
G-CSF: Granulocyte-colony stimulating factor	PMN: Polymorphonuclear neutrophils
GIT: Gastrointestinal tract	PRR: Pathogen recognition receptor
GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor	PTCs: Post-treatment controllers
HMCV: Human cytomegalovirus	PTMs: Pigtail macaques
HSCs: Hematopoietic stem cells	RBC: Red blood cell lysis
HIV: Human immunodeficiency virus	RIG-I: Retinoic acid-inducible gene I
HLA-DR: Human leukocyte antigen – DR isotype	RMs: Rhesus macaques
ICS: Intracellular staining	RNA: Ribonucleic acid
IFN α : Interferon alpha (interferon type I)	ROS: Reactive oxygen species
IFN γ : Interferon gamma (interferon type II)	SHIV: Simian human immunodeficiency virus
IFNAR: Interferon alpha/beta receptor	SIV: Simian immunodeficiency virus
IL: Interleukin	SM: Sooty mangabey
IL-1RA: Interleukin-1 receptor antagonist	ssRNA: Single-strand ribonucleic acid
IP-10 (CXCL10): Interferon gamma-induced protein 10	TAN: Tumor-associated neutrophils
	TCR: T cell receptor
	TLR: Toll-like receptor
	TNF α : Tumor necrosis factor alpha
	WHO: World Health Organization

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FOREWORDS

This work is part of the graduating process for obtaining the PhD diploma delivered by the doctoral school Biosigne, from the Paris-Saclay University.

This work focuses on the characterization of polynuclear neutrophils and their interactions with T lymphocytes in the pathophysiology of the simian immunodeficiency virus infection in cynomolgus macaques. This PhD was performed in the research unit Infectious Diseases Models for Innovative therapies (IDMIT, INSERM UMR1184, Université Paris-Saclay, Commissariat aux Energies Atomiques et alternatives of Fontenay-Aux-Roses) under the supervision of Pr. Olivier Lambotte, Dr. Roger le Grand and Dr. Julien Lemaitre. Funding for this project was partially provided by the ANRS RHIVIERA (Remission of HIV Infection Era) consortium through a public-private partnership. This consortium aims to develop new tools and strategies in the search of a scalable sustained remission of HIV infection.

INTRODUCTION

Infections due to human immunodeficiency viruses (HIV) lead to chronic diseases in humans, characterized by a progressive depletion of peripheral CD4+ T lymphocytes and chronic immune activation. Without treatment, people living with HIV (PLWHIV) progress to acquired immunodeficiency syndrome (AIDS) within several years, a syndrome defined by profound immunodeficiency and elevated risks of opportunistic infections. In PLWHIV, antiretroviral therapy (ART) administration blocks HIV replication and prevents disease transmission but does not completely resolve inflammation.

I. Chronic inflammation in people living with HIV: a remaining challenge

In the absence of a cure for HIV infection, ART remains necessary for patients to control viral replication and maintain normal blood CD4+ T cell counts. Although current therapies are highly effective in PLWHIV, patients are exposed to systemic inflammation. This inflammatory condition can be attributed to different factors, including low levels of HIV replication in tissues, persisting antigenic stimulation induced by bacterial translocation from the gut, and ongoing chronic activation of immune cells. In the long term, metabolic, neurologic, or cardiac diseases are shown to be more frequent in PLWHIV compared to the general population, increasing morbidity and mortality risks in these patients. Tackling inflammation in PLWHIV by addressing early and late immune events in the disease therefore remains of major importance to improve patients' life expectancy and quality of life.

i) Brief overview of HIV infection in 2025

1) HIV, a disease that remains deadly despite effective ART

In the early 1980s, several cases of patients suffering from fungal infection *Pneumocystis pneumonia* were reported in the US Centers for Disease Control [1].

Rapidly, an epidemic started to spread in the northern countries, and a T-lymphotropic retrovirus was identified in 1982 to be responsible for this deadly disease. The pathogen was isolated in 1983 and was progressively characterized as a retrovirus, then called lymphadenopathy-associated virus (LAV), renamed later HIV [2]. As the epidemic was spreading around the globe, new techniques to identify and quantify HIV in patients were urgently required. Hence, researchers and doctors progressively developed diagnostic assays for patients and isolation methods to characterize HIV. In the mid 1980's, the viral sequence of HIV-1 was described for the first time [3]. From this work, a phylogenetic description of HIV allowed the identification of two viral lineages, HIV-1 and HIV-2. Later, these human strains were found to result from several inter-species transmissions from non-human primates (NHP) species to the human species, likely due to hunting or meat preparation [4,5]. After HIV started to circulate among humans, different viral groups emerged. A large majority of human infections during the pandemic have been caused by HIV-1 group M. HIV-1 and HIV-2 both are pathogenic, share a common structure, and present similar replicative mechanisms. Nonetheless, HIV-2 is characterized by lower transmission rates and slower progression to immunodeficiency in patients. In this manuscript, we will use the term "HIV" to designate HIV-1 and HIV-2, unless specified differently.

HIV can be transmitted through the exchange of body fluids such as vaginal secretions, sperm and blood, and then establishes in mucosal tissues. Most horizontal transmissions of HIV occur during barrier-free vaginal and anal intercourse, but also after unsafe injection drug use. Vertical transmissions from mother to child are also frequent during pregnancy, birth or breastfeeding.

Until today, infections due to HIV have affected more than 80 million people worldwide and caused the death of more than 40 million. Approximately 40 million people were living with HIV in 2024: among them, 77% were receiving ART, and about 15% remained unaware of their status. The development of new antiretroviral drugs and efficient prevention strategies such as pre-exposure prophylaxis (PrEP) or post-exposure

prophylaxis (PEP) have considerably reduced HIV transmission rate. Despite a 60% reduction in new HIV infections since 1995, 1.3 million people were newly diagnosed with HIV in 2023, and approximately 630,000 HIV-related deaths were reported the same year, which represented a death every minute in 2023 according to the World Health Organization (WHO). Overall, HIV still represents a threat to public health in 2025 and many challenges remain to prevent, cure and eradicate HIV-related diseases.

2) HIV structure and cell targets

HIVs belong to a family of viruses called Retroviridae or retroviruses. Viruses in this family are characterized by enveloped virions with an inner core containing the viral genome and replicative enzymes, allowing pathogens to integrate their DNA into the genome of infected cells. More specifically, HIV is a member of the lentivirus genus, which presents long periods of incubation, is cytopathogenic, and leads to chronic infections [6]. HIV genome is composed of 2 copies of 9kb single strand RNAs, and contains 9 different genes resulting in the synthesis of 15 different proteins. Common retroviral genes gag, pol and env encode for structural, enzymatic and envelope proteins respectively. The viral genome is protected inside a tridimensional structure called capsid, which plays an essential role in the replicative cycle of HIV (**Figure 1**). Besides the HIV genome, different enzymes necessary for infection of the target cells are contained in the capsid: protease, reverse transcriptase, and integrase [6]. HIV is also characterized by a lipid bilayer, an envelope covered with various proteins. The two glycoproteins gp120 and gp41 are crucial for viral protein detection and for the binding of HIV to target cell receptors.

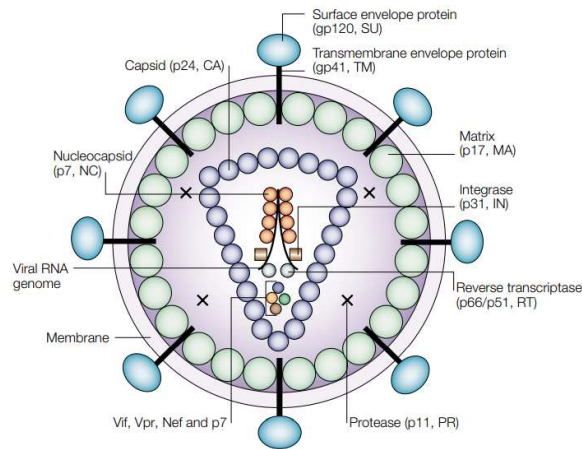


Figure 1: Structure of HIV-1, from H.L. Robison, *Nature Reviews Immunology* 2002 [7]

HIV-1 is composed of two single-strand RNAs and presents an envelope with gp120 and gp41 proteins, which are crucial for HIV fusion with target cell membranes.

In the 1980s, the protein cluster of differentiation 4 (CD4) was identified as the main cell receptor for HIV [8]. CD4 is mainly expressed by CD4⁺ T cells and binds to class II major histocompatibility complexes (MHC II) on antigen-presenting cells (APCs) during immune responses, leading to T cell activation. The CD4 protein is also expressed in macrophages, monocytes and dendritic cells (DCs) at lower levels [9]. Co-receptors CCR5 and CXCR4 are involved in the fusion between the viral envelope and the target cell membrane, and determine the nature of the cell target [10]. After the binding and fusion of HIV to the target cell membrane, genome replication occurs by reverse transcription of the RNA genome into a double-stranded DNA, which can eventually integrate into the host genome (**Figure 2**).

Rare individuals presenting homozygous CCR5 Δ 32 mutations are resistant to HIV-1 infection as the viral entry is blocked from target cells [11]. Several remissions from HIV-1 infection were recently achieved in patients after transplantation of hematopoietic stem cells from HIV-1-resistant individuals, including one French patient who was recently cured in Marseille [12].

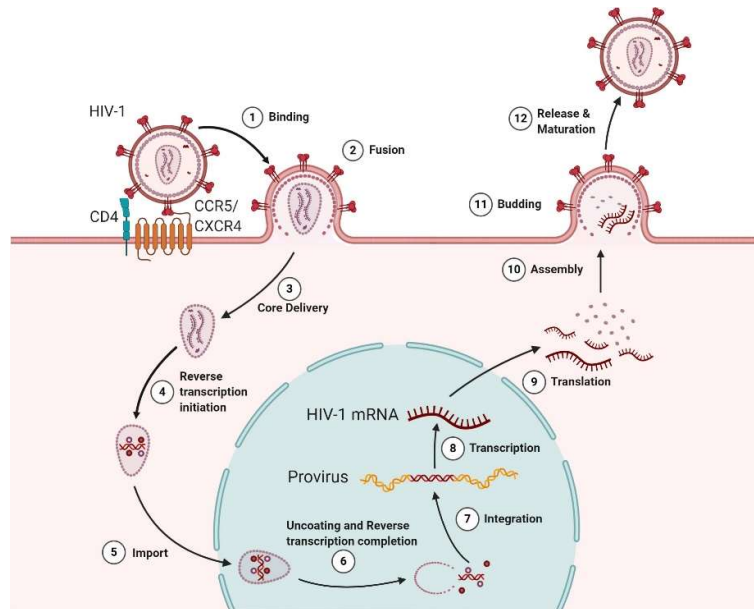


Figure 2: The HIV life cycle, from Ramdas et al., Frontiers in microbiology, 2020 [13]

HIV uses CD4 as a target cell receptor and CCR5 or CXCR4 as co-receptors. After the fusion between the HIV envelope and the target cell membrane, a reverse transcription step leads to the importation of HIV DNA inside the cell nucleus. There, using integrase, HIV DNA can integrate the hosts' DNA. From there, the infected cell can sustain the viral replication by producing new virions.

3) HIV drugs: effective treatments & remaining challenges

After the pandemic started, many trials began to find efficient antiviral treatments against HIV. Azidothymidine (AZT) was the first drug approved in 1987 [14]. AZT is a molecule targeting the reverse-transcriptase of HIV, thus blocking viral replication. Even though the administration of AZT increased the life expectancy of patients by several months, the efficiency of this molecule was limited, and resistant HIV variants appeared rapidly [15]. Additionally, adherence to AZT was low because of side effects. Later, at the end of the 1990s, the life expectancy of patients increased considerably with the development of combined antiretroviral therapies, composed of at least 3 different molecules targeting different steps of the viral replicative circle [16,17]. In fact, HIV drug molecules are divided into several categories depending on their target [17]:

- Nucleoside reverse transcriptase inhibitors (NRTIs) or nucleotide reverse transcriptase inhibitors (NtRTIs)

- Non-nucleoside reverse transcriptase inhibitors (NNRTIs)
- Co-receptor inhibitors and fusion inhibitors
- Protease inhibitors
- Integrase inhibitors (INIs)

ART combinations can be adapted for each patient depending on different parameters, such as their immunological status. For PLWHIV, ART administration induces an undetectable viral load (less than 50 RNA copies of HIV per mL of blood) and restoration of blood CD4+ T cell counts after several weeks [18]. Early administration of ART after exposure to HIV induced a higher efficiency of the treatment compared to a late administration. Early ART was associated with lower viral expansion, lower inflammation and better preservation of CD4+ T cells [19]. Recently, Passaes et al. also showed in a cynomolgus macaque model of HIV infection that a treatment administered early after pathogenic exposure favored the control of viral replication and a higher number of memory CD8+ T cells after treatment interruption [20].

Indeed, a small fraction of PLWHIV can control viral replication after ART interruption. These patients, called post-treatment controllers (PTCs), show no signs of HIV disease and very low plasma viral loads after several months of analytical treatment interruption [21,22]. Currently, many research projects focus on understanding the virological and immunological characteristics of PTCs to investigate new therapy approaches for an HIV cure [20,23]. So far, several factors have been associated with better control of HIV infection without treatment, such as high CD4+/CD8+ T cell ratios, low cell-associated HIV DNA levels, and even specific transcriptomic and metabolomics signatures compared to non-controllers [20,24].

Despite their high efficacy, some ART molecules, such as Dolutegravir, have been associated with long-term toxic effects in patients, such as obesity or metabolic disturbances, and even neurological effects [17]. Other than side effects, another remaining challenge related to ART is emerging drug resistance in PLWHIV. Indeed,

drug-resistant HIV variants can be transmitted, increasing the risk of treatment failure among patients [25].

4) Non-human primate models remain necessary to study HIV and develop new cures

Phylogenetic studies identified HIV-1 and HIV-2 as descendants of simian immunodeficiency viruses (SIV), which are retroviruses endemic to African apes and Old World monkeys. As such, HIV-1 was derived from SIVcpz in chimpanzees [26], while HIV-2 and SIVmac descended from a sooty mangabey strain, SIVsmm [27], and animals from about thirty different non-human primate (NHP) species were found carrying different strains of SIV [28]. Many African monkey species and apes are natural hosts for SIV, including African green monkeys (AGMs) and sooty mangabeys, two species that do not develop AIDS when exposed to SIV. On the other hand, several Asian NHP species are susceptible to certain strains of SIV, inducing a pathophysiology close to HIV-1 infection in humans. Besides, HIV-1 is unable to replicate in macaques due to cellular blocks such as restriction factors TRIM5 or APOBEC3A [1]. This is why Asian macaques have been mainly studied as animal models for HIV infection over the last 40 years.

In fact, in vitro assays using primary cells and cell lines exposed to HIV particles have considerably improved our knowledge of HIV structure, replicative cycle and molecular infectious mechanisms. However, these experiments do not thoroughly reflect the complexity of HIV pathology, with systemic alterations and clinical symptoms [29]. Therefore, animal models remain necessary and complementary to these in vitro assays and current mathematical models.

Although NHP models are the most commonly used for the study of HIV infection, humanized mice also constitute prevalent models, and each model presents benefits and drawbacks. Humanized mice are immunodeficient mice engrafted with human cells and/or tissues, mainly investigated to decipher anti-HIV immune responses, T cell

activation and exhaustion. These models are also frequently used to develop innovative therapies such as gene editing or immunotherapies [29]. However, humanized mice do not fully recapitulate all the features of HIV disease, such as the different phases of infection or progression to AIDS, and also lack mature functional human neutrophils, although this issue is now being tackled [30].

As previously mentioned, simian models for HIV infection have been of major importance for the study of HIV pathophysiology, as they present great proximity with humans regarding their genetic background, their immune system and their organ architecture. In NHP studies using pathogenic SIV exposure, animals are usually inoculated with simian strains such as SIVmac251 and SIVmac239, or humanized strains called SHIV [31]. Historically, rhesus macaques (RMs), pig-tailed macaques (PTMs) and cynomolgus macaques (CyMs) have been the most studied NHP species to investigate HIV pathophysiology. Indeed, these Asian macaque species develop many symptoms of the human disease: progressive CD4⁺ T cell depletion, persistent plasma viremia, and progression to AIDS after months or years of SIV infection. These three macaque species present some differences in the pathophysiology induced by SIV, and further details with a focus on cynomolgus macaques are provided in the Methods section p80-83.

Despite the cost of NHP studies, these animal models present several advantages compared to clinical and mice studies. First, macaques can be monitored closely before and at all stages of the disease. Longitudinal follow-ups allow precise characterizations of innate and adaptive immune cells, especially in the primary phase of infection, during which immune events might affect the long-term disease [32]. The early acute phase of HIV is difficult to monitor in patients. Hence, results from NHP studies are complementary to extensive prospective studies in at-risk healthy volunteers, which can be heavy and demanding for patients. NHP models are also needed for the study of chronic untreated infection and progression to AIDS because samples from untreated patients are rare. Besides, studying immunological behaviors from natural

NHP hosts is crucial to decipher the characteristics of individuals controlling SIV infection [33].

Another critical aspect relies on the study of resident immune cells and HIV-infected cells in tissues, which usually requires access to lymphoid organs from patients, a particularly invasive procedure. On the other hand, NHP models allow the sampling of lymphoid and non-lymphoid tissues from NHPs upon euthanasia (spleen, gut or lung and brain) or during SIV infection by performing ex vivo biopsies (lymph nodes). Studies on NHP tissues have brought significant insights into the characterization of the structure, distribution and organization of SIV-infected cells and associated organs, as well as local immune responses and inflammation [34]. Finally, NHPs usually present good responsiveness to common antiretroviral drugs, which supports their involvement in preclinical therapies and HIV vaccine studies.

All in all, studying SIV infection in NHPs remains crucial to understand early and late immune events occurring during HIV infection and to evaluate the efficacy of innovative therapies against HIV, as well as vaccines. That being said, one must underline the use of animals for research should be refined, reduced and replaced progressively with computer modeling developments or organoids as much as possible.

ii) The acute phase of HIV infection is associated with local and systemic inflammatory responses

HIV infection is characterized by 3 phases: a primary/acute infection, a chronic phase and, after several years, AIDS. As we will see in this chapter, the acute phase of infection is crucial because early immune events greatly impact HIV disease progression. Few days after exposure to HIV, innate and adaptive immunity orchestrate anti-HIV responses.

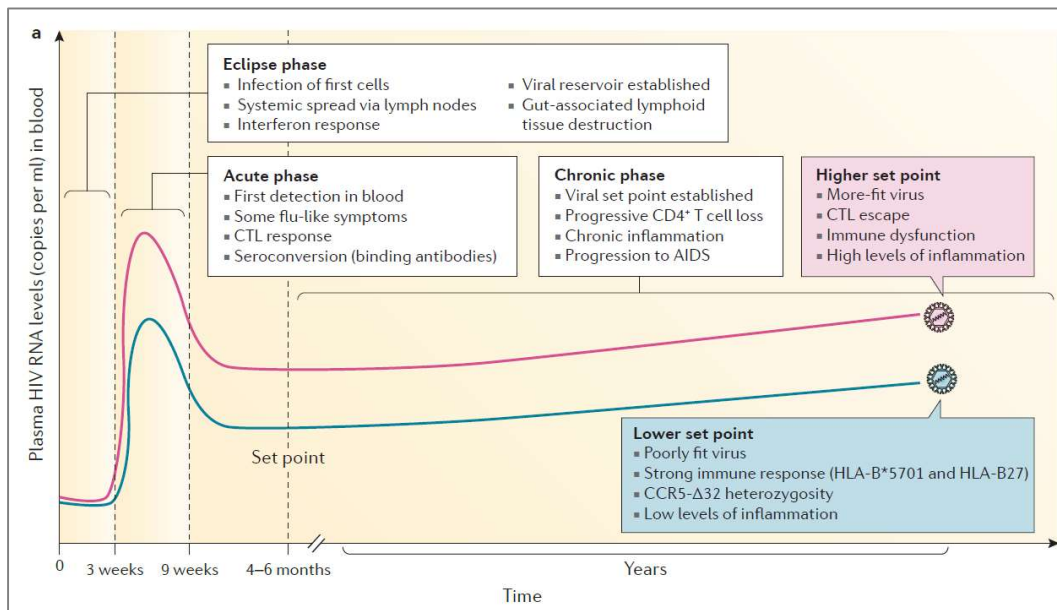


Figure 3: Phases of HIV infection, from Deeks et al., Nature Reviews Disease Primers, 2015 [35]

In patients, HIV infection leads to a peak in plasma viral loads approximately one month after exposure, followed by a period of latency that leads to progressive immunodeficiency (AIDS) after several years in the absence of ART.

Primary HIV infection is characterized by a rapid rise of viral RNA in the plasma, associated with non-specific clinical manifestations in about half of the patients with symptoms such as fever, headache and malaise, however resolved rapidly [36].

Immune responses developed in the first 10 days are particularly critical in HIV-1 infection. Indeed, that time window is characterized by an eclipse phase, during which HIV struggles to escape innate immune responses and begins to spread at the local foci of infection (**Figure 3**). This eclipse phase is followed by a strong amplification of the virus at the site of infection and in local lymphoid tissues before dissemination [37]. In patients, the viral replication usually peaks around 20-30 days after infection, while in NHP models, viremia peaks around 10-14 days after SIV inoculation.

During the acute phase of infection, HIV induces a depletion of tissue CD4⁺ T cells: Th17 and Th1 subsets in gut-associated lymphoid tissues were shown to be strongly depleted due to HIV cytopathic effects [38]. Among infected cells, only a few integrate

HIV DNA and enter a long-term resting state in which the viral genome persists indefinitely, even with therapy, constituting viral reservoirs [39]. Remarkably, peripheral CD4⁺ T cells from patients with acute HIV infection could produce inflammatory molecules such as IFN γ , IL-17 or IL-2 when stimulated non-specifically, and this capacity was inversely correlated with CD8⁺ T cell activation [40].

Concurrently to T cell depletion in tissues, early immune events following HIV or SIV infection are characterized by robust innate responses associated with systemic pro-inflammatory signals [41]. In the first days after exposure, a systemic cytokine cascade is induced, characterized by higher plasma levels of IL-1RA, IFN α , but also IP-10, TNF α , sCD14 and MCP-1. Some of these markers, including IP-10, were associated with rapid disease progression and low CD4⁺ T cell counts [42]. Concentrations of several pro-inflammatory interleukins were also found to be more elevated in the plasma of patients, including IL-6, IL-8, IL-18 and IL-15 [43,44]. Interestingly, the suppressive cytokine IL-10 was also transiently elevated. This cytokine cascade was not as strong in non-progressive or attenuated models of SIV infection compared to pathogenic infection in RMs [45], and was reduced with an early treatment [44]. Additionally, a prolonged systemic inflammatory response and continuous expression of interferon-stimulated genes (ISG) were associated with disease progression in RMs. On the contrary, inflammatory responses in non-pathogenic infection in AGM returned to baseline around 30 days after infection [46]. In patients, systemic inflammatory markers such as IP-10 have also been associated with disease progression [19]. Overall, these results show how the magnitude, duration and nature of early immune responses impact chronic HIV disease and evolution to AIDS.

Within two weeks post-infection, adaptive responses are developed with a strong expansion of CD8⁺ T cells HIV-specific following the viremia peak [44]. The magnitude of cytotoxic T lymphocytes in early infection was negatively correlated with the viral setpoint during the chronic phase [47]. Yet, HIV's rapid generation of viral escape mutants and reservoirs make cytotoxic responses insufficient to eradicate the virus.

iii) How chronic inflammation occurs and persists in HIV infection

With current therapies, most PLWHIV receiving ART present undetectable viral loads and restored CD4+ T cell counts, although we can observe incomplete restoration of T lymphocytes in case of late diagnosis [48]. Globally, PLWHIV develop more comorbidities than the overall population and are exposed to higher risks to develop cardiovascular, neurological or metabolic complications [49–51]. Different factors can explain the frequent occurrence of these comorbidities, and one of them is chronic inflammation.

Indeed, PLWHIV present higher concentrations of systemic inflammatory molecules such as IL-6, IL-8 or sCD14, which may reflect a persisting activation of immune cells. Globally, the level of immune activation strongly correlated with disease progression, morbidity and mortality [52,53]. Even though the precise mechanisms underlying chronic inflammation remain poorly understood, various causes have been identified. First, PLWHIV present markers of gut barrier dysfunction, which is associated with increased risks of bacterial translocation from the gut microbiota to local tissues and inflammation. Second, the persistence of HIV in viral reservoirs in lymphoid organs can support a low-level antigenic stimulation for local immune cells. Finally, co-infections with *Mycobacterium tuberculosis*, cytomegalovirus or hepatitis viruses are also frequent in PLWHIV, increasing immune perturbations. Other cofactors, such as at-risk behaviors, including tobacco or alcohol consumption, also participate in inflammation and will not be detailed here.

1) Gut tissue alteration leads to bacterial translocation

The gastrointestinal tract (GIT) is a tissue with many crucial functions during homeostasis and during infections. This organ is at the interface between the outside, mainly microbiota and nutrients, and the inside of the body, the bloodstream. Epithelial and immune cells reside in this tissue and constitute the mucosal barrier necessary to ensure the integrity and protection of the host by preventing external agents from

penetrating the mucosa. Adaptive cells such as T and B lymphocytes are the most abundant immune cells in this lymphoid tissue, but innate cells such as macrophages, natural killer (NK) cells and neutrophils are also present during homeostasis [54]. Among T cell subsets, Th17 cells play a crucial role in mucosal immunity by producing IL-17, an important cytokine for the defense against bacteria or fungi [55], and IL-22, which regulates host defense at barrier surfaces.

In patients evolving to AIDS, gastrointestinal symptoms such as diarrhea are observed [56]. As a matter of fact, one of the major sites for HIV replication and pathogenesis is the GIT [57], and infection with HIV was shown to impair the gut barrier integrity [58,59]. Indeed, HIV is responsible for the infection and depletion of many CCR5+ CD4+ T cells in the GIT. In particular, resident Th17 cells are preferentially lost, mostly by cytolytic events in the mucosa [60], which endangers mucosal surveillance. Additionally, the loss of tight junctions in epithelial cells favors the risk of translocation of antigens, including microbial products, from the lumen into the tissue and peripheral circulation [61]. The rupture of the intestinal barrier can be seen in patients with gut epithelial dysfunction markers such as elevated sCD14 and LPS plasma levels, which have been associated with a higher risk of mortality in chronic HIV patients who are not treated with ART [62,63]. External bacteria in the mucosa induce a strong recruitment of other immune cells from the circulation, exacerbating local inflammation [64]. During chronic HIV infection, imbalances of the microbial flora also participate in inflammation in patients [65]. A recent study showed that administering integrase inhibitors to patients could reverse the effects of bacterial translocation, systemic inflammation, gut permeability, and microbiota [66].

2) HIV reservoirs may favor persisting inflammation

a. HIV reservoir establishment

During early HIV infection, in lymphoid organs, a fraction of HIV-infected cells integrate the viral genome. This integration of viral DNA in tissue-infected cells is responsible for the long-term persistence of HIV despite efficient therapy. Therefore, even with efficient ART, PLWHIV present a very low viremia (about 1 HIV RNA copy/milliliter of plasma) and viral DNA in tissue reservoirs [67]. This is why, when ART is interrupted, most PLWHIV experience viral rebounds. In this case, we usually observe a reactivation of some HIV-infected cells that support HIV replication [68]. Although definitions can differ, viral reservoirs can be defined as persisting HIV-infected cells in PLWHIV receiving ART, regardless of the mechanism [69]. Reservoirs are mainly constituted of CD4+ resting memory T cells that integrated HIV DNA in their genome and became latent. In HIV-infected cells, latency is a state where cells become non-productive for new virions, but remain capable of producing viral particles when they are activated [69]. Besides, the integration of defective HIV genomes in target cells is highly frequent. The resulting production of viral antigens may contribute to the induction of local immune responses and favor inflammation in tissues [70].

The establishment of HIV reservoirs occurs in the first days after viral transmission, before the apparition of clinical symptoms in patients, making ART insufficient to prevent a total eradication of the virus in the tissues [71]. In SIV-infected RMs, reservoir cells were found in the GIT 3 days after inoculation. However, viral reservoirs decreased when therapy was administered a few days after SIV inoculation [72]. These beneficial results of early therapy on reducing reservoirs were confirmed in cohorts of children living with HIV [73,74].

Characterizing HIV reservoirs in patients remains highly challenging. Although current tools such as quantitative viral outgrowth assay (QVOA) allow the estimation of resting CD4+ T cells containing HIV DNA [75], this technique was shown to underestimate total viral reservoirs because latent inducible reservoir cells cannot be quantified [76]. Indeed,

some cells infected with HIV continue to be transcriptionally active with a production of HIV DNA, proteins and virions, constituting the active reservoir. On the contrary, some infected cells remain completely silent and do not replicate the virus: these cells constitute the latent reservoir. Several mechanisms support the persistence of HIV in reservoir cells, such as quiescence, cell proliferation and residual replication.

To target HIV reservoirs, one barrier is to identify HIV-infected cells, which is still challenging due to their rarity and the lack of specific markers [69]. Recently, VLA-4 was proposed as a new marker for HIV-replication-competent reservoir cells [77]. HIV reservoir CD4⁺ T cells have also been shown to express higher levels of immune checkpoint proteins such as PD-1 and TIGIT [78]. Medical history, gender, age and other factors also contribute to HIV reservoir heterogeneity, which constitute a challenge for the development of universal cure strategies [79,80].

Studies of HIV tissue reservoirs are key for localizing and characterizing infected cells. Over the last few years, various tools have been developed to decipher lymphoid tissue organization and whole-body reservoir distribution during HIV infection. The use of in-situ hybridization (ISH), immunohistochemistry and immunofluorescence provided considerable knowledge on viral persistence and provided insights into new therapeutic strategies [81]. NHP models are particularly suited for tissue reservoir analysis [69]. Interestingly, Estes et al. have shown a different repartition of viral reservoirs before and after ART administration [82]. Before treatment, HIV-infected cells were distributed in the main lymphoid tissues: lymph nodes, spleen, gut and lung, whereas in ART-treated animals, 99% of HIV-infected cells were located in the gut [69,82], and similar results were described in patients [83]. It is possible that HIV persistence in viral reservoirs could favor a prolonged antigenic stimulation, which could enhance chronic immune activation and inflammation in patients with ART.

Altogether, HIV persistence in tissue reservoirs could participate in chronic inflammation in PLWHIV. Targeting and eliminating viral reservoirs still constitute the main challenge to curing HIV in 2025.

b. Pharmacological sanctuaries

As previously described in this manuscript, efficient ART does not completely eliminate HIV in patients due to latently infected cells, which remain capable of expressing viral particles under certain conditions. One of the reasons why ART is not sufficient for HIV elimination is the low penetration of pharmacological molecules in certain organs containing HIV-infected cells, such as the central nervous system [84]. The absorption, distribution and metabolic disruption of antiretroviral drugs vary greatly depending on the nature of the molecules. Tissues with subtherapeutic ART exposure, which can support active replication of HIV, constitute pharmacological sanctuaries, and improving ART distribution in lymphoid tissues remains a challenge.

Because accessing tissues from patients is particularly difficult and relies on invasive procedures, the distribution of antiretroviral drugs was mainly studied in mice and NHPs. In 2010, Bourry et al. compared the effects of early or late administration of ART in pathogenic SIV infection on SIV replication in tissues [85]. In this model, the administration of ART in SIV-infected CyMs just after the viremia peak did not lower SIV replication in lymph nodes and spleen in the early chronic phase, which suggested these organs could constitute pharmacological sanctuaries. On the contrary, ART induced lower levels of viral replication in gut-associated lymphoid tissues, which suggested an efficient drug penetration in this organ. However, Estes et al. demonstrated in 2014 that the intracellular concentration of ART in PBMCs was significantly lower in the gut, lymph nodes and the rectum compared to the blood cells [82]. Similar results were found in human samples comparing ART concentration in PBMCs from lymphoid tissues or blood [86]. Additionally, the low penetration of drugs in some deep organs, such as the central nervous system or the genital tract, was associated with drug resistance in PLWHIV [87,88]. Of note, other mechanisms than poor diffusion were described to explain the low efficiency of ART in tissues. Some antiretroviral molecules were found to interact with drug transport proteins by acting as substrates, inducers or inhibitors. Metabolic enzymes and expression of drug efflux

transporters by immune cells could also contribute to inadequate drug distribution in PLWHIV receiving ART [89].

3) Co-infections with HIV increase inflammation in PLWHIV

a. HIV/HCMV co-infection

Infections due to the human cytomegalovirus or HCMV are quite common in the global population, as the prevalence goes from 40% to 100%, with usually no clinical symptoms. PLWHIV are particularly exposed to co-infections with HCMV. After the primary phase of infection, HCMV infection is characterized by a life-long viral persistence in humans and induces continuous exposure to viral antigens, similar to HIV [90]. There were evidence supporting HCMV replication in mucosal tissues, which could participate in T cell dysfunction and impaired immunity [91]. Although interactions between HIV and HCMV are complex and not fully understood, it is known that HCMV favors inflammation during co-infections. Besides soliciting the T cell compartment by mobilizing and maintaining HCMV-specific T cells [91], HCMV participates in worsening inflammation in patients by several mechanisms. Indeed, HCMV replication is enhanced by inflammatory stimuli, and as we described before, HIV patients are exposed to persistent inflammation. By developing various molecular strategies, HCMV can increase the level of inflammation in tissues: increased expression of inflammatory mediators such as IL-6 or IL-1 β [92,93], disruption of chemokine trafficking by altering the expression of IL-8 or RANTES, or even contribution to gut barrier disruption [91,94]. Overall, even with ART, HIV-CMV co-infections increase the risks of being exposed to chronic diseases such as cardiovascular or neurological affections [95].

b. HIV/Mtb co-infection

Tuberculosis, caused by *Mycobacterium tuberculosis* (Mtb), affects about 25% of the global population, and about 5-10% of people infected with Mtb develop symptoms

[96]. Tuberculosis was responsible for approximately 1.25 million deaths in 2023 according to the WHO. The currently available vaccine, Bacillus Calmette–Guérin (BCG), is poorly efficient in adults and does not completely prevent Mtb transmission. In PLWHIV, the risk of acquiring Tuberculosis is increased by 26 compared to people who do not live with HIV. In 2015, about 1.14 million people were estimated to live with both HIV and Mtb, with 400,000 related deaths, setting Mtb as the leading cause of mortality in PLWHIV [97]. Of note, receiving ART considerably reduces the risk of mortality in HIV/Mtb co-infections [98] but does not allow a full restoration of the immune compartment. While host-pathogen interactions are particularly complex in HIV/Mtb coinfections, considerable efforts are ongoing to understand the pathophysiology leading to an exacerbated chronic inflammation in co-infected patients.

c. HIV/HCV co-infection

Another frequent co-infection in PLWHIV is the hepatitis C virus, which is transmitted through the same routes as HIV (sexual contact, drug use or mother-to-child transmission during pregnancy or birth) [99]. Hepatitis C infection does not affect HIV disease progression in patients or their response to ART, however the co-infection leads to increased risks of liver-related deaths compared to PLWHIV without coinfection [100].

iv) Innate and adaptive immune cells are altered during chronic HIV infection

During acute HIV infection, patients exhibit high-magnitude CD8⁺ T cell responses specific to the virus [101]. While antiviral roles of CD8⁺ T cells against HIV are crucial and well-established, chronic untreated HIV disease progressively leads to alteration of CD8⁺ T cell differentiation, proliferation, as well as cytokine production and cytotoxicity, in contrast to CD8⁺ T cells from elite controllers [102]. These dysfunctions

are also associated with a loss of naïve CD8⁺ T cells and an exhausted phenotype of blood CD8⁺ T⁺ cells, characterized by the expression of immune-checkpoint inhibitor proteins such as PD-1, which expression was correlated positively with CD8⁺ T cell function impairment and elevated viral loads in patients [103]. Conversely, chronic HIV infection is also associated with higher frequencies of CD38⁺ HLA-DR⁺ CD8⁺ T cells compared to healthy individuals, a phenotype associated with an immune activation [104,105]. Although the mechanisms leading to T cell dysfunction are still under investigation, inflammation, Treg depletion and lymphoid fibrosis may be involved in the process [106]. Only partially restored CD8⁺ T cell phenotype and functions have been observed in patients who initiated ART [107,108]. Strikingly, elite controllers, who are chronic HIV patients maintaining a low/undetectable viral load without medication, present HIV-specific CD8⁺ T cells with more cytokine polyfunctionality and higher suppressive capacities towards HIV [109].

Because elite controllers elicit better T cell responses than non-controllers, many current strategies to eradicate HIV rely on the restoration of CD8⁺ T cell functions, however, these strategies have remained unsuccessful so far.

Besides CD8⁺ T cells, CD4⁺ T cells are also qualitatively and quantitatively affected during chronic HIV. In the absence of ART, CD4⁺ T cell counts remain low, and memory T cells are dysfunctional, with a lower expression of IL-2 and defects in proliferation [110,111] and higher expression of immune checkpoint inhibitors. The expression of PD-1 is indeed more elevated on HIV-specific CD4⁺ T cells during chronic HIV, with a significant reduction when ART was administered to the patients [112], and the expression of CTLA-4 on CD4⁺ T cells from PLWHIV correlated with disease progression [110]. Importantly, blood CD4⁺ T cells from PLWHIV also exhibit higher expression of CD38, similar to CD8⁺ T cells [113].

Additionally to T cells, B cells are also perturbed during chronic HIV infection, with higher immature B cell subsets in the blood of PLWHIV, and alterations in the memory

B cell compartment. The administration of ART only partially restored these defects in PLWHIV [114].

In parallel with adaptive cell alterations, innate immune cells have also been shown to be activated during chronic HIV, in particular monocytes and macrophages. Indeed, several inflammatory molecules found in the plasma of chronic PLWHIV are related to monocyte/macrophage activation, including IP-10 (CXCL10), MCP-1, sCD14 or sCD163 [115]. Nevertheless, the overall responsibility of innate immune cells in favoring or lowering local and systemic inflammation remains to be fully elucidated. Yet, understanding the role of innate cells in early and late inflammation during HIV infection may bring to light novel strategies to diminish inflammation and improve life patients' expectancy. Among innate immune actors, the neutrophil population has been particularly understudied in HIV, while their involvement in other inflammatory chronic diseases is now well established.

II. Neutrophils as key actors of inflammation, in HIV and other diseases

Neutrophils are the most abundant leukocytes in the human blood. For many years, these innate immune cells were considered short-lived cells with only functions to detect and, after a massive recruitment from blood to tissues, to eliminate pathogens. However, over the last 20 years, the research on neutrophils has highlighted many more roles of these cells in different inflammatory and infectious contexts. Recent investigations on the neutrophil life cycle, phenotype and subsets brought new insights into these innate cells, which are becoming of great interest in designing innovative therapeutic strategies. Even though neutrophils were shown to be involved in the defense against HIV, a lot remains unknown about their impact on early and chronic inflammation and how they interact with other immune cells in tissues.

i) Neutrophils, innate specialists of inflammation

Polymorphonuclear neutrophils (PMNs) represent the majority of leukocytes in human peripheral blood, counting for 50-70% of circulating immune cells. Emerging from the bone marrow to the blood as terminally differentiated mature cells, the neutrophil lifespan has reached approximately 5.6 days in the blood [116]. Neutrophils display many pathogen recognition receptors (PRRs). Additionally, they are equipped with ready-to-release proteins which are pre-formed and stored in granules, a characteristic that makes neutrophils particularly fast responders when exposed to pathogens. As they present potent capacities to respond to chemotactic signals from tissues, a high number of neutrophils can be recruited rapidly in tissues to detect and eliminate pathogens as soon as they penetrate the body.

Apart from the described antimicrobial functions of neutrophils, many other capacities of PMNs have been emerging over the last years, putting these cells at the heart of an immune balance, inducing beneficial effects or detrimental consequences depending

on the nature of the disease, the local microenvironment or the subtype of neutrophils. Recent studies have shown that neutrophils are involved in a large spectrum of processes, including regulating innate and adaptive responses, tissue repair, or even homeostasis maintenance.

1) From bone marrow to tissues

a. Granulopoiesis

Human neutrophils originate from hematopoietic and progenitor stem cells (HSPCs) in the bone marrow (BM), with about 10^{11} neutrophils produced daily in a healthy individual. Along differentiation and maturation processes, neutrophils undergo structural and functional changes such as the acquirement of granules, a process called granulopoiesis. Historically, neutrophil development was studied through microscopy and cell coloration, which allows the analysis of cellular shape, size, and nucleus morphology [117,118].

In the BM, the production process of mature neutrophils is divided into two phases: neutrophil lineage determination and committed granulopoiesis. The very first step consists of the differentiation of HSCs in CD34+ multipotent progenitors (MPs), induced by the homing of stem cells in vascular niches in the BM under the regulation of chemokines such as CXCL12 produced by endothelial cells [119]. CD34+ cells then evolve in many different immune cells, but the expression of PU.1 transcriptional factor commits them to common myeloid progenitors (CMPs). Further, PU.1 and C/EBP alpha transcription factors drive CMPs to become granulocyte and monocyte progenitors (GMPs). The stimulation of GMPs by IL-3 and IL-6 drives them to become proliferative neutrophil progenitors, starting with myeloblasts, the first step of committed granulopoiesis [120]. From myeloblasts, stages of granulocyte differentiation are myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and polymorphonuclear neutrophils (**Figure 4**). All of these differentiation steps highly rely on the evolution of expression and concentration of soluble growth factor granulocyte colony-stimulating factor (G-CSF), as well as transcription factors of the C/EBP family, GFI1, and PU.1.

Usually, the term “pre-neutrophils” gathers neutrophil proliferative subsets, while immature neutrophils refer to non-proliferative band neutrophils, and mature neutrophils correspond to the fully segmented nuclear neutrophils.

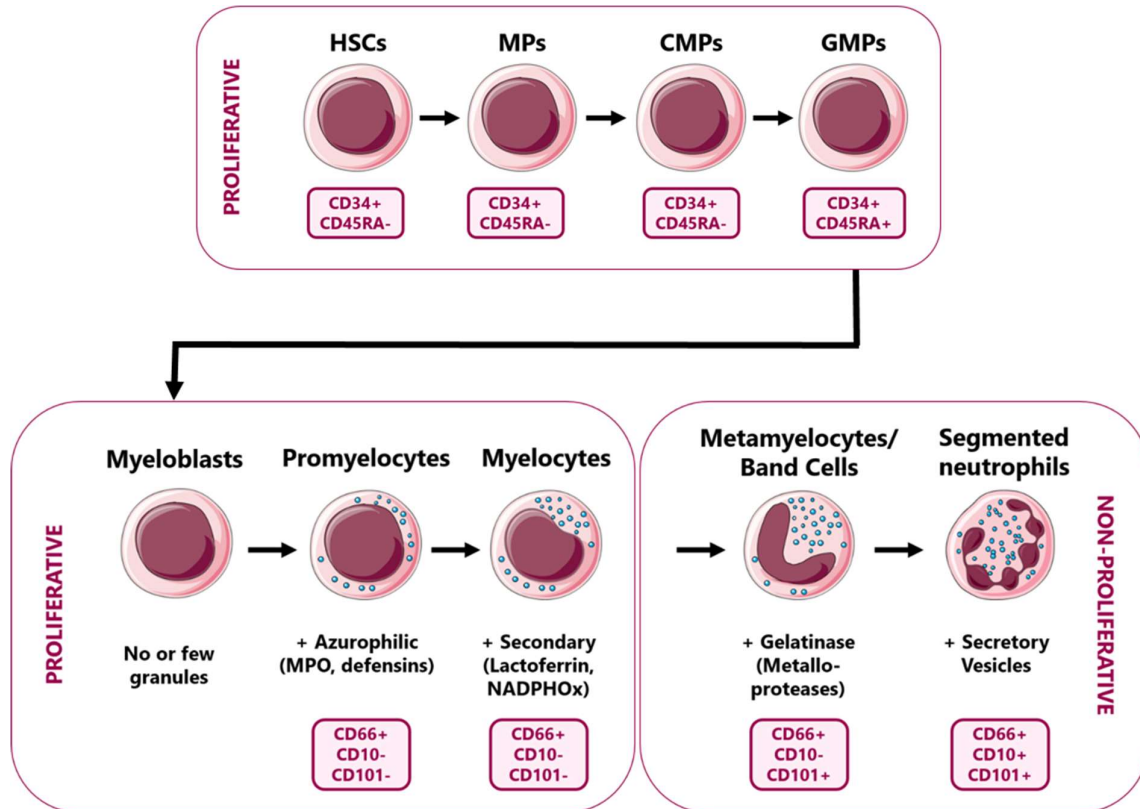


Figure 4: Main differentiation steps during human granulopoiesis, created with Powerpoint, adapted from Yvan-Charvet et al., Trends in Immunology, 2019 [120] and Amulic et al., Annual Reviews of Immunology, 2012 [121]

Neutrophil differentiation from hematopoietic stem cells occurs in the bone marrow. Each differentiation step depends on the expression of transcription factors and local gradients of cytokines or chemokines. Along this maturation process, neutrophils progressively acquire different types of granules and undergo phenotypic changes, ending in the generation of mature neutrophils with a segmented nucleus, then released in the blood.

Through granulopoiesis, neutrophils develop three types of cytoplasmic granules: primary or azurophilic granules are the first to be formed and are the largest (0.3µm diameter) [121]. They notably contain myeloperoxidase (MPO), a crucial enzyme for reactive oxygen species reaction (ROS) cascade, and central antimicrobial products like defensins. Secondary or specific granules contain lactoferrin and lysozyme, but also compounds of NADPH oxidase, which are involved in ROS production. Tertiary

granules contain metalloproteases, like gelatinase and matrix metalloproteinase 9 (MMP-9). Secretory vesicles are the last to be formed. These vesicles are also considered as neutrophil granules, although they do not come from the Golgi compartment but are formed through endocytosis at the last maturation stages [121].

Primary granules appear during the transition from myeloblasts to promyelocytes, which are characterized by a round nucleus. When promyelocytes evolve in myelocytes, cells progressively acquire specific granules. As they move to metamyelocytes or band neutrophils, the chromatin condenses, and the nucleus becomes more segmented. From this stage, neutrophils are non-proliferative and generate terminally differentiated polymorphonuclear neutrophils containing all three granule types plus secretory vesicles [121].

Extensive work has been done to characterize neutrophils phenotypically during their maturation steps, leading to the identification of markers differentiating pre-, immature and mature neutrophils. In humans, the markers CD10 and CD101, and in mice, cKit, CXCR4, Ly6G and CXCR2 are commonly used to separate the three subsets [117].

Upon acute or chronic stress, immature neutrophil frequencies are increased in patients' blood [122]. This early release of immature neutrophils from the BM upon stress, called emergency granulopoiesis, was observed in a large diversity of inflammatory contexts, such as bacterial infections or autoimmune diseases. A possible explanation is the massive recruitment of mature neutrophils from the circulation to the injured tissue, creating an imbalance in the maturation process in the BM. When emergency granulopoiesis occurs, neutrophils can emerge from other organs, such as the spleen, a process called extramedullary granulopoiesis [123]. Recent mice studies have stressed that emergency granulopoiesis is complex and tightly regulated to respond to threatening agents [122].

b. Neutrophil life cycle

During maturation, neutrophils are retained in the BM by CXCL12, a chemotactic ligand produced by perivascular cells and osteoblasts that binds to neutrophil membrane receptor CXCR4. As they enter the circulation, mature neutrophils lose the expression of CXCR4 [124].

In Mammalians, the immune system is sensitive to the circadian rhythm: light and dark periods alternation is necessary for good health, as shown by a higher exposition to diseases and infections when this rhythm is broken. Depending on the timing of pathogen exposure, different immune responses are induced: in mice, when pathogen exposure happens during their active phase, at night, total blood leukocyte counts and cytokine levels are reduced, inducing a less efficient immune response and higher mortality. Among regulators of circadian immunity, BMAL1 is one of the major transcription factors responsible for chemotactic signals and immune cell recruitment in mice and humans [124].

As a matter of fact, neutrophils are particularly sensitive to the circadian rhythm. Indeed, the expression of CXCL12, which regulates neutrophil homing or migration from the BM to the blood, is also submitted to daily oscillations. As such, neutrophil egress from BM to blood, and homing from blood to tissues depend on the time of the day or night. The number of neutrophils and their phenotype vary with the circadian rhythm: neutrophils sampled at night expressed lower CD62L and CXCR4 expression compared to "aged" neutrophils in mice [124].

Depletion or dysregulation of neutrophils also induce deleterious effects on other circadian-related parameters, underlining the role of these cells in the regulation of immune balance and homeostasis in general [125].

Although neutrophils have always been considered as short lived-cells with a reported lifespan of 4-18h, a study in 2010 challenged this idea by demonstrating a neutrophil lifespan of 5.4 days [116]. However, these results were questioned because BM

neutrophils may also have been stained in these experiments, increasing the resulting lifespan [126]. Recently, it was confirmed that neutrophil production in the bone marrow lasts approximately 6 days, and the neutrophil half-life in blood circulation is a few hours [127]. Importantly, neutrophil survival was shown to be increased in chronic inflammatory contexts, including HIV [128,129], which suggests that neutrophils may participate in maintaining inflammation and tissue damage.

Homeostatic mechanisms leading to neutrophil death are still under investigation. Nevertheless, macrophages have been shown to engulf apoptotic neutrophils through efferocytosis in tissues [130,131]. In some diseases, neutrophil efferocytosis induces macrophage polarization and promotes tissue healing [132,133]. On the other hand, dysfunctional efferocytosis of infected neutrophils can lead to bacterial dissemination [134,135]. In zebrafish and mice models, neutrophils were shown to migrate from tissues back to the blood circulation and end up in lung tissue or BM through reverse transendothelial migration. This process could contribute to the resolution of inflammation by decreasing the number of neutrophils in tissues. Yet, it may also be detrimental by spreading inflammation in other compartments in inflammatory contexts [136].

Overall, recent studies on neutrophil differentiation, emergence from niches, recruitment in tissues and cell death have brought considerable insights into how these cells behave during homeostasis, but also in inflammatory and pathogenic contexts.

c. Neutrophil heterogeneity

Recent investigations of neutrophils from different species have evidenced the phenotypic diversity and great plasticity of these cells. Methods allowing deep characterization of neutrophil phenotypes, such as mass cytometry or transcriptomic studies, identified many different neutrophil subsets. These neutrophil subsets display various phenotypical or functional properties, depending on the species, pathogenic

context, and even isolation method [137]. In this paragraph, we will evoke briefly current neutrophil subsets which are commonly described and investigated in diseases.

One of the most described neutrophil subsets is low-density neutrophils (LDN), which are obtained from the mononuclear fraction after a density separation from human blood, contrary to “normal density neutrophils” (NDN), which are found in the erythrocyte fraction. LDN are composed of mature and immature neutrophils and their low density has been attributed by some authors to a degranulated state. Increased frequencies of these cells have been reported in many diseases, including rheumatoid arthritis, lupus, cancer and sepsis [138].

Another subset of neutrophils widely identified are myeloid cells displaying immunosuppressive capacities, which have been identified and particularly studied in cancer and are called “myeloid-derived suppressor cells” (MDSC). Two MDSC populations have been identified: monocytic (Mo-MDSC) and granulocytic (G-MDSC) subsets [139]. Of note, G-MDSCs are not phenotypically or morphologically different from classically described human neutrophils. They are usually defined in humans as CD14⁻CD11b⁺CD15⁺HLA-DR⁻CD33^{dim} cells [139] and include a mix of mature and immature neutrophils. This subset is mostly described in oncology, but they were recently identified in other inflammatory contexts, such as HIV infection or sepsis [140].

Over the last decade, there has been tremendous investigation into the role of neutrophils in tumors and cancers. Neutrophils infiltrating solid tumors are called tumor-associated neutrophils (TAN). In many cancer studies, neutrophils have been shown to be detrimental to the host by facilitating tumor spread and T cell exhaustion, yet they can also present anti-tumor properties. In mice, two subsets have been described for TAN, similar to macrophages: N1 for anti-tumor and N2 for pro-tumor neutrophils [137].

Finally, a lot of work has also been done regarding the characterization of neutrophils “aging”, which corresponds to the evolution of neutrophil phenotypes and functions from their BM emergence to homing in tissues, as previously evoked.

Despite being widely used in neutrophil biology, the precise definition of these different neutrophil subsets remains quite controversial. In particular, some authors wrote critical papers about the identification of these neutrophil populations because some of them are not mutually exclusive, such as LDN and G-MDSCs for example, which can be confusing when reading articles from different fields. Hence, several authors called researchers to unify the definitions of neutrophil subsets [137].

To better define neutrophil subsets, one major question remains: are neutrophils diverse and specialized since their differentiation in the BM, or do they acquire capacities when recruited to tissues? Several teams have focused on defining neutrophil fates and subsets over the last few years, and this field of research is currently very active. Because mice experiments are more easily reproduced and flexible, most of the corresponding data come from mice neutrophil studies.

As such, using transcriptomics, Grieshaber-Bouyer et al explored mice neutrophil differentiation and subsets from bone marrow to tissues [141]. Using different dimensionality reduction methods, such as Uniform Manifold Approximation and Projection (UMAP) clustering and diffusion mapping, authors were able to reconstitute the chronology of gene expression in mice neutrophils. They could map neutrophil transcripts from early immature neutrophils in the BM to mature neutrophils in the blood and spleen (**Figure 5**). Then, they used the RNA velocity method to confirm that the mapped transcripts followed a continuous distribution and could reconstitute chronological events in neutrophil maturation. This led authors to propose a model based on a continuum between neutrophil subsets across time, called “neutrotime”. Remarkably, authors found a similar trajectory for human neutrophils using human RNA sequencing datasets from the Human Cell Atlas [141].

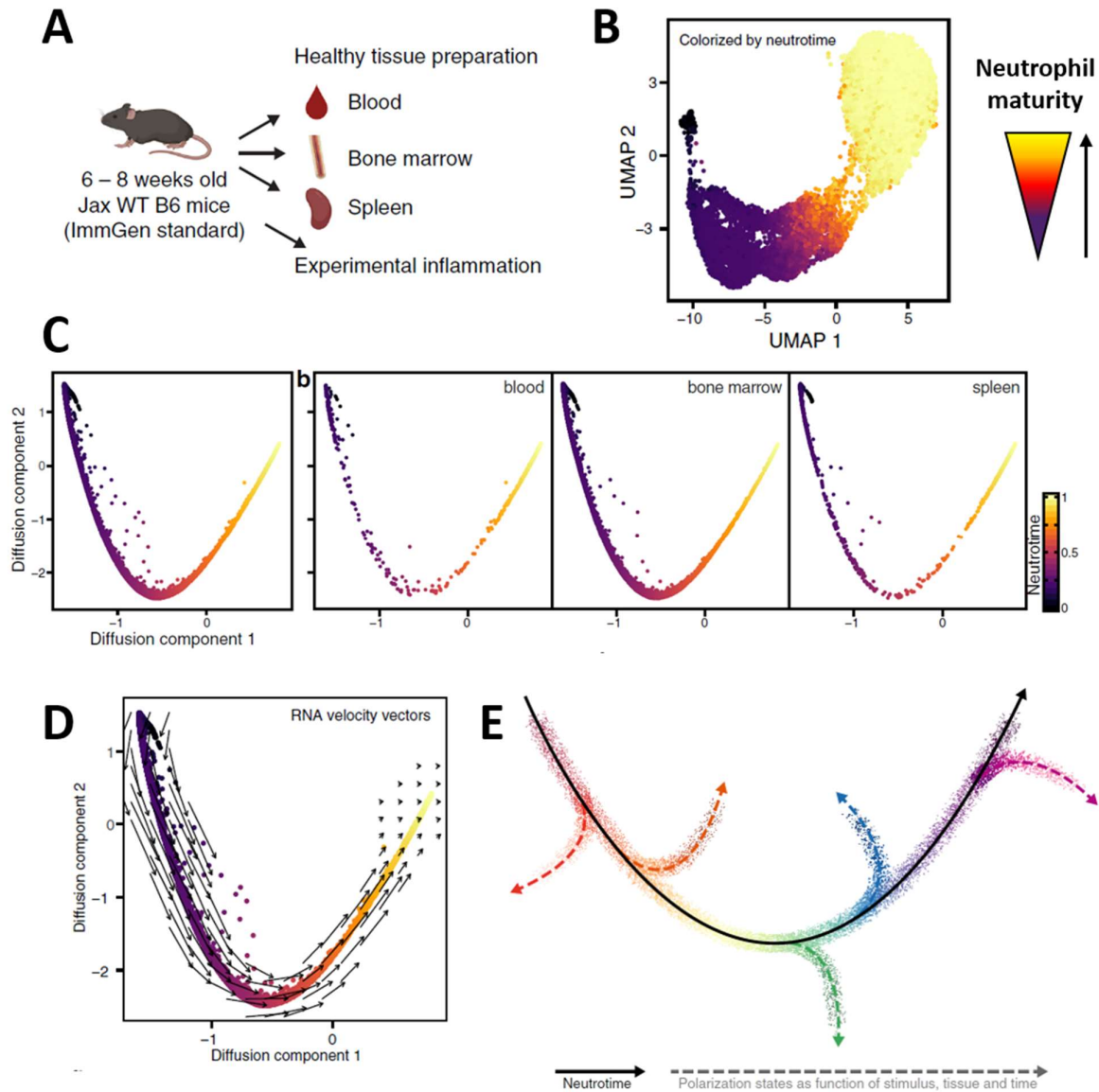


Figure 5: The differentiation and specialization of mice neutrophils from bone marrow to tissues rely on a continuum called « Neutrotime », adapted from Grieshaber-Bouyer et al., Nature Communications, 2021 [141]

(A) Experiments were run on neutrophil samples from mice blood, BM and spleen; (B) Concordance between two dimensional reductions methods for the analysis of neutrophil transcripts: diffusion mapping and Uniform Manifold Approximation and Projection (UMAP) clustering. On this graph, we can see the distribution of neutrophils depending on their maturity status; (C) Distribution of neutrophil transcripts using the diffusion mapping approach. On these graphs, we can see that neutrophils from blood displayed a more mature phenotype (yellow), while bone marrow neutrophils were more immature (purple); (D) Representation of time vectors for transcriptomic data from neutrophils using the RNA velocity method. This figure demonstrates that neutrophil gene expression relies on a continuum; (E) Final model proposed by authors: neutrophils are organized along neutrotime and can deviate from it across time and in case of inflammation

In this work, authors also investigated how exposing mice to inflammatory or infectious stimuli could affect neutrophil transcriptional activity, hence the distribution of neutrophils in the continuum. To observe how neutrophil gene expression was modified in a pathological context, mice were submitted to three different types of sterile inflammations induced by IL-1 β injections (inducing peritonitis, pneumonitis) or exposition to serum from arthritic mice (inducing arthritis). In these inflammatory contexts, neutrophil gene expressions were consistently modified compared to homeostasis and suggested recruitment of mature PMNs from circulation to inflamed tissues [141]. Interestingly, genes related to the expression of IL-1RA, CD14 and chemokines were upregulated in neutrophils during inflammation. Strikingly, the different types of inflammations induced heterogeneous gene expression in neutrophils from inflamed sites, which suggests that neutrophils not only are transcriptionally active but may also specialize in tissues during inflammation. Interestingly, similar results were found by another team in 2020 [142].

Conversely, one should note that despite the consistent results obtained through these experiments, authors found discrepancies between neutrophil gene expression and protein content, reinforcing the necessity for caution when extrapolating transcriptomic results to protein expression.

These results support a high plasticity of neutrophils, which undergo molecular and cellular changes in response to systemic and local stresses. These changes also depend on the nature and localization of the injury. It is now accepted that neutrophils constitute a heterogeneous immune population and that these cells can reprogram when entering tissues (**Figure 6**). A full comprehension of the neutrophil life cycle, responses to injuries, and systemic and tissue fates will be necessary in the future to elucidate the role of neutrophils in health and disease.

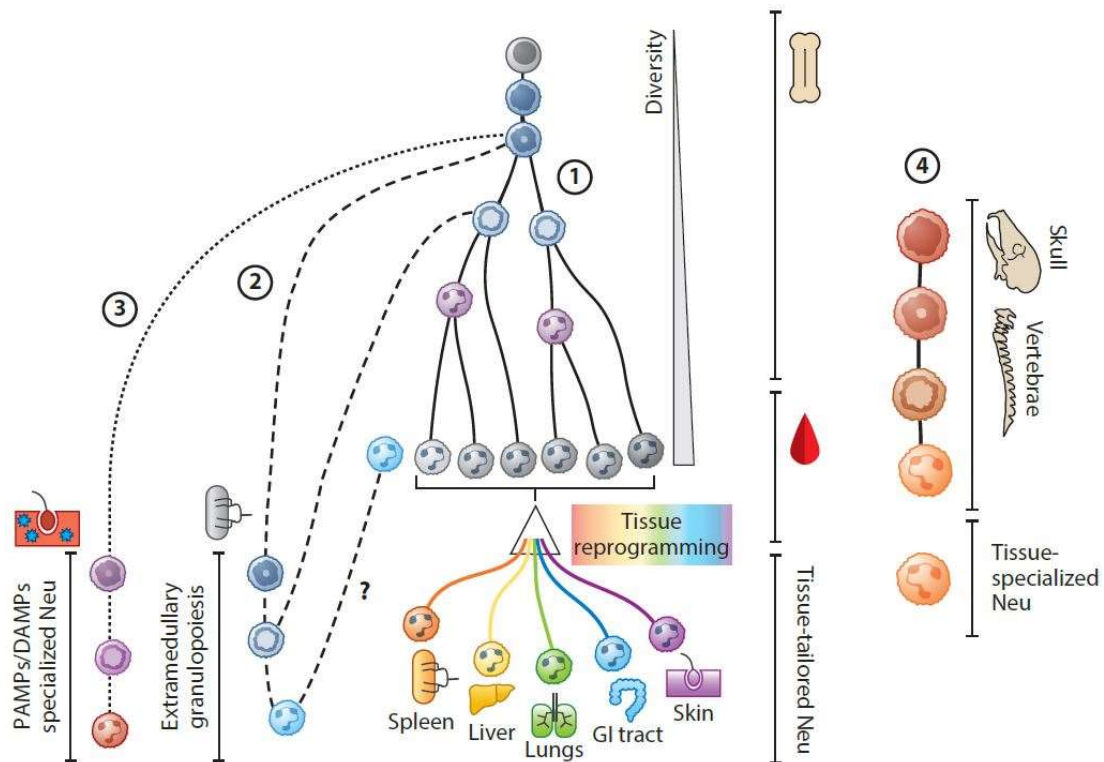


Figure 6: Diversity in neutrophils from bone marrow to tissue compartments, from Arca-Crevillén et al., Annual Review of Pathology: Mechanisms of Disease, 2024 [143]

(1) Neutrophils acquire diversity and become more heterogeneous during their development. The diversification of neutrophils increases as they infiltrate into tissues; (2) In inflammatory conditions, granulopoiesis may occur in other tissues than the BM, including the spleen (extramedullary granulopoiesis); (3) Neutrophils precursors could be recruited directly in tissues where they become mature in case of inflammatory or infectious stimuli; (4) Depending on the hematopoietic niche, neutrophil diversification differs

2) Neutrophils are equipped to detect danger signals, infiltrate into tissues and eliminate pathogens

Neutrophils play crucial roles during infections, especially in the first hours after pathogen exposure. In patients, depletion of neutrophils or neutropenia can lead to extended pathogen spreading and the development of comorbidities [144]. After pathogen exposure, neutrophils are rapidly mobilized at the site of infection, eliminating pathogens and/or infected cells using a broad spectrum of functions such as ROS production, enzymes and antimicrobial molecules release, or phagocytosis.

a. Rolling and recruitment

Over the last years, intravital imaging in mice has provided many insights into the mechanisms and chronology of neutrophils recruitment from the circulation to injured or inflamed sites [145,146]. In response to injury signals in tissues, local resident innate cells rapidly produce cytokines and chemoattracting signals, which induce surface alterations in endothelial cells, ending in the upregulation of P and E-selectins at their surface [147]. Circulating neutrophils express high levels of selectin receptors such as P-selectin glycoprotein ligand-1 (PSGL-1), which bind to endothelial cells' selectin receptors. This binding then induces neutrophil rolling at the surface of endothelium, in capillaries. As neutrophils undergo rolling in vessels, they progressively slow down and are more likely to receive chemoattractant signals expressed by endothelial cells. Further activation of neutrophils through CXCL8 or CXCL2 leads to switches in neutrophil integrin conformation and binding of neutrophil $\beta 2$ integrins LFA-1 (CD11b/CD18, $\alpha L\beta 2$) and Mac-1 (CD11b/CD18, $\alpha M\beta 2$) to endothelial ICAM-1 and ICAM-2 proteins. This process of neutrophil adhesion to endothelial cells also induces a reorganization in neutrophil morphology to initiate transmigration to tissues, called swarming, driven by a gradient of chemoattractants [147]. Neutrophils are usually exposed to a pro-inflammatory environment once they have passed the endothelial barrier. In tissues, neutrophils can respond to various chemoattractants, including cytokines or chemokines like IL-8 or IFN γ , but also complement like C5A, lipids like eicosanoids (LTB $_4$), and pathogen-derived peptides such as N-Formylmethionyl-leucyl-phenylalanine (fMLP). The binding of chemoattractant molecules to neutrophil receptors leads to neutrophil cytoskeleton rearrangement and triggers cell motility in tissues along the gradient concentration of chemoattractants. Neutrophils are also chemoattractant producers. Notably, they can produce high levels of IL-8, which activates the recruitment of other neutrophils from the circulation and is linked to inflammation.

b. Pathogen and danger detection

As innate host defenders, neutrophils are equipped with pathogen recognition receptors (PRRs), including toll-like-receptors (TLRs), nod-like receptors (NLRs), C-type lectin receptors (CLRs) and RNA sensors RIG-I-like receptors (RLRs) [148] (**Figure 7**). These receptors detect many different pathogen-derived molecules like bacterial products, viral particles, and fungi or parasites.

TLRs are constitutively expressed and recognize different natures of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs): for instance, lipopolysaccharide (LPS) binds to TLR4, while TLR9 detects pathogenic DNA. Neutrophil TLR ligation induce the activation of signaling pathways such as the MyD88 pathway, which leads to the transcription of pro-inflammatory genes and cell activation. Depending on the nature of the stimuli, TLR responses can elicit different neutrophil responses such as degranulation, NET formation or increased cell migration. Human neutrophils express all TLRs except for TLR3, and TLR7 expression remains controversial [148].

NLRs are cytoplasmic sensors of pathogen and danger signals. The role of NLR family pyrin domain-containing 3 (NLRP3) was particularly studied because this receptor is involved in the formation of inflammasomes [149,150]. Inflammasomes are cytosolic protein complexes which are formed upon activation induced by pathogens or danger signals. When formed, inflammasomes recruit caspase-1 and activate gasdermin D, which usually leads to pyroptosis, a lytic form of cell death. However, neutrophils were shown to be resistant to pyroptosis. Inflammasome complexes are extensively involved in the defense against pathogens, but their dysregulation can participate in inflammation, as shown in COVID-19 infection [151].

CLRs are primarily involved in the defense against fungi and their binding leads to neutrophil degranulation, phagocytosis and production of ROS.

RLRs are receptors detecting intracytoplasmic double-stranded RNA. These PRR play major roles against viral infections by triggering innate immune responses. RLRs include Retinoic Acid Inducible Gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). The activation of RLRs trigger the expression of antiviral genes, including type I interferons and pro-inflammatory cytokines [152].

Among the complement receptors (CRs), CR3 also called CD11b/CD18 or Mac-1 is particularly abundant on neutrophils engaged in chemotactic and phagocytic activities [153].

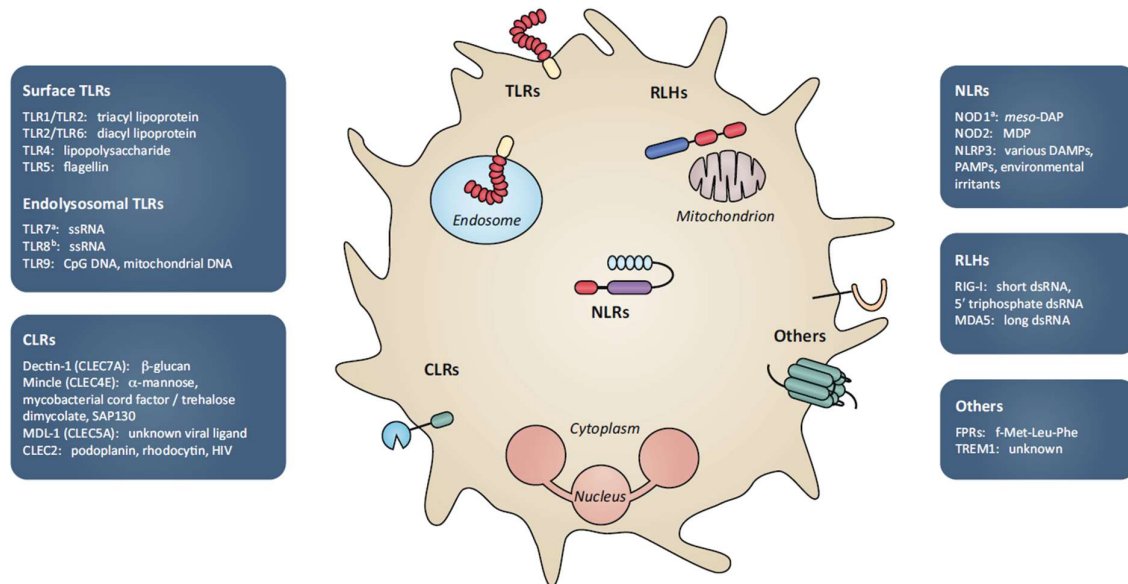


Figure 7: Pattern recognition receptors in neutrophils, from Thomas et al., Trends in Immunology, 2013

Neutrophils are equipped with various receptors to detect DAMPs and PAMPs. Some PRRs are located at the cell membrane, such as TLR4, while others are cytoplasmic, such as RIG-I. The diversity and abundance of these PRRs explain why neutrophils are particularly fast responders in tissues.

Neutrophils also express receptors for the constant region of IgG immunoglobulins (Fc γ Rs), which are primarily involved in recognizing opsonized pathogens or immune complexes. In humans, neutrophils express six types of Fc γ Rs: Fc γ RI/CD64, Fc γ RIIA/CD32a, Fc γ RIIB/CD32b, Fc γ RIIC/CD32c, Fc γ RIIIA/CD16a, and Fc γ RIIIB/CD16b [154]. The glycosylphosphatidylinositol (GPI)-anchored protein CD16b is the most

expressed FcγR on human mature neutrophils. Depending on the context of inflammation or infection, the intensity of expression of neutrophil FcγRs is modulated. For instance, the exposition of neutrophils to IFN γ induces upregulation of CD64, which leads to increased antibody-dependent cell-mediated cytotoxicity (ADCC) capacities in neutrophils or the release of ROS. Interestingly, CD64 was proposed as a biomarker for several inflammatory conditions, such as sepsis [154,155] and COVID-19 [156]. Another example is CD32a, whose affinity for IgG is higher in activated than resting neutrophils. Chronic activation of neutrophils by immune complexes can lead to auto-immune disorders through the unregulated release of inflammatory molecules such as IL-8 [157].

c. Antimicrobial mechanisms and tissue repair

- *Neutrophil degranulation*

Considering their number in circulation and their capacity to be recruited in high numbers at inflammatory sites, neutrophils are able to respond quickly to a number of threatening agents. Most of the neutrophil protein arsenal is pre-stored inside granules, contrary to adaptive cells, which usually synthesize many proteins for their activity after receiving activating or pro-inflammatory signals from other cells.

Neutrophil degranulation, leading to the release of antimicrobial molecules in the tissue microenvironment, is tightly regulated and depends on many parameters, such as the nature and strength of the stimuli. Usually, granule release is triggered by antigen detection through PRRs or FcγR binding: intracellular granules fuse with either the cell membrane or the phagosome and release their content in the microenvironment [121]. Usually, secretory vesicles, which contain molecules to facilitate neutrophil extravasation on their membrane, are the first to be mobilized, while azurophilic granules are the last. Degranulation mainly relies on the influx/efflux of intracellular calcium levels [121].

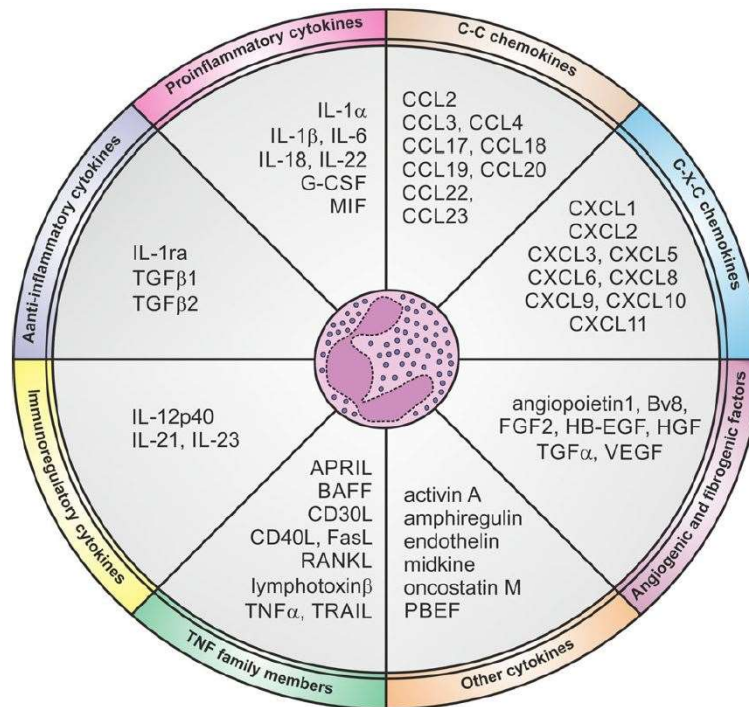


Figure 8: Cytokines and chemokines potentially produced by human neutrophils, from Tamassia et al., European Journal of Clinical Investigation, 2018 [158]

Neutrophils were shown to release many different cytokines and chemokines. Some of these proteins are involved in cell signaling (IL-1 β , TNF α , IL-6), highlighting the capacity of neutrophils to influence other immune and non-immune cell activities. While some of these molecules are involved in inflammation, such as IP-10 (CXCL10), others display anti-inflammatory properties, such as IL-1RA. These results highlight the plasticity of neutrophils.

When degranulating, neutrophils not only release antimicrobial proteins, but also various molecules for cell signaling, cytokines, chemoattractants and alarmins. Indeed, neutrophils have been shown to produce a large diversity of molecules, including anti-inflammatory (IL-1RA, TGF β 1 and TGF β 2) and pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-6, IL-8), but also immunoregulatory cytokines such as IL-12 or chemokines such as CXCL10 [143] (**Figure 8**). The expression of IL-10 by neutrophils is controversial [159].

- *Reactive oxygen species (ROS)*

One of the most potent neutrophil mechanisms to eliminate infectious agents is the release of ROS in the tissue microenvironment or inside intracellular phagosomes [143].

The production of ROS relies on the assembly of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a protein complex composed of several NOX enzymes. NOX2 is the main catalytic subunit of this complex and assembles in the plasma or

phagosomal membrane, then induces the precursor superoxide anion O_2^- by oxygen reduction [143]. This superoxide is then converted to oxygen metabolites, including hydrogen peroxide H_2O_2 , through dismutation. Secondary oxidants such as hydroxyl radicals, hypochlorous acids, chloramines and hypothiocyanites are generated through myeloperoxidase (MPO) activity: among oxygen species, HOCl presents a particularly strong antimicrobial effect [143]. NADPH complexes can form at the plasma membrane to induce extracellular ROS or at the phagosomal membrane during phagocytosis (see the paragraph dedicated to phagocytosis).

- *Neutrophil extracellular traps (NETs)*

Another primary function of neutrophils is their capacity to produce extracellular traps (NETs) in response to various stimuli, including PAMPs, inflammatory mediators, and synthetic molecules [160]. In that context, neutrophils can release DNA structures composed of decondensed chromatin fibers associated with citrullinated histones and cytoplasmic or degranulation proteins such as MPO or elastase. By trapping pathogens, NETs enhance their elimination and limit pathogens spreading in the micro-environment by concentrating them in the DNA structure and destroying virulence factors by releasing proteases. NETosis can be triggered by activation stimuli involving FcγR, TLRs, or other receptors such as advanced glycation end-products (RAGE) and PSLG-1 receptors. According to some studies, the formation of NETs could involve ROS by triggering the release of neutrophil elastase, which would degrade F-actin. This molecule partially cleaves DNA histones, leading to chromatin decondensation [161].

NETosis is mainly described in neutrophils, but the release of extracellular traps has also been shown in other innate immune cells, including eosinophils, basophils, or macrophages [162].

- *Phagocytosis*

During phagocytosis, pathogens are engulfed in cell-membrane-derived vacuoles called phagosomes, structures that fully mature through the fusion of neutrophil

granules to the phagosome. The recruitment of antimicrobial granule proteins such as MPO and NADPH oxidase components in the phagosome leads to the formation of ROS, which induces pathogen death [121] (**Figure 9**). Phagocytosis can be triggered by the direct interaction of pathogens/PRR with PAMPs; however, this mechanism is less efficient than binding opsonized particles to FcγR or complement receptors such as complement receptor 3 (CR3) [163].

- *Wound healing and tissue repair*

In parallel with these antimicrobial functions, neutrophils were recently shown to participate in tissue repair by releasing enzymes degrading the extracellular matrix locally and by producing of vascular endothelial growth factor (VEGF), two mechanisms which favor neoangiogenesis within the restored tissue [143].

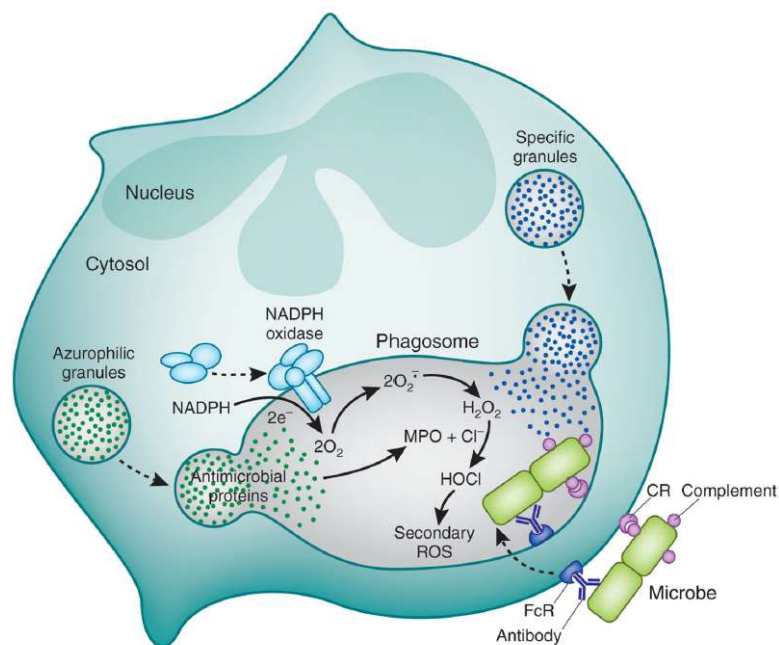


Figure 9: Neutrophil activation during phagocytosis, from Kobayashi et al., Immunological Reviews, 2023 [164]

Phagocytosis is triggered by the engulfment of opsonized pathogens in phagosomes, where neutrophils release ROS after NADPH oxidase assembly. Intracellular granules also fuse with the phagosomal membrane, adding antimicrobial proteins to eliminate pathogens.

ii) Role of neutrophils in inflammation: insights from non-HIV diseases

The antimicrobial functions of neutrophils are now well-established in many viral, bacterial, and fungal pathogenic contexts. However, as previously evoked, the dysregulation of neutrophil functions can lead to deleterious consequences for the host, such as ongoing inflammation and tissue damage. In this chapter, we aim to raise the detrimental role of neutrophils in a few diseases and illustrate the involvement of PMNs in different inflammatory contexts.

1) Neutrophils in lung diseases

Acute and chronic respiratory infections and diseases are frequent in adults and children and are responsible for a consequent number of deaths globally. Although neutrophils only represent 1% of lung immune cells at steady state, respiratory diseases can be associated with increased neutrophil infiltration. An increasing number of studies report that neutrophils infiltrating lungs could be detrimental to the host by favoring inflammation and inefficient pathogen clearance. Here, we present briefly the roles of neutrophils in several lung diseases.

- *Influenza viruses*

Influenza are acute viral respiratory infections that cause significant morbidity and mortality globally and still represent an important threat to human and animal health in 2025 [165]. If a therapy is provided rapidly after the onset of symptoms, patients usually do not undergo severe complications. However, there are currently no effective treatments for severe influenza. In 2019, Tang et al. analyzed hosts factors associated with severe influenza. They found that severely infected patients presented increased NET formation, delayed apoptosis and neutrophil-related inflammatory pathways [166]. Yet, neutrophils also participate in viral clearance, as experiments with neutrophil depletion induced a severe form of the disease in a mice model of influenza [167].

These results highlight that neutrophil responses need to be balanced and can lead to severe complications if their activity is not tightly regulated.

- *COVID-19*

Recently, the role of neutrophils has been particularly investigated in the respiratory tract disease due to COVID-19 infection. Patients severely affected by this disease develop respiratory distress and organ failure, which can lead to death. Interestingly, proteomics analysis from the blood of patients who developed a severe disease presented neutrophil-related immune signatures with increased expression of G-CSF and IL-8 [168]. These signatures were predictive of the mortality of patients. In another study, COVID-19 infection induced transcriptomic signatures of neutrophil degranulation and interferon-related responses in the lungs of infected RMs [169]. Similar to COPD, the formation of NETs was increased in the blood and lungs of critically ill patients, increasing thrombosis and ischemic stroke risks [170,171]. The neutrophil-to-lymphocyte ratio was also established as a predictive marker for COVID-19 patients [172].

- *Mycobacterium tuberculosis*

Tuberculosis, an infection due to *Mycobacterium Tuberculosis* (Mtb) represented the 6th cause of death in lower-middle-income countries in 2021. Challenges to cure and eliminate this infection are increasing bacterial resistance to antibiotics and the low efficacy of current vaccines. The roles of neutrophils in Mtb infection are still under investigation. However, there is accumulating evidence that neutrophils play dual roles during Tuberculosis, which may depend on the stage and the severity of infection. In the acute phase, neutrophils show a critical role in Mtb clearance and display antibacterial activities, including phagocytosis of infected macrophages, and limit bacterial dissemination [173]. Nonetheless, during latent TB, neutrophil infiltration in granulomas was associated with local inflammation due to neutrophil oxidative burst, necrosis and NETosis in lungs [174].

- *Chronic obstructive pulmonary disease, an example of non-infectious lung disease*

According to the WHO, Chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death worldwide. This respiratory disease is characterized by restricted airflow and breathing issues with multifactorial causes, including tobacco consumption. The involvement of neutrophils in this disease has been demonstrated in patients and mice models. Indeed, in broncho-alveolar lavages from patients, neutrophil counts have been shown to be more elevated, in association with elevated concentrations of IL-8 and TNF α , which might be produced by local macrophages and epithelial cells [175,176]. Neutrophil elastase, one of the major proteinases of neutrophils with a role in bacterial clearance, was shown to participate in local inflammatory processes during COPD [177]. This protein was shown to contribute to airway mucus obstruction, IL-8 upregulation, and NET formation, which also participate in inflammation and tissue damage [178]. Patients suffering from COPD are often exposed to bacterial infection, which also supports inflammation in the lung [179].

2) Neutrophils in inflammatory bowel diseases

Inflammatory gut diseases are characterized by chronic inflammation in the gastrointestinal tract (GIT), with various symptoms such as weight loss and abdominal pain. The two major forms are ulcerative colitis (UC) and Crohn's disease (CD), which are distinct by their clinical and histological aspects [180]. Causes of these diseases are multiple and include genetic, environmental, microbiotic, and immune factors. In both diseases, disruption of the gut tolerance and signals of tissue damage may be responsible for maintaining a high level of inflammation, and both innate and adaptive compartments are dysregulated.

In UC, infiltration of neutrophils in the GIT was correlated with disease severity [181], and the dosage of fecal calprotectin is frequently used to evaluate inflammation in patients [182]. A recent proteomics analysis also showed elevated neutrophil proteins

and proteins related to NETs in biopsies from patients [183]. The recruitment and activation of neutrophils from the circulation were maintained by IL-8 production from endothelial cells, and elevated NETs and ROS productions were reported in neutrophils from patients [184]. The release of NETs in UC has been particularly studied, and NETosis is one of the mechanisms described as participating in gut damage in UC [184]. On the other hand, CD results from an inefficient immune response after exposition to an infectious pathogen. Among other factors, there is a lack of recruitment of neutrophils to the gut during the acute phase of the disease which may result from a defect in the secretion of pro-inflammatory molecules from the gut. Additionally, neutrophil functions seem to be impaired, with reduced phagocytic capacities that lead to immune tolerance against gut bacteria [185]. In summary, if neutrophils are involved in both diseases, the underlying mechanisms of how they fuel inflammation in gut tissues seem to differ. Although the role of neutrophils in these diseases is not fully elucidated, there is accumulating evidence showing neutrophil participation in chronic inflammation in patients.

3) Neutrophils in auto-immune diseases

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by systemic inflammation associated with antibodies against the rheumatoid factor and citrullinated peptides. Synovial joints are particularly affected, leading to a progressive erosion of the bones and cartilage. Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by an aberrant activation of the immune system and the generation of auto-antibodies against nucleic acids [186].

In both of these diseases, neutrophils exhibit an aberrant activation, with delayed apoptosis [186], increased ROS production [187,188], and neutrophil degranulation [189]. In RA, the binding of neutrophils on immune complexes from the joints leads to increased ROS production and induces local damage; a similar process was also described in SLE, with immune complexes binding to the blood vessels [186]. By

inducing auto-antibodies against NETs and increasing local damage, NETosis was also identified as participating in inflammation. In RA, the presence of citrullinated proteins on NETs is also responsible for autoantibody generation [190], and NET debris can be found in synovial fluids and serum from patients [186]. Interestingly, transcriptomic analysis revealed neutrophils presented strong signatures related to IFN α responses in both RA and SLE [186]. Notably, several strategic therapies lead to a reduction in neutrophil activation and inflammation in RA and SLE. For instance, Jak inhibitors led to reduced neutrophil migration, while anti-TNF biological agents decreased TNF α expression in neutrophils and NF- κ B activation in patients living with RA [191]. In SLE, a combination of Rituximab and Belimumab lead to decreased NET production and auto-antibody titers [192].

Overall, the impact of neutrophils in inflammatory diseases relies on a balance between protection and damage to the host, and the consequences highly depend on the nature of the disease and host-related parameters, including their age, gender and co-morbidities. Neutrophils constitute a heterogeneous population, which can be difficult to isolate and analyze in tissues from patients in pathogenic contexts. Some neutrophil mechanisms identified as deleterious are common in several of the diseases we referenced, such as the production of NETs. Understanding neutrophil dynamics during diseases will help develop relevant therapies for the future, but for that, we will also need to elucidate how neutrophils impact other immune cells.

III. Neutrophils modulate adaptive cell responses in acute & chronic stress situations (in non-HIV diseases)

Over the last two decades, there has been accumulating evidence showing that neutrophils can modulate T cells in contexts of infection, cancer or chronic inflammation. Depending on the context, neutrophils have been shown to either activate or suppress T cell activities through different mechanisms. The interactions between neutrophils and T cells can enhance T cell responses and favor pathogen clearance. Still, on the other hand, neutrophil-T cell interactions can also contribute to unresolved inflammation for the host. This chapter will focus on neutrophil subsets associated with T cell modulation, the mechanisms involved, and the consequences of neutrophil-T cell interactions for the host.

i) Neutrophils participate in T cell recruitment and polarization through direct or indirect interactions

1) Recruitment of T cells by neutrophils at the inflammatory sites with chemotactic molecules

As mentioned in this introduction, neutrophils can express a broad spectrum of pro and anti-inflammatory molecules in some infectious or inflammatory contexts. These proteins released by neutrophils can affect the microenvironment and constitute signals for proximal cells.

For instance, some proteins released by activated neutrophils present chemoattractant properties, such as α -defensins, Li-37 and azurocidin [183,184]. Indeed, defensins have been shown to attract human naïve T cells and dendritic cells in ex vivo assays [195]. Importantly, exposure to IFN α combined with TNF α , LPS, or IL-1 β induced a high IP-10 production from neutrophils, a chemoattractant of T lymphocytes [196]. Additionally,

after exposure to IP-10 released by neutrophils, T cell migration was abrogated in the presence of anti-IP-10 antibodies.

In a mice model of influenza infection, the depletion of neutrophils induced a reduction of CD8+ T cell recruitment to the trachea [197]. By studying cytokines expressed by murine neutrophils, authors showed that CXCL12 induced T cell chemoattracting effects. Further investigation showed that when moving, neutrophils leave chemoattracting-trails, which accumulate in tissues, increase CD8+ T cell recruitment, and favor bacterial clearance.

2) Neutrophils can induce polarization and proliferation of T cells during infections

Over the last two decades, evidence has accumulated for neutrophil capacity to polarize T cells depending on the immune context. In a mice model of Legionella pneumonia, neutrophil depletion led to loss of Th1-responses characterized by IFN γ and IL-12 signatures in the lungs of infected mice, and strongly increased mice susceptibility to this bacterial infection [198]. On the other hand, neutrophils were suggested to drive early Th2 polarization in a mice model of Leishmania major infection, a response associated with higher susceptibility to this infection [199]. The crosstalk between neutrophils and Th17 cells has been mainly explored in humans and mice. In 2010, Cassatella et al showed that adding culture supernatants of human activated neutrophils using LPS and IFN γ induced chemotaxis of Th17 clones. These chemoattracting effects were blocked with anti-MIP-3 α and anti-MCP-1 antibodies. In parallel, activated neutrophils also promoted direct chemotaxis of Th1 clones through IP-10 and MCP-1, while Th2 cells were not affected [200]. More recently, neutrophil peptide cathelicidin was shown to enhance Th17 differentiation in human and mouse CD4+ T cell cultures. In the presence of cathelicidin, frequencies of IL-17A+IL-17F+ CD4+ T cells and CD4+ T cell survival were increased [201]. The neutrophil-Th17 crosstalk was also investigated in auto-immune diseases [202], and further studies are

required to understand how this interaction could affect disease outcomes. Of note, induction of DC maturation by neutrophils has also been described, participating indirectly in T cell activation [203].

Lastly, the release of NETs was also shown to induce T cell priming in ex vivo assays from healthy human cells. Indeed, CD4⁺ and CD8⁺ T cells exposed to NETs expressed higher levels of CD25 and CD69, an effect absent in the presence of NETosis inhibitor diphenyleneiodonium chloride (DPI). Additionally, authors showed that NETs reduced the T cell activation threshold [204].

3) Neutrophils: involved in antigen presentation?

It is widely known that DCs and macrophages are professional antigen-presenting cells (APCs). APCs are characterized by the expression of MHC II and co-stimulatory proteins such as CD80/CD86, as well as the capacity to process and present antigen peptides to T cells in lymphoid organs such as lymph nodes. Although neutrophils do not present such features at steady state, several teams have recently demonstrated that neutrophils can present an APC-like phenotype in various pathological contexts. These results mostly rely on mice models, yet there is growing evidence for a similar phenotype in human neutrophils [205]. Indeed, human neutrophils are able to express MHC II molecules following IFN γ stimulation [206] and also present intracellular stores of costimulatory molecules CD80/CD86, which can be brought to the membrane after Mac-1 binding [207,208]. In chronic inflammatory contexts such as rheumatoid arthritis, neutrophils from patients have been shown to express HLA-DR, which may be induced by IFN γ exposition produced by local T cells [209]. MHC-II^{pos} and MHC-II^{neg} neutrophil phenotypes in bovines have been identified in the context of mastitis, a bacterial infection, but also at steady state. Interestingly, those two phenotypes were also characterized by different ROS production and phagocytic properties [210,211]. In resting animals, MHC-II^{pos} neutrophils represented less than 5% of total neutrophils in mice BM and cattle blood, while this phenotype represented 40% and 60% of

neutrophils in mouse and bovine lymph nodes, respectively. Similarly, 10-20% of human neutrophils in lymph nodes expressed HLA-DR in a study published in 2019 [212]. These data suggest neutrophils may present antigens to T cells in these lymphoid organs.

The expression of HLA-DR and co-stimulatory molecules on human neutrophils is higher in the presence of memory T cells compared to naïve T cells, which may be due to increased IFN γ production by memory cells [213]. The capacity of neutrophils to integrate and process antigens with proteases in lysosomes also makes them interesting cells to present antigens at their surface; however, this presentation is much shorter in time than in macrophages [205]. Moreover, to this day, there is no clear evidence for prolonged cell-cell contacts supporting the hypothesis of neutrophils presenting antigens to T cells. Besides, cross-contaminations of neutrophil fractions by professional APCs can bias studies investigating ex-vivo antigen presentation by neutrophils. Lastly, neutrophils could favor antigen presentation by activating DCs. In a study from Van Gisbergen et al, the binding of human neutrophil Mac-1 and DC-SIGN on dendritic cells led to the maturation of DCs through TNF α release, which induced Th1 responses [214].

Overall, although neutrophils do present APC features in some contexts, it is unlikely that they participate in antigen presentation to the same extent as macrophages and DCs. On the other hand, in inflammatory contexts where neutrophil survival is increased in tissues, and considering their capacity to express HLA-DR and costimulatory molecules, it is possible that neutrophils could be involved in presenting antigens to antigen-specific memory T cells in lymphoid organs [205].

ii) Suppression of T cell responses by neutrophils and induction of immune tolerance

There is emerging evidence that neutrophils are also capable of suppressing T cells. One of the major described mechanisms for T cell suppression by neutrophils is the production of arginase-1, which induces L-arginine depletion, suppressing T cell proliferation and cytokine synthesis in human samples [215]. The neutrophil production of arginase-1 has been widely described in cancer micro-environments since the early 2000s [216], and arginase is now considered by many teams as a therapeutic target for cancer therapy [217,218]. Using an arginase-1 inhibitor, Gross et al showed significant reductions in tumor growths in several mice models of tumors. This reduction was associated with better infiltration of T cells in tumors, higher levels of inflammatory cytokines and increased interferon-inducible genes [219].

Immune checkpoint proteins take part in the regulation of immune responses and are involved in the crosstalk between T cells and other immune cells or cancer cells. These immune checkpoint proteins have been at the center of many studies and clinical trials because their inhibition can lead to increased T cell responses, especially in cancers. Among these proteins, we can cite PD-1, CTLA-4 or TIM-3.

In some contexts, neutrophils have been shown to express ligands to immune checkpoints proteins, including PD-L1 that binds to PD-1 on T cells, and CD80 that binds to CTLA-4 on T cells. The expression of PD-L1 on neutrophils has been shown in HIV patients (see Introduction, part. IV), but also in contexts of cancer, sepsis and bacterial diseases such as Tuberculosis. Furthermore, the expression of PD-L1 on neutrophils was associated with capacities to suppress T cell activation and proliferation in these contexts [220–222].

Additionally, some authors show neutrophils can release the suppressive cytokine IL-10, leading to the suppression of T cell immune responses [223]. However, IL-10

production from neutrophils has been a subject of controversies that is not fully elucidated [223].

Lastly, it is worthy to note that in other contexts such as pregnancy, the suppressive role of neutrophils may be beneficial to the host. For instance, neutrophils could participate in regulating T cells and favor immune tolerance during pregnancy. The impairment of this mechanism could lead to inflammatory conditions such as pre-eclampsia [224].

An overview of the mechanisms involved in T cell modulation by neutrophils described above is presented in **Figure 10**. This overview indicates that neutrophils can take part in the immune balance in many pathogenic or stress contexts. Indeed, on the one hand, neutrophils can participate in increasing immune responses, either leading to pathogen clearance or favoring detrimental inflammation. On the other hand, can suppress T cell functions, which can lead to inefficient antimicrobial responses, or, on the contrary, induce immune tolerance that is beneficial in contexts such as pregnancy.

Overall, the impacts of neutrophils on T cell modulation are highly dependent on the context. A recent article also underlines how the level of activation of both T cells and neutrophils can impact their interactions [225].

How neutrophil-T cell interactions impact infections and chronic inflammation is not fully understood. Yet, deciphering the mechanisms underlying neutrophil-T cell interactions in blood and tissues could lead to the development of new therapeutic strategies in a board diversity of pathologies. For instance, considering the inflammatory properties of neutrophils, investigating the role of these cells in HIV infection could provide new insights into reducing chronic inflammation in patients.

ACTIVATION OF T CELLS

SUPPRESSION OF T CELLS

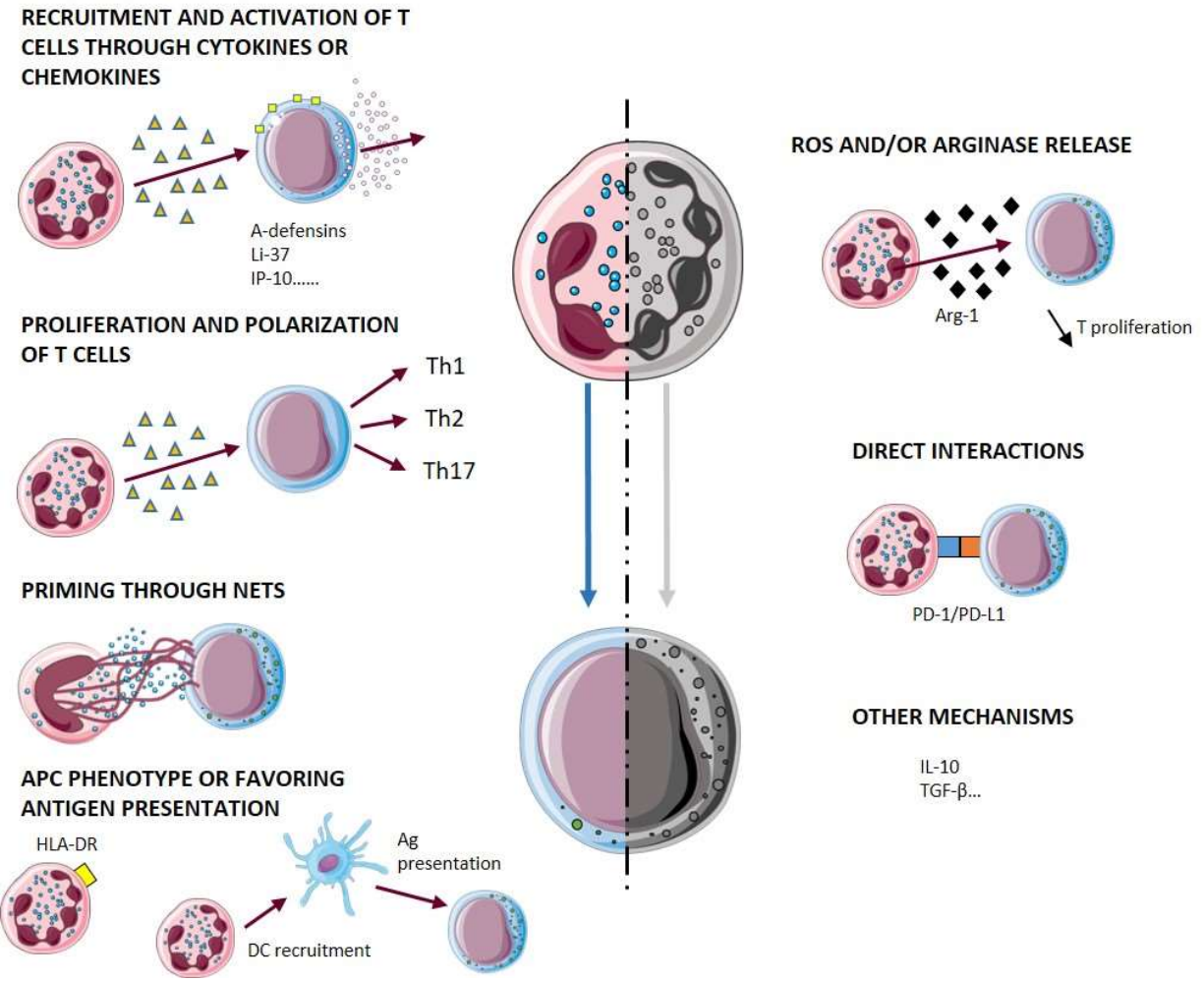


Figure 10: Mechanisms involved in T cell modulation induced by neutrophils, adapted with Powerpoint from Moffat et al., Blood, 2024 [205]

Neutrophils can modulate T cell phenotype, activation and functions through different mechanisms that require cell-cell contacts or soluble molecules. Depending on the immune context, neutrophils can increase or suppress T cell activities, which could influence pathogen clearance and/or chronic inflammation

IV. Neutrophils show dual roles in HIV infection

In the history of HIV research, neutrophils have been neglected for a long time, as they were considered poorly involved in the development or evolution of this disease. Nonetheless, over the last 20 years, many proofs emerged and unveiled how neutrophils are implied in eliminating HIV in tissues and more importantly, how this cell population can impact viral spread and global immune responses. The inflammatory nature of neutrophils and their capacity to modulate their microenvironment place them at the heart of a complex balance between the containment of the disease and the development of unrelated-to-AIDS comorbidities.

i) Neutrophils in HIV acquisition or protection

The involvement of neutrophils in favoring or reducing HIV acquisition has mostly been studied through the investigation of the composition of the female genital tract (FGT) in clinical studies. In this tissue, neutrophils usually represent 10 to 20% of total immune cells and present a different phenotype from peripheral neutrophils, including increased capacities to produce NETs and different levels of expression for the usual markers [226–228]. By creating a microenvironment that favors HIV replication, inflammation in the FGT has been shown to increase the risk of HIV acquisition. Indeed, enrichment in cytokines such as MIP-1 α , MIP-1 β , and IP-10 in cervicovaginal lavages have been correlated with a higher risk of seroconversion in women [229]. However, the role of neutrophils in exacerbating or limiting genital inflammation during HIV infection has been controversial. As a matter of fact, some authors showed a protective role of mucosal PMNs against HIV through the release of neutrophil extracellular traps. In one study, neutrophils from the FGT produced NETs within a few minutes after exposure to HIV viral particles (VLP), and resulting NETs were able to induce HIV-VLP entrapment and inactivation [227]. These results underline the role of neutrophils as effector cells in the FTG. Interestingly, neutropenia has also been associated with higher risks of HIV acquisition in a South African clinical study [230].

On the other hand, inflammatory neutrophil-related markers such as LL-37, IL-8 and α -defensins were found to be linked to a higher risk of acquiring HIV [231,232]. This may result from neutrophil-related inflammation that enhances T cell recruitment locally.

ii) Neutrophils exert antiviral responses against HIV

Even though neutrophils were largely studied and described for their role during bacterial infections, these innate cells also have been shown to develop antiviral responses. However, only a few papers focused on neutrophil capacities to block or eliminate HIV or HIV-infected cells, especially during the acute phase of HIV infection, considering the difficulty of having access to samples at that time. As a consequence, many results measuring neutrophils' antiviral capacities against HIV focus on ex vivo neutrophils from healthy donors incubated with HIV particles or HIV-infected cells.

One of the first works showed in vitro neutrophils could inactivate or block HIV-1 entry in target cells through the release of α -defensins [233,234]. While this capacity was first attributed to CD8+ T cells by mistake, a paper was retracted two years later as authors found α -defensin production was due to neutrophils [234]. Another team showed neutrophils stimulated with Phorbol 12-myristate 13-acetate (PMA) from healthy donors could block HIV-1 replication in a T cell line through MPO and ROS release [235].

NETosis was also described as a mechanism for blood neutrophils to capture HIV-1 after direct recognition through TLR7/8, but this process was found to be countered after HIV-1 triggered IL-10 production by DCs [236]. In another study, neutrophils exposed to HIV-1 infected macrophages were able to inhibit the viral replication by releasing NETs, and the effect was revoked in the presence of DNase I [237]. Additionally, the presence of NETs elicited the production of RANTES and MIP-1 β from primary macrophages, which show potent anti-HIV-1 effects [237,238]. Some studies also report neutrophil from tissues exert antiviral activities against HIV. As mentioned

previously, genital neutrophils are capable of capturing and inactivating HIV particles through NET production. In pig-tailed macaques infected with SIV, Sivanandham et al visualized and quantified NETs isolated from unstimulated and PMA-stimulated neutrophils: in this work, blood neutrophils isolated during acute and chronic phases of SIV infection produced significantly more NETs compared to before infection. Plasmatic NETs were also increased in SIV-infected macaques, and both plasmatic NETs and NET production were only partially reduced in ART-treated animals [239]. Strikingly, histological analysis of SIV-infected animals revealed the presence of NETs in various tissues such as gut crypt abscesses, lungs, liver, and in some blood vessels. This paper is particularly noteworthy as there are only a few works on NETs from PLWHIV or SIV-infected NHPs, especially focusing on tissues.

More recently, Reif et al developed a culture of healthy donors tonsil lymphoid cells infected with an HIV variant and measured viral titers by evaluating the reverse transcriptase activity. When neutrophils were added to the culture, they found viral titers were declining progressively, and CD4+ T cell depletion was abrogated. This inhibitory effect of HIV-1 by neutrophils depended on cell-cell contact, but interestingly, neither NETosis, ROS, nor TLR7/8 blockade could explain the observed effect [240].

Other mechanisms participating in limiting HIV spreading are Antibody-Dependant Cellular Cytotoxicity (ADCC) and Antibody-Dependant Cellular Phagocytosis (ADCP). Both of these functions rely on the binding of the constant part of specific monoclonal antibodies (Fc), to receptors (FcγR) expressed by innate immune cells, including neutrophils. Several studies on HIV vaccines in NHPs showed that FcγR-mediated responses could increase the level of protection against the virus [241,242]. Although neutrophils were less effective than macrophages and natural killer cells to eliminate HIV-infected cells [236], they responded faster [243].

iii) Neutrophils and their roles in chronic inflammation during HIV infection

1) Neutrophil counts, phenotypes and functions are altered during HIV infection

- *Neutrophil counts*

Many studies report neutropenia in PLWHIV, characterized by a prolonged decrease in neutrophil blood counts. In clinical studies, low neutrophil counts were associated with disease progression with lower CD4+ T cell counts and higher viral loads [244]. In this study, ART could restore PMN numbers. Low neutrophil counts have been associated with higher risks of contracting bacterial infections, especially during HIV infection [245].

Several factors can explain neutropenia. One of the hypotheses relies on a direct cytotoxic effect on neutrophils caused by HIV, yet no evidence has been raised for this phenomenon. In fact, the virus was shown to affect progenitor cells in the BM, but not differentiated mature neutrophils. By studying the presence of HIV gag protein in sorted CD34+ from patients, Christoph C Carter and al. found that after 3 days of HIV (without its envelope) exposure, 6% of hematopoietic stem cells were infected, and these cells were more susceptible to cell death compared to uninfected cells. In 3/7 patients living with the virus, gag proteins were detected in CD34+ cells, and all of the 7 patients' HPCs produced gag protein after stimulation. Of note, HIV proteins were also present in patients treated with ART for more than 6 months [246].

Anti-neutrophil antibodies detected in the blood associated with neutropenia in patients also support higher neutrophil-induced death [247,248].

Finally, several studies showed neutrophils from PLWHIV to be more apoptotic than healthy humans and NHPs [6-9]. Indeed, a decreased number of neutrophils associated with a higher expression of annexin V was observed during the acute phase of SIV infection in RMs [249]. The same observation was done in chronically infected

macaques and correlated with the evolution of the disease [250]. Genes related to neutrophil intrinsic apoptotic pathways were also more elevated in PLWHIV, and ART administration did not restore the expression of these genes at baseline levels [251]. Ex vivo measurement of neutrophil viability from PLWHIV supported these results, and GM-CSF partially reduced neutrophil apoptosis [252].

- *Neutrophil phenotypes*

During HIV infection, neutrophils present a modified phenotype compared to healthy individuals. In 2020, Lemaitre et al found blood neutrophils from chronically SIVmac251-infected CyMs were more heterogeneous and more immature than uninfected macaques. During chronic SIV infection, blood neutrophils showed higher expression of CD11b and decreased expression of CD62L, a phenotype associated with activation that was partially resolved by ART [253].

Blood neutrophils from PLWHIV also display an activated phenotype, even in patients receiving ART. Elbim et al demonstrated that rested neutrophils from PLWHIV showed decreased CD62L expression during the early phase of infection, as well as higher expression of CD11b/CD18 at all stages of HIV infection, independently of the treatment [254]. These results were confirmed more recently with a lower neutrophil expression of CD62L and CD16b in PLWHIV receiving ART compared to healthy donors [255]. Strikingly, levels of neutrophil activation were more elevated in HIV+ patients suffering from chronic diseases related to inflammation [255]. In another study, the expression level of the FcγR CD64 in neutrophils was also shown to be more elevated in PLWHIV compared to healthy individuals. Interestingly, the expression of CD64 was the highest during early HIV infection and remained elevated in ART-naïve patients. The expression of CD64 was not entirely resolved in patients on successful ART, suggesting this protein could be used as a marker for chronic inflammation [256].

- *Neutrophil functions*

Despite their antiviral role in acute infection, several studies demonstrated dysfunctions in neutrophils, especially during HIV infection. First studies showing abnormal responses from granulocytes during HIV infection were published in the 1990s, showing neutrophil responses such as bactericidal activity against *Staphylococcus aureus* were decreased in children and adults living with HIV compared to uninfected individuals [257,258]. Similarly, chemotactic responses and fungicidal activities of blood neutrophils from chronically infected PLWHIV were significantly reduced compared to healthy donors [259]. Of note, several articles show contradictory results regarding neutrophil ROS production in PLWHIV [254,260–262]. In 2000, Vecchiarelli et al found a higher production of IL-12 in neutrophils from PLWHIV than in healthy donors, but an impaired response to LPS [263]. Several studies also showed monocyte dysfunctions in phagocytosis, and a few mechanisms responsible for impaired phagocytosis were proposed, such as altered formation of phagosomes or down-regulation of the signaling chain involved in FcγR-mediated phagocytosis [264,265].

Interestingly, subcutaneous injection of GM-CSF to patients suffering from AIDS temporarily improved neutrophil functions in one study [266] and increased neutrophil cytotoxicity against HIV-infected cells in vitro, independently of HIV status [267].

ART has been shown to partially reverse neutrophil dysfunction, even though PMN responses were not completely restored [259,268]. However, the major parameter correlating with restored neutrophil functions was the immunological status (higher CD4+ T cell counts, lower viral loads). Even more so, patients with AIDS presented the strongest impairment of neutrophils [269].

To this day, few mechanisms have been explored and described to explain defects observed in neutrophils from HIV patients. Recently, Piatek et al investigated changes in the chromatin of neutrophils in blood PMNs from PLWHIV in comparison with healthy donors. They demonstrated neutrophils in PLWHIV presented different post-transcriptional histone modifications compared to healthy donors, with an increased expression of histone modification processes associated with methylation, while

histone acetylation was inhibited. Increased expression of histone methylation was linked to increased global activation in neutrophils with a higher expression of adhesion molecules CD11b/CD18 and enhanced ROS production. On the other hand, the expression of several genes coding transcription factors related to the NFκB pathway, involved in fast immune responses, was decreased. This dysregulation may be responsible for lower neutrophil responses to stimuli such as LPS despite a higher basal level of activation [270].

Globally, blood neutrophils from PLWHIV present an activated phenotype associated with dysfunctional activities. Alterations in neutrophils are only partially resolved with ART. Although the precise mechanisms involved in neutrophil changes remain unknown, the prolonged exposition to inflammatory mediators related to HIV persistence, including IFN γ or TNF α , may participate in maintaining PMNs activated during the chronic phase of the disease. On the other hand, the involvement of activated neutrophils in chronic inflammation in patients is still unclear.

2) Neutrophils in gut mucosa/tissues during HIV infection

Even though the study of neutrophils in tissues remains difficult due to limited access to samples, the involvement of neutrophils in tissue inflammation and gut mucosa alteration has been investigated by several teams in patients and NHP models. In SIV-infected RMs, Estes et al. observed a neutrophilic infiltration in the colon using MPO staining on histological slides, and the presence of PMNs was considerably increased when animals progressed to AIDS [271]. In 2015, Somsouk et al also showed a higher infiltration of neutrophils in biopsies of ART-treated PLWHIV, with a trend for correlation with lower CD4⁺ T cell counts [272]. In 2019, Klatt et al showed frequencies of fresh neutrophils from the gastrointestinal tract (GIT) of PLWHIV receiving ART were increased compared to healthy individuals [6]. In patients receiving ART, the frequencies of CD16^{high} Caspase-3^{low} neutrophils, corresponding to a non-apoptotic functional phenotype, were also increased in the GIT compared to healthy individuals. These results suggest a higher neutrophil lifespan in the gut of patients compared to

healthy controls, which could contribute to persisting inflammation in this organ [6]. Recently, increased neutrophil infiltration has also been shown in gut biopsies from PLWHIV, with a similar intensity during the early or late stages of HIV infection [273]. Similar results had been obtained in the past from sigmoid biopsies in early [274] and chronic HIV despite ART, with a reduction of neutrophil infiltration after recombinant IL-7 administration in patients [275]. Additionally, the investigation of the gut microbiome demonstrated bacteria from the GIT could affect neutrophil lifespan in patients, either by increasing or decreasing it depending on the nature of the dominating bacteria [129].

During chronic HIV, increased neutrophil infiltration with an extended lifespan could contribute to inflammation, tissue damage and favor microbial translocation. Indeed, neutrophils have been shown to be deleterious in patients with inflammatory bowel disease, where IL-8 is overexpressed, and ROS production is increased in the colonic tissues of patients [180].

3) Neutrophil modulation of T cells during HIV infection

Despite accumulating proof of neutrophils' capacity to modulate T cells in acute and chronic stress situations, only a few studies investigated PMN-T cell interactions during HIV infection. However, several authors have shown neutrophils from HIV patients or SIV-infected animals either expressed modulatory molecules or could directly modulate T cell activity through various mechanisms.

First, several teams have shown an increase in arginase-1 expression in blood neutrophils from PLWHIV, a protein that inhibits T cell responses, as described in our previous chapter.

In 2010, Cloke et al. reported elevated arginase-1 activity in the PBMC fraction of patients with ≤ 350 CD4⁺ T cells/ μ L of blood compared with healthy controls [276]. Levels of arginase-1 activity were negatively correlated with CD4⁺ T cell counts and CD8⁺ T cell counts in untreated patients, but not in patients receiving ART. Authors

further identified arginase-1 positive cells as CD15+CD14- low-density neutrophils (LDN), suggesting this subset could be partially involved in T cell suppression during HIV infection. A confirmation of this LDN subset identification was brought in 2012 by the same team [140], which reported a higher frequency of LDN in patients with CD4+ T cell counts $\leq 350/\mu\text{L}$. Recently, Marsile-Medun et al. also demonstrated a higher frequency of LDG in PLWHIV. This LDG population was characterized by a decreased expression of Fc γ R, including CD16 and CD32, while CD64 expression was increased compared to normal-density granulocytes (NDG). Additionally, LDN from patients displayed a more activated phenotype compared to NDN with decreased CD62L expression [277].

In 2021, Marquez-Coello et al evaluated the expression of various immunomodulatory molecules on blood neutrophils from HIV patients. Through this study, they showed neutrophils from ART-naïve PLWHIV expressed significantly more intracellular arginase-1, PDL-1 and IL-10 compared to healthy donors. Interestingly, the expression of these markers was not resolved after ART administration and even increased compared to untreated patients [278]. These observations are aligned with previous results obtained by Bowers et al. in 2014, who also demonstrated a higher expression of PDL-1 on blood neutrophils from patients living with HIV compared to healthy donors, independently of ART [279]. In co-cultures of neutrophils and PBMCs, blood neutrophils from patients induced a strong reduction in IFN γ + T cells using HIV-specific and non-specific stimulation. Through blocking assays using PDL-1 antibodies and ROS scavengers, authors identified PD-1/PDL-1 interaction and ROS production by neutrophils as mechanisms partially involved in the immunosuppression exerted by neutrophils on T cells. After showing that PLWHIV had higher LDN frequencies than healthy donors, they depleted LDN from the PBMC fraction, which resulted in an increase of IFN γ + CD8+ T cells, suggesting LDN could be partly responsible for T cell immunosuppression. Neutrophils with high PDL-1 expression correlated positively with CD3 downregulation on T cells and elevated concentrations in arginase-1 in HIV patients [279]. Overall, these results suggest HIV infection induces neutrophils with an

immunosuppressive phenotype, which may participate in T cell exhaustion during the chronic phase of HIV infection.

Recently, another study investigated neutrophil aging in the blood of treated and untreated PLWHIV. In this work, authors report elevated frequencies of “aged” neutrophils, characterized by a CD62L_{low}CXCR4⁺ phenotype, in untreated patients, with a partial resolution with ART [280]. This neutrophil subset was positively correlated with viral loads and inversely with CD4⁺ T cell counts. Interestingly, ROS production of aged neutrophils was decreased compared to non-aged neutrophils upon LPS stimulation, and total neutrophils from untreated patients produced more spontaneous ROS and less after LPS stimulation. These results indicate neutrophils from untreated patients were less functional. Finally, using co-cultures of neutrophils and PBMCs from patients, authors showed immunosuppressive effects of neutrophils on T cells that were partially reversed using blocking antibodies against PD-L1 and arginase-1 [280].

Conversely, neutrophils have also been shown to activate T cells in the context of HIV infection. Using co-cultures between blood neutrophils and PBMCs for 48 hours, Dunsmore et al. have shown that neutrophils induced increased expressions of CD69, HLA-DR and CD25 on T cells in ART-treated patients with low CD4⁺ T cell counts. Authors identified the mechanism associated with T cell activation by neutrophils relied on the shedding of galectin-9 on neutrophils from these patients. This soluble molecule binds to CD44 on T cells and induces activation [281]. In PTMs infected with SIV, neutrophils were shown to trap T cells in NETs, which increased annexin V expression in T cells, indicating cell death [239]. Authors suggest neutrophils could increase T cell depletion in tissues, as T cells were found trapped in NETs in gut and lung tissues.

In our laboratory IDMIT, Lemaitre et al. also studied the impact of SIV infection on blood neutrophils from SIV-infected CyMs that received ART after 28 days, 6 months, or were left untreated [282]. SIV infection induced an increase in blood immature and activated blood neutrophils with elevated CD11b and lower CD62L expressions compared to uninfected animals. During the acute phase of infection, adding

neutrophils to PBMCs in co-culture assays induced an increase in intracellular T cell cytokine production, including higher expression of IL-2 and IFN γ in CD8 $^+$ and CD4 $^+$ T cells, as well as increased IL-17A production in CD4 $^+$ T cells. In parallel with this activating role of neutrophils, T cell proliferation was decreased in the presence of neutrophils during acute SIV infection. In the chronic infection, no significant effects were observed when neutrophils were added to PBMCs. In summary, SIV infection in CyMs was associated with changes in blood neutrophil phenotype and increased modulatory function toward T cell activation during the acute phase. The consequences of this crosstalk between these neutrophils and T cells on inflammation are yet to be addressed, and further investigation is needed to identify the cellular and molecular mechanisms at stake.

Overall, these results indicate that during SIV/HIV infection, neutrophils undergo phenotypical and functional changes that impact their capacity to modulate other immune cells, including T cells, which play a central role during this infection. Yet, the precise effects of neutrophils on T cells and the repercussions of these cell-cell interactions on inflammation, organ dysfunction, and viral persistence during HIV infection are unknown.

The roles of neutrophils are now being investigated in many diseases, and studies have established that these cells constitute a plastic, heterogeneous, and complex cell population with a broad range of functions. However, how neutrophils are involved in HIV infection remains to be fully elucidated, and insights from findings in other inflammatory diseases must be taken into account.

OBJECTIVES AND METHODS

I. Global objectives

Chronic inflammation leads to increased risks of comorbidities in PLWHIV. Hence, enhancing our knowledge of the mechanisms leading to chronic inflammation remains a major challenge to improve patients' quality of life. Processes leading to chronic inflammation in PLWHIV are complex and induced by many different biological and immune parameters. In chronic HIV infection, a prolonged and dysregulated activation of innate and adaptive immune cells is observed. While the roles of mononuclear inflammatory immune cell populations, such as macrophages and dendritic cells, have been extensively studied, the involvement of neutrophils throughout HIV infection is still unclear. Yet, neutrophils' potent antimicrobial functions and their capacity to enter tissues massively make them potential actors in early and chronic inflammation during HIV infection. Apart from their classical antimicrobial and inflammatory functions, the capacity of neutrophils to modulate T cell activation and proliferation is getting growing attention in the immunology field. Previous works from several teams support that such neutrophil-T cell interactions could occur in HIV and SIV infections. In particular, Julien Lemaitre et al have demonstrated an increase in blood immature neutrophil fraction associated with immunomodulatory properties during the acute phase of pathogenic SIV infection in cynomolgus macaques. However, the nature of the interaction between neutrophils and T cells, the impacts of neutrophils on the PBMC cytokine network, and the role of neutrophils in inflammation during SIV infection are unknown.

Studying neutrophils at the different stages of HIV disease in patients is complex, and sample availability is limited. Here, we investigated neutrophil changes in a cynomolgus macaque (CyM) model of HIV infection using a simian strain of the virus, SIVmac251. This model of HIV infection has been developed and well characterized in IDMIT over the last decades [32,283]. For our work, this model is relevant as CyMs present strong

similarities regarding immune cells compared to humans and develop a pathophysiology similar to HIV-1 when infected with SIVmac251.

In this work, we hypothesized that SIVmac251 infection could induce immune events affecting neutrophil phenotypes and functions, especially their capacity to modulate T cells, in early and late infection in CyMs. We also postulated that neutrophils and their interactions with T cells during SIV infection could contribute to chronic inflammation or viral persistence, despite ART.

Hence, our main objectives were to 1) characterize more deeply neutrophil phenotypes and functions during SIV infection, 2) investigate how neutrophils modulate T cell and PBMC activities, and 3) study the impact of neutrophils in inflammation in the early and late stages of SIV infection in CyMs.

For this work, we had access to blood samples from healthy CyMs and at different times of SIVmac251 infection. Animals were followed for a minimum of 6 months, and we included one group that received ART 28 days after exposure to evaluate if the treatment had an effect on our observations. The animals we followed were part of different SIVmac251 studies in IDMIT. Tissue samples were also collected upon euthanasia for further analyses of tissue neutrophils.

Blood neutrophil phenotype was assessed throughout SIVmac251 infection using classical flow cytometry. Markers for lineage, activation and maturity were included, allowing a robust characterization of neutrophil subsets in CyMs. In parallel, we addressed neutrophil modulation of soluble cytokines and chemokines released by PBMCs during SIVmac251 infection. To do so, we quantified the concentrations of pro and anti-inflammatory cytokines and chemokines in supernatant co-cultures of PBMCs and neutrophils. Next, using similar co-cultures, we evaluated the direct effects of neutrophils on T cell activation using intra and extracellular flow cytometry. We also studied the nature of neutrophil-T cell interactions using Transwells.

Additionally, to characterize neutrophil-related immune signatures associated with SIV infection, we carried out Partial Least-Squares Discriminant Analysis (PLS-DA) in early-infected and chronically-infected CyMs with or without ART. We used the data from whole blood neutrophil phenotypes and intracellular stainings in neutrophil-T cell co-cultures for this analysis.

Lastly, we performed a mass spectrometry analysis to investigate changes in blood neutrophil proteins and proteome signatures before and during SIVmac251 infection.

II. Methods

i) *Cynomolgus macaques infected with SIVmac251, a relevant model for the study of neutrophils in HIV-1 infection*

- ***Pathogenic SIVmac251 in CyMs induces a disease similar to HIV-1***

As accessing samples from patients can be quite challenging, the use of animal models remains necessary to study the pathophysiology of HIV infection. The main animal models for HIV infection are NHPs and mice models. Humanized mice are genetically immunocompromised mice engrafted with human tissues to reconstitute the human immune system [31]. While these mice models bring many insights into HIV infectious mechanisms, they only partially reconstitute the human immune system and HIV-1 pathophysiology. Moreover, mice models do not allow the production of functional human neutrophils, and the co-existence of mouse and human neutrophils is observed [30].

In parallel, NHPs have also been extensively studied as models for HIV-1 infection because some species develop symptoms similar to HIV when exposed to certain simian strains of the virus. As mentioned in the introduction (part I.4), commonly used NHP models for HIV infection are rhesus macaques (RMs), pigtailed macaques (PTMs)

and *Cynomolgus* macaques (CyMs). The Mauritian CyM infected with pathogenic SIVmac251 has been well characterized in IDMIT as a model for HIV infection for many years [284]. After exposure to SIVmac251, CyMs develop a pathophysiology very close to HIV-1 in humans, with features such as CD4+ T cell depletion, chronic immune activation and persistence of a high viremia when animals remain untreated. After several months of infection, CyMs develop AIDS, a state reached after six months post-infection or more, which is less rapid than RMs and PTMs infected with pathogenic SIV. Of note, SIVmac251 was historically isolated from RMs and is genetically closer to HIV-2 than HIV-1. Contrary to RMs and PTMs, the Mauritian population of CyMs is characterized by a low genetic diversity of major histocompatibility complex (MHC) with only seven MHC haplotypes. Some of these haplotypes are associated with resistance against SIVmac251 infection [285], and a screening of animals allows the identification of SIVmac251-susceptible macaques. Additionally, CyMs are sensitive to various antiretroviral molecules used in PLWHIV. For our study, ART+ animals received daily intracutaneous injections of a tritherapy composed of one integrase inhibitor (Dolutegravir) and two NRTI (Emtricitabine and Tenofovir).

Finally, there are many similarities between CyMs and humans regarding their immune systems. Most of the markers used to define T and B lymphocytes, as well as innate myeloid cells, are common to the two species [286–288]. These immune markers have been cross-validated using flow and mass cytometry, and most of the antibodies used in NHP studies are clones targeting human epitopes (**Table 1**).

Accordingly, markers to evaluate T cell activation in CyMs are also similar to those used in human studies, including CD38, HLA-DR and CD69, as well as IFN γ , TNF α and interleukins (IL) such as IL-2, IL-6 or IL-17 [20,282]. Nonetheless, some differences exist between human and CyM protein epitopes, requiring antibodies or reagents specific to NHPs such as the non-human primate Cytostim for T cell stimulation.

Cell population	Human markers	Cynomolgus macaque markers
Eosinophils	CD66+ CD125+	CD66+ CD125+
Basophils	CD66- CD123+ HLA-DR-	CD66+/- CD123+ HLA-DR-
Monocytes	CD14+ HLA-DR+	CD14+ HLA-DR+
pDC	CD19- CD14- HLA-DR+ CD123+	CD20- CD14- HLA-DR+ CD123+
mDC	CD19- CD14- HLA-DR+ CD11c+	CD20- CD14- HLA-DR+ CD11c+
NK cells	CD66- CD3- Granzyme B+	CD66- CD3- CD8+
CD8+ T cells	CD3+ CD8+	CD3+ CD8+
CD4+ T cells	CD3+ CD4+	CD3+ CD4+
B cells	CD19+	CD20+
Neutrophils	CD66+ CD16+	CD66+

Table 1: Markers for immune cell phenotyping in humans and cynomolgus macaques, adapted from Elhmouzi-Younes et al, Cytometry. Part A: the journal of the International Society for Analytical Cytology, 2017 [286]

- **Neutrophils from CyMs are similar to human neutrophils**

We are currently writing a review on neutrophils and infectious diseases in non-human primate models. Here are some elements addressed in this future review with a focus on CyM neutrophils.

First of all, neutrophils represent approximately 60% of the total leukocyte population, similar to humans [286]. Besides, studies showed that neutrophils from CyMs are mostly similar to human neutrophils regarding their production, differentiation, and release in circulation. Cytology of BM cells allowed the identification and quantification of the different neutrophil maturation stages in CyMs: myeloblasts, promyelocytes, myelocytes, metamyelocytes, band and segmented neutrophils [253,289].

In NHPs, the global characteristics of neutrophils are common to human neutrophils, including cell structure and granularity. However, blood neutrophil phenotyping through flow or mass cytometry highlighted some differences in the expression of neutrophil proteins from the two species. In humans, segmented neutrophils are often identified through the expression of CD66, CD11b, CD15, and CD16b, among many other markers. Many of these markers are expressed in neutrophils and were validated

in RMs and CyMs, but some of them are absent on neutrophils, or their expression has not been confirmed due to a lack of cross-reactivity. For instance, CD16 is not expressed on neutrophils from NHPs except for sooty mangabeys [290,291], and CD15 presents a very low cross-reactivity in NHPs. For neutrophil differentiation, some markers such as CD49d are also less relevant in NHPs than in humans for the identification of the different neutrophil subpopulations [253]. Moreover, CyM neutrophils present an activation phenotype similar to humans, with CD62L downregulation and/or CD11b, CD32a, or CD64 upregulation when exposed to stress or stimuli [282]. Palgen et al. observed a more activated neutrophil phenotype in healthy CyMs compared to human neutrophils through mass cytometry analysis, with a higher basal expression of CD11b and CD32 [286]. Additionally, CyM neutrophils respond to common stimuli such as PMA, LPS, TNF α or R848 (unpublished data from IDMIT) and can perform phagocytosis [253]. Although, to our knowledge, there is no comparative study of the expression of PRRs from human and CyM neutrophils, these data suggest that neutrophils from both species display similar activation pathways.

Overall, CyMs represent a relevant model for this study because they develop a disease similar to HIV-1 when infected with SIVmac251, and their immune system is close to the human immune system, in particular for our main populations of interest, which are neutrophils and T lymphocytes.

ii) Experimental techniques

- ***Neutrophil isolation from the blood***

To study the role of neutrophils during SIV infection, the isolation of pure neutrophils was necessary. Indeed, the cell density separation from CyM whole blood only allows the segregation of mononuclear cells in a ring below plasma, and granulocytes in the tube pellet. Although neutrophils represent the major cell population in the granulocyte fraction, eosinophils constitute an important contaminant population, as

they can represent 5-15% of the granulocyte fraction. This is why we developed a magnetic cell sorting to deplete CD125+ eosinophils from the granulocyte fraction. We also added magnetic beads targeting CD2+ cells to remove any remaining T lymphocytes or natural killer cells.

- ***Analysis of supernatant soluble molecules in neutrophil-PBMC co-cultures***

To study the impact of neutrophils on different soluble molecules produced by PBMCs, we set up a model of co-cultures between autologous PBMCs and blood neutrophils in the presence of a non-specific T cell stimulation, or in the absence of stimulation. In this assay, we did not add any agent to block secretion, allowing prolonged exchanges of small molecules, paracrine and autocrine effects. We used a commercial kit from Milliplex, which allows the detection of 38 cytokines and chemokines in NHPs. Experiments were run before infection, after 10 days post-infection, 15 days post-infection, and 6 months post-infection. For this experiment, we had access to more animals during the chronic phase compared to the other timepoints.

- ***Effects of neutrophils on T cell activation***

To study the impact of neutrophils on T cell activation, we performed similar co-cultures as described in the previous paragraph. Because we studied T cell intracellular cytokine production, we used brefeldin A to block cell secretion after 1 hour of co-culture. Additionally, intracellular protein recycling was blocked using monensin to study the expression of CD107a, a protein expressed in cytotoxic granules. Indeed, CD8+ T cell degranulation induces the expression of CD107a at the cell surface, which usually is then recycled intracellularly and degraded.

To study T cell activation, we built a flow cytometry panel for cell staining after an overnight incubation. T cell activation was measured through CD69 expression, a standard extracellular early activation marker in CyMs, and the expression of intracellular cytokines IFN γ , TNF α , IL-2 and IL-17A. For CD8+ T cells, we added the marker CD107a. We performed co-cultures with three different neutrophil-to-PBMC

ratios (1:1, 3:1, 5:1) to evaluate if the number of neutrophils impacted the effects on T cells.

In order to investigate the contact-dependent effects of neutrophils on T cells during SIV infection, neutrophils and PBMCs were co-cultured in classical wells or separated by a 0.4 μm membrane, which blocks the circulation of large molecules.

- ***PLS-DA analysis***

Because principal component analysis (PCA) analysis is not informative on the parameters responsible for the discrimination of different groups, we rather performed Partial Least Squares Discriminant Analysis (PLS-DA). For each of these analyses, we identified the parameters contributing the most to the discrimination of each group. We performed such analysis for the whole blood flow cytometry and ICS datasets.

- ***Proteomics***

As previously evoked in the introduction of this manuscript, neutrophils are particularly enriched in proteins of different natures, stored in granules, vesicles or cytoplasm. On the contrary to proteins, neutrophils present low-levels of RNAs, which enhances the risk of false results linked to contamination by other cells in transcriptomic studies. This is why we decided to perform a proteome analysis instead of a transcriptional analysis of neutrophils.

For proteomics analysis, neutrophils were isolated and purified using cell sorting, as described in the first paragraph. In other experiments we performed, neutrophils were processed in RPMI medium supplemented with fetal bovine serum (FBS) which brings protein contamination. During neutrophil isolation for proteomics, we used Hank's Balanced Salt Solution (HBSS) without calcium or sodium to limit neutrophil activation and avoid external protein contamination. The acquisition of samples by mass spectrometry was done in collaboration with Jean Armengaud's team at the Progénomix platform in CEA Marcoule.

RESULTS

I. Neutrophil isolation from macaque whole blood by magnetic cell sorting

i) Impact of magnetic cell sorting on neutrophil activation

- *Rational approach and methodology*

Neutrophils are short-lived cells which are particularly sensitive to external stimuli. Changes in the media, long experiments and multistep processes have been shown to trigger neutrophil CD62L shedding and increase CD11b expression, two receptors linked to neutrophil activation [292]. Here, we aimed to study pure blood neutrophils from CyMs, and this is why we developed a cell sorting on the granulocytic fraction obtained after Ficoll separation. Since the most abundant granulocytic cells after neutrophils are eosinophils, representing up to 15% of the granulocyte fraction, we decided to remove eosinophils through magnetic sorting using beads. Our main concern with this technique relied on the impact of this process on neutrophil phenotype and function.

In these additional results, we evaluated the purity of isolated neutrophils. We analyzed the phenotype of neutrophils from whole blood and after cell sorting in six healthy CyMs to assess their activation level. We stained 200,000 neutrophils using a flow cytometry panel with common neutrophil markers for phenotype and activation, as shown in **Table 2**. We also evaluated neutrophil phenotype after TNF α stimulation to assess the capacity of neutrophils to respond to such stimuli after isolation.

Antibody	Clone	Fluorochrome	Laser	Company
BlueVid	-	-	355-450/50	Thermofisher
CD64	10.1	BUV737	355-740/35	BD
CD10	HI10a	BV421	405-420/10	Biologend
CD45	REA1023	Viogreen	405-525/50	Miltenyi
CD3	SP34.2	BV650	405-660/20	BD
CD123	7G3			BD
CD8a	RPAT8			BD
CD20	2H7			BD
CD62L	SK11	BV711	405-710/50	BD
CD11b	REA713	FITC	488-530/30	Miltenyi
CD14	REA599	PerCP-Vio700	488-695/40	Miltenyi
CDw125	REA705	PE	561-585/15	Miltenyi
PD-L1	29E.2A3	PE-Dazzle 594	561-610/20	Biologend
CD101	REA954	PE-Vio770	561-780/60	Miltenyi
CD32a	IV.3	AF647	640-670/14	Stemcell
HLA-DR	L234	AF700	640-730/45	Biologend
CD66abce	TET2	APC-Vio770	640-R775 / 50	Miltenyi

Table 2: Flow cytometry panel for the characterization of neutrophils from CyMs

This panel was created and adapted from previous results obtained in IDMIT. It includes markers for lineage (CD45, CD66, CD3, CD123, CD8, CD20, CD14, CD125), markers for neutrophil maturity (CD10, CD101) and markers for neutrophil activation (CD64, CD62L, CD11b, CD32a and HLA-DR). This panel was used for all experiments assessing neutrophil activation in our study.

• Results

First, we observed that the magnetic cell sorting resulted in >95% neutrophil purity and efficient removal of CD125+ eosinophils (**Figure 11A and 11B**). We then studied the level of neutrophil activation after isolation, and we found that the mean fluorescence intensity (MFI) of several activation markers were increased after purification compared to whole blood neutrophils, including CD11b, CD64 and CD10. A similar tendency was observed for CD62L, but it was not significant. The expression of CD32a was not affected (**Figure 11C-11I**)

We then studied the capacity of isolated neutrophils to respond to a mild activation, TNF α . We found that a 1 ng/mL concentration of TNF α induced a strong and significant decrease of CD62L expression (**Figure 11C and 11D**), but the expression of CD11b was unchanged (**Figure 11E and 11F**).

Overall, we were successful in purifying neutrophils from CyM whole blood. Although this multistep process induced a significant increase in CD11b expression, neutrophils were still sensitive to TNF α stimulation, as shown by the decrease in CD62L expression. We also concluded that with our technical process, CD11b was not an ideal marker to measure neutrophil activation after isolation, on the contrary to CD62L.

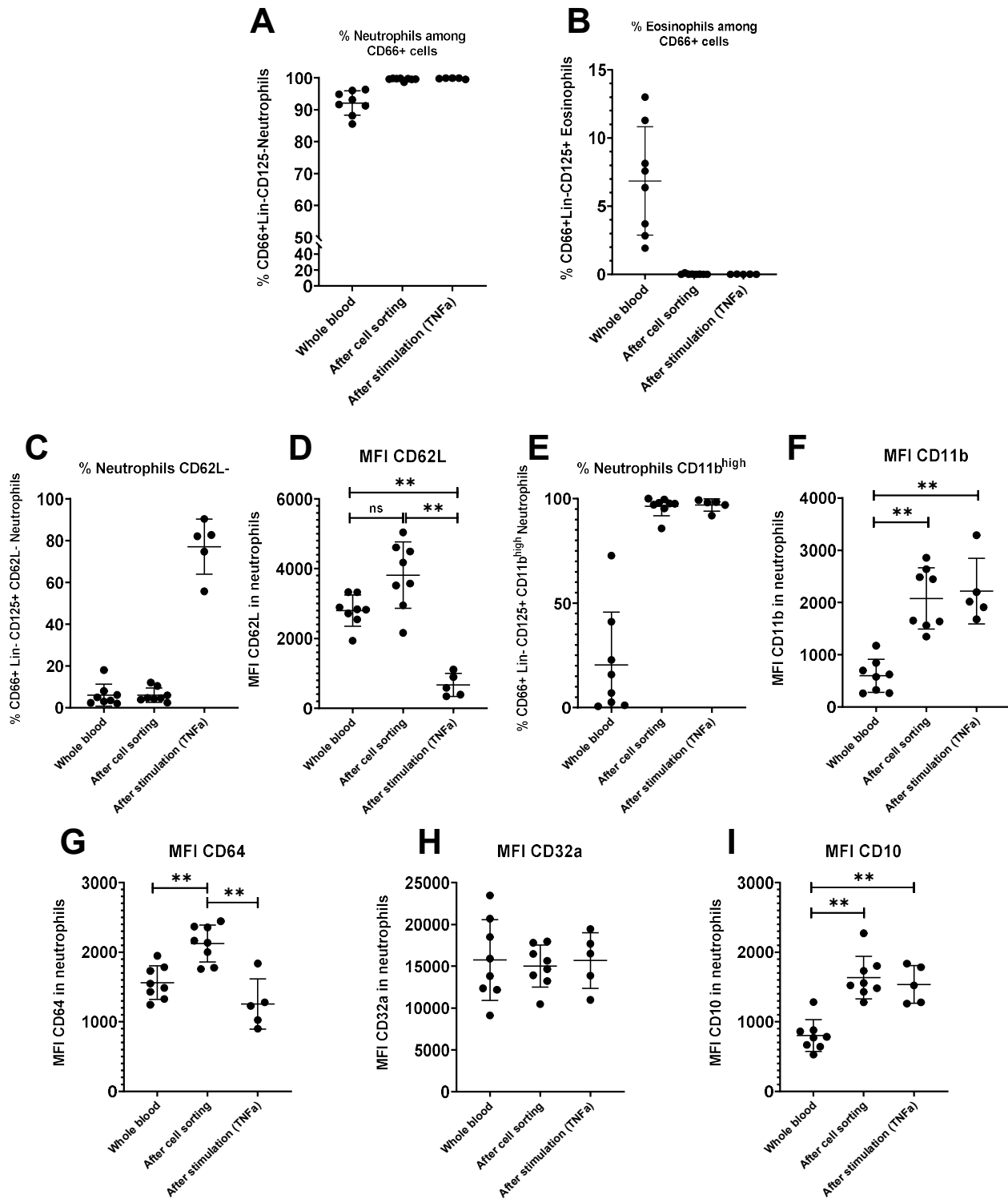


Figure 11: Effects of isolation techniques on purity and activation of neutrophils from CyMs

(A) Scatter dot plots of the neutrophil frequency among CD66+ cells before and after PMN isolation from the blood; (B) Scatter dot plots of the eosinophil frequency among CD66+ cells before and PMN isolation from the blood; (C-I) Scatter dot plots of frequencies or mean fluorescence intensities of indicated marker in neutrophils before and after PMN isolation from the blood and after TNF α stimulation of isolated PMNs. Statistics: Wilcoxon tests were run, * $p < 0.05$, ** $p < 0.01$

ii) Identification of the technical step inducing neutrophil activation during cell isolation

We further aimed to identify which step of the process was responsible for the observed neutrophil activation. To do so, we performed similar flow cytometry stainings as previously described on the neutrophil fraction from the whole blood of CyMs after the following steps: Ficoll/red blood cell (RBC) lysis, and magnetic cell sorting. These experiments were run on four animals, one was healthy and three were SIV+ART+ animals treated for five months.

Through these experiments, we identified that the Ficoll/RBC lysis step was the step inducing neutrophil activation, and not the neutrophil magnetic sorting (**Figure 12A-12D**). Considering that this Ficoll/RBC lysis step could not be avoided, we proceeded further with this experimental process while being aware that neutrophils are exposed to some activation during this step.

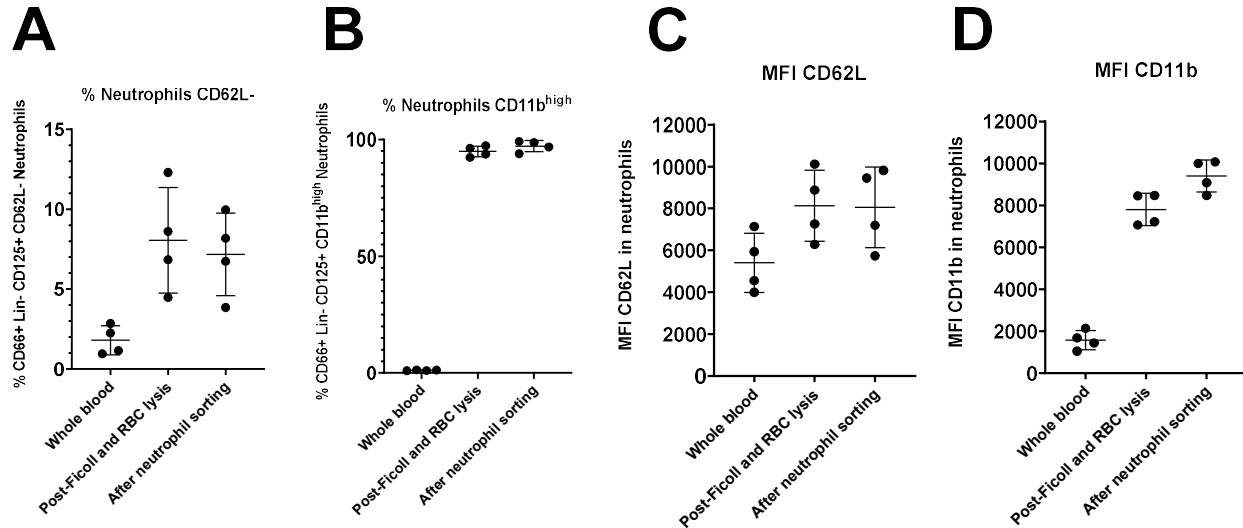


Figure 12: Level of neutrophil activation in whole blood of CyMs, after Ficoll/RBC lysis and after magnetic cell sorting

(A-B) Frequencies of CD62L- and CD11b^{high} in neutrophils from whole blood, after Ficoll/RBC lysis or after neutrophil sorting; (C-D) Mean fluorescence intensities of CD62L and CD11b in CyM neutrophils from whole blood, after ficoll/RBC lysis or after neutrophil sorting. No statistics were performed due to a small number of animals.

II. Main results: NEUTROPHILS INDUCE CONTACT-DEPENDENT MODULATION OF T CELL ACTIVATION AND INFLAMMATORY MOLECULES DURING SIV INFECTION IN NON-HUMAN PRIMATES

i) Context and objectives of the study

Previously, Julien Lemaitre et al. investigated the changes induced by SIVmac251 infection in CyMs in whole blood neutrophils. First, using mass cytometry, Julien Lemaitre and colleagues showed that blood neutrophils were more heterogeneous and displayed an immature phenotype after 18 months of SIV infection compared to uninfected macaques. Further analyses showed that chronic SIV infection was also associated with activated neutrophils, characterized by a higher expression of CD11b and a lower expression of CD62L, and impairment of neutrophil phagocytic capacities. In a second study in 2022, Julien Lemaitre et al. explored neutrophil changes in both acute and chronic SIV infection in a larger group of SIV-infected animals. He found that acute SIV infection (10 days post-infection) was associated with increased frequencies of CD10⁻ immature and CD62L^{low}, CD11b^{high} activated neutrophils. This phenotype persisted after 6 months of untreated infection, but was partially resolved in animals that received ART after 28 days of infection. In this study, Julien Lemaitre et al. found that neutrophils increased T cell activation during the acute phase of SIV infection. Indeed, when co-cultured with neutrophils, intracellular productions of IFN γ and IL-2 were increased in CD4⁺ and CD8⁺ T cells. In parallel, neutrophils also modulated T cells by increasing CD4⁺ T cell proliferation and decreasing CD8⁺ T cell proliferation. Altogether, these data indicated that SIV infection in CyMs was associated with phenotypical changes in neutrophils along with capacities to modulate T cell activation and proliferation. However, how these phenotypic changes and neutrophil-T cell interactions could play a role in inflammation during SIV infection was not addressed.

As previously explained in this manuscript, chronic inflammation in PLWHIV leads to increased risks of developing comorbidities. In this work, we wanted to investigate if neutrophils could participate in chronic inflammation during SIV infection in CyMs. To do so, we investigated how neutrophils modulate pro- and anti-inflammatory cytokines and chemokines produced by PBMCs. We also investigated the nature of interaction between neutrophils and T cells in early and late SIVmac251 infection in CyMs. Additionally, we explored the changes in neutrophil proteomes along SIV infection with and without ART.

In this work, animal blood withdrawal, clinical monitoring, complete blood counts, SIV inoculation, and organ sampling upon euthanasia were performed by the Animal Welfare team platform (ASW) in association with project managers Laure Fournier-Le-Ray and Tatiana Botin. Plasma viral loads were followed by polymerase chain reaction, by the Laboratory for Immunology and Infections (L2i) team platform. Longitudinal follow-ups of CD4+ T cell counts were performed by the Flow Cytometry team platform (LFC) and Florian Meurisse, using flow cytometry.

After blood withdrawal from CyMs, I was able to perform the following technical steps, before and during SIV infection:

- Whole blood neutrophil flow cytometry stainings
- PBMC isolation and/or neutrophil cell sorting followed by:
 - Co-cultures of PBMCs and neutrophils for Luminex assays
 - Co-cultures of PBMCs and neutrophils for intracellular flow cytometry stainings to measure T cell activation
 - Sampling of neutrophil proteomes from purified neutrophils
 - ROS production assays (see additional results)
- BM and spleen neutrophil flow cytometry stainings (see additional results)

The quantification of soluble molecules from PBMC/neutrophil co-culture supernatants and related quality controls were processed by Julie Morin using Luminex kits. PLS-DA analyses were performed with Paul Mazet, who is a bioinformatician in IDMIT. Paul also

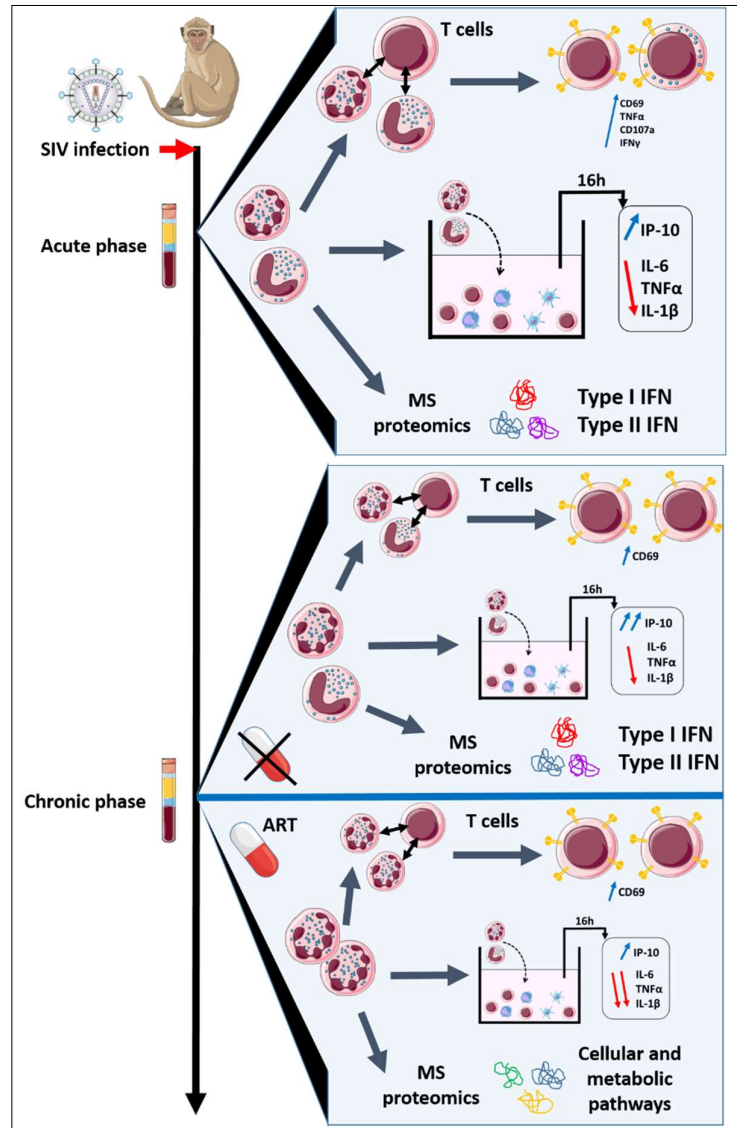
participated in the analysis of the proteomics dataset. Mass spectrometry acquisition for neutrophil proteomics was performed by the Laboratoire Innovations technologiques pour la Détection et le Diagnostic (LI2D), at the CEA center in Marcoule, France.

With the support from all the colleagues cited here, I was able to analyze most of the data presented in this article. I also mainly wrote this article, which was largely revised by Julien Lemaitre, Olivier Lambotte and Roger Le Grand.

ii) Article (in preparation)

Targeted journal:
Cell reports medicine

Graphical abstract (created with Powerpoint)



1 **NEUTROPHILS INDUCE CONTACT-DEPENDENT MODULATION OF T CELL**
2 **ACTIVATION AND INFLAMMATORY MOLECULES DURING SIV INFECTION IN**
3 **NON-HUMAN PRIMATES**

4 G. Sandillon¹, L. Fournier Le Ray¹, Paul Mazet¹, T. Botin¹, F. Misplon¹, J. Jhon¹, J. Morin¹,
5 L. Bossevot¹, O. Dissake¹, F. Relouzat¹, A-S. Gallouet¹, N. Bosquet¹, J. Armengaud², R. Le
6 Grand¹, O. Lambotte^{1,3} and Julien Lemaitre¹

7 ¹ Université Paris-Saclay, Inserm, CEA, Center for Immunology of Viral, Auto-immune,
8 Hematological and Bacterial diseases » (IMVA-HB/IDMIT/UMRS1184), Fontenay-aux-
9 Roses & Le Kremlin-Bicêtre, France

10 ² Université Paris-Saclay, CEA, Département Médicaments et Technologies pour la
11 Santé (DMTS), INRAE, Bagnols-sur-Cèze, France

12 ³ AP-HP, Université Paris Saclay, department of internal medicine and clinical
13 immunology, Bicêtre Hospital, Le Kremlin Bicêtre, France

14

15 *Corresponding Author: Julien Lemaitre, julien.lemaitre@cea.fr

16 **Abstract**

17 Despite antiretroviral therapy (ART), people living with HIV are exposed to a persisting
18 chronic inflammation, leading to increased risks of developing comorbidities. How
19 polynuclear neutrophils, the most abundant blood immune population with potent
20 antimicrobial and inflammatory properties, participate in this chronic inflammation, is
21 poorly described. Here, we investigate how neutrophils are modified at the cellular and
22 protein levels, and how they modulate other immune cells during SIVmac251 infection
23 in cynomolgus macaques (CyMs), a model for HIV infection. Using co-cultures of
24 autologous neutrophils and PBMCs from CyMs, we studied how neutrophils modulate
25 soluble cytokines and chemokines levels and T cell activation in early and late
26 SIVmac251 infection. We show that during SIVmac251 infection, neutrophils regulate
27 pro-inflammatory molecules such as IP-10 and increase T cell expression of activation
28 markers in a contact-dependent manner. Additionally, mass spectrometry analysis of
29 neutrophil proteomes revealed that during SIVmac251 infection, neutrophils express
30 higher levels of proteins linked to interferon pathways, a phenotype partially resolved
31 with ART.

32 INTRODUCTION

33 In the absence of a cure, antiretroviral therapy (ART) remains necessary for people living
34 with HIV (PLWHIV) to control viral replication and maintain high blood CD4+ T cell
35 counts [1]. Despite the high efficiency of ART, unresolved immune activation persists
36 in association with low-grade chronic systemic inflammation [2,3]. Long-term
37 consequences are increased morbidity and mortality risks in PLWHIV compared to the
38 general population [4–6]. Inflammation, resulting from a combination of diverse
39 mechanisms, is initiated during HIV primary infection, persists in the long term, and
40 does not resolve despite ART [4].

41 Historically described as short-lived cells participating in acute inflammation, emerging
42 evidence suggest polymorphonuclear neutrophils are involved in many chronic
43 diseases, including HIV infection-related disorders such as metabolic, cardiovascular
44 and neurological diseases [7,8]. Neutrophils are the most abundant cells in the human
45 blood and represent potent defenders against pathogens, including HIV [9]. However,
46 they can become detrimental to the host when their functions are dysregulated,
47 inducing damage to the microenvironment and tissues [10]. Although neutrophils are
48 not mainly targeted by HIV, their phenotype and functions are affected during HIV
49 infection. In chronic HIV and non-human primate models for HIV infection, studies have
50 reported blood neutrophils are of more diverse phenotypes than in non-HIV-infected
51 individuals, in particular with increased immature and activated phenotypes,
52 characterized by elevated CD11b and decreased CD62L expressions [11,12]. These
53 phenotype changes are only partially resolved with ART. In addition, in PLWHIV on ART,
54 blood neutrophil survival is increased, contributing to their accumulation in tissues
55 such as the gut, indicating neutrophils could participate in HIV pathophysiology and
56 chronic inflammation [13].

57 Besides these changes in phenotype, there is growing evidence for neutrophil ability
58 to modulate T cells through various mechanisms during HIV infection, including the
59 expression of immunomodulatory proteins or the release of reactive oxygen species

60 [14]. However, the precise implication of neutrophils in exacerbating or limiting
61 inflammation by modulating T cells is unclear. Indeed, neutrophils are described as
62 playing dual roles in HIV infection, either participating in T cell exhaustion through PD-
63 L1 and arginase-1 expression or, on the contrary, increasing T cell activation by the
64 release of neutrophil extracellular traps (NETs) or soluble galectin 9 [15–18].

65 Non-human-primate (NHP) models such as cynomolgus macaques (CyMs) infected
66 with pathogenic SIV represent valuable approaches for the study of HIV infection, as
67 they recapitulate most features of the human disease and are sensitive to ART [19].
68 Additionally, neutrophils from CyMs are close to human neutrophils, with similar
69 differentiation in bone marrow and comparable expression of activation and
70 differentiation markers in circulation and tissues [11]. Recently, Lemaitre et al have
71 shown modulatory capacities of neutrophils on T cell activity in CyMs infected with
72 SIVmac251 [19]. Frequencies of T cells expressing IL-2, IFN γ and TNF α were increased
73 in the presence of autologous blood neutrophils during the acute phase of infection.
74 These immunomodulatory properties of blood neutrophils in SIV-infected CyMs were
75 also associated with a more immature and activated phenotype.

76 This study explored how neutrophils participate in inflammation at different timepoints
77 of SIVmac251 infection in CyMs. Through co-cultures of neutrophils and PBMCs, we
78 investigated neutrophil modulation of soluble PBMC cytokine/chemokine network,
79 direct contact-dependent interactions with T cells, and dynamics of neutrophil
80 proteomes in early and late pathogenic SIVmac251 infection.

81

82 RESULTS

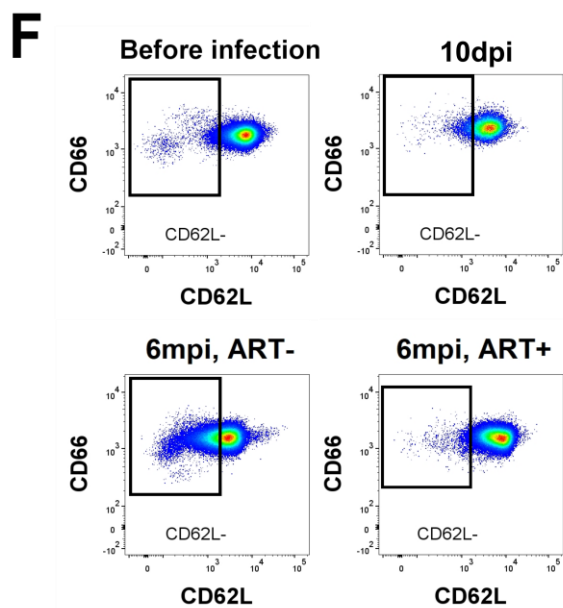
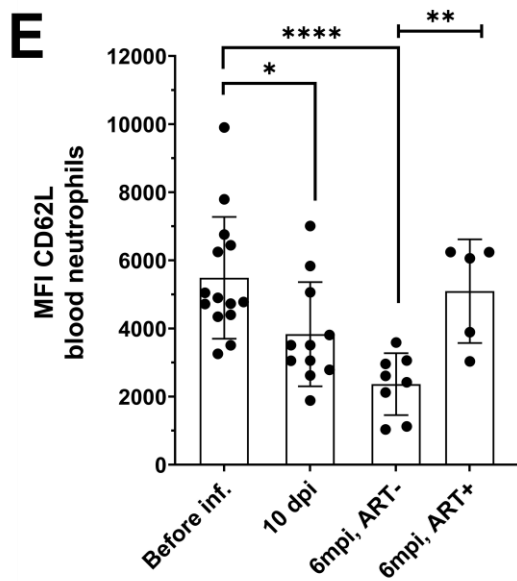
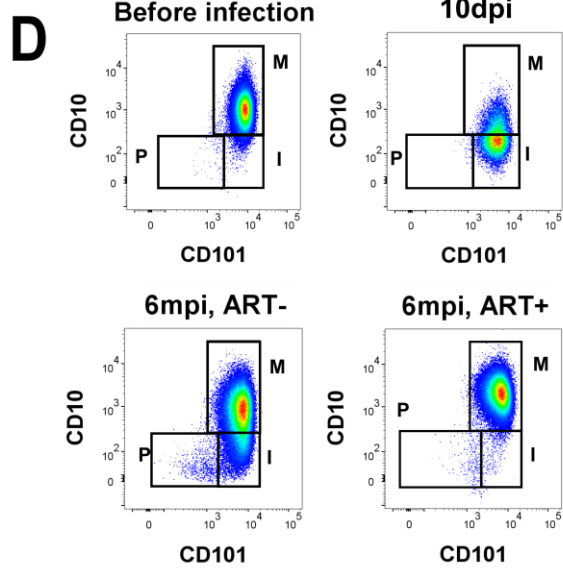
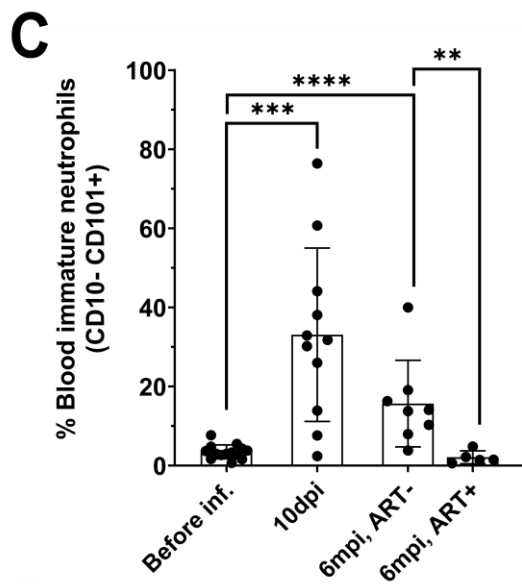
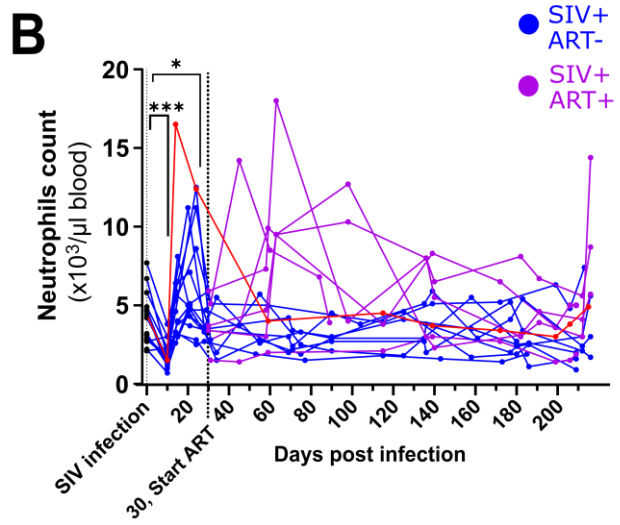
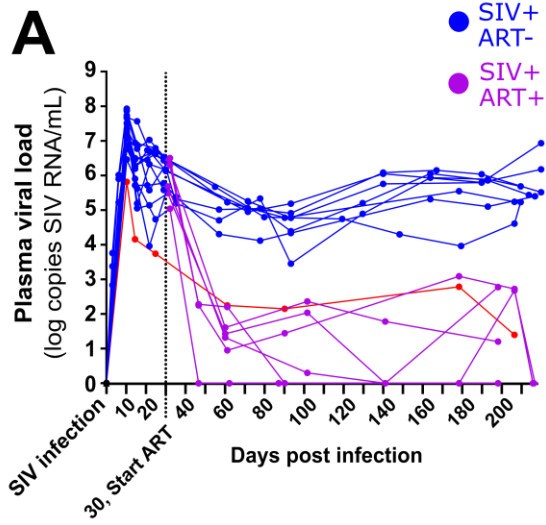
83 SIV infection in CyMs is associated with immature neutrophils and 84 increased neutrophil activation

85 To interrogate the role of neutrophils in SIVmac251 disease pathophysiology, fifteen
86 CyMs were challenged intravenously (I.V.) with 1000AID50 of SIVmac251 and
87 antiretroviral therapy (ART) was initiated in six infected animals at 28 days post-
88 infection (dpi) with a combination comprising Tenofovir, Emtricitabine and
89 Dolutegravir (SIV+ ART+). Nine other macaques were left untreated (SIV+ ART-). As
90 expected, CyMs developed a primary infection following I.V. challenge, with plasma
91 SIVmac251 RNA load spiking at approximately 10dpi in all animals (**Figure 1A**) [20],
92 and associated with a transient CD4+ T cell depletion (**Figure S1A**). After ART initiation,
93 rapid reduction of plasma viral load occurred in all treated animals with a few blips.
94 Despite the exclusion of CyMs bearing an MHC-H6 haplotype, who spontaneously
95 control SIV replication [21], one animal in the SIV+ ART- group exhibited a low viral
96 load compared to the other untreated animals. Therefore, this animal was excluded
97 from further comparative analysis between uninfected and SIV-infected CyMs. During
98 primary infection, there was a significant decrease in blood neutrophils at 10 dpi
99 followed by a peak between 20 and 24 dpi compared to baseline (**Figure 1B**). When
100 ART was initiated at 28 dpi, neutrophil counts had returned to baseline levels, with no
101 significant difference from baseline counts. We did not observe significant differences
102 at later time points between ART+ and ART-animals. These results indicate that primary
103 SIV infection in CyMs is associated with an early depletion followed by a spike of
104 circulating neutrophils that resolved at 28 dpi.

105 Changes in neutrophil counts have been associated with the modulation of activation
106 status and maturity. We further characterized neutrophil subpopulations based on their
107 maturation stage by flow cytometry. We previously identified that the combination of
108 CD101 and CD10 delineates macaque pre-neutrophils (CD101- CD10-), immature
109 (CD101+ CD10-) and mature neutrophils (CD101+ CD10+) similarly to humans. Along

110 with maturation-based neutrophil subpopulations, we monitored neutrophil activation
111 by following the expression of CD62L, CD64, CD11b, CD32a and PD-L1. Before
112 infection, circulating neutrophils were mainly CD10+ mature neutrophils (**Figure 1C,**
113 **1D and S1B**) and displayed low levels of activation based on CD62L and CD11b
114 expression. Following infection with SIV, neutrophil subpopulations and phenotypes
115 rapidly changed with a significant increase of CD10- neutrophils and increased
116 activation in primary infection (**Figure 1C-1F**). At 10 dpi, frequencies of CD101+ CD10-
117 immature neutrophils significantly increased (**Figure 1D**). In parallel, we found a
118 decrease in CD62L expression in blood neutrophils (**Figure 1E-1F and S1C**). We
119 analyzed CD62L expression in neutrophil subpopulations, and we observed that the
120 CD62L downregulation was mainly driven by mature neutrophils (**Figure S1D**). In the
121 absence of treatment, at 6 mpi, immature neutrophil frequencies remained elevated
122 along with neutrophil activation compared to baseline. ART initiation at 28 dpi in the
123 treated group was associated with frequencies of immature neutrophils and levels of
124 activation similar to baseline. All other activation markers were not significantly
125 changed during infection (**Figure S1E-S1H**).

126 Altogether, SIV infection of CyMs resulted in increased activated and immature
127 neutrophils in blood during primary infection. This phenotype persisted in chronic
128 infection of CyMs that were not given ART. Meanwhile, ART restored this phenotype
129 with levels of immature and activated neutrophils comparable to baseline.



131 **Figure 1. Increased immature and activated circulating neutrophils during**
132 **SIVmac251 infection in CyMs**

133 (A) Plasma viral loads in $n = 15$ SIVmac251-infected CyMs over time (blue lines), with $n = 6$ being treated at 1mpi
134 to 6mpi with ART (switching to purple lines). Red line indicates $n = 1$ infected animal that controlled infection; (B)
135 Total neutrophil counts from blood during the course of SIVmac251 infection in the same $n = 15$ CyMs; (C)
136 Frequencies of immature CD101+ CD10- neutrophils in blood before infection or during acute SIVmac251 infection
137 (10dpi), and with (+) or without (-) ART beginning at 1mpi, individual values (dots) or group averages (bars) \pm
138 standard deviation (SD); (D) Expression of CD101 versus CD10 at indicated times during infection, defining M
139 (mature), I (immature) or P (pre) neutrophil subsets; (E) Mean fluorescence intensity (MFI) of CD62L expression by
140 total blood neutrophils from individual animals (dots) or group average MFI (bar) \pm SD; (F) Expression of CD66
141 versus CD62L by blood neutrophils at indicated times. Wilcoxon tests were performed for all paired datasets, and
142 Mann-Whitney tests were performed for unpaired datasets; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$
143

144

145 **Neutrophils modulate PBMC cytokine and chemokine networks in SIV infection**

146 Neutrophils can modulate adaptive immunity through different mechanisms, in HIV
147 infection and other diseases [14,22]. The early and chronic changes in neutrophil
148 activation and the increase of circulating immature neutrophils could be associated
149 with capacities to modulate other immune cells and inflammation.

150 Thus, we further analyzed how neutrophil changes during SIV infection could impact
151 PBMCs and inflammation. We set up an in vitro assay to measure the impact of
152 neutrophils on different pro and anti-inflammatory molecules produced by activated
153 autologous PBMCs in infected CyMs.

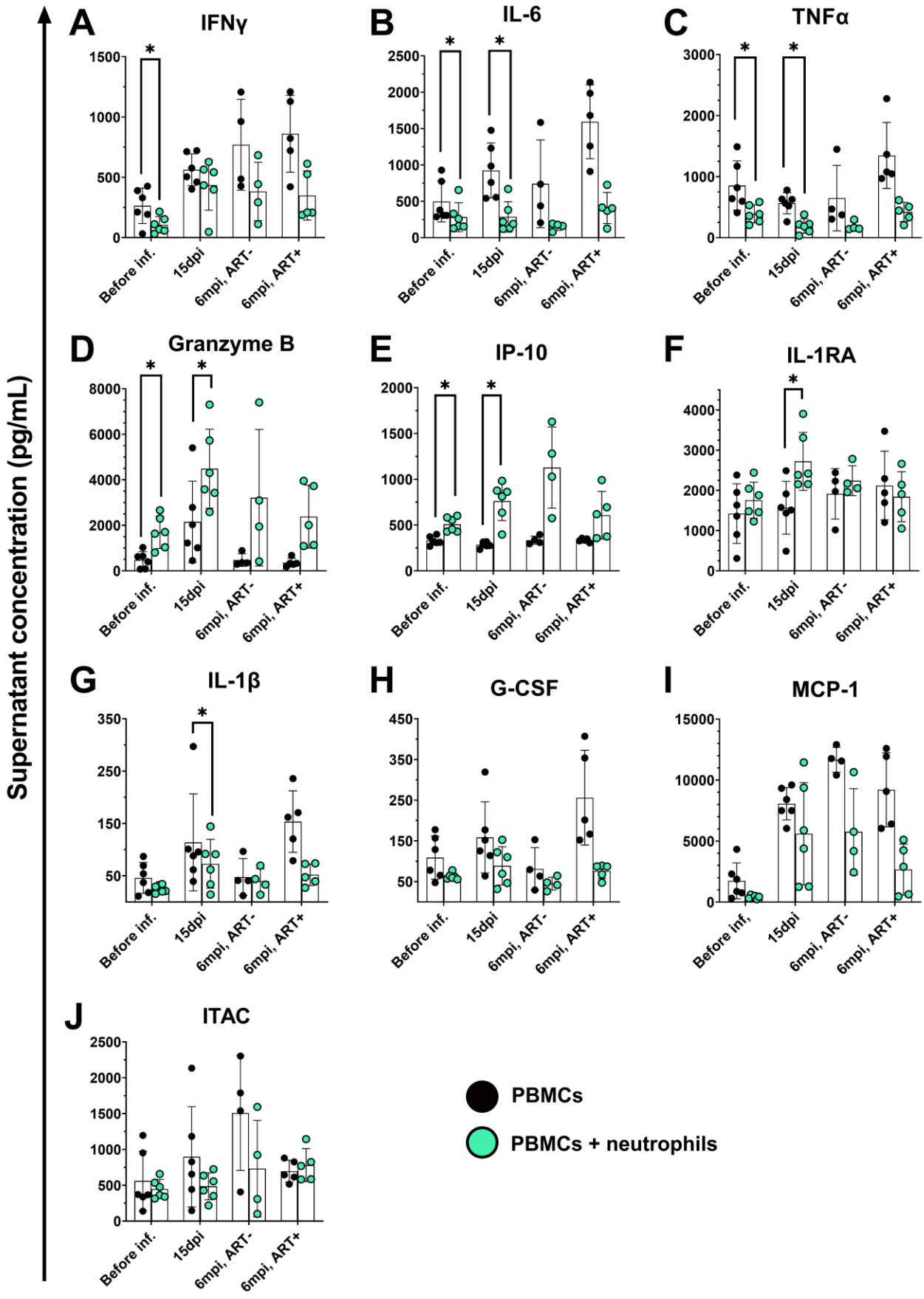
154 After overnight stimulation of T cells with Cytostim, a non-specific stimulation that
155 crosslinks the TCR of T cells and MHC molecules, we quantified the concentration of
156 38 pro and anti-inflammatory chemokines and cytokines in the supernatant of PBMC
157 cultures in the presence or absence of neutrophils, before infection and in primary and
158 chronic phases of SIV infection. Before infection, overnight stimulation of PBMCs in the
159 absence of neutrophils resulted in the production of molecules in culture supernatants,
160 including IFN γ , TNF α or IL-6 (**Figure S2**). Neutrophils induced decreases in IFN γ , IL-6
161 and TNF α concentrations and increases in granzyme B, IP-10, IL-4, IL-17A, VEGF-A and
162 TGF α concentrations (**Figure 2A-2E and Figure S3**).

163 In primary infection, stimulated PBMCs from SIV-infected CyMs produced higher levels
164 of numerous molecules in culture supernatants than before infection, including IFN γ ,
165 IL-1 β , MCP-1, granzyme B, sFasL and IFN α 2. On the contrary, IL-2 and IL-4 levels were
166 significantly decreased in PBMC cultures compared to before infection (**Figure S2**). The
167 presence of neutrophils increased the concentration of granzyme B, IP-10 and IL-1RA
168 in co-culture supernatants (**Figure 2D-2F**) compared to PBMCs cultured alone, while
169 concentrations of IL-6, TNF α and IL-1 β were decreased (**Figure 2A, 2C and 2G**).

170 In the chronic phase, PBMCs produced higher levels of soluble IFN γ , MIG, MCP-1 and
171 IL-22 compared to before infection in both SIV+ ART- and SIV+ ART+ groups.
172 Additionally, PBMCs from treated animals produced significantly higher levels of IL-6,
173 IL-1 β , G-CSF, IFN α 2 MIP-1 α , sFasL and IL-10 compared to baseline, which was not the
174 case for untreated animals (**Figure S2**). At this timepoint, we did not observe significant
175 changes in cytokine production due to neutrophils, as the low number of macaques
176 precluded statistical analyses. However, we found that the concentrations of pro-
177 inflammatory proteins such as IFN γ , IL-6, TNF α and MCP-1 tended to decrease in the
178 presence of neutrophils compared to PBMCs cultured alone in both groups (**Figure**
179 **2A-2C and 2I**), while IL-1 β , G-CSF and IFN α 2 levels tended to decrease in the SIV+
180 ART+ group, but not for SIV+ ART- animals (**Figure 2G and 2H, Figure S3**). In parallel,
181 concentrations of IP-10 and granzyme B tended to increase in co-cultures with
182 neutrophils compared to PBMCs alone in both SIV+ ART+ and SIV+ ART- groups
183 (**Figure 2E**). Finally, the cytokine ITAC tended to be higher in SIV+ ART- animals, and
184 the concentration decreased in the presence of neutrophils (**Figure 2J**).

185 Overall, we observed that neutrophils cultured with PBMCs from SIV-infected CyMs
186 modulated the concentration of key molecules related to T cell and innate cell
187 activation during acute and late phases. These results suggest that neutrophils could
188 modulate PBMCs, and especially T cells, by inducing decreases of IL-6, TNF α and IL-1 β ,
189 while increasing IL1-RA. On the other hand, neutrophils also induced elevation of IP-
190 10, an inflammatory chemokine that attracts T cells. These results suggest an impact of

191 neutrophils on molecules produced by PBMCs and inflammation during early and late
192 pathogenic SIV infection.



194 **Figure 2: Neutrophils modulate the concentration of cytokines and chemokines**
195 **produced by PBMCs in early and late SIVmac251 infection in CyMs**

196 (A-J) Supernatant concentrations of indicated molecules in overnight cultures of stimulated PBMCs without (black
197 dots) or with neutrophils (blue dots) from CyMs before infection ($n = 6$) during the acute (15dpi, $n = 6$) or chronic
198 (6mpi, $n = 4$ SIV+ ART-, $n = 5$ SIV+ ART+) phase of SIVmac251 infection. Individual values are represented by dots
199 and group averages by bars \pm SD. Wilcoxon tests were run for all paired datasets; *: $p < 0.05$

200
201

202 **Neutrophils modulate T cell activation through contact-dependent**
203 **mechanisms in early and late SIV infection**

204 Since neutrophils can modulate PBMC cytokine and chemokine networks during SIV
205 infection, we next focused on their direct impact on T cells. We previously showed that
206 neutrophils increased CD8+ and CD4+ T cell intracellular cytokine production during
207 the acute phase of SIV infection. Thus, we explored if a contact between neutrophils
208 and T cells is required for these interactions, by performing co-cultures of neutrophils
209 and T cells separated or not by a 0.4 μ m Transwell membrane. After overnight
210 stimulation with Cytostim, in the presence or absence of neutrophils, we used
211 intracellular staining and flow cytometry to analyze CD4+ and CD8+ T cell activation
212 (CD69), cytotoxic activity (CD107a) and intracellular cytokine production (TNF α , IFN γ ,
213 IL-17A and IL-2).

214 Before SIV infection, neutrophils had mild effects on T lymphocyte activation with no
215 significant impact on CD69, IFN γ , CD107a or IL-17A expression in T cells (**Figure S4A-**
216 **S4F**). Neutrophils reduced TNF α production significantly in CD4+ T cells and CD8+ T
217 cells and IL-2 production in CD4+ T cells, but not CD8+ T cells. These results suggest
218 neutrophils can decrease T cell production of TNF α and IL-2 at steady state (**Figure**
219 **S4G-S4J**).

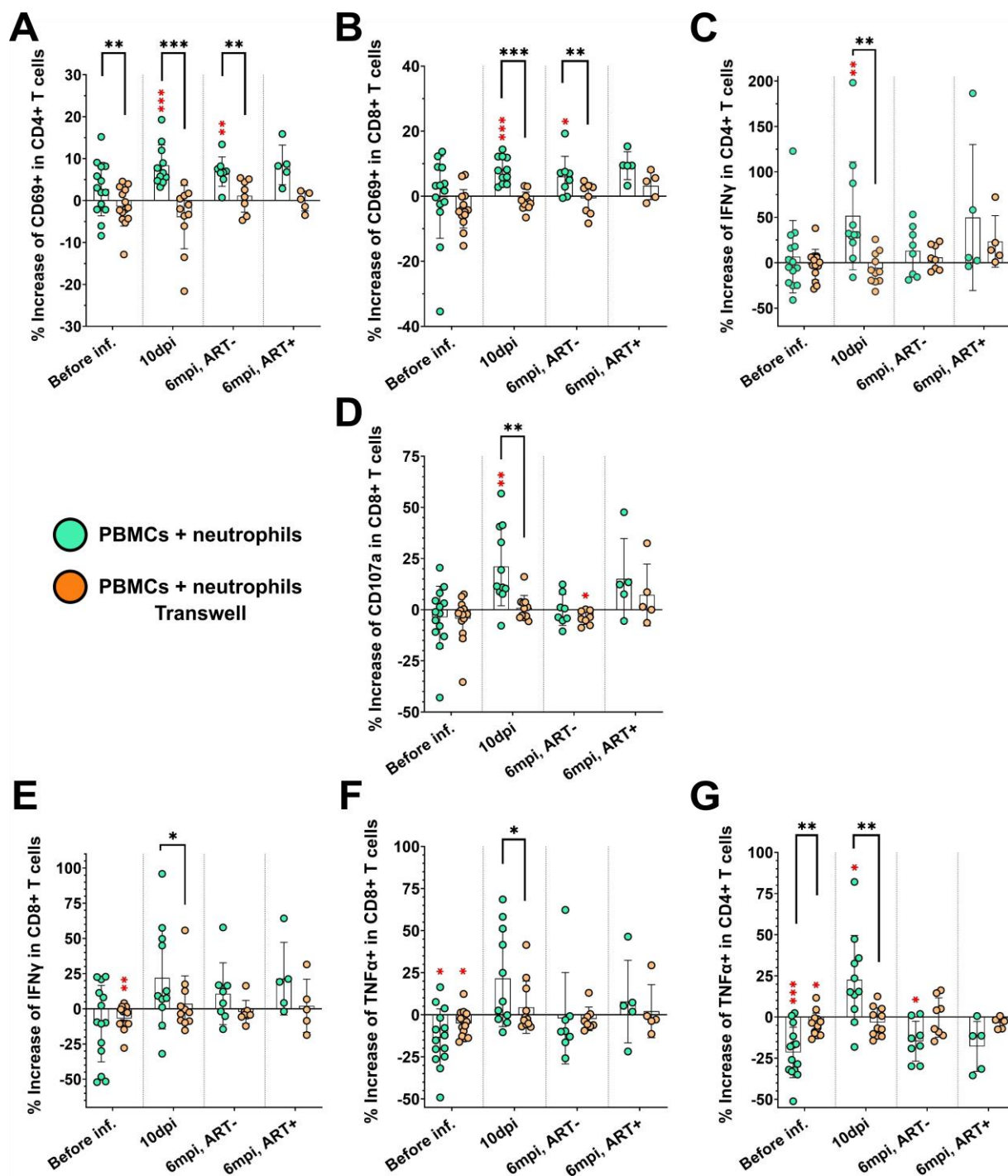
220 In primary infection, both frequencies of CD69+ CD8+ and CD69+ CD4+ T cells
221 significantly increased in the presence of neutrophils compared to PBMCs cultured
222 alone (**Figure S4A and S4B**). We normalized the data by calculating percentages of
223 increased activated T cells in the presence of neutrophils compared to PBMCs alone,

224 with or without Transwells. We found that CD69 expression was not increased when
225 neutrophils and T cells were not in contact (**Figure 3A and 3B**). Similarly, neutrophils
226 increased IFN γ in CD4 $^+$ T cells (**Figure S4C**), CD107a expression in CD8 $^+$ T cells (**Figure**
227 **S4F**) and TNF α production in CD8 $^+$ and CD4 $^+$ T cells (**Figure S4G and S4H**). We
228 found a similar trend of IFN γ expression in CD8 $^+$ T cells, but it did not reach statistical
229 significance (**Figure S4D**). When PBMCs and neutrophils were separated by Transwells,
230 no significant effects of neutrophils were observed on T cell activation (**Figure 3C-3G**).
231 Additionally, neutrophils did not affect IL-17A in CD4 $^+$ T cells, IL-2 production in CD4 $^+$
232 T cells or CD8 $^+$ T (**Figure S4E, S4I and S4J**).

233 At 6mpi, in the SIV $^+$ ART $^-$ group, neutrophils increased CD69 expression on CD8 $^+$ and
234 CD4 $^+$ T cells when cells were in contact, but not in the Transwell condition (**Figure 3A**
235 **and 3B**). No significant effects were observed during the chronic phase regarding the
236 production of TNF α , IFN γ and CD107a on CD4 $^+$ and CD8 $^+$ T cells (**Figure S4**). Similar
237 to before infection, TNF α $^+$ CD4 $^+$ and IL-2 $^+$ CD4 $^+$ T cells were decreased in the
238 presence of neutrophils in SIV $^+$ ART $^-$ animals, but not in the Transwell condition
239 (**Figure 3G, S4G and S4I**).

240 Here, we show that neutrophils directly impacted T cells by increasing CD4 $^+$ and CD8 $^+$
241 T cell activation, cytokine production and CD8 $^+$ T cell cytotoxicity during the acute
242 phase of SIVmac251 infection in CyMs. Neutrophils still increased CD69 expression on
243 T cells after 6 months of SIV infection, but not the other markers.

244 Altogether, these results demonstrate that direct activation of T cells by neutrophils
245 during acute and chronic phases of SIVmac251 infection relies on contact-dependent
246 mechanisms.



247

248 **Figure 3: Neutrophils increase T cell activation in a contact-dependent manner**
 249 **during SIV infection**

250 (A-G) Percentages of increase of positive T cell frequencies for indicated markers after overnight cultures of
 251 stimulated PBMCs with neutrophils in contact (blue dots) or separated by a 0.4 μ m transwell membrane (orange
 252 dots). Experiments were performed before infection ($n = 14$) during the acute (10dpi, $n = 11$) or chronic (6mpi, $n =$
 253 8 SIV+ ART-, $n = 5$ SIV+ ART+) phase of SIVmac251 infection. Individual values are represented by dots and group
 254 averages by bars \pm SD. Wilcoxon tests were performed for paired datasets (black stars) *: $p < 0.05$; Non-parametric
 255 one sample Wilcoxon tests were performed with theoretical median $Me = 0$ (red stars)

256 **Neutrophil signatures discriminate between uninfected, acute and**
257 **chronic SIV infection +/- ART**

258 Our findings show that neutrophils are phenotypically changed during SIV infection in
259 CyMs, and these changes are associated with contact-dependent T cell modulation in
260 acute and chronic SIV infection. We then interrogated if the observed changes in
261 neutrophils during SIV infection could constitute biomarkers that distinguish the
262 different groups of CyMs using partial least-squares discriminant analysis (PLS-DA). We
263 based this discriminant analysis on two datasets: whole blood neutrophil phenotypes
264 and direct effects of neutrophils on T cell activation (Figure 3). Direct modulatory effects
265 of neutrophils were expressed as percentages of increases of expression for each T cell
266 marker, as reported in Figure 3. In this analysis, we identified parameters contributing
267 the most to the separation between uninfected and SIV+ macaques at the different
268 time points of infection, according to their variable importance score (**Figure S5A-**
269 **S5C**).

270 With this approach, we found that the most discriminating features between SIV-
271 infected CyMs at 10dpi and uninfected animals were related to the neutrophil
272 modulation of T cell activation (**Figure 4A and 4B**). Increased TNF α +, CD107a+ and
273 IFN γ + CD8+ T cells in the presence of neutrophils were the parameters that
274 contributed the most to the discrimination of early infected animals, as well as the
275 frequency of CD62L- neutrophils. On the contrary, uninfected animals were
276 characterized by a high frequency of mature neutrophils and by parameters unrelated
277 to neutrophils, including T cell activation in the absence of neutrophils (**Figure 4B**).

278 Likewise, we performed a PLS-DA analysis to compare uninfected animals, SIV+ ART-
279 and SIV+ ART+ CyMs. From this analysis, the first component allowed us to
280 discriminate uninfected animals against both groups of chronically infected macaques,
281 while the second component allowed us to compare SIV+ ART+ versus SIV+ ART-
282 animals (**Figure 4C**). Variables that discriminated the most chronically infected CyMs
283 from uninfected macaques were related to direct neutrophil modulation of T cells,

284 including modulation of IFN γ + and TNF α + CD8+ T cell frequencies, and CD69+ CD8+
285 and CD69+ CD4+ T cell frequencies (**Figure 4D**). Lastly, by analyzing component 2 of
286 the PLS-DA, we found that these neutrophil-related signatures were more discriminant
287 in SIV+ ART+ CyMs than SIV+ ART- CyMs (**Figure 4E**). Additionally, untreated CyMs
288 were characterized by a high frequency of CD62L- neutrophils, while SIV+ ART+ CyMs
289 showed a higher proportion of mature neutrophils.

290 Together, these results indicate that in early or late SIV infection, SIV-infected CyMs
291 were characterized by elevated signatures related to neutrophil phenotype and
292 capacities to modulate directly T cell activation, compared to uninfected animals.
293 Furthermore, neutrophils had higher capacities to modulate T cell function in treated
294 animals than untreated animals during the chronic phase of infection.

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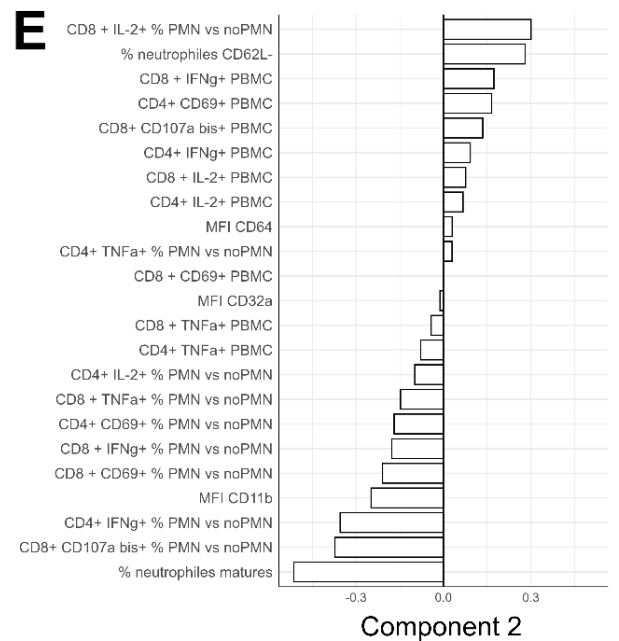
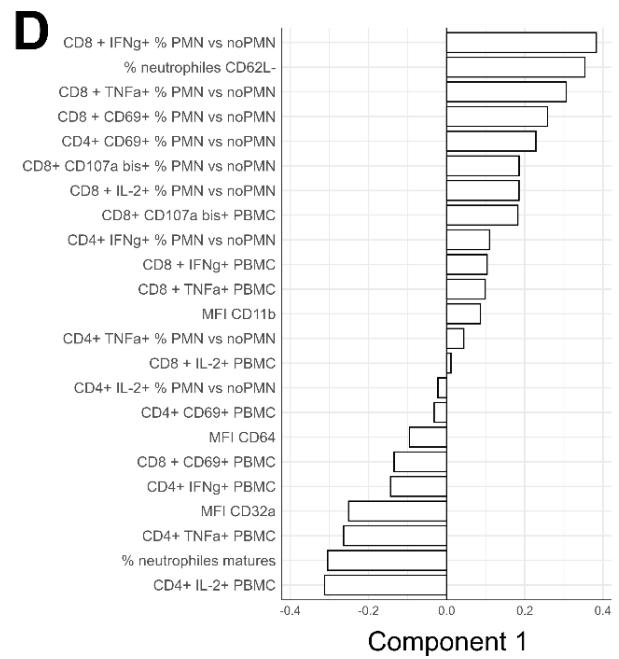
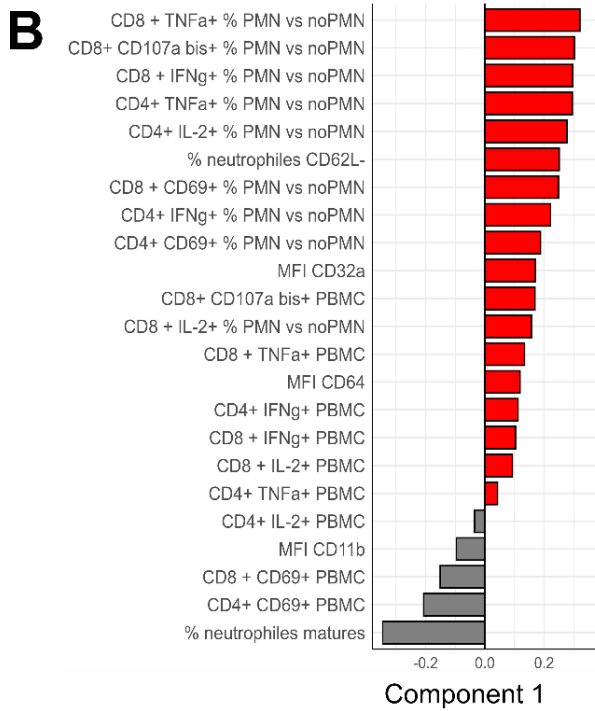
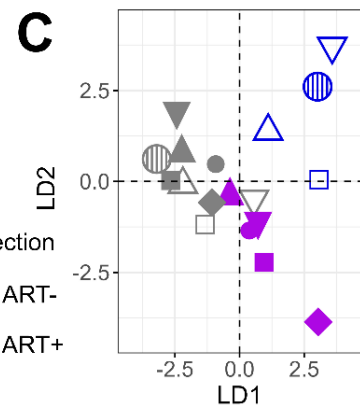
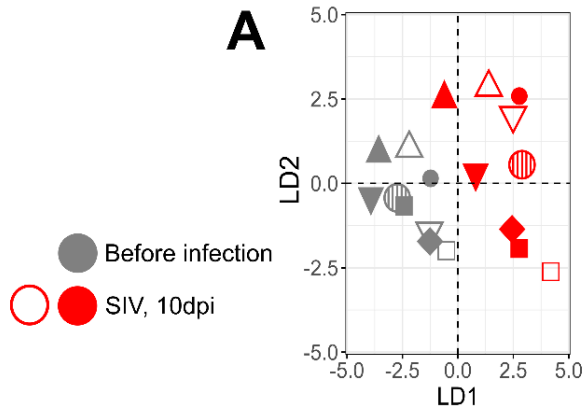
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303 **Figure 4: Neutrophil modulatory properties are key features of SIVmac251**
304 **infection in macaques**

305 (A) Partial Least-Squares Discriminant Analysis (PLS-DA) comparing uninfected (grey) and SIV-infected CyMs at
306 10dpi (red); (B) Parameters contributing the most to the 1st component of the corresponding PLS-DA; (C) PLS-DA
307 comparing uninfected (grey), SIV+ ART- CyMs (blue) and SIV+ ART+ CyMs (purple) at 6mpi; (D) Parameters
308 contributing the most to the 1st component of the corresponding PLS-DA; (E) Parameters contributing the most to
309 the 2nd component of the corresponding PLS-DA. Annotations: CD8+CD69+% PMN vs noPMN: percentage of
310 increase of CD69+CD8+ T cells frequency in the presence of neutrophils. CD8+CD69+ PBMC: frequency of
311 CD69+CD8+ T cells without neutrophils. MFI: mean fluorescence intensity

312

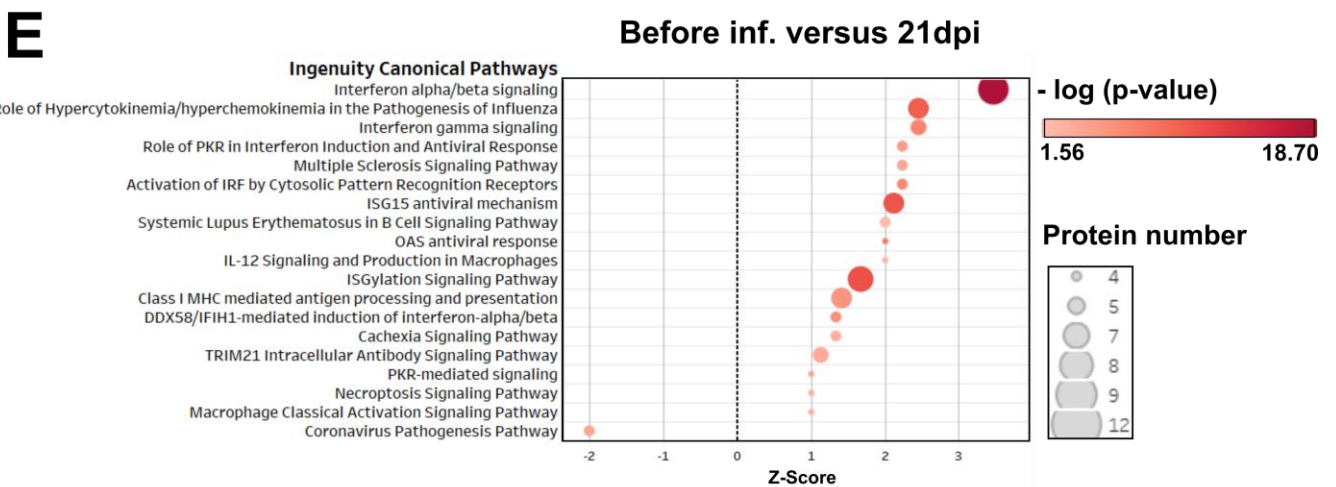
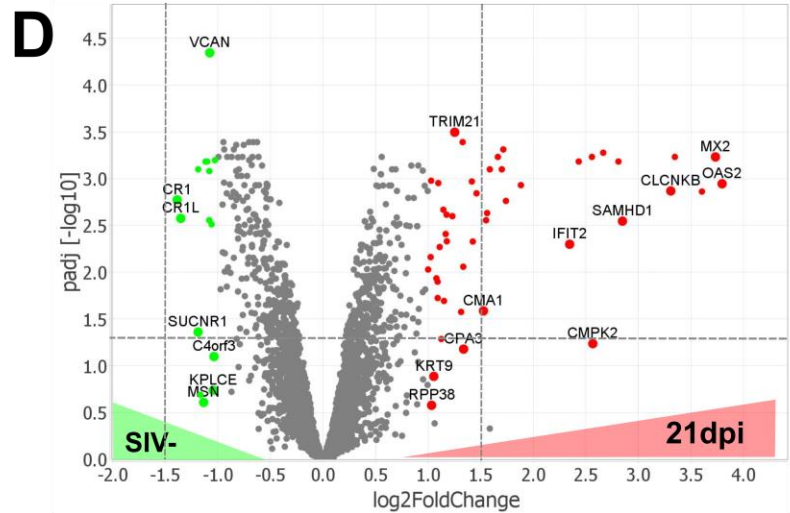
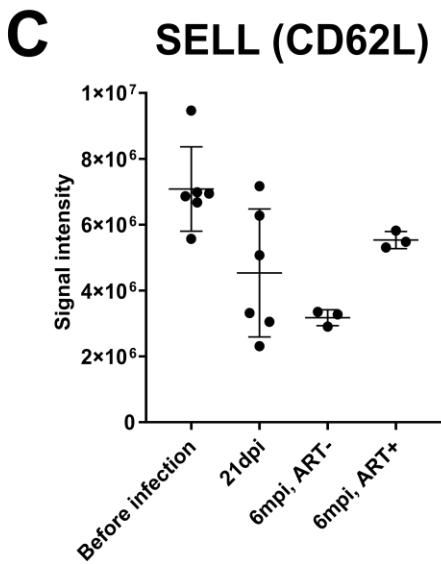
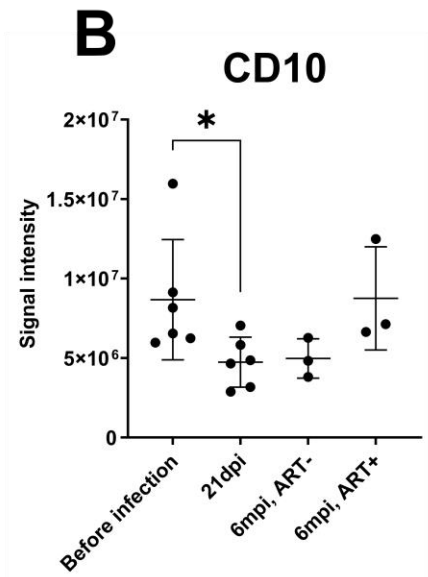
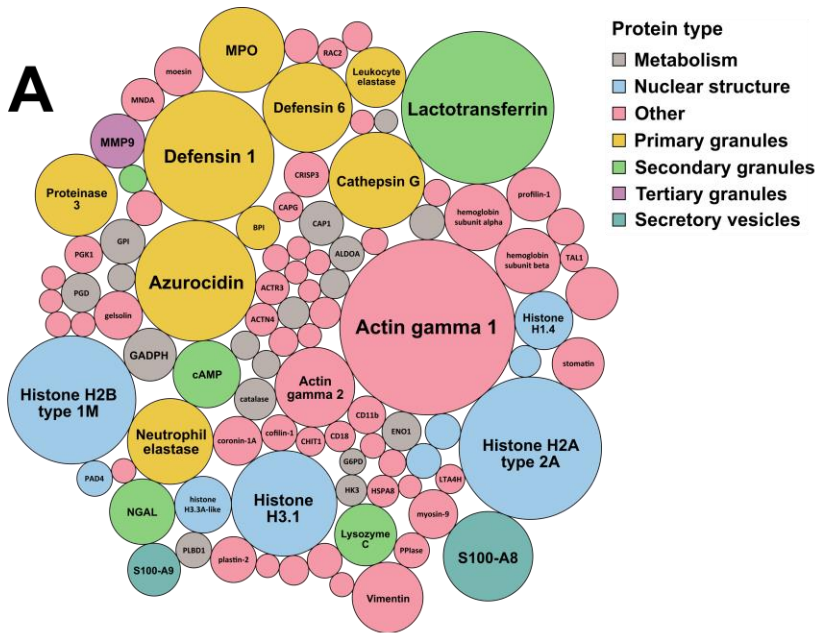
313 **Type I interferon pathways are upregulated in neutrophil proteomes**
314 **in primary and chronic SIV infection**

315 Because neutrophils express low levels of RNA but display high protein content, we
316 decided to perform proteomic assays rather than transcriptomic assays [23]. Hence, we
317 explored proteome changes in purified blood neutrophils induced during SIV infection
318 in CyMs. We used data-independent acquisition mass spectrometry (DIA-MS)
319 proteomic analysis on purified blood neutrophils and assessed the impacts of SIV
320 infection and ART on neutrophils. We identified 5767 proteins in CyM neutrophils, with
321 few sample variations (**Figure S6A**). Before infection, the most abundant proteins in
322 macaque neutrophils were similar to those previously described in human neutrophil
323 proteomes [24], including lactotransferrin, azurocidin, neutrophil defensins, cathepsin
324 G, S100-A8, elastase and myeloperoxidase. We regrouped the most abundant proteins
325 into categories: metabolism, nuclear structure and granules or vesicles (**Figure 5A and**
326 **Figure S6B**). To confirm our previous results on the increase of immature CD10-
327 neutrophils and downregulation of CD62L following SIV infection, we analyzed CD10
328 and CD62L expression and observed a similar dynamic (**Figure 5B and 5C**).

329 We then performed differentially expressed proteins (DEPs) analysis between
330 timepoints and groups of macaques. In primary infection (21dpi), we identified 23 DEPs,
331 which were all upregulated in neutrophils compared to baseline (**Figure 5D**). These
332 DEPs included proteins related to antiviral activities, such as RIG-I, MX2 or SAMHD1,
333 and proteins linked to interferon pathways, such as IFIT2. We further performed a
334 pathway analysis and found that neutrophils from SIV-infected animals showed

335 upregulated pathways associated with type I and type II interferon signaling, antiviral
336 responses and class I MHC-mediated antigen processing and presentation (**Figure 5E**).

337



339 **Figure 5: Neutrophils from untreated CyMs display increased expression of**
340 **proteins related to interferon pathways**

341 (A) Mass spectrometry signal intensities of the fifteen most abundant proteins in neutrophil proteomes of $n = 6$
342 uninfected CyMs with individual values (dots) or average values (bars) \pm SD; (B-C) Mass spectrometry signal
343 intensities for CD10 and CD62L in neutrophils from CyMs before infection ($n = 6$) during the late acute (21dpi, $n =$
344 6) or chronic (6mpi, $n = 3$ SIV+ ART-, $n = 3$ SIV+ ART+) phase of SIVmac251 infection; (D) Volcano plot of DEPs in
345 neutrophils from CyMs infected with SIVmac251 at 21dpi (right) compared to before infection (left); (E) Pathways
346 associated with DEPs in neutrophils from CyMs infected with SIVmac251 at 21dpi. Pathways were obtained using
347 Ingenuity Pathway Analysis. Wilcoxon tests *: $p < 0.05$
348

349 In the chronic phase (6mpi), we found 30 DEPs in neutrophils from SIV+ ART- animals
350 and 25 DEPs in neutrophils from SIV+ ART+ animals compared to uninfected animals
351 (**Figure 6A and 6B**). Signal intensities of proteins related to antiviral defense, such as
352 RIG-I, OASL or MX1, and proteins linked to type I interferon pathways, such as IFIT1
353 and IFI16 increased at 21pi remained high at 6mpi in SIV+ ART- animals (**Figure 6C-**
354 **6E, Figure S7A and S7B**). We then compared DEPs in neutrophils from SIV+ ART- and
355 SIV+ ART+ CyMs to baseline. We found that some of the DEPs were common in the
356 two groups, including proteins related to type I interferon pathways such as IFIT1, and
357 IFIT3, but also antiviral proteins such as OASL and MX2 (**Figure 6A and 6B**). For these
358 proteins, neutrophils from SIV+ ART+ animals presented intermediate intensities
359 between uninfected and SIV+ ART- animals at 6mpi (**Figure 6C-6E**). Additionally,
360 neutrophils from SIV+ ART+ animals showed DEPs specific to their group, with
361 ribosomal proteins such as FAU and several large ribosomal subunit proteins (RPL).
362 These proteins were not increased in neutrophils from SIV+ART- animals, or to a lesser
363 extent (**Figure 6B and 6F**).

364 To assess the effect of ART on neutrophil proteomes, we next compared neutrophil
365 DEPs from treated and untreated animals at 6mpi. First, we identified significantly
366 upregulated or downregulated proteins in both groups compared to uninfected
367 animals. Then, we established proteins with fold changes significantly different
368 between ART+ and ART- groups (**Figure S8A**) and proceeded to pathway analyses.
369 SIV+ ART- neutrophils were characterized by the upregulation of type I interferon
370 signaling, interferon gamma signaling and mitochondrial protein degradation (**Figure**
371 **6G and S8B**), while neutrophils from SIV+ ART+ animals displayed an upregulation of

372 pathways related to RNA processing, integrin signaling, EIF2 signaling and neutrophil
373 degranulation (**Figure 6G and S8C**). Importantly, SIV+ ART+ animals did show
374 upregulation of IFN α / β signaling, but these signatures were not dominant compared
375 to untreated animals (**Figure S8C**).

376 These results indicate acute SIV infection-induced changes in neutrophil proteomes,
377 which were characterized by a higher expression of proteins related to type I/type II
378 IFNs and antiviral pathways. These pathways persisted in neutrophils after 6 months of
379 infection in CyMs, while an administration of ART induced a partial reversion of these
380 neutrophil signatures, with a return to metabolic and homeostatic activities.

381

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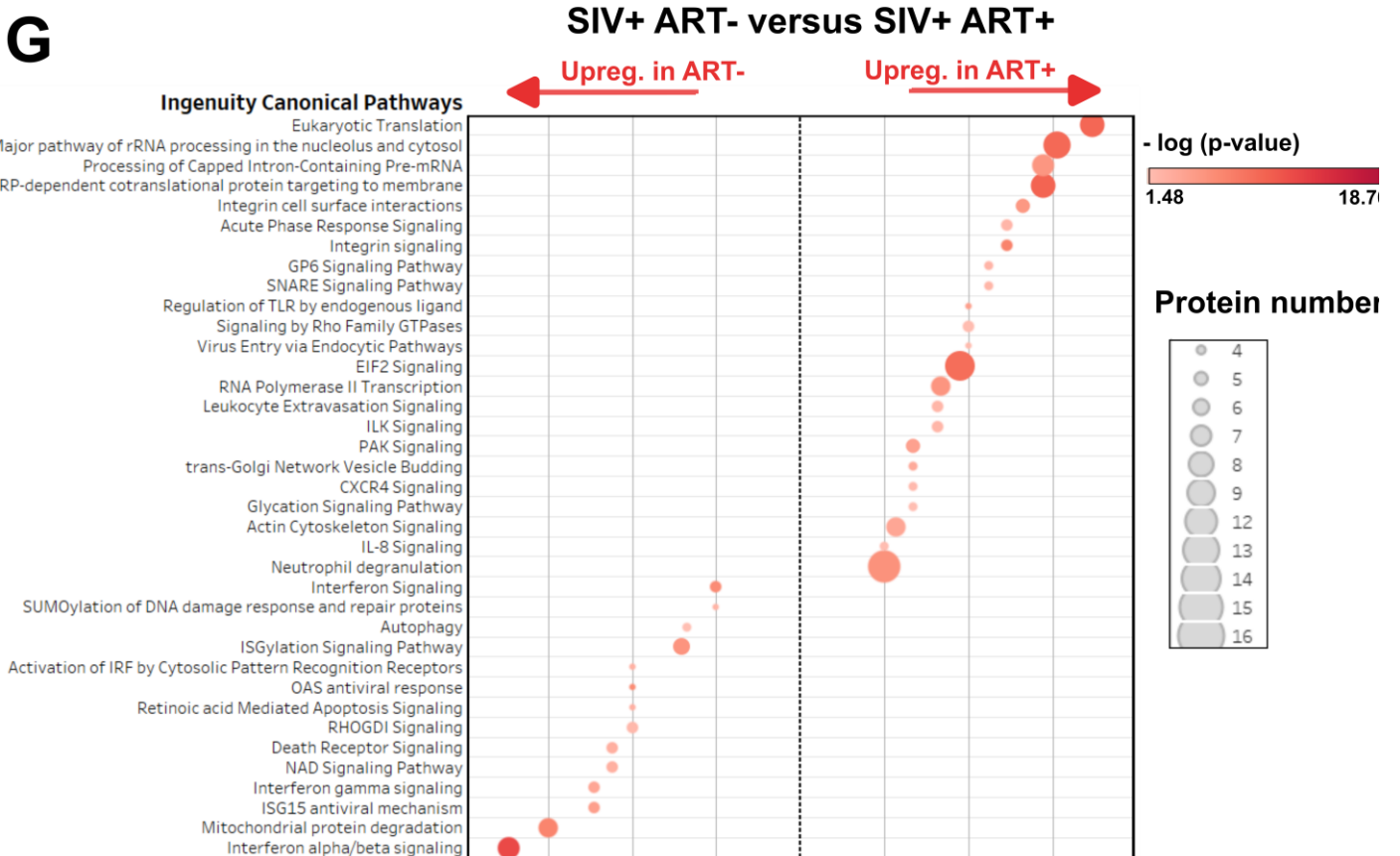
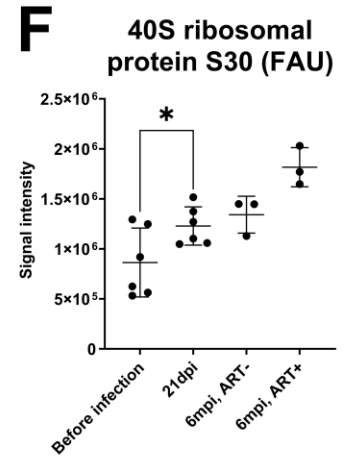
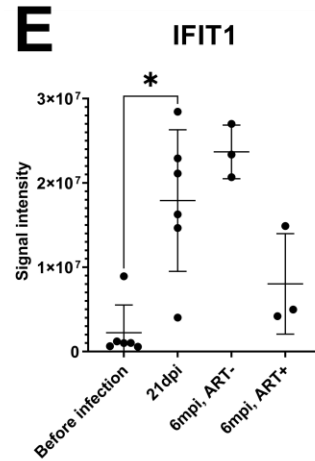
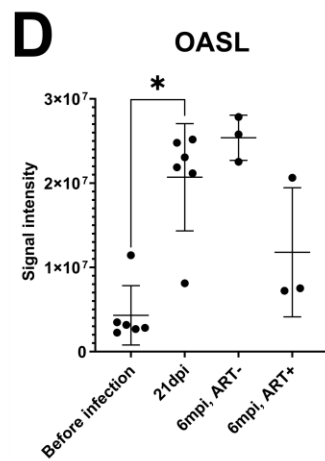
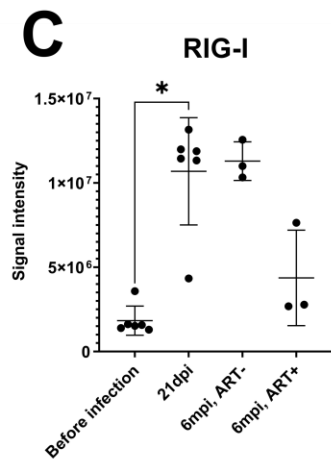
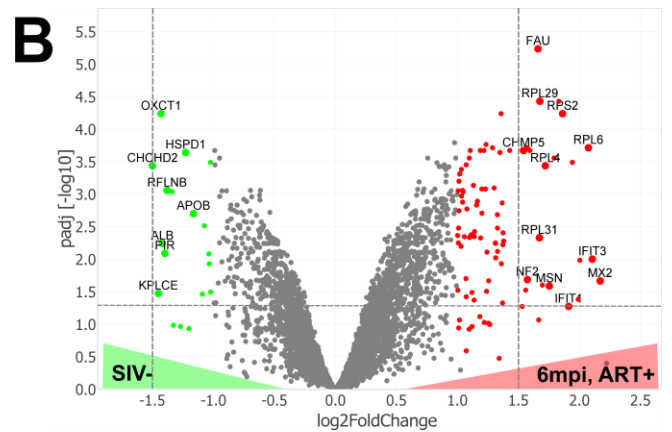
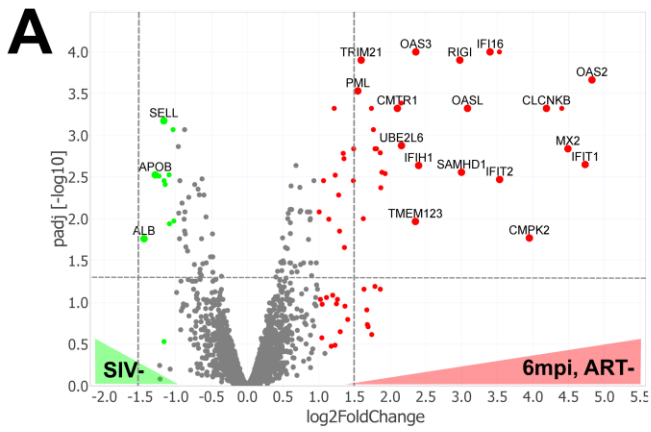
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384

385 **(Fig. next page)**

386 **Figure 6: Upregulation of antiviral and interferon-related pathways in CyMs**
387 **neutrophils in early and late SIV infection, partially resolved with ART**

388 (A) Volcano plot of DEPs in neutrophils from CyMs infected with SIVmac251 without ART at 6mpi; (B) Volcano plot
389 of DEPs in neutrophils from CyMs infected with SIVmac251 with ART at 6mpi; (C-F) Scatter plots of mass
390 spectrometry signal intensities for RIG-I, OASL, IFIT1 and FAU proteins in uninfected and SIVmac251-infected CyMs;
391 (G) Using Ingenuity Pathway Analysis, pathways associated with DEPs in neutrophils from chronically SIV-infected
392 CyMs, with or without ART, compared to before infection. Statistics: Wilcoxon tests, *: $p < 0.05$



394 **DISCUSSION**

395 Investigation of the immune events leading to inflammation in HIV infection remains
396 challenging. Here, we took advantage of the cynomolgus macaque model infected with
397 SIVmac251, which shares most features of HIV physiopathology, to investigate the role
398 of neutrophils in shaping T cell activities and inflammation. In this work, we
399 demonstrate that SIV in CyMs leads to changes in neutrophil phenotype and function,
400 particularly in their capacity to modulate T cell activation and cytokines or chemokines
401 produced by PBMCs.

402 In HIV infection, neutrophils display an activated phenotype associated with altered
403 functions [12], and have been shown to infiltrate gut tissues in patients and NHP
404 models during chronic infection [13,25]. Yet, their roles in inflammation are not fully
405 elucidated, and neutrophils' beneficial versus detrimental effects are discussed [15].

406 Here, using flow cytometry on whole blood cells, we report that SIVmac251 acute
407 infection was associated with increased frequencies of CD10⁻ CD101⁺ immature
408 neutrophils in the blood of infected macaques, confirming previous results [19]. The
409 simultaneous decrease in neutrophil counts may suggest accelerated hematopoiesis
410 due to neutrophil recruitment in tissues or increased neutrophil apoptosis, as described
411 by others [26]. We also found that CD62L shedding was mainly driven by mature
412 neutrophils, and not immature, suggesting that mature subsets may be more
413 responsive to activation than immature neutrophils. We also assessed the impact of
414 ART on neutrophil phenotype and modulatory functions during chronic SIV, and found
415 that treated animals showed a restored neutrophil phenotype compared to untreated
416 animals. Additional analysis of CD10 and CD62L expressions using proteomics also
417 confirmed these observations. Increased frequencies of immature neutrophils were
418 found in HIV patients in the low-density granulocytes subset (LDG), yet were not
419 assessed in normal-density granulocytes (NDG) [26].

420 Only a few studies were conducted to investigate neutrophil modulation of other
421 immune cells in HIV infection or their impact on inflammation. In chronic HIV infection,

422 several teams have shown that PD-L1 and arginase-1 expressions were increased in
423 neutrophils and associated with suppressive functions towards T cell activation [16,27].
424 On the other hand, Dunsmore et al demonstrated that blood neutrophils from PLWHIV
425 receiving ART were associated with T cell activation through the release of soluble
426 galectin-9 [18]. Here, we showed neutrophils could modulate other immune cell
427 activities during acute and chronic SIVmac251 pathology in CyMs. We demonstrate
428 that neutrophils exerted effects both on soluble effector molecules and directly on T
429 cells, hence that neutrophils can impact PBMC cytokine/chemokine networks and T cell
430 activation directly.

431 To our knowledge, it is the first report on neutrophil modulation of soluble cytokines
432 and chemokines produced by PBMCs during SIV/HIV infection. In our work, neutrophils
433 mostly induced decreased concentrations of soluble cytokines and chemokines
434 released by T cells, including IL-6, IL-1 β , and TNF α . Additionally, during the acute phase
435 of infection, IL-1RA levels were increased in the presence of neutrophils. IL-1RA is an
436 antagonist of IL-1 that mostly plays an anti-inflammatory role and can be produced by
437 various immune cells, including neutrophils [28]. Elevated plasma concentrations of IL-
438 1RA were described during acute SIV infection in PLWHIV and untreated macaques,
439 but not in macaques receiving ART [29,30]. This points out that in our model,
440 neutrophils could regulate inflammation during the acute phase of SIV infection. In ex-
441 vivo assays, neutrophils were shown to process and regulate different inflammatory
442 cytokines such as inactivation of IL-1 β through proteases release [31] or processing of
443 TNF α and IFN γ , resulting in oxygen species release [32,33]. Nonetheless, in our study,
444 neutrophils also induced higher levels of the T-cell chemoattracting chemokine IP-10
445 during acute SIV infection. Similar trends were observed in chronic SIV infection, even
446 in treated macaques. This chemokine was shown to be highly involved in HIV infection
447 in early and chronic HIV infection [34]. Indeed, high plasmatic levels of IP-10 were
448 found in PLWHIV during early infection [35] but also in chronic patients receiving ART
449 and elite controllers [36]. Plasma IP-10 levels were recently linked to increased mortality
450 in HIV patients [37]. Additionally, Ramirez et al showed in 2014 that stimulated PBMCs

451 from ART-treated PLWHIV exposed to recombinant IP-10 secreted lower levels of IFN γ
452 and IL-6 than unexposed PBMCs [38]. As such, we may hypothesize that neutrophils
453 could play dual roles in local and systemic inflammation by increasing IP-10 levels in
454 HIV infection, while decreasing inflammatory cytokine concentrations. Whether IP-10
455 plays a role in decreasing TNF α , IL-6 and IL-1 β levels remains to be elucidated.

456 In a previous work, Lemaitre et al. showed that neutrophils increased T cell activation
457 during the acute phase of SIVmac251 infection in CyMs [19]. Here, we confirmed these
458 results and extended this observation to a cytotoxic CD8⁺ T cell marker, CD107a, which
459 expression also increased in the presence of neutrophils co-cultured with PBMCs.
460 Additionally, we demonstrate that these direct effects of neutrophils on T cell activation
461 were only found when neutrophils and PBMCs were in contact, but not separated by
462 Transwell membranes. These results suggest that the mechanisms involved in the
463 modulation of T cell activation are triggered by cell-cell contacts. Although we did not
464 test this hypothesis, others have reported direct T cell priming by NET release [39]. In
465 this intracellular cytokine assay, we did not find any clear difference between untreated
466 and SIV⁺ ART⁺ animals during chronic SIV. This may be due to the low number of
467 macaques included in the study, or because 5 months of treatment were not long
468 enough to assess differences between groups. Indeed, several ART⁺ animals presented
469 viral loads above the detection threshold, which may reflect a low-grade inflammation
470 in these CyMs.

471 In HIV infection, interferon-related signatures have been described in patients [40] and
472 the roles of type I IFNs during the infection are still under investigation [41], but the
473 involvement of neutrophils in these signatures is unknown. By analyzing proteomes
474 from purified neutrophils, we showed that neutrophils from SIV-infected CyMs
475 displayed interferon type I and type II signatures after the acute phase of infection.
476 Type I IFN pathways persisted after 6 months of infection in treated and untreated
477 animals. However, in ART-treated animals, neutrophils were characterized by pathways
478 related to cellular and transcriptional activities. Contrary to transcriptional analyses,

479 only a few studies focused on in-depth proteomic analyses of isolated human
480 neutrophils. A study of 2024 reported transiently elevated type I IFN pathways in
481 critically severe COVID-19 patients, associated with increased expression of MX1 and
482 MX2 [42]. Similar signatures were reported in transcriptional studies in macaques
483 infected with SARS-Cov2 [43], but also in other diseases such as tuberculosis and auto-
484 immune disorders [44,45]. To our knowledge, this is the first report of IFN pathway
485 signatures in neutrophils from SIV or HIV-infected individuals. Additionally, our data
486 suggest that neutrophils could be involved in antiviral mechanisms against SIVmac251,
487 as we found increased expressions of proteins that detect or induce SIVmac251
488 restriction such as RIG-I and SAMHD1 respectively. Human neutrophils were also
489 previously reported to detect and restrict HIV infection in ex-vivo assays [46,47].

490 We are aware this work presents some limitations. First, it is known that isolating fresh
491 neutrophils can be challenging, and so far, there are no existing methods to purify
492 neutrophils directly from whole blood of non-human Primates. Here, we developed a
493 method to obtain a >95% pure population of neutrophils that involves several steps,
494 including the use of Ficoll-Paque, which may have induced neutrophil activation.
495 Besides, we could not identify a specific mechanism or molecule involved in the
496 neutrophil modulation of T cells, nor did we perform any T-cell suppressive assay of
497 SIVmac251-infected cells to assess if T cell functionality was changed in the presence
498 of neutrophils. Finally, we worked on a small number of macaques, which prevented us
499 from performing statistical analyses during chronic SIVmac251 infection.

500 Further analysis will be required to study neutrophil-T cell interactions. For the next
501 experiments, we could use confocal microscopy to perform protein staining of cellular
502 synapses and potential T cell entrapment in NETs. To study the crosstalk between
503 neutrophils and T cells in tissues during SIV infection, analyzing their cellular
504 distribution in different organs such as the spleen, a lymphoid organ reservoir for SIV,
505 will also be necessary. In colorectal cancer, neutrophils were shown to increase T cell
506 activation, and cells were found to co-localize in biopsies from patients [48].

507 Overall, we report that neutrophils modulate inflammatory molecules and induce
508 contact-dependent T cell activation during SIVmac251 infection in CyMs. Additionally,
509 our work suggest neutrophils' involvement in inflammation as they displayed IFN type
510 I pathways and were associated with increased IP-10 concentrations in the presence of
511 PBMCs. In the future, deciphering the role of neutrophils in HIV infection could lead to
512 the identification of new therapeutic targets to lower chronic inflammation in PLWHIV.

513

514 **ACKNOWLEDGEMENTS**

515 We thank all members of the Animal Science and Welfare, FlowCyTech, Immunology
516 and Infectiology core facilities of the IDMIT infrastructure for their excellent expertise
517 and outstanding contributions: Quentin Sconosciuti, Maxime Potier, Victor Magneron,
518 Eleana Navarre, Jean-Marie Robert, Emma Burban, Sebastien Langlois, Kyllian Lheureux,
519 Matthieu Van Tilbeurgh, Marco Leonec, Wesley Gros, Laetitia Bossevot and Sylvie
520 Legendre. We also thank Oscar Haigh for his contribution to proteomic data analysis.

521

522 **METHODS**

523 **Non-human primates and sampling**

524 Fifteen cynomolgus macaques (*Macaca fascicularis*) imported from Mauritius were
525 housed at the IDMIT infrastructure animal facility at the CEA, Fontenay-Aux-Roses,
526 France. Animals expressing the H6 MHC haplotype were excluded because of their
527 increased natural capacity to control SIVmac251 infection [21]. All animals were
528 infected by intravenous inoculation with 1000 animal infectious dose 50% (AID50) of
529 pathogenic SIVmac251 isolate and followed for 6 months. Six SIV-infected animals
530 were treated daily subcutaneously, starting from 28 days post-infection, with a
531 combination of two nucleoside reverse-transcriptase inhibitors, emtricitabine (40
532 mg/kg) and tenofovir disoproxil fumarate (5.1 mg/kg) and one integrase inhibitor,

533 dolutegravir (2.5 mg/kg) [49]. Nine other animals remained untreated. Blood was drawn
534 and collected in heparin-lithium tubes under anesthesia, consisting of an intramuscular
535 injection of zolazepam/tiletamine (10mg/kg; Virbac, Carros, France). The macaque
536 study was approved by the « Ministère de l'Éducation Nationale, de l'Enseignement
537 Supérieur et de la Recherche » (France) and the Ethic Committee « Comité d'éthique en
538 expérimentation animale n°44 ». Animals were handled in accordance with national
539 regulations (CEA accreditation number D92-032-02) and European Directive (2010/63,
540 recommendation n°9) in compliance with the standards for Human Care of the Office
541 for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-
542 01 and #F22-00556.

543 ***Immunophenotyping of whole blood neutrophils***

544 Heparinized whole blood was processed within 1h following blood draw to preserve
545 neutrophils. For phenotypic analysis, antibody staining mix was added to 100µL of
546 heparinized whole blood for 15min at room temperature. Samples were lysed and fixed
547 using BD FACs Lysing (BD Bioscience) for 15min at room temperature. Cells were
548 washed three times with PBS and fixed (Fixation Buffer, Biolegend). Acquisition was
549 performed with a ZE5 BioRad cytometer and analysis using FlowJo software.

550 CD64 (10.1, BD Bioscience), CD10 (HI10a, Biolegend), CD45 (REA1023, Miltenyi Biotec),
551 CD3 (SP34.2, BD Bioscience), CD123 (7G3, BD Bioscience), CD8a (RPAT8, BD Bioscience),
552 CD20 (2H7, BD Bioscience), CD62L (SK11, BD Bioscience), CD11b (REA713, Miltenyi
553 Biotec), CD14 (REA599, Miltenyi Biotec), CDw125 (REA705, Miltenyi Biotec), PD-L1
554 (29E.2A3, Biolegend), CD101 (REA954, Miltenyi Biotec), HLA-DR (L234, Biolegend),
555 CD32a (IV.3, Stemcell) and CD66abce (TET2, Miltenyi).

556 ***Granulocyte and PBMC isolation***

557 To separate the different cell fractions, 6mL of freshly collected heparinized blood was
558 diluted 1:3 with PBS and was layered on Ficoll-Paque solution. After a 20min
559 centrifugation, the mononuclear cell layer and the pellet containing granulocytes were

560 collected separately and processed with pre-warmed lysis solution for 8min to
561 eliminate erythrocytes. To enrich the granulocytes fraction in neutrophils, a magnetic
562 depletion was performed: cells were stained with anti-CD125 antibodies conjugated to
563 PE (Miltenyi Biotec) and washed with AutoMACs Buffer (Miltenyi Biotec). Anti-PE and
564 anti-CD2 microbeads (Miltenyi Biotec) were added to the granulocyte fraction to target
565 eosinophils and remaining CD2+ cells respectively, before performing magnetic
566 depletion using LD columns (Miltenyi Biotec). Neutrophil purity was >95% using this
567 technique.

568 ***Neutrophil/PBMC co-culture for intracellular cytokine assay***

569 Isolated PBMCs were stimulated (TCR stimulation with Cytostim NHP, Miltenyi Biotec)
570 and cultured in presence or absence of autologous neutrophils at 1:1, 3:1 and 5:1
571 neutrophil-to-PBMC ratio, together or separately using Transwell plates (Corning). A
572 mix of co-stimulating antibodies (CD28/CD49d, BD Bioscience) and CD107a antibody
573 (Miltenyi Biotec) were added to the wells. To study the intracellular cytokine
574 production, Brefeldin A (10µg/mL, Sigma-Aldrich) and Monensin (1000X, Biolegend)
575 were added to the wells after a 1-hour incubation at 37°C, 5% CO₂. After overnight
576 incubation, viability staining was performed for 20min at 4°C (Live/Dead, Thermofisher),
577 cells were washed and permeabilized/fixed with Cytofix/Cytoperm reagent (BD
578 Bioscience). Cells were stained with antibody mix for 30min at 4°C, washed and
579 resuspended in fixation buffer (Biolegend).

580 ***Measurement of soluble cytokines & chemokines***

581 PBMCs and neutrophils were co-cultured overnight. After 5mn centrifugation at
582 2200rpm, supernatants were collected and stored at -20°C. For cytokines
583 quantification, NHP 38-plex (Luminex) was performed. The cytokine and chemokine
584 profiles in coculture supernatants were analyzed using a bead-based multiplex assay.
585 Measurements were performed before infection, 10 days post-infection, 15 days post-
586 infection and 6 months post-infection.

587 With the Milliplex system (MILLIPLEX MAP Non-Human Primate Cytokine Magnetic
588 Bead Panel, MilliporeSigma), sCD40L, G-CSF, GM-CSF, sFasL, TNF- α , IFN- γ , granzyme
589 A, granzyme B, IFN α 2, IP-10, MCP-1, ITAC, MIG, MIP1 α , MIP3a, MIP1 β , TGF α , VEGF α ,
590 perforin, sCD137, IL-1RA, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12/IL-13 (p40),
591 IL-15, IL-17A, IL-18, IL-21, IL-22, IL-23, IL-33 and RANTES levels were measured.

592 The assays were performed according to the manufacturer's instructions. The various
593 timepoints were evaluated once. Seven standards as well as negative controls provided
594 in the kits were included. In addition, an in-house internal control was generated by
595 pooling supernatants of CyMs PBMCs that were cultivated in RPMI + 10% FCS and
596 activated with either PMA-ionomycin, concanavalin A, or LPS, to assess the efficiency
597 and reproducibility of the kit.

598 ***Partial Least Squares Discriminant analyses***

599 The Partial Least Squares Discriminant Analysis (PLS-DA) was performed on data from
600 the Intracellular Staining assays (ICS) and whole blood neutrophil phenotype assays
601 using R software (version 4.2.1). The mixOmics package was employed for model
602 development, while the ggplot2 package was used for visualization. The interpretation
603 of the PLS-DA model was guided by the examination of variable importance in
604 projection (VIP) scores, which identify the most influential variables contributing to
605 group separation. Based on their VIP scores (provided in the supplementary data,
606 SFig.5), the top variables were further ranked according to their contribution to the
607 discriminant components, as indicated by their loading coefficients.

608

609 ***Sample preparation and Proteomics and Data-independent acquisition shotgun*** 610 ***proteomics***

611 200,000 neutrophils were isolated as previously described in HBSS medium, and placed
612 in Eppendorf tubes. After a 400g centrifugation for 7min at 4°C, cells were resuspended
613 in a mix of PBS and SDS-page and heated at 99°C for 5min. Samples were stored at -
614 20°C and processed at the end of the study. Before acquisition, samples were

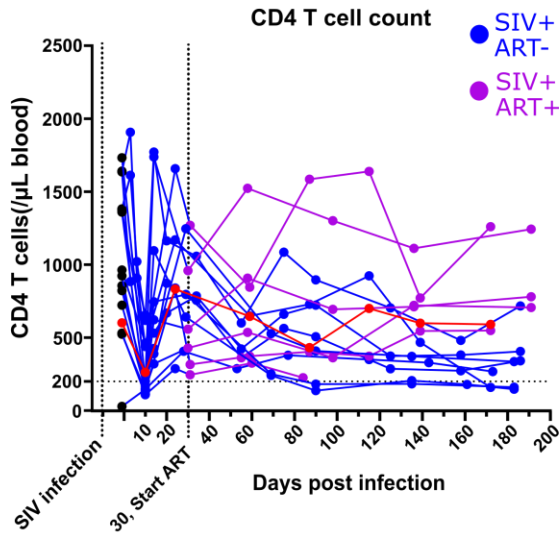
615 denatured again for 5 min at 99°C, briefly centrifuged, and then a volume of 25 µL of
616 each sample was subjected to NuPAGE 4–12% Bis-Tris gel (Thermo) electrophoresis for
617 5 min. Proteins were stained for 5 min with Coomassie SimplyBlue SafeStain (Thermo)
618 prior to in-gel trypsin proteolysis with Trypsin Gold (Promega) using 0.011%
619 ProteaseMAX surfactant (Promega), as described [50]. Peptides were characterized with
620 an Orbitrap Exploris 480 (Thermo Scientific) tandem mass spectrometer coupled to a
621 Vanquish Neo UHPLC (Thermo Scientific) as previously described [51]. Peptides were
622 loaded on a reverse-phase Acclaim PepMap 100 C18 precolumn (5 µm, 100 Å, 300 µm
623 i.d. × 5 mm, Thermo) and then resolved on a EasySpray PepMap 100 Neo C18 column
624 (2 µm, 100 Å, 75 µm i.d. × 50 cm, Thermo) at a flow rate of 250 nL.min⁻¹ using a 90-
625 min gradient (4-25% B in 75 min, and 25-40% B in 15 min), with 0.1% HCOOH/100%
626 H₂O as mobile phase A and 0.1% HCOOH/100% CH₃CN as mobile phase B. The mass
627 spectrometer was operated in data-independent acquisition mode as previously
628 described [52]. Peptides and proteins were identified from the dataset using the DIA-
629 NN software version 1.8.2 [53] against the *Macaca fascicularis* NCBI fasta database. The
630 following parameters were used for peptide assignment: trypsin as enzyme, two missed
631 cleavages allowed, carbamidomethylated cysteines as fixed modification, methionine
632 oxidation as variable modification, peptide length comprised between 7 and 30, charge
633 precursor of +2 or +3, and false discovery rate at 1%. Highly heuristic protein grouping
634 was used to reduce the number of protein groups obtained and high accuracy
635 quantification mode was enabled.

636

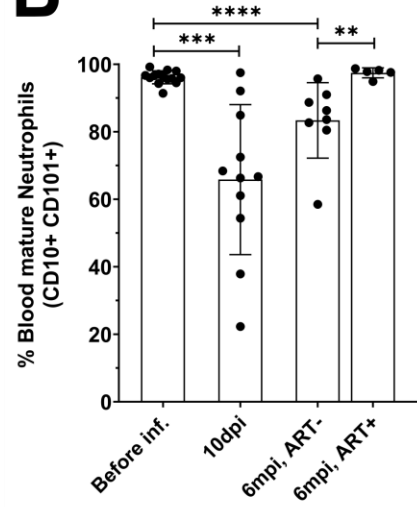
637 ***Statistical analysis***

638 Statistical analysis was carried out using GraphPad Prism version 10.0. Comparison
639 between two groups was carried out using non-parametric Wilcoxon tests for paired
640 datasets and Mann-Whitney tests for unpaired datasets. All the details of the statistical
641 analysis including the type of test used can be found in the legends of the figures. Data
642 represented as mean ± SD (Standard Deviation). *p < 0.05, **p < 0.01, ***p < 0.001,
643 ****p < 0.0001, ns = not significant.

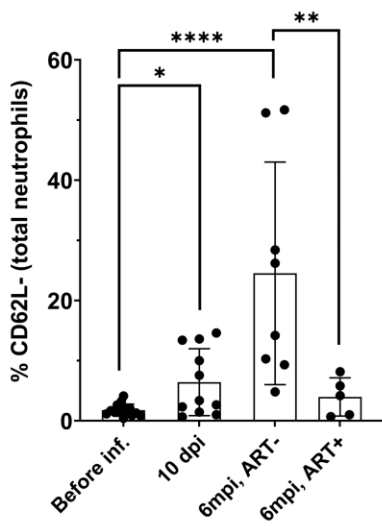
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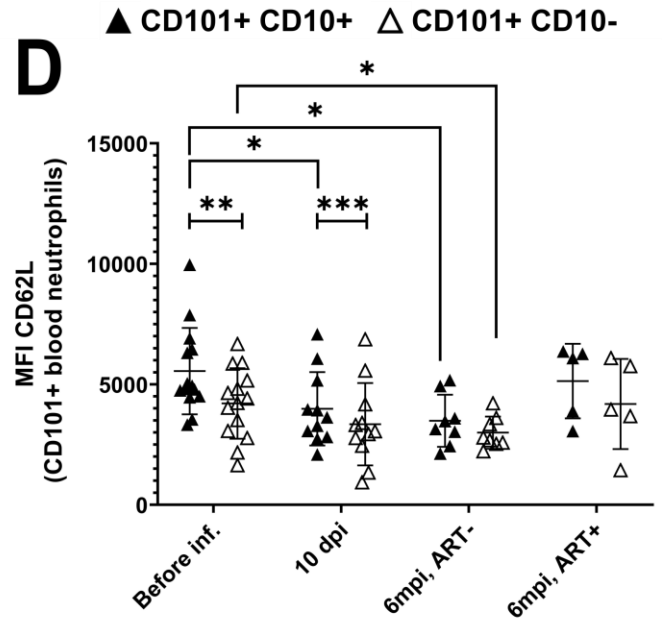
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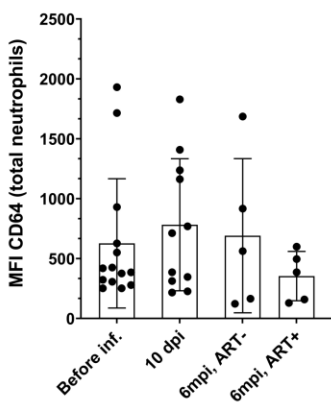
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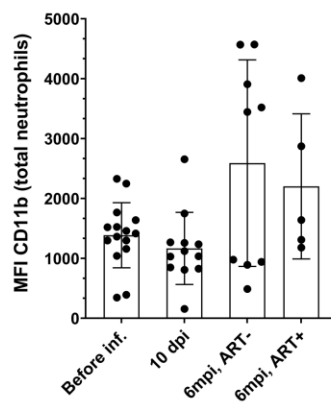
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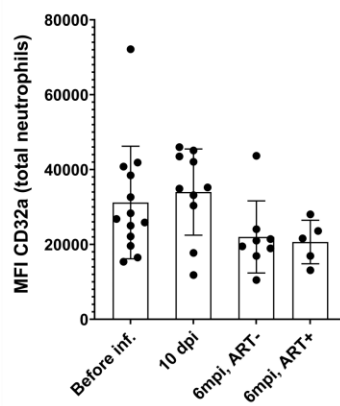
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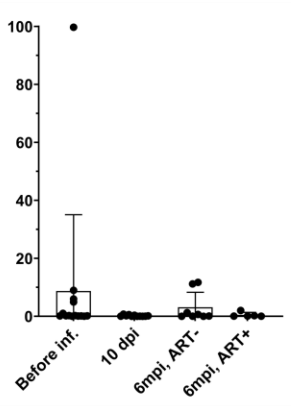
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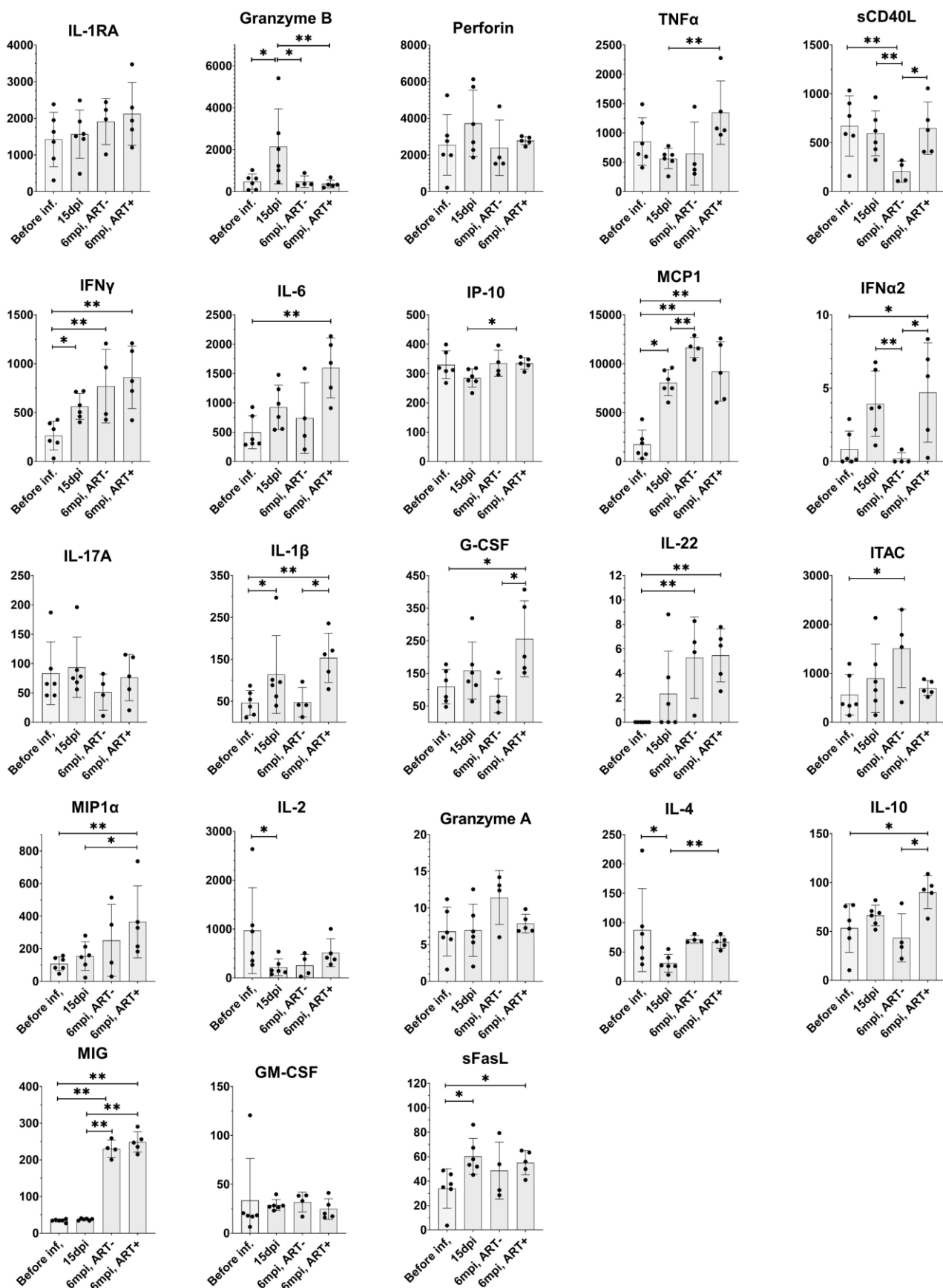
H



646 **Figure S1: CD4+ T cell count and blood neutrophil phenotypes during SIV**
647 **infection in CyMs**

648 (A) Absolute counts of CD4+ T cells $n = 15$ SIVmac251-infected CyMs over time (blue lines), with $n = 6$ being treated
649 at 1mpi to 6mpi with ART (switching to purple lines). Red line indicates $n = 1$ infected animal that controlled
650 infection; (B) Frequencies of mature CD101+ CD10+ neutrophils in blood before infection or during acute
651 SIVmac251 infection (10dpi), and with (+) or without (-) ART beginning at 1mpi, individual values (dots) or group
652 averages (bars) \pm standard deviation (SD); (C) Frequencies of CD62L- blood neutrophils before and during
653 SIVmac251 infection; (D) Mean fluorescence intensities of CD62L expression by CD10+ mature or CD10- immature
654 blood neutrophils before and during SIVmac251 infection; (E-G) Mean fluorescence intensities of CD64, CD11b,
655 CD32a expression by blood neutrophils before and during SIVmac251 infection; (G) Percentages of PD-L1+
656 neutrophils during SIVmac251 infection. Wilcoxon tests were performed for paired datasets, and Mann-Whitney
657 tests were run for unpaired datasets (ART+ versus ART-); **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

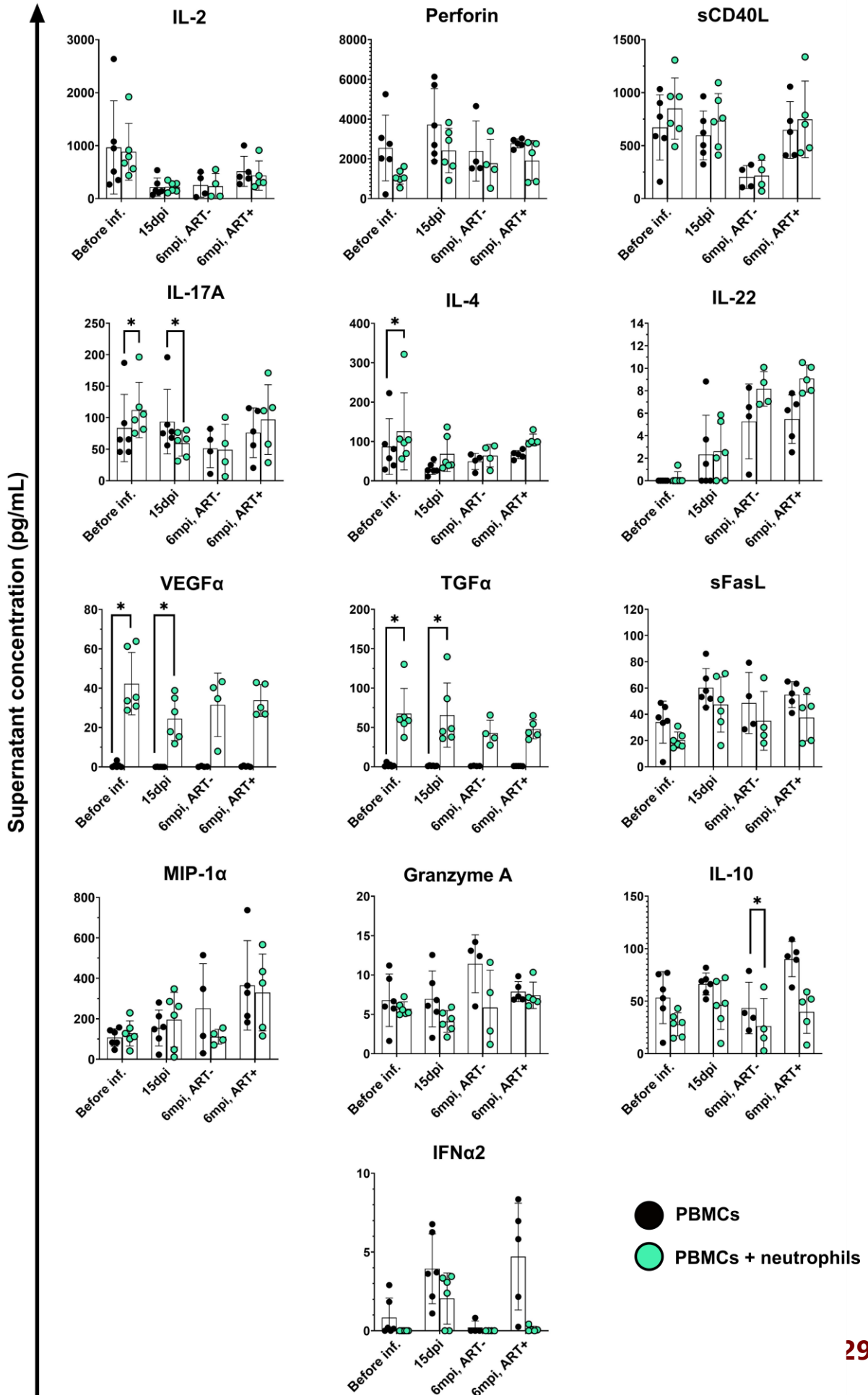
Supernatant concentration (pg/mL)



659 **Figure S2: Supernatant cytokine and chemokine levels produced by stimulated**
660 **PBMCs during SIV infection**

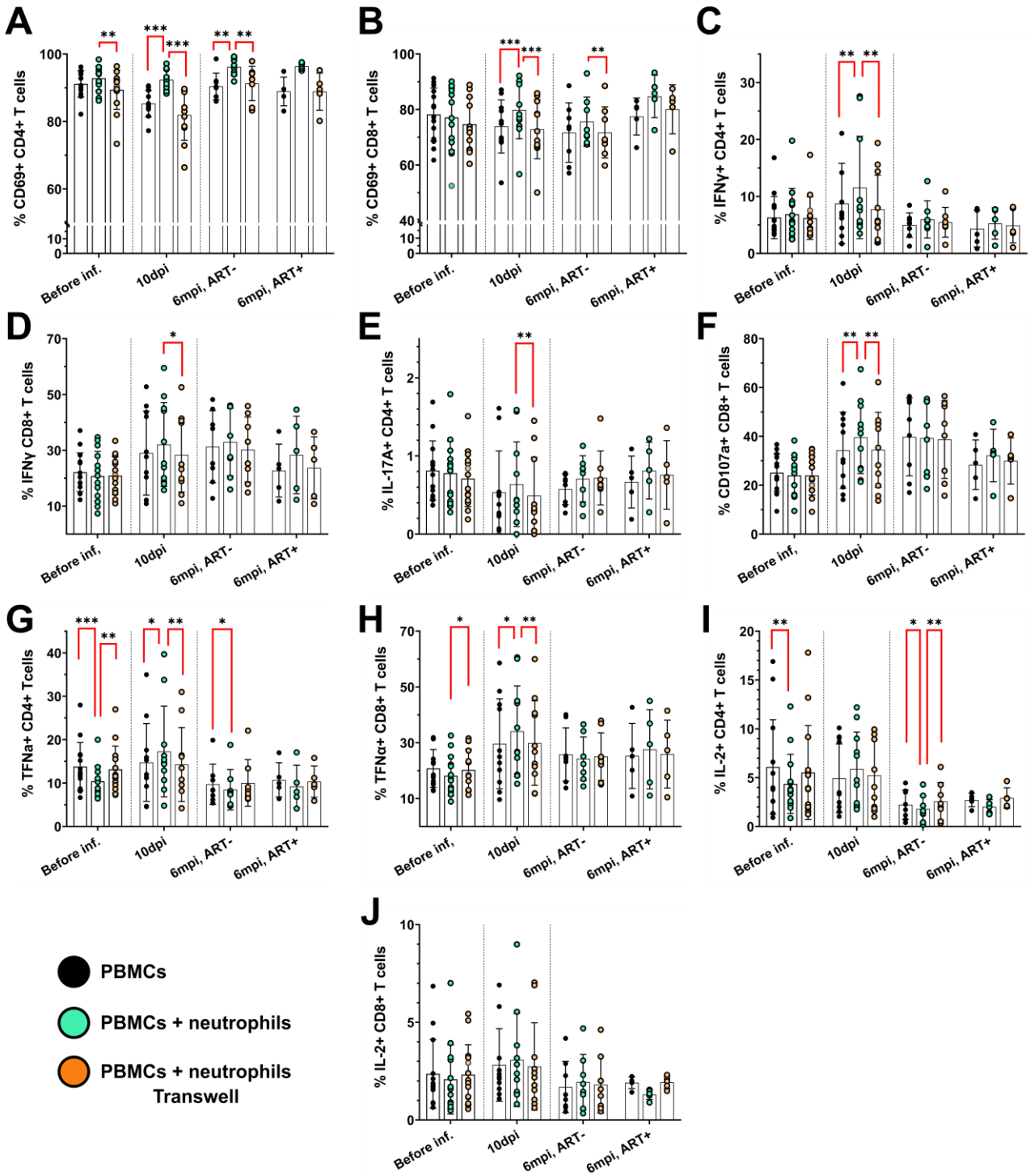
661 Supernatant concentrations of indicated molecules in overnight cultures of stimulated PBMCs from CyMs before
662 infection ($n = 6$) during the acute (15dpi, $n = 6$) or chronic 208 (6mpi, $n = 4$ SIV+ ART-, $n = 5$ SIV+ ART+) phase of
663 SIVmac251 infection. Individual values are represented by dots and group averages by bars \pm SD. Wilcoxon tests
664 were run for all paired datasets; *: $p < 0.05$; **: $p < 0.001$

665



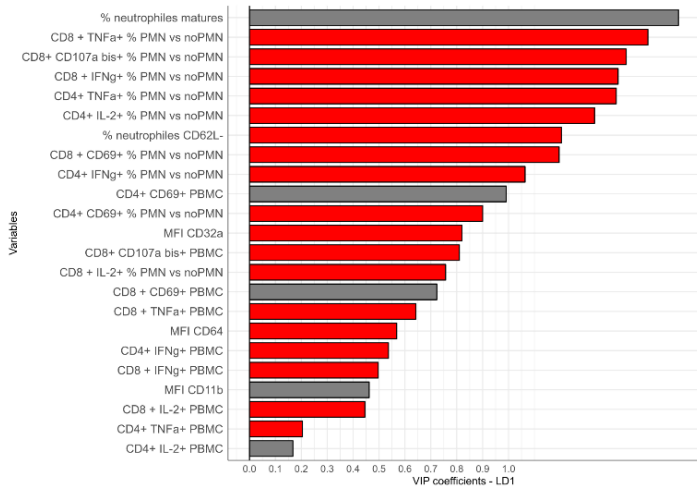
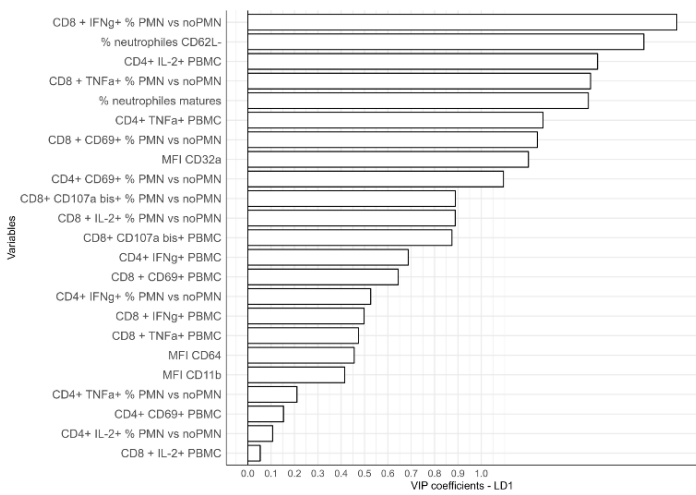
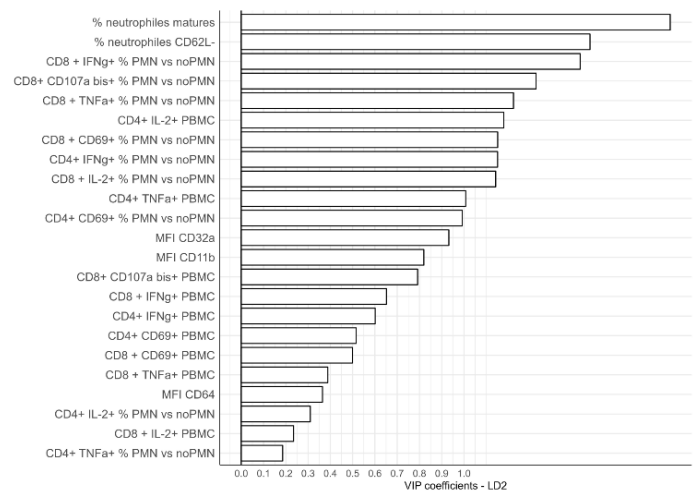
667 **Figure S3: Supernatant cytokine and chemokine levels produced by stimulated**
668 **PBMCs in the presence of neutrophils during SIV infection**

669 Supernatant concentrations of indicated molecules in overnight cultures of stimulated PBMCs without (black dots)
670 or with neutrophils (blue dots) from CyMs before infection ($n = 6$) during the acute (15dpi, $n = 6$) or chronic (6mpi,
671 $n = 4$ SIV+ ART-, $n = 5$ SIV+ ART+) phase of SIVmac251 infection. Individual values are represented by dots and
672 group averages by bars \pm SD. Wilcoxon tests were performed for all paired datasets; *: $p < 0.05$



673 **Figure S4: Activation of T cells in the presence of neutrophils during SIV infection**
 674 **in classical or transwell conditions**

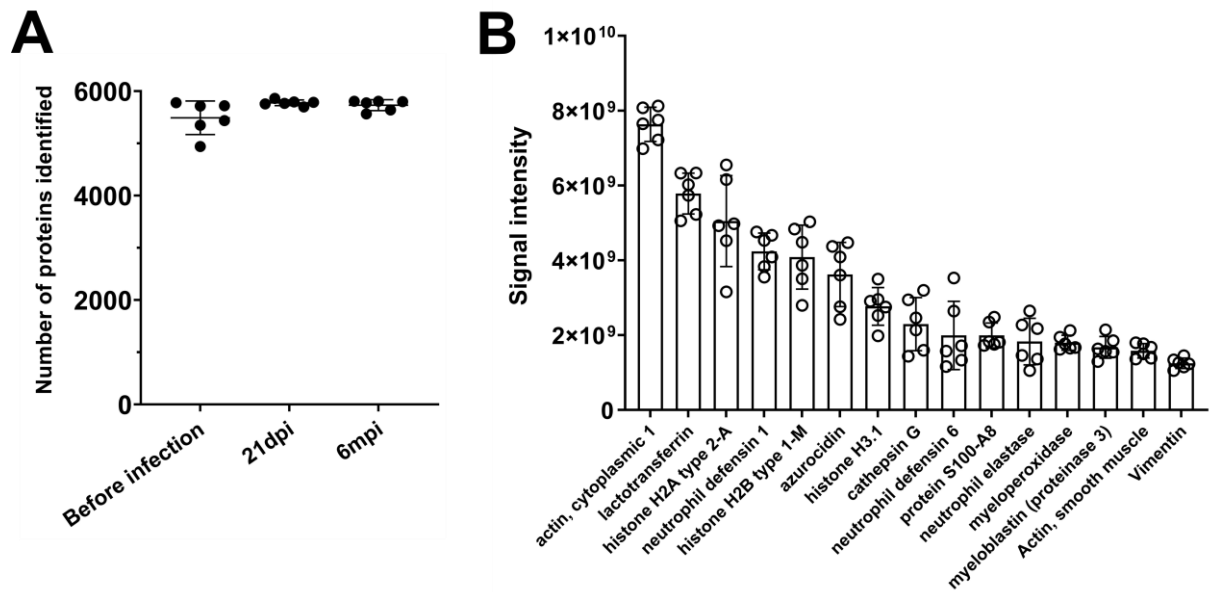
675 (A-J) Frequencies of positive T cells for each indicated marker. Positive cells were quantified by intra and extracellular
 676 flow cytometry in the absence (black dots) or presence of neutrophils with a 5:1 ratio (neutrophil:PBMC). PBMCs
 677 and neutrophils were in contact (blue dots) or separated by a Transwell membrane (orange dots) to measure
 678 contact-dependent effects. Wilcoxon tests were performed *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$
 679

A**B****C**

680 **Figure S5: Variable Importance scores for top variables of PLS-DA from Fig.4**

681 (A-B) Variable Importance scores (VIP) for the variables composing component LD1 in the PLS-DA from Fig 4.A
 682 comparing data from uninfected and SIVmac251-infected CyMs at 10dpi; (C-D) VIP scores for the variables
 683 composing component LD1 and component LD2 respectively, in the PLS-DA from Fig 4.C comparing data from
 684 uninfected, SIV+ ART- and SIV+ ART+ CyMs

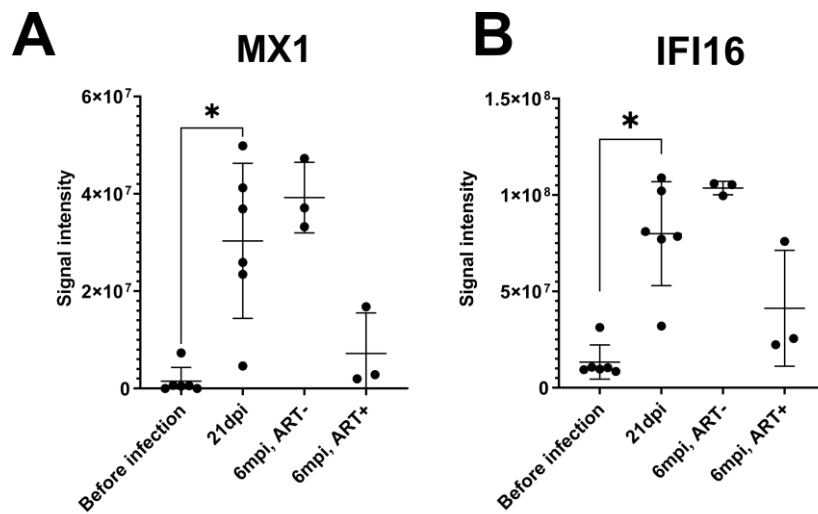
685



686
 687 **Figure S6: Number of proteins identified and changes in mass spectrometry signal**
 688 **intensities of MX1 and IFI16 before and during SIVmac251 infection in CyMs**

689 (A) Number of proteins identified with mass spectrometry analysis in purified neutrophils from CyMs before and
 690 during SIVmac251 infection ($n = 6$); (B) Mass spectrometry signal intensities for indicated proteins in uninfected and
 691 SIVmac251-infected CyMs.

692



693 **Figure S7: Mass spectrometry signal intensities of MX1 and IFI16 before and**
 694 **during SIVmac251 infection in CyMs**

695 (A-B) Mass spectrometry signal intensities of indicated proteins in uninfected and SIVmac251-infected CyMs.;
 696 Wilcoxon tests were performed for paired datasets *: $p < 0.05$

697

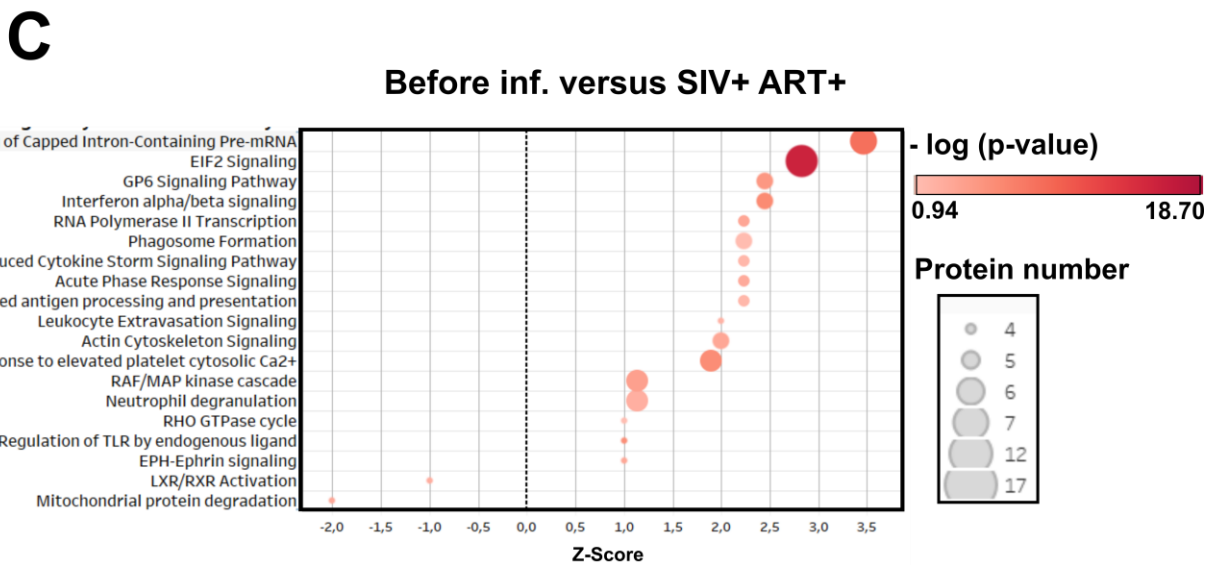
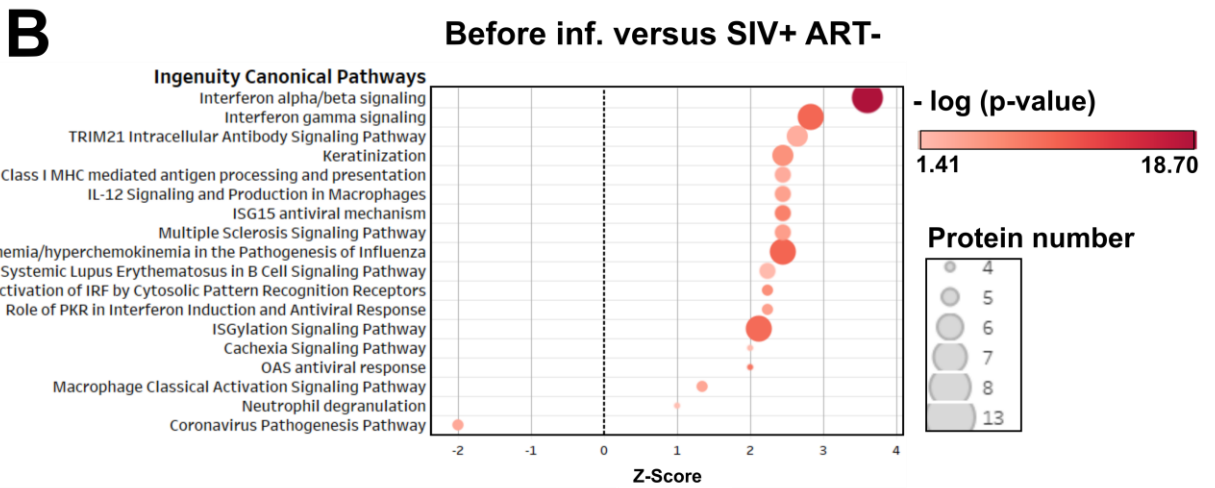
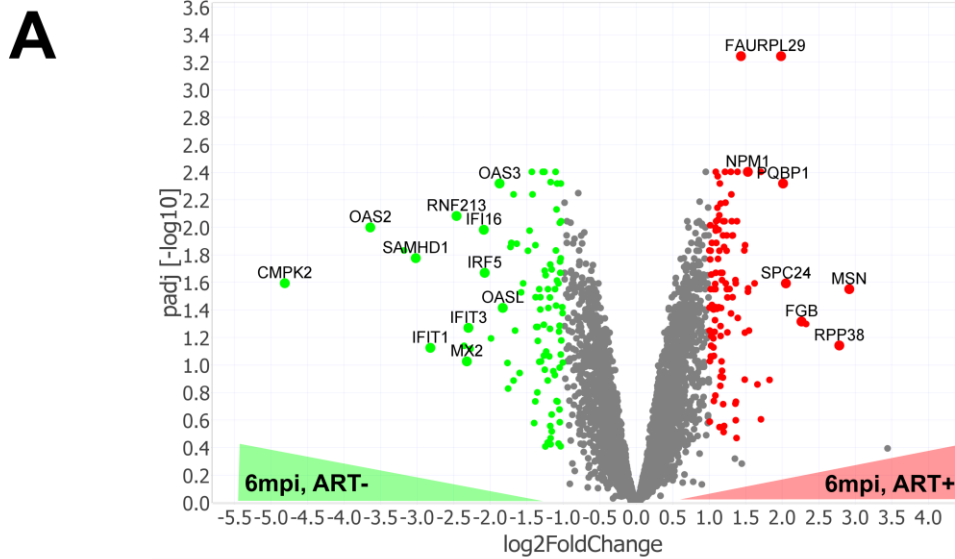
698

699 Figure next page

700 **Figure S8: Up and down-regulated pathways associated to differentially**
701 **expressed proteins of neutrophils during chronic SIV infection with or without**
702 **ART**

703 (A) Ingenuity Pathways Analysis (IPA) of upregulated pathways in neutrophils from SIV+ ART- CyMs compared to
704 baseline; (B) Ingenuity Pathways Analysis (IPA) of upregulated pathways in neutrophils from SIV+ ART+ CyMs at
705 6mpi compared to baseline; (C) Volcano plot of DEPs in neutrophils from SIV+ ART- CyMs (left) or SIV+ ART+ CyMs
706 (right) at 6mpi compared to baseline.

707



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III. Complementary results

i) Evaluation of neutrophil ROS production in the course of SIV infection

- **Rational approach and methodology**

As mentioned in the introduction of this manuscript, the impact of HIV/SIV infection on neutrophil ROS production is unclear, as studies showed contradictory results. Neutrophil ROS production leads to the release of arginase-1, a protein shown to suppress T cell function. Hence, we decided to measure the intracellular production of ROS using CellROX (Thermofisher) in 200,000 pure neutrophils isolated from 6 SIV-infected macaques using different stimuli such as TNF α , fMLP and PMA. Because we also aimed to evaluate neutrophil capacity to respond to these stimuli and because we previously showed the neutrophil response could be evaluated by studying CD62L rather than CD11b, we also performed flow cytometry stainings on neutrophils to follow the expression of CD62L.

- **Results**

First, we evaluated the robustness of our test before infection by measuring the production of ROS with stimuli of different natures or left at 4° or 37°C for 1 hour. In this experiment, we observed that the intensity of ROS production, measured through the mean of fluorescence intensity (MFI) of the CellROX probe, differed depending on the strength of the stimuli. In particular, the combination of TNF α and fMLP induced a significant increase in ROS production compared to unstimulated neutrophils. The highest ROS production was found with PMA stimulation, as expected in view of the literature (**Figure 13A**) [293].

We then compared the production of ROS from neutrophils before infection and at different timepoints of SIV infection in CyMs in three different conditions. We evaluated the fold change of the CellROX MFI at 37°C without stimulation, a mild stimulation with

TNF α and fMLP, or a strong stimulation, PMA. After 21 days of SIV infection, we distinguished two groups of animals with different intensities of ROS responses, independently of the condition (**Figure 13B-13D**).

At 2 months post-infection, the ROS production of neutrophils from untreated animals tended to be higher compared to animals receiving ART, in the 37°C and the mild stimulation conditions (**Figure 13C**). However, we could not perform statistical analyses because there were only three animals in each group. Interestingly, this tendency reversed after 4 months post-infection, with a higher ROS production in ART+ animals than untreated animals.

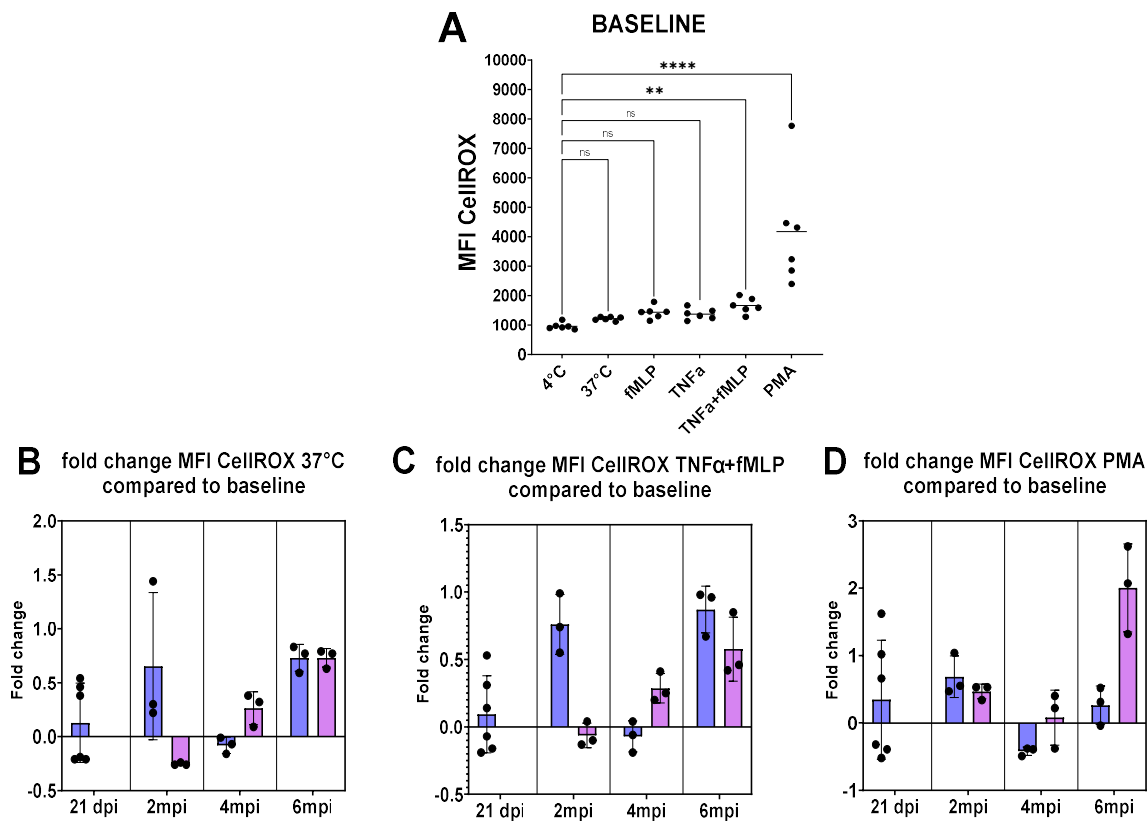


Figure 13: Neutrophil ROS production before and during SIV infection in CyMs, with stimulations of different nature

(A) Mean Fluorescence intensity of the CellROX probe for ROS measurement by flow cytometry in neutrophils from healthy macaques exposed to different conditions. Stats: Friedman test. **: $p < 0.01$, ****: $p < 0.0001$; (B-D) Fold change of CellROX MFI compared to baseline, in neutrophils exposed to 37°C, TNF α +fMLP or PMA before and during SIV infection.

Lastly, after 6 months of SIV infection, the production of ROS tended to be more elevated compared to the baseline condition for all stimulations. Depending on the nature of the stimulation, untreated and treated animals showed different responses (**Figure 13B-13D**).

All in all, even if we saw variations in the production of ROS along SIV infection, we did not identify a clear tendency for neutrophil defects or increased ROS capacities. In the future, including more animals may help identify such tendencies.

ii) Additional experiments to identify mechanisms underlying neutrophil modulatory effects on T cells

- **Rational approach and methodology**

As we identified that neutrophils modulate T cell activation and intracellular cytokine production during SIV infection, we aimed to investigate these interactions more deeply. First, as different modulatory capacities have been described depending on the state of maturation of neutrophils [294], we developed a sorting of CD10⁺ mature and CD10⁻ immature fractions of blood neutrophils and co-cultured these separate fractions with autologous PBMCs.

Secondly, we evaluated CD14⁺ blood monocytes as potential partners in the PMN–T cell interactions, and we used magnetic cell sorting to remove these cells from the PBMC fraction before co-culturing neutrophils and PBMCs.

As we performed these experiments during the chronic phase of SIV infection, only few samples could be processed for these two tests.

- **Results**

First, we validated the efficiency of this sorting. To do so, we performed May Grunwald-Giemsa (MGG) stainings on Cytospin spreads of each cell fraction. We were able to confirm the separation of CD10⁺ mature neutrophils from CD10⁻ immature neutrophils according to nucleus shapes (**Figure 14F-14G**).

We then compared T cell activation in the presence of CD10⁺ or CD10⁻ neutrophils using similar co-cultures as previously described in the Methods section. We found that CD10⁻ immature neutrophils exerted lower effects on T cells than CD10⁺ neutrophils. Indeed, CD69 expression on CD8⁺ T cells was increased in the presence of mature neutrophils, while immature neutrophils induced a lower increase of CD69 expression on CD8⁺ T cells (**Figure 14A**). However, we did not see a similar tendency in CD4⁺ T cells regarding CD69 expression (**Figure 14D**). Additionally, intracellular TNF α expression on CD8⁺ and CD4⁺ T cells decreased in the presence of mature and immature neutrophils. This decrease in TNF α expression was expected regarding our results on the modulatory effects of neutrophils during the chronic phase of SIV infection. However, we found that the decrease in TNF α expression in T cells tended to be stronger in the presence of mature neutrophils compared to immature neutrophils (**Figure 14B and 14E**). Moreover, we saw a similar tendency for CD107a expression in CD8⁺ T cells (**Figure 14C**).

Overall, these results could suggest that during chronic SIV infection, mature neutrophils are more responsible than immature neutrophils for the modulatory effects of neutrophils on T cells in this intracellular cytokine assay. However, we are aware that these results should be considered very carefully due to the very low number of animals (only three). Furthermore, these results should not be extended to the acute phase of SIV infection. Yet, we were successful in sorting CD10⁺ versus CD10⁻ neutrophils, a process that could be useful for studying both subsets in the future.

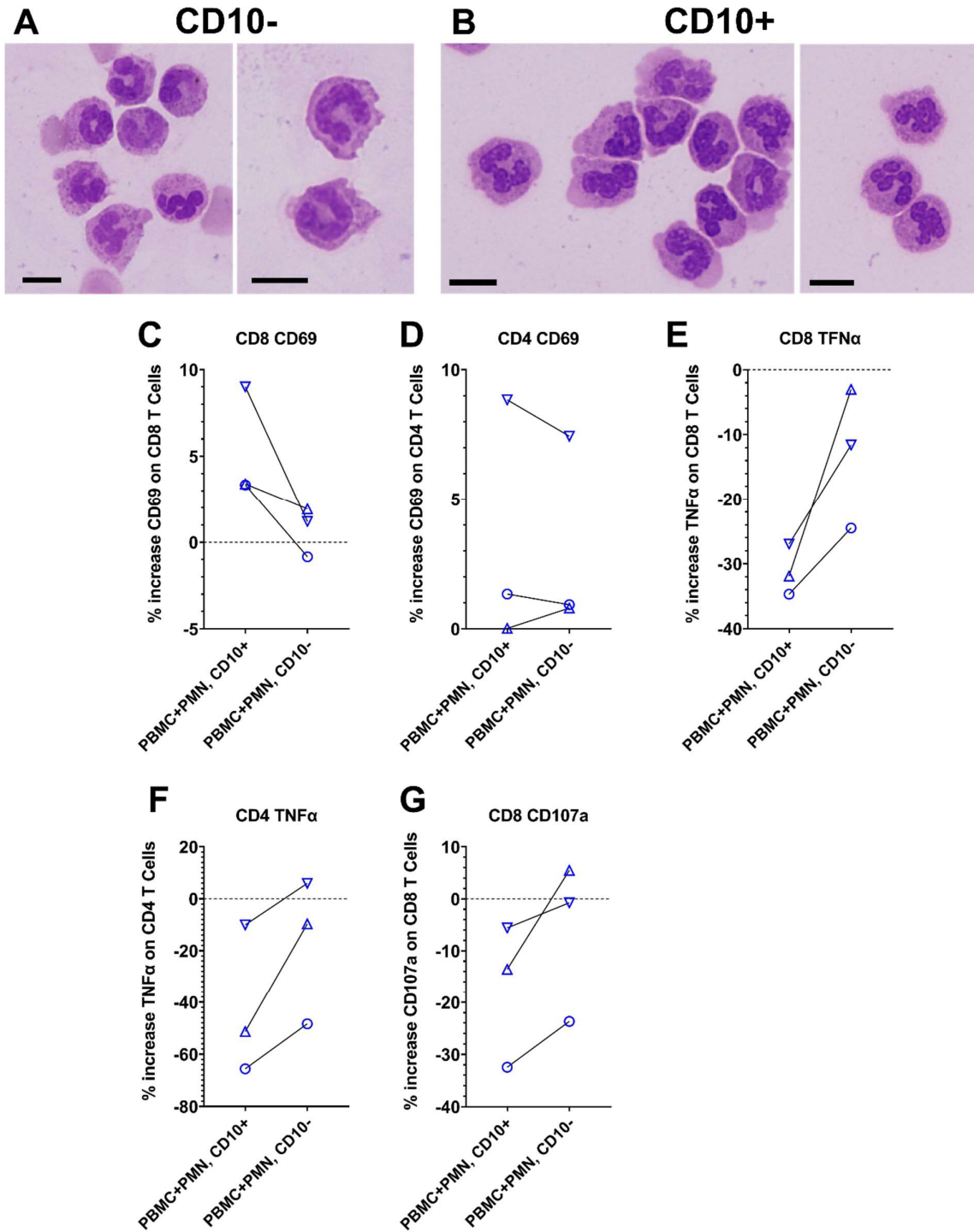


Figure 14: Effects of CD10+ or CD10- neutrophils on T cell activation during the chronic phase of SIV infection without ART

(A-B) MGG stainings of CD10- and CD10+ neutrophil fractions after cell sorting. Bar: 10 μ m (C-G) Percentage of increase of expression of indicated marker on T cells in presence of CD10+ or CD10- of neutrophils from SIV-infected CyMs; NB: "PMN" designates total neutrophils, which contains mature and immature neutrophils.

Then, we studied if CD14⁺ monocytes from PBMCs were important partners in the modulatory effects of neutrophils on T cells. We used negative magnetic depletions to remove CD14⁺ cells from PBMCs and performed similar co-cultures as previously described in the Methods section (**Figure 15A**). As expected, when we removed the CD14⁺ cells from PBMCs without adding neutrophils, CD69 decreased (**Figure 15B and 15C**). When we added neutrophils to total PBMCs, as expected regarding our previous results, CD69 expression increased in both CD8⁺ and CD4⁺ T cells. When we removed CD14⁺ cells from the PBMCs and added neutrophils, we did not observe any major changes on CD69 expression in T cells. However, when we looked at TNF α expression, it seemed that removing CD14⁺ cells from neutrophil-T cell co-cultures induced a decrease in TNF α expression compared to the condition with total PBMCs (**Figure 15D and 15E**).

Again, we should take these results with care due to the low number of animals. Yet, it seems that CD14⁺ cells could be participating in the modulatory effects of neutrophils on T cells regarding some activation markers (TNF α) and not others (CD69). Further investigation would be needed to confirm these observations on more animals.

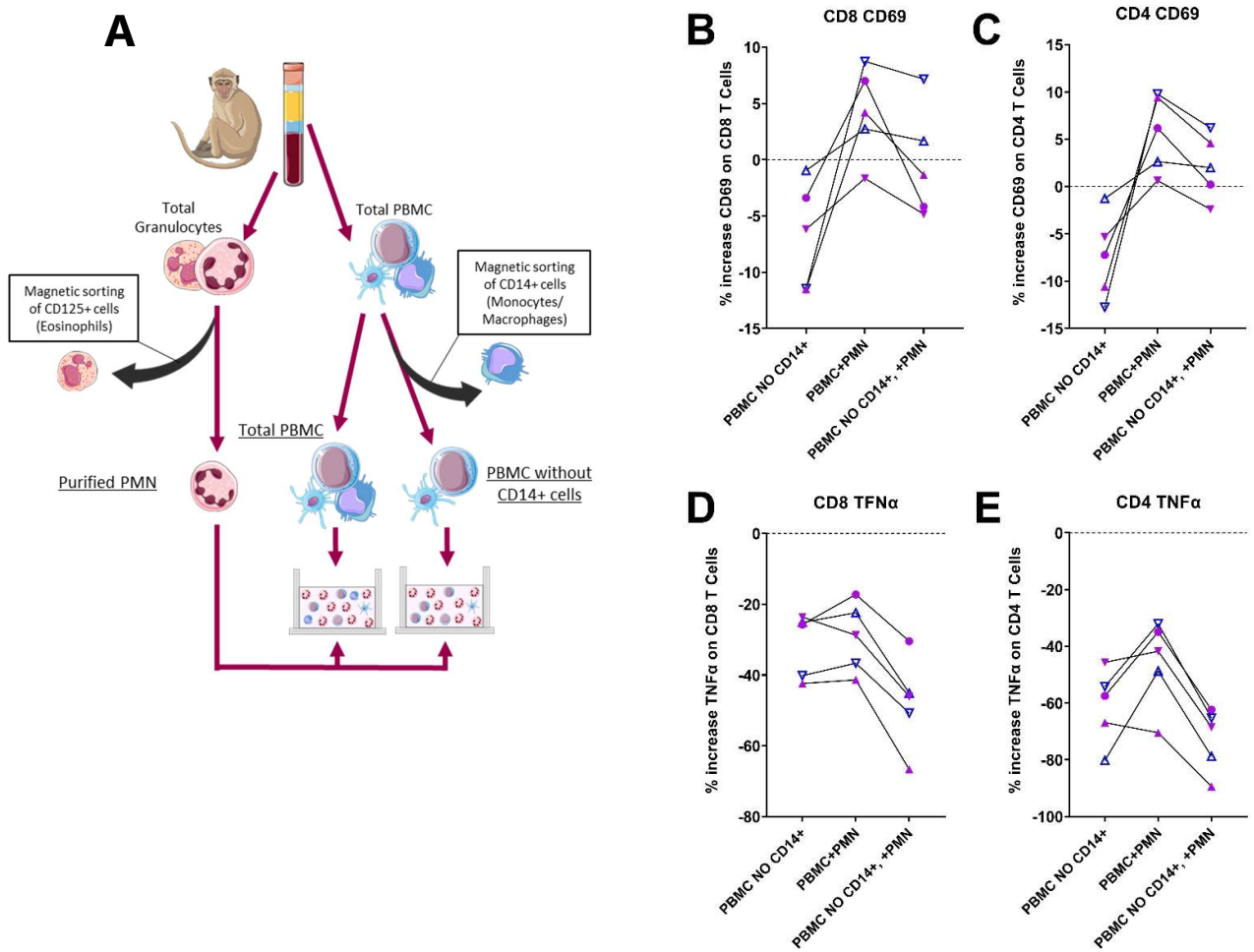


Figure 15: Study of CD14+ monocytes as a partner in neutrophil-T cell interactions during chronic SIV infection

(A) Experimental protocol for co-cultures with or without CD14+ cells; (B-E) Percentages of increase of indicated marker on T cells in presence or absence of CD14+ monocytes and in the presence or absence of autologous neutrophils. NB: "PMN" designates total neutrophils, which contain mature and immature neutrophils

iii) Phenotyping of neutrophils in tissues of SIV-infected animals

- **Rational approach and methodology**

Finally, we aimed to study the phenotype of neutrophils from bone marrow and spleen in SIV-infected CyMs upon euthanasia. We had access to these tissues upon euthanasia, from which we isolated total cells. To obtain splenic cells, we first performed a mechanical dissociation of the spleen using GentleMacs dissociator (Miltenyi) and then filtered the cell suspension with a 100µm filter. Then, we performed a red blood cell lysis for bone marrow and splenic cell suspensions for 8mn, similarly to blood samples. Then, after several washes, we performed flow cytometry stainings on one million cells for each tissue using the same flow cytometry panel for neutrophils as previously described in this manuscript. We performed these experiments in four chronically infected SIV animals that had been left untreated and 5 animals that had been receiving ART for several months.

- **Results**

We were able to identify mature, immature and pre-neutrophils in bone marrow and splenic samples. As expected, bone marrow neutrophils mostly comprised immature cells (**Figure 16A and 16B**). As previously evoked in the work of Lemaitre et al., SIV+ ART- animals presented a lower proportion of mature neutrophils and more pre-neutrophils than SIV+ ART+ animals (**Figure 16A**). On the other hand, we found no difference between the proportions of mature, immature and pre-neutrophil frequencies in the spleen of both groups (**Figure 16C and 16D**)

These results may indicate that BM granulopoiesis is still perturbed after several months of SIV infection without treatment, while ART seems to resolve granulopoiesis. However, bone marrow stainings from healthy animals will be required to find if ART+ animals returned to baseline regarding their neutrophil phenotype. Secondly, the frequencies of neutrophil subsets were not different in the two groups. Two hypotheses

can be raised: either these neutrophil subsets are unaffected during SIV infection or shifted in ART+ and ART- animals. Again, similar stainings from animals that were not infected with SIV could help us unveil this question.

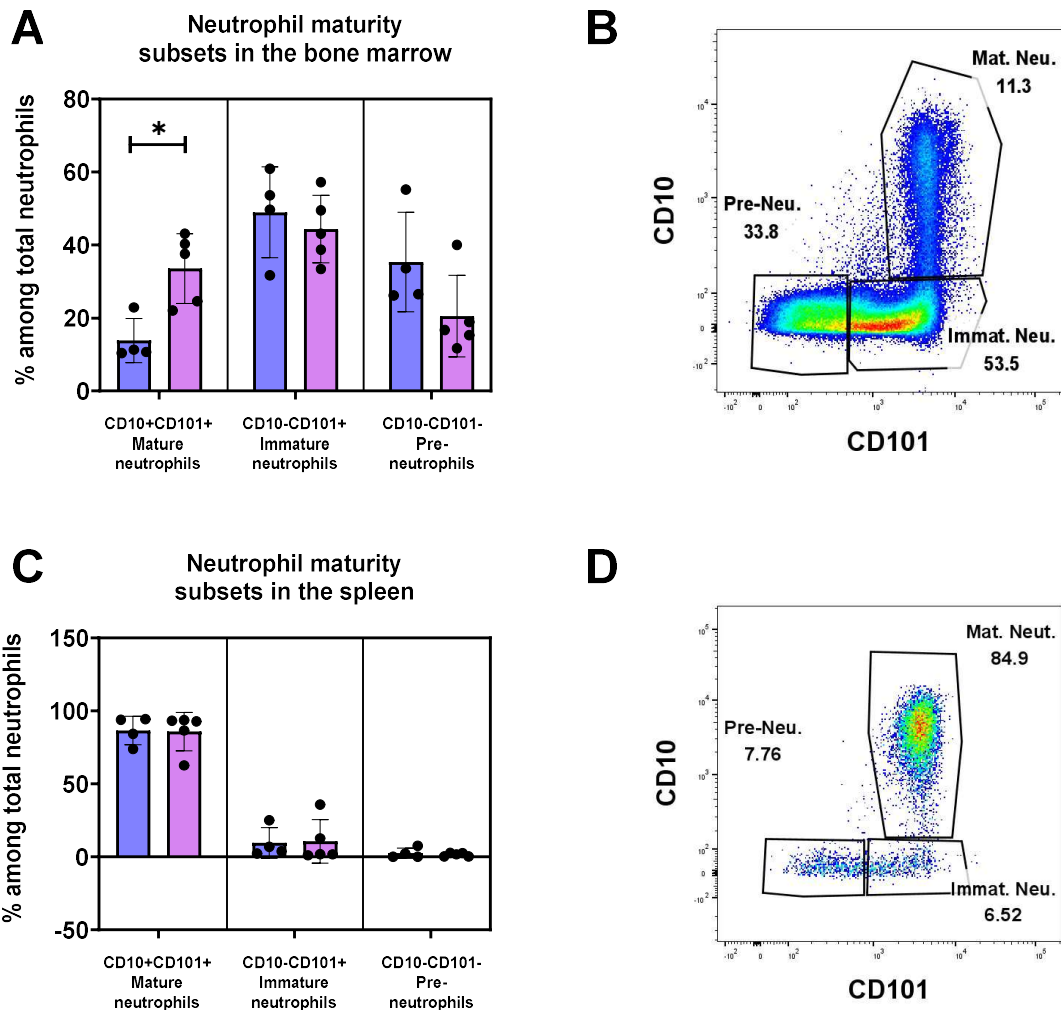


Figure 16: Identification and quantification of mature, immature and pre-neutrophil subsets in bone marrow and spleen of chronically infected CyMs

(A) Frequencies of mature, immature and pre-neutrophil subsets in bone marrow from SIV-infected CyMs identified by flow cytometry. Statistics: Mann-Whitney tests, * $p < 0.05$ (B) Representative graph of neutrophil subsets in the bone marrow of SIV+ ART- CyMs at 6mpi; (C) Frequencies of mature, immature and pre-neutrophil subsets in the spleen from SIV-infected CyMs identified by flow cytometry; (D) Representative graph of neutrophil subsets in the spleen of an SIV-infected CyM at 6mpi

iv) Neutrophils modulate T cells during Mycobacterium tuberculosis infection in cynomolgus macaques (from Dinko et al., manuscript in preparation)

Different infectious diseases are studied in IDMIT, mainly using NHP models. Recently, Julien Lemaitre, Candie Joly and colleagues developed a CyM model for tuberculosis infection. Tuberculosis, which is caused by *Mycobacterium tuberculosis*, is still one of the global leading causes of death. Interestingly, neutrophils were shown to play dual roles in this infection. On the one hand, neutrophils participate in innate immune responses against the bacteria through the release of antimicrobial peptides and cytokines, but on the other hand, they were shown to exacerbate inflammation and bacterial survival in tissues by producing ROS and NETs. However, the precise implication of neutrophils in this disease and their phenotypical or functional changes at the different phases of tuberculosis infection are not fully elucidated.

Another PhD student from IDMIT, Stanley Dinko, investigated the neutrophil dynamics in CyMs infected with *Mycobacterium tuberculosis*. Hence, I had the opportunity to investigate if neutrophils could modulate T cells in this model of bacterial infection. This work was done in collaboration with Stanley Dinko et al., whose study is partially presented above. For this work, I performed co-cultures of PBMCs and purified neutrophils from 4 CyMs infected with Mtb before and at 9 weeks post-infection. I also took charge of the analysis of these results and participated in creating the resulting figure. I also participated in reviewing the article presented above.

Here, I will present this study shortly and the associated results from the neutrophil-T cell experiments we ran.

1 **DISEASE PROGRESSION IS ASSOCIATED WITH DIFFERENTIAL NEUTROPHILS**
2 **MATURATION IN *MYCOBACTERIUM TUBERCULOSIS* INFECTED MACAQUES**

3 Stanley B. Dinko¹, Candie Joly¹, Paul Mazet¹, Gaëlle Sandillon¹, Victor Magneron¹,
4 Natalia Nunez², Céline Mayet¹, Ségolène Diry², Cassandra Gaspar², Marco Leonec¹,
5 Sophie Luccantoni¹, Camille Ludot¹, Benoit Delache¹, Emma Jouglu¹, Julie Morin¹, Wafa
6 Zouaoui-Frigui³, Roland Brosch³, Vanessa Contreras¹, Anne-Sophie Galloüet¹, Nathalie
7 Bosquet¹, Francis Relouzat¹, Quentin Pascal¹, Baptiste Jean¹, Marion Holzapfel¹, Olivier
8 Lambotte¹, Thibaut Naninck¹, Roger Le Grand^{1*} and Julien Lemaître^{1*}

9 ¹Université Paris-Saclay, INSERM, CEA U1184 IMVA-HB/IDMIT, Fontenay-aux-Roses,
10 France, ²Life and Soft, Fontenay-aux-Roses, France, ³Institut Pasteur, Université Paris
11 Cité, Unit for Integrated Mycobacterial Pathogenomics, Paris, France.

12 ***Corresponding authors** Julien.lemaître@cea.fr and roger.le-grand@cea.fr

13 **Summary**

14 In tuberculosis (TB), neutrophils have been described as both harmful and helpful. This
15 duality suggests that the diversity of neutrophils has an impact on the control or the
16 progression of the disease . In our study involving cynomolgus macaques infected with
17 *Mycobacterium tuberculosis*, we identified transcriptomic signatures of neutrophil
18 degranulation and the emergence of immature neutrophils (CD101+ CD10-) circulating
19 in the blood of macaques with fast progression to active TB, which were not observed
20 in animals with slow TB progression. In addition, circulating neutrophils from infected
21 animals displayed new capacities to modulate TNF- α production and cytotoxic function
22 of CD8 T cells in a contact-dependant mechanism. At the infection site, our findings
23 reveals that neutrophils of animals with fast progressive TB significantly infiltrate the
24 lung parenchyma and T cell rich area of granulomas, in comparison with
25 latent/subclinical TB animals. Our study uncover the complex role of neutrophil
26 heterogeneity in TB pathogenesis and modulation of CD8 T cells functions.

27 **Keywords**

28 Tuberculosis, non-human primate, neutrophils, active tuberculosis, CD8+ T cells

29

- **Context and objectives of the study**

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is the leading cause of death among infectious diseases (WHO-global TB report 2024). In 2024, about ¼ of the global population was infected with Mtb [96]. Although the bacillus of Calmette and Guérin vaccine (BGC) is available and quite efficient in infants, it induces low protection in adults [295]. Besides, current treatments face increasing Mtb multi-drug resistance. As a consequence, there is an urgent need for developing innovative therapies against Mtb infection. Understanding immune responses and correlates of

immune protection induced during TB remains a major challenge to finding new therapeutic targets.

In the defense against Mtb infection, adaptive immune responses have been extensively studied to the detriment of innate immune responses. In this work, Dinko et al. hypothesized that early innate myeloid immune responses and polymorphonuclear neutrophil dysfunctions might impact the progression of Mtb infection. The objective was to evaluate myeloid cell heterogeneity associated with disease progression at both systemic and tissue levels. The study of early Mtb infection in humans is challenging owing to ethical considerations, late diagnosis and inaccessibility to tissues. As such, Mtb infection was studied in the cynomolgus macaque model, which recapitulates the different spectrum of human TB, ranging from latent to active tuberculosis.

- **Methodology**

Recent exploration of non-human primate (NHP) models such as rhesus macaques, marmosets and cynomolgus macaques has come in handy. NHPs are now considered the gold standard for studying and modeling M. tuberculosis infection owing to the fact that they better reflect human pathology than other animal models [296–298]. Among NHP species, the CyM (*Macaca fascicularis*) model serves well for modeling tuberculosis owing to its ability to reproduce both active and latent infection, even with virulent strains of Mtb [299,300]. Here, this model of Mtb infection was developed and fully established, and allowed the study of early immune events and their impact on the progression of infection. Ten CyMs were exposed to 25 CFU of Mtb Erdman strain through bronchoscopic instillation and were followed for up to 18 weeks. The exposure resulted in two profiles of Mtb infection, fast progressors (FP) and slow progressors (SP), based on clinical symptoms such as weight loss, dyspnea, hypoxia and severe coughing, bacterial load, IGRA and PET-CT imaging. Innate myeloid responses were assessed using whole blood transcriptomic analysis, mass cytometry immunophenotyping, and neutrophil functional assays including measurements of

ROS and NET production, as well as evaluation of T cell modulation by neutrophils in co-cultures.

- **Results**

First, the model of Mtb infected was validated in CyMs. Low-dose Mtb infection led to different outcomes in CyMs, ranging from latent to active TB, providing a favorable experimental situation to explore the link between disease progression and early innate immune responses. After bacterial exposure, all animals became infected, as evidenced by positive ESAT-6/CFP-10 ELISpot responses, lung granulomas observed at 4 weeks post-exposure on PET/CT imaging, and symptoms developed by some animals (**Figure 16A-16C**). Six out of ten animals progressed quickly to severe disease and reached the endpoint from 12 to 20 weeks after exposure (**Figure 16B**). These animals had a significantly higher clinical score, especially due to weight loss, dyspnea, hypoxemia and severe coughing (**Figure 16B**). On the other hand, four macaques exhibited slower disease progression with no or mild symptoms of transient weight loss or blood oximetry reduction. Because disease progression and clinical presentation differ in kinetic and magnitude between animals, we grouped them as fast progressors (FP) and slow progressors (SP). Three-dimensional reconstructions of serial PET/CT scans evidenced the differences in terms of lesions' initial formation, kinetic and distribution, showing few isolated hyper-metabolic (¹⁸F-FDG positive) lung granulomas in SP and more diffuse lung lesions with lobular or lobar pneumonia in FP (**Figure 16A, 16C and 16D**). Tracheobronchial lymph node inflammation was also observed (**Figure 16E**).

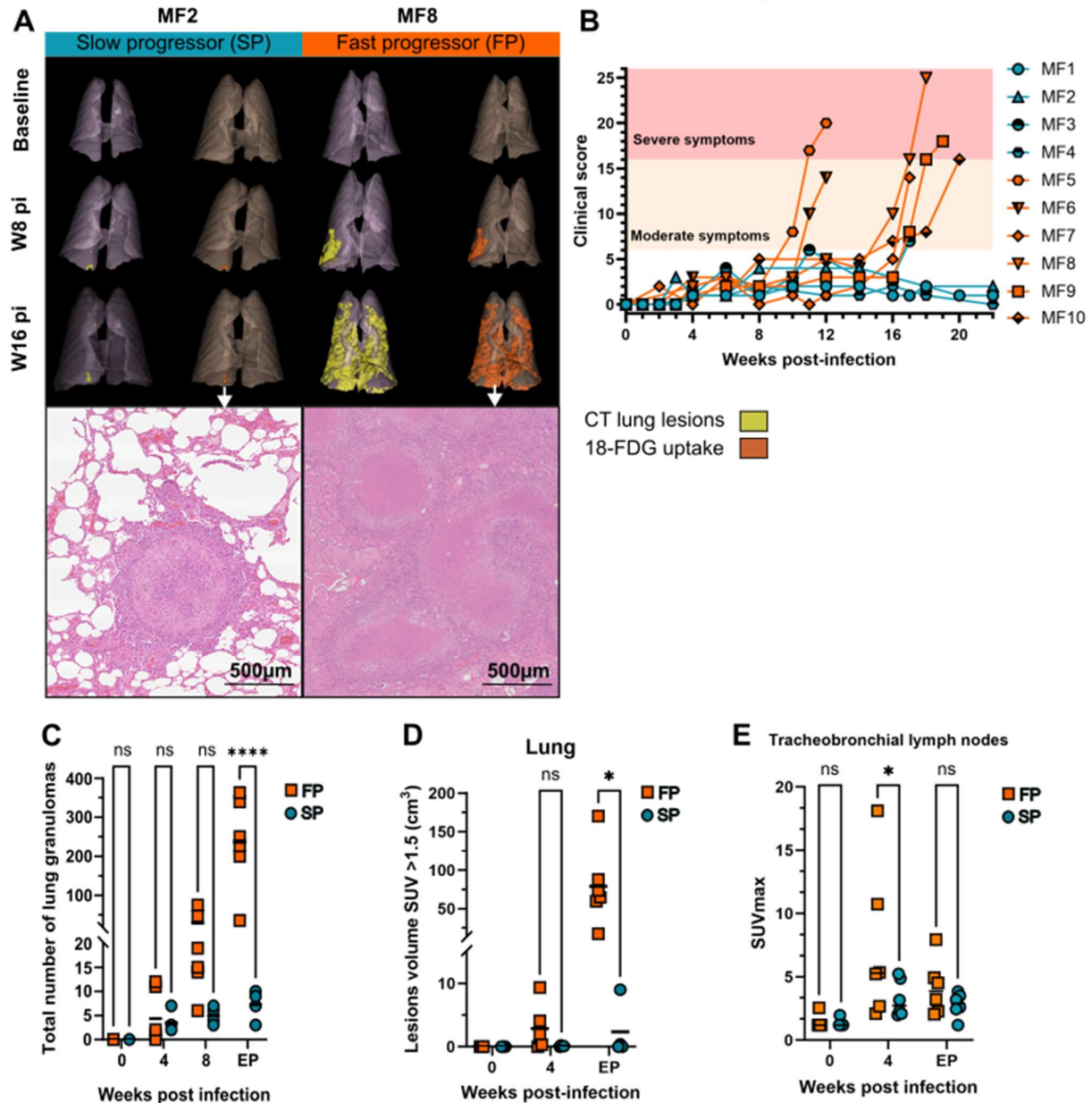


Figure 16. Low dose *Mtb* infection leads to heterogeneous clinical evolution, lung inflammation and bacterial burden; from Dinko et al (in preparation)

(A) Three dimensional reconstructions of lungs using PET/CT scans of two representative *Mtb* infected NHPs at different time points. CT opacities are depicted in yellow and 18-FDG uptake (SUV > 1.5) in orange. (B) Clinical scores of fast and slow progressor animals. (C) The total number of lung granulomas counted on CT scans. (D-E) The amount of ¹⁸F-FDG uptake volume in the lung (with SUV > 1.5 corresponding to FDG positive threshold) and in tracheobronchial lymph nodes (SUVmax). Data are median, and * pvalue < 0.05, ** pvalue < 0.01 and *** pvalue < 0.001, **** pvalue < 0.0001, 2 way ANOVA test with Sidak's multiple comparison test. BL: baseline, EP: end point, SUV: standard update value. Dark cyan: Slow progressors (SP) and Orange: Fast progressor (FP).

Because Mtb infection was also associated with a shift in blood neutrophil maturation and neutrophil signatures in transcriptomic data, further analyses of neutrophil functions were performed. To determine whether changes in neutrophil maturation were associated with the modulation of their functions, NETosis and ROS production were assessed in four CyMs infected by Mtb. NET production by blood neutrophils was quantified by determining the amount of myeloperoxidase-DNA (MPO-DNA) complex produced after stimulation with peptidoglycan (PGN) or phorbol myristate acetate (PMA). With this system, unstimulated neutrophils released no or negligible amounts of NETs, while PMA stimulation induced a high amount of NETs with no significant differences between pre-infection and post-infection (**Figure 17A and 17B**). PGN was used as a physiological stimulation inducing NET production in neutrophils. Compared to uninfected macaques, NETosis was significantly increased at 4 weeks pi ($p < 0.05$), indicating that the infection induces increased NET release capacities by neutrophils. The level of NET production was not significantly different in later time points, with data showing more heterogeneity. We also measured reactive oxygen species (ROS) using a whole blood flow cytometry assay based on cellROX technology. Capacities to produce ROS by neutrophils in response to PMA and N-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) were both significantly increased at 4 weeks pi ($p < 0.01$ and $p < 10^{-3}$ respectively) (**Figure 17C and 17D**). Neutrophils stimulated with PMA or fMLP produced significantly less ROS at 8 weeks pi compared to 4 weeks pi ($p < 0.05$ and $p < 0.01$, respectively). At 12 weeks pi, neutrophils ROS response to PMA remained significantly increased compared to baseline. These data indicate that neutrophils' capacities to produce NETs and ROS are increased in the early phase of the infection.

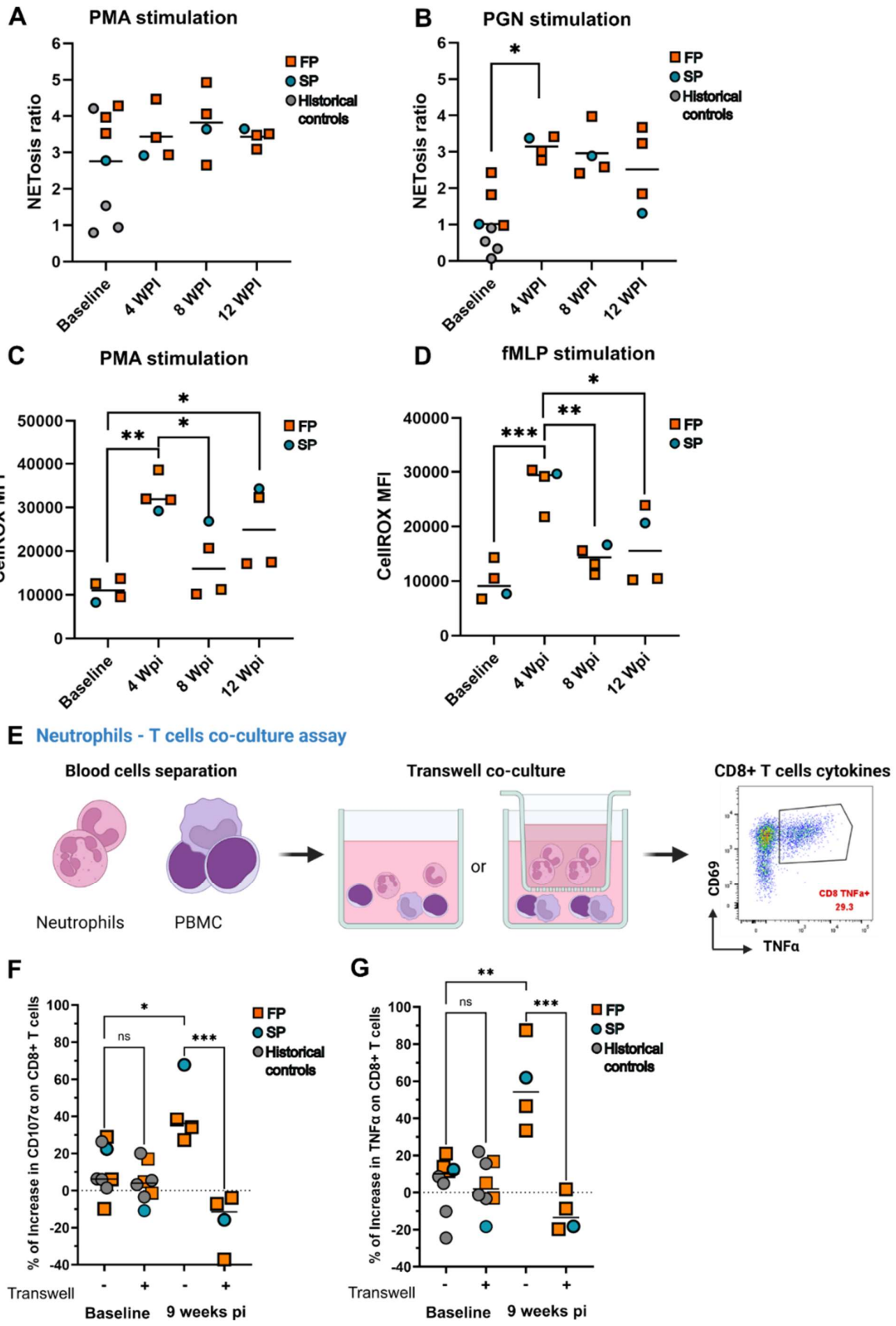
In other studies, changes in neutrophil phenotype or neutrophil subsets have been sometimes associated with capacities to modulate T cells. We previously showed that neutrophils could modulate T cells during acute and chronic Simian Immunodeficiency Virus infection in cynomolgus macaques. Hence, we assessed the capacities of autologous purified neutrophils to modulate CD4⁺ and CD8⁺ T cell activation, cytotoxicity and intracellular cytokine production using an in vitro co-culture assay

(**Figure 17E**). Due to the blood volume needed, we only focused our analysis on baseline and 9 weeks pi in four CyMs infected by Mtb. Before infection and in historical uninfected controls, neutrophils had a limited impact on T cell functions. At 9 weeks post-infection, neutrophils from infected animals increased CD107a expression and production of TNF- α in autologous CD8+ T cells compared to baseline (**Figure 17F and 17G**). To investigate the mechanism of T cell modulation by neutrophils, we performed a transwell assay, in which neutrophils were separated from PBMC into two medium-filled compartments with a 0.4 μ m porous membrane. In the presence of a transwell membrane, T cell modulation by neutrophils was reduced, underlying a contact-dependent mechanism (**Figure 17F and 17G**). Overall, these results indicate that neutrophils positively modulated T cells' cytotoxicity and TNF- α production during Mtb infection through a contact-dependent mechanism. Further analysis with more animals will be required to assess if this neutrophil modulation of T cells is different between fast and slow progressors.

(Figure next page)

Figure 17. Functional characterization of neutrophils during Mtb infection in CyMs, from Dinko et al (in preparation)

(**A-B**) NETosis ratio of blood neutrophils stimulated by phorbol myristate acetate (PMA) or peptidoglycan quantified MPO-DNA ELISA. Bar graph of data represents the mean. * $p < 0.05$ by Kruskal-Wallis test with Tukey's correction. (**C-D**) Reactive oxygen species (ROS) production by whole blood neutrophils stimulated by PMA or fMLP measured using CellROX probe by flow cytometry. Bar graph of data represents the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Kruskal-Wallis test with Tukey's correction. (**E**) Schematic of neutrophils-T cells co-culture and transwell assay design. Sorted neutrophils were co-cultured with autologous PBMC in absence or presence of transwell membrane. Activation markers and cytokine production by T cells was measure by intracellular staining coupled with flow cytometry after overnight stimulation with Cytostim. BioRender.com. (**F-G**) Percentage of increase of CD107a and TNF α production by CD8+ T cells representing the increase or decrease of percentage of CD107a or TNF α positives cells in presence of neutrophils compare to PBMC alone. Data from animals at 9 weeks post-infection were compared with baseline and historical controls, ie healthy animals from other internal studies. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Kruskal-Wallis test with Tukey's correction.



DISCUSSION & PERSPECTIVES

DISCUSSION

In our study, we chose cynomolgus macaques (CyMs) infected by SIVmac251 as a non-human primate model of HIV. This model was particularly relevant regarding several aspects. First, SIVmac251 infection in CyMs is well characterized in our laboratory (and elsewhere) regarding the disease's pathophysiology and evolution. We ensured all animals developed the infection through the following of CD4+ T cell counts and SIV plasma viral loads. Of note, one animal developed AIDS early, while another was able to control its viral load without any treatment.

Thanks to the animal facility and the Animal & Welfare (ASW) team in IDMIT, we performed all experiments very rapidly after animal blood withdrawal, which allowed us to limit neutrophil activation or damage. We followed animals closely and performed experiments at different key stages of SIV infection. We also had access to chronically infected animals with or without ART, and we assessed the impact of treatment on our observations. Such a follow-up in patients would be particularly challenging. Another major asset of our model is that neutrophils from CyMs and humans are very similar regarding their phenotype and responses to stimuli. Because many neutrophil markers are common between humans and CyMs, we built a flow cytometry panel to follow the expression of CD66, CD62L, CD11b and FcγR, as well as proteins whose expression change with maturation like CD101 and CD10 [253,282].

However, contrary to humans, neutrophils from NHPs do not express CD16 [290], a protein often used as a specific marker for this cell population. To this day, there are no specific markers for neutrophils in CyMs. Hence, one major constraint in this work was the proper isolation and purification of fresh blood CyM neutrophils. As such, a drawback of the study is the potential neutrophil activation/modification due to the isolation process. In our work, we designed a protocol to purify neutrophils from CyM whole blood, but the process involved many steps: Ficoll, red blood cell lysis and

magnetic depletion to remove eosinophil contamination. Although we demonstrated that CD62L expression was relatively poorly changed during the process, we saw that the expression of CD11b was considerably increased, which may suggest neutrophil degranulation or activation. Additionally, we found that the technical step inducing this CD11b upregulation was the Ficoll separation/red blood cell lysis, and not the magnetic sorting. This observation emphasizes the need to develop neutrophil sorting from CyM whole blood, a tool commonly used for human samples in recent studies that does not exist for NHP models. In IDMIT, many efforts have been collectively made to isolate neutrophils from CyM whole blood or to separate them from the erythrocyte fraction, using sedimentation, percoll, and even dextran sedimentation. Unfortunately, none of these experiments were conclusive regarding neutrophil purification, and many techniques induced neutrophil activation. Collaborating with a company might be necessary for the development of neutrophil sorting from CyM whole blood.

In the literature, LDN/G-MDSCs were shown to exert T cell modulation in various infectious contexts, including HIV infection [140]. Interestingly, a few teams have managed to purify neutrophils from rhesus macaque whole blood using Ficoll [301], while we did not find any report on LDN isolation from CyMs. These differences may suggest neutrophils or erythrocytes from CyMs present a different density than human or rhesus macaque neutrophils.

Immature neutrophils are known to be increased in the blood in response to chronic or acute stress. Recently, Marini et al. demonstrated that daily injections of G-CSF for 5 days in healthy patients induced an increase in CD10⁻ blood neutrophils and showed that CD10⁻ or CD10⁺ neutrophils modulated differently T cells [294]. Increased CD10⁻ neutrophils were also observed in the blood of sepsis patients [302,303] and were associated with increased T cell proliferation, while CD10⁺ mature neutrophils inhibited T cell proliferation. In acute myocardial infarction, CD10⁻ neutrophils were also associated with modulatory functions of T cells, with an increased IFN- γ production by CD4⁺ T-cells [304]. These observations tend to show that after an exposition to stress,

immature neutrophils are released early from the bone marrow, and that these cells modulate T cells differently compared to mature neutrophils.

Therefore, we developed a protocol to purify CD10⁺ and CD10⁻ neutrophils from CyM whole blood using magnetic depletion cell sorting. With cytopins, we showed this cell sorting successfully separated CD10⁻ neutrophils from the CD10⁺ fraction. However, the expression of CD10 on neutrophils forms a smear: as such, some CD10_{low} or CD10_{mid} neutrophils were selected in the CD10⁺ fraction, which may create a bias when comparing the functional roles of CD10⁺/CD10⁻ neutrophils. As we performed these experiments at the end of the study, the number of animals was too low to draw clear conclusions.

Indeed, two important limits for our study are the number of animals and the variable number of animals depending on the experiments. The latter point is due to the fact that some animals came from studies conducted by other teams in IDMIT, thus limiting the blood volume we had access to, especially during the viremia peak at 10 days post-infection. This timepoint is particularly studied during SIV infection because it is difficult to investigate in patients.

In our work, we demonstrated SIV infection in CyMs was associated with higher frequencies of immature and activated neutrophils. These results confirm previous observations obtained by Lemaitre et al. in IDMIT and also corroborate results from patients living with chronic HIV [253,305]. In parallel with this phenotype, we found a significant decrease in total blood neutrophils at 10 days post-infection. These results could suggest either a recruitment of mature blood neutrophils in the tissues or an elevated neutrophil cell death, which has been described in the literature [249]. In our study, we did not assess neutrophil apoptosis markers; however, adding cell death markers such as caspase-3 or annexin V could be relevant to tackle this question.

To investigate how neutrophils can modulate T cell activity at steady state or during SIV infection, we set up co-culture assays of isolated neutrophils and PBMCs from CyMs.

Using these co-cultures, we first investigated how neutrophils modulate soluble cytokines and chemokines produced by PBMCs before and during SIV infection using a multiplex assay to quantify supernatant molecule concentrations in co-cultures. Strikingly, in uninfected animals, neutrophils modulated several cytokines produced by PBMCs. Cytokines related to T cell activation $\text{TNF}\alpha$, $\text{IFN}\gamma$ and IL-6 were decreased, which suggests that at steady state, neutrophils, which are mostly mature cells, are already capable to modulate T cells by reducing local pro-activating cytokines. Few studies explored neutrophil modulation of T cells in healthy individuals. In 2011, Thewissen et al. showed that CD69 expression on T cells decreased in the presence of neutrophils and that these suppressive effects were mediated by arginase-1 [306]. More recently, Minns et al. demonstrated that the impact of neutrophils on T cells depended on each cell type's activation level in healthy volunteers [225]. In this work, neutrophils did not affect T cell proliferation, PD-1 expression, or soluble production of $\text{TNF}\alpha$ / $\text{IFN}\gamma$ in naïve T cells. However, when neutrophils and T cells were cultured in presence of CD3/CD28 stimulation for 24h, neutrophils decreased soluble $\text{TNF}\alpha$ and $\text{IFN}\gamma$ productions by T cells in supernatants. Moreover, if neutrophils were added after T cells were previously exposed to CD3/CD28 stimulation for 24h, this suppressive activity did not occur. In our CyM model, we co-cultured neutrophils and T cells together in the presence of T cell stimulation and also found suppressive effects of neutrophils at steady state. In the presence of neutrophils, supernatant concentrations of $\text{TNF}\alpha$, $\text{IFN}\gamma$ and IL-6 were decreased, as well as the intracellular T cell production of $\text{TNF}\alpha$ in CD4+ and CD8+ T cells. Altogether, these observations suggest that neutrophils may have a regulatory role in T cell activation in the absence of infection or inflammation.

During SIV infection, after 15 days, interactions between neutrophils and PBMCs resulted in increased IL-1RA concentrations in co-cultures. This protein is usually induced in inflammatory contexts and displays anti-inflammatory properties by blocking the binding of IL-1 on its receptors. Hence, two hypotheses can be raised from this result: either that anti-inflammatory pathways are induced in PBMCs in response to neutrophil inflammatory effects, or that neutrophils themselves can adopt an anti-inflammatory phenotype after several hours of culture with PBMCs. Importantly, IL-1RA was shown to be more elevated in the plasma during the acute phase of HIV/SIV infection, which underlines the relevance of analyzing the role of this molecule [307]. Besides, IL-1RA can be produced by neutrophils [308], but also by other cells such as monocytes and macrophages [309]. This may suggest that neutrophils tend to lower local inflammation in the presence of PBMCs, which could be beneficial to the host to avoid over-inflammation and local damage in tissues.

During the chronic phase of SIV infection, neutrophils in co-cultures with PBMC induced decreased concentrations of many T-cell pro-inflammatory molecules in the media, including IFN γ , TNF α , IL-6 or IL-1 β . Again, one may hypothesize either that neutrophils present or acquire anti-inflammatory properties in the presence of PBMCs, or that neutrophils are able to bind/capture these inflammatory molecules, which can induce signaling pathways that are yet to be defined. The latter hypothesis is supported by the fact that neutrophils express receptors to IFN γ , TNF α and IL-1, and are responsive to these molecules. For instance, neutrophils were shown to produce ROS in response to TNF α [310], which in turn could lead to T cell immunosuppression through arginase-1, as evoked in the introduction of our manuscript. Multiple studies showed neutrophils respond to IFN γ [209,311], and neutrophils were shown to process and degrade IL-1 β [312]. Notably, chemokines related to macrophage/monocyte activation were also decreased in the presence of neutrophils in our study, including MCP-1. It suggests that neutrophils not just regulate T cells locally, but also other mononuclear cells such as monocytes.

While several pro-inflammatory molecules were decreased in co-culture supernatants, one major chemokine was particularly increased in the presence of neutrophils, which is interferon gamma-induced protein 10 (IP-10 or CXCL10). IP-10 is an interferon-induced molecule that participates in inflammation and immune cell recruitment, including T cells. IP-10 levels were shown to be more elevated in the plasma during the chronic phase of HIV in patients, even in ART+ patients [115]. In 2014, Noel et al also demonstrated that HIV elite controllers presented a lower plasmatic concentration of IP-10 compared to other patients living with HIV. However, plasma IP-10 concentrations from elite controllers remained more elevated than those of healthy volunteers [313]. As such, our results suggest neutrophils may be involved in the elevation of IP-10 in patients, hence participating in inflammation.

To our knowledge, this is the first investigation of the effects of neutrophils on soluble molecules produced by PBMCs in the course of SIV or HIV infection. Although this assay is only partially informative on the cellular sources of molecules and associated targets, this data underlines the capacity of neutrophils to interact with PBMCs and modulate the local microenvironment. Importantly, neutrophils exerted modulatory effects related to T cell activation, but also inflammation, which led us to investigate the direct effects of neutrophils on T cells by studying their intracellular cytokine production and activation.

To evaluate direct effects of neutrophils on T cell activation, we studied T cells after overnight co-cultures between PBMCs and isolated neutrophils using extra and intracellular cytokine staining (ICS). At the resting state, neutrophils induced some effects on T cell activation and cytokine production. Notably, intracellular expressions of TNF α in CD8+ and CD4+ T cells were decreased, while CD69 expression slightly increased in CD4+ T cells. Again, this shows that neutrophils have a potential to regulate T cell activity even at steady state.

During the acute phase of infection, the presence of neutrophils induced a consistent increase of intracellular expression for all the markers we observed on CD4+ and CD8+

T cells, except of IL-2 and IL-17. Although the consequences of this activation of T cells by neutrophils remain unknown yet, these results suggest neutrophils during the peak of infection, with neutrophils increasing T cell defense capacities against viral spread. During the chronic phase of infection, neutrophils could still consistently increase the expression of CD69 on CD4+ and CD8+ T cells, but not intracellular cytokine production. These observations could reflect T cell exhaustion: in HIV infection, T cells' activation level remains more elevated compared to healthy donors, but CD8+ T cells are dysfunctional [103]. However, we were not able to study common immune checkpoint inhibitors such as PD-1 or CTLA-4 on T cells in our study and we did not observe any increase in PD-L1 expression on blood neutrophils.

Additionally, we demonstrated that the impact of neutrophils on T cells relied on contact-dependent mechanisms, as we observed no effects of neutrophils when cells were separated by a 0.4µm membrane. As mentioned in the introduction of this manuscript, neutrophils have been shown to exert T cell activation or suppression through several mechanisms during chronic HIV infection [279,281].

Interestingly, the "T-cell activating" role of neutrophils observed during the acute phase of SIV infection did not reflect in the levels of chemokines and cytokines we measured in PBMC/PMN co-culture supernatants. At first, this discordance surprised us, as we expected similar effects of neutrophils on intracellular cytokine production in T cells and soluble molecules. When we analyzed more deeply ICS and Luminex assays, we realized that these two assays are actually complementary. Indeed, ICS assays relied on a 1-hour incubation during which neutrophils and PBMCs, including stimulated T cells, interact freely in the presence of other cellular partners. After the 1-hour incubation, cell secretion was blocked using BFA and monensin. On the other hand, secretion remained active in the Luminex assays, which allowed the exchange of soluble factors and activation of cellular pathways, and we obtained the "final" result of these interactions after overnight incubation. As such, ICS assays gave us information on the direct effects of neutrophils on T cells, while multiplex assays informed us on how

neutrophils impact cytokine/chemokine concentration in PBMCs in the presence of T cell stimulation without knowing which cell produced which molecule.

In our study, we could not identify a cellular or molecular mechanism to explain how neutrophils modulate T cells during SIV infection. Yet, we performed supplementary experiments during the chronic infection to characterize more precisely the interaction between neutrophils and T cells, by (1) evaluating the role of blood CD14+ monocytes/macrophages as potential partners in the interaction and (2) evaluating the roles of CD10+ or CD10- neutrophils on T cells. Although these experiments were conducted only on a few animals, the results suggested that CD14+ cells do not play a major role in neutrophil/T cell interactions after 6 months of SIV infection. In parallel with these assays, we found that CD10+ mature neutrophils were the most responsible for increasing T cell activation compared to CD10- cells. These results remain to be confirmed in a higher number of animals. They should also be performed at the peak of infection, during which the frequencies of CD10- blood neutrophils are the highest, and the direct effects of neutrophils the strongest.

It is also necessary to underline that we only assessed T cell activation through extracellular and intracellular expression of common T cell markers, but we did not study the functional aspects of T cells with suppressive or cytotoxic assays, for example. Here, we used a non-specific stimulation for a broader activation. However, further investigation remains necessary to evaluate if neutrophils also increase specific CD8+ T cells capacities to respond to SIV peptides or kill SIV-infected cells. One difficulty would be to use tetramers to isolate such specific cells, which are rare after only 10 days of SIV infection.

To identify potential neutrophil-related signatures in SIV-infected animals, we performed PLS-DA analyses to identify the data contributing the most to differentiating the groups of animals we studied. These analyses allowed us to integrate data of different natures at different timepoints of SIV infection. When we built these analyses, we identified the importance of having complete datasets, and we learned how to

handle missing values. Indeed, we could not integrate datasets with missing values because PLS-DA analyses rely on linear regressions, which restricted our analysis to the complete datasets. Nonetheless, this method highlighted signatures related to neutrophil modulation, especially in the acute phase of infection, reinforcing the importance of studying these cells during SIV infection.

Considering that about 75% of PLWHIV had access to antiretroviral therapy in 2023, including animals receiving ART in our study seemed relevant. Hence, we included animals receiving ART after 1 month of SIV infection, which allows both the study of untreated acute and treated chronic phases of infection in these animals. When we compared treated versus untreated animals, we did not observe clear differences in the modulation of T cell activation and intracellular cytokine expression between ART+ and ART- animals after 6 months of infection. Several reasons can explain this lack of differences. First, a 5-month ART duration is relatively short to stabilize the effects on SIV-infected macaques completely. Indeed, after 5 months of therapy, only one animal treated with ART out of 5 presented a viral load close to the undetectability threshold, while others still presented viral loads between 1 and 3 log SIV RNA copies/mL blood. Another point is that animals received daily intradermal injections, which may have caused a low-grade inflammation that could create bias. Finally, we performed the experiments on 5 animals ART+ during the chronic phase of SIV, a relatively low number of animals that prevents strong statistical analyses.

On the other hand, the production of IP-10 by PBMCs tended to be more elevated in SIV+ ART- animals in the presence of neutrophils compared to SIV+ ART+ animals. This may suggest that untreated animals present a higher inflammatory potential than animals that received ART, and that neutrophils could participate in this inflammation, a hypothesis which is relevant to literature [115].

After investigating how neutrophils can modulate T cells in SIV infection, we wanted to further analyze neutrophil changes at cellular levels. Because neutrophils express low

levels of RNA but high protein content, we decided to perform proteomic assays instead of transcriptomic assays. To our knowledge, this is the first report of a proteomic analysis of pure neutrophils from cynomolgus macaques at homeostasis and during SIV infection. Proteins were identified using a cynomolgus macaque database, and we found that the most abundant neutrophil proteins from macaque and human neutrophils were similar. Currently, many transcriptomic studies of neutrophils rely on mice datasets. In a recent study, Sollberger et al. demonstrated that mice neutrophils showed fewer primary granule proteins than human neutrophils and a higher global protein mass [314]. This result also strengthens the relevance of using macaque models to study neutrophil changes during homeostasis and infections, and for future pre-clinical trials for neutrophil modulation.

In the course of SIV infection, we found that neutrophils were strongly imprinted with type I IFN responses. A higher expression of different proteins than uninfected animals, such as RIG-I, IFIT1B, or IFIT3 drove these signatures. Our analysis supports persisting interferon-related signatures in neutrophils during untreated SIV infection, which may participate in chronic inflammation. Although animals receiving ART displayed fewer proteins related to interferon pathways, some proteins remained elevated compared to baseline in this group, such as IFIT1, suggesting that the phenotype of neutrophils is not fully restored with ART.

Type I IFN are proteins and glycoproteins interfering with viral replication that can be produced by all nucleated cell types. Nonetheless, plasmacytoid DCs and monocytes were shown to produce high amounts of these proteins compared to other cells. The production of type I IFN is triggered by the recognition of PAMPs by PRRs such as TLRs or RLRs. In HIV infection, type I interferon responses were described in patients, but studies gave mixed results on their roles in HIV control and systemic inflammation [315,316]. In 2023, Mackelprang et al. showed that interferon-stimulated genes (ISG) were upregulated during the acute phase of HIV infection in PLWHIV, and that these signatures persisted in untreated chronic infection [317]. Additionally, both non-HIV

controllers and PTCs presented upregulated type I IFN responses after analytic ART interruption, but these signatures were resolved after several months in PTCs and not in non-controllers [24]. These observations suggest that unresolved type I interferon responses could be involved in chronic inflammation and immune exhaustion [318]. This hypothesis is also supported by our observations, and suggest that neutrophils could be involved in that process. Two hypotheses can be raised in light of our results. First, the presence of proteins linked to ISG proteins in neutrophils could be reflecting an ongoing inflammation that could be partially sustained by unresolved type I IFN pathways sustained by monocytes and plasmacytoid DCs. This hypothesis is relevant because neutrophils express receptors to type I IFN (IFNAR) and can trigger inflammatory responses when binding to IFNs. A second hypothesis would be that neutrophils could favor type I IFN responses by producing high levels of these proteins in the context of SIV or HIV infection. A deeper analysis of neutrophil responses to type I IFNs in the context of HIV or SIV infection could provide new insights into the roles of these cells. In other contexts, the studies of omics and disease-induced pathways in neutrophils are recent, and we only found a few studies. Recently, type I interferon pathways signatures were described in proteomes of neutrophils from COVID-19 patients in early disease [319], and in transcriptomic signatures in neutrophils from patients living with Sjögren's syndrome [320]. In parallel with these pathways, our results reinforce the hypothesis that neutrophils could exert antiviral functions against SIV, as shown by upregulated expressions of SAMHD1 or MX2, for instance. Investigating neutrophil proteomes from early and chronic PLWHIV and re-investigating direct and indirect interactions between neutrophils and HIV/SIV would be of interest to confirm our observations.

Currently, most proteomics analyses are built using transcriptomic pathway datasets, and more specifically human and mouse cellular pathways. Because we worked on protein samples from CyMs, we are aware this could constitute a limit for our analysis, even more so because neutrophil transcriptomics is still under investigation. With the proteomics dataset we provide, we hope to fill some existing gaps in neutrophil

knowledge in both human and CyMs proteome databases and that this dataset will be helpful for future studies on neutrophils.

Finally, we are aware this work presents limitations. First, we were sometimes limited in our statistical analyses due to the low number of animals. The animal model we studied, CyMs, is expensive and limits the number of individuals we can include. As previously mentioned, we are also aware that our results can present some bias due to neutrophil activation because of the process. Moreover, many of our results rely on neutrophil-PBMC co-cultures that model a micro-environment such as a tissue. Yet, we know that tissues are complex environments with many other actors, such as epithelial cells, that we could not include in this model. Lastly, we could not identify any molecular or cellular mechanisms responsible for the modulation induced by neutrophils on T cells. However, now that we have identified that neutrophils are involved in the regulation of T cell activation and soluble effectors in a contact-dependent way, we will be able to target interleukins and neutrophil functions such as the release of ROS to decipher neutrophil-T cell interactions.

Overall, in this work, we identified neutrophil changes at the cellular and protein levels before and at different phases of SIV infection in cynomolgus macaques. We found neutrophils could modulate T cells differently before and during SIV infection, both through soluble cytokine/chemokine modulation and directly in co-cultures. Neutrophil-related signatures in SIV-infected macaques were also confirmed using PLS-DA analysis. Additionally, neutrophils were involved in the modulation of inflammatory proteins by decreasing or increasing them, especially in the chronic phase of infection. We also report for the first time (type I) interferon-related responses in neutrophils that were partially resolved in animals treated for several months with ART. We believe our work brings evidence to integrate neutrophils in strategies targeting inflammation in patients living with HIV, especially in therapies focusing on interferon-related responses.

PERSPECTIVES

In the future, we should extend our investigations to other infectious diseases, especially lung diseases in which neutrophils have been shown to play a role (COVID-19, tuberculosis, influenza). Preventing neutrophils from fueling inflammation and activating other immune cells could lower organ damage and mortality in patients. In light of our results, we believe that studying how neutrophils can shape T cell activation and functions could lead to a better understanding of immunity in pathophysiology and inflammation.

One of the major perspectives for this project is the investigation of neutrophil-T cell interactions in tissues from SIV-infected macaques. Indeed, several teams have shown that neutrophils undergo changes in phenotype or transcriptional profile when entering tissues, which may also affect their functional capacities and their roles [141,321]. Therefore, in my opinion, it would be particularly relevant to investigate the bio-distribution of tissue neutrophils in organs of interest in SIV infection, including the splenic and intestinal SIV reservoirs, using imaging techniques on embedded tissues. More specifically, we would like to stain CD8+ T cells, CD4+ T cells and neutrophils to evaluate a potential co-localization of these types of cells in target tissues, which would reinforce the hypothesis of a direct interaction between neutrophils and T cells and support the data we obtained from blood cells.

Even more importantly, I think a great perspective would be to develop imaging on living cells, to study specifically neutrophil-T cell interactions on a precise moment, or over time. This could be done on ex-vivo cell cultures like in our study, but we can also imagine performing in vivo videomicroscopy in macaque tissues, which has been performed on rhesus macaque granulomas [322]. This sort of experiment could bring much information on the mechanisms involved in neutrophil-T cell interactions during homeostasis and in pathogenic contexts. Nonetheless, this would require developments to perform multiple stainings on co-culture cells to identify NETosis (with MPO/elastase/histones) or synapses between cells.

In parallel, further investigating the impact of neutrophils on T cells in ex-vivo assays could also help decipher cell-cell mechanisms. For instance, we would like to evaluate the effects of adding different neutrophil-induced components to T cells on their activation. Isolating and adding NETs from stimulated neutrophils to T cell cultures could be interesting. Finally, characterizing more precisely the cytokines and chemokines specifically produced by macaque neutrophils in response to various signals in SIV infection may help us identify the molecular actors involved in PMN-T cell interactions. Investigating the responses of neutrophils to the pro-inflammatory cytokines produced by T cells, such as IL-6, IFN γ and TNF α , could be one of the first steps to unveil this crosstalk. In particular, as previously described, we could investigate the potential upregulation or release of arginase-1 or galectin-9 by neutrophils.

Considering that IP-10 has been linked to the severity of the disease in PLWHIV, investigating the role of neutrophils in favoring IP-10 release would be of great interest. More specifically, identifying if neutrophils are a source of IP-10 or if this chemokine is released by another cell type after interaction with neutrophils, such as macrophages, could help in understanding how this molecule is produced. Indeed, another axis of this work could be the study of neutrophil-macrophage interactions.

By deepening our proteomics dataset analysis, we will also improve our knowledge of the characterization of neutrophils from cynomolgus macaques. This will help test specific neutrophil markers and identify common neutrophil markers between cynomolgus macaques, other NHP species, and humans. This could also be done in parallel with single cell-transcriptomic assays to have a complete overview of neutrophils in our model. As previously mentioned, improving neutrophil isolation by developing a cell sorting from the whole blood of CyMs/NHPs in collaboration with industry would be of major importance for further studies of neutrophils in these models. Indeed, isolating neutrophils properly would not only increase the relevance of using this animal model to study the role, but also the relevance of our results for translational studies to more clinical aspects.

In the long term, we could imagine finding neutrophil-related biomarkers for inflammation that could be targeted to reduce pain and comorbidities in patients. For instance, therapies targeting NETs with DNase I or heparin have been shown to improve outcomes in several diseases, including lung diseases, and such drugs are now considered for COVID-19 patients' clinical trials [323]. Another field in which neutrophils are becoming of interest in innovative therapies is cancer. Neutrophils are now integrated into immunotherapies, and chimeric antigen receptor (CAR)-neutrophils have been developed recently using engineered human pluripotent stem cells delivering anti-tumor drugs [324]. We could imagine using such CAR-neutrophils to deliver ART drugs to organs that constitute pharmacological sanctuaries, or drugs regulating inflammation and metabolism in organs involved in comorbidities. However, direct or indirect targeting of neutrophils should be carefully considered because, as evoked in the introduction, these cells are involved in many regulatory and defense pathways crucial for host homeostasis and survival. This gathers all the considerations we must take to appreciate how neutrophils are at the center of a “good cop/bad cop” balance.

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APPENDIX

Posters

- *Role des neutrophiles dans l'infection SIV : effets immunomodulateurs T et impact tissulaire sur la persistance virale*, Gaëlle Sandillon, Julien Lemaitre, Delphine Desjardins, Roger Le Grand, Olivier Lambotte.
Presented during the **Université des Jeunes Chercheur.e.s de SIDACTION**, Novembre 2022, Carry-le-Rouet, France
- *Expansion of immature neutrophils during SIV infection is associated with their capacity to modulate T cell function*, Gaëlle Sandillon, Julien Lemaitre, Delphine Desjardins, Anne-Sophie Gallouët, Mario Gomez-Pacheco, Christine Bourgeois, Benoit Favier, Asier Saez-Cirion, Roger Le Grand and Olivier Lambotte.
Presented during the **Neutrophil symposium**, December 2022, Mexico City, Mexico
- *Immunomodulation of T Cells by Neutrophils during SIV infection in cynomolgus macaques*, Gaëlle Sandillon, Julien Lemaitre, Laure Fournier-Le-Ray, Tatiana Botin, Delphine Desjardins, Roger Le Grand and Olivier Lambotte.
Presented during the **Biosigne Doctoral school day**, April 2023, Le Kremlin-Bicêtre, France

Oral communications

- *Immunomodulation of T lymphocytes by neutrophils during SIV infection in Cynomolgus macaques*. Presented during the **Biosigne Doctoral school day**, April 2024, Saclay, France
- *Immunomodulation of T lymphocytes by neutrophils during SIV infection in Cynomolgus macaques*. Presented during the **Neutrophil symposium**, September 2024, Munich, Germany

Training courses

Here are two courses I followed that particularly enriched my knowledge of HIV on the one hand, and helped me gain confidence throughout my PhD on the other hand.

- **Université des Jeunes Chercheur.e.s de SIDACTION 2022**: This program is a 1-week multidisciplinary formation presenting all HIV-related research domains. In

this program, we built a multidisciplinary group project focusing on improving antiretroviral therapy acceptance in children living with HIV.

- **Mentoring program « Women in Science » 2023:** This one-year program aims to guide, advise and support young female researchers in their personal and professional development. Through this program, I was mentored by Dr. Carine Van Heijenoort, and I participated in several formations, such as “How to Speak in Public” and “Improve your Self-knowledge”

Scientific animations and participation in IDMIT’s life

During my time at IDMIT, I wanted to get involved in the organization of scientific and student gatherings inside and outside the research unit. Taking part in the organization of these events allowed me to extend my network and increase my knowledge of various scientific subjects.

- I was the vice-president of the ACTIF association in 2022/2023 (Association du CEA des Thésard.e.s d’Ile de France): during this time, I co-organized various activities for PhD students from the CEA. I also co-organized and/or co-animated conferences for M2 students on climate change in 2022 and 2023, with the topics “Renewable energies” in 2022 and “Water” in 2023.
- I co-organized the “Doctoral and Post-doctoral Day” at the CEA Research Institute of Biology “François Jacob” (about 100 students): I was part of the organizing committee and the jury who attributed prizes for best posters and oral presentations.
- I co-organized the “Doctoral and Post-doctoral Day” at IDMIT in 2022 and 2024 (about 30 students)
- I co-animated a workgroup during the IDMIT seminar in 2023 on the theme of ecology in laboratories
- I was elected as the representative of fixed-term employees for the IDMIT council in 2024