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Heterogeneity of polymorphonuclear neutrophils in HIV-1 infection. Study of SIV-infected cynomolgus macaque model.

Julien Lemaitre

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Heterogeneity of polymorphonuclear neutrophils in HIV-1 infection Study of SIV-infected cynomolgus macaque model

Thèse de doctorat de l'Université Paris-Saclay
préparée à l'Université Paris-Sud

École doctorale n°569 Innovation thérapeutique :
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Spécialité : Immunologie

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List of abbreviations

3TC: Lamivudine

AAV: Antibody-associated vasculitis

ACPA: Anti-citrullinated protein antibodies

ADCC: Antibody-dependent cellular cytotoxicity

ADCP: Antibody-dependent cellular phagocytosis

AGM: African green monkey

AIDS : Acquired immunodeficiency syndrome

ANRS: Agence nationale de recherche sur le sida et les hépatites virales

ARDS: Acute respiratory distress syndrome

AZT: Azidothymidine

BAFF: B-cell activating factor

C/EBP- α : CCAAT/enhancer-binding protein alpha

cART: Combination antiretroviral therapy

CD: Cluster of differentiation

CLR: C-lectin receptor

CMP: Common myeloid progenitor

COPD: Chronic obstructive pulmonary disease

CRP: C-reactive protein

CTLA4: Cytotoxic T-lymphocyte-associated protein 4

DAMP: Danger-associated molecular pattern

DC: Dendritic cell

DNA: Deoxyribonucleic acid

DRV: Darunavir

DTG: Dolutegravir

Fc-R: Fragment crystallizable receptor

FDC: Follicular dendritic cell

fMLP: N-formylmethionine-leucyl-phenylalanine

FTC: Emtricitabine

G-CSF: Granulocyte-colony stimulating factor

G-MDSC: Granulocytic-myeloid-derived suppressive cell

GALT: Gut-associated lymphoid tissue

GM-CSF: Granulocyte-monocyte colony stimulating factor

GMP: Granulocyte-monocyte progenitor

HBV: Hepatitis B virus

HCMV: Human cytomegalovirus

HCV: Hepatitis C virus

HESN: HIV-exposed seronegative

HIV: Human immunodeficiency virus

HLADR: Human leukocyte antigen DR type

HSC: Hematopoietic stem cells

HTLV: Human T cell leukemia/lymphoma virus

I-FABP: Intestinal fatty-acid binding protein

ICP-MS: Inductively coupled plasma mass spectrometry

IDV: Idinavir

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

IP-10: Interferon gamma-induced protein 10

ISG: Interferon-stimulated genes

ITAM: Immunoreceptor tyrosine-based activation motif

LDN: Low-density neutrophil

LN: Lymph node

LPS: Lipopolysaccharide

MALT: Mucosa-associated lymphoid tissues

MDA5: Melanoma differentiation-associated protein 5

MHC: Major histocompatibility complex

MMP: Matrix metalloproteinase

MPO: Myeloperoxidase

Mtb: Mycobacterium tuberculosis

NADPHox: Nicotinamide adenine dinucleotide phosphate oxidase

Nef: Negative regulatory factor

NET: Neutrophil extracellular trap

NK: Natural killer

NLR: Nucleotide-binding oligomerization domain-like receptor

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PCR: Polyclonal chain reaction

PD-1: Programmed cell death 1

pDC: Plasmacytoid dendritic cell

PMA: Phorbol myristate acetate

PMN: Polymorphonuclear neutrophil

PPAR γ : Peroxisome proliferator-activated receptor gamma

QVOA: Quantitative viral outgrowth assay

RA: Rheumatoid arthritis

RIG-I: Retinoic acid-inducible gene I

RNA: Ribonucleic acid

ROS: reactive oxygen species

rRNA: Ribosomal ribonucleic acid

SIV: Simian immunodeficiency virus

SLE: Systemic lupus erythematosus

SM: Sooty mangabey

SNS: sympathetic nervous system

SPADE: Spanning-tree Progression analysis of density-normalized events

ssRNA: Single-strand ribonucleic acid

TAN: Tumor-associated neutrophil

TB: Tuberculosis

TCM: T lymphocyte central memory

TCR: T cell receptor

TDF: Tenofovir disoproxil fumarate

TEM: T lymphocyte effector memory

Tfh: T follicular helper cell

TGF- β : Transforming growth factor beta

Th: T helper cell

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TNFR: Tumor necrosis factor receptor

Treg: T regulatory cell

WHO: World health organization

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Forewords: brief history of a zoonosis

“There will be new diseases. It is a fatal fact. Another fact, as fatal, is that we will never be able to detect them from their origin. When we have a notion of these diseases, they will all be formed, adults could we say. They will appear as Athena appeared, coming out of the brain of Zeus. How would we recognize these new diseases, how would we suspect their existence before they donned their costume of symptoms?” – Charles Nicolle, Destin des maladies infectieuses

In 1981, first acquired immunodeficiency syndromes (AIDS) were reported among five young men affected by pneumocystis and severe T cells depletion¹. Quickly more new cases were reported and the lentivirus involved, human immunodeficiency virus-1 (HIV-1), was identified in a lymph node biopsy from a patient². HIV-1 is a retroviridae lentivirus characterized by a bilayer envelope containing glycoproteins and two single RNA strands genome. Gp120 glycoprotein determines the range of host cells and targets CD4 receptor present in CD4+ lymphocytes, macrophages and monocytes. Interaction of Gp120 with CD4 needs a co-receptor to enter the cell, such as CXCR4 and CCR5, which also determines virus targets. Basically, T cell tropic HIV-1 viruses use CXCR4, whereas macrophage tropic viruses use CCR5 receptor. Another important characteristic of retrovirus is the reverse transcription, allowing HIV to integrate its genome in host cells, thus generating reservoirs and long-term infection.

Infection by HIV is divided in 3 different phases: primary infection, chronic phase and AIDS. Only 50% of the patients present symptoms during primary infection, especially flu-like syndrome, asthenia, lymphadenopathies and digestive symptoms³. Primary-infection lasts in median 2 weeks with sustained viral replication and CD4+ T cells depletion. Then, with partial control of viral replication by adaptive immune response starts the chronic phase, lasting years in which clinical signs are reduced but transmission risk is elevated. As the CD4+ T cell count decreases, non-AIDS defining events appear as oral candidiasis, herpes zoster. When the CD4+ T cell count is below 200/mm³, the risk of opportunistic infection strongly increases, with an elevated risk of bacterial infection (tuberculosis), oesophageal candidiasis, pneumocystosis, toxoplasmosis and some cancers (lymphoma, Kaposi sarcoma). AIDS is defined in the United State by a CD4+ T cell count below 200/mm³ and in France by the occurrence of various AIDS defining conditions. Immune restoration by antiretroviral treatment with the increase of CD4+ T cell counts is fundamental to control AIDS related diseases.

The emergence of HIV-1 pandemic raised question about its origin. Identification of closely related lentivirus in African primates, Simian Immunodeficiency Viruses (SIV), supports the hypothesis of zoonotic transmission from primate to human⁴. Indeed, many African monkeys and apes harbor SIV but rarely develop AIDS, and are so called natural hosts. On the contrary, Asian macaques are not found to be naturally infected by SIV. Experimental infection of Asian macaques with some strains of SIV results in opportunistic infections, cancers directly related to high viral loads and CD4 T cell depletion. Non-natural SIV hosts have been essential to better understand HIV-1 physiopathology and for vaccine and treatment development. Besides, natural host highlighted mechanisms involved in resistance to disease development, which basically relies on control of immune activation. Phylogenetic analyses of SIV obtained from 40 primates species reveal that HIV-1 came from SIVcpz or SIVgor isolated from Chimpanzee (*Pan troglodytes*) and Western gorilla (*Gorilla gorilla*) respectively⁴. Molecular clock analyses had dated the beginning of HIV-1 infection (group M and O) epidemic in early twentieth century in west central Africa⁵, where cross-species transmission may occurred between apes and human⁶. Bushmeat hunters being exposed to apes blood and body fluids is the best explanation of transmission to human⁴. Cross-species transmission was not a matter of chance and happened independently four time for HIV-1, giving rise to the four independent groups. HIV-1 (group M and O) spreads for 50-70 years from colonial western Africa before becoming worldwide and its first recognition in western countries. Colonial exploitation of western Africa's resources and populations in early twentieth century had created perfect conditions for HIV-1 emergence and worldwide spreading^{7,8}. Since start of the pandemic, UNAIDS estimated 77.3 million people have been infected with HIV and 35.4 million died from AIDS-related illnesses⁹. Globally in 2017, 36.8 million people were living with HIV with 59% accessing antiretroviral therapy.

Introduction

I. The unsolved chronic inflammation in HIV-1 infection

In absence of a cure, lifelong antiretroviral treatment is necessary to control virus replication because of the inability of combinational antiretroviral treatment (cART) and host defenses to eradicate the virus. cART has increased life expectancy and the quality of life of HIV-infected patients, but is still far from a cure. Despite cART, non-AIDS related co-morbidities (metabolic, neurodegenerative and cardiovascular diseases) normally associated with aging have been shown to be more frequent in HIV-infected population than aged-matched population¹⁰. This increased frequency of co-morbidities was soon associated with the persistence of chronic inflammation (Figure 1)¹¹. Even under long-term cART, HIV-infected patients have a higher level of soluble inflammatory markers (ex. IL-6) and gut epithelial dysfunction markers (ex. I-FABP)¹². Several hypotheses have been explored to explain the persistence of chronic inflammation in on-ART patients (Figure 1). First, because of their physicochemical properties, drugs generally do not distribute throughout the whole body. cART was shown to be reduced in gut and lymphoid tissues, leading to residual replication suspected to maintain local inflammation¹³. Second, gut mucosal damages and associated microbial translocation, which are initiated in primary infection, were shown to be also elevated in on-ART patients. Since markers of epithelial barrier integrity (ex. zonuline), microbial translocation (ex. 16S RNA) correlate with myeloid cells activations (ex. soluble CD14) and pro-inflammatory cytokines (ex. IL-6)¹⁴⁻¹⁶, this phenomenon is likely to drive chronic inflammation. Third, co-infections with *Mycobacterium tuberculosis*, hepatitis viruses and cytomegalovirus are common in HIV-infected patients and have been also associated with chronic inflammation¹⁷. Lastly, behavior and lifestyle are strongly linked with morbidity and mortality, notably through inflammation¹⁰.

Nevertheless, exact mechanisms and cells involve in the persistence of increased levels of soluble mediators are not clear. Microbial translocation and residual replication are both potent to induce innate immune cells activation, which may further release pro-inflammatory mediators¹⁸. In turn, innate immune activation in tissues promote tissue fibrosis and hypercoagulability, which are suspect to lead to non-AIDS comorbidities¹⁹. Monocytes and macrophages were shown to be involved in chronic inflammation in HIV-infection since they are producers of IL-6 and release sCD14 under microbial product stimulation.

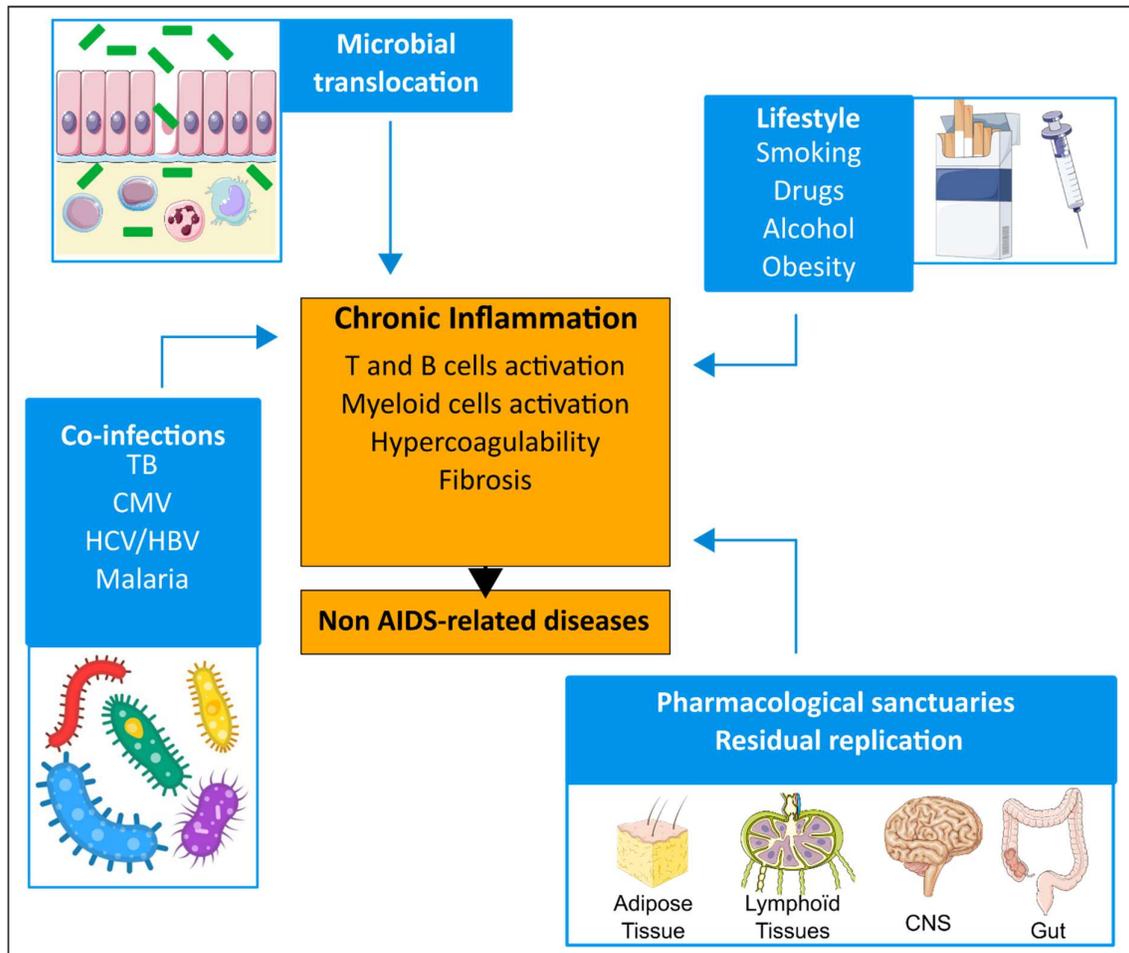


Figure 1: Major causes of chronic inflammation in HIV-infected patients under cART and association with non-AIDS-related diseases. Unrestored gut mucosal homeostasis under cART leads to persistent microbial translocation, which maintains inflammatory state. Sustain immune activation induces tissue fibrosis especially in adipose tissue, in lymph nodes, in the liver, which reduces clearance of microbial products and leads to liver diseases. Lifestyle influences also inflammatory state and impacts organs functions such as the liver (alcohol) and the lungs (tabacco). The existence of pharmacological sanctuaries allows virus persistence and residual replication inducing local and systemic inflammation and organs dysfunction. Co-infections are common in HIV-infected patients and are contributors to chronic inflammation.

In the first part, I will focus on causes and consequences of chronic inflammation under cART with the emphasis on the role of myeloid cells. But one of the most potent pro-inflammatory myeloid cells, polymorphonuclear neutrophil (PMN), has been overlooked in HIV-infection²⁰. Neutrophils have been involved in most of non-AIDS-related comorbidities as an effector of chronic inflammation²¹, notably potent to release IL-6 and also to modulate coagulation cascades leading to thrombosis. In addition, PMN activation was higher in HIV-infected patients having non-AIDS-related comorbidities, suggesting a relationship between neutrophils and level of systemic inflammation²². Thus we hypothesize that PMN dysfunction could be responsible of chronic inflammation in HIV infection^{23,24}. In the last part, I will review the literature on the role of PMN in physiology and chronic inflammatory diseases.

II. Causes of chronic inflammation in treated HIV-infection and consequences

In HIV infection, chronic inflammation originates from viral persistence and inability of the host to resolve inflammation. Natural hosts, such as African green monkeys, demonstrate that virus-host interaction could lead to the resolution of inflammation despite viral persistence, thus protecting against AIDS. In absence of functional cure, cART is the best option to reduce disease progression, but inflammation remains elevated. In the first part, we will present HIV infection as a chronic inflammatory disease. In the second part, we will describe cause and consequences of chronic inflammation under cART.

A. HIV infection as a chronic inflammatory disease

In primary infection, immune response starts with acute phase molecules such as serum amyloid A, detectable in plasma 3 to 5 days after transmission. Increasing of viremia is associated with growing innate immune response and important inflammatory cytokines release. This inflammatory cytokine burst starts with high levels of IFN- α , interleukin -15 (IL-15), followed by a rise of IP-10, TNF- α , IL-18 and IFN- γ ²⁵. Studies conducted in both SIV-infected macaques and HIV-infected patients showed a rapid and barely irreversible depletion of memory CD4+ T cells in mucosa, mostly in CCR5+ cells (Figure2) ^{26,27}. HIV-1 infection leads to progressive depletion of memory and naïve CD4+ and CD8+ T cells via direct and indirect mechanism²⁸. Direct virus killing and NK cells or CD8 T cells cytotoxic activity were thought to be the major mechanism of CD4 T cells depletion, but Quingsheng L. and colleagues showed that only 7% of lamina propria CD4+ T cells were infected by SIV²⁹. The virus indirectly impairs thymus output early in SIV infection, where a decrease of thymocyte number is associated with cell apoptosis³⁰. In addition, immune activation is an important indirect mechanism of uninfected CD4 T cell depletion via the Fas-FasL and the TRAIL-DR5 apoptosis pathways³¹. The decrease half-life of exhausted cells contributes to the reduction of the memory CD4 T cell pool³².

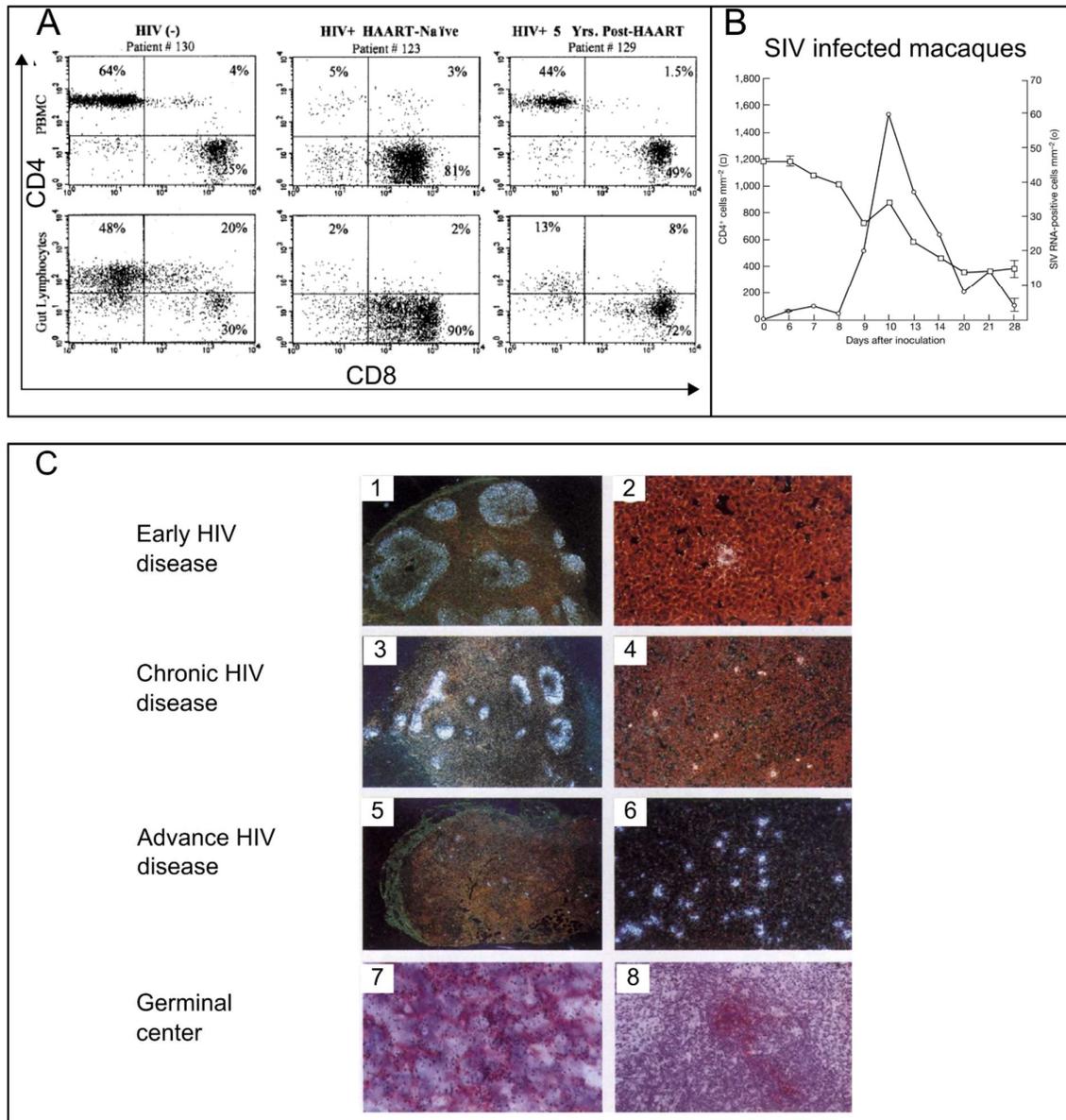


Figure 2: Gut CD4+ T cell depletion and lymph node histopathology during HIV-1 infection. **A**-Flow cytometry and immunohistochemical analysis showing CD4 T cells depletion in colon GALT from chronically HIV-1 infected patients, and partial restoration under cART. **B**-Massive CD4 T cells depletion within lamina propria occurs during primary infection in SIV infected macaques. The peak of viral replication in GALT coincides with plasma viral load. **C**- LN histology associated with HIV-1 RNA in situ hybridization from patients at different stage of the disease. 1, 2- In early HIV disease, HIV RNA is located in follicles (1) and paracortical area (2) as indicated by white dots of silver grains. 3, 4- In chronic HIV disease, follicles are disrupted with intense signal of HIV RNA and increasing staining in paracortical zone. 5, 6- In advance HIV disease, LN architecture was lost with an absence of follicle and HIV RNA located in paracortical zone. 7- FDC network in pink (anti-CD21) coinciding with HIV RNA appearing as black dots in chronically infected patient. 8- Impaired FDC network and HIV RNA in a involute follicle. Adapted from Guadalupe, M. et al (*J. Virol* 2003)³³, Li Q. et al. (*Nature* 2005)²⁹, Pantaleo G. et al (*Nature* 1993)³⁴.

From chronic SIV/HIV infection to AIDS, secondary lymphoid organs undergo progressive histological changes associated with CD4+ T cell depletion and chronic immune activation. Indeed, lymphadenopathy (enlarge lymph node) seen in first description of AIDS patients reflect the importance of secondary lymphoid tissues. Viral replication in LN is generally 10 to 100 fold higher than in PBMC, making lymphoid tissue the major site of viral production³⁴. Histological evaluation of lymph nodes (LN) demonstrated a progressive destruction of architecture. Follicular hyperplasia with focal coalescence of follicles explained lymphadenopathy observed in AIDS patients. As the disease progresses, follicles atrophy progresses and is associated with fibrosis and paracortical hyperplasia. Viral particles were found in LN, notably in germinal centers associated with follicular dendritic cells (FDC)³⁵ (Figure 2). Disappearance of FDC plays a central role in follicular atrophy, because their organizational role in follicle architecture is lost.

B. Failure of antiretroviral treatments to resolve inflammation

1) Reservoirs and pharmacological sanctuaries maintain immune stimulation

i. HIV persistence in cellular and tissue reservoirs

Antiretroviral treatments efficiently reduce viral replication to undetectable viremia but are not curative since cART interruption mostly leads to viral replication return. Recovery of viral replication is possible thanks to viral reservoirs established in first days of infection. A reservoir is defined by persisting HIV-infected cells under treatment, regardless the mechanism allowing persistence (quiescence, cell proliferation or residual replication)³⁶. Evaluation of HIV reservoirs mostly relies on the detection of HIV-specific nucleic acids, which is the easiest and the most sensitive method. Presence of HIV DNA within cells is the most used reservoir quantification, but do not make the difference between replication-competent and defective virus³⁷. Quantitative viral outgrowth assays (QVOA) makes this distinction, and represents a gold standard to measure frequency of CD4+ T cells infected by replication-competent proviruses³⁸. At the cellular level, researcher had identified few markers on CD4+ T cells associated with enrichment of chromosomally integrated HIV-1 provirus such as PD-1, CTLA-4, HIV-specific TCR, some integrins and more recently CD32a. Unfortunately, these markers

are still unable to gather all infected cells, because none of them is universal for latently infected CD4 T cells, and they miss infected non-CD4+ T cells.

Reservoir is also defined by its anatomical localization, each tissue or organ owing its specific cell reservoir distribution. HIV/SIV DNA has been detected in several lymphoid and non-lymphoid tissues³⁹. Primary and secondary lymphoid tissues are reservoir for HIV (Figure 2). In bone marrow, HIV infects resident CD4 T cells and macrophages^{34,40}. Studies on ART-suppressed patients did not detect HIV DNA in CD34+ progenitor but there is evidence that mast cell precursor could be latently infected by the virus⁴⁰⁻⁴². Spleen and lymph nodes contain huge numbers of infected cells and free virions trapped in FDC network⁴³. Even after years of cART HIV RNA and DNA are still detected in lymph nodes⁴⁴. Germinal centers harbored viral particles HIV p24, p17 and gp120/gp41 abundantly detected from patients under effective cART⁴⁵. In patients under ART, central memory and transitional memory represent the largest CD4+ T cells reservoir in peripheral blood, whereas effector memory CD4+ T cells had a greater contribution in lymphoid tissues³⁶. Recently, studies on SIV-infected macaques and HIV-infected patients have identified another important contributor of lymphoid tissue reservoir: CD4+ T cells follicular helpers (Tfh). Persistence of infected Tfh is due to the impossibility of cytotoxic T lymphocytes to enter germinal centers⁴⁶. Tfh were associated with high level of HIV-1 RNA in patients after up to 12 years of treatment duration, thus representing a source of residual replication and participating to chronic inflammation⁴⁷. In ART-suppressed patients gut, HIV-1 DNA and RNA were identified in both CD4+ T cells and CD13+ myeloid cells, mostly macrophages^{48,49}. Indeed, in on-cART individuals, gut is one of the organs comprising the highest number of infected cells. By analyzing 4 different regions of the gut from on-ART individuals, Yulk *et al.* showed HIV DNA levels per CD4+ T cells were 5-10 fold higher in gut compared to peripheral blood⁴⁸(Figure3).

Myeloid cells, especially macrophages, represent an important reservoir cells in other organs. HIV DNA and RNA were detected in lung tissue, mostly in macrophages and comparison of HIV-1 sequence evolution in the lung showed a compartmentalization from the blood^{50,51}. In central nervous system from on-ART patients, HIV DNA and RNA are localized to perivascular macrophages and microglial cells⁵². Male reproductive tract, especially testis is an immune-privilege site in which specific humoral and cellular response are reduced against HIV-1^{53,54}.

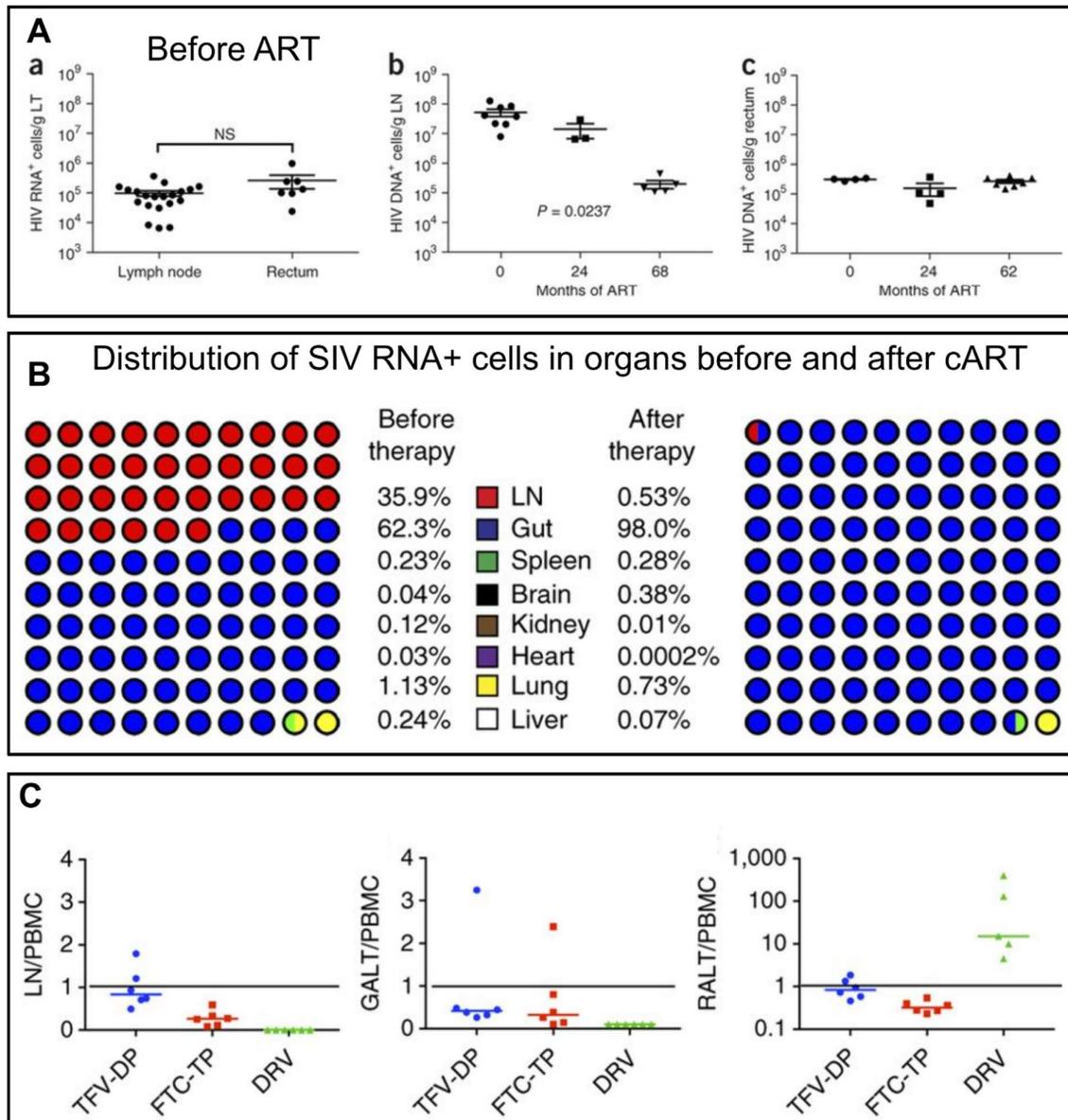


Figure 3: Persistence of viral reservoir and residual replication are associated to poor antiretroviral drugs distribution. **A**-Evaluation of HIV RNA⁺ cells by *in situ* hybridization within LN and rectum from HIV infected patients before and after cART. **a**, HIV reservoir in LN and rectum are similar before treatment initiation. **b-c**, cART reduced HIV reservoir in LN but had no effect in rectum. **B**-Representation of SIV RNA⁺ cells distribution in SIV-infected macaques before and under cART. Gut represent the major site of residual replication under cART. **C**- Intracellular concentrations of Tenofovir-DP, emtricitabine-TP and darunavir in different tissues compared to PBMC from SIV-infected macaques. Reduce intracellular drug concentrations in LN, gut and rectum associated with residual replication support the hypothesis of pharmacological sanctuaries. Intracellular tenofovir concentration in LN explained the reduction of SIV⁺ cells in this compartment after cART. Adapted from Siddiqui S. et al. (*Viruses* 2019) and Estes, J. D. et al. (*Nat. Med.* 2017)^{55,56}.

SIV infected macaques treated with cART had both HIV DNA and RNA positive cells in prostate, vas deferent, epididymis and seminal vesicle due to persistence in macrophages⁵⁷. Lastly, skin and adipose tissue constitute also reservoirs for HIV-1. Few studies detected competent HIV DNA in dermis, epidermis, especially Langerhans cells. Replication competent HIV was found in CD4+ T cells from adipose tissue^{58,59}.

ii. Pharmacological sanctuaries as a source of chronic inflammation

HIV-1 RNA is still detectable in several tissues from HIV-infected patients receiving cART. HIV replication under cART, called residual replication, remains controversial since most of studies aiming to find evolution of blood HIV failed⁶⁰. Using ultra-sensitive PCR, very low viremia (median 3.1 copies/ml) was still detectable among patients under cART and for years of follow-up^{61,62}. A recent study showed evidence of residual viral evolution, suggesting ongoing replication, in lymph nodes during cART⁶³. This underline the importance to explore lymphoid tissues, where residual replication might take place, rather than blood. The existence of residual replication indicates effective cART is not able to suppress HIV-1 replication in some compartment, called pharmacological sanctuaries.

Because of physicochemical properties, drugs generally do not distribute throughout the whole body. Depending on the molecule, absorption, distribution, metabolism and elimination affect drug concentration in tissues. In vivo imaging using positron emission tomography conducted in rats allowed to measure distribution of radiolabeled FPMPA (tenofovir analog). Spleen and submandibular lymph nodes had a two-fold lower FPMPA penetration than blood, mesenteric lymph nodes and testis 4 fold lower, and brain up to 25 fold lower than blood concentration⁶⁴. Bourry et al. showed that SIV-infected macaques treated with AZT/3TC/IDV had a persistent viral replication in peripheral lymph nodes and spleen, but efficiently suppressed viral replication in gut and GALT⁶⁵. Using *in situ* hybridization of viral RNA, J Estes et al. evaluate the distribution of productively infected cells within the body in SIV-infected macaques before and after cART (darunavir, ritonavir, emtricitabine and tenofovir)⁵⁵. After 20 weeks under cART, virus-productive cells in tissues were still detected at a lower level. In macaques under cART, gut comprises 98% of all viral RNA+ cells within tissues, and it was associated with a poor treatment diffusion in this compartment (Figure 3). Lamivudin concentration was reduced in secondary lymphoid tissue and correlated with level

of viral RNA in tissue. Another study showed presence of HIV RNA in all lymph nodes samples from HIV-infected patients receiving protease inhibitors⁶⁶. HIV RNA was associated with lower ARV concentration in lymphoid tissue compared to plasma. In fact all tissue reservoirs (lymphoid tissues, central nervous system, genital tract and liver) are also suspected to host residual replication since HIV RNA is detected¹³. Beside suboptimal drug distribution in tissue, cellular mechanisms are also responsible to reduce treatment effectiveness. Transporters expressed by monocytes and tissue macrophages could decrease intracellular drug concentrations of ARV and substrates. Even if treatment reaches infected macrophages, efflux transporters make tissue macrophages pharmacological sanctuaries^{67,68}. Multidrug resistant proteins (MRP) and P-glycoprotein were shown to be expressed by several tissue specific macrophages, such as microglia, and decreased anti-HIV activity of lamivudine and zidovudine.

2) Maintenance of type I interferon response under cART

Type I interferons are beneficial cytokines during viral infections by inducing hundreds of interferon-stimulated genes (ISG) which protect other uninfected cells⁶⁹. Nevertheless, persistent HIV-1 replication maintains activation of ISG, leading to chronic immune activation. Higher levels of IFN α correlated with ISGs up-regulation in CD4+ T cells from HIV-1 infected individuals, and was also associated with CD4+ T cell depletion^{70,71}. Initiation of cART reduced plasmatic inflammatory markers such as TNF- α and IFN- γ but not IP-10 and sCD14⁷². Moreover, ultrasensitive CRP (CRP_{us}) is rarely normalized in patients on ART. Interestingly, following cART there was a lesser reduction of plasmatic inflammatory markers in women compared to men in a study⁷³. Sex-related differences in term of inflammation and response to cART in HIV infected patients have been explained by hormones and type I interferon production⁷⁴. At the same viral load, women had a higher up-regulation of ISGs compared to men. Under TLR7 stimulation, female's pDC express more mRNA of all IFN α and IFN β . HIV-1 derived ssRNA induce higher IFN α production associated with CD8+ T cells activation in women compared to men⁷⁴. Hormonal cycle also influences TLR7-responsiveness and IFN α production, making more complex to analyze sex differences⁷⁰.

C. Unrestored mucosal immune response under cART

The gastrointestinal tract is an important site for HIV pathogenesis regardless the route of acquisition, since >40% of all body lymphocytes are located in gut associated lymphoid tissues (GALT)⁷⁵. Lamina propria contains a large population of resident memory CD4+ T cells, which is largely depleted in primary infection, before efficient adaptive response is generated²⁷. Indeed, first case reports of AIDS already described gastrointestinal symptoms with diarrhea and weight loss⁷⁶. HIV-1 preferentially depletes gut Th17 cells as shown by Paiardini, Brenchley and colleagues⁷⁷. In this study, bronchoalveolar lavages, peripheral blood and terminal ileum biopsies obtained in HIV-1 infected patients revealed infection of Th17 cells and a tissue dependent depletion of Th17 cells. This CD4+ T helper cells producing IL-17 was first described in respiratory mucosal response to bacteria, with IL-17 known to promote rapid recruitment of monocytes and neutrophils, to induce G-CSF production and IL-8^{78,79}. Indeed, Th17 cells are enriched in human GALT and participate to gut homeostasis by inducing epithelial cells regeneration, mucin production and by maintaining tight junction integrity⁸⁰. Thus, depletion of Th17 is thought to be responsible of epithelial barrier breakdown observed in HIV-infected patients, leading to microbial translocation.

Microbial translocation exposes lamina propria to microbial product and fuels intestinal inflammation, which in turn conducts to microbiota alteration⁸¹. Work conducted by Lynch S. has shown an enrichment of Proteobacteria and a decrease abundance of Bacteroidetes in chronically infected patients, underlying a dysbiosis associated with HIV-1 infection⁸². In the phylum of Proteobacteria there was a high representation of Enterobacteriaceae family, containing proinflammatory pathobionts. Dysbiosis was correlated with IL-6 and tryptophane catabolism, both linked to inflammation and unbalance immune response. So, HIV-1 infection induces dysbiosis because of inflammation and metabolism alteration, in turn altered microbiota may also participate to gut inflammation as seen in inflammatory bowel diseases.

Recently, neutrophil infiltration has been associated with colonic epithelial cells apoptosis and microbial translocation in HIV and SIV infections, especially in chronic phase^{83,84}. Regardless HIV-infected patients were receiving cART, there was an increase PMN infiltration in colonic lamina propria^{83,85}. PMN infiltration in colon was significantly higher in chronic phase compare to primary infection⁸⁶. (Figure 4) Whether neutrophils are a cause or a consequence of gut epithelium damage remains to be determined.

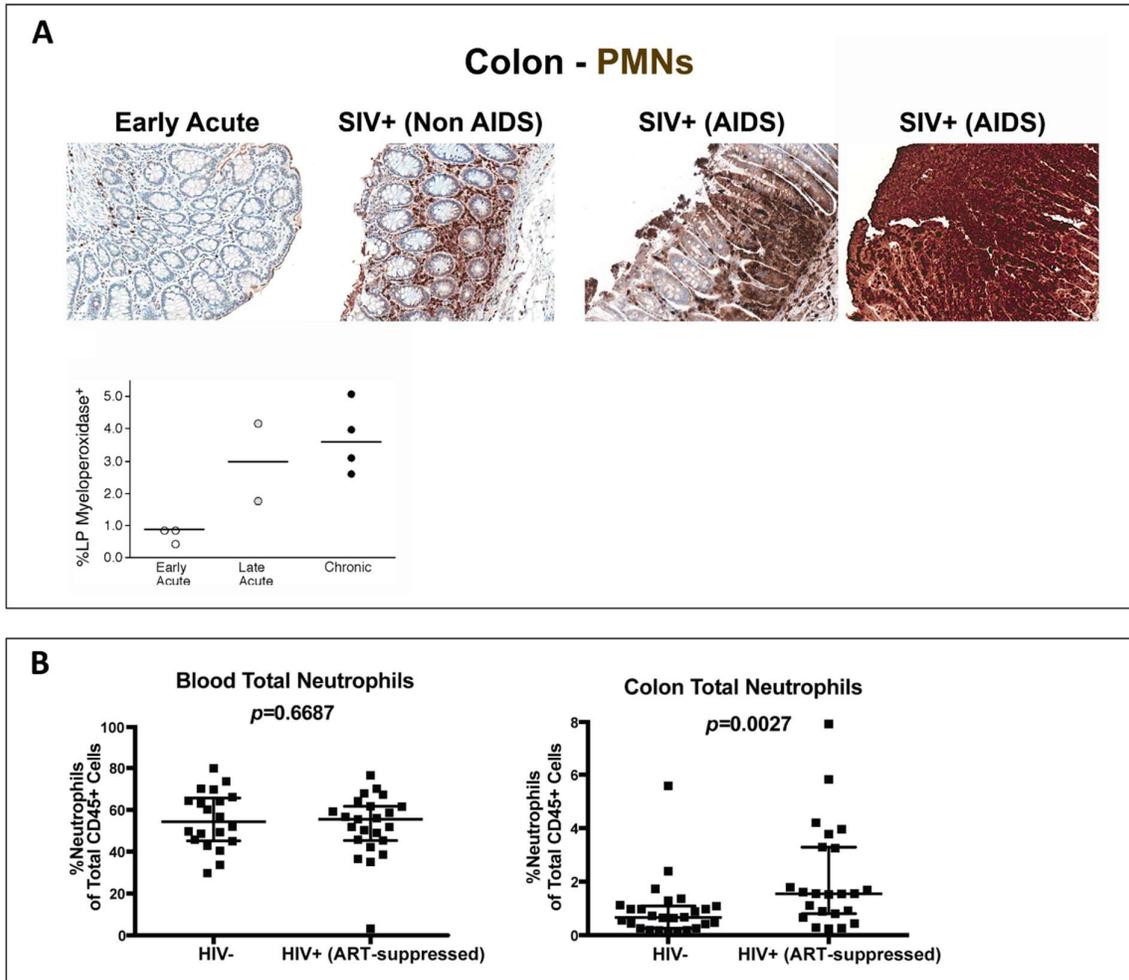


Figure 4: Neutrophils infiltrate gut during HIV infection. *A*-SIV-infected RM colon stained for myeloperoxidase (brown) to quantify PMN infiltration. PMN infiltration increased with epithelial lesion and with the progression of the disease. In AIDS, neutrophils infiltration was important. Graphic showing neutrophil infiltration in few SIV infected RM. *B*-Neutrophil frequency in blood from HIV-infected patients under cART did not differ from healthy controls. Besides, neutrophil frequency measured in colorectal biopsies showed increased infiltration HIV-infected patients under cART. Adapted from Hensley-McBain T. et al. (PLoS Pathog. 2019).

In chronic inflammatory disease, increase lifespan of PMN is associated with tissue damages. A recent study shed light on the link between PMN lifespan and gut microbiota⁸⁷. Using flow cytometry, authors confirmed the increase of neutrophils in colorectal biopsies. To evaluate gut neutrophils survival, decrease of CD16 expression and increase of active Caspase-3 were used and demonstrated a higher percentage of surviving PMN in HIV-infected patients, regardless they received cART. Gut microbiota imbalance observed in HIV-infected individuals was characterized by enrichment of *Prevotella* and decrease abundance of *Lactobacillus*. Neutrophil stimulation with *Prevotella* was capable to increase PMN survival whereas *Lactobacillus* species increased PMN apoptosis. Thus, neutrophil survival is associated with pro-inflammatory microbiota and environment in HIV infection. Like inflammatory bowel diseases, gut mucosa damage in HIV infection might be mediated by neutrophil persistence and activation⁸⁸. It will be necessary to evaluate neutrophil function in mucosae to better understand their contribution to tissue damage and immune activation.

Tissue analysis of SIV-infected rhesus macaques indicate presence of microbial products which reached draining and peripheral lymph nodes and liver, where myeloid cells induce local immune responses⁸⁴(Figure 5). Microbial translocation, measured directly by plasma LPS, and indirectly by LBP or sCD14, was correlated with viral load and CD8+ T cells activation, regardless if patients were or not taking cART^{89,90}. Therefore, microbial translocation participates to systemic inflammation. Gut translocated microbial products arrive first to the liver from portal vein, where macrophages, dendritic cells and Kupffer cells are activated and release cytokines such as TNF, IL-6 and IL-8⁸⁴. The number of Kupffer cells has been shown to decrease in HIV-infected patients, which impairs the liver's ability to clear microbial products^{91,92}. The resulting liver inflammation promotes hepatocyte cell death and fibrosis⁹³. Co-infections with hepatitis B or C viruses and alcohol consumption worsen the fibrosis. Interestingly, liver diseases in HIV-infected patients represent 13-18% causes of non-AIDS-related death⁹⁴.

Residual replication and HIV persistence are linked with inflammation⁹⁵. Despite cART, treated HIV-infected patients have lymph nodes abnormalities such as damage of follicular structure, poor CD4+ T cell restoration and follicular abnormalities from hyperplasia to regression^{96–98}. The detection of microbial products in axillary and mesenteric lymph nodes from SIV-infected RM, suggests microbial translocation may participate to lymph nodes impairment⁸⁴. Local immune activation induced by LPS triggers TGF- β 1 pathways, and thus collagen deposition in lymphoid tissue^{99,100}. Collagen preferentially deposits in T cell zone and correlates with disease stage⁹⁷. Collagen deposition has dual deleterious role: by reducing niches for CD4+ T cells reconstitution, and by limiting immunological synapses formation.

In this vicious circle, generalized fibrosis disrupts normal lymphoid tissue structure and function, and thus participates to myeloid cells activation. By inducing inflammation and fibrosis, microbial translocation might be also a mechanism to consider to explain lymphoid tissue damage in patient under cART. Nevertheless, it is still hard to conclude on whether inflammation comes from previous tissue lesions acquired before treatment initiation, or ongoing inflammatory processes occurring under cART or both.

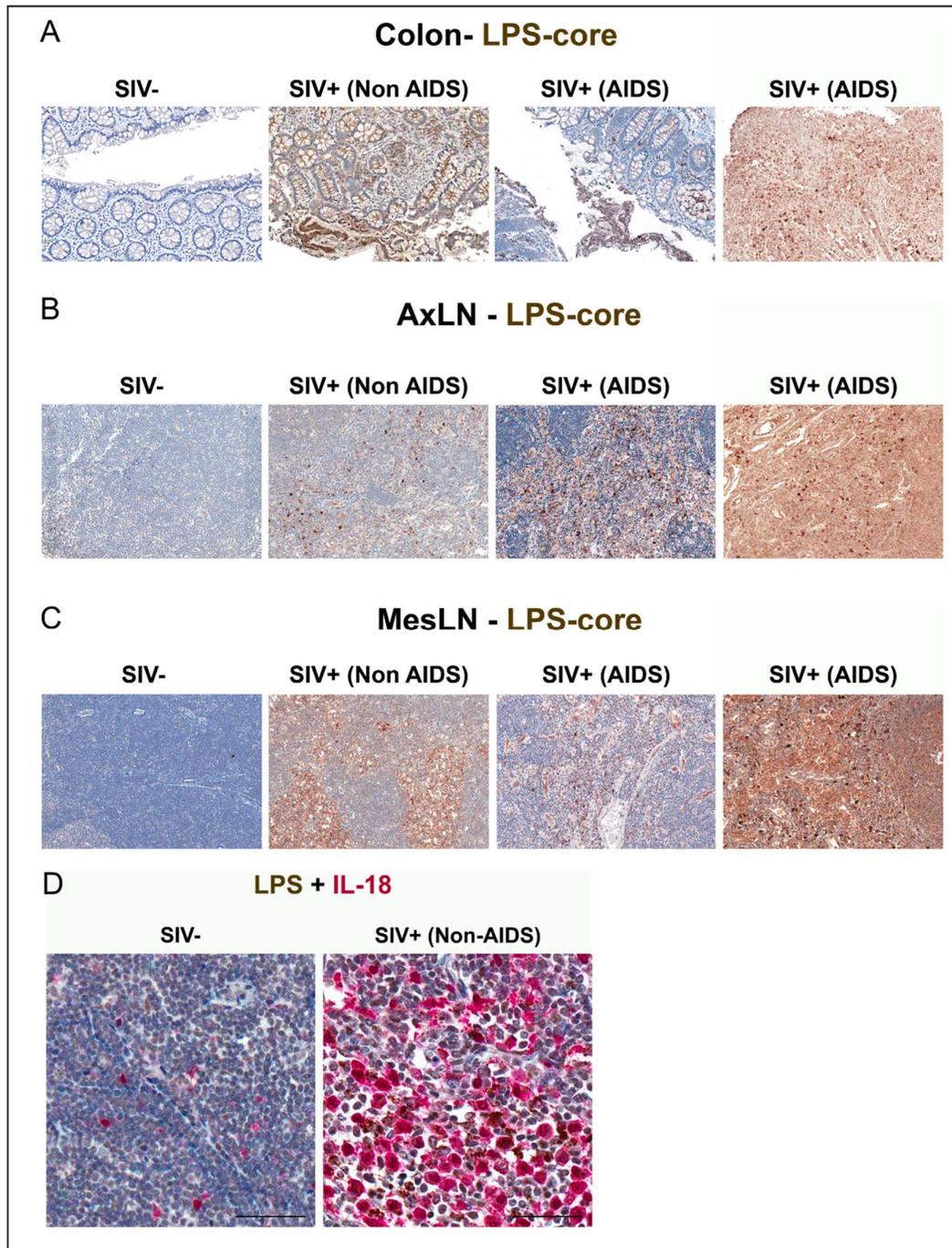


Figure 5: Translocated microbial products reach secondary lymphoid tissues and drive distant inflammation. **A,B,C**-Images of colon stained for LPS-core antigens (brown) from SIV-uninfected and SIV-infected macaques (non-AIDS and AIDS). The two last images show the array of staining observed in AIDS. **A**-Histology showing an increase of LPS+ cells in lamina propria associated with epithelial damages. **B**-Images of paracortical T-cell zone of mesenteric LN with LPS accumulation increasing with disease progression. **C**-Microbial translocation reaches distant axillary LN with accumulation of LPS within germinal centers, paracortex and medullary cord. **D**-Co-localization of LPS and IL-18 inflammatory cytokine in mesenteric LN from SIV-uninfected and SIV-infected macaques. Adapted from Estes, J. D. et al (PLoS Pathog. 2010)⁸⁴.

D. Co-factors associated with amplification of inflammation

1) Co-infections as additional sources of inflammation

HIV infection, by weakening the immune system, is associated with co-infections by reactivation of pathogens or increase susceptibility to newly acquired pathogens. Even after initiation of cART, co-infections fuel chronic inflammation and are associated with increase morbidity and mortality¹⁰¹. Tuberculosis, viral hepatitis and cytomegalovirus infection are the most prevalent HIV-coinfections and have been studied for their impact on chronic inflammation.

i. Tuberculosis strongly activates immune cells

Tuberculosis (TB) co-infection has been largely studied because, globally, 1 in 4 individuals are living with latent TB infection¹⁰². In 2015, it has been estimated that 1.14 million of HIV-1 infected individuals suffer from active TB. Active TB accelerates HIV disease progression, increasing plasma¹⁰² and cerebrospinal¹⁰³ viral loads associated with increased viral quasispecies evolution¹⁰³. In active TB infection, *M. tuberculosis* contributes to higher immune activation in HIV-1 co-infected patients. In TB/HIV-1 infected patients, T-cell (HLA-DR and CD38) and macrophages activation markers (sCD163, sCD14 and IL1-RA) were elevated in both pleural fluids and plasma^{104,105}. These inflammatory markers in pleural fluid were also higher in TB/HIV-1 than TB mono-infected patients, suggesting a synergy on pulmonary inflammation. In the same studies, comparison of plasma LPS or IFABP in HIV/TB co-infected and HIV mono-infected patients shown similar level of microbial translocation, without impact of TB. Interestingly, even latent TB exacerbates HIV-1 associated immune activation¹⁰⁶. TB reactivation is increased in HIV-infected patients because of memory CD4+ T cells decline and reduced polyfunctionality^{107,108}. In HIV infection, tissues infected by *M. tuberculosis* show depletion of CD4+ T cells and large infiltration of neutrophils¹⁰⁹ with increase of TNF- α activity. In active tuberculosis, patients had a distinct transcriptional signature involving interferon-inducible genes expressed by neutrophils¹¹⁰. This neutrophil-driven signature was correlated with lung radiographic severity, making neutrophils associated with tissue damages. Indeed, neutrophils infiltration and neutrophilia are associated with poor prognosis and are correlated with serum pro-inflammatory markers^{111,112}. Thus, TB increases pulmonary and lymph nodes damages and recruit PMN increasing systemic inflammation.

Restoration of TB immune response after cART initiation is globally associated with a decreased risk of reactivation and primary TB¹⁰⁸. Nevertheless, within the first 3 months of cART incidence of TB reactivation is increased in patients¹¹³. This ART-associated TB relies on the increase of Th1 response against *M. tuberculosis*, characterized by IFN- γ , CXCL10, CXCL9 and IL-18 production^{114,115}. Indeed, cART initiation induces systemic inflammation because of immune reconstitution inflammatory syndrome.

ii. Cytomegalovirus modulates neutrophils responses

Human cytomegalovirus (HCMV) is a common β -herpesvirus acquired during childhood or early adulthood, with about 70% seroprevalence in adults having good socioeconomic conditions¹¹⁶. Primary CMV infection induces a robust immune response and the virus persists in latent form, or may replicate at low levels thanks to immune evasion mechanisms. HCMV is able to stimulate and maintain high level of specific T cells, with up to 50% of circulating CD8+ T cells and 30% of CD4+ T cells¹¹⁷. With aging, HCMV progressively selects memory CD8 T cells (CD28- CD57+) having a poor proliferation capacities and producing large amount of IFN- γ , TNF- α , IL-1 β and IL-6 upon stimulation¹¹⁸. This effect on CD8 T cells selection may contribute to immunosclerosis observed with aging¹¹⁷. Most of HIV-infected patients are co-infected with CMV, with both viruses being associated with inflammation. In a recent study, HIV/CMV co-infected patients had a lower CD4+/CD8+ T cells ratio compared to mono-infected patients, maybe because of differential impact of both virus on CD4 and CD8 T cells¹¹⁹. Compared to HIV mono-infection, co-infection with HCMV significantly increases inflammatory soluble factors, such as IP-10 (CXCL10), TNF-RII and D-dimer¹¹⁶. Interestingly, HCMV has been shown to modulate neutrophils at its advantage promoting viral dissemination. Production of viral CXCL-1 by HCMV was suspected to attract neutrophils at the site of infection and further attracting target cells, such as monocytes¹²⁰. In a study, HCMV-stimulated neutrophils released IL-8, IL-6, TNF α and MIP-1 α , which promoted monocyte migration and activation to a permissive HCMV phenotype¹²¹. Neutrophils had also an increase survival after HCMV stimulation, which is frequently associated with inflammation in other diseases. In the same study, authors hypothesize that HCMV shapes neutrophil response in a pro-inflammatory state, promoting its own persistence and dissemination.

In absence of treatment, HCMV accelerates HIV disease progression and also participates to AIDS-related infections¹²². For example HCMV retinitis, potentially leading to blindness, is one of the most common manifestations in advanced HIV infection¹²³. Nevertheless, the inflammatory role of HCMV in HIV-infected patients under cART is not clear. Co-infection with HCMV has been shown to increase non-AIDS related complications¹²⁴. In this large study, HIV/CMV co-infection was associated with an increased risk of cardiovascular and cerebrovascular diseases. Treatment with valganciclovir performed in a small clinical trial reduced CD8+ T cell activation, suggesting that treatment of herpesvirus infections can reduce immune activation¹²⁴.

iii. Viral hepatitis co-infections and liver fibrosis

Worldwide more than 30% of HIV infected patients are co-infected with hepatitis B (HBV) or C (HCV) virus. Sharing the same route of transmission than HIV, HBV and HCV have been both implicated to development of liver diseases, increasing morbidity and mortality. Mono-infection with HCV or HBV are also associated with microbial translocation, showing elevated levels of plasma LPS. In multiple studies on HIV/HCV co-infected patients, elevated levels of plasma sCD14, LPS or 16s rRNA were associated with higher risk of severe liver disease¹²⁵⁻¹²⁷. Similarly, HBV/HIV co-infected patients demonstrated higher LPS and sCD14 levels than HBV mono-infected patients. Microbial translocation leads to liver fibrosis mainly through Kupffer cells activation and depletion, especially observed in HCV/HIV co-infected patients¹²⁸. Both viruses are responsible of liver inflammation by inducing IP-10 (CXCL10) production by hepatocytes, a cytokine which is known to correlate with liver fibrosis in HIV/HCV co-infection¹²⁹. Neutrophils participate to liver damage by secreting matrix metalloproteinase which remodel extracellular matrix allowing mononuclear infiltration¹³⁰. In a HBV mouse model, targeting neutrophils by matrix metalloproteinase blockade, depletion of neutrophils or neutralization of chemokines (CXCR1/2) reduce liver mononuclear infiltration and reduce liver disease¹³⁰⁻¹³². CXCR1/2 chemokine axis may be a common mechanism of pathologic neutrophils recruitment in liver cirrhosis and failure¹³³. Without cART, co-infections of hepatitis viruses with HIV lead to an increase of microbial translocation, acting at the same time on gut permeability and liver blood filtration of microbes from portal vein. Microbial translocation participates to liver inflammation mainly through IP-10 which increase the risk of liver disease.

Taken together, HBV or HCV infection in HIV-infected patients, along with aging and heavy alcohol consumption contribute to an accelerated liver disease with progression to cirrhosis¹²⁹. HBV-active cART (tenofovir, emtricitabine) has reduced globally liver-related mortality, but liver disease still progresses in some patients, especially when CD4 count is low¹³⁴. cART also reduces liver-related mortality in HCV/HIV co-infected patients, but they have still a higher risk of liver disease acquisition than matched control population¹³⁵. Most of studies analyzed immune activation and inflammation in the context of untreated co-infection¹³⁶. Even if cART globally reduces immune activation, hepatitis/HIV co-infected patients have still higher inflammatory biomarkers levels than uninfected population¹³⁷. In HIV-infected patients under cART, HCV co-infection was shown to increase T cells immune activation and to reduce immune reconstitution, which were worsen in case of liver disease¹³⁸. Thus, liver damages induced by viral hepatitis increase chronic inflammation in HIV patients.

2) Lifestyle and inflammation

Behavior and lifestyle are strongly linked with morbidity and mortality, notably through inflammation. Alcohol has been shown to induce Kupffer cells and liver macrophages to M2 polarization which trigger hepatocyte senescence. Besides, cigarette smoke leads to lung inflammation and chronic lungs diseases. Deleterious lifestyles are frequently observed in people living with HIV, with nearly 50% of which have an history or a current usage of drugs, tobacco or alcohol disorder¹³⁹. These detrimental lifestyles have been associated with chronic inflammation in HIV-infected individuals. Methamphetamine usage in HIV-infected ART-treated patients increased CD4+ and CD8+ T cells activation, exhaustion and proliferation compared to non-methamphetamine users¹⁴⁰. In the same study, the increase of immune activation was associated with higher HIV reservoir among PBMC. In a study on 352 HIV-infected patients, smoking was strongly correlated with an increased level of IL-6 and body mass index was related to C-reactive protein level¹⁴¹. Along with chronic inflammation, HIV-infected smokers has a higher CD4+ and CD8+ T cells activation, exhaustion and an increased microbial translocation (sCD14 and LPS) compared to HIV non-smokers¹⁴². Maybe because of liver toxicity, heavy alcohol consumption increases plasma sCD14, surrogate of myeloid cells activation in case of microbial translocation¹⁴³.

In HIV-infected patients under cART, obesity has been shown to impair glucose metabolism and increase sensitivity to IL-6, CRP and plasma TNFR1 levels¹⁴⁴. A recent study also demonstrated that adipose tissue is a viral reservoir with a pro-inflammatory environment in both SIV infected macaques and HIV infected patients under cART¹⁴⁵. Taken together, these studies demonstrate the importance of behavior and lifestyle in chronic inflammation management.

E. Consequences of chronic inflammation

So even under long-term cART, HIV-infected patients have still higher chronic inflammation mostly evidenced by an increase of soluble inflammatory markers (IL-6, sTNFR1/2, CRP, sCD14,IDO) and gut epithelial dysfunction markers (zonulin and I-FABP). The increase levels of these biomarkers are associated with non-AIDS co-morbidities¹¹. In patients under cART, non-AIDS related diseases normally associated with aging, have been shown to be higher in HIV-infected population than aged matched population¹⁰. This comprises diabetes mellitus, cardiovascular disease, arterial hypertension, non-AIDS-related cancers, neurocognitive dysfunction, hyperlipidemia, kidney diseases and osteoporosis¹⁴⁶. Persistent inflammation in chronic HIV-infection has been shown to cause premature aging of immune system¹⁴⁷. Aging of immune system, termed immunosenescence, characterized by reduced response to vaccine and increase sensibility to infectious diseases, was thought to be responsible of non-AIDS related diseases¹⁴⁸. Differentiation of CD8+ T cells from naïve to effector memory is associated with telomere shortening and CD57 expression for highly differentiated effector memory, which characterize senescent cell¹⁴⁹. CD8 T cells CD28- CD57+ accumulates in HIV-infected patients, demonstrating telomere shortening¹⁵⁰. Telomere shortening, has been also linked to immune activation in HIV disease¹⁵¹. But immunosenescence markers did not predict the development of non-AIDS related disease¹⁵², and might be more a consequence of immune activation rather than a cause of non-AIDS co-morbidities.

Myeloid cells and especially PMN, which are producers of inflammatory cytokines¹⁵³, modulators of coagulations cascades²³ and effectors of tissue damages and fibrosis¹³³, may greatly participate to chronic inflammation²¹. Under cART, innate and myeloid markers were strongly associated with non-AIDS related comorbidities, whereas T cells activation were not¹⁵². In several studies, level of coagulation markers (D-dimer, tissue factor), monocyte activation and inflammation are predictive of cardiovascular (eg. acute myocardial infraction), thromboembolic diseases (eg. venous thromboembolism)^{19,154,155} and non-AIDS-defining cancer¹⁵⁶. Generalized tissue fibrosis observed in lymphoid tissues, adipose tissue and liver from HIV-infected patients might be also a cause of development of non-AIDS related diseases¹⁸. Indeed, initiation of cART slow down liver fibrosis progression notably decreasing mortality due to liver diseases¹⁵⁷.

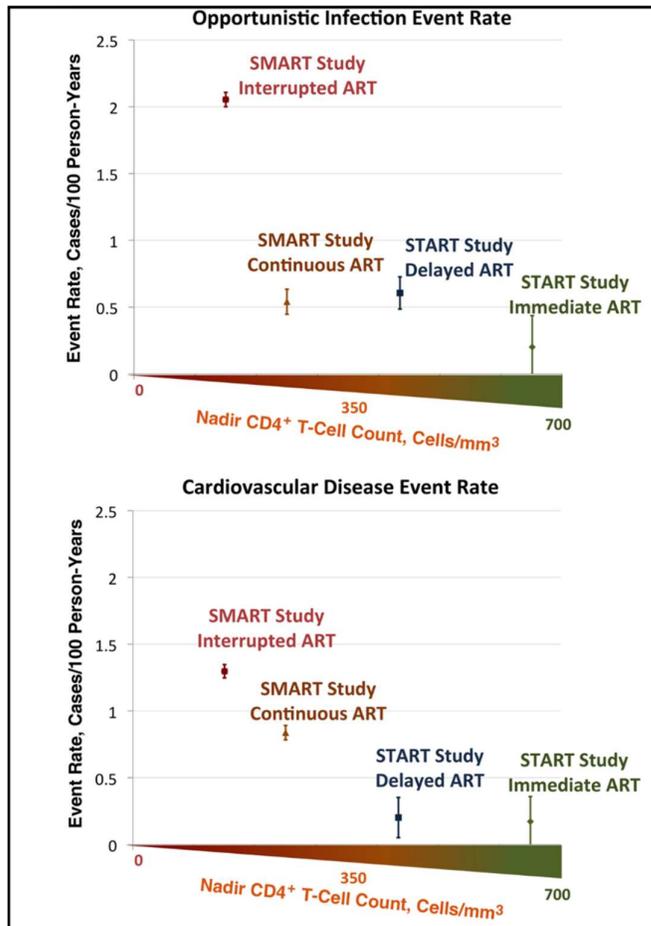


Figure 6: Incidences of non-AIDS-related comorbidities (cardiovascular diseases and opportunistic infections) according to the Nadir CD4+ T cell count before cART in START and SMART studies. Adapted from Hunt P. W (J. Infect. Dis. 2016)¹¹.

It is clear that early cART initiation is beneficial to reduce chronic inflammation and viral reservoirs. Nevertheless, START (Strategic Timing of Antiretroviral Treatment) trial showed that not all non-AIDS comorbidities are reduced in a group of patients treated during primary infection (Figure 6)¹⁵⁸. Compared to delay ART initiation, early treatment significantly reduced cancer and infections but had no effect on cardiovascular diseases. The SMART (Strategies for Management of Antiretroviral Therapy) trial compared continuous treatment with intermittent treatment based on CD4+ T cells count¹⁵⁹. Treatment interruption was associated with an increased risk of non-AIDS related disease compare to continuous ART. But delay ART arm of START trial demonstrated 4-fold lower rates of cardiovascular compared to continuous arm ART of SMART¹¹.

One explanation is maybe the follow-up duration of patients, which had not yet developed these comorbidities. Also, there was differences in term of nadir CD4+ T cells counts between studies. Thus, early cART initiation is not sufficient to reduce non-infectious comorbidities, and systemic inflammatory threshold acquired in first weeks of infection may determine long term organ diseases.

F. Resolution of chronic inflammation by natural hosts

HIV-1 came from zoonotic transmission occurred in the beginning of twentieth century from chimpanzee infected with SIV_{cpz}. Chimpanzees were thought to be resistant to SIV_{cpz}, but natural infection leads to immunodeficiency in this specie, as observed in human¹⁶⁰. In fact, SIV_{cpz} was not found in all four subspecies of chimpanzees, suggesting that SIV acquisition was a recent event, occurring before subspecies divergence¹⁶¹. SIVs have been detected in most of African primates from genus of *Ceropithecus*, African green monkeys (AGM, *Chlorocebus*), mandrills and drills (*Mandrillus*), the mangabeys (*Cercocebus*), which are natural host of their respective SIV¹⁶². For natural hosts, homologous SIV isolate lacks of virulence and the infection rarely progress to AIDS¹⁶². Nevertheless, they are still virulent for other species and SIV_{smm} is an interesting example, since it gave rise to human HIV-2 and Asian macaques SIV_{mac}^{163,164}. Depending on the strain, SIV infection of Asian macaques leads to high viral load, development of progressive CD4+ T cell depletion and opportunistic infection¹⁶⁵. Comparison of pathogenic with nonpathogenic primate models had permit to better understand mechanisms of protection, acquired thanks to thousands years of virus-host co-evolution¹⁶⁶. Sooty mangabeys (SMs) and African green monkeys (AGMs) were intensively studied to understand mechanisms of nonpathogenic SIV infection, so I will mainly focus on these models.

As pathogenic infection in human and Asian macaques, natural hosts demonstrate a high viremia in primary infection and chronic phase, and depletion of CD4+ T cells occurring only in primary infection in which immune activation level is high^{167,168}. Interestingly, AIDS resistance mechanisms of natural hosts do not rely on immune suppression of viral replication, meaning that host evolution selected another way for protection. First, they keep healthy levels of peripheral CD4+ T cells on the long term particularly by preserving central memory CD4+ T cells (TCM)¹⁶⁹, T cell regeneration¹⁷⁰, and lymph node architecture and function^{171–173}. Second, mucosal immunity is long term preserved with normal ratio between Th17 and Treg in GALT, explaining the absence of microbial translocation in AGM and SM^{174,175}. Lastly, despite ongoing viral replication there is a lack of chronic immune activation^{173,176,177}.

In natural host, primary SIV infection is similar to HIV and SIV pathogenic infection with increasing viremia reaching a peak around 12 days post-infection (Figure 7). Depletion of CD4+ T cells is observed in periphery and is more severe in gut and lung lymphoid tissues¹⁷³.

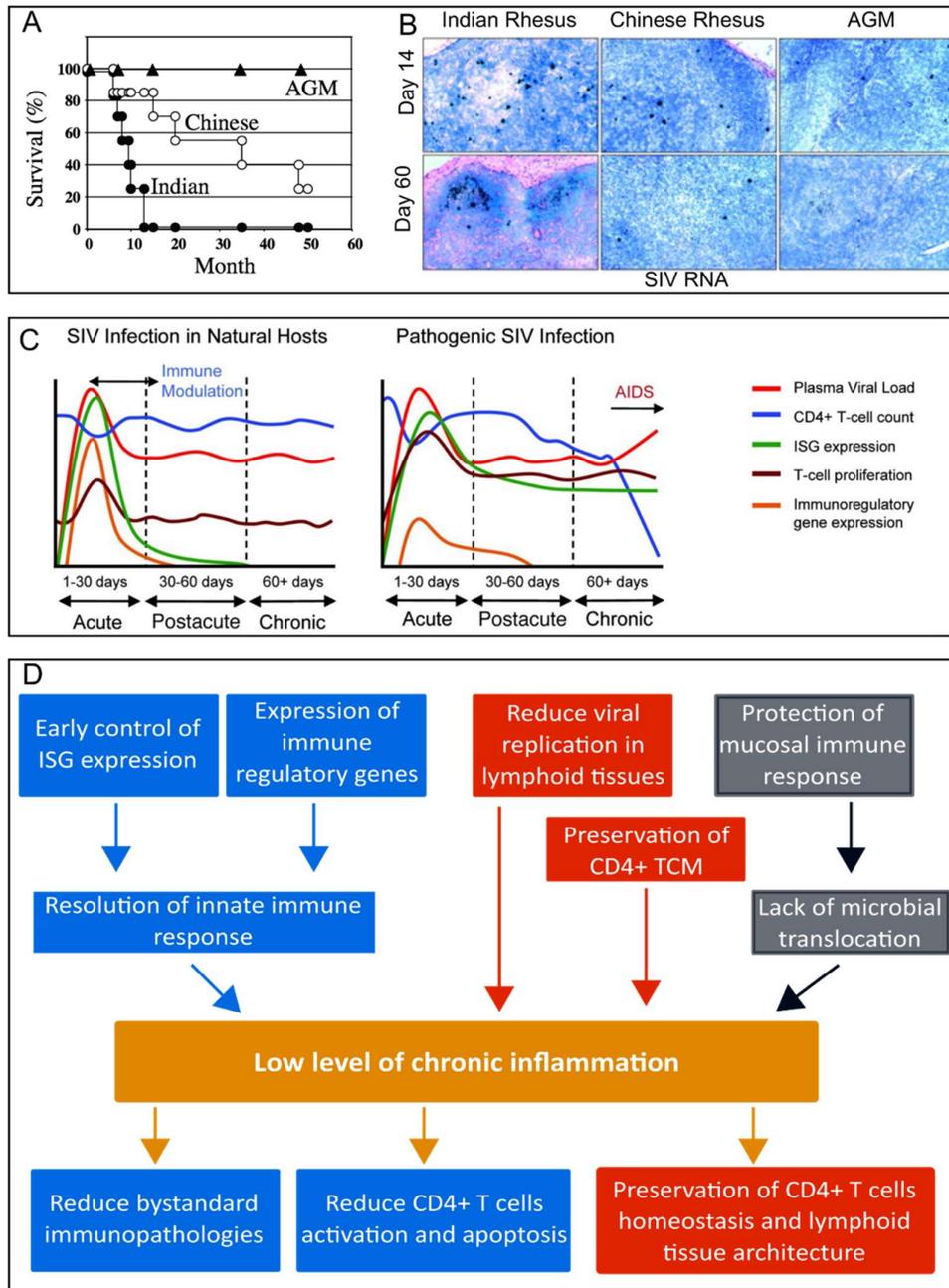


Figure 7: Mechanisms involve in control of chronic inflammation by natural hosts.

A-Percentage of survival of SIVsmm-infected AGMs and SIVmac251-infected RMs of Indian and Chinese origins. Despite persistent viral replication, AGM did not develop AID-related symptoms. **B**-In situ hybridization of SIV RNA in LN from RMs or AGMs. In the peak of replication, AGMs and RMs demonstrated similar SIV RNA+ cells in T-cell zone, but at day 60 viral replication in LN was extremely low in AGMs, despite similar levels of plasma viral load. **C**- S. E. Bosinger *et al.* model of immunomodulation during SIV infection in natural and pathogenic hosts. Despite persistent viral replication, natural host demonstrated reduce ISG expression, T-cell proliferation and elevated immunoregulatory genes expression protecting from CD4+ T cells depletion and AIDS. Adapted from Cumont M.-C. *et al.* (J. Virol. 2008) and Bosinger S. E. *et al.* (J. Clin. Invest. 2009)^{173,178}. **D**-Mechanisms of control of chronic inflammation and consequences for the physiopathology of SIV infection in natural hosts. Adapted from Chahroudi A. *et al.* (Science 2012) and Ploquin M. J. *et al.* (Current Opinion in HIV and AIDS 2016)^{12,179}.

Cellular immune response, especially CD8+ T cells, allows a control of the viremia which is maintain during chronic phase¹⁸⁰. In fact, strong innate and adaptive immune response are initiated in primary infection, mostly characterized by type I interferon signatures, T cells proliferation and pro-inflammatory cytokines¹⁷⁸. Despite ongoing viral replication mucosal CD4+ T cells stabilize or recover in chronic phase. One explanation is the resolution of inflammation observed from 4 to 8 weeks post-infection, which protects the natural host against all immune mediated pathologies, such as T cell apoptosis, lymph node fibrosis and impaired immune functions¹⁸¹. Four mechanisms have been described to explain the control of chronic inflammation: reduced microbial translocation, reduce ISG signature, down-regulation of CD3-T cell receptor, reduction of immune response against the virus and low level of replication in lymphoid tissues (Figure 7).

In natural host reduction of chronic inflammation could be explain by protection of mucosal immune response, avoiding microbial translocation observed in pathogenic infection. Even if there is a significant depletion of mucosal CD4+ T cells in acute infection⁷⁷, Th17 cells are protected and intestinal epithelial integrity is preserved in natural host⁸⁴. In acute SIV infection both natural and pathogenic host demonstrated elevated ISG signatures¹⁸². However, in natural hosts (AGM and SM) ISG expression levels return to baseline 30 days post-infection, showing a resolution of acute inflammation. By limiting type I interferon, AGM and SM may reduce detrimental chronic immune activation. Another factor reducing immune activation relies on the capacity of Nef protein from SIVsmm and SIVagm to down-regulate CD3-TCR expression on T cell surface¹⁸¹. The hypothesis is that reduction CD3-TCR expression may reduce further T cells activations. But Nef protein from SIVmac pathogenic infection is also able to down-regulate CD3-TCR, so this mechanism alone is not sufficient to explain the control of immune activation. AGM and SM have a reduced immune response to SIV infection, contributing also to limited inflammation and immune activation. Limiting viral replication in LN is another protective mechanism of natural host. The ability of NK cells to infiltrate germinal centers in primary infection, thanks to IL-15 expression by FDC, is a good evidence for the effective innate immune response¹⁸³. Thus, killing CD4+ T follicular helper cells infected by SIV reduce viral reservoir, participating to control of immune activation. The implication of neutrophils in the resolution of inflammation in natural hosts has not been studied yet.

Myeloid cells are important effectors of chronic inflammation and are involved in all mechanisms at its origin. Neutrophils are of particular interest in chronic inflammatory diseases since they are the largest circulating leukocyte population, equipped with deadly weapons and arriving first to injury site. Our understanding of neutrophils has been restricted for a long time by their fragility and dogma defining them as expendable phagocytic cells. As we are going to see, abundant recent studies demonstrate plasticity and several new functions carried by neutrophils.

III. Neutrophils plasticity in response to inflammation

For long, neutrophil function in the immune response was restricted to defense against invading pathogens using efficient killing weapons. Indeed, in case of neutropenia or leukocyte adhesion deficiency syndrome, patients are more prone to bacterial and fungal infections. Recently, this dogma has been challenged by the demonstration that neutrophils lifespan in circulation reaches 5.4 days¹⁸⁴. Neutrophils live enough to respond to their environment thus creating phenotypical heterogeneity depending on the disease. Soon this phenotypic diversity was associated with unexpected function of neutrophils, modulating adaptive immune response and promoting resolution of inflammation. In the first part, we will present the life cycle of neutrophils from bone marrow production to controlled apoptosis, while passing by physiological functions. But in unresolved inflammation, neutrophils homeostasis is disrupted. As we are going to see in the second part, neutrophil has a pathogenic role by enhancing inflammation and leading to tissue damages.

A. Neutrophil life from granulopoiesis to efferocytosis

1) Feedback loop of neutrophils production

Regulation of neutrophils production relies on neutrophils apoptosis in tissues through a multistep process (Figure 8). Tissue macrophages and DC sense and respond to their environment (TNF- α , TLR agonist (LPS) or CD40-CD40L interactions) by secreting IL-23¹⁸⁵. IL-23 induces production of IL-17A by specialized T cells called neutrophil regulatory cells (Tn)¹⁸⁶. Thus, IL-17A is an important stimulus for granulocyte colony stimulating factor (G-CSF) secretion, promoting neutrophils production and mobilization. A feedback negative loop allows the control of neutrophil production by sensing an increased number of neutrophils in tissues. Phagocytosis of apoptotic neutrophil by macrophages and DC (called efferocytosis) leads to a decrease secretion of IL-23, ending with decrease production/mobilization of neutrophils¹⁸⁵. Lymphoid cells producing IL-17A regulates neutrophils production and gather a continuum from innate lymphoid cells to helper CD4 T cells, called Th17¹⁸⁷. Th17 represent less than 15% of IL-17A producing cells in the body¹⁸⁶, which are all characterized by expression of RAR-related orphan receptor gamma (ROR γ t). The vast production of IL-17A in the body, occurring in MALT, has been shown to be supported by $\gamma\delta$ T cells representing up to 60% of IL-17A production¹⁸⁷.

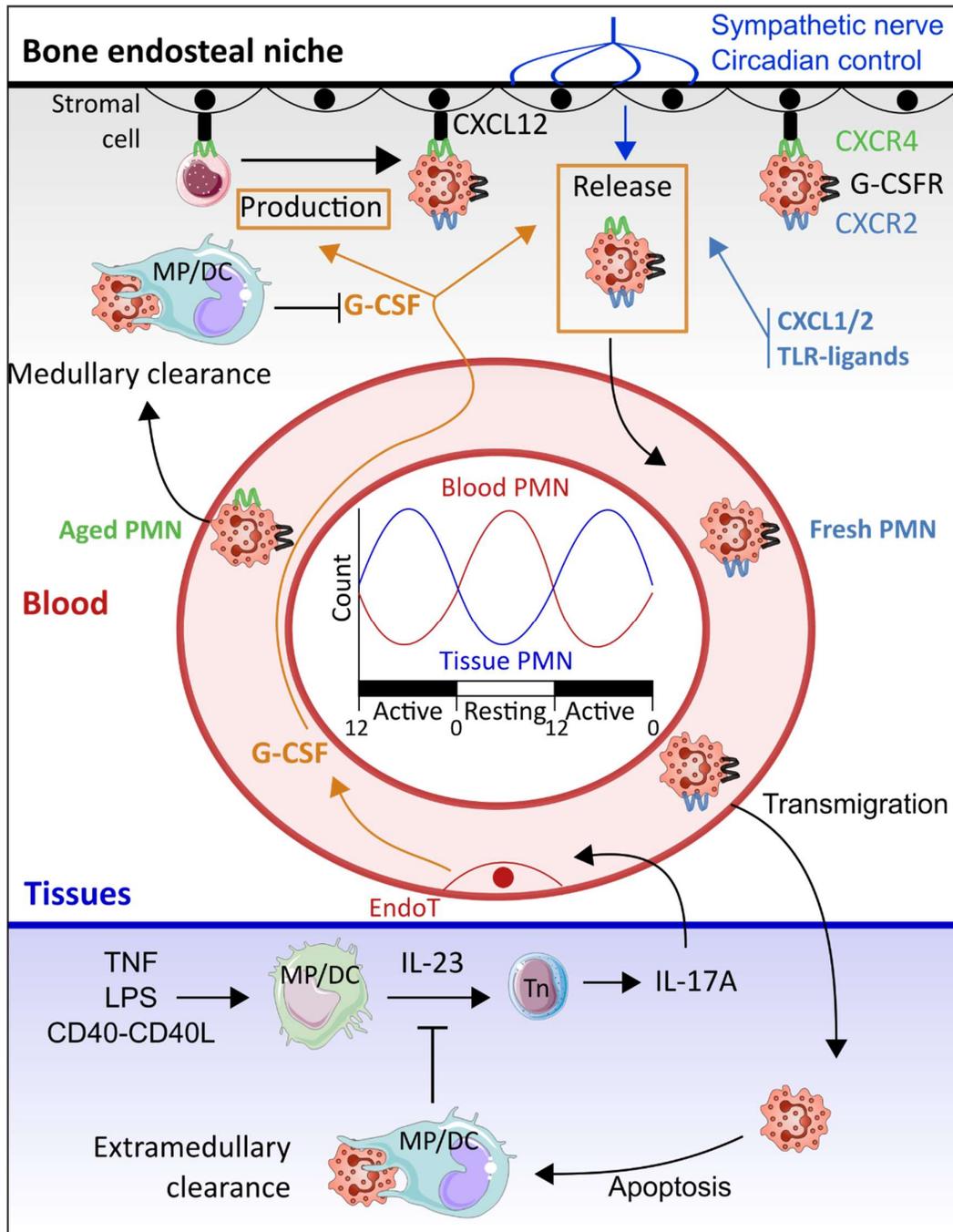


Figure 8: Regulation of neutrophils production and circulation. Neutrophils are produced in endosteal niches within bone mainly under G-CSF signaling. In steady state, G-CSF, TLR signaling, CXCR2 agonists, and SNS mediate mature neutrophils release from bone marrow pool. Neutrophils blood mobilization and tissue migration follows circadian oscillation mediated by CXCL12/CXCR4 expression, under control of SNS. Circulating aged-neutrophils migrates back to bone marrow and tissue neutrophils are both clear by mononuclear phagocyte system. IL-17A targets numerous cells, such as endothelial cells, inducing G-CSF secretion. Neutrophils phagocytosis negatively regulates IL-23/IL-17A/G-CSF axis, which decrease neutrophils production and mobilization. In case of inflammation, macrophages or DC activate IL-23/IL-17A/G-CSF axis, which, along with TLR agonist and pro-inflammatory chemokines, increase neutrophils production and mobilization. MP: macrophage, DC: dendritic cell, Tn: neutrophil regulatory cells, EndoT: endothelial cell.

IL-17A maintains mucosal barrier integrity through enhancing tight junction synthesis and by inducing microbicidal agent synthesis¹⁸⁷. IL-17A also induces G-CSF secretion by epithelial cells, bone marrow macrophages, endothelial cells, fibroblasts and mesothelial cells. G-CSF binding to its receptor on neutrophils precursors induces proliferation, differentiation and activation in bone marrow¹⁸⁸. Nevertheless, in steady state other factors participate to neutrophils production since in G-CSF deficient mice residual granulopoiesis was observed¹⁸⁹.

Cell retention in the bone marrow relies on the chemokine receptor CXCR4, express by both stem cells and neutrophils¹⁹⁰. CXCR4 binds CXCL12 (SDF1, stromal derived factor 1) which is expressed on bone marrow stromal cells: vascular endothelial cells, mesenchymal stem cells, fibroblasts, adipocytes and osteoblasts¹⁹¹. Neutrophils form a pool of cells maintained by CXCR4, which could be released on demand by CXCR2, G-CSFR or TLR signaling¹⁹². G-CSF induces neutrophils mobilization to circulation by reducing CXCL12 expression on endothelial cells, which reduce adherence and promote signaling by CXCR2.

2) Differentiation and maturation of neutrophils

Production of myeloid cells within bone marrow represent two-thirds of hematopoietic activity, with a major part devoted to neutrophils and each day in a healthy adult, around 1 to 2 x10¹¹ neutrophils are produced¹⁹³. Granulopoiesis starts from hematopoietic stem cells (HSCs) which are located close to osteoblasts, forming a niche along the endosteal border where blood flow and oxygen level are reduced¹⁹⁴. A variety of osteoblast adherence factors are expressed to maintains HSCs quiescent in endosteal niche¹⁹⁵. Commitment and differentiation of HSCs to granulocytes relies on HSC homing to vascular niches in vascular sinuses of central bone marrow. Expression and secretion of CXCL12 by endothelial cells attract HSC to vascular niches where they are bound to vascular niche via P and E-selectin¹⁹⁶. This environment facilitates differentiation to CD34+ multipotent progenitors (MPs). Subsequent to this, expression of PU.1 transcription factor commit MMPs to common myeloid progenitors (CMPs) potent to give rise to myeloid cells, megakaryocyte and erythroid lineage¹⁹⁷. Transition from CMPs to granulocyte/monocyte progenitors (GMPs) depends on co-expression of PU.1 and C/EBP- α transcription factors. Further commitment to neutrophil precursors is based on decrease PU.1 expression along with maintenance of C/EBP- α expression.

Stimulation by IL-3, GM-CSF and IL-6 allow differentiation from CMPs to GMPs. G-CSF drives commitment of GMPs to neutrophils production by proliferation and maturation of myeloblast to segmented neutrophil.

Within bone marrow, neutrophil production is divided into three compartments: proliferation, maturation and storage¹⁹⁴. Myeloblast is the first step of granulopoiesis, in which all cellular steps are characterized by nuclear shape, granules content and cytoplasm using May-Grünwald Giemsa bone marrow aspirate staining¹⁹⁸. (Figure 9) From Myeloblast to promyelocyte stage, azurophilic granules number increase and progressively becomes less apparent to light microscopy with maturation. The next myelocyte stage shows condensation chromatin and apparition of secondary (or specific) granules. Myelocyte is the last stage undergoing cell division in granulopoiesis. Metamyelocyte and further band cell progressively mature with increasing chromatin condensation and tertiary (gelatinase) granules are stored. Nuclear segmentation is classically formed of 3-5 lobes and defines mature (segmented) neutrophil. At this last maturation stage, neutrophil produce secretory vesicles. Mature neutrophils are stored in bone marrow before on demand mobilization in blood. In human, neutrophils differentiation takes 6-7 days from the last cell division to release into the circulation¹⁹⁹.

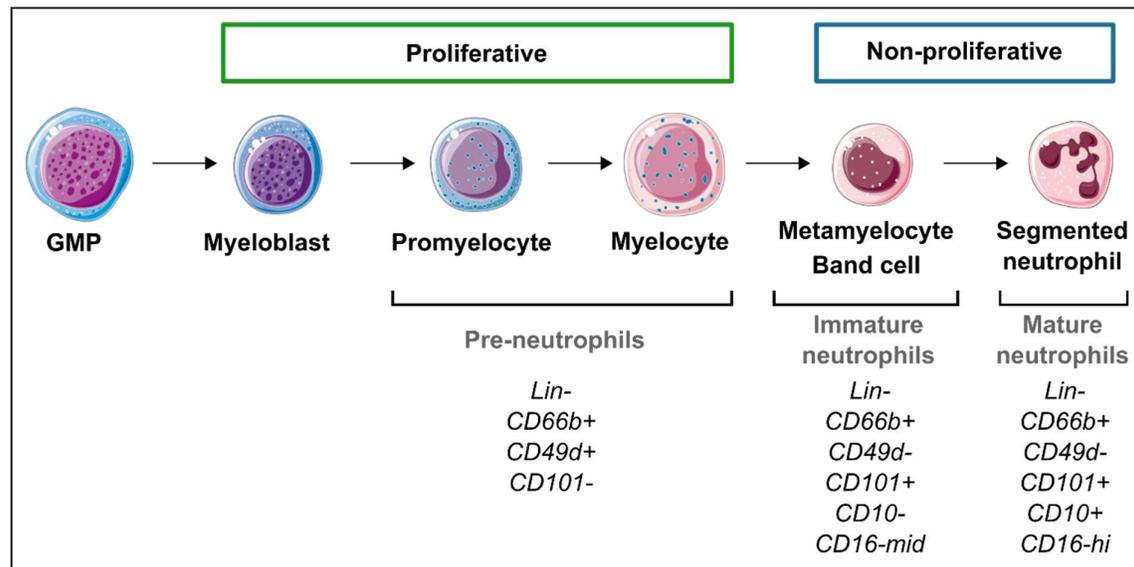


Figure 9: Neutrophil differentiation in human bone marrow. Actual definition of neutrophils differentiation stages in human bone marrow with proposed phenotypic definition of neutrophils stages defined based on literature. GMP: granulocyte-monocyte progenitor.^{199,200}

3) Circadian cycle orchestrates neutrophils circulation and tissue migration

Several immune parameters have a circadian dependent variation, and neutrophil blood count is one of those^{201,202}. Beside mechanisms I presented before, sympathetic nervous system (SNS) plays a central role in coordinating immune cells oscillation observed within 24 hours²⁰¹. Briefly, light is the principal synchronizing stimulus captured by retina and is transmitted through retinohypothalamic tract to hypothalamus, in which suprachiasmatic nuclei form the central clock. SNS innervates most of organs, especially lymphoid organs, where locally it synchronizes immune cells using noradrenaline²⁰³. Through hypothalamic-pituitary-adrenal axis, SNS has also a systemic synchronization of immune response by secretion of glucocorticoids and catecholamines by adrenal glands. Oscillation of circulating neutrophils are based on activity phase which is diurnal for human. At night during resting phase, neutrophils peak because of a release of mature cells from bone marrow. SNS innervation induces a rhythmic decrease of CXCL12 expression on endothelial cells at night²⁰⁴. During active phase, circulating neutrophils decrease because of a reduction of bone marrow liberation and also by migration to tissue (Figure 8). Sympathetic nerves modulate expression of adhesion molecules and chemokines on endothelial cells, guiding neutrophils to tissues²⁰⁵. Catecholamines are known to increase circulating neutrophils, predominantly released from marginated pool present in capillaries of lung, liver, spleen and bone marrow²⁰³. Marginated pools have been estimated to represent 51% of all blood granulocytes, representing a large inaccessible neutrophil compartment by blood drawn²⁰⁶. Maturation and activation state of neutrophils seem to influence their body distribution. Studies performed in mouse showed an increase expression of CXCR4 before neutrophil apoptosis. It is proposed that increase CXCR4 expression leads neutrophils to bone marrow where macrophages phagocytosis takes place. A recent study using plerixafor (CXCR4 agonist) demonstrated in both mice and non-human primate models the existence of a large marginated pool of neutrophils in lungs²⁰⁷. Lung endothelial cells express CXCL12 and trap aged neutrophils.

To enter the tissue, neutrophils interact with endothelial cells at post-capillary venules. Classically, leukocytes recruitment is divided in multiple steps: tethering, rolling, adhesion, crawling and transmigration. Endothelial cells determine the attachment of neutrophil by expression of E and P-selectin, in response to TNF- α , IL-1 β and IL-17 or by direct pathogen recognition by pattern-recognition receptors (PRR)^{208,209}. Neutrophils constrictively express

PSGL-1 and L-selectin (CD62L) allowing interaction with E and P-selection on endothelial surface. CD44 and PSGL-1 interaction slower rolling and mediate PLC- γ and PI3K activation leading to neutrophil cytoskeletal rearrangement. Firm adhesion to endothelium is mediated by CD18 β integrin on neutrophils. Transmigration could be paracellular (between endothelial cells) or transcellular depending on endothelium-neutrophils interactions. Tissue migration increase neutrophil transcriptional activity, NADPH oxidase activity and susceptibility to degranulation^{190,208,209}. This state of partial activation, termed priming, prepares neutrophil to respond rapidly to invaded pathogen present in tissue.

4) Anti-microbial function of neutrophils

During acute inflammation neutrophils accumulates in injury sites thanks to diapedesis, and following chemokine gradients. Environment along with endothelium interaction prime and activate neutrophils making them more potent to respond to infection. Human neutrophils express most of Toll-like receptors (TLR), with the exception of TLR3 and TLR7, leading to pro-inflammatory cytokines secretion²¹⁰. Depending on the environment, neutrophils have been shown to produce multiples molecules (anti-/pro-inflammatory cytokines, chemokines, TNF superfamily and angiogenic/fibrogenic factors) thus modulating immune response²¹¹.

Phagocytosis is the most recognized antimicrobial function of neutrophils. Neutrophils are consider to be “professional phagocytes” since they can engulfed up to 10-12 particles²¹². Detection of pathogens is performed by receptors detecting microbial polysaccharides (Dectin-1) or by sensing opsonized particles with Fc-receptors (CD89, CD16, CD32, CD64) or complement receptor (eg. CD35). Activation of these receptors induce membrane invagination forming cytoplasmic vesicle called phagosome²¹². Fusion with granules later form phagolysosome, a deadly compartment full of proteolytic enzymes and antimicrobial molecules. The NADPH oxidase assembly and activation are essential in microbial killing by producing ROS within phagolysosome. Briefly, NADPH oxidase is a tertiary structure composed of cytoplasmic p47^{phox}, p67^{phox}, p40^{phox} and membranous NOX2, p22^{phox}, acting together as an electron transferase from NADPH to oxygen (O_2), generating superoxide anion (O_2^-) within the phagolysosome²¹³. Granules proteins convert O_2^- to hydrogen peroxide (superoxide dismutase) then metabolize into hypochlorous acid (myeloperoxidase). Patient with chronic granulomatous disease, characterized by a defective mutation on NADPH subunit, have

neutrophils unable to generate ROS and to kill bacteria. So, ROS production is a central mechanism in pathogen killing. Granules produced during granulopoiesis are subdivided in 4 types: primary (or azurophil) granule, secondary (specific) granule, tertiary (gelatinase) granule and secretory granules. Granules are released depending on the intensity and the type of stimulus in a hierarchical order, called graded exocytosis²¹⁴. Secretory granules are formed by endocytosis during nucleus segmentation in bone marrow and are the first mobilized to the plasma membrane. Secretory granules contains NOX2/p22^{phox} NADPH oxidase making neutrophils more prone to produce ROS, activation state called priming²¹³. They contain also numerous membrane receptors (eg. CD14, CD16, CD65) and adhesion molecules (eg. CD11b/CD18, CD67) allowing neutrophils to better sense pathogens²¹². Tertiary granules are the second most easily released granules, containing matrix degrading enzymes (eg. arginase 1, gelatinase) and adhesion molecules involved in diapedesis. Gelatinase are also potent to destroy tissue during neutrophil response. Specific granules are full of antibacterial protein such as lactoferrin, haptoglobin, lysozyme, which target different essential pathways for microbial survival²¹⁴. Matrix metalloproteinase are also overrepresented in specific granules and act on structural components of pathogens, leading to bacterial killing. Azurophil granules have a limited exocytosis and contribute mostly to phagolysosome formation. They contain metalloproteinases, forming hypochlorous acid, matrix metalloproteinase and serine proteases (eg. elastase, cathepsin G) which degrade a variety of extracellular matrix²¹². Antimicrobial peptides are also found in azurophilic granules, such as α -defensin, active on bacteria, fungus and some enveloped viruses.

First described as a new microbicidal mechanism capable to capture and kill microorganisms, neutrophil extracellular traps (NETs) were formed by chromatin extrusion decorated with granules protein by neutrophils²¹⁵. After 2h hours of incubation with PMA, authors showed liberation of NETs associated with necrotic cell death of neutrophils, a mechanism called NETosis²¹⁶. Further research using PMA stimulation showed that NETs are formed under NADPH oxidase activation cascade, leading to chromatin decondensation by conversion of arginine to citrulline on histones²¹⁷. Azurophilic granules are then translocated to nucleus, associating neutrophil elastase and myeloperoxidase with chromatin, further release into extracellular space. Nevertheless, the use of a non-physiologic stimuli (PMA) raised questions about the mechanism of NET formation in vivo and NETosis cell death process²¹⁸⁻²²¹.

Observation of NET liberation without cell death, using GM-CSF primed neutrophils further stimulated by TLR4 agonists or C5a, suggested that NET production and NETosis necrotic cell death were independent mechanisms²²¹. In addition, NET were formed by mitochondrial DNA rather than nuclear DNA, in a mechanism dependent on ROS activation and glycolytic ATP production allowing cytoskeletal rearrangement²²⁰. Liberation of mitochondrial DNA may also play a role as a danger associated molecular pattern, potentially modulating inflammation²²¹. Besides, killing capacities of NETs have been also questioned. NETs are capable to trap microorganism, limiting dissemination but live organism were still recover from NETs²¹⁹. The importance of NETs as an antimicrobial mechanism was also challenged by study of Papillon-Lefèvre syndrome (PLS), involving a lack of elastase in neutrophils, an enzyme required for NET production²²². Neutrophil from PLS patient were potent to kill *K. pneumonia* without forming NETs and PLS patient did not shown elevated frequency of infections. NETs formation is of particular interest in auto-immune diseases, in which they may provide a source of auto-antigens²¹⁸.

Neutrophils contribute also to resolution of inflammation by switching their pro-inflammatory cytokines secretion to lipoxine and resolvines which inhibit transendothelial migration and chemotaxis. They also produce anti-inflammatory IL-1ra and TGF- β . Neutrophils elimination by efferocytosis also generate anti-inflammatory M2 macrophages, producing IL-10 and TGF- β ²²³.

5) Neutrophil compartmentalization and death to control inflammation

Classical model of neutrophil circulation in the body postulated that tissue was the final destination. This has been challenged by number of studies showing the ability of neutrophils to go back to circulation after migration to tissue in zebra fish and in mouse models^{224,225}. In this phenomenon, called reverse transmigration, neutrophils have an increase life time, are more potent to produce ROS but cannot transmigrate again to tissue. Intravital microscopy on a ischemia reperfusion model showed the importance of JAM-C (endothelial cell junction molecule) degradation by neutrophils under Leukotriene B4 activation²²⁵. These reverse-transmigrated neutrophils harbor an activated profile potentially involved in systemic inflammation. Neutrophils are also able to emigrate from tissue using lymphatic vessels. Indeed in several bacterial infections, pathologists have identified neutrophil infiltration in

draining lymph nodes²²⁶. In mouse models neutrophils have been shown to infiltrate lymph nodes using both blood and lymph and expressed a specific activated phenotype (CD11b^{high} CD62L^{low} CXCR2^{low})²²⁷. In the same study, they demonstrated that trafficking to LN only occurred under inflammation and neutrophils stimulation by immune complexes. In lymph nodes, neutrophils can shape immune responses by promoting T cells proliferation and undergoing antigen presentation²²⁷.

Since neutrophils are involved in tissue damage by releasing enzymes and pro-inflammatory components, clearance of apoptotic neutrophils is essential. Phagocytosis of apoptotic neutrophils by tissue macrophages, termed efferocytosis, participates to resolution of inflammation and wound healing. Macrophages undergoing efferocytosis produce anti-inflammatory mediators TGF- β and PGE₂. Efferocytosis has been demonstrated in vivo to occur in lung, spleen, liver and bone marrow²²⁸⁻²³¹. Bone marrow and spleen are important compartments performing efferocytosis in steady state^{228,232}. Aged neutrophils expressing CXCR4 and SDP-1 α home back to bone marrow to be phagocytosed by macrophages²²⁹. Under inflammatory conditions, liver has a major role in neutrophil clearance as shown in a endotoxemia model and patients with inflammatory diseases^{230,232}. In that study, gadolinium chloride blockade of Kupffer cells induced a neutrophilia and a relocation of neutrophils in lung and spleen. Liver is an important organ in elimination of blood-borne bacteria coming from the gut. Kupffer cells were shown to phagocyte neutrophils which have engulfed bacteria, leading to resolution of inflammation in a listeriosis model²³³. As explain above, efferocytosis leads to a negative feed-back loop, which reduces neutrophil production by reducing IL-23 production by macrophages.

B. Heterogeneity and function of neutrophils in chronic inflammation

In acute inflammation, neutrophils production and functions are adapted to both respond to the invader and resolve inflammation to avoid tissue damages. Remodeling of stromal cells and endosteal niches under inflammation modulate myelopoiesis, thus producing neutrophils heterogeneity^{234,235}. In numerous chronic diseases, such as atherosclerosis, myocardial infarction or neurodegenerative disorders, neutrophils have been involved in tissue damages and maintenance of inflammation¹⁹⁹. Neutrophils heterogeneity in cancers, autoimmune diseases and infectious diseases have been intensively studied in recent years, which are inspiring to study neutrophils in HIV infection. In the first part, we will present the impact of neutrophils on chronic inflammation observed in autoimmune diseases. Neutrophils heterogeneity has been demonstrated in autoimmune diseases, in which NETosis has a central role in physiopathology²³⁶. In the second part, we will present neutrophil heterogeneity in cancer in which MDSC have been demonstrated to suppress T cell responses since 1984²³⁷. The third part is dedicated to neutrophils and tuberculosis. Tuberculosis is a particular chronic infection in which recent studies have demonstrated a role of neutrophils in immune regulation as well as granuloma reaction²³⁸. Neutrophils phenotypes and functions are less understood in chronic viral infections. Trying to make a parallel with HIV infection, in the last part we will present the current knowledge on neutrophils heterogeneity in chronic viral infections.

1) Auto-immune diseases

Autoimmune disease are multifactorial diseases characterized by a loss of tolerance against autoantigens. Environmental factors are suspected to trigger autoimmune diseases in genetically predisposed individuals, leading to abnormal innate and adaptive immune responses. Neutrophils have been involved in various aspects of autoimmune diseases, participating to chronic inflammation and tissue lesions. Rheumatoid arthritis (RA) is a destructive joint inflammation characterized by synovitis, systemic inflammation and presence of autoantibodies, such as rheumatoid factor (IgA/IgM against IgG) and anticitrullinated protein antibodies (ACPA)²³⁹. Pannus represents the active lesion in RA, a synovial invasive tissue formed by activated fibroblasts, lymphocytes, macrophages and neutrophils²⁴⁰. TNF is a central cytokine in RA driving systemic inflammation. As consequence, sustained exposure to TNF leads to abnormal neutrophil functions in RA, inducing priming and

genes transcription²⁴¹. Circulating neutrophils from RA patient showed a higher capacity to produce ROS, compare to healthy individuals²⁴². Within joints, neutrophil degranulation and ROS production are mediated by immune complexes, targeting articular cartilage²⁴³. In RA synovial fluid, neutrophils survive for several days because of hypoxic environment and anti-apoptotic cytokines (TNF, GM-CSF and IL-8)^{244,245}. Neutrophils amplify lesions and inflammation by releasing numerous pro-inflammatory mediators, comprising RANK-L and BAFF, which activate respectively osteoclasts and B lymphocytes²⁴⁰. Neutrophils have been shown to release NET in RA, with blood and synovial fluid neutrophils more prone to release NETs. Interestingly, NETs contain citrullinated histones H4 which are consider as auto-antigenes²¹⁸. Neutrophils sustains ACPA production and may initiate production of new auto-antibodies²⁴¹. Anti-TNF therapy is efficient to reduce disease activity and inflammation, and TNF inhibitors were shown to decrease neutrophils activation notably by downregulating NF- κ B pathways²⁴⁶.

Association between neutrophils and systemic lupus erythematosus (SLE) has been made in 1986 with the first description of low-density neutrophils (LDN) in peripheral blood mononuclear cells (PBMC), obtained after density gradient centrifugation²⁴⁶. In SLE, B and T cells responses have a prominent role with production of immune complexes and autoantibodies against nuclear antigens²⁴⁷. The role of neutrophils in SLE have been suggested since patients had abnormal interferon and neutrophil signatures in blood²⁴⁸. LDN represent a pathogenic subset of neutrophils producing pro-inflammatory cytokines, with enhanced capacity to form NETs and activate endothelial cells²⁴⁹. Neutrophils participate to tissue damages and were observed to infiltrate and to form NETs in skin and kidneys²⁴⁹. The release of NET participates actively to type I interferon production by stimulating pDC, and amplifies inflammation in tissues²⁵⁰. As in RA, LDN maintain inflammation and auto-antibody production through NETs which are composed of potent nuclear auto-antigens.

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is characterized by vascular inflammation with or without granulomatosis²⁵¹. AAV have polymorphic manifestations, affecting vessels from kidney, skin, respiratory tract and peripheral nerves. The presence of MPO and PR3 auto-antibodies have been identified in vasculitis patients, and are important to the diagnosis of these diseases. These auto-antibodies underline the role of neutrophils which are source of auto-antibodies and induce endothelial cells damage^{249,252}.

These auto-antibodies were shown to activate neutrophils previously primed by inflammatory stimuli such as TNF or G-CSF²⁵³. NETs formation have been involved in pathologic mechanism of ANCA vasculitis, with neutrophils more prone to release NETs²⁴⁹. Also, sera from patients contained circulating MPO-DNA and calprotectin, which were consider as markers of NETs production^{254,255}. In renal biopsy obtained from patients demonstrating necrotizing glomerulonephritis, DNA and granules proteins are located in inflammatory foci or around necrotizing area, suggesting a relationship between NETs and tissues damages²⁵⁶. Nevertheless, most of studies identified NETs using DNA in combination granule proteins, which could be also the results of cell necrosis combined with neutrophils degranulation²²¹. MPO and PR3 are key proteins with pro-inflammatory properties²¹⁸. Disease activity is positively correlated with serum PR3 and neutrophils count in patients²⁵⁷. In association with ANCA neutrophils induce vascular damages and granuloma, perpetuating inflammation and auto-immunity.

2) Cancers

Cancer are chronic inflammatory disease among which neutrophils diversity has been widely studied. Inflammatory environment, partially driven by cancer cells, promotes tumor initiation, growth, angiogenesis and metastasis²⁵⁸. Neutrophils were shown to favor all these steps of tumorigenesis, but also demonstrated antitumoral function. Depending on the stage the tumor and the compartment analyzed, different subsets of neutrophils have been identified according to their function, phenotype and sedimentation after density gradient²⁵⁸. Ligands for CXCR2 were observed early in human tumor development, leading to neutrophils recruitment. In early stage of lung cancer, tumor-associated neutrophils (TAN) were activated and express different chemokine receptors (CXCR3, CXCR4 and CCR7) compared to peripheral neutrophils²⁵⁹. TAN produced proinflammatory factors (IL-8, TNF- α and IL-6) and were able to stimulate T cell proliferation and IFN- γ production. Referred as N1, these TAN have a hypersegmented nucleus with a more mature phenotype. But in advanced carcinoma or in head and neck cancers, elevated levels of TAN increased the risk of recurrence or death by 66%²⁶⁰. From early stage antitumoral to late stage pro-tumorigenic TAN, there is a switch of functions apparently induced by tumor environment²⁶¹. TAN promoting tumor growth, called N2, displayed an immature phenotype characterized by increase arginase activity. Demonstrated in a breast cancer mouse model, tumor cells activate IL-17/G-CSF axis using $\gamma\delta$ T

cells²⁶². This mechanism increases TAN infiltration, promoting tumor progression and metastasis. Interestingly, polarization to pro-tumorigenic N2 relies on TGF- β anti-inflammatory cytokine present in tumor environment²⁶³. One mechanism by which TAN participate to tumor growth and metastasis is by their angiogenic capacities, normally observed in resolution of inflammation and wound healing²⁶⁴. MMP9 secreted by TAN degrade extracellular matrix and thereby releases VEGF and FGF2²⁶⁵.

Another populations described in cancer are myeloid derived suppressor cells (MDSCs), having phenotype related to monocytes (M-MDSC) or granulocytes (PMN-MDSC) and capable to suppress T cell responses²⁶⁶. There is still not a clear phenotypical definition of MDSC and true definition relies on demonstration of suppressive activity. A transcriptomic analysis of naïve neutrophil, TAN and PMN-MDSC demonstrate these 3 populations were different, but naïve neutrophil being more closely related to PMN-MDSC²⁶⁷. TAN had downregulated genes associated with cell-cytotoxicity compared to other populations, whereas PMN-MDSC had increased genes expression of MHC class II and cytokines. In tumor and peripheral blood from cancer patients, LDN are also heterogenous neutrophil populations overlapping with PMN-MDSC and TAN, with immunosuppressive capacities^{268,269}. LDN contain both immature and mature cells but with different origins. Like PMN-MDSC, mature LDN seems to originate from normal density neutrophils, acquiring suppressive function after TGF- β stimulation²⁶⁹ and activation-induced degranulation²⁷⁰. Immature LDN are also suspected to originate from an incomplete maturation within bone marrow²⁶⁸. Globally tumor environment and chronic inflammation lead to bone marrow and tumor neutrophil reprogramming, protecting from efficient immune response and promoting development of tumor.

3) Tuberculosis

Infection by *Mycobacterium tuberculosis* (Mtb) is a leading cause of death worldwide, with 10 million people declaring an active tuberculosis in 2017²⁷¹. Despite tuberculosis is a bacterial infection, most of studies on innate immune response have been focused on macrophages, NK cells and DC. Neutrophil has a dual role in tuberculosis with a protective or detrimental role, depending on the stage of infection²³⁸. Since fundamental Koch's work in 19th century on guinea pig, several animal models have been used to study tuberculosis with different inoculation routes, strains and disease evolution, conducting to misleading interpretation of the role of neutrophils in physiopathology²³⁸. Of note mouse model fails to reproduce latent

infection and show different pathology compare to human. In fact, neutrophils seem to have a differential role depending on the stage of the infection. Neutrophils are first responders, along with alveolar macrophages, accumulating within 1st day to 1st week in lungs following intranasal or intratracheal inoculation. Elimination of Mtb or induction of latent infection relies on early immune response and clearance by phagocytes. Neutrophil priming seems to influence their capacity to phagocyte and kill bacteria. Repeated Mtb challenges lead to increase infiltrating neutrophils thank to IL-17 producing T cells. Capacity to kill Mtb has been shown to be variable among human, thus explaining elimination capacities in early infection by some individuals²⁷². Resistant individuals may have circulating primed neutrophils, more prone to rapidly respond to mycobacterial infection. If neutrophils fail to kill Mtb they may undergo apoptosis followed by macrophages phagocytosis, necrosis or migrate to draining lymph nodes where they disseminate bacilli. Neutrophils also modulate immune response against Mtb notably by secretion of IL-12, IP-10, MIP-1 α and MCP-1, attracting and polarizing T cells. Neutrophils also promote T cell proliferation and IFN- γ , IL-5, IL-6 and IL-10 through HNP1-3 secretion. Neutrophil-DC interactions have been suspected since neutrophils increased DC maturation and further T cell activation. In some circumstances, neutrophils were shown to present antigen, especially to memory T cells requiring a lower activating signal than naïve T cells²⁷³. This antigen presenting function was suspected in tuberculosis, with the identification of neutrophils expressing MHC class II and CD83 and CD86 in patients' pleura effusions²⁷⁴. These neutrophils expressed also more TLR2 and CD64 but were more prone to apoptosis compare to circulating neutrophils, making questionable their antigen presenting capacity, which remain to be demonstrated.

Compared to uninfected and latently infected patients, active tuberculosis was characterized by distinct transcriptional signature involving interferon-inducible genes expressed by neutrophils¹¹⁰. This neutrophil-driven signature was correlated with lung radiographic severity, making neutrophils associated with tissue damages. Indeed, neutrophils infiltration and neutrophilia are associated with poor prognosis and correlated with serum pro-inflammatory markers^{111,112}. Less is known about neutrophils heterogeneity tuberculosis, but some authors suggested that epigenetic reprogramming of neutrophils may explained detrimental neutrophil functions, whereas other suggest that neutrophilia only reflect failure of Th1 immunity to control Mtb^{238,275}.

4) Neutrophils in chronic viral infections

In acute viral infection, active replication increases virus chances of transmission but rapidly face an evolving immune response. This battle continues until the infection resolves, the virus kills its host or, the infection becomes chronic. Chronic infection is established when virus evades immune clearance and the immune system finds an equilibrium between avoiding tissue damages and controlling of viral replication²⁷⁶. Function of neutrophils during viral infection has been studied lately, compared to bacterial or fungal infections. Being the first immune cells infiltrating the infection site, neutrophils modulate and participate to anti-viral response²⁷⁷. Human neutrophils are able to respond to a broad array of pathogen-associated molecular patterns (PAMPs). Neutrophils express pattern recognition receptors (PRRs) scanning extracellular environment (eg. Toll-like receptors, TLRs; C-lectin receptors, CLRs), cytoplasmic acid nucleic (eg. RIG-I) and intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs)²⁷⁸ (Figure 10).

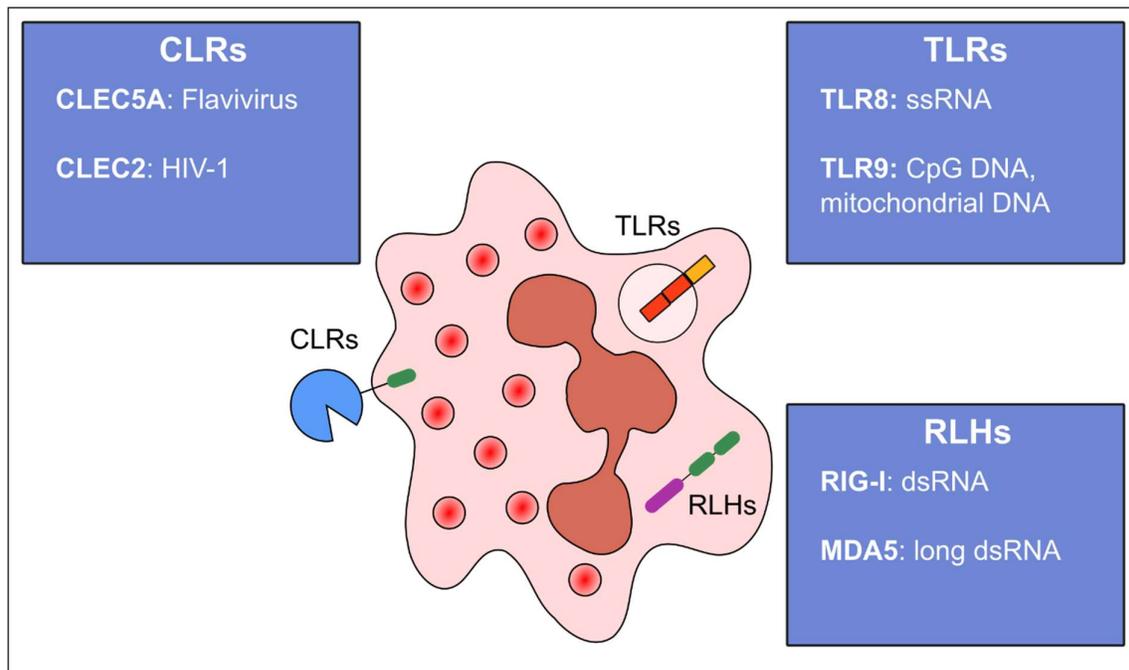


Figure 10: Pattern recognition receptors sensing viruses in human neutrophils.

Human neutrophils express almost all TLR except TLR7 and TLR3, but they can respond to imiquimod (R837, TLR7 agonist) and polyI:C (TLR3 agonist) in TLR-independent pathways^{279,280}. Stimulation of human neutrophils with ligand of TLR8 (ssRNA) and TLR9 (CpG DNA), present in viruses, induces broad responses such as degranulation, cytokine production, phagocytosis and respiratory burst²⁷⁸. In addition, all TLR agonist have been shown to promote neutrophils survival, through NF-KB and MAPKs activation, which participate to neutrophil-associated immunopathology in chronic inflammation²⁸¹. CLRs are a family of diverse proteins recognizing carbohydrate moieties, with some acting as PRRs thanks to immunoreceptor tyrosine-based activation-like motif (ITAM). Some CLRs express in neutrophils sense flaviviruses (CLEC5A) or HIV-1 (CLEC2) eliciting proinflammatory cytokines production and phagocytosis respectively²⁷⁸.

Other PRRs express by neutrophils are intracellular sensors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), which detect cytoplasmic double-stranded RNA (dsRNA). Neutrophil stimulation by polyI:C induces expression of type I IFN, cytokines (TNF- α , IL-12p40) and chemokines (CXCL10, CXCL8, CCL4 and CCL20)²⁷⁹. Chronic viral infections induce neutrophils activation and survival leading to tissue damages. Human neutrophils were shown to be primed in HSV-1, hCMV, HCV and HTLV-1 infections, associated with increase production of pro-inflammatory cytokines²⁸²⁻²⁸⁵. Another common phenomenon in chronic viral infection is the increase neutrophils survival observed in hCMV and HCV infections^{121,133}. In HCV and HBV infections, neutrophils participate to liver damage leading to cirrhosis by secreting matrix metalloproteinase and by recruiting more immune cells^{130,131}.

C. Neutrophils in HIV infection

1) Dynamic of neutrophils in HIV infection

i. *HIV infection and neutropenia*

Neutropenia is a common hematological abnormality seen in early study in 1987, and was presented in 30% of seropositive asymptomatic males²⁸⁶. A more recent study showed in HIV-infected women 44% of neutropenia prevalence (<2000 cells/ μ l) in a 7.5 years survey²⁸⁷. Neutrophil count correlated with disease progression, CD4+ T cells loss and was inversely correlated to HIV viral load²⁸⁸. However, in pediatric patients neutropenia appears even earlier in HIV infection, and is also observed in patient during primary infection²⁸⁸. Left-shift (reduce neutrophil's nuclear segmentation) is common in neutropenic HIV-infected patients, indicating redistribution of neutrophil population²⁸⁹. Globally, cART reduces incidence of neutropenia. Many mechanisms have been proposed to explain neutropenia in HIV-infected patients, notably bone marrow impairment induced by Nef and inflammation, along with neutrophil apoptosis. Nef protein was shown to inhibit proliferation of granulo-monocytic precursor cells and activated transcriptional suppressor PPAR γ in CD34+ cells^{290,291}. Indeed, HIV-infected individuals and SIV-infected macaques both demonstrated a reduced number of bone-marrow CD34+ progenitors with decreased differentiation capacity^{292,293}. In multiple studies, there was no evidence of CD34+ progenitors infection by HIV or SIV²⁹²⁻²⁹⁴. Pro-inflammatory bone marrow environment (TNF α , MIP-1 α and MIP-1 β) may decrease clonogenic activity and progenitors maintenance²⁹⁵. The ability of marrow stromal cells in HIV infection to release G-CSF was also reduced after stimulation²⁹⁶. In addition, growth factors (IL-3, GM-CSF and SCF), which are in reduced level in HIV-patients sera, also participate to neutropenia^{297,298}. Antibodies against MAC-1 (CD11b/CD18), myeloperoxidase and elastase have been detected in HIV patients, with up-to 41% of HIV individuals having anti-MAC-1 in a study^{299,300}. These antibodies might be the result of NET production presenting new auto-antigens to adaptive immune cells. Neutropenia has been linked to increased susceptibility to apoptosis of circulating neutrophils in chronically SIV-infected rhesus macaques³⁰¹. In this study, increased percentage of apoptotic neutrophils was associated with disease progression and neutrophils priming (CD11b upregulation and ROS production). As we are going to see, neutropenia has been also attributed to neutrophil mobilization to tissues, especially in mucosa.

ii. Mucosal neutrophils in HIV infection

Mucosal dysfunction, especially in gut, is an important characteristic of HIV infection which is associated to focal breach in the gastro-intestinal epithelial barrier leading to microbial translocation. Neutrophil infiltration has been used as a marker of gastrointestinal epithelium damage. Immunohistochemistry analysis of recto-sigmoid biopsies taken from HIV-infected patients (treated and untreated) reveals an increased neutrophil infiltration in lamina propria, even under cART^{83,85} (Figure 4). In the same study, early cART (initiated in Fiebig I/II) improved GI tract integrity after 96 weeks follow-up. When patients discontinued their treatment, PMN infiltration increased along with gastrointestinal damage. Whether neutrophils are a cause or a consequence of gut epithelium damage remain to be determined. Relationship between microbiota and neutrophils in HIV infection has been recently highlighted⁸⁷. In this study, they showed an increase of PMN survival in lamina propria associated with microbiota, which may potentially lead to mucosal damages (see part I-C.).

2) Neutrophil function in HIV infection

i. Neutrophil's antiviral response

Human neutrophil peptides (HNP), also called α -defensins, were widely studied because of their antiviral activity, capable to inactivate HIV-1 or to block virus entry and replication in vitro. HNP activity were first identified as a CD8+ T cells soluble antiviral factors in long-term HIV nonprogressors³⁰². The publication was further retracted because HNP production came from neutrophil contamination, but this work was still interesting by showing the importance of PMN in control of HIV infection³⁰³. Besides HNP, release of MPO by degranulation and ROS production was shown to inactivate HIV-1³⁰⁴. Neutrophils from HIV-infected patients were able to kill infected cells using antibody-dependent cell-mediated cytotoxicity (ADCC), mediated by antibody to env glycoprotein³⁰⁵. Killing mechanism is still unknown but did not rely on ROS formation. Compared to monocytes and NK cells, neutrophils were less effective to mediate ADCC killing of HIV-infected cells³⁰⁶, but were much faster to respond³⁰⁷. Since neutrophils are more numerous than monocytes and NK cells, this killing mechanism might be underestimate. In addition, NK cells from gastrointestinal tract and female reproductive tract express negligible Fc-receptors compare to macrophages and neutrophils. Antibody-dependent cellular phagocytosis (ADCP) of infected cells or immune complexes is part of

antibody response observed in elite controllers and can be performed by neutrophils³⁰⁸. Neutrophils mediated higher phagocytosis of gp120 coated beads or immune complexes, suggesting that inducing ADCP would be an interesting correlate of protection in vaccine development³⁰⁹.

NET formation in HIV/SIV infection was also explored, since these structures concentrate toxic neutrophils enzymes and peptides. HIV-1 can stimulate NET production by human neutrophils through TLR8 signaling, leading to capture and inactivation of the virus³¹⁰. In acute SIV_{sab}-infected pigtailed macaques (14 dpi) neutrophils had an increase of NET production, which was related to intense viral replication prior to alteration in gut integrity³¹¹. In chronic phase (50 dpi), there was an increase NETosis of unstimulated neutrophils, which was associated with microbial translocation. In the same animals, co-localization of MPO and neutrophil elastase in tissue revealed the presence of NET in gut, lung, heart and kidney. Like in ulcerative colitis, there were crypt abscesses in colon with the presence of NETs capturing CD3+ lymphocytes and CD68+ macrophages. Around cryptic abscesses, neutrophils infiltrated *lamina propria* and were in close interaction with T cells. In the same study, in vitro analysis using PMN and PBMC co-cultures demonstrated that NETs trapped T cells, B cells and macrophages, leading to cells apoptosis. In glomerular capillaries and other kidney's small blood vessels, NETs were also detected and were suspected to participate to microthrombi formation. Thus, neutrophil infiltration in HIV/SIV infection induce tissue damage and immune cell depletion.

ii. Dual role in HIV acquisition and protection

The role of neutrophils in term of protection versus acquisition is not well understood and may depend on the tissue and the context studied. Neutropenia (<2500 cells/mm³) was associated with a 3-fold increased risk of HIV acquisition in South African female sex workers³¹². In female reproductive tract neutrophils are associated with local inflammation and increase risk of HIV acquisition. In cervicovaginal secretion collected from Kenyan sex workers, higher α -defensins and LL-37 levels were associated with increased risk of HIV acquisition³¹³. In CAPRISA 004 microbicide trial, elevated level of IL-8 in cervicovaginal secretion was associated with genital inflammation and increased risk of HIV acquisition³¹⁴. IL-8 is highly produced by activated neutrophils, which are important effectors in genital inflammation. In the same cohort, neutrophil proteases were increased and were associated

with an increase of endocervical CD4+T cells and IL-17 expression³¹⁵. Neutrophils may increase HIV acquisition by recruiting target cells through inflammatory cytokines and participate to mucosal damage in case of dysbiosis or genital infection.

In contrast, neutrophils were associated indirectly with protection against HIV acquisition. In foreskin of HIV-exposed but seronegative individuals (HESN), levels of neutralizing Ig-A and α -defensins were elevated compared to unexposed controls³¹⁶. In fact, neutrophil response to inflammatory stimulus was an important characteristic of HESN. Circulating neutrophils from HESN presented reduced expression of PRR (TLR4, TLR9, NOD1, NOD2, NLCR4 and RIG-I) along with reduced cytokine production (IL-1 β , IL-6, IL-18 and TNF- α). When stimulated with HIV-1, TLR2 and TLR4 ligands neutrophils from HESN produced lower levels of ROS compared to seropositive individuals³¹⁷.

iii. Neutrophils function and chronic inflammation

In chronically HIV-infected patients, circulating neutrophils demonstrated CD62L shedding and up-regulation of CD11b, suggesting neutrophils priming²². Chronic exposure to pro-inflammatory mediators in HIV-infection may also explain neutrophil priming and impairment. In patients under cART, soluble pro-inflammatory markers persist. Neutrophil priming measured by steady state ROS production and surface expression of CD62L, CD11b and CD16 were associated with IL-18 serum level in HIV infected patients, which is known to prime neutrophils²². In addition, PMNs priming was more elevated in patients having non-AIDS-related comorbidities. In this study, circulating IL-17+ lymphocytes were increased in HIV-infected group and correlated with PMN priming. Thus, neutrophil priming may be driven by IL-17-producing cells. By measuring CD62L shedding and CD11b translocation, authors also shown normal PMN responses to TLR agonists and TNF- α in these patients compare to healthy donors²². But in other studies, neutrophil priming was associated with impaired antimicrobial function. Elbim C. *et al.* shown also an increase of ROS production by unstimulated neutrophils and ,using DCFH-DA as a specific fluorescent marker of H₂O₂ production, observed an impaired oxidative burst in response to fMLP, IL-8 and TNF³¹⁸. Authors also separated patients according clinical stage or presence of opportunistic infection, and correlated disease progression with PMN impairment. In chronically infected children fMLP chemotaxis and *S. aureus* bactericidal activity were also impaired³¹⁹. Under cART, PMN phagocytosis and oxidative burst were shown also to be impaired and correlated negatively with viral load, and positively CD4 T cell count,

and treatment success³²⁰. In this study, treatment initiated in advance chronic infection did not restore phagocytosis function. Impairment of phagocytosis and bacterial killing have been demonstrated for *S. aureus*, *M. tuberculosis*, *C. albicans* and *E. coli*, explaining partially the increased susceptibility of HIV-infected individuals to those pathogens^{319,321–323}. Exact mechanisms responsible for phagocytosis impairment are unknown, but abnormal NADPHox complex distribution within PMN have been proposed to explain reduced ROS production and bacterial killing³²⁴.

Few studies have evaluated neutrophil immunomodulatory functions in HIV infection. First observation of immunomodulatory neutrophils was made on HIV-infected individuals with low CD4+ count showing abnormally high arginase activity in PBMC³²⁵. Arginase is produced by neutrophils and capable to inhibit T cell function by degrading L-arginine, so the authors looked for an immunomodulatory PMN population within PBMC. Absent in healthy donors, LDN were significantly increased in chronically HIV infected patients (CD4 count below 350/mm³), but represented less than 5% of PBMC. They were segmented CD33^{high} CD16^{low} CD11b^{high} neutrophils demonstrating a phenotype close to G-MDSC. Demonstration of suppressive activity of both low-density and normal density neutrophils in HIV-infection were made by Bowers N.L. *et al*³²⁶. In this study, neutrophils from HIV-infected individuals expressed PD-L1, which correlated with T cell immune activation and plasma markers of degranulation (arginase 1, NGAL and MPO). Both NDN and LDN from chronically HIV-infected patients were potent to suppress T cells IFN- γ production in PD1/PD-L1 and ROS dependent mechanisms. Since plasma markers of degranulation were higher, they hypothesize that neutrophil priming and degranulation under inflammatory environment promote LDN/G-MDSC function. Neutrophil heterogeneity is a matter of definition and latter description of suppressive neutrophils were termed G-MDSC in HIV/SIV infection. In primary HIV infected patients, G-MDSC were increased in PBMC compared to healthy controls, but representing less than 1% of PBMC³²⁷. Functional analysis demonstrated suppressive activity of G-MDSC against CD8+ T cell proliferation and IFN- γ production, which was in part mediated by PD-L1. Another study confirms the increased frequency of G-MDSC in primary HIV infection (Fiebig II/III)³²⁸. Authors showed a higher level in chronic phase correlating with TRAIL plasma level but without correlation with viral load, suggesting an association with chronic inflammation. In chronically SIV infected rhesus macaques, G-MDSC were also increased in blood but

depleted in bone marrow whereas animals controlling viral replication had normal levels³²⁹. Nevertheless, they did not demonstrate suppressive activity of G-MDSC, but still showed that chronic infection changed granulocytes population in bone marrow. Taken together these results indicates circulation of suppressive LDN, especially when CD4 count is low. Bowers N.L. *et al*³²⁶ suggested that generation of suppressive LDN is a physiological process occurring in case of chronic inflammation and which may participate to T cells immune exhaustion using PD-L1/PD-1 axis.

But LDN represented a small population of PBMC in HIV-infected patients compare to systemic lupus erythematosus (SLE), in which LDN represent up to 15% of PBMC³³⁰. In autoimmune diseases, neutrophils are pro-inflammatory cells and they participate to chronic inflammation^{249,331,332}. Pro-inflammatory functions of neutrophils in HIV infection remain to be determine, since other subpopulations have not been explored yet and could demonstrate opposite immunomodulatory functions³³³. Thus, neutrophils could be also involved in HIV chronic inflammation. Recently, Hensley-McBain T. *et al.* reported an accumulation of neutrophils in colonic lamina propria from chronically HIV infected patients⁸⁷. Increased neutrophils survival in the gut was associated with changes in microbiota, such as *Lactobacillus: Prevotella* ratio. Since neutrophils were associated with gut epithelial damage, microbial translocation in SIV/HIV infections⁸⁴ and increase of neutrophil survival is associated with pro-inflammatory function in inflammatory bowel diseases³³⁴, authors hypothesizes that neutrophils could participate to gut inflammation in HIV infection.

In sum, neutrophils seem to be involved in HIV-1 acquisition and progression. Less is known about neutrophil heterogeneity and their functions in the course of HIV-1 infection. LDN were shown to be immunosuppressive cells in chronic HIV infection. But there are also evidence that neutrophils participate to chronic inflammation and tissue damages. Neutrophil is the most abundant circulating leukocyte with efficient pro-inflammatory and potentially destructive functions, which could participate to HIV-1 chronic inflammation. Thus targeting neutrophils could also give rise to new therapeutic strategies to resolve chronic inflammation.

Methodology

I. General objectives

The infected host fails to eradicate HIV-1, despite significant control of viral replication by combinational antiretroviral therapy. HIV persistence is associated with chronic inflammation in many infected patients, leading to an increased risk of comorbidities, such as cardiovascular diseases, neurocognitive diseases, and cancers. In addition, events occurring during HIV primary infection determine the progression of the disease and notably innate myeloid response is the first to sense and respond to the virus. Monocytes, dendritic cells and macrophages have been widely studied especially because they are antigen presenting cells initiating adaptive immune response and because they are productively infected. Neutrophils are the first myeloid cells arriving in the site of any infection and they are also potent proinflammatory cell in chronic diseases, which participate to tissue damages. Nevertheless, PMN have been overlooked in the context of HIV infection, but there are few evidence of their role during chronic inflammation. The lack of information about neutrophils in HIV physiopathology is partially explained by their fragility. Polymorphonuclear are sensitive cells needed to be analyzed within hours to avoid priming and cell death, furthermore cryopreservation is impossible and lead to cells death.

Our hypothesis is that events occurring during primary HIV infection shape neutrophil function and differentiation, which in turn lead to their participation to chronic inflammation and immune dysfunction, even under cART.

It is still difficult to study fist days of HIV infection and to perform tissue analysis in patients because of limited sample availability. In this project we performed all analysis in non-human primate models of AIDS. Cynomolgus macaques infected with SIVmac251 are a suitable model to overcome such limitations, because many aspects of this model parallel those of HIV-1 physiopathology and the hematopoietic system of macaques is similar to humans.

What is the impact of chronic SIV infection on myeloid cells heterogeneity and neutrophils function?

We first wanted to evaluate the diversity of myeloid cells during late chronic SIV infection. We analyzed blood and bone marrow using multidimensional mass cytometry to maximize the chance of unraveling changes induced by SIV infection to myeloid population. This analysis highlighted neutrophils heterogeneity in blood and bone marrow. In late chronic SIV infection, blood neutrophils had a phenotype close to bone marrow, suggesting circulation of immature neutrophils. Based on markers identified by mass cytometry, we looked for circulating immature neutrophils in early chronic SIV-infected macaques, receiving or not cART. Based on available marker at that time, we could not identify immature neutrophils but we demonstrated that SIV infection leads to neutrophil priming and impairment of phagocytosis. Under cART, neutrophil priming was partially decreased but phagocytosis remained impaired.

What is the dynamic of neutrophils during primary and chronic SIV infection? Do neutrophils modulate T cells responses during SIV infection?

We first wanted to determine when immature neutrophils are circulating during SIV infection and if it is associated with changes in bone marrow granulocytes differentiation. To follow neutrophil differentiation in cynomolgus macaque, we combined flow cytometry cell sorting with cytology to validate in our model markers newly described in human. Thereby, we developed two flow cytometry panels, the first one making differences between pre-neutrophils (myeloblast, promyelocyte and myelocyte), immature neutrophils (metamyelocyte and band cell) and segmented neutrophils. The second one was dedicated to evaluate relationships between neutrophil priming, viral replication, CD4⁺ T cell depletion and T cells activation. Since LDN have been detected in people living with HIV, we followed LDN frequencies in PBMC in our model. To evaluate if neutrophils can modulate T cell proliferation and cytokine production during SIV infection, we developed a co-culture assay between PBMC and neutrophils.

In primary SIV infection, there was a decrease of circulating segmented neutrophils replaced by immature neutrophils having a primed phenotype. Changes in neutrophil maturation stage and level of activation matched with dynamic of viral replication in primary infection. In chronic phase, these phenotypes were maintained to a lesser extent and were correlated with T cell immune activation. Blood neutrophils were also capable to modulate T cell response and

demonstrated pro-inflammatory function. Early initiation of cART partially restored neutrophil phenotype.

Which tissues are infiltrated by neutrophils during SIV infection? Is there a tissue related phenotype of neutrophils?

Lymphoid and mucosal tissues are major site of immune dysfunction and HIV reservoir, in which there are few evidence of neutrophil involvement. Phenotype and function of neutrophils in tissue during HIV infection have not been explored yet. So, we evaluate in the cynomolgus model, neutrophil infiltration and phenotype in blood, bone marrow, spleen, mesenteric lymph nodes, ileum, colon and bronchoalveolar lavages using flow cytometry and minimal tissue preparation. We first analyzed tissues from healthy cynomolgus macaques and we plan to analyze tissue from chronically infected animals. In addition, we collected tissues from previous studies to perform multiparametric immunohistofluorescence in order to localize PMN, T cells and productively infected cells. Here, we will present unpublished analysis made on tissues, showing a phenotype related to each tissue.

II. Study model

Cynomolgus macaques (*Macaca fascicularis*) are Asian primates, which are not natural host of lentivirus, and develop AIDS after exposure to SIVmac251. Physiopathology of SIV infection in cynomolgus macaques is close to HIV infection in humans regarding the dynamic of viral replication, CD4 T cells depletion and chronic immune activation^{335,336}. As in HIV-1 infected human, SIVmac251-infected macaques slowly progress to AIDS when compared to rhesus macaque (*Macaca mulatta*) and pig-tailed macaques (*Macaca nemestrina*), which develop AIDS in 70 weeks and 42 weeks respectively¹⁶⁵. Furthermore, cynomolgus macaques from Mauritius Island demonstrate a limited MHC diversity (7 MHC haplotypes), allowing selection or exclusion of haplotypes associated with control of viral replication³³⁷. Cynomolgus macaques cannot be productively infected by HIV-1 because of TRIM5 genes polymorphisms, which restrict the viral cycle³³⁸.

In 1985, SIV was isolated from rhesus macaques, from New England Primate Research Center (USA), presenting transmissible immunodeficiency characterized by opportunistic infections and lymphoma³³⁹. In fact, SIVsmm from captive mangabeys, housed in United States primate centers, was unintentionally transmitted to rhesus macaques in mid-1960^{339,340}. Thus, first descriptions of immunodeficiency in macaques preceded HIV-1 emergence. SIVmac251 (name from rhesus macaque from which it was isolated) is a virus isolate with genetic heterogeneity (viral quasispecies), which use CCR5 as a co-receptor but rarely evolve to use CXCR4³⁴¹. Thus, replication predominates in memory CD4+ T cells present in mucosal tissues, as seen in most HIV-1 isolates³³⁶. So, even if SIVmac251 is genetically related to HIV-2, host-virus interaction leads to physiopathology mimicking HIV-1 infection such as CD4 T cells depletion, dynamic of viral replication and cellular and humoral responses (Figure 11).

In cynomolgus macaques, pharmacodynamics / pharmacokinetic of integrase inhibitors (dolutegravir) and NRTIs (emtricitabine, and tenofovir), administered *per os* or subcutaneously, are similar to human (Figure 11)^{342,343}. These drugs are commonly used in human against HIV-1 infection, and also efficiently inhibit integrase and reverse transcriptase from SIVmac251. Therefore, cynomolgus macaques infected by SIVmac251 is suitable model to study the impact of long-term treatment on reservoirs, residual replication and chronic immune activation.

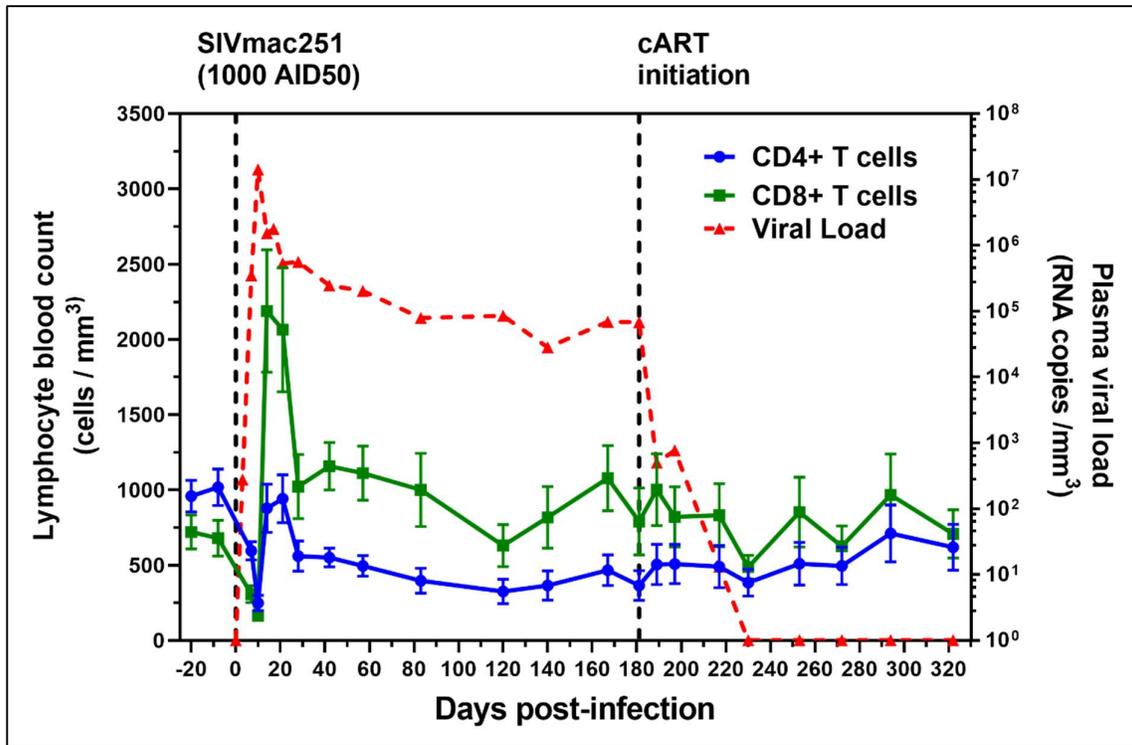


Figure 11: Dynamic of viral replication along with CD4+ T cells and CD8+ T cells in SIVmac251-infected cynomolgus macaques. Intravenous inoculation of 1000 AID50 leads to exponential viral replication, peaking at 10 days post-infection, like in human HIV-1 infection, which decreases with CD8+ T cells response. Blood CD4+ T cell count decreases in primary infection and chronic phase. Administration of dolutegravir, tenofovir and emtricitabine subcutaneously efficiently suppresses plasma viral load and restores CD4+ T cell count.

III. Principles of mass cytometry

Classical flow cytometry allowed a deeper understanding of hematopoietic cells phenotype and function in steady and pathological states. Advances in polychromatic flow cytometry enable to quantify up-to 18 markers per cells along with size and granularity, but have limitation of the number of spectrally resolvable fluorochromes (ie. spectral overlap)³⁴⁴. Single cell analysis has recently turned a corner with the development of mass cytometry. Inductively-coupled mass spectrometry (ICP-MS) was first designed to precise heavy elements analysis and quantification, and have been hijacked by Baranov et al. for single cell multiparametric analysis³⁴⁵. Cell staining relies on antibodies coupled with pure metal isotope (mainly lanthanide), elements which are not present in biological samples³⁴⁶. Briefly, cell suspension is first aerosolized, then atomized and ionized in a 7000K plasma (Figure 12). Heavy elements coming from each cell are then separated and analyzed by mass spectrometry. The latest mass cytometer can theoretically measure up-to 135 metal per cell, with the limitation of production of each isotopes and classical mass cytometry reach 40 markers. In the last ten years, this technology allowed deep phenotyping of cells without performing compensation with similar sensitivity to flow cytometry. Nevertheless, mass cytometry is an expansive powerful tool devoted to research with slow throughput (500 cells/s) compared to flow cytometry³⁴⁷.

Mass cytometry generates complex data which cannot be analyzed by conventional biaxial dot plots, at the risk of losing discovery capabilities of such data set. Thus, many algorithms have been developed to meet the need of data representation (eg. vi-SNE) or cluster analysis (eg. SPADE)³⁴⁸. We are going to focus on the algorithm we used in this project: spanning-tree progression analysis of density-normalized events (SPADE). SPADE performs an unsupervised analysis of mass cytometry data, which creates a 2D minimum spanning tree summarizing relationships between clusters of cells (nodes). SPADE represents equally rare and abundant cell type by first performing density-dependent down-sampling³⁴⁹. Second, cells selected by down-sampling with similar phenotypes are gather into a cluster. Third, SPADE summarizes the data by creating a tree representing relationships between cluster phenotypes, the minimum spanning tree.

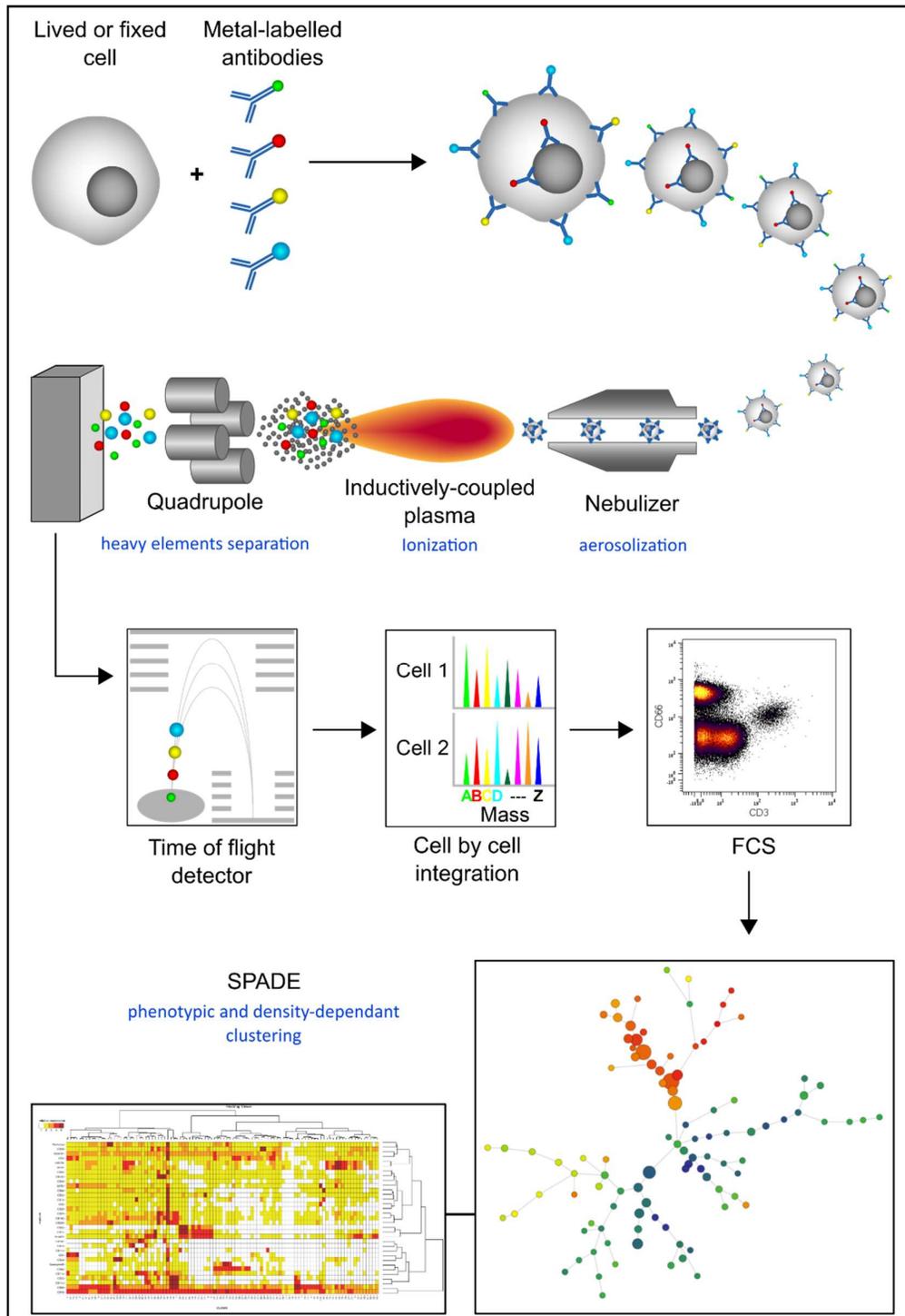


Figure 12: Mass cytometry operation and data analysis. Mass cytometry relies on metal-labelled antibodies used for cell staining. Fluidic and nebulizer inject cell by cell into plasma, which atomizes and ionizes sample. Heavy isotopes are then selected and launched by the quadrupole to time-of-flight detector. Mass cytometer integrates all isotopes signals from each cell and generate FCS file containing all cells from the sample. We performed deep phenotyping using SPADE algorithm, allowing phenotype-based clustering while preserving rare populations.

Finally, SPADE locates each cell from the data set in the cluster it belongs, which is a process called up-sampling. So, this approach reveals cell diversity and does not lose rare population, giving a new way to analyze hematopoietic populations. To better analyze SPADE results such as cluster phenotypes, differences between groups and perform follow-up of cluster abundance in multiple time-points, bioinformatics team from the lab developed an R-package, called SPADE-VizR³⁵⁰.

Mass Cytometry Reveals the Immaturity of Circulating Neutrophils during SIV Infection

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Keywords

AIDS · Antiretroviral treatment · Mass cytometry · Neutrophils · Nonhuman primate · Phagocytosis

Abstract

The infected host fails to eradicate HIV-1, despite significant control of viral replication by combinational antiretroviral therapy. Here, we assessed the impact of HIV infection on immune-cell compartments in a SIVmac251 nonhuman primate infection model, which allowed the choice of contamination route, time of infection, and treatment follow-up. We performed high-throughput multiparameter single-cell phenotyping by mass cytometry to obtain a global vision of the immune system in blood and bone marrow. Circulating polymorphonuclear neutrophils (PMNs) with impaired phagocytosis had altered surface expression of CD62L and CD11b during early chronic infection. The initiation of combinational antiretroviral treatment during primary infection did not restore PMN function. The maturation state of PMNs was highly altered during late chronic SIV infection, showing a primarily immature phenotype. Our results provide new

insights into PMN involvement in the pathogenesis of HIV infection and may play a role in the establishment and maintenance of chronic immune activation.

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Introduction

Combinational antiretroviral treatment (cART) has increased life expectancy and the quality of life of HIV-infected patients. Nevertheless, lifelong treatment is necessary to control virus replication because of the inability of cART and host defenses to eradicate the virus. HIV persistence is associated with chronic inflammation in many infected patients, leading to an increased risk of comorbidities, such as cardiovascular diseases [1], neurocognitive disease [2], kidney disease [3], and cancer [3]. The proinflammatory cytokine IL-6 and soluble (s)CD14 and sCD163 levels are associated with an increased risk of mortality for both treated and untreated HIV-infected patients [4–6]. Inflammatory markers can also be directly linked to the monocyte and

macrophage activation associated with microbial translocation that occurs in HIV infection [7]. Finally, chronic inflammation contributes to exhaustion of the adaptive immune system and T-cell activation. Polymorphonuclear neutrophils (PMNs) are myeloid cells that are also associated with chronic inflammation in autoimmune diseases, such as rheumatoid arthritis [8]. However, few studies have been conducted on their role in HIV infection. In chronic HIV-1 or SIV infection, neutrophils show an activated profile [9], increased sensitivity to apoptosis [10], and impaired phagocytosis [11, 12]. Neutrophils have been shown to suppress interferon- γ production by CD8+ T cells in chronic HIV-1 infection via the PD-L1/PD-1 axis and were more prone to degranulation, contributing to chronic immune activation in patients [13].

It is currently difficult to obtain a global view of the impact of HIV infection on the immune system, especially on myeloid cells. Available studies have several limitations. Bone marrow, which is the differentiation and maturation compartment of myeloid cells, is still poorly characterized in HIV infection. In addition, innate immunity plays a major role in the first steps of any infection, but the study of the first days of infection is rare in humans because of limited sample availability. Cynomolgus macaques infected with SIVmac251 are a suitable model to overcome such limitations, because many aspects of this model parallel those of HIV-1 physiopathology [14–17] and the hematopoietic system of macaques is similar to that of humans [18, 19].

We characterized myeloid-cell heterogeneity in cynomolgus macaque blood and bone marrow using recently developed mass cytometry technology, based on metal isotope-conjugated antibodies, that enables the detection of more than 40 markers per cell [20], providing major advantages over conventional flow cytometry. The background is very low because heavy metals are not present in biological specimens and there is no need for channel compensation, as required for laser-based flow cytometry. Our analysis suggests significant modification of the myeloid-cell compartment, especially circulating PMNs. During the early chronic phase, PMNs had surface expression of PMN CD62L decreased and that for CD11b increased, suggesting that PMNs were primed in SIV infection. In parallel, circulating PMNs demonstrate a mature phenotype but phagocytosis was impaired. In late chronic SIV infection, blood PMNs consisted of mostly immature neutrophils. These results highlight the need for further study of tissue myeloid-cell dynamics in an HIV infection model to better understand chronic inflammation and design new treatments for HIV remission and a functional cure.

Materials and Methods

Nonhuman Primates and Whole-Blood Collection

Twenty-nine cynomolgus macaques (*Macaca fascicularis*), imported from Mauritius, were housed at the IDMIT infrastructure animal facility at the CEA, Fontenay-aux-Roses, France. Animals expressing the H6 MHC haplotype were excluded because of their increased natural capacity to control SIVmac251 infection [21]. Seventeen animals were infected by intravenous inoculation with 1,000 animal infectious dose 50% (AID50) of pathogenic SIVmac251 isolate and followed for 18 months [22]. Six SIV-infected animals were subcutaneously treated daily, starting from 28 days after infection, with a combination of two nucleoside reverse-transcriptase inhibitors, emtricitabine (40 mg/kg) and tenofovir disoproxil fumarate (5.1 mg/kg), and one integrase inhibitor, dolutegravir (2.5 mg/kg) [23]. Twelve healthy macaques were used as an uninfected control group.

Three healthy animals and 2 SIV-infected animals, which were sampled 18 months after inoculation, were used for mass cytometry analysis. In addition, 15 animals in the early chronic phase of SIV infection (120–288 days after inoculation) were used, along with 9 uninfected macaques (online suppl. Table 1; for all online suppl. material, see www.karger.com/doi/10.1159/000499841), for flow cytometry analysis. Blood was drawn, and bone marrow aspirates were collected under anesthesia, consisting of an intramuscular injection of 10 mg/kg ketamine (Rhône-Mérieux, Lyon, France). Blood was collected into lithium-heparin tubes (Vacutainer BD, USA) and bone marrow aspirates from the iliac crest using a 10-mL 18-G syringe containing citrate dextrose (10 mg/mL).

The macaque study was approved by the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche (France) and the ethics committee Comité d'éthique en expérimentation animale No. 44 under reference 2015102713323361.02 (APAF-IS#2453). Animals were handled in accordance with national regulations (CEA accreditation No. D92-032-02) and European Directive (2010/63, recommendation No. 9) in compliance with the Standards for Human Care of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance No. A5826-01.

Reagents and Cell Preparation and Storage for Mass Cytometry

Blood and bone marrow samples were processed using a previously described procedure, consisting of fixation, red blood cell lysis, and freezing [19], that allows the recovery of all blood leukocyte populations, especially PMNs, which are highly labile and cryosensitive. Briefly, 1 mL of whole blood or bone marrow aspirate was mixed with 10 mL fixation mixture and incubated on ice for 10 min. After centrifugation, red blood cells were lysed by adding 10 mL Milli-Q water at room temperature (RT) and incubating the samples for 20 min. After two washes with 1 \times modified Dulbecco's phosphate-buffered saline (DPBS), the cells were counted and stored at -80°C in fixation mixture at a final concentration of 15×10^6 fixed leukocytes/mL and distributed into aliquots containing 3×10^6 cells.

Staining and CyTOF Acquisition

Three million fixed cryopreserved cells were thawed at 37°C , washed twice with PBS/0.5% bovine serum albumin and incubated on ice for 30 min with the metal-labeled surface antibodies listed in online supplementary Table 2. All antibodies were previously tested for cross-reactivity against cynomolgus cells [19]. After two washes with 1 \times modified DPBS, the cells were fixed with PBS/1.6% paraformaldehyde (PFA) at RT for 20 min and permeabilized with

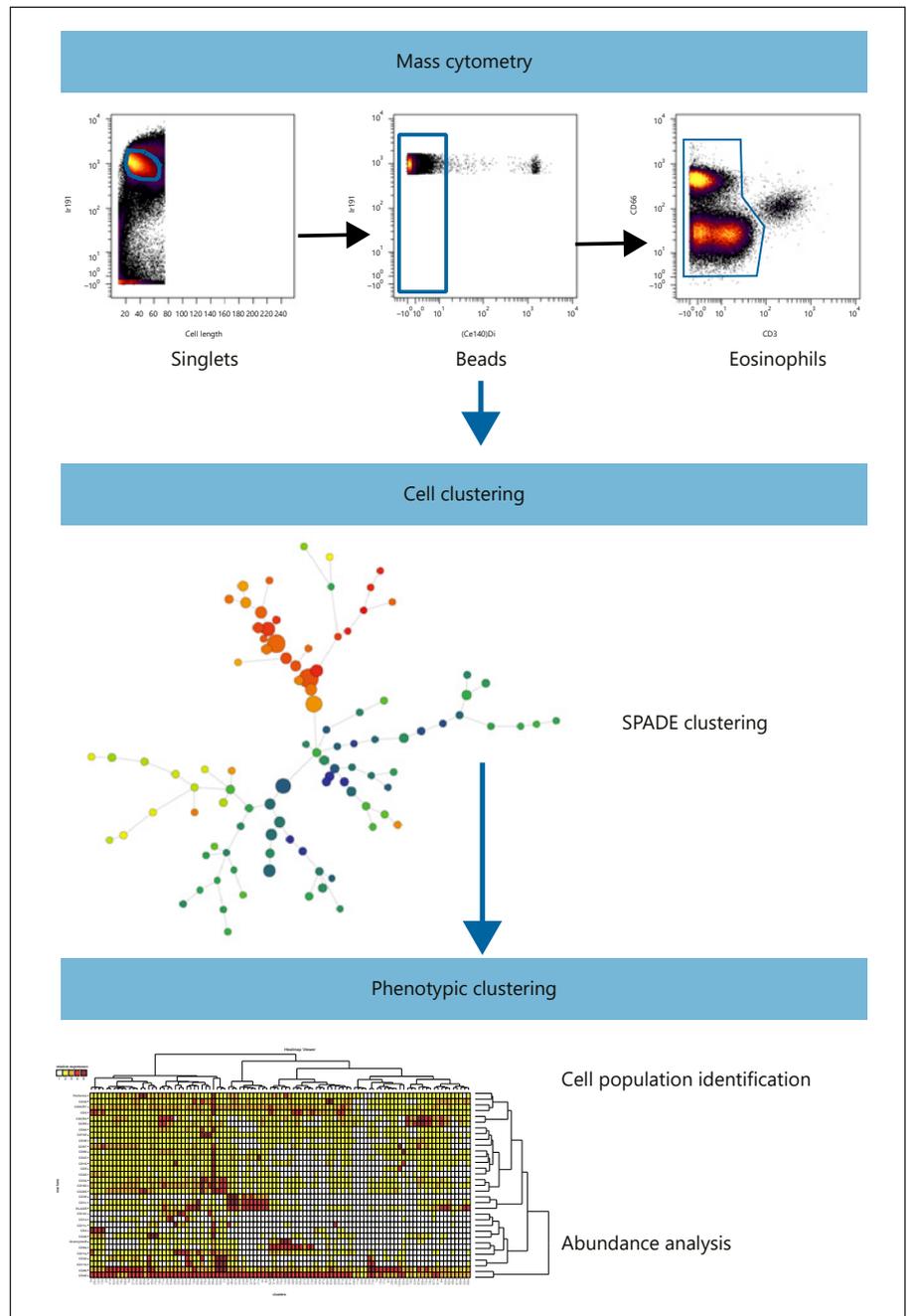


Fig. 1. Mass cytometry data analysis pipeline. Data were first normalized using internal metal beads to correct instrument variation during the time of acquisition. Cell events were selected within singlets and normalization beads excluded using iridium-191 nucleus staining. Then, cell events were manually gated on the CD66abce/CD3 bivariate plot to exclude CD3/CD66abce double-positive cells, eosinophils that bind all metal complexes on the antibodies. Cell clustering was performed using the SPADE algorithm, grouping phenotypically related cells in a homogenous cluster. The clusters were annotated, and abundance variation was analyzed using the SPADRVizR R-package.

1× Perm/Wash Buffer (BD Biosciences) at RT for 10 min. Intracellular antibodies and iridium nucleic acid intercalator were incubated on ice for 30 min. After two washes with 1× modified DPBS, the cells were fixed with PBS/1.6% PFA at RT for 20 min, centrifuged, and stored overnight in 0.1 μM iridium nucleic acid intercalator in PBS/1.6% PFA. The following day, the cells were washed with Milli-Q water, resuspended in 1 mL Milli-Q water, and filtered through a 35-μm nylon mesh cell strainer (BD Biosciences), before the addition of EQTM Four-Element Calibration Beads (Fluidigm, San Francisco, CA, USA), according to the manufacturer’s instructions. Each sample was split in half and run the same

day to reduce instrument variation. The number of acquired cells is given in online supplementary Table 3 for each sample.

Data Processing and Analysis for CyTOF

Data were normalized using Rachel Finck’s MATLAB normalizer [24]. Replicates were concatenated using the FCS file concatenation tool (Cytobank, Mountain View, CA, USA). Four-Element Calibration Beads were excluded by manual gating on the Ce¹⁴⁰ channel. Singlets were selected on an ¹⁹¹Ir DNA intercalator/cell length bivariate plot (Fig. 1). We consistently observed a clear population of cells showing a strong background in all channels and in

all samples, but absent from the CD66abce/CD3 bivariate plot. Recently, Rahman et al. [25] reported generalized nonspecific metal-conjugated antibody binding by eosinophils during the staining of fixed whole-blood cells, resembling the pattern observed in our fixed whole-blood samples, suggesting that these double-positive cells were eosinophils. We further excluded CD66abce/CD3 double-positive eosinophils from mass cytometry analysis.

One spanning-tree progression analysis of density-normalized events (SPADE) [26] was performed on both blood and bone marrow data sets using 33 clustering channels. Cell nodes generated by SPADE were manually annotated accordingly to the rules shown in online supplementary Table 4. We performed a deeper analysis of these nodes using our recently released SPADEVizR R-package [27], dedicated to cytometry data analysis and focusing primarily on SPADE-derived data. The SPADE nodes were categorized by calculating the 1st and 99th percentiles of the expression distribution of the total cells for each marker and dividing this range into five expression categories (online suppl. Fig. 1). The mean of the median marker expression of cells contained in each node was then used to assign the expression of each marker to one of the five categories. Data for individuals with less than 50 events in a given node were excluded from the calculation to avoid biasing the phenotypical characterization of these small cell nodes. Hierarchical clustering was performed using the Euclidean metric and complete linkage method we recently published [27].

Flow Cytometry Cell Preparation and Phagocytosis Assay

Briefly, heparinized whole blood was processed within 1 h following blood draw to preserve neutrophils. For the phagocytosis assay, 100 μ L whole blood was incubated for 15 min on ice or in a 37°C water bath with *Escherichia coli* fluorescent bioparticles (pHrodo™ BioParticles, ThermoFisher) at a 20:1 ratio of particles to phagocytes. Phagocytosis was stopped by transferring the samples to ice and the addition of 20 ng cytochalasin D, inhibiting actin polymerization and further stopping phagocytosis of bioparticles. For phenotypic analysis and the phagocytosis assay, viability staining was first performed for 15 min at room temperature, according to the manufacturer's protocol (Live Dead, ThermoFisher). Then, antibody staining was performed with the following antibodies for 15 min at RT: CD64 BUV732 (10.1, BD Bioscience), CD11b V450 (ICRF144, BD Bioscience), CD3 BV605 (SP34.2, BD Bioscience), CD8a BV605 (RPAT8, BD Bioscience), CD20 BV605 (2H7, BD Bioscience), CD62L BV711 (SK11, BD Bioscience), CD32abc BV786 (FLI8.26, BD Bioscience), CD14 FITC (M5E2, BD Bioscience), CD16 PerCP-Cy55 (3G8, BD Bioscience), CDw125 PE (A14, BD Bioscience), CXCR4 PE-Dazzle 594 (12G5, Biolegend), CD89 PE-Cy7 (A59, Biolegend), HLADR AF700 (L234, Biolegend), and CD66abce APC-Vio770 (TET2, Myltenyi Biotec). Samples were then lysed and fixed using BD fluorescence-activated cell sorting (FACS) Lysing (BD Bioscience) for 15 min. After an additional wash with PBS, acquisition was performed on a BD FORTESSA flow cytometer (BD Bioscience) and analyzed

Fig. 2. Bone marrow and blood leukocyte characterization by mass cytometry. The SPADE tree shows the global analysis of leukocyte populations in blood and bone marrow from uninfected and chronically SIV-infected macaques. **a** The topology of the SPADE tree is shown with the cluster number associated with each node and color according to manually annotated leukocyte populations.

using FlowJo software. The fluorescence of the bioparticles increases with acidification of the phagolysosome. Thus, we measured neutrophil phagocytosis by determining the percentage of pHrodo-positive PMNs. Then, we calculated the phagocytosis ratio to remove background fluorescence as follows:

$$\frac{\%pHrodo\ PMN\ at\ 37^{\circ} - \%pHrodo\ PMN\ at\ 0^{\circ}C}{\%pHrodo\ PMNs\ at\ 0^{\circ}C}$$

Neutrophil Cell Sorting and Cytology

For cell sorting by flow cytometry, whole blood or bone marrow from two uninfected animals were first NH4Cl lysed, then FcR were blocked using cynomolgus macaque serum. Cells were counted and incubated 30 min with the following antibodies: CD11b (ICRF44), CD45 (D058-1283), CDw125 (A14), CD3 (REA994), CD20 (LT20), CD8a (BW135/80), CD14 (TUK4) CD32a (IV.3), and CD66 (TET2). Cell sorting was performed on FACSaria I flow cytometer (Becton Dickinson). Sorted populations were cytopspined and then stained by May-Grünwald-Giemsa. Pictures were taken by a Nikon Eclipse 80i with Dxm 1200C digital camera at 60 \times magnification. Cells were identified by morphological criteria by a cytologist. Myeloblasts, promyelocytes, and myelocytes were considered as pre-neutrophils, metamyelocytes, and band cells as immature neutrophils and segmented neutrophils as mature.

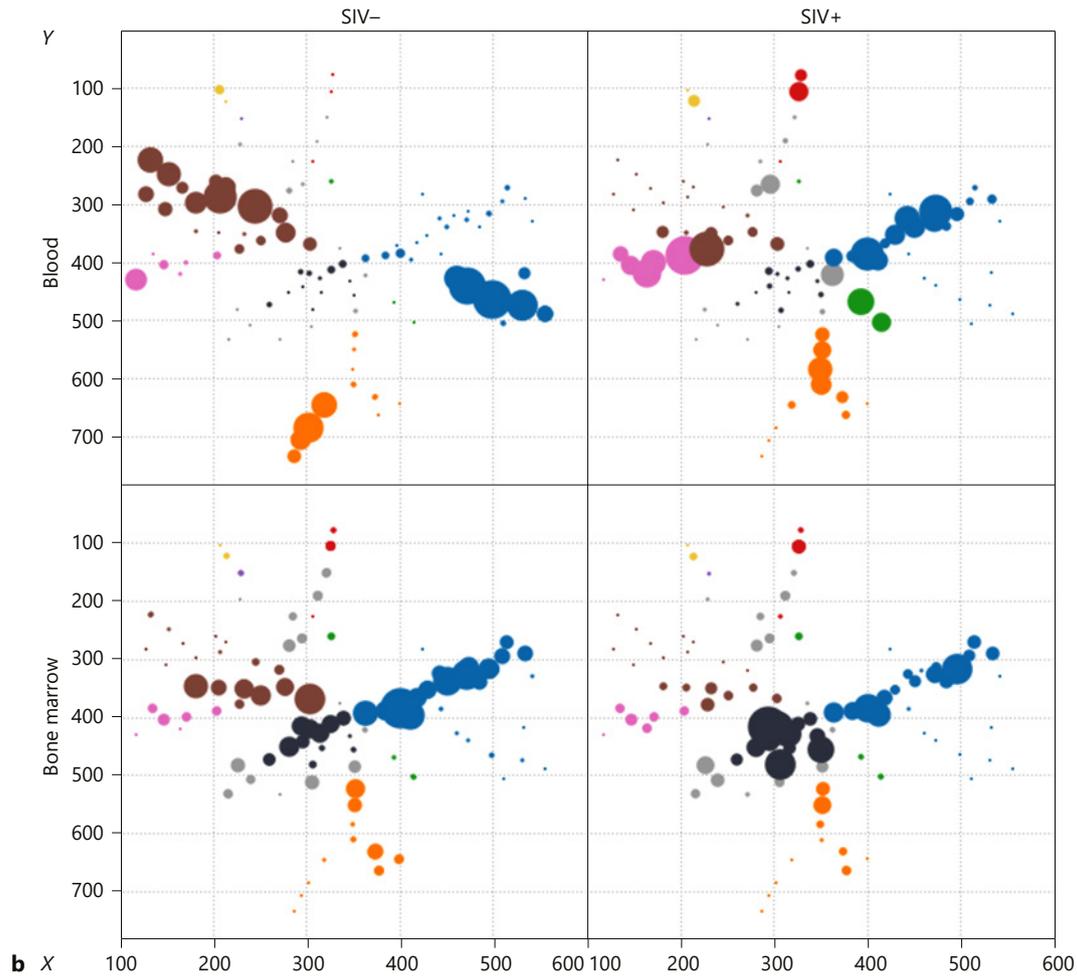
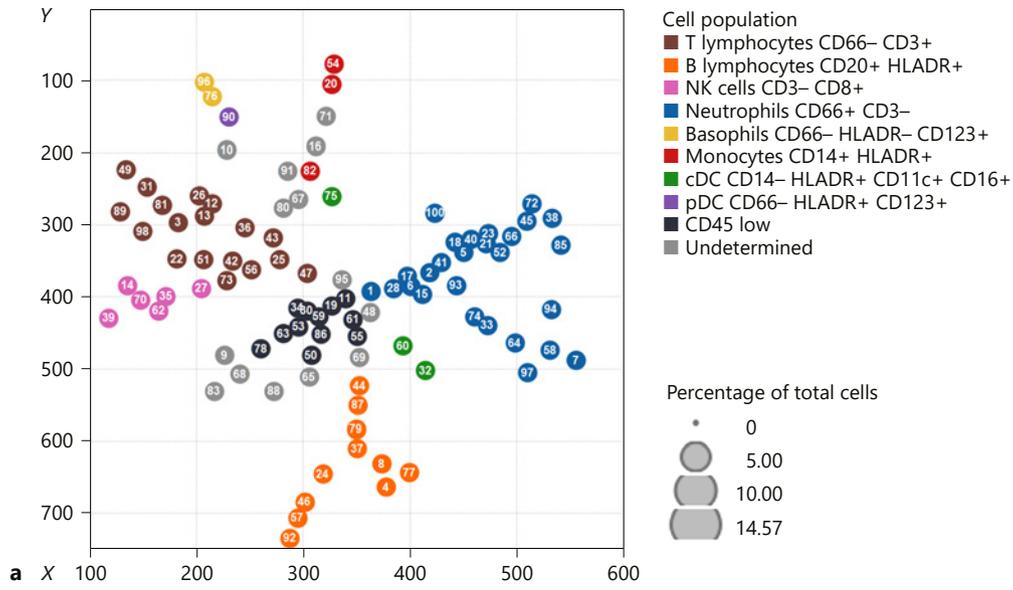
Results

Identification of Leukocyte Changes during SIVmac251 Infection by Mass Cytometry

We first performed a pilot study in animals in the late chronic phase of SIV infection (18 months) to maximize the chance of unraveling major changes among cell subsets by multidimensional mass cytometry analysis. We used an unsupervised computational approach to objectively uncover cellular phenotypic heterogeneity from single-cell high-dimensional data (Fig. 1). SPADE analysis allows the organization of cells into a hierarchy of related phenotypes, forming cell clusters with close phenotypic profiles [26]. We created a 100-cluster common SPADE tree, which recapitulates the phenotypes of blood and bone marrow cell populations, to investigate the impact of SIVmac251 infection in the macaques. Clusters were grouped based on major cluster determinant markers (Fig. 2 and online suppl. Table 4), such as neutrophils, basophils, T lymphocytes, B lymphocytes, monocytes,

cDC, classical dendritic cells; pDC, plasmacytoid dendritic cells. **b** The tree was built using all samples and split according to the compartment and SIV status. Each dot represents a cluster with the size proportional to the cell abundance among all cells. SIV infection led to changes in the abundance of neutrophil clusters in both blood and bone marrow.

(For figure see next page.)



classical dendritic cells, and plasmacytoid dendritic cells. The study focused on myeloid cells. Thus, few markers for lymphoid cells were included, resulting in a limited number of T-, B-, and NK-cell clusters determined by the SPADE algorithm.

Despite the lack of lymphocyte markers, we observed already described CD4 T-cell, CD8 T-cell, and NK-cell phenotypes that characterize chronic SIV and HIV infection, thus validating the mass cytometry approach and analysis pipeline we used. Late chronic SIV infection affected peripheral T cells, decreasing the abundance of five clusters of CD4 T cells (#3, 12, 13, 26, and 36) in SIV-infected individuals (Fig. 2), as expected. Clusters 3, 12, and 13 were characterized by CD25^{high} CCR7^{high} CD29^{mid/high} cells, which disappeared from the blood (online suppl. Fig. 1). CD8 T-cell clusters (#73, 42, and 22) that increased during late chronic SIV infection were CX3CR1^{high}, a marker previously correlated with cytotoxic function and effector differentiation [28]. NK-cell clusters enriched during late chronic SIV infection (#14, 35, and 70) expressed granzyme and perforin, displaying a cytotoxic profile. Peripheral B-lymphocytes had a specific phenotypic pattern during SIV infection (Fig. 2). The bone marrow of infected monkeys showed a striking decrease in T cells, associated with an increase in CD45 cell subsets.

Mass Cytometry Shows Neutrophils to Be Diverse and Immature during Late Chronic SIV Infection

We focused our analysis on PMNs, as mass cytometry associated with SPADE analysis revealed strong heterogeneity of PMN populations in blood and bone marrow. Indeed, 28 of 100 clusters were classified as PMNs, based on the expression of CD66 and the lack of other lineage markers.

In the uninfected control group, PMN clusters were separated into two distinct branches, depending on blood or bone marrow origin (Fig. 2). Bone marrow and blood PMNs had clearly distinct phenotypes, as expected. Peripheral PMNs appeared to be highly affected by infection, showing a clear enrichment of bone-marrow pheno-

type. Neutrophil granulopoiesis can be divided into three different maturation stages based on proliferation capacities, phenotype, and cell morphology [29]. Pre-neutrophils are PMN precursors with proliferative capacities, which further differentiate into immature and mature neutrophils. We were able to associate cluster phenotypes by neutrophil maturation stage in bone marrow from the uninfected control group based on CD66, CD45, CXCR4, CD32, and CD11b expression profiles (Fig. 3a) [30, 31]. Using FACS, in combination with cytochrome, we were able to confirm cell morphology associated with maturation phenotypes identified by mass cytometry in blood and bone marrow (Fig. 3b). Pre-neutrophil phenotype was associated with myeloblasts, promyelocytes, and myelocytes in May-Grünwald-Giemsa-stained cytology. Immature phenotype was mainly associated with metamyelocyte/band cell cytology, and the mature fraction contained segmented neutrophils. Frequency of pre-neutrophils, immature and mature neutrophils obtained in FACS analysis were consistent with mass cytometry data. Bone marrow had highly abundant pre-neutrophils and immature neutrophils (Fig. 3b, c) [29, 32], as expected. Mass cytometry analysis was also able to detect a small number of pre-neutrophils and immature neutrophils in the blood of uninfected macaques, with the dominant population being mature neutrophils, as expected. Late chronic SIV infection led to a redistribution of neutrophils in the blood and bone marrow. Mature neutrophils were no longer present in the circulation and were replaced by immature neutrophils (Fig. 3c). This global change in neutrophil maturation was characterized by low expression of CD11b, CD32, CD45, and CD66 (Fig. 3a). In addition, bone marrow pre-neutrophils were more abundant in chronically SIV-infected animals.

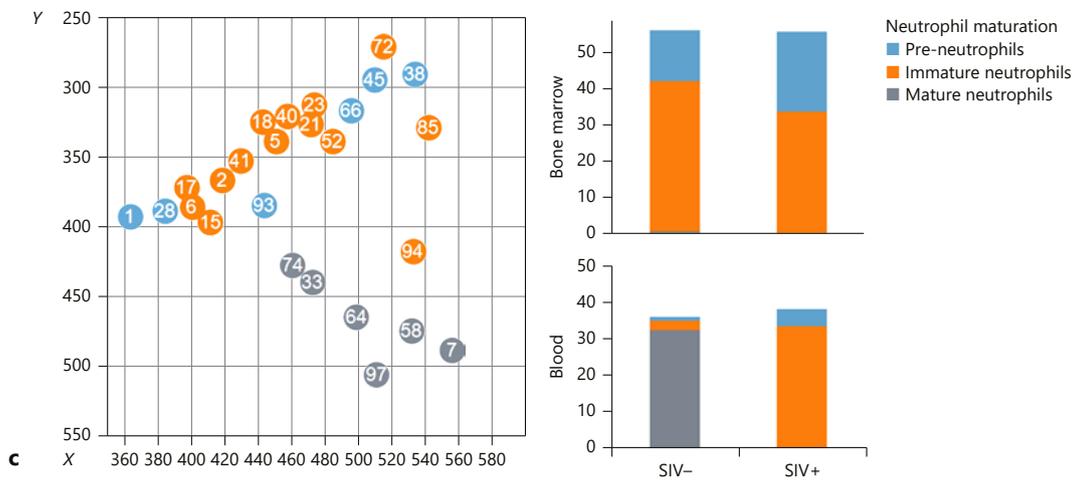
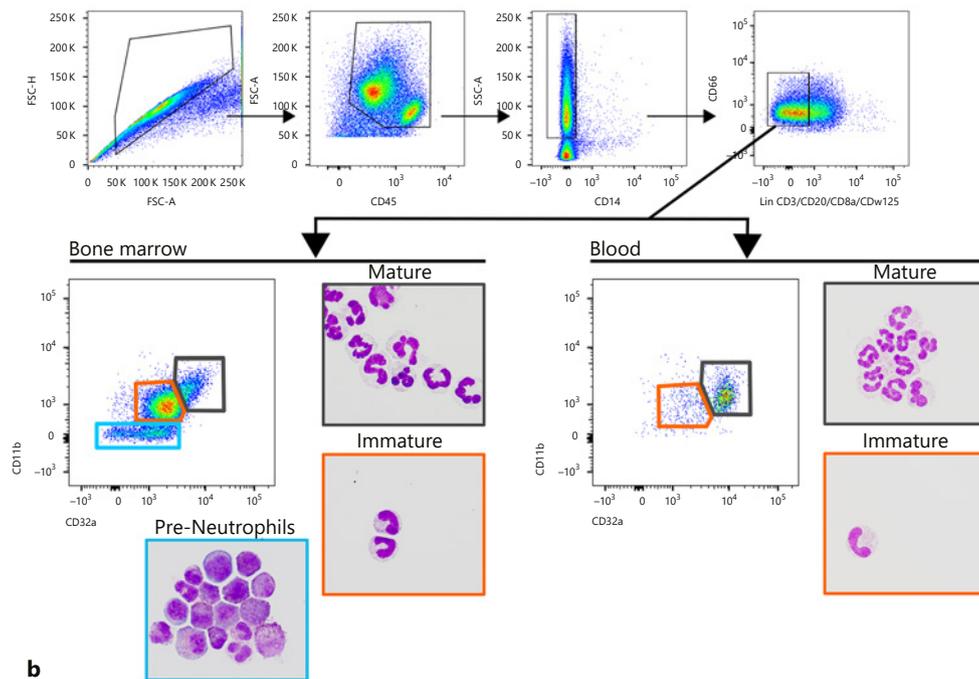
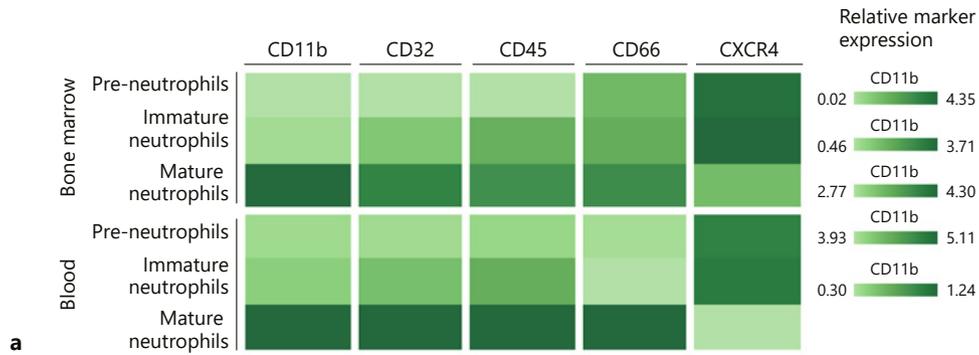
Neutrophil Priming Occurs during Early Chronic SIV Infection, Shown by the Impairment of Phagocytosis

We then characterized the redistribution of neutrophils at earlier stages of infection and the impact of cART by analyzing peripheral blood from 15 SIV-infected and 9 un-

Fig. 3. Determination of neutrophil maturation stage during SIV infection. **a** Neutrophil maturation stage and phenotype according to compartment. The relative expression of CD11b, CD32, CD45, CD66, and CXCR4 allow us to split neutrophils into three populations according to maturation: pre-neutrophils, immature neutrophils, and mature neutrophils. **b** FACS gating strategy and representative May-Grünwald-Giemsa-stained cytochrome of sorted CD11b⁻ CD32a⁻ pre-neutrophils, CD11b⁺ CD32a^{mid} immature and CD11b⁺ CD32a^{high} mature neutrophils. **c** SPADE tree from

Figure 1, focusing on neutrophil clusters with the associated cluster number and colored by maturation stage. Abundance of each neutrophil maturation stage among leukocytes from the uninfected control group and SIV-infected animals. SIV infection induced a decrease in the abundance of mature neutrophils in blood and an enrichment of immature neutrophils in blood. Clusters with less than 150 cells were excluded, and cluster annotation was performed according to online supplementary Table 4.

(For figure see next page.)



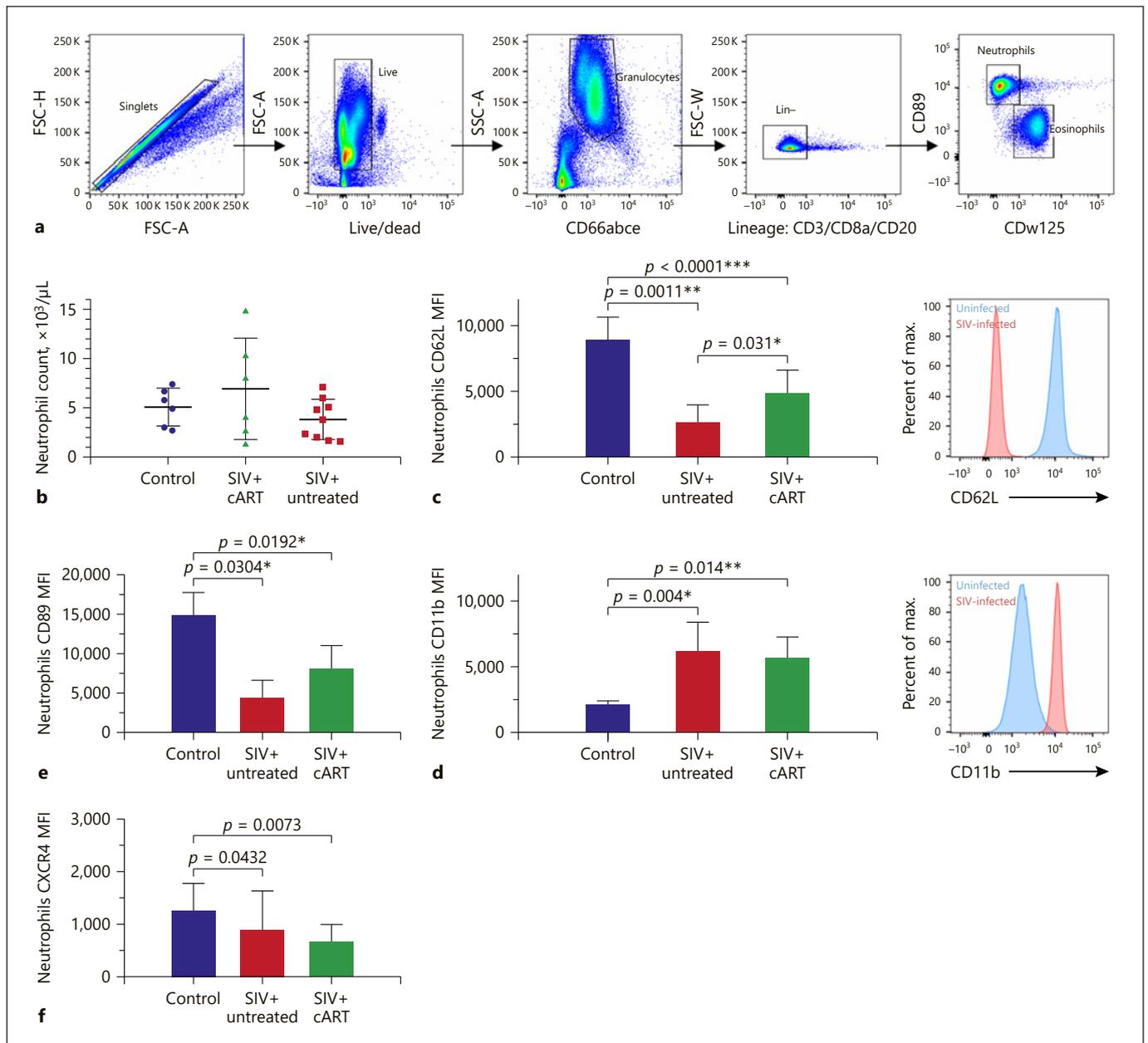


Fig. 4. SIV infection induces neutrophil priming during the early chronic phase, which is partially restored by treatment. **a** Gating strategy used in cynomolgus macaques to isolate neutrophils from eosinophils by flow cytometry. **b** Neutrophil blood count in three groups. **c, d** CD11b and CD62L marker expression in blood neu-

trophils. SIV infection-induced neutrophil priming during the early chronic phase was partially restored by cART. **e, f** CXCR4 and CD89 MFI of blood neutrophils from uninfected control, untreated SIV-infected, and treated SIV-infected macaques. Kruskal-Wallis test and Dunn's multiple comparison test.

infected macaques by conventional flow cytometry. They included 12 macaques 120 days after infection, with 6 animals under cART from day 28 of infection; the remaining 3 macaques were at 288 days of infection, without cART. We identified neutrophils as CD66abce positive, lineage negative (CD3, CD20, and CD8a), CD89 positive, and

CDw125 negative, due to the lack of a CD66b antibody that cross-reacts with cynomolgus macaque cells (Fig. 4a).

There was no difference in the neutrophil count from whole blood between control, SIV+ cART and SIV+ untreated groups (Fig. 4b). Neutrophil CD66 and CD32 expression was similar between groups, showing no differ-

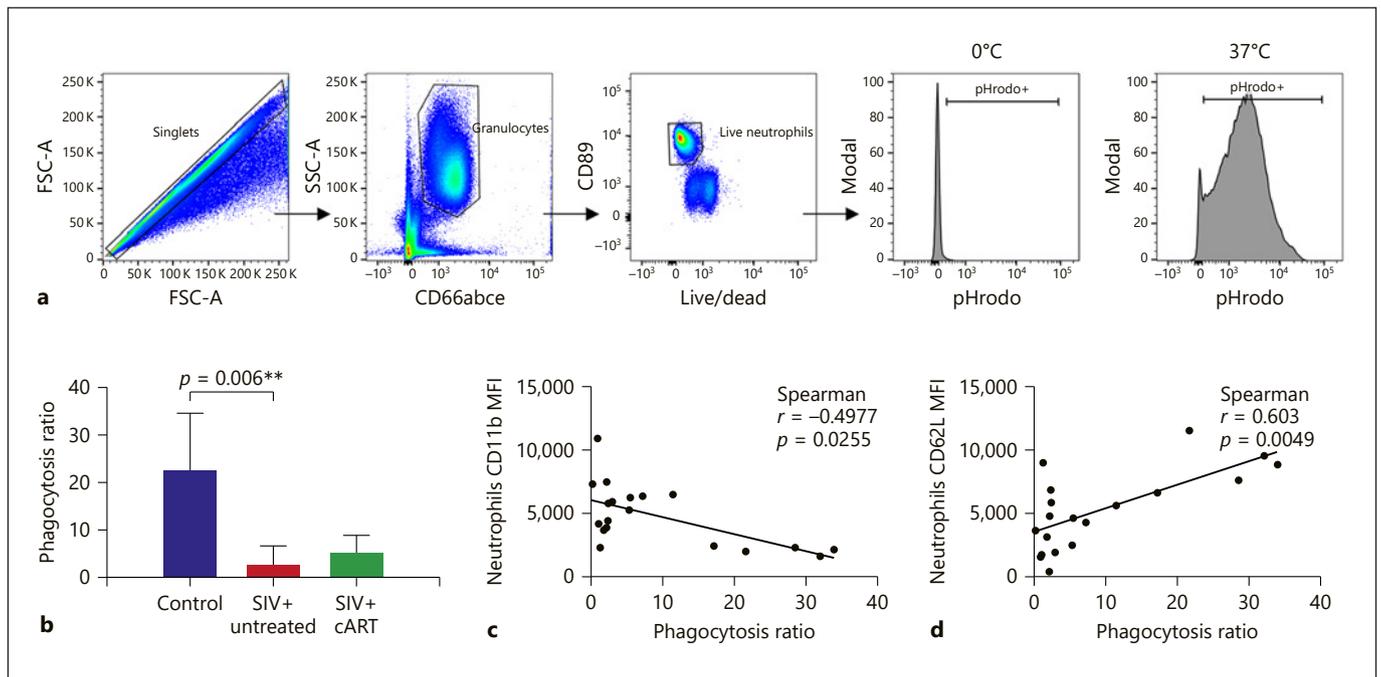


Fig. 5. Neutrophil phagocytosis during early chronic infection. **a** Gating strategy used to analyze neutrophil phagocytosis in cynomolgus macaques and *E. coli* bioparticle fluorescence measurement. **b** Phagocytosis ratio of blood neutrophils in the three groups. Neutrophil phagocytosis decreased during early chronic

SIV infection, and treatment initiation during primary infection could not restore phagocytosis. **c** Correlation between the MFI of CD11b and CD62L of neutrophils with phagocytosis. Spearman test and Kruskal-Wallis associated with Dunn's multiple comparison test.

ence in terms of maturation during the early stages of infection (online suppl. Fig. 2). We analyzed CD62L shedding and the increase in CD11b expression, which have been linked to priming [33], between PMN of treatment groups in order to identify changes in their activation state associated with SIV infection. Neutrophil CD11b expression was significantly higher for both SIV-infected groups (treated, $p = 0.014$, and untreated, $p = 0.004$, Fig. 4d) than that for uninfected controls. In parallel, CD62L expression was lower for all infected animals, suggesting an increased PMN priming during the early stages of SIV infection (treated: $p < 0.0001$, untreated: $p = 0.0011$, Fig. 4c) [34]. Differential expression of CD62L and CD11b was partially reduced by cART, as shown by higher CD62L expression, but CD11b expression was unaffected. Neutrophils from early treated and untreated SIV-infected macaques showed a significantly lower expression of CXCR4 than those of uninfected macaques ($p = 0.0073$, Fig. 4f). Moreover, SIV infection induced an increase in CD89 (Fc α -RI) expression, which was not corrected by cART (Fig. 4e). Thus, neutrophils displayed altered expression of surface molecules, with modulation of the chemokine receptor CXCR4 and CD89 Fc receptor levels.

We characterized phagocytosis of whole blood neutrophils to determine whether priming modulates neutrophil function. We used bacterial particles coated with a pH-dependent fluorescent dye, allowing us to measure phagocytosis by following acidification of the phagosome (Fig. 5a). The phagocytic capacity of neutrophils of untreated SIV-infected animals was significantly less than that of the uninfected control group (Fig. 5b). This decrease in phagocytosis correlated inversely with CD11b expression (Fig. 5c) and positively with CD62L expression (Fig. 5d) but not with CXCR4 or CD89 expression, a finding consistent with the idea that exposure of neutrophils to SIV causes functional impairment. Early cART initiation failed to restore PMN phagocytosis in the treated group.

Discussion

We describe, for the first time, the diversity of the leukocyte population in the blood and bone marrow of SIV-infected macaques using multiparametric mass cytometry. Such analysis highlights numerous modifications of the repartition of leukocyte subsets. Neutrophils were par-

ticularly heterogeneous, with distinct complex phenotypes in blood and bone marrow. We used myeloid markers and published phenotypes [35–38] to differentiate pre-neutrophils, with proliferative capacities, from immature and mature neutrophils in a cynomolgus macaque model of HIV infection. During late chronic SIV infection, immature bone marrow neutrophils were found in the blood, showing mobilization of the medullary neutrophil pool, as described in emergency granulopoiesis and sepsis [37]. Immature neutrophils were the most abundant circulating granulocyte populations found in SIV-infected animals. Moreover, mature neutrophils almost completely disappeared from the blood and bone marrow, probably undergoing apoptosis or tissue mobilization [39]. It will be necessary for future studies to determine whether functions differ between mature and immature PMN subpopulations, and if so, whether they are functionally different populations during SIV infection.

Mass cytometry showed elevated levels of the chemokine receptor CXCR4 in bone marrow neutrophils and subsets of peripheral neutrophils from chronically SIV-infected macaques. At steady state, CXCR4 maintains neutrophils in the bone marrow by interacting with CXCL12 expressed on endothelial cells [30]. CXCL12 is downregulated when inflammation occurs or upon G-CSF signaling in endothelial cells, resulting in neutrophil mobilization. CXCR4 expression decreases in young circulating neutrophils [40] and aged neutrophils re-express CXCR4, allowing them to home to specific sites and be cleared by macrophages in the spleen and bone marrow [41]. It is difficult to determine whether neutrophils are immature or aged based on CXCR4 expression alone. However, the association of strong CXCR4 expression with CD66, CD32, and CD45 suggests that the circulating neutrophils were immature, just having been mobilized from the bone marrow. Mature neutrophils were no longer present in the blood of chronically infected macaques, indicating apoptosis of aged neutrophils or mobilization to tissues where viral replication sustains inflammation.

Reducing the number of markers in flow cytometry abolished the ability to differentiate immature from mature neutrophils in blood as the neutrophil population was homogeneous in terms of CD66 and CD32 expression. Other markers are necessary to better delineate neutrophil maturation, such as CD49d, CD101, and CD10, recently described to distinguish between pre-neutrophils, immature neutrophils, and mature neutrophils in human blood and bone marrow [32].

The combination of mass cytometry and flow cytometry results suggests that CXCR4 expression on neutro-

phils evolves, depending on the stage of SIV infection and the neutrophil subset: CXCR4 downregulation during early phases, probably due to the presence of young primed neutrophils, followed by an increase in CXCR4 expression during the late stages of infection to normal levels or higher, due to the massive egress of immature neutrophils from the bone marrow and the presence of rare aged mature neutrophils.

Neutrophils observed during the early chronic phase of SIV infection had changes in surface expression of CD62L and CD11b, which have been previously associated with priming [33]. There is a continuum of PMN activation from priming to full activation depending on the stimulus intensity [42]. Priming is a complex mechanism in which the environment induces changes in neutrophil responsiveness to stimuli. Priming relies on mobilization of secretory vesicles and gelatinase granules, whereas fully activated neutrophils are characterized by NADPH oxidase activation along with azurophilic granule release [43]. In this study, the analysis of CD62L and CD11b alone did not allow conclusions to be drawn about the extent of PMN activation following exposure to SIV.

In the early chronic phase, PMN phagocytic capacity was also impaired. The oxidative burst in neutrophils and phagocytosis correlated with viral load, CD4 T-cell count, and treatment success, as reported in a recent study [12]. In the same study, the phagocytic activity in HIV-infected patients was significantly less than that of healthy individuals, but without a beneficial effect of cART. However, patients from this cohort had advanced chronic HIV infection, and treatment was initiated long after transmission. We demonstrated that neutrophil phagocytosis was impaired from early chronic SIV infection using *E. coli* bioparticles. Treatment initiated 1 month after inoculation did not restore neutrophil phagocytosis in SIV-infected animals. Early cART partially reduced the impact on CD62L and CD11b expression, but neutrophil phagocytosis was still impaired, probably because of chronic immune activation. Since the phagocytosis assay is based on acidification, it is also possible that neutrophils lost their capacities to acidify their vacuoles during SIV infection, making them less potent pathogen killers. Neutrophil priming must be explored in further detail, to understand the inflammatory environment associated with SIV infection which shapes their functionality and in turn fuels chronic immune activation.

The use of mass cytometry allowed us to identify numerous neutrophil subsets and show the presence of immature populations during the chronic phases of SIV infection. The monitoring of immature circulating neutrophils is performed for the diagnosis and prognosis of patients with sys-

temic inflammatory response syndrome [44]. Patients with high levels of myelocytes and metamyelocytes (called myelocytosis) had a poor outcome, regardless of whether the origin was infectious or noninfectious. Whether myelocytosis is associated with HIV progression or treatment escape could be determined, as a blood smear could be a simple and valuable bedside prognostic tool. Moreover, we demonstrated that cART initiated during primary infection could not reduce neutrophil priming and function. Whether neutrophils play a role in the establishment and maintenance of chronic immune activation should be explored, as neutrophils represent 40–60% of circulating leukocytes.

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Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

R.L.G., O.L., and J.L. designed and supervised the study. J.L. performed experiments and discussed the data. R.L.G., O.L., D.D., and A.C. discussed the data and read the paper. J.L. wrote the paper.

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II. Immature CD10- neutrophils exert immunostimulatory function during primary and chronic SIV infection

A. Summary

In chronic HIV infection, neutrophils have been classically described as homogenous cell population impaired by the infection. In the first article, using multidimensional analysis of all leukocytes, we observed globally neutrophils heterogeneity with circulation of immature neutrophils in late chronic SIV infection (>1 year of infection). But the moment in which neutrophils diversification occurs during HIV/SIV infection is unknown. In this second article in preparation, the objective was to characterize neutrophil heterogeneity in primary and chronic SIV infection. Using conventional flow cytometry and flow cytometry cell sorting, we first developed tools to differentiate neutrophils according to their maturation stage in cynomolgus macaque: pre-neutrophils (promyelocyte and myelocyte), immature neutrophils (metamyelocyte and band cell) and mature neutrophils²⁰⁰. In parallel, we also evaluate neutrophil priming using CD62L expression.

Neutrophil heterogeneity is a question of phenotype, but above all a question of function. There are few descriptions of functional heterogeneity in HIV infected patients, where authors described low-density neutrophils with immunosuppressive functions against T cells IFN- γ production^{325,326}. But less is known about the impact of other neutrophils subsets, gathered in normal-density neutrophil population. Recent studies demonstrated the co-existence of neutrophils subpopulation with opposite immunomodulatory function against T cells responses^{333,351}. Indeed, G-CSF-treated donors demonstrated circulation of both mature and immature neutrophils having respectively immunosuppressive and immunostimulatory effects on T cells proliferation and IFN- γ production. This study underlines the need to study function with phenotype of all subpopulations to comprehend the impact of neutrophils in diseases: suppressive or pro-inflammatory. Immunostimulatory function of neutrophils has not been studied in HIV/SIV infection, while it has been identified in autoimmune diseases and cancers³⁵². In this study, we used an autologous co-culture between normal density neutrophils and T cells to evaluate the impact of neutrophils on T cells proliferation and cytokine production during SIV infection.

In primary infection, even if total neutrophil count was not affected, sub-population of neutrophils changed, with an increase of primed CD62L-low and immature CD10- neutrophils. This acute response was associated with immunostimulatory functions evidenced by increase CD4 T cell proliferation, along with CD4 and CD8 T cells IL-2 and IFN- γ production. But interestingly, neutrophils demonstrated an opposite role on CD8 T cells by suppressing proliferation. In chronic SIV infection, the frequency of primed and immature neutrophils was still significantly elevated. Neutrophils had no impact on autologous T cells proliferation, IL-2 and IFN- γ production. But interestingly, neutrophils increased TNF- α production by CD4 T cells. We also evaluate the frequency of LDN in the course of SIV infection and we observed only a slight increase in primary infection but not in chronic phase.

This suggest a potent immunostimulatory function of normal-density neutrophils against T cells in SIV infection, which could in turn participate to immune activation. Initiation of early treatment within first month of SIV infection reduced frequencies of primed and immature neutrophils to basal levels, whereas delayed treatment (6 months post-infection) had limited impact on these subpopulations' frequencies. Thus, our hypothesis is that chronic inflammation shaped neutrophil functions, and maintained pathologic pro-inflammatory immune modulation.

To better characterize immunomodulatory functions of mature and immature neutrophils against T cells we are currently developing a cell sorting assay, using magnetic beads coated with antibodies to negatively sort neutrophils. This technic will allow multiple functional analysis of subpopulations, without disadvantage of density gradient separation. This last experiment for this study will draw conclusion on whether CD10+ or CD10- neutrophils have similar or different immunomodulation properties.

B. Manuscript

Title: Immature CD10- neutrophils are pro-inflammatory cells during primary and early chronic SIV infection

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Short title: Neutrophil heterogeneity in SIV infection.

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Key words: AIDS, antiretroviral treatment, neutrophils, immunomodulation, non-human primate

1) Abstract

Even under combinational antiretroviral treatments (cART), HIV-1 persistence is associated with chronic inflammation in infected patients, leading to an increased risk of comorbidities, such as cardiovascular diseases, neurocognitive disease, and cancer^{11,353}. Under cART, occurrence of non-AIDS-related comorbidities is associated with increased levels of innate immune markers, notably myeloid cells activation, whereas T cells activation was not correlated to outcomes¹⁵². Myeloid cells, mainly monocytes and macrophages, have been involved in immune activation observed in HIV-1 infection. But neutrophil (PMN), the most abundant circulating myeloid cell, have been less studied in HIV infection²⁰ whereas they were associated with chronic inflammation in autoimmune diseases, such as rheumatoid arthritis³⁵⁴. Most available studies shown an impact of HIV infection on PMNs function with decrease of microbicidal functions^{318,355}. PMNs are also primed in chronic phase with CD62L shedding, CD11b translocation and increased ROS production in unstimulated cells²². In this study, authors shown neutrophils activation in HIV-infected individuals having a non-AIDS-related comorbidity. Thus, HIV-1 infection has a deleterious effect on neutrophils but less is known about the role of neutrophils on chronic inflammation. To evaluate if PMNs are associated with HIV chronic inflammation, we characterized neutrophils heterogeneity in blood and bone marrow from SIV-infected cynomolgus macaques. In primary infection, even if PMN count was not significantly changed, frequency of circulating mature CD10+ PMN was decreased, and they were replaced by immature CD10- PMN. At the same time, PMN demonstrated a CD62L-low CD64+ HLADR+ primed phenotype. After 6 months of infection, CD62L-low primed and CD10- immature PMN were significantly higher in bone marrow and blood. Depending on the time of infection, PMN demonstrated variable immunomodulatory function against T cells proliferation and cytokine production. Notably, neutrophils were capable to increase CD4 and CD8 T cells production IL-2, IFN- γ and CD4 T cells proliferation, suggesting an immunostimulatory function. But in chronic phase, PMN increased TNF- α production by CD4 T cells. In this study, we provide unprecedented insight into PMN heterogeneity in the course of SIV infection. Since PMN represent 40-70% of circulating leukocytes and primed PMN are more potent to release pro-inflammatory cytokines and to transmigrate, they should be considered as a new player in HIV chronic inflammation.

2) Introduction

In absence of a cure for HIV infection, lifelong antiretroviral treatment is necessary to control virus replication because of the inability of combinational antiretroviral treatment (cART) and host defenses to eradicate the virus. cART has increased life expectancy and the quality of life of HIV-infected patients, but is still far from a cure^{13,356}. Despite cART, non-AIDS related co-morbidities (metabolic, neurodegenerative and cardiovascular diseases) normally associated with aging have been shown to be higher in HIV-infected population than aged-matched population¹⁰. This increased frequency of co-morbidities was soon associated with the persistence of chronic inflammation¹¹. Even under long-term cART, HIV-infected patients have a higher level of soluble inflammatory markers (ex. IL-6) and gut epithelial dysfunction markers (ex. I-FABP)¹².

Nevertheless, exact mechanisms and cells involve in the persistence of increased levels of soluble mediators are still not clear. Residual replication and microbial translocation are potent to activate monocytes, dendritic cells and macrophages, leading to chronic inflammation^{357–359}. But one of the most potent pro-inflammatory myeloid cells, polymorphonuclear neutrophil (PMN), has been overlooked in HIV-infection²⁰. Consider as short-lived phagocytic cells, new studies demonstrate immunomodulatory functions of PMNs and a life expectancy reaching 5,4 days in certain conditions^{184,191,199,333}. In addition, PMN have been involved in pathogenesis of metabolic, cardiovascular and neurological diseases which are also non–AIDS related comorbidities²¹. Recently, PMN infiltration have been shown in patients under cART and was associated with colonic epithelial cells apoptosis and microbial translocation in SIV-infected macaques^{83–85}. PMN survival in gut have been shown to be increase in chronic HIV infection, which is associated with pro-inflammatory function in other diseases⁸⁷. Beside, both normal density and low-density neutrophils expressing PD-L1 were potent to suppress specific T cells IFN- γ production in PD1/PD-L1 and ROS dependent mechanisms. Depending on the disease studied, PMN may has a pro-inflammatory or suppressive function, which immunomodulatory function is impossible to predict based on currently available phenotyping approaches³⁵².

Here we analyzed PMN heterogeneity in a non-human primate model infected by SIVmac251. We demonstrated that circulating PMNs in SIV primary infection were mostly constituted of primed (CD62L-low) immature (CD10-) PMNs. This immature phenotype was associated with

a positive pro-inflammatory function of PMNs against CD4+ and CD8+ T cells IL-2 and IFN-g production but suppressed CD8+ T cells proliferation. In chronic SIV infection, percentage of PMN of immature phenotype was lower compare to acute phase but still elevated with the regard to non-infected animals, and PMN demonstrated positive modulation of CD4+ T cells TNF-a production. After 3 months under cART, immunostimulatory role of PMN was return to basal levels. Thus, our results suggest the implication of immature PMN on T cells activation in SIV infection.

3) Material and method

Nonhuman primates and sampling

Twelve cynomolgus macaques (*Macaca fascicularis*), imported from Mauritius, were housed at the IDMIT infrastructure animal facility at the CEA, Fontenay-aux-Roses, France. Animals expressing the H6 MHC haplotype were excluded because of their increased natural capacity to control SIVmac251 infection³⁶⁰. All animals were infected by intravenous inoculation with 1,000 animal infectious dose 50% (AID50) of pathogenic SIVmac251 isolate and followed for more than 12 months³⁶¹. Six SIV-infected animals were subcutaneously treated daily, starting from 28 days after infection, called group 1, with a combination of two nucleoside reverse-transcriptase inhibitors, emtricitabine (40 mg/kg) and tenofovir disoproxil fumarate (5.1 mg/kg), and one integrase inhibitor, dolutegravir (2.5 mg/kg)³⁶². Six remaining animals, called group 2, were treated with same route and posology 6 months after infection. Blood was drawn and bone marrow aspirates collected under anesthesia, consisting of an intramuscular injection of 10 mg/kg zolazepam/tiletamine (Virbac, Carros, France). Blood was collected into lithium-heparin tubes (Vacutainer BD, USA) and bone marrow aspirates from the iliac crest or humeral head using a 10-ml 18G-syringe containing citrate -dextrose (10 mg/ml). The macaque study was approved by the « Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche » (France) and the ethics committee « Comité d'éthique en expérimentation animale n°44 » under reference 2015102713323361.02 (APAFIS#2453). Animals were handled in accordance with national regulations (CEA accreditation number D92-032-02) and European Directive (2010/63, recommendation N°9) and in compliance with the Standards for Human Care of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-01.

Reagent and cell preparation for flow cytometry

Briefly, heparinized whole blood and citrated bone marrow were processed within 1 h following blood draw to preserve neutrophils. For phenotypic analysis viability staining was first performed for 15 min at room temperature, according to the manufacturer's protocol (Live Dead, Thermofisher). Then, antibody staining was performed with the following antibodies for 15 min at room temperature. Samples were then lysed and fixed using BD FACS Lysing (BD Bioscience) for 15 min. After an additional wash with PBS, acquisition was performed on a BD FORTESSA flow cytometer (BD Bioscience) and analyzed using FlowJo software. Gating strategies are detailed in supplementary figure 1.

Cell isolation

Mononuclear cells and granulocytes were isolated by density gradient centrifugation (Ficoll-Paque; GE Healthcare Life Sciences) of blood. Using this technic, we obtained 95% of neutrophil purity in globular fraction. Isolation was further shown to not induce PMN activation using CD62L and CD11b expression.

Neutrophils cells sorting and cytology

For cell sorting by flow cytometry, whole blood or bone marrow from two uninfected animals were first NH₄Cl lysed, then FcR were blocked using cynomolgus macaque serum. Cells were counted and incubated 30min with the following antibodies. Cell sorting was performed on FACSaria I flow cytometer (Becton Dickinson). Sorted population were cytopspined and then stained by May-Grünwald Giemsa. Picture were taken by Nikon Eclipse 80i with Dxm 1200C digital camera at 60x magnification. Cell were identified by morphological criteria by a cytologist. Promyelocytes and myelocytes were considered as pre-neutrophils, metamyelocytes and band cells as immature neutrophils, and segmented neutrophils as mature.

Neutrophils / PBMC co-culture for cytokine and proliferation assay

TCR-stimulated (Cytostim NHP, Miltenyi) T-cells were cultured in presence or absence of autologous PMN at 1:1 or 5:1 PMN to PBMC ratio. Concerning intracellular cytokines, after 1 hour of stimulation brefeldin A (Sigma) was added to block protein transport to Golgi. For proliferation assay, PBMC were stained with 1 μ M CFSE (Cell Trace, Invitrogen) for 5 min and reaction was stopped using cold FCS. Co-culture were maintained 16 hours for cytokine assay and 96 hours for proliferation assay at 37°C 5% CO₂, before flow cytometry analyses.

For both assays, 20 min of viability staining at 4°C (Live Dead, Thermofisher) was done, and for cytokine assay, cells were permeabilized and fixed (Cytotfix/cytoperm, BD Bioscience). Then cell staining was performed for 30 min at 4°C with the following antibodies.

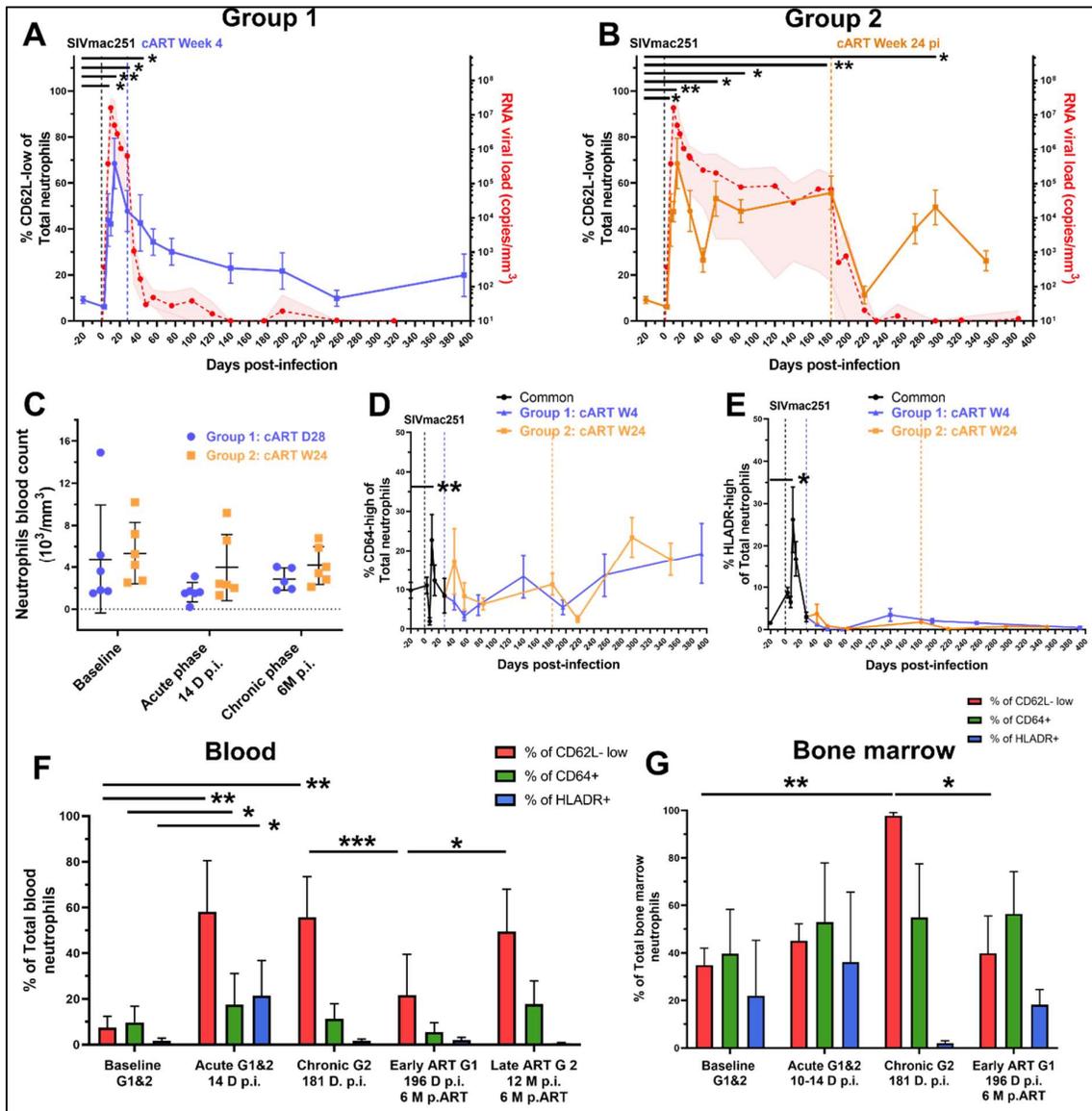
Statistical analysis

Statistical analyses were carried out with GraphPad Prism 8 software (GraphPad Software Inc.). We used Friedman test with Dunn's multiple comparison for statistical analyses of multiple time points from the same animal. A Mann-Whitney non-parametric test was used to compare data from Group 1 and Group 2. We performed a Wilcoxon matched-pair signed rank test to analyze the impact of PMNs on T cells proliferation and cytokine production. In graphs, the thresholds for statistical significance are indicated as follows: * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

4) Results

Neutrophil priming in acute and chronic SIV infection

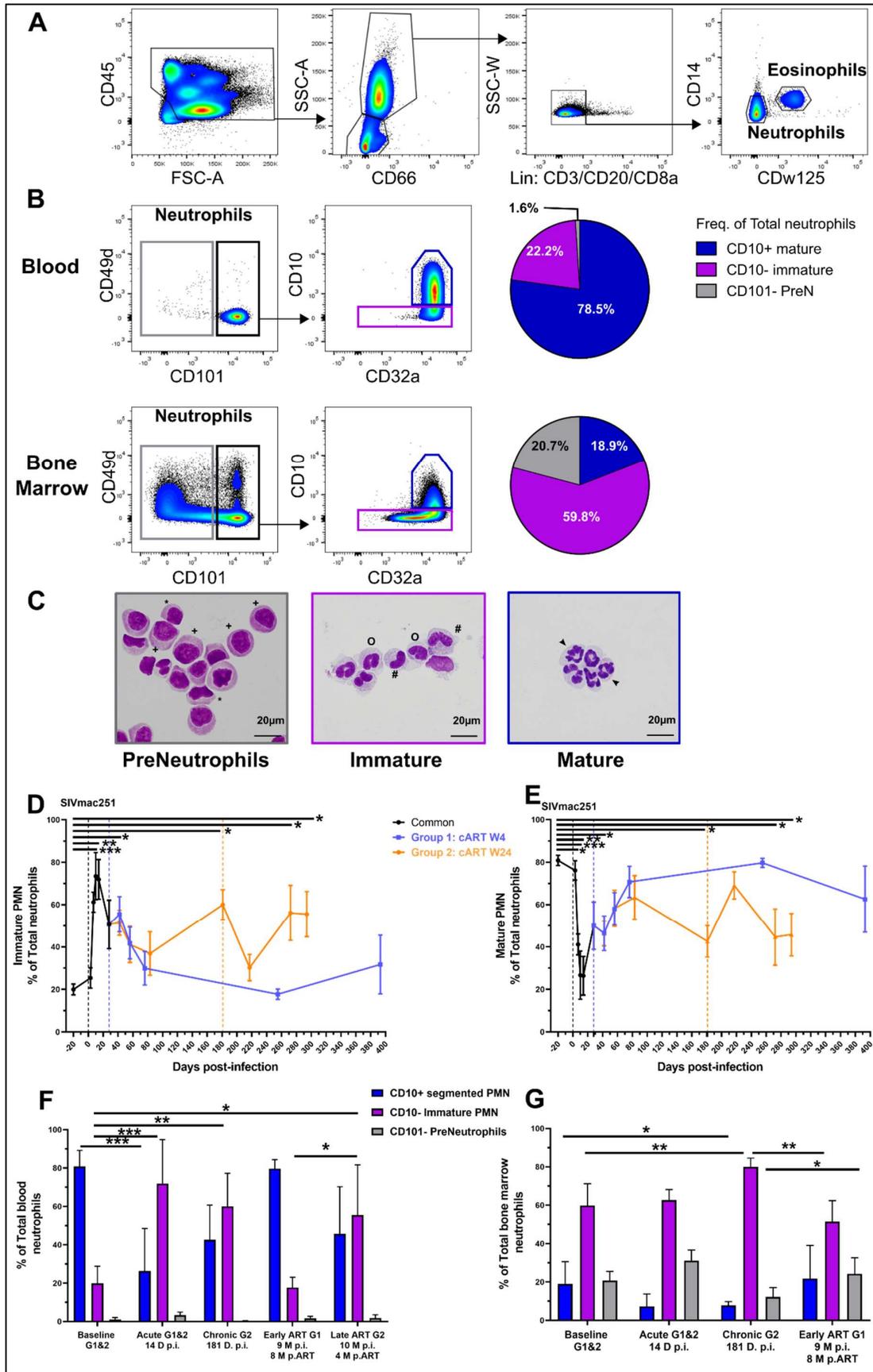
Neutrophil priming have been identified in chronic SIV and HIV infections^{22,318,363}, but the dynamic of neutrophil in the course of infection is poorly defined. Therefore, we characterized the dynamic of neutrophils priming in SIV-infected non-human primates in acute, chronic phase and under cART. We analyzed both fresh blood and bone marrow with multiparameter flow cytometry to explore these two important neutrophil compartments (Figure 1 A-B). We used several surface markers expression as surrogate of priming, including CD62L shedding along with upregulation of CD11b, CD64 and HLA-DR. To evaluate if early cART initiation could affect PMNs priming, animals were separated in two groups accordingly to time of treatment initiation (Group 1 cART initiated at 28 days p.i.; Group 2 cART initiated at 180 days p.i.). Taking all time points together, there was no significant differences of neutrophils blood count between groups and time point ($p=0.915$) (Figure 1C). Nevertheless, we could identify neutrophils subpopulation on the basis of CD62L, CD64 and HLADR expression. In primary infection, both groups demonstrated a significant increase of CD62L-low neutrophil frequency in association with increasing viral replication, and reaching its maximum at the peak of viral replication (Figure 1 A, B). Previous study have demonstrated an association between CD62L-shedding and neutrophils priming in HIV-1 infection³¹⁸, we therefore considered CD62L-low population as primed neutrophils. In absence of cART, primed neutrophil population is maintained significantly elevated in group 2 during chronic phase (Figure 1 B). In addition, CD64 and HLADR surface expression on neutrophils was significantly elevated only at the peak of viral replication, and showed non-significant fluctuation during the follow-up, independently from cART time of initiation (Figure 1 D, E). In group 1, percentage of CD62L-low neutrophils (primed) was reduced almost to basal levels after 6 months of cART. (Figure 1 F). When initiated in chronic phase (6 months p.i.), cART reduced primed PMNs frequency after one month of treatment (Figure 1B). However, in the same group neutrophil priming increased thereafter, being significantly elevated 6 months after cART initiation. In bone marrow, frequency of CD62L-low neutrophils was significantly increased only during chronic phase (Figure 1G). In group 1, cART completely restore normal values after six months of treatment, but we are currently evaluating bone marrow from group 2. Changes in CD62L expression among bone marrow may indicates priming or increase of CD62L-negative neutrophil precursors, affecting neutrophils maturation.



SIV infection impact blood neutrophils maturation

We previously reported circulation of immature neutrophils in late stage of chronic phase of SIVmac251 infection using mass cytometry³⁶³. However, gold standard to differentiate neutrophil populations according to maturation stage remains the cytology characterization. As recently described in humans, CD101 and CD10 expression allows to segregate pre-neutrophils (CD101⁻ CD10⁻) from immature neutrophils (CD101⁺ CD10⁻) and segmented neutrophils (CD101⁺ CD10⁺)²⁰⁰. Using cell sorting by flow cytometry associated with evaluation of cell morphology, we demonstrated here that these markers combined with CD32a, delineate same neutrophils subpopulations in cynomolgus macaque's blood and bone marrow (Figure 2 A, B). In bone marrow, CD101⁻ population was a mixed population gathering promyelocytes and myelocytes, and CD101⁺ CD10⁻ population was composed of metamyelocytes and band cells (Figure 2 C). Then, using these markers we evaluated the changes in neutrophil maturation stages associated with SIV infection. Indeed, in primary infection circulating mature PMNs are replaced by immature PMNs (Figure 2 D, E). Interestingly, the dynamic of immature neutrophils followed viral replication, reaching its maximum at 10-14 days post-infection. In early chronic phase, frequency of immature PMNs decreased with viral replication but was still significantly increased after 6 months of infection. Nevertheless, early cART initiation proved to restore durably neutrophils maturation balance. In group 2, after cART initiation (6 months p.i.) the frequency of immature PMNs was reduced within first month, but then increased after 3 and 4 months of treatment.

Similar changes were observed in bone marrow only during chronic phase (Figure 2 F). Immature neutrophil frequency increased, along with a decrease of mature and pre-neutrophils. Under cART distribution between neutrophils maturation stages in bone marrow was restored.



(Figure description on the next page)

Figure 2: Circulation of immature neutrophils in primary and chronic SIV infection.

A) Gating strategy used to identify PMNs in blood and bone marrow from cynomolgus macaques. **B)** Identification of PMN's maturation stages, corresponding distribution and cytology in blood and bone marrow. CD101- Pre-neutrophils population is composed of promyelocyte (+) and myelocytes (*). CD101+ fraction is composed of immature CD10- (#: metamyelocytes, o: band cell) and mature CD10+ (arrow: segmented PMN). **D-E)** Frequency of immature and mature PMNs in blood during SIV infection and treatment. Before cART initiation for group 1, both groups are represented together in a black line and are then separated. Graphs values indicates mean with SEM for both groups, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ by paired Friedman test with Dunn's multiple comparison. **F-G)** Frequency in blood and bone marrow of each PMN's maturation stages in acute, chronic untreated and treated SIV infection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by paired Friedman test with Dunn's multiple comparison and Mann Whitney test for unpaired comparison between groups.

Neutrophils stimulated T-cells cytokine production and modulates T-cells proliferation in acute and chronic SIV infection

As reported for several distinct diseases in human, PMN can have pro-inflammatory or immunosuppressive function. In order to evaluate T cells immunomodulation by PMN in SIV infection, we analyzed cytokine production by flow cytometry of TCR-activated T cells, in presence or absence of autologous PMNs. We used density gradient centrifugation to isolate neutrophils from blood. This approach, however, do not allow to retain low-density neutrophils (LDN), which remain in the mononuclear fraction. Nevertheless, we did not observe significant changes in LDN frequency in the PBMC fraction of SIV infected macaques. We therefore surmise that impact of LDN on T cells function may be minimal, although this hypothesis remains to be confirmed (Supplementary Fig. 2). Before infection, PMN had no effect on CD4+ and CD8+ T cells IL-2, IL-17A, TNF α production, but were able to significantly increase IFN- γ production by CD8+ T cells (Figure 3 A-F). In primary infection, neutrophils significantly increased IL-2 and IFN- γ production by both CD4+ and CD8+ T cells (Figure 3 A, B, C, D). Interestingly, PMN also increased IL-17A production by CD4+ T cells in acute phase. This immunomodulatory function is associated with high frequency of primed immature PMN observed in acute phase. In chronic phase, PMN did not modulate IL-2 production by T cells, but they induced a significant increase of TNF- α production by CD4+ T cells, with a trend to increase IFN- γ production by CD8+ T cells (Figure 3 C-D). This suggest a switch in PMN immunomodulatory function from acute to chronic phase, with globally, a pro-inflammatory effect on CD4+ and CD8+ T cells cytokine production.

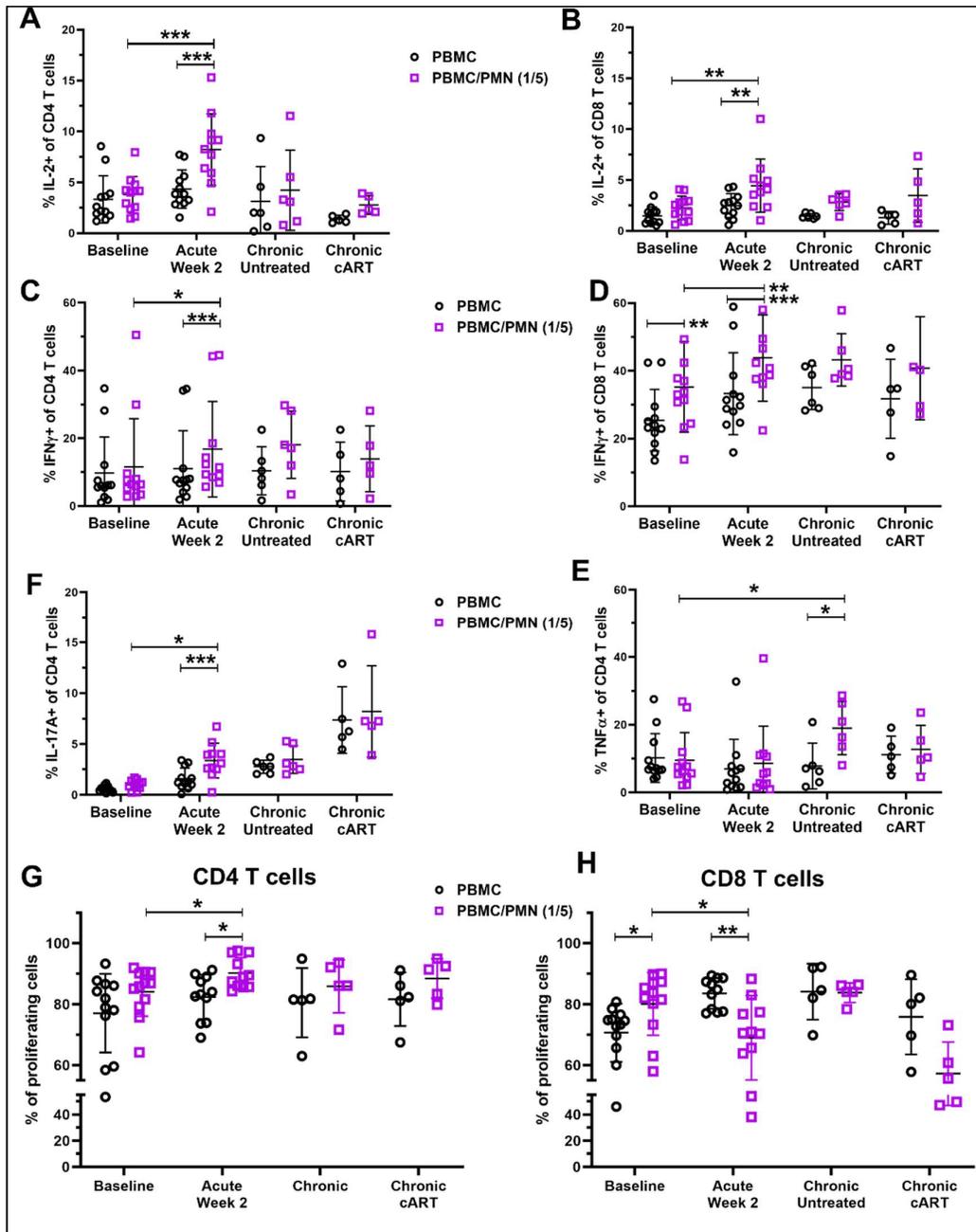


Figure 3: T cells immunomodulation by neutrophils during SIV infection.

A-F) TCR-stimulated T cells were cultured 16h in presence or absence of PMN at a 5:1 PMN to T-cell ratio. T cell cytokines production was measured by intracellular cytokine staining of IL-2, IFN-g, TNF α and IL-17A after co-culture. Graphs represent frequency of CD4+ T cells or CD8+ T cells positive for each cytokine in absence (black) and presence of PMN (purple). G-H) TCR-stimulated T cells were cultured 96h in presence or absence of PMN at a 5:1 PMN to T-cell ratio. T-cell proliferation was measured by CFSE dilution and populations identified by flow cytometry. Graphs represent frequency of CD69+ CD4+ T cells or CD8+ T cells with CFSE dilution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by Wilcoxon or Mann-Whitney tests depending on pairing. Baseline: group 1&2, Acute week 2: group 1, Chronic: group 2 (6M p.i.), Chronic cART: Group 1 (7M p.i., 6M pART).

We also measured proliferation of TCR-activated T-cells in presence or absence of PMN using CFSE dilution after 96 hours of ex-vivo stimulation (Figure G, H). During acute phase, PMN stimulated CD4+ T cells proliferation but had no significant effect during chronic phase and under cART. Before infection, PMNs stimulated CD8+ T cells proliferation, whereas in acute phase they demonstrated a suppressive effect on proliferation. Interestingly, in non-treated animals, during chronic infection (6 months p.i.), this effect on proliferation was lost, whereas basal proliferation of CD8+ T cells without PMNs remain elevated compare to pre-infection level. In cART treated animals (group 1, 9M p.i., 8M p.ART) CD8+ T cells proliferation is suppressed by PMNs, although the low number of studied animals may be insufficient to reach statistically significant values ($p=0,0625$). These results suggest a T cells-dependent immunomodulatory function of PMNs in SIV infection which evolves in the course of infection and with a differential effect on CD4+ and CD8+ T cells.

5) Discussion

Description of neutrophils heterogeneity in peripheral blood, such as different maturation and activation status, have been made in cancers and autoimmune diseases¹⁹⁹. In these chronic inflammatory diseases, new neutrophil functions have been described such as modulation of T-cell responses. Neutrophils are also equipped with mediators potent to induce tissue damages and maintain chronic inflammation. Chronic HIV-1 infection, even under cART, could be considered as a chronic inflammatory disease, in which neutrophils had received little attention. A recent study has demonstrated the link between neutrophils and microbial translocation, a source of HIV-associated chronic inflammation⁸⁷. Thus, characterization of neutrophils heterogeneity and immunomodulatory function in HIV-1 infection would help to better understand their roles in the maintenance of inflammation.

Here we provide the first description of neutrophil's heterogeneity associated with T-cells immunomodulatory functions during SIV infection. Using the association of CD101, CD10 and CD32a expression, we were able to distinguished precursors, immature and mature neutrophils in blood and bone marrow from SIV-infected macaques. In primary infection, circulating PMNs were predominantly composed of immature neutrophils. This immature phenotype was associated with an increased level of priming markers HLADR, CD64 and CD62L shedding. Definition of PMN priming relies on partial activation of NADPH-oxidase associated

with an increase responsiveness to stimulus. We evaluated PMN priming using CD62L-low shedding since it has been associated with NADPH-oxidase activation^{364,365}, but we did not directly evaluate NADPH-oxidase function. In peripheral blood, CD62L shedding has been classically associated with primed hypersegmented neutrophils, notably in acute bacterial infection³⁶⁶. Here immature PMNs had similar dynamics than CD62L-low PMNs, and to confirm priming it would be necessary to evaluate NADPH-oxidase function in the course of SIV infection. HIV infection has also a different physiopathology compared to bacterial infections which may explain major differences in terms of neutrophil heterogeneity. Changes in peripheral blood neutrophil populations observed in primary infection partially resolved in early chronic phase, but frequency of CD62L-low PMN and immature PMN increased in late chronic phase. These results are consistent with bone marrow neutrophils analysis in which primed immature PMNs are increased in chronic SIV infection. Early initiation of cART (4 weeks post-infection) protected against long term PMN changes observed in untreated chronic SIV infection. Indeed, after 6 months under cART frequency of immature and primed PMN was back to basal levels. Neutrophils are also potent to store and produce several cytokines under stimulation²⁴. As an immune cell involved in chronic inflammation, it will be interesting to evaluate their cytokine production capacities in the course of SIV-infection and under cART.

We showed that neutrophils could differentially modulate CD4+ and CD8+ T cells. Primed CD62L-low immature CD10- neutrophils demonstrated an immunostimulatory function against T cells in primary infection. Notably, PMNs increased IL-2 and IFN- γ production by CD4+ and CD8+ T cells, and increased CD4+ T cells IL-17A production. PMNs differentially modulate T cells by increasing CD4+ T cells proliferation, but interestingly decreased CD8+ T cells proliferation. It will be necessary to evaluate the impact of PMNs on SIV-specific CD8+ T cells function, considering that PMNs could impair antiviral response. In chronic SIV infection, we observed immunostimulatory function restricted to CD4 T cells, in which PMNs increased TNF- α production. Thus, depending on the phase of infection and which cell PMNs interact with (CD4 vs CD8 T cell), neutrophils show different immunomodulation.

Acute bacterial and viral infections or LPS and G-CSF administration induce all circulation of immature neutrophils, known as "left shift", and PMN priming^{333,367-369}. Primary SIV infection mimics this phenomenon with mobilization apparently from bone marrow mobilization. But persistent inflammation in chronic SIV infection has been shown to alter hematopoiesis^{370,371}

and may lead to neutrophils impairment. Another hypothesis is that chronic viral infection lead to reprogramming of neutrophil precursors, producing different granulocytes compare to steady state. Indeed, SIV/HIV chronic inflammation affects neutrophils function, as it has been demonstrated for phagocytosis, ROS production, and in this study, for T-cells immunostimulatory properties³⁶³³⁷²³¹⁹. In group 1, we also explored the impact of cART on immunomodulatory properties of PMNs. Nevertheless, due to the number of animals we lack of statistical power, but identified an interesting trend to suppressive function against CD8+ T cells proliferation. Under cART, PMNs did not demonstrated significant immunomodulatory properties against T cells. We hypothesize that early cART restored PMN function by reducing systemic inflammation.

Here, we used a co-culture assay thanks density-based neutrophil sorting because of the lack of available sorting methods in cynomolgus macaques. This method retrieves normal-density neutrophils (NDN) and had no impact in term of PMN priming, but it missed the effect of LDN which are located in PBMC fraction. Bower NL. , Helton ES. *et al* have shown the capacity of LDN to suppress CD8+ T cells proliferation and IFN- γ production in chronically HIV-infected patients, which was mediated by PD-L1³²⁶. In our study we did not showed significant increase of LDN in the course of SIV infection. In addition, we did not observe nor CD8+ T cells suppression of IFN- γ production in chronic SIV infection, neither PD-L1 expression on neutrophils (supplementary Fig3). In this study HIV-infected individuals were chronically infected for years, and authors selected patients on the basis of PD-L1 expression to perform co-culture assay. Here, we followed only for 6 months SIV infection and PD-L1 expression on neutrophils may necessitate a longer time of infection to be up-regulated. In G-CSF-treated patients, Marini O. *et al* demonstrated opposite immunomodulatory function of immature CD10- and mature CD10+ neutrophils, being immunostimulatory and immunosuppressive respectively³³³. In our study we identified significant PMN immunostimulatory function in primary and chronic SIV infection, when frequency of immature CD10- and primed CD62L-low PMN were significantly increased. To confirm that immunostimulatory function is restricted to immature CD10- PMN in SIV infection, it will be necessary to evaluate separately the function of CD10- immature and CD10+ mature PMN.

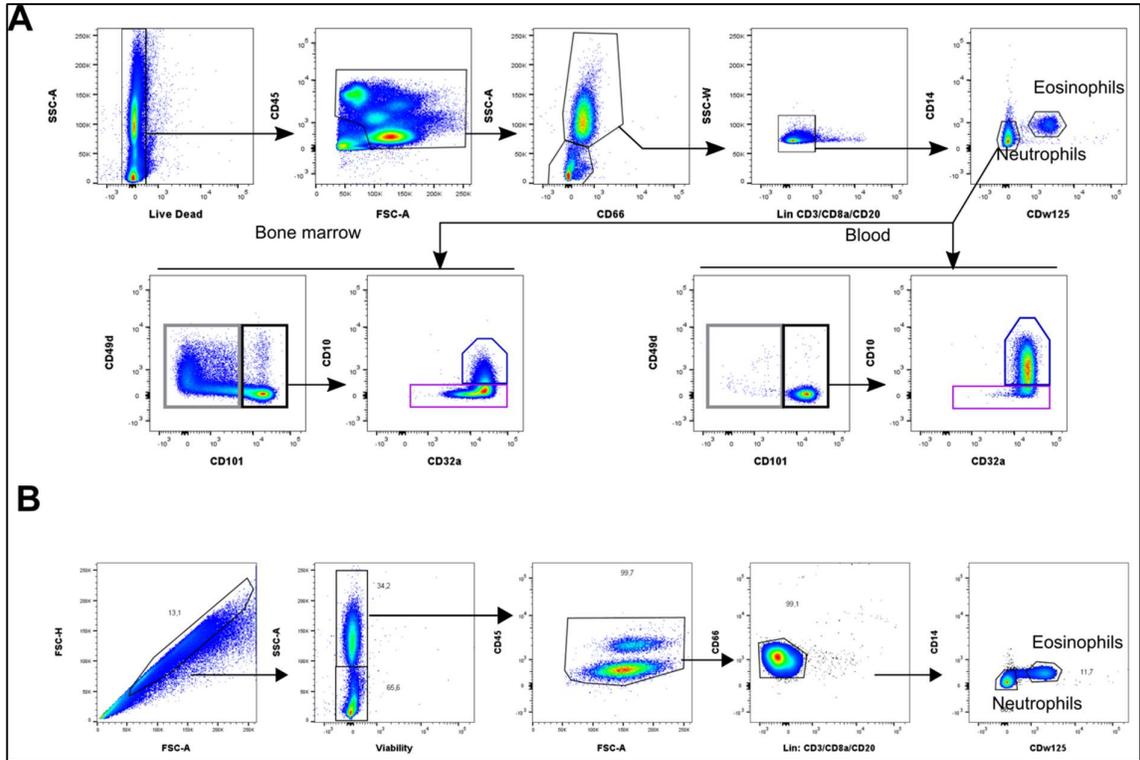
In sum we demonstrated neutrophils heterogeneity in SIV infection and its impact on T cells function. Primed CD62L-low and immature CD10- neutrophils were increased in primary and

chronic SIV infection. This phenotype was associated with immunostimulatory function against T-cells proliferation and cytokine production. In turn, PMNs could participate to chronic inflammation by modulating T cells response. But deep functional characterization is needed in both macaque models and patients to better understand the role of immature neutrophils in HIV-1 infection. If they turn to be important as pro-inflammatory cells, treatments aiming to modulate neutrophils could be used as strategies to reduce chronic inflammation patients.

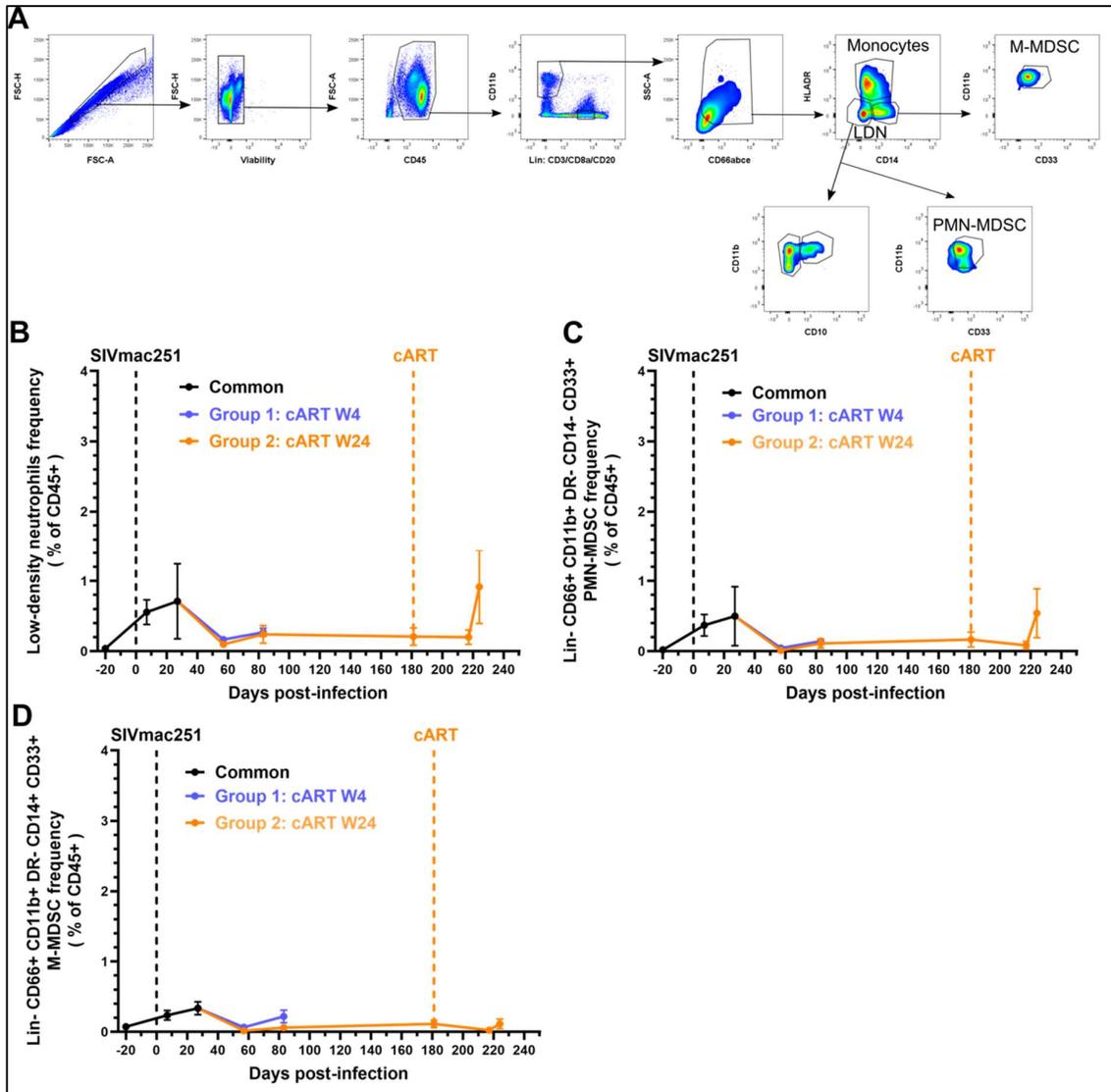
Acknowledgements

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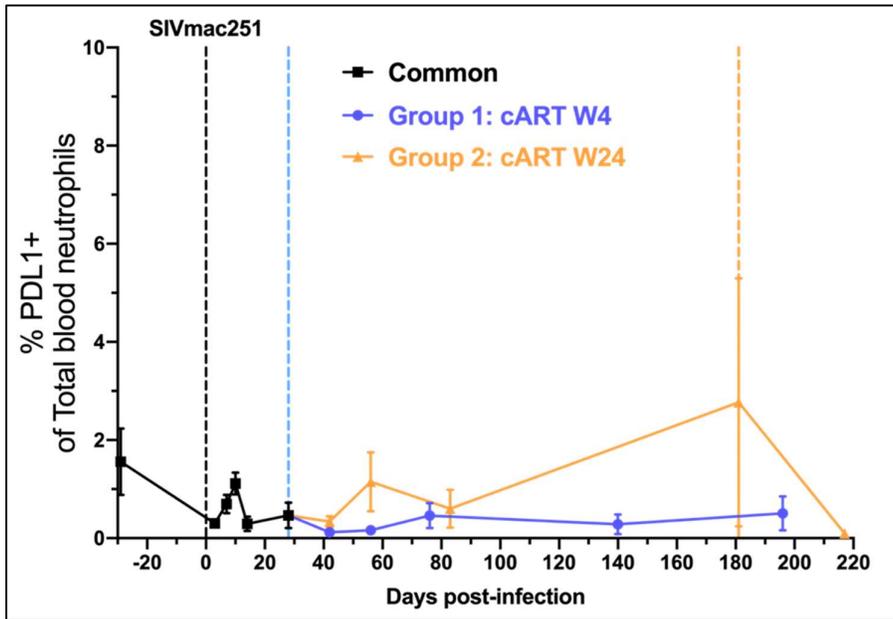


Supplementary Figure 1: Gating strategies used to identify neutrophils in cynomolgus macaques. Gating strategy used in conventional flow cytometry to identify neutrophils according to their maturation stage (A) and priming (B).



Supplementary Figure 2: SIV infection did not affect low-density neutrophils.

A) Gating strategy used to identify LDN (Lin- CD11b+ CD66+ DR- CD14-), PMN-MDSC (CD33+ LDN) and M-MDSC (Lin- CD11b+ CD66+ DR- CD14+) in mononuclear fraction. B-C-D) Frequency of LDN, PMN-MDSC and M-MDSC in CD45+ PBMC from group 1&2 (Black) in primary infection and both separate groups in chronic phase. There was no statistical differences using paired Kruskal-Wallis test with Dunn's multiple comparison.



Supplementary Figure 3: Frequency of PDL1+ PMN in the course of SIV infection

III. Unpublished results: tissue neutrophils heterogeneity in cynomolgus macaques

A. Introduction

HIV-1 infection takes place mainly in lymphoid tissues where the virus replicates, even under antiretroviral treatment. Residual replication, microbial translocation and co-infections are major sources of chronic inflammation observed in HIV-1 infected patients under cART³⁷³. Thus, to better understand causes and consequences of chronic inflammation it is necessary to explore at the tissue level. Myeloid cells, especially macrophages, have been implicated in the setting and the maintenance of chronic immune activation. Since neutrophils are potent pro-inflammatory cells, it is important to better characterize their phenotype and function in tissues during HIV infection. Nevertheless, neutrophil heterogeneity has been studied mainly in blood and bone marrow but less is known about tissues. Recently, neutrophil heterogeneity was associated with tissue-related neutrophil function and depends on inflammatory environment³⁵². This has been demonstrated in inflammatory bowel disease, in which there is an increase of circulating and *lamina propria* CD177+ neutrophils, releasing more ROS, MPO and NETs under stimulation compare to CD177⁻³⁷⁴.

To study phenotype of tissues neutrophils from cynomolgus macaque, we used flow cytometry associated with fast and gentle digestion protocol to retrieve leucocytes. Here we present preliminary data from healthy animals, which validate the methodology used and gives for the first time distribution and phenotype of tissue's neutrophils from cynomolgus macaque. In the future, we will use this approach along with histology to characterize neutrophils in SIV infected macaques.

B. Material and method

We analyzed samples from five cynomolgus macaques (*Macaca fascicularis*), imported from Mauritius, housed at the IDMIT infrastructure animal facility at the CEA, Fontenay-aux-Roses, France. Blood was drawn and bone marrow aspirates collected under anesthesia, consisting of an intramuscular injection of 10 mg/kg zolazepam/tiletamine (Virbac, Carros, France). Blood was collected into lithium-heparin tubes (Vacutainer BD, USA) and bone marrow aspirates from the iliac crest or humeral head using a 10-ml 18G-syringe containing citrate -

dextrose (10 mg/ml). Bronchoalveolar lavages were collected using 20ml of NaCl injected using endotracheal tube and were kept at 4°C with RPMI. At necropsy, spleen was kept on RPMI at 4°C, processed using gentleMACS dissociator, and cell suspension was filtered with 70µm mesh. Red blood cell lysis was performed using NH₄Cl hypotonic lysis. For the gut, 3cm of distal ileum and descendant colon were opened and washed to remove fecal content. To focus on lamina propria and Payer patches, epithelial layer was removed for 20 min using 6.4 mM EDTA. Tissue digestion was performed using DNase I and Collagenase D for 20 min at 37°C. Cell suspension obtained from tissues were resuspended at 3 million cells per 100µL before staining.

Viability staining was first performed for 15 min at room temperature, according to the manufacturer's protocol (Live Dead, Thermofisher). Then, antibody staining was performed with the following antibodies for 30 min at 4°C Bone marrow and blood samples were then lysed and fixed using BD FACS Lysing (BD Bioscience) for 15 min. After an additional wash with PBS, acquisition was performed on a BD FORTESSA flow cytometer (BD Bioscience) and analyzed using FlowJo software.

C. Preliminary results

Flow cytometry allowed to delineate neutrophils from all tissue samples (Figure 1A-B), except from mesenteric lymph nodes (draining ileum) in which we identified less than 100 cells. We first evaluated neutrophils frequency among leucocytes from bone marrow, blood, bronchoalveolar lavages (BAL), ileum and colon (Figure 1B). In the spleen and BAL, neutrophils represented 11.9% and 7.8% of total CD45+ leucocytes respectively. We also identified significant neutrophil population in ileum and colon, representing approximately 1% of total leucocytes. We also evaluated maturation stages in blood, bone marrow and spleen (Figure 1C). In the blood and bone marrow we obtained a distribution of maturation stages equivalent to previous studies.

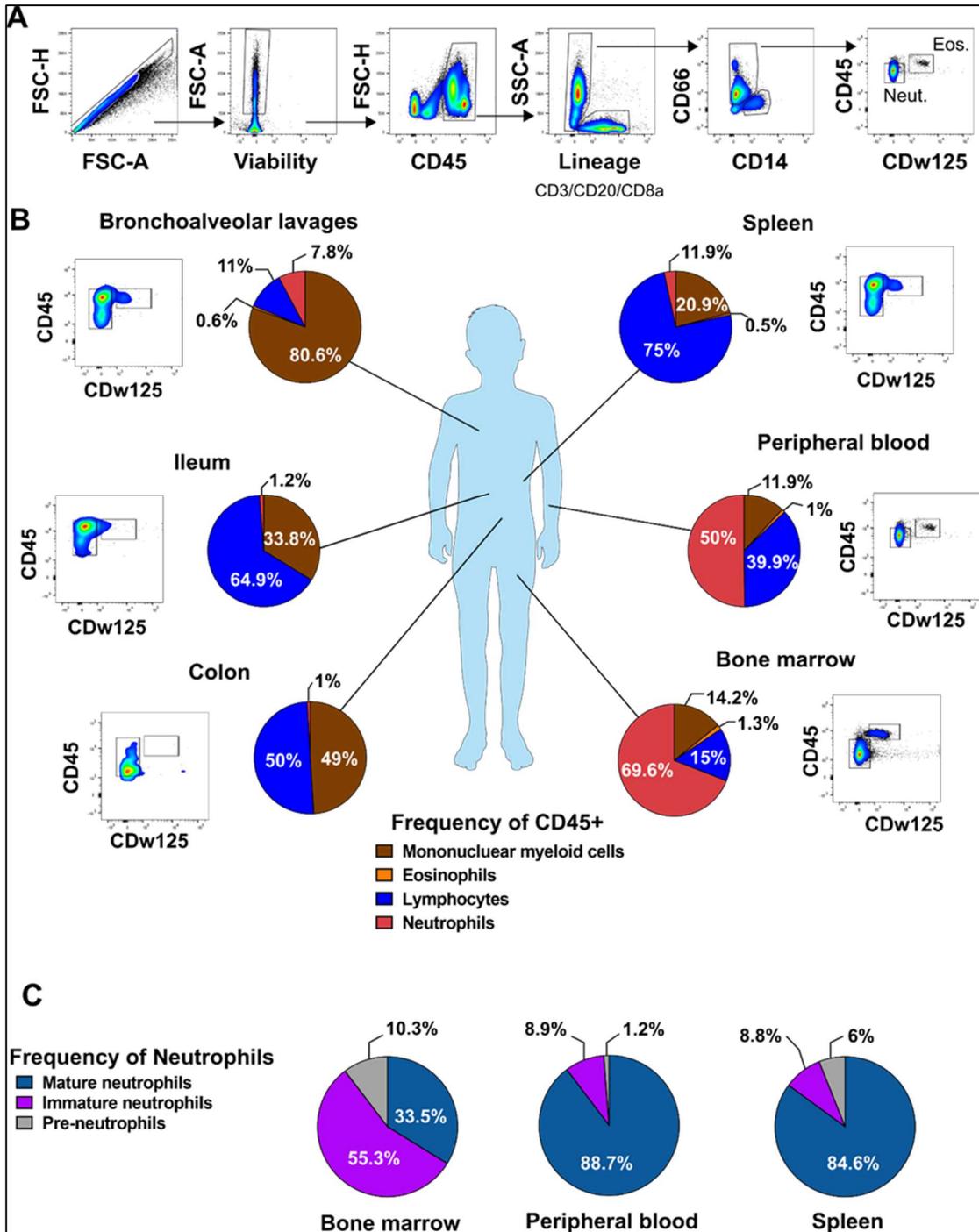


Figure 1: Characterization of tissues neutrophils in cynomolgus macaque.

A) Gating strategy used to identify neutrophils from all compartment analyzed. **B)** Pie charts represent frequency in CD45+ of each leucocyte population identified in tissues. B cells, T cells and NK cells were considered as lymphocytes and monocytes/macrophages with dendritic cells were considered as mononuclear myeloid cells. Corresponding gate showing neutrophils is also represented. **C)** Pie charts representing repartition of neutrophils by maturation stages in bone marrow, blood and spleen.

Interestingly, we observed elevated proportion of pre-neutrophils in the spleen, suggesting a potent extra-medullary hematopoiesis as observed in other mammal species¹⁹⁴. Nevertheless, this remains to be confirmed by cell sorting by flow cytometry associated with cytology. In addition, neutrophils heterogeneity has been described in spleen from mouse model, having B cell help or anti-bacterial function according to their maturation stages³⁷⁵. Thus, it would be interesting to evaluate neutrophil's functions in the spleen according to their maturation stage in SIV infection.

We also evaluated priming markers and Fc-receptors expression on neutrophils from tissues. Neutrophils demonstrated a primed phenotype in all tissues with elevated frequency of CD62L-low, HLADR+ and PD-L1+ neutrophils in spleen, BAL, ileum and colon. This phenotypic changes have been already described in mouse models where neutrophil transmigration had an activated phenotype^{268,369}. We also analyzed expression of CD89 (Fc- α R), CD64 (Fc- γ RI) and CD32a (Fc- γ RIIA) on tissue neutrophils. CD64 was more expressed on bone marrow and BAL neutrophils. In bone marrow this is due to the coexistence of different neutrophil maturation stages expression this marker. In BAL, CD64 up-regulation maybe due to phagocytic function in alveolar space³⁷⁶. These results strongly suggest that tissue specific neutrophil phenotypes in the healthy Cynomolgus macaque. It will be very informative to study tissues in SIV-infected macaques.

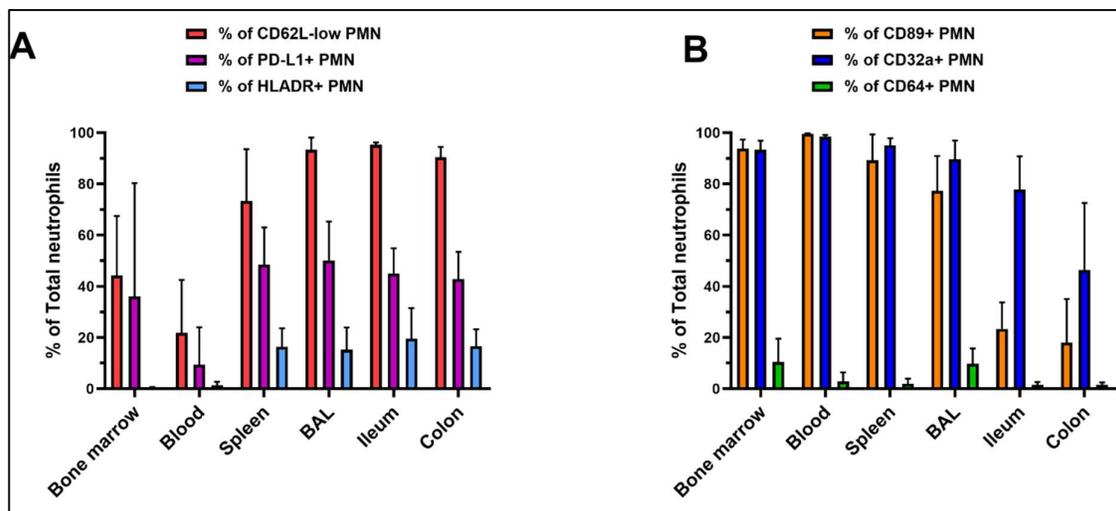


Figure 2: Neutrophils had a tissue specific phenotype in healthy cynomolgus macaque.
A) Frequency of neutrophils expressing priming markers HLADR, PD-L1 and CD62L-shedding in each tissue. **B)** Frequency of neutrophils expressing Fc-receptors CD89, CD32a and CD64 in each tissue.

Discussion and perspectives

I. Validation of experimental approach

In these studies, we used cynomolgus macaques infected by SIVmac251 as a model for HIV-1 infection. We injected intravenously 1000 animal infectious dose 50% (AID50) allowing to infect all animals, and reproducing physiopathology observed in HIV-1 infected patients, with progression from primary infection to long-term chronic infection, and lastly AIDS^{165,336}. Cynomolgus macaques from Mauritius have the advantage to show a limited genetic diversity, but possess multiples MHC haplotypes associated with differential progression to AIDS³³⁷. SIVmac251 is phylogenetically related to HIV-2, but in cynomolgus macaques notably because of restriction factors virus-host interaction leads to a dynamic of infection close to human. This model is also useful to study pharmacological sanctuaries and reservoirs because most of antiretroviral treatment used in patients suppress SIVmac251 replication³⁷⁷. Lastly, compared to mouse models, immune system of cynomolgus macaque is closer to human, making nonhuman primates more suitable models for infectious diseases pre-clinical research^{378,379}. In this study, we also performed a long-term follow-up of immunological parameters from primary infection to advanced chronic infection. Therefore, we reduced interindividual heterogeneity and sample size by using paired statistical analyses.

Our work was focused on macaques neutrophils, which demonstrated similar differentiation in bone marrow compare to human³⁸⁰. But compared to human, cynomolgus macaque's neutrophils lack CD16b expression which may lead to different responses to antibodies such as ADCC³⁸¹. To analyze neutrophil immunomodulatory functions, we used density gradient sedimentation, in the absence of validated kit to specifically purify these cells. Thus, globular fraction used was contaminated by few basophils and by 3-10% of eosinophils depending on the animal. For this reason, we are currently developing a negative sorting protocol based on magnetic microbeads. This will permit to work on highly purified neutrophils as well as neutrophils subpopulations from cynomolgus macaque for functional analysis. Furthermore, this method will allow a fast recovery of neutrophils, which are fragile cells.

In sum, cynomolgus macaque is a relevant preclinical model to better understand physiopathology of HIV-1 infection and response to treatments. Existence of few differences in term of leukocytes population's markers is one limitation of this model. In addition,

functional analyses of neutrophils were limited by available sorting procedure for non-human primates. For this reason, we are developing a purification method to refine our understanding of neutrophils.

II. Discussion

Here we provided the first description of neutrophil heterogeneity in SIV infected macaques. Taking advantage of multiparametric mass cytometry, we identified circulating immature neutrophils in advanced chronic SIV infection, underlying mobilization of bone marrow cells as described in sepsis³⁸². Based on human markers delineating maturation stages, mass cytometry analysis was able to identify pre-neutrophils, immature neutrophils and mature neutrophils. In early chronic phase, reducing the number of markers for flow cytometry, we abolished the ability to differentiate immature from mature neutrophils in blood as the neutrophil population was homogeneous in terms of CD66 and CD32 expression. Other markers were necessary to better delineate neutrophil maturation, such as CD49d, CD101, and CD10, recently described in human blood and bone marrow²⁰⁰. We used these markers in our second work. In advanced HIV-1 infection, neutrophils were already known to be impaired, showing priming, reduce phagocytosis and oxidative burst in correlation with viral load and CD4 T cell count^{372,383}. Our analysis also showed that neutrophils were primed earlier in chronic infection, with reduced phagocytic capacities. These results suggest that changes of neutrophils phenotype and functions occur earlier in the course of infection.

To better characterize neutrophil priming and maturation in the course of SIV infection, we tested new markers and combined classical flow cytometry cell sorting with cytology in our second study. We had the opportunity to study animals in a longitudinal study, called p-VISCONTI, on SIV infection and impact of early or delay treatment on immune response. The p-VISCONTI project aimed also to induce post-treatment control of infection in cynomolgus macaques, as observed in VISCONTI cohort³⁸⁴. Interestingly, SIV infection had no significant impact on neutrophil blood count, which was highly variable among animals and time points. But when analyzing neutrophil subpopulations, we revealed the impact of SIV infection. In primary infection, CD10- immature and CD62L-low primed neutrophils were increased in peripheral blood, following the dynamic of viral replication. Despite a partial resolution after

primary infection, frequencies of immature and primed neutrophils were maintained elevated after 6 months of infection. We observed also a global decrease of mature neutrophils in bone marrow and blood suggesting a mobilization to tissues, an increase of apoptosis or a lack of differentiation. Indeed, the increase of neutrophil apoptosis has been shown in SIV primary infection, but other mechanisms may explain what we observed in chronic phase. Indeed, we observed increased frequency of immature neutrophils in bone marrow only in chronic phase, suggesting an impact on bone marrow production. In sum, primary SIV infection mimic a classical acute infection with elevated activation of neutrophils, mobilization of mature to tissues and emergency granulopoiesis liberating immature cells into the circulation²¹. But persistence of viral replication and inability of immune system to clear the virus in chronic infection was associated with maintenance of phenotypic changes, notably by impacting bone marrow production.

Immunomodulatory function of neutrophils is a recent discovery which has been linked to maturation stage by Marini O. *et al*³³³. In G-CSF treated donors, they demonstrated opposite role of CD10+ mature and CD10- immature neutrophils with respectively immunosuppressive and immunostimulatory effect on T cells. Here we have also seen an association with CD10- immature neutrophils and immunostimulatory function on T cells cytokine production and CD4 T cells proliferation. This effect was more pronounced in primary infection than in chronic phase notably because of a higher frequency of CD10- immature neutrophils at this time point. By using density-based sorting, we retrieved only normal-density neutrophils (NDN) and we missed the effect of low-density neutrophils (LDN) which are located in the PBMC fraction. Brower NL. , Helton ES. *et al* have shown the capacity of LDN and NDN to suppress T cells proliferation and IFN- γ production in chronically HIV-infected patients, which was mediated by PD-L1³²⁶. In our study we did not showed significant increase of LDN in the course of SIV infection. In addition, we did not observe nor suppression of IFN- γ production by T cells in chronic SIV infection, neither PD-L1 expression on neutrophils. Nevertheless, in Brower's study HIV-infected individuals were chronically infected for years whereas we analyzed neutrophil functions at only 6 months post-infection. In advanced HIV-1 infection, neutrophil alteration may be exacerbated and LDN circulation may increase with specific immunomodulatory properties.

When cART was initiated early (4 weeks post-infection), neutrophil phenotype progressively returns to basal level. On the contrary, initiation of cART after 6 months of infection did not fully restore phenotype. The later the treatment is initiated, the more persistent is chronic inflammation and its impact¹⁵⁸. So, two mechanisms may explain the impact of delay cART on neutrophils phenotype and function: 1- SIV infection and chronic inflammation had increased bone marrow production and neutrophils turnover leading to circulation of immature cells, 2- persistence of inflammation change neutrophils precursors, giving rise to functionally different cells. Both mechanisms may also coexist, but bone marrow analysis showed elevated proportion of immature neutrophils and reduction of pre-neutrophils in chronic phase, supporting the first hypothesis. We are currently analyzing bone marrow from animals treated after 6 months of infection to evaluate if these changes are maintained under cART. To explore the second hypothesis, it would be necessary to evaluate epigenetic modifications of neutrophils precursors, which could explain the generation of functionally different neutrophils³⁸⁵.

III. Perspectives

Chronic inflammation occurs when the host is unable to eliminate the initiating stimuli, or when the host fails to resolve inflammation. Neutrophils have been associated with metabolic, cardiovascular and neurological diseases, which are all chronic inflammatory diseases²¹. Interestingly, these diseases constitute non-AIDS related co-morbidities. Our work has highlighted a role of neutrophils in modulation of T cell response in SIV-infected macaques, leading to an increase of pro-inflammatory secretion. We have shown neutrophil heterogeneity, but we did not analyze function of each subpopulation. For this reason, we are developing new tools to better sort neutrophils in cynomolgus macaque. Thus, to confirm that immunostimulatory function is restricted to immature CD10- PMN in SIV infection, we plan to evaluate separately the function of CD10- immature and CD10+ mature PMN. Using this sorting tool, phagocytosis, ROS production, cytokine production, ADCC, NETosis, DC immunomodulation are potential neutrophils function we could analyze in these subpopulations. But we must learn and develop most of these functional assays we did not use yet. In parallel, to obtain a precise definition of mature and immature PMN during SIV

infection, we will perform a transcriptomic analysis on sorted cells from blood and bone marrow. This will increase our knowledge on neutrophils in the course of infection, and it will reveal pathways to focus our work on specific functions.

In addition, our results suggest mobilization of mature neutrophils in tissues, and except for the gut, the role of tissue neutrophils has not been explored in HIV infection. With cynomolgus macaque model, we plan to first explore neutrophils phenotype in tissues using the combination of flow cytometry and multiparametric immunohistology. To exert immunomodulation of T cells, neutrophils must be present in the same tissue than T cells. White pulp of the spleen and GALT are of particular interest because neutrophils were shown to infiltrate these lymphoid tissues. Thus, immunohistofluorescence will provide *in-situ* colocalization and neutrophils phenotype. Collecting neutrophils from tissue is still a difficult task to harvest enough cells, so we will focus on spleen, bone marrow and broncholaveolar lavages for functional analyzes. Taking advantage of positron-emission tomography associated to tomodesitometry present in our facility, it will be interesting to use neutrophil-specific radiotracer recently developed in order to identify tissues infiltrated by neutrophils in the course of SIV infection³⁸⁶. This whole-body imaging technology will give access to tissue neutrophils dynamic, allowing to visualize marginated pool, lung, spleen and liver neutrophils.

More importantly, it will be also necessary to confirm these results in HIV-infected patients at different stages of the disease. For this purpose, thanks to the access to ANRS cohorts of patients, we planned to analyze blood samples from HIV-1 infected patient in primary infection, chronic phase and under cART to evaluate neutrophils phenotype and function.

Neutrophils appear to participate to chronic inflammation. If indeed they are important modulators of inflammation it would be interesting to analyze PMN in natural host, such as African green monkey, in which resolution of inflammation seems to be the answer against AIDS. Tools we developed for cynomolgus macaques could be adapted for this specie. Pathogenic models and natural hosts could be used to evaluate new therapeutic strategies targeting neutrophils to reduce chronic inflammation.

If neutrophils represent an important contributor in HIV chronic inflammation, targeting them could reduce inflammation. Research to modulate neutrophil activation in other chronic inflammatory diseases may inspire new therapeutic strategies for HIV infection, since they are sharing common mechanisms. Few drugs modulating neutrophils activity have been developed recently. In mouse models of atherosclerosis or lupus, peptidyl arginine deiminase 4 (PAD4) inhibition (Cl-amidine) reduced NET formation and related tissue damages^{387,388}. Blocking leukocytes recruitment is another strategy targeting mainly adhesion molecule, but they are not neutrophil specific²¹. Reducing neutrophil infiltration in tissues to resolve inflammation is another interesting strategy, which could be interesting for HIV-infection. Inducing neutrophil apoptosis would decrease tissue damages and phagocytosis of apoptotic neutrophils by macrophages (efferocytosis) would induce resolution of inflammation. R-Roscovotine (inhibitor of cyclin-dependent kinase) was shown to induce caspase-dependent neutrophil apoptosis in mice³⁸⁹. It would be interesting to test these new drugs in preclinical models of HIV-infection to reduce chronic inflammation and restore immune function.

For long, neutrophils have not been studied for their immunomodulatory properties, considered as a homogenous phagocyte population. Modulation of the immune system by neutrophils must be explored in other infectious diseases to better understand the link between their heterogeneity and immune response against pathogens. Acute and chronic respiratory diseases are one of the most important causes of severe illness and death worldwide, with chronic obstructive pulmonary disease (COPD), acute lower respiratory tract infections and tuberculosis are top three³⁹⁰. Interestingly, there are relationships between neutrophils, lungs, and respiratory diseases. Tuberculosis is an interesting example of chronic respiratory disease, but only few studies have demonstrated in tuberculosis a cross-talk between neutrophils, DC and T cells²³⁸. Beside, acute respiratory distress syndrome (ARDS) is a fatal complication of influenza infection due to epithelial cells damage and exaggerated innate immune response, especially neutrophils³⁹¹. In ARDS, neutrophils have been shown to release NETs, ROS and pro-inflammatory cytokines leading to lung damage. But less is known about their immunomodulatory properties, which could also participate to uncontrolled immune response to influenza. Tuberculosis and ARDS are examples among respiratory infections where neutrophil heterogeneity could participate to impair immune response. We are currently developing programs to study physiopathology and vaccines against respiratory

infectious diseases in our facilities. Particularly, non-human primate models are available to study tuberculosis, influenza infection and respiratory syncytial virus infection, which will be used for these purposes. Taking advantage of these models, tools we developed to study neutrophils in SIV infection could be used to explore neutrophil heterogeneity in these diseases.

Neutrophil heterogeneity may originate from inflammation diversity, inducing modulation of cell ontogeny and leading to different cellular functions. A better understanding of neutrophils heterogeneity in infectious diseases will be necessary to better understand immunopathology and to design new therapeutic strategies to block or enhance functions of this abundant and efficient immune cell.

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Hétérogénéité des polynucléaires neutrophiles dans l'infection par le VIH-1. Etude du modèle macaque cynomolgus infecté par le SIV.

Introduction

Persistance de l'inflammation chronique chez les patients recevant une thérapie antirétrovirale combinée

Les thérapies antirétrovirales disponibles ne permettent pas d'éradiquer le VIH-1 de l'organisme et nécessitent une prise quotidienne, afin de réduire la réplication virale jusqu'à des niveaux indétectables. Les thérapies antirétrovirales combinées (TARC) ont permis d'augmenter l'espérance de vie et la qualité de vie des personnes vivant avec le VIH-1, mais ne sont toujours pas curatives. En cas d'interruption thérapeutique, la réplication reprend grâce à l'existence de réservoirs viraux établis dès la primo-infection¹. Le réservoir est défini comme étant l'ensemble des cellules et tissus infectés par le VIH-1, qui persistent malgré le traitement². La persistance de l'infection malgré le traitement est associée à un risque accru de développer des comorbidités non associées au SIDA, telles que des maladies métaboliques, neurodégénératives et cardiovasculaires³. Ainsi, comparée à une population séronégative d'âge équivalent, la fréquence de ces maladies normalement associées au vieillissement est plus élevée chez les individus infectés par le VIH-1 et recevant un traitement. Chez ces patients, plusieurs études ont montré une élévation du niveau de biomarqueurs inflammatoires (CRP, IL-6 et IL-18) et de dysfonctionnement de la muqueuse digestive (I-FABP, Zonuline)⁴. Le niveau de ces biomarqueurs est directement corrélé avec le risque de développer des comorbidités non associées au SIDA, ce qui suggère une persistance pathologique de l'inflammation même avec la TARC⁵. L'inflammation chronique, caractéristique majeure de l'infection par le VIH-1, persiste donc à bas niveau chez les individus sous TARC et serait associée au développement de comorbidités. Plusieurs mécanismes expliquent la persistance de l'inflammation chez les individus recevant une TARC. Premièrement, du fait de leurs propriétés physicochimiques, les molécules antirétrovirales ne sont généralement pas distribuées de façon homogène dans l'ensemble du corps⁶. Les organes dans lesquels la concentration des antirétroviraux est réduite (intestin, organes lymphoïdes) sont appelés sanctuaires pharmacologiques et sont le siège d'une réplication virale résiduelle alimentant l'inflammation. Deuxièmement, les lésions de la muqueuse digestive induites par l'infection par le VIH-1 persistent sous traitement⁷⁻⁹. Cette atteinte de la barrière intestinale entraîne la translocation de produits microbiens (ARN 16S) dans la circulation sanguine, provoquant l'activation des cellules myéloïdes (sCD14) et la production de cytokines pro-inflammatoires (IL-6 et IL-18). Troisièmement, chez les personnes infectées par le VIH-1, les co-infections par *Mycobacterium tuberculosis*, par les virus hépatiques et par le cytomégalovirus sont fréquentes et participent au maintien de l'inflammation chronique¹⁰. Enfin, le mode de vie est fortement associé à la morbidité et la mortalité, en particulier la consommation excessive d'alcool et de drogues, qui sont impliquées dans l'inflammation³.

Les mécanismes exacts et les cellules impliquées dans la persistance de l'inflammation sont mal compris. L'élévation de biomarqueurs inflammatoires, tels que sCD14, IL-6, IL-18 et CRP,

suggèrent que l'activation de l'immunité innée joue un rôle majeur¹¹. En effet, la translocation microbienne, les co-infections et la réplication résiduelle induisent l'activation des cellules myéloïdes, qui libèrent en retour des cytokines pro-inflammatoires. Ce mécanisme est suspecté de promouvoir la fibrose tissulaire, ainsi qu'un état d'hypercoagulabilité¹². Il a été décrit que les monocytes et les macrophages, en réponse à la translocation microbienne, participent à l'inflammation associée au VIH-1, notamment en produisant de l'IL-6¹³. Ainsi, mieux comprendre les cellules et les mécanismes impliqués permettrait de développer de nouvelles stratégies thérapeutiques visant à réduire l'inflammation chronique chez les patients sous traitement. Mais les cellules myéloïdes ne désignent pas uniquement les macrophages et monocytes, et le rôle des granulocytes dans la persistance de l'inflammation reste peu étudié. Les polynucléaires neutrophiles (ou neutrophiles) sont les granulocytes les plus abondants de la circulation sanguine (40-70% des leucocytes circulants), aux capacités pro-inflammatoires exceptionnelles dont l'implication dans l'inflammation associée au VIH-1 est mal comprise¹⁴.

Hétérogénéité des polynucléaires neutrophiles au cours de l'inflammation et implication dans l'infection par le VIH-1

Pendant longtemps, les neutrophiles ont été considérés comme une population homogène de cellules phagocytaires, ayant une courte durée de vie et dont le rôle était restreint à la défense contre les microorganismes pathogènes¹⁵. Les neutrophiles sont des cellules immunitaires complexes à étudier du fait de leur fragilité et doivent être analysées dans les heures suivant le prélèvement, ce qui a été longtemps un frein à leur caractérisation. Récemment, de nombreux dogmes ont été contestés avec des études montrant que les neutrophiles sont formés de différentes sous-populations, qui participent à la modulation des lymphocytes T, la résolution de l'inflammation et la cicatrisation¹⁶. De plus, une autre étude suggère que l'espérance de vie d'un neutrophile peut atteindre 5 jours en conditions inflammatoires, soutenant l'hypothèse de la plasticité des granulocytes.

En cas d'inflammation aiguë, la production et la fonction des neutrophiles s'adapte pour éliminer l'envahisseur tout en permettant de résoudre l'inflammation, afin d'éviter tout dommage tissulaire¹⁷. Si le système immunitaire échoue à éliminer l'agent pathogène, il doit trouver un équilibre entre le contrôle de l'envahisseur et la réduction des dommages tissulaires. Dans ce contexte, l'inflammation chronique a des conséquences pathologiques importantes. Dans de nombreuses maladies inflammatoires chroniques, les neutrophiles semblent jouer un rôle fondamental. Les maladies auto-immunes sont caractérisées par une perte de tolérance envers les antigènes du soi, induisant une inflammation chronique. Dans la polyarthrite rhumatoïde, le lupus érythémateux systémique ou encore les vascularites auto-immunes, les neutrophiles participent aux lésions tissulaires et perturbent la réponse immunitaire¹⁸. La découverte de neutrophiles de faible densité (LDN, pour « low-density neutrophils ») fut un des premiers éléments démontrant l'existence de sous-populations de neutrophiles. Les LDN sont obtenus après centrifugation du sang par gradient de densité et se retrouvent avec les cellules mononucléées du sang périphérique. La fréquence des LDN augmente dans le sang des patients atteints de lupus où ils présentent des capacités pro-inflammatoires, supportant la formation d'auto-antigènes en libérant des « pièges extracellulaires » (NET, pour « neutrophils extracellular traps »). Plus récemment, les travaux

de Marini *et al* montrent que la diversité fonctionnelle des neutrophiles est fonction de leur stade de maturation¹⁹. En étudiant l'impact d'un traitement au G-CSF, utilisé pour mobiliser les cellules souche hématopoïétiques, cette équipe a identifié des sous-population de neutrophiles pouvant moduler la réponse des lymphocytes T. Chez les donneurs, le G-CSF induit la circulation de neutrophiles matures et immatures possédant des nouvelles propriétés immunomodulatrices des lymphocytes T. Les neutrophiles matures sont alors capables d'augmenter la prolifération et la production d'IFN- γ des lymphocytes T, alors que les neutrophiles immatures montraient un effet opposé. La fonction immunomodulatrice des neutrophiles a été mise en évidence par dans différent contextes pathologiques comme certains cancers, maladies infectieuses et auto-immunes. Néanmoins, l'impact d'une sous-population de neutrophile sur la fonction des lymphocytes T peut différer d'une maladie à l'autre, ce qui démontre la grande plasticité des granulocytes et souligne notre méconnaissance de ce mécanisme.

Peu d'informations sont disponibles sur le rôle des neutrophiles dans l'infection par le VIH-1¹⁴. Des analyses immunohistologiques ont montré une augmentation du nombre de neutrophiles dans le colon des individus infectés chroniquement par le VIH-1, recevant ou non une TARC²⁰. Cette infiltration neutrophilique est associée à des lésions de l'épithélium intestinal, suggérant un rôle des neutrophiles dans l'inflammation digestive. Plus récemment, Lynch S. *et al* ont montré que les neutrophiles qui infiltrent le colon voient leur survie augmentée. Les neutrophiles présentent alors une dérégulation de leur apoptose, qui est fondamentale pour la résolution de l'inflammation. Dans la même étude, les auteurs observent également un lien entre la perturbation du microbiote digestif, la translocation microbienne et l'infiltration de neutrophiles dans le colon. Les neutrophiles sont donc des nouvelles cellules effectrices de l'inflammation chronique associée au VIH-1. D'autres études montrent également une activation des neutrophiles circulants, avec des capacités antimicrobiennes réduites chez des individus chroniquement infectés par le VIH-1. Il est donc probable que, lors de l'infection par le VIH-1, les neutrophiles infiltrent des tissus comme l'intestin où ils ne peuvent éliminer les bactéries intestinales qui traversent la muqueuse. Enfin, Bower *et al* démontrent pour la première fois le rôle immunomodulateur des neutrophiles chez des patients infectés chroniquement par le VIH-1²¹. Les neutrophiles seraient capables de réduire la production d'IFN- γ par les lymphocytes T, participant au dysfonctionnement de la réponse immunitaire antivirale.

Méthodologie

Le système immunitaire de l'hôte échoue à éradiquer le VIH-1 malgré le contrôle de la réplication virale par la TARC. Cette persistance est associée au maintien de l'inflammation chronique, qui entraîne un risque accru de développement de comorbidités.

Notre hypothèse est que les événements précoces suivant l'infection par le VIH-1 ont un impact à un long terme sur la fonction et la différenciation des neutrophiles, qui vont participer et entretenir l'inflammation chronique, et ce malgré le traitement antirétroviral.

Néanmoins, il est toujours difficile d'étudier les premiers jours de l'infection par le VIH-1 et de réaliser des analyses tissulaires chez les personnes vivant avec le VIH-1, pour des raisons éthiques et d'accès aux prélèvements. Dans ce projet nous avons donc réalisé ces analyses grâce à un modèle primate non-humain, possédant un système hématopoïétique similaire à celui de l'homme. Le modèle du primate non-humain a permis de grandes avancées dans notre compréhension de la physiopathologie de l'infection par le VIH-1, dans le développement des outils de diagnostic et la mise au point des stratégies thérapeutiques et préventives²². Le macaque cynomolgus (*Macaca fascicularis*) infecté par le SIVmac251 est l'un des modèles le plus pertinent, car il reproduit de nombreux aspects de la physiopathologie du VIH-1^{23,24}. De plus, la plupart des traitements antirétroviraux utilisés chez les patients sont efficaces contre le SIV, ce qui permet d'étudier la persistance des réservoirs viraux et de l'inflammation. Pour ces raisons nous avons utilisé ce modèle de l'infection par le VIH-1.

Quel est l'impact de l'infection chronique par le SIV sur la diversité des populations myéloïdes et la fonction des neutrophiles ?

Dans un premier temps, nous avons analysé le sang et la moelle osseuse pour évaluer la diversité des cellules myéloïdes au stade chronique tardif de l'infection par le SIVmac251. Pour maximiser nos chances de découvrir des changements dans les populations myéloïdes nous avons utilisé la cytométrie de masse²⁵. Pour ce faire, nous avons développé un panel de 35 anticorps ciblant des marqueurs d'intérêt et possédant une réactivité croisée pour les cellules du macaque cynomolgus. L'analyse multidimensionnelle non supervisée des données obtenues par cytométrie de masse nous a permis d'identifier la circulation de neutrophiles immatures durant la phase chronique tardive de l'infection²⁶. Cette découverte surprenante nous a amené à nous focaliser sur la caractérisation des neutrophiles. Avec la cytométrie en flux nous avons évalué le niveau d'activation et la fonction phagocytaire des neutrophiles au stade chronique précoce de l'infection par le SIV sur 18 macaques cynomolgus. En absence de TARC, neutrophiles était activés avec une fonction phagocytaire réduite. La mise en place de la TARC n'a pas permis de rétablir la fonction et le phénotype des neutrophiles.

Quel est la dynamique des neutrophiles durant les phase aiguë et chronique de l'infection par le SIV ? Les neutrophiles ont-ils la capacité de moduler la fonction des lymphocytes T au cours de l'infection ?

Nous avons décrit pour la première fois la circulation de neutrophiles immatures en phase chronique de l'infection par le SIV. Nous souhaitons donc déterminer à quel moment de l'infection se produisent ces changements de répartition des sous-population neutrophiles.

La cytométrie de masse nous a permis d'identifier les différents stades de maturation des neutrophiles sur la base de nombreux marqueurs. Néanmoins cette approche reste complexe et couteuse, nous souhaitons donc pouvoir identifier les stades de maturation des neutrophiles plus simplement. Le travail fondamental de Evrard M. *et al*, récemment publié, montre que la combinaison de CD10, CD16 et CD101 permet de séparer les neutrophiles humains en trois populations : pré-neutrophiles (promyélocytes et myélocytes), neutrophiles immatures (métamyélocytes et cellules en bande) et neutrophiles matures²⁷.

En associant tri cellulaire assisté par cytométrie en flux et cytologie, nous avons pour la première fois démontré que les marqueurs découverts chez l'homme délimitent les même sous-populations de neutrophiles chez le macaque cynomolgus, exception faite de CD16 qui fut remplacé par le CD32a. Ensuite à l'aide de la cytométrie en flux, nous avons suivi longitudinalement la fréquence des neutrophiles matures et immatures, ainsi que leur niveau d'activation, dans le sang et la moelle osseuse de 12 macaques cynomolgus infectés par le SIVmac251. Afin d'évaluer l'impact d'une TARC (ténofovir, emtricitabine et dolutégravir) initié précocement sur le phénotype et la fonction des neutrophiles, les animaux ont été séparés en deux groupes : le premier groupe traité après 28 jours d'infection et le second traité après 6 mois d'infection.

Afin de caractériser la fonction immunomodulatrice des neutrophiles sur les lymphocytes T, nous avons développé un test de co-culture entre des cellules mononuclées du sang périphérique et des neutrophiles. A l'aide de ce test et après 16 heures de co-culture avec des neutrophiles autologues, nous avons évalué la production de cytokines des lymphocytes T CD4+ et T CD8+ par marquage intracellulaire. La capacité de prolifération des lymphocytes T a été également mesuré par dilution du CFSE après 96 heures de co-culture avec des neutrophiles autologues. Par ailleurs, les paramètres biologiques caractérisant l'infection par le SIV, tels que la charge virale, le comptage des lymphocytes T CD4+ et l'activation lymphocytaire ont également été mesurés au cours de cette étude.

Quels tissus sont colonisés par les neutrophiles durant l'infection par le SIV ? Existe-il un phénotype de neutrophile associé au tissu ?

Les tissus lymphoïdes et muqueux sont des sites majeurs du dysfonctionnement du système immunitaire, où s'établissent les réservoirs du VIH-1. La distribution et la fonction des neutrophiles dans ces tissus est encore mal connue, seulement une étude récente montre un lien entre inflammation, colonisation du colon par les neutrophiles et translocation microbienne au cours de l'infection par le VIH-1. Dans le but de mieux comprendre la dynamique et la distribution tissulaire des neutrophiles au cours de l'infection par le SIV, nous avons développé des outils pour isoler et caractériser les neutrophiles tissulaires (issus de la rate, des nœuds lymphatiques mésentériques, de l'iléon, du colon, de lavage bronchoalvéolaire et des poumons) en associant cytométrie en flux et immunohistologie. Dans ce manuscrit, nous présentons également des résultats préliminaires sur la répartition des neutrophiles dans les tissus de macaques cynomolgues sains.

Résultats

La cytométrie de masse révèle l'immaturité des neutrophiles circulants au cours de l'infection par le SIV

Notre but premier était de comprendre l'impact de l'infection sur l'ensemble des populations de cellules myéloïdes. Afin d'obtenir une vision globale des populations leucocytaires du sang et de la moelle osseuse, nous avons utilisé la cytométrie de masse. L'analyse non supervisée par l'algorithme SPADE des données obtenues a mis en évidence à la diversité des populations de neutrophiles²⁶. L'infection chronique avancée par le SIV entraîne des changements majeurs des neutrophiles sanguins, qui sont principalement constitués de sous-populations phénotypiquement immatures (CXCR4⁺, CD32a^{low} et CD11b^{low}). Néanmoins, l'identification précise du stade de maturation des neutrophiles est effectuée classiquement en analysant leur morphologie. Pour confirmer que ces sous-population correspondent à des neutrophiles immatures, nous avons combiné le tri cellulaire assisté par cytométrie avec la cytologie. Cette analyse nous a permis de valider que les neutrophiles CD32a^{low} et CD11b^{low} identifiés par cytométrie de masse correspondent à des neutrophiles immatures.

Ces observations ont été effectués à un stade avancé de l'infection chronique par le SIV, nous souhaitons donc identifier si ces modifications apparaissent plus précocement. Nous nous sommes donc focalisés sur la caractérisation phénotypique et fonctionnelle des neutrophiles au début de la phase chronique de l'infection par le SIV. De plus, les patients infectés par le VIH-1 sont traités précocement grâce aux outils diagnostiques disponibles. Nous avons donc analysé deux groupes d'animaux infectés par le SIV, recevant, ou non, une TARV. Sur la base des marqueurs identifiés en cytométrie de masse, nous avons développé un panel simplifié de cytométrie en flux afin d'analyser les neutrophiles sanguins. En parallèle, nous avons utilisé une méthode de mesure des capacités phagocytaires des neutrophiles. Nous avons observé une augmentation de l'expression de CD11b et une diminution de CD62L à la surface des neutrophiles chez les animaux non traités, ce qui suggèrent un état d'activation supérieur. Cet état d'activation était également associé à une réduction des capacités phagocytaires des

neutrophiles. Chez les animaux traités par la TARC, l'état d'activation des neutrophiles était limité mais persistant et les capacités phagocytaires n'étaient pas restaurées.

Dans cette étude nous avons montré que l'initiation du TARC ne permet pas de réduire le niveau d'activation des neutrophiles et de restaurer leur fonction phagocytaire. Nous avons également mis en évidence la circulation de neutrophiles immatures au cours de l'infection chronique avancée par le SIV. Sachant que les neutrophiles représentent 40 à 70% des leucocytes circulants, il apparaît primordial de déterminer si les neutrophiles jouent un rôle dans l'établissement et le maintien de l'inflammation chronique.

Les neutrophiles possèdent une fonction immunomodulatrice durant la phase aiguë et chronique de l'infection par le SIV

Il existe peu d'articles décrivant l'impact de l'infection par le VIH-1 sur les neutrophiles. Dans toutes ces publications, les neutrophiles sont analysés durant la phase chronique de l'infection et sont décrits comme une population homogène. Nous avons précédemment démontré, grâce à une analyse multidimensionnelle, que les neutrophiles forment en réalité une population hétérogène, et qu'une population de neutrophiles immatures est mobilisée au cours de la phase chronique avancée de l'infection par le SIV. Cependant, le stade précis de l'infection par le SIV/VIH-1 auquel se produit la diversification des neutrophiles reste indéterminé. Dans cette seconde étude, notre objectif était de caractériser la diversité des neutrophiles durant les phases aiguë et chronique de l'infection par le SIV. En utilisant la cytométrie en flux et le tri cellulaire, nous avons tout d'abord développé une méthode de suivi des sous-populations de neutrophiles suivant leur stade de maturation chez le macaque cynomolgus : les pre-neutrophiles CD101- (promyélocytes et myélocytes), les neutrophiles immatures CD10- (metamyélocytes et cellules en bande) et les neutrophiles matures CD10+. En parallèle, nous avons également évalué le niveau d'activation des neutrophiles en mesurant l'expression des marqueurs CD62L, CD11b et HLA-DR.

La diversité des neutrophiles est une question de phénotype, mais surtout de fonction. Il existe peu de description de la diversification fonctionnelle des neutrophiles au cours de l'infection par le VIH-1. Dans la littérature, les auteurs décrivent des neutrophiles de faible densité possédant une fonction immunosuppressive sur la production d'IFN- γ par les lymphocytes T. Mais on ne connaît pas les fonctions des autres sous-populations de neutrophiles. De récentes études montrent que les donneurs de cellules souches traitées au G-CSF possèdent à la fois des neutrophiles matures et immatures dans leur sang. Ces deux stades de maturation qui coexistent démontrent respectivement des fonctions immunosuppressives et immunostimulatrices envers les lymphocytes T. Cette étude souligne la nécessité d'étudier simultanément le phénotype et la fonction des sous-populations pour comprendre le rôle des neutrophiles dans une maladie. La fonction immunostimulatrice des neutrophiles au cours de l'infection par le VIH/SIV n'a pas encore été étudiée, alors qu'elle a été mise en évidence dans d'autres maladies inflammatoires chroniques comme les maladies auto-immunes et certains cancers. Dans cette étude, nous avons utilisé une technique de co-culture entre des neutrophiles purifiés par gradient de densité et des lymphocytes T autologues, afin d'évaluer

l'impact des neutrophiles sur la prolifération et la production de cytokines par les lymphocytes T durant l'infection par le SIV.

En primo-infection, même si le comptage des neutrophiles sanguins n'est pas modifié, la répartition des sous-populations de neutrophiles a changé, avec une augmentation de la fréquence des neutrophiles activés CD62L^{low} et immatures CD10-. La circulation de neutrophiles immatures et activés en phase aiguë est associée à une fonction immunostimulatrice, induisant une augmentation de la prolifération des lymphocytes T CD4+, ainsi qu'une augmentation de la production d'IL-2 et d'IFN- γ par les lymphocytes T CD4+ et CD8+. Néanmoins, les neutrophiles ont également montré une capacité à réduire la prolifération des lymphocytes T CD8+. Lors de la phase chronique de l'infection par le SIV, la fréquence des neutrophiles activés et immatures a toujours été significativement élevée. Fonctionnellement, les neutrophiles n'ont pas eu d'impact sur la prolifération des lymphocytes T autologues et la production d'IL-2 et d'IFN- γ . Néanmoins, les neutrophiles ont contribué à l'augmentation de la production de TNF- α par les lymphocytes T CD4+. Parallèlement, nous avons également mesuré la fréquence des neutrophiles de faible densité au cours de l'infection. Celle-ci n'a pas varié significativement au cours du suivi. Ces résultats suggèrent que les neutrophiles possèdent une activité immunostimulatrice envers les lymphocytes T au cours de l'infection par le SIV, ce qui pourrait contribuer à l'activation immunitaire.

L'initiation de la TARC un mois après l'infection a permis de réduire la fréquence des neutrophiles activés et immatures. Cependant, un traitement initié 6 mois après l'infection a eu un effet limité sur ces fréquences, ce qui suggère que la persistance de l'inflammation chronique à long terme pourrait irréversiblement modifier les neutrophiles. Ainsi, notre hypothèse est que l'infection par le VIH-1/SIV façonne les fonctions des neutrophiles, qui en retour participent au maintien de l'état inflammatoire.

Diversité des neutrophiles tissulaires et impact de l'infection par le SIV

Afin de mieux comprendre la dynamique et l'impact des neutrophiles sur l'inflammation chronique au cours de l'infection par le VIH-1, il est nécessaire d'explorer les tissus. Pour ce projet, nous avons développé des méthodes d'analyse par cytométrie en flux et par immunohistologie, permettant la caractérisation phénotypique et la numération des neutrophiles dans les tissus.

Les résultats préliminaires obtenus à partir de prélèvements sur animaux sains ont. Dans la rate et les lavages bronchoalvéolaires, les neutrophiles représentent respectivement 11,9% et 7,8% des leucocytes de ces compartiments. De plus, nous avons observé une fréquence élevée de pré-neutrophiles dans la rate, suggérant une capacité d'hématopoïèse extra-médullaire chez le macaque cynomolgus. En se basant sur l'analyse de l'expression des marqueurs CD62L, HLA-DR et PD-L1, nous avons pu mettre en évidence que les neutrophiles tissulaires ont un profil plus activé que les neutrophiles du sang périphérique. Ces changements phénotypiques ont été décrits dans des modèles murins où les neutrophiles ayant transmigrés montrent un profil activé. Enfin, l'expression des récepteurs au Fc (CD64, CD32a et CD89) est dépendante du tissu étudié. Par exemple, les neutrophiles intestinaux expriment plus faiblement le

marqueur CD89. Ces résultats démontrent la diversité des neutrophiles tissulaires et soulignent la nécessité d'approfondir cette analyse afin de mieux comprendre le rôle des neutrophiles tissulaires dans l'infection par le VIH-1.

Discussion

Avec ce travail nous apportons la première description de la diversité des neutrophiles au cours de l'infection par le SIV. Grâce à une approche d'analyse multiparamétrique et non supervisée, nous avons mis en évidence la présence de neutrophiles immatures dans le sang périphérique à un stade avancé de l'infection chronique par le SIV. Ces résultats suggèrent la mobilisation de neutrophiles immatures depuis la moelle osseuse, tel qu'observé durant le sepsis. Les neutrophiles de patients à un stade avancé de l'infection par le VIH-1 étaient connus pour être altérés et activés, avec une réduction des capacités phagocytaires et de la production d'espèces réactives de l'oxygène. Notre analyse démontre que les neutrophiles sont activés dès le début de la phase chronique, avec des capacités phagocytaires réduites. Ces résultats suggèrent aussi que ces changements phénotypiques et fonctionnels se produisent précocement lors de l'infection. Afin de mieux caractériser la dynamique des neutrophiles au cours de l'infection par le SIV, nous avons exploré de nouveaux marqueurs et combiné le tri cellulaire par cytométrie en flux avec la cytologie dans notre seconde étude. Nous avons l'opportunité de suivre longitudinalement 12 macaques cynomolgus infectés par le SIV. L'infection n'a pas eu d'impact significatif sur la numération des neutrophiles, mais a eu un impact majeur sur la répartition des sous-populations de neutrophiles. Dès la primo-infection, les fréquences des neutrophiles immatures CD10⁻ et activés CD62L^{low} sont augmentés dans le sang périphérique, et deviennent maximales 14 jours post-infection, simultanément au pic de la réplication virale. La fréquence des neutrophiles immatures et activés reste élevée 6 mois après l'infection. La présence de neutrophiles immatures dans la circulation peut être expliquée par une augmentation de l'apoptose des neutrophiles ou une mobilisation de ceux-ci vers les tissus. En effet, il a été montré que lors de la primo-infection la fréquence des neutrophiles apoptotiques est augmentée. Mais d'autres mécanismes peuvent expliquer ce que nous observons en phase chronique. En résumé, la primo-infection s'apparente à une infection aiguë avec une activation et une mobilisation des neutrophiles matures vers les tissus, sièges de la réplication virale. Une hématopoïèse d'urgence se met en place pour compenser les pertes, libérant des cellules immatures dans le sang. La persistance virale et l'incapacité du système immunitaire à éliminer le virus lors de la phase chronique entraîne la persistance des changements phénotypiques des neutrophiles.

La fonction immunomodulatrice des neutrophiles est une découverte récente. Le travail de Marini O. *et al* a mis en évidence le lien entre stade de maturation des neutrophiles et orientation de la fonction immunomodulatrice¹⁹. Ainsi, chez des donneurs sains traités avec du G-CSF, ils démontrent que les neutrophiles matures CD10⁺ et immatures CD10⁻ possèdent des rôles opposés sur la modulation de la réponse T. Notre travail suggère que les neutrophiles immatures ont une fonction immunostimulatrice au cours de l'infection par le SIV, à la fois sur la production de cytokines et sur la prolifération des lymphocytes T CD4⁺. Cette fonction est plus forte en primo-infection, au moment où la réplication virale est intense et la fréquence des neutrophiles immatures est élevée. Du fait de l'absence de méthodes de purification des

neutrophiles chez le macaque cynomolgus, nous avons trié les neutrophiles en utilisant une centrifugation par gradient de densité. Grâce à cette méthode nous avons isolé les neutrophiles de densité normale, mais pas les neutrophiles de faible densité (LDN). Bower NL *et al* ont démontré chez des patients infectés chroniquement par le VIH-1, que les LDN peuvent réduire la prolifération et la production d'IFN- γ par les lymphocytes T²¹. Dans cette étude, la fonction suppressive des neutrophiles est médiée par PD-L1. Dans notre étude, nous n'avons pas observé d'augmentation significative de la fréquence des LDN. De plus, les neutrophiles ne présentaient pas d'augmentation d'expression de PD-L1 et ne possédaient pas de fonction suppressive sur la réponse T. Cette différence de résultats s'explique certainement par le fait que la phase chronique de l'infection par le SIV que nous avons analysé est plus précoce comparée à celle de patients infectés par le VIH-1 depuis des années. Une inflammation chronique persistante pourrait induire de nouveaux mécanismes de diversification des neutrophiles avec l'apparition de LDN aux propriétés immunosuppressives.

L'initiation précoce de la TARC permet un retour progressif du phénotype des neutrophiles au phénotype initial. Au contraire, l'initiation du traitement en phase chronique 6 mois après l'infection a eu un effet limité sur la restauration du phénotype des neutrophiles. Plus le traitement est initié tardivement, plus l'inflammation chronique est persistante et elle modifie durablement cette population cellulaire. Deux mécanismes pourraient expliquer l'impact du traitement tardif sur le phénotype et la fonction des neutrophiles. La première hypothèse est que l'infection entraîne une hématopoïèse d'urgence, qui produit et libère dans la circulation un nombre important de neutrophiles immatures. La seconde hypothèse est que l'infection et l'inflammation, même chez les patients sous TARC, agissent sur l'ensemble des étapes de l'hématopoïèse des neutrophiles, conduisant à la production de neutrophiles fonctionnellement différents. Ainsi, Les neutrophiles matures et immatures produits lors de l'infection seraient différents de ceux générés à l'état basal. Pour confirmer cette seconde hypothèse, il serait intéressant d'évaluer les modifications épigénétiques des précurseurs de neutrophiles.

Perspectives

Nous avons donc mis en évidence une diversification des neutrophiles induite lors de l'infection par le SIV. Néanmoins, nous n'avons pas exploré la fonction immunomodulatrice de chacune des sous-populations de neutrophiles. Pour cette raison, nous développons actuellement une méthode de séparation des neutrophiles chez le macaque cynomolgus. Cette approche nous permettra d'étudier séparément la fonction des neutrophiles matures et immatures au cours de l'infection par le SIV. En effet, Il serait important d'avoir une vision complète des fonctions supportées par ces sous-populations, telles que la production de molécules réactives de l'oxygène, la sécrétion de cytokines, ainsi que la modulation de la fonction d'autres cellules immunitaires. L'analyse transcriptomique des sous-populations de neutrophiles est l'approche que nous avons sélectionnée pour explorer l'impact de l'infection par le SIV sur les fonctions et le métabolisme des neutrophiles. Nous procédons actuellement à cette analyse, qui nous permettra d'accroître nos connaissances sur la biologie du neutrophile au cours de l'infection par le SIV.

Nos résultats, associés aux quelques études disponibles dans littérature, suggèrent que les neutrophiles sont mobilisés dans les tissus lors de l'infection par le VIH-1. A l'exception de l'intestin, le rôle des neutrophiles dans les tissus n'a pas encore été exploré. En combinant la cytométrie en flux à l'immunohistologie, nous prévoyons de caractériser le phénotype et la localisation des neutrophiles dans les tissus. De plus, en mettant à profit la tomographie à émission positron couplé à la tomodensitométrie, (technologie à disposition au sein de notre institut), nous serons capables d'évaluer la distribution des neutrophiles tissulaires au cours de l'infection par le SIV, en utilisant un radio-traceur spécifique de cette population²⁸. L'imagerie corps entier, rendu possible grâce à cette technique d'imagerie, permettrait d'obtenir une vision globale de la répartition des neutrophiles dans l'ensemble des organes simultanément et de manière non invasive.

Il sera également important de confirmer ces résultats chez des patients infectés par le VIH-1 et ce, à différents stades de l'infection. Dans ce but, nous planifions une étude pilote afin de caractériser la diversité des neutrophiles sanguins chez des patients infectés par le VIH-1.

Le singe vert d'Afrique (AGM) est un hôte naturel du virus SIV, contrairement au macaque cynomolgus, qui est un modèle pathologique de l'infection reproduisant la physiopathologie de l'infection humaine par le VIH-1²⁹. En effet, l'AGM est capable de résoudre la réponse inflammatoire induite par la persistance du virus, ce qui le protège d'une évolution vers un stade SIDA. Il serait donc intéressant de caractériser les neutrophiles au cours de l'infection de l'AGM par le SIVagm. Pour cela, les outils développés pour l'étude des neutrophiles chez le macaque cynomolgus pourraient être adaptés à cette espèce. Si les neutrophiles s'avèrent participer activement à l'inflammation chronique, l'étude comparative de l'hôte naturel et du modèle pathologique de l'infection par SIV pourrait être très instructive. Cette analyse permettrait potentiellement d'élaborer des stratégies thérapeutiques ciblant les neutrophiles.

Notre travail a démontré l'importante diversité phénotypique et fonctionnelle des neutrophiles, dont le rôle dans l'infection par le SIV/VIH-1 a été longtemps sous-estimé. Ce travail ouvre la voie vers l'étude des neutrophiles dans de nombreuses autres maladies infectieuses chroniques, où ils sont encore peu étudiés. La tuberculose, le paludisme ou encore les infections à *Chlamydia* sont des infections chroniques dans lesquelles les neutrophiles sont impliqués mais dont le rôle est mal compris³⁰⁻³². Une analyse comparative permettrait d'appréhender l'hétérogénéité des neutrophiles et leurs fonctions modulatrices de la réponse immunitaire lors d'une infection. La caractérisation des neutrophiles dans des modèles expérimentaux de la tuberculose, du paludisme ou de l'infection à *Chlamydia* permettrait une meilleure compréhension du rôle des neutrophiles dans la physiopathologie de ces infections.

De la diversité des réponses inflammatoires semble émerger la diversité des neutrophiles. Une meilleure compréhension de cette diversification dans les maladies infectieuses est nécessaire pour comprendre la physiopathologie des infections et élaborer des stratégies thérapeutiques innovantes, capables de moduler la fonction de cette cellule immunitaire abondante et performante.

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Titre : Hétérogénéité des polynucléaires neutrophiles dans l'infection par le VIH-1.
Etude du modèle macaque cynomolgus infecté par le SIV

Mots clés : SIDA, VIH-1, neutrophile, immunité innée, traitement antirétroviral

Résumé : La persistance du VIH-1 est associée au maintien de l'inflammation chronique chez les patients infectés, malgré la mise en place de combinaison de traitements antirétroviraux. L'inflammation chronique est associée à un risque augmenté de développer des comorbidités, non associées au SIDA. Les polynucléaires neutrophiles (PNN) sont des cellules myéloïdes qui ont été impliqués dans de multiples maladies inflammatoires chroniques. Néanmoins, leur rôle dans l'infection par le VIH-1 est moins bien connue. Afin de pallier ce manque de connaissances, nous avons évalué l'hétérogénéité des PNNs dans le modèle macaque cynomolgus infecté par le SIVmac251. L'analyse phénotypique par cytométrie de masse a révélé la circulation de PNNs immatures en phase chronique de l'infection.

En caractérisant l'hétérogénéité des PNNs au cours de l'infection par le SIV, nous avons observé une augmentation des fréquences des neutrophiles immatures et activés dans le sang dès la primo-infection. En phase chronique, les PNNs immatures et activés étaient toujours significativement augmentés dans le sang et la moelle osseuse. Au cours de l'infection, les PNNs avaient une fonction immunostimulatrice envers la prolifération et la sécrétion cytokinique des lymphocyte T. L'initiation d'un traitement antirétroviral précoce a permis de restaurer le phénotype des PNNs. Les PNNs sont des cellules à fort potentiel pro-inflammatoire qui devraient être ainsi considérés comme de nouveaux effecteurs de l'inflammation chronique associée au VIH-1.

Title : Heterogeneity of polymorphonuclear neutrophils in HIV-1 infection.
Study of SIV-infected cynomolgus macaque model.

Keywords : AIDS, HIV-1, neutrophil, innate immunity, antiretroviral treatment

Abstract: Even under combinational antiretroviral treatments (cART), HIV-1 persistence is associated with chronic inflammation in infected patients, leading to an increased risk of non-AIDS-related comorbidities. Polymorphonuclear neutrophils (PMN), have been less studied in HIV infection whereas they were associated with chronic inflammation diseases. To evaluate PMN heterogeneity in SIVmac251 nonhuman primate infection model, we first performed multiparameter single-cell phenotyping by mass cytometry giving a global vision of the immune system. This analysis demonstrated circulation of immature PMN with impaired during chronic infection. Then, we characterized neutrophils heterogeneity in the course of SIV infection.

In primary infection, there was an increased frequency of CD10- immature and CD62L-low primed PMNs in peripheral blood. In chronic phase, CD10- immature PMNs were significantly higher in bone marrow and blood, maintaining a primed profile. During SIV infection, PMNs demonstrated variable immunomodulatory function against T cells proliferation and cytokine production. Early cART allowed to restore PMN phenotype. In this study, we provide unprecedented insight into PMN heterogeneity in the course of SIV infection. Since PMN represent 40-70% of circulating leukocytes and primed PMN are more potent to release pro-inflammatory cytokines and to transmigrate, they should be considered as a new player in HIV-1 chronic inflammation.