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ANALYSIS OF TRANSCRIPTIONAL AND FUNCTIONAL CAPACITY OF HUMAN NEUTROPHILS DURING AGING

S.S.D. MED/04

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SOMMARIO

L'invecchiamento è associato ad un progressivo declino della funzione del sistema immunitario con conseguente aumento della suscettibilità alle infezioni virali e batteriche. Questo fenomeno, noto come immuno-senescenza, è stato definito "*inflammaging*" da C. Franceschi nel 2000, termine coniato dall'unione delle parole inglesi *inflammation* (infiammazione) e *aging* (invecchiamento). Con questo termine ci si riferisce ad uno stato di infiammazione cronica a bassa intensità e persistente, che è caratterizzato da aumentati livelli sierici di IL-6, TNFα e proteina C-reattiva. Ci sono evidenze sperimentali che questo status influenzi il sistema immunitario (e viceversa) e si è, inoltre, ipotizzato che questo ambiente infiammatorio possa predisporre l'organismo allo sviluppo di varie malattie legate all'età. Mentre le alterazioni dell'invecchiamento che coinvolgono le cellule del sistema immunitario adattativo sono ben documentate, quelle legate all'immunità innata sono meno note. Un numero crescente di studi suggerisce che negli anziani vi possa essere una compromissione di molteplici funzioni dei granulociti neutrofili come, per esempio, la capacità fagocitaria, la produzione di anione superossido e la capacità di sopravvivenza dopo attivazione.

Sulla base di queste premesse, il mio studio mira a caratterizzare varie proprietà funzionali dei neutrofili isolati da individui anziani sani (> 65 anni) e, a compararle, con quelle di neutrofili di soggetti giovani sani (25-35 anni) usando diversi approcci metodologici. Perciò, neutrofili isolati da sangue intero con un altissimo livello di purezza (> 99.7 %) sono stati incubati con vari stimoli per studiarne le funzioni e la capacità trascrizionale, per poi valutare eventuali differenze nei due gruppi. Il gruppo degli anziani sani è stato selezionato sulla base di parametri, definiti dal protocollo SENIEUR, e sono stati esclusi i soggetti non completamente sani o non rientranti nelle caratteristiche definite nel suddetto protocollo. I donatori sono stati, inoltre, sottoposti ad analisi del sangue complete, per valutare il loro stato di salute. Tra i risultati ottenuti con le analisi del sangue vi è un'alta concentrazione plasmatica della proteina C-reattiva negli anziani sani, dato che conferma la letteratura. Per determinare se i neutrofili di individui anziani sani possano avere funzionalità compromesse rispetto a quelli isolati da donatori sani giovani è stata misurata la capacità di produrre specie reattive

dell'ossigeno (ROS), in particolare l'anione superossido (O_2^-), sia dopo stimolazione che non. In cellule non stimolate, la generazione spontanea di O_2^- è risultata essere leggermente più elevata negli anziani rispetto ai giovani, suggerendo così che neutrofili allo stato stazionario possano produrre una maggiore quantità di ROS con l'invecchiamento. Al contrario, confermando alcuni dati presenti in letteratura, è stato scoperto che dopo stimolazione con fMLF, PMA e dopo *priming* con GM-CSF, la produzione di anione superossido da parte dei neutrofili non cambia con l'avanzare dell'età. È stata poi esaminata l'espressione di marcatori di membrana relativi allo stato di attivazione, quali CD11b, CD62L e CD16, non sono state osservate differenze statisticamente significative tra neutrofili isolati da donatori anziani e giovani. Al contrario, studi di espressione genica hanno indicato che l'induzione di geni pro-infiammatori, come IL-6, CXCL8 e TNFα, viene leggermente compromessa con l'invecchiamento in neutrofili stimolati con LPS e R848, ligandi rispettivamente del TLR4 e del TLR8. Per quanto riguarda l'espressione di geni antinfiammatori, non è stato rilevato nessun effetto diretto, almeno per quanto riguarda i geni esaminati SOCS3 e IL-1ra. Inoltre, utilizzando tecniche di sequenziamento di nuova generazione (Smart-seq^2), abbiamo eseguito un'analisi globale del trascrittoma allo scopo di identificare eventuali geni differenzialmente espressi in soggetti anziani rispetto a quelli dei giovani. L'analisi è stata fatta sui neutrofili e, per paragone, sui monociti autologhi, sia appena isolati, sia dopo stimolazione con R848 per 20 h. Dai risultati ottenuti esaminando il trascrittoma si evince un'alterazione dell'induzione di geni pro-infiammatori, nei neutrofili degli anziani come, per esempio, IL-6, CXCL8 e TNFα, dopo stimolazione con R848, confermando quanto ottenuto dall'analisi dell'espressione genica effettuata tramite RT-qPCR. L'analisi necessita di essere approfondita ad altri geni, oltre a quelli elencati in precedenza, per poi poter essere, eventualmente, correlata a difetti funzionali presenti in neutrofili negli anziani, con lo scopo di poter trovare possibili meccanismi patogenici legati all'invecchiamento.

ABSTRACT

Aging is associated with a progressive decline of the immune system function, that ultimately results in an increased susceptibility to viral and bacterial infections. This phenomenon, known as *immunosenescence*, has been defined as "*inflammaging*" by C. Franceschi in 2000, a term coined by joining the word inflammation with aging. This term refers to a state of low-grade chronic inflammation, which is characterized by raised serum levels of IL-6, TNFα and C-reactive protein. There is experimental evidence that this status can influence the immune system and it has also been hypothesized that this inflammatory environment may influence the organism to the development of various age-related diseases. Whereas age-related alterations of adaptive immunity are well documented, detailed analysis of the impact of advancing age on innate immunity remains, however, unresolved. An increasing number of studies suggests that, in the elderly, there is an impairment of multiple functions of neutrophils, such as for instance, phagocytic capacity, superoxide anion production and survival capacity after activation.

Based on these premises, this study aims at characterizing the different functional properties of neutrophils from healthy aged individuals (> 65 years) compared with those of healthy young participants (25-35 years), using multiple methodological approaches (RT-qPCR, smart-seq^2, flow cytometry, respiratory burst assay). Hence, neutrophils, isolated from whole blood of the two enrolled cohorts with a high degree of purity (> 99.7 %) were incubated with various stimuli to evaluate the presence of eventual functional and/or transcriptional differences between the two groups. The elderly group was selected on the basis of criteria defined by the SENIEUR protocol, with exclusion of samples if donors not completely healthy. To assess the health status of donors, they all performed a complete blood test. Among the results of the blood tests, I found high plasma levels of C-reactive protein (CRP), in line with the literature. Furthermore, it has been evaluated the production of reactive oxygen species (ROS) by neutrophils, in particular the superoxide anion (O_2^-), both under resting conditions and after stimulation. Under resting conditions, O_2^- generation was found to be slightly higher in neutrophils from the elderly than in the younger group, thus suggesting

that steady state neutrophils produce higher amounts of O_2^- during aging. On the contrary, in line with some of the data of the literature, I found that, upon stimulation with fMLF or PMA, as well as after priming with GM-CSF, the production of superoxide anion by neutrophil sdoes not change with age. Furthermore, I examined the expression of surface membrane markers related to the activation state of neutrophils such as CD11b, CD62L and CD16. However, I observed no statistically significant differences between the neutrophils isolated from elderly and young donors. Gene expression studies indicated that the induction of proinflammatory genes such as IL-6, CXCL8 and TNFα, was slightly impaired by aging in neutrophils stimulated with LPS and R848, TLR4 and TLR8 ligands, respectively. No changes in the anti-inflammatory SOCS3 and IL-1ra mRNA expression could be instead detected in the same samples. In addition, using new generation sequencing techniques (Smart-seq^2), I performed transcriptome analysis for the purpose of identifying genes differentially expressed in elderly subjects as compared to those of young subjects. The analysis was made in neutrophils and, for comparison, autologous $CD14^+$-monocytes, freshly isolated or after stimulation with R848 for 20 h. The results obtained by examining the transcriptome show that there is an alteration of the induction of proinflammatory genes, such as IL-6, CXCL8 and TNFα in the elderly, after stimulation with R848. These data are in line with what was found in the analysis of gene expression carried out by RT-qPCR. The analysis must be extended to more genes, besides those listed before, in order to eventually correlate them to functional defects present in neutrophils in the elderly and, finally, to find possible pathogenic mechanisms in aging.

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ABBREVATIONS

APC	allophycocyanin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
CBC	complete blood count
CCL	chemokine CC motif ligand
cDC	common dendritic cells
CD	Cluster of differentiation
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP followed by high throughput sequencing
CLR	membrane-bound C-type lectin receptors
CLEC	C-type lectin domain family
CMP	common myeloid progenitor
COPD	chronic obstructive pulmonary disease
CpG	5'- cytosineguanine-3' dinucleotides
CRP	C-reactive protein
CXCL	chemokine CXC motif ligand
CXCR	CXC-chemokine receptor
DC	dendritic cells
DNA	Deoxyribonucleic acid
EDTA	ethylenediamine tetra-acetic acid
eGFR	estimated glomerular filtration rate
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
fMLF	Formyl-Methionyl-Leucyl Phenylalanine
FPKM	Fragments per kilobase of transcript per million mapped reads
FPR	formyl peptide receptor
G-CSF	granulocyte colony stimulating factor
GADPH	glyceraldehyde phosphate dehydrogenase
GFR	Glomerular filtration rate
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GMP	Granulocytes myeloid progenitors
H_2O_2	Hydrogen peroxide
H3K27Ac	Histone 3 Lysin 27 Acetylation
HBSS	Hank's Balanced Salt Solution
HDL	High density lipoprotein
HLA-DR	major histocompatibility complex class II
HOCl	hypochlorous acid
HOBr	hypobromous acid
HOI	hypoiodous acid
HBG	haemoglobin
HCT	haematocrit
ICAM	Intercellular Adhesion Molecule
IF	immunofluorescence
IFN β	Interferon β
IHC	immunohistochemistry
IL	Interleukin
iNKT	invariant natural killer T cells
IP	Immunoprecipitation
kbp	kilo base pairs
LDL	Low density lipoprotein
LDTFs	lineage-determining TFs
LPS	lipopolysaccharide
MAC	macrophage receptor 1
MAPK	mitogen-activated protein kinase
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MMP	matrix metalloproteinase
MNE	mean normalized expression
MPO	myeloperoxidase
MPV	mean platelet volume
mRNA	messenger RNA

MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NGS	next generation sequencing
NKT	natural killer T cells
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
nt	nucleotide
O_2	singlet oxygen
O_2^-	Superoxide anion
O_3	Ozone
O.D.	Optical density
•OH	hydroxyl radical
PAF	platelet activating factors
PAMPs	pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer salt
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PCR	Polymerase Chain Reaction
Phox	phagocyte oxidase
PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophils
PRR	pattern recognition receptors
RA	rheumatoid arthritis
RDW	red cell distribution width
RIG-I	Retinoic acid-inducible gene I
RNA	Ribonucleic acid
RNAi	RNA interference
RNA-seq	RNA - followed by high throughput sequencing
ROI	reactive oxygen intermediates
ROS	reactive oxygen species

RPL32	Ribosomal Protein L32
RT-qPCR	reverse transcription quantitative PCR
SASP	Senescence-associated secretory phenotype
SEM	Standard error mean
SENIEUR	Senior Europeans
SOCS3	Suppressor of cytokine signaling 3
TF	Transcription factor
Th	T helper
TLR	Toll-like receptor
TNF	tumour necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN β protein
TRIF	TIR domain-containing adaptor protein inducing interferon β
TREM	triggering receptor expressed on myeloid cells
TSO	template-switching oligos
VCAM	vascular cell adhesion molecule
WBCs	white blood cells

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INTRODUCTION

NEUTROPHILS

Neutrophils, also called polymorphonuclear leukocytes, are the most abundant population of circulating white blood cells representing the 40% - 60% of total leukocytes [1]. They play a major role in mediating the earliest phases of the inflammatory response. The appropriate initiation and resolution of inflammation is crucial for the clearance of infection and the prevention of nonspecific tissue damage, which otherwise can lead to chronic inflammatory diseases such as obstructive pulmonary disease (COPD), cystic fibrosis, rheumatoid arthritis (RA) [2].

The name "polymorphonuclear leukocytes" is due to the multi-lobed shape of the nucleus of these cells, which distinguishes them from other white blood cells of lymphoid or myeloid origin, such as lymphocytes and monocytes. Neutrophils are generated in the bone marrow during granulopoiesis from the granulocyte-monocyte progenitor precursors (GMPs), a myeloid-committed progenitor cell. Neutrophils are produced from GMPs by subsequent differentiation stages from the most immature to the most mature cells: myeloblasts, promyelocytes, myelocytes, metamyelocytes, band cell and segmented neutrophils [3]. Under normal homeostatic conditions, neutrophils are released into the systemic circulation only when fully mature.

Neutrophils have a short lifespan (approximately a half-life of 8 h) and are estimated to be produced at a rate of 1-2 × 10^{11} cells per kilogram body weight per day in a healthy adult human [4], surviving in the blood for hours or a few days. After the release from the bone marrow, neutrophils circulate in systemic blood as spherical cells of about 12 to 15 μm in diameter, displaying high levels of physical plasticity, a characteristic that allows them to move through capillary networks [5]. Neutrophils are activated by pathogen-associated molecular patterns (PAMPs), such as formyl-methionyl-leucocyl-phenylalanin (fMLF), endotoxin, and other Toll-like receptor (TLR) ligands. Alternatively, they can be activated by cytokines such as granulocyte-monocyte-colony stimulating factor (GM-CSF), IL-

15, IL-18 or ligands triggering receptor expressed on myeloid cells (TREM-1). To increase their bactericidal potential, their life-span is extended at the site of infection by bacterial components such as LPS, complement factors, platelet activating factors (PAF) and pro-inflammatory cytokines such as TNFα, IL-6, IL-1β [6-8], as well as granulocyte colony-stimulation factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) [9].

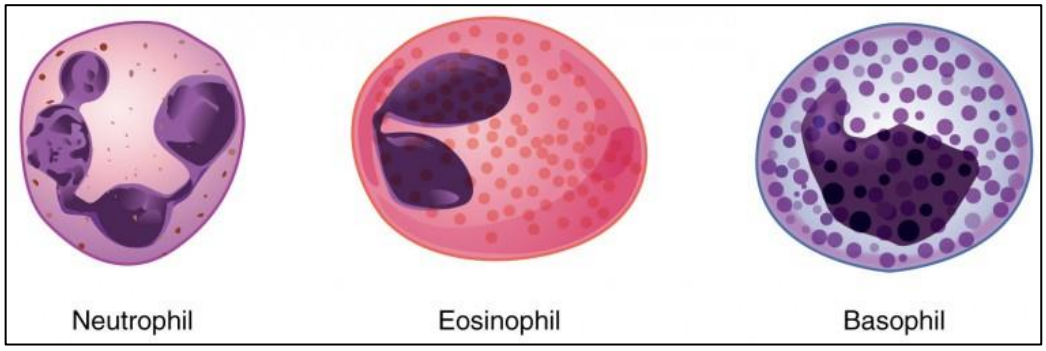

Figure 1 | Granulocytes: neutrophils, eosinophils and basophils.
The specific granules of neutrophils stain weakly, as they are "neutral", hence the term neutrophil. The nucleus of eosinophils usually has only two lobes, while almost all of the cytoplasm appears filled with the specific granules. As the term "eosinophil" indicates, these granules are not neutral but stain red or pink when eosin or a similar dye is used in the staining process. On the right, a basophil having nucleus lobes usually not as well defined as in neutrophil granulocytes but appearing S-shaped. The specific granules of basophils are stained deeply bluish or reddish-violet.

Neutrophils, basophils and eosinophils belong to the granulocytes, so called because their cytoplasm is characterized by the presence of numerous granules (**Figure 1**). In neutrophils, the granules are traditionally divided into three sub-types: azurophilic (primary), specific (secondary) and gelatinase (tertiary), and it is possible to discern between them based on the enzymes each type of granule contains. The nomenclature primary, secondary and tertiary is due to the time in which each type of granules is formed during the maturation of neutrophil in the bone marrow. Azurophilic granules contain neutral serine proteinases, (elastase, proteinase 3, and cathepsin G), myeloperoxidase (MPO) and other antimicrobial proteins (e.g. α-defensins). Specific granules are also predominantly bactericidal and contain products such as lactoferrin [10].

Gelatinase granules contain metalloproteases (MMPs) such as gelatinase which digest extracellular matrix and aid neutrophil migration [11] (**Figure 2**). These granules weakly stain with either basic or acidic dyes (hematoxylin and eosin, respectively), which distinguishes neutrophil granules from those of basophils and eosinophils (**Figure 1**).

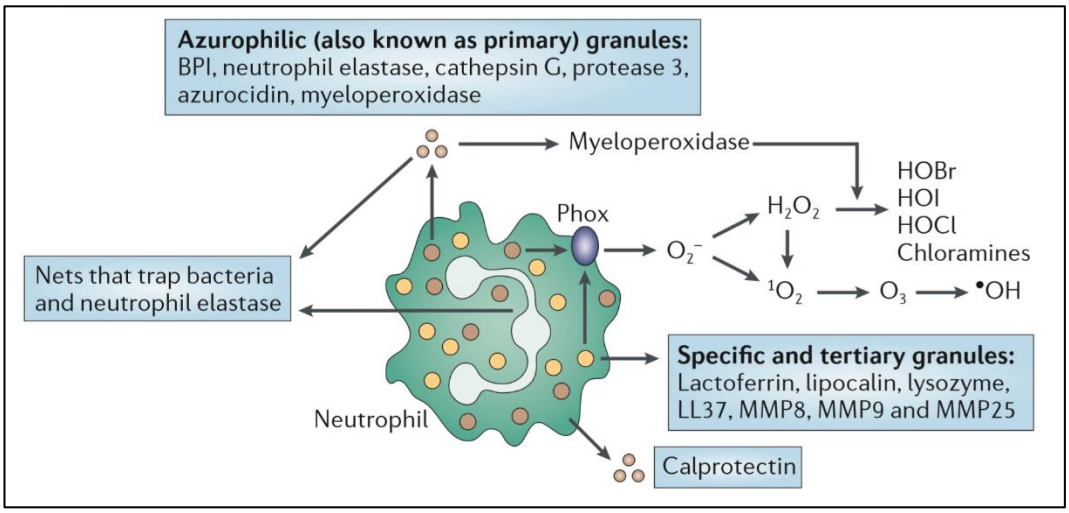

Figure 2 | Granules and anti-microbial molecules of neutrophils.
Microbicidal products arise from most compartments of the neutrophil: azurophilic, specific and tertiary granules, plasma and phagosome membranes, the nucleus and the cytosol. (Nathan, C., Nat Rev Immunol, 2006. **6**(3): p. 173-82.) [2]

NEUTROPHILS AND INFLAMMATION

Neutrophils are key component of the innate immunity and they are particularly known for their potent anti-microbial functions which make them the first line of defense against pathogens [12]. Following release from bone marrow, neutrophils circulate in the vasculature and upon inflammation or infection, they rapidly migrate in high numbers toward the injured site (e.g., infection, tissue damage), where they exert classical defensive functions, including degranulation, release of reactive oxygen species (ROS) and lipid mediators, pathogen phagocytosis and elimination [4, 13]. Degranulation of specific granules and the extrusion of NETs create an antimicrobial environment at the inflammatory site that contributes to the killing of pathogens.

Neutrophils extravasate through endothelia of post capillary venules [2] in response to chemotactic agents, amongst which interleukin-8 (CXCL8) is the most potent, that are produced in response to proinflammatory stimuli by macrophages, mast cells, epithelial cells, keratinocytes, fibroblasts, endothelial cells and neutrophils as well [14]. In addition, many bacterial products, such as fMLF and peptidoglycan, directly function as chemo-attractants and contribute to neutrophil recruitment [15]. To enter the infected tissue, neutrophils slowly roll along the endothelial surface through interactions of a family of C-type lectin glycoproteins known as selectins (type L, P, E- selectins), establishing a low-affinity adhesive interaction [15]. L-selectins, in particular CD62L, are transmembrane glycoproteins present on the surface of neutrophils, while E and P- selectin are expressed by endothelial cells. The endothelium has an active role in this step, in fact, the interaction of selectins with their ligands enables leukocytes to adhere to the inflamed endothelium under condition of blood flow (**Figure 3**).

Subsequently, extracellular chemotactic factors, mostly activating G-protein coupled receptors (GPCR) on the neutrophil, initiate β_2-integrin inside-out signaling, shifting integrins into an active state. These active β_2-integrins subsequently bind to their ligand, for instance intercellular adhesion molecule 1 (ICAM-1) for CR3 ($\beta_2\alpha_M$, also known as CD11b/CD18), which enables neutrophils to stop rolling and adhere to the vessel wall [16]. The arrest phase is mediated by the binding of integrins to immunoglobulin superfamily members, such as intracellular adhesion molecule-1 (ICAM-1) and ICAM-2, expressed by epithelial cells (**Figure 3**). After firm interaction, neutrophils crawl over the vessel endothelium in the search for a suitable location to transmigrate, either paracellularly or in rare cases transcellularly, into the underlying tissues. Several neutrophil surface molecules, including CD31, CD54, CD44, and CD47, facilitate the penetration into the junctions and the underlying basement membrane without damaging these structures, as well as and the transmigration through the endothelium into tissues [15]. Once in the tissues, neutrophils migrate in an integrin-independent manner, and ultimately employ their anti-microbial capacity by mechanisms that include (1) the powerful oxidative burst, (2) the release of

proteolytic enzymes stored in granules, (3) the phagocytosis, and (4) the formation of neutrophil extracellular traps (NETs) [17] (**Figure 3**).

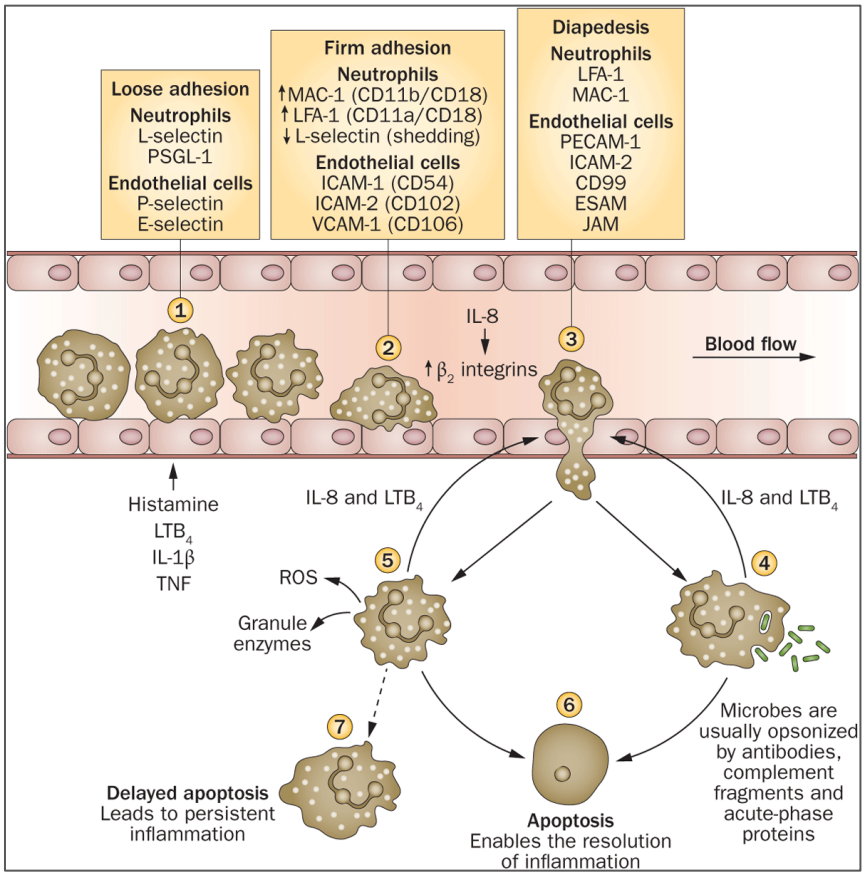

Figure 3 | Migration of circulating neutrophils to sites of inflammation or infection.
(1) Neutrophil rolling. (2) Firm adhesion of neutrophils to endothelial cells. (3) Paracellular migration of neutrophils through intercellular junctions of endothelial cells. (4) Transmigrated neutrophils phagocytose and kill microbes. (5) Activation of neutrophils by soluble immune complexes induces the release of ROS and granule enzymes. (6) Apoptotic neutrophils are removed by macrophages and other phagocytic cells. (7) Delayed apoptosis results in persistent inflammation and tissue damage, due to the continued release of ROS, granule enzymes and cytokines [18].

Neutrophils move towards a chemotactic gradient until they localize microbes. If the chemotactic gradient disappears, the cells execute themselves by apoptosis. Although neutrophils appear to be committed to apoptosis, recent evidence demonstrates that the *in vitro* life span and functional activity of mature

human neutrophils can be significantly extended by their incubation with proinflammatory mediators, bacterial products and cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), lipopolysaccharide (LPS) and interferon-γ (IFN□). These active biological signals also regulate PMN function in order to strengthen PMN activity under conditions that require increased neutrophil function, such as bacterial infection. Therefore, a large body of experimental evidence indicates that PMN apoptosis plays a fundamental role in the regulation of host defense and inflammatory response [19].

PATTERN RECOGNITION RECEPTORS

Recognition of microbial pathogens is an essential condition for the initiation of the innate immune response, and it is mediated by germline-encoded pattern-recognition receptors (PRRs). PRRs are present on the cell surface, in endosomal vesicles and in the cytoplasm of a cell, and they are able to recognize molecular structures that are shared by pathogens, known as pathogen-associated molecular patterns (PAMPs). PRRs comprise Toll-like receptors (TLRs), membrane-bound C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and RIG like receptors (RLRs) [20] (**Figure 4**). TLRs were the first PRRs to be identified, and for this reason they are the most well-characterized. To date, 10 functional TLRs have been identified in humans (TLR1-10), which are able to recognize a wide range of PAMPs derived from viruses, bacteria, mycobacteria, fungi, parasites. These PAMPs includes lipoproteins, dsRNA, lipopolysaccharide, flagellin, ssRNA and DNA. Each TLR detects distinct PAMPs [21, 22].

TLRs belong to the type I transmembrane proteins and they are composed of a leucine-rich repeats ectodomain, which is involved in ligand binding, a transmembrane region and a cytosolic Toll/IL-1 receptor (TIR) domain, which is essential for signaling. TLRs are found on the cell surfaces and on intracellular membranes. For instance, TLR1/6, TLR2, TLR4, and TLR5 are expressed on the cell surface for sensing extracellular lipids, lipoproteins, and proteins derived from cell membrane components, whereas TLR3, TLR7/8, and TLR9 are mainly

localized inside cells on endoplasmic reticulum (ER) and endosomal membranes where they detect nucleic acids.

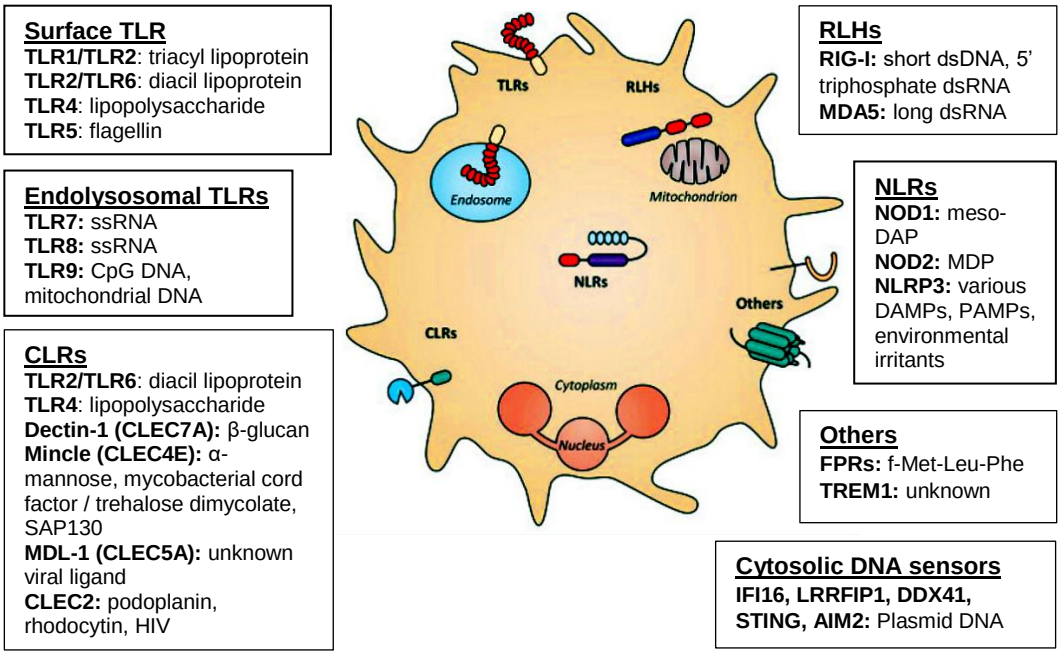

Figure 4 | Pattern recognition receptors expressed by neutrophils.
Pattern recognition receptor families and respective members expressed by neutrophils. Adapted from [23] with the data from [24].

Human neutrophils are known to express almost all TLRs (**Figure 4**), with the exception of TLR3 and TLR7 [25, 26]. Among the various TLRs, the most studied in human neutrophils is TLR4 - which mediates responses to Gram-negative bacteria, recognizing the lipid A component of lipopolysaccharide (LPS) - and TLR8 - which recognizes single strand RNA (ssRNA) of viral origin and is also strongly activated by imidazoquinoline compounds such as R848 (Resiquimod). The latter displays potent antiviral activity, activating immune cells via endosomal TLR7/TLR8, and it represents a very potent activator of survival of, and prolonged gene expression changes and cytokine expression in, human neutrophils. In this context, our group has reported that human neutrophils can *de novo* express and produce biologically active amounts of IL-6, but only after an overnight incubation with TLR8 ligands, which indeed display the capacity to remodel chromatin at the *IL-6* locus [27].

In monocytes, macrophages and dendritic cells, recognition of LPS by TLR4 and the co-receptors CD14 and MD-2 triggers two signaling pathways that rely on the adaptor proteins MyD88 and TRIF (TIR domain-containing adapter inducing IFNβ) [28]. By contrast, TLR4 activation by LPS in human neutrophils fails to directly trigger the TRIF-dependent pathway. The latter pathway is known to activate, throughout downstream signals, interferon regulatory factor 3 (IRF3), a transcription factor (TF) crucial for the induction of transcription of interferon beta (IFNβ) messenger RNA (mRNA). Moreover, endogenous IFNβ is known to promote the expression of interferon-stimulated genes (ISG), such as, for instance, CXCL9/CXCL10/CXCL11. Thus, since neutrophils are unable to activate the TRIF-signaling pathway in response to LPS, consequently they do not express IFNβ and/or other ISG mRNAs [29].

PRODUCTION OF CYTOKINES, CHEMOKINES, AND ANTIMICROBIAL AGENTS BY NEUTROPHILS

As already mentioned, once released into the circulation, neutrophils proceed to seek signs of infection and inflammation which lead to a series of events culminating in the migration through the vessel wall and inside the tissue to the site of microbial invasion, where they begin a professional antimicrobial killing program [30, 31]. Among the antimicrobial activities by neutrophils, there is the extrusion of NETs, which represents an important strategy to immobilize and kill invading microorganisms. NETs consist of a scaffold of chromatin fibers, among which DNA and histones represent the major constituents, and granular proteins such as neutrophil elastase, cathepsin G, and myeloperoxidase (MPO). Subsequently, neutrophils phagocytose the infectious particle they have encountered [32] and generate various antimicrobial agents, including reactive oxygen species which are produced by the activity of the NADPH oxidase [33]. Then, the contents of intracellular granules are released into the phagosome, resulting in killing and digestion of the microorganisms [34]. Once infection is cleared, neutrophils die by apoptosis, to be subsequently recognized and ingested by macrophages to get resolution of inflammation [35] (**Figure 5)**. Recent

evidence suggests that neutrophils are directly involved in the active induction of resolution through the production of pro-resolving lipid mediators [36, 37].

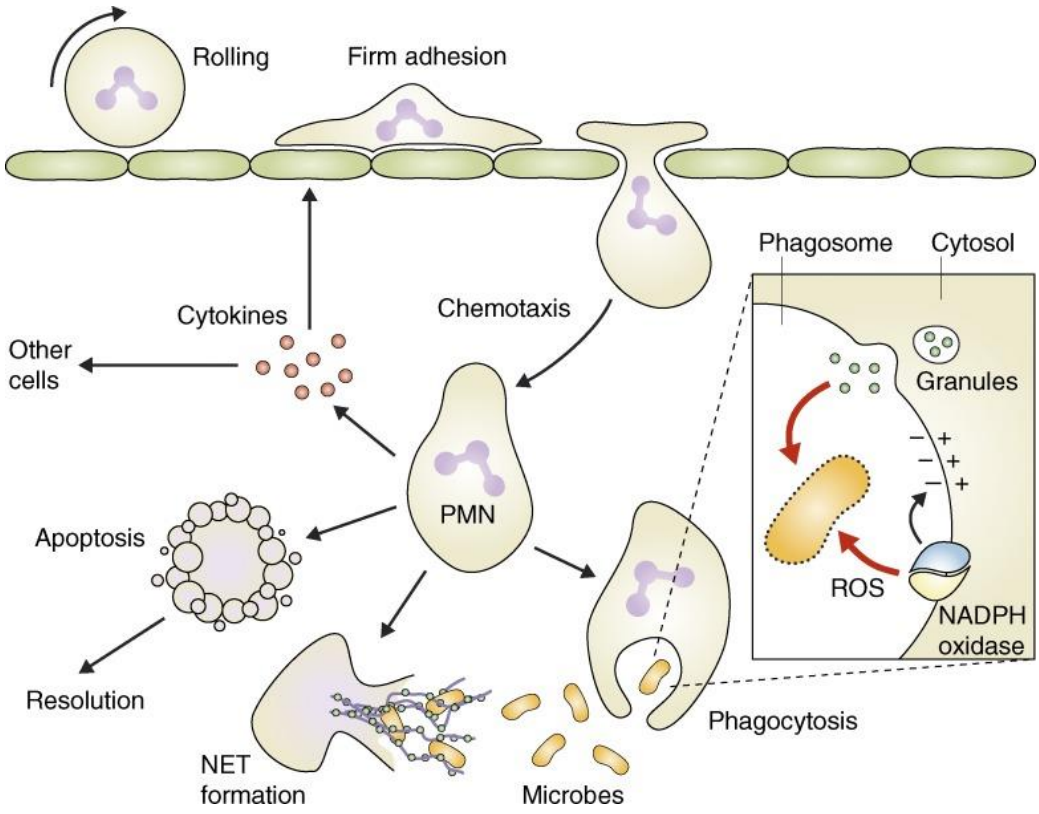

Figure 5 | Functions of neutrophils at the site of inflammation.
After migrating to the site of inflammation, neutrophils phagocytose and digest the invading microbes, as well as release NETs and produce cytokines, which contribute to the inflammatory process. Once infection is cleared, neutrophils die by apoptosis. Pathogen killing inside the phagosome occurs by ROS generated by the NADPH oxidase, as well as by granule enzymes released from intracellular granules. Adapted from [38].

Superoxide anion

Neutrophils generate reactive oxygen species (ROS) during phagocytosis and in response to pathogenic and host-derived inflammatory signals. This functional response, also referred to as “respiratory burst”, plays an important role in innate immunity against invading microorganisms. It is performed by the Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme which is only activated when its cytosol-based and membrane-based components assemble together [39]. NADPH oxidase is a multiprotein complex that is dormant in quiescent cells since its components are segregated into the cytosolic

and membrane compartments. In response to stimulation, these components rapidly assemble at cell membranes [33]; for instance, neutrophils are able to assemble NADPH oxidase complex on the phagosome membrane allowing it to catalyze the reduction of O_2 to form superoxide anions (O_2^-) [40]. The superoxide anion is not itself a reactive oxidant, but represents a substrate for the production of other reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and hypochlorous acid (HClO) [41], and these species can interact with an unlimited number of macromolecules. Although ROS production contributes to host defense, being critical for the killing and degradation of internalized bacteria and particles, it can also cause collateral damage to host tissues in case of unbalanced response [42].

Cytokines

Cytokines are proteins secreted by a broad range of cells, including immune cells, endothelial cells, fibroblasts, and various stromal cells. Cytokines mediate many of the functions of these cells and take part to direct important biological processes such as inflammation, immunity, repair, and angiogenesis [43]. Human neutrophils, particularly in response to microbial antigens and cytokines, not only synthesize numerous proteins that directly participate in their effector functions, including complements components, Fc receptors, but also produce a variety of pro- and anti-inflammatory cytokines (including, IL-1β, IL-1ra, IL-6), chemokines (including CXCL1, CXCL8, CXCL10, CCL2, CCL3, CCL4 and CCL23 [44]), TNF superfamily members (including TNFα, TRAIL, FasL, and BAFF), colony-stimulating and angiogenic factors (HB-EGF, G-CSF, VEGF) [45, 46] (**Figure 6**). In this context, the cytokines that human neutrophils are able to express and release, either constitutively or upon activation by microenvironmental stimuli [45], are crucial for the role of neutrophils in innate and adaptive immune responses and for their role in defense and pathology.

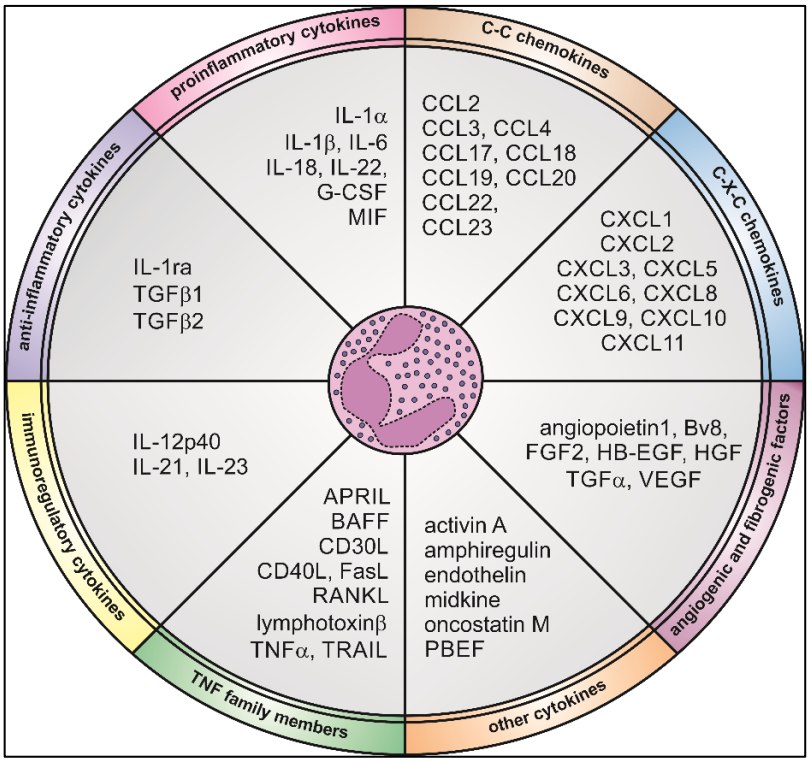

Figure 6 | Cytokine production by human neutrophils.
Expression and/or production of the listed cytokines have been validated in human neutrophils by gene expression techniques, immunohistochemistry, enzyme-linked immunosorbent assays (ELISAs) or biological assays. Adapted from [47].

Cytokine production by neutrophils is controlled by regulatory mechanisms that act at different levels, including mRNA transcription (as in the case of CXCL8 and CCL3 mRNA in neutrophils treated with LPS), stability (as in the case of IL-1ra mRNA in neutrophils treated with IL-13) or translation (as in the case of TNFα mRNA in neutrophils treated with GM-CSF), as well as protein secretion. In addition, epigenetic mechanisms are involved in the control of cytokine expression [48]. If compared to monocytes/macrophages, lymphocytes or dendritic cells (DCs), neutrophils produce lower amounts of cytokines per cell basis, at least *in vitro* [45, 49]. This is because they possess 10-20 times less total RNA than other leukocytes [50]. However, even though a single neutrophil generally produces very little amount of cytokines, neutrophils overtake in numbers mononuclear leukocytes by 1-2 orders of magnitude in case of acute infections or in inflammatory site [47], thus implying that the sum of cytokines

produced by activated neutrophils can certainly shape the inflammatory response. Furthermore, neutrophils can *de novo* synthesize cytokines that can be stored in intracellular compartments and then secreted in response to appropriate stimuli, for example: B-cell activating factor (BAFF), TNF-related apoptosis-inducing ligand (TRAIL), CXCL8, CCL20, and interleukin-1 receptor antagonist (IL1-ra) [51, 52].

Cross-talk between neutrophils and innate immune cells

There is emerging evidence indicating that neutrophils not only are involved in the killing of extracellular pathogens but also contribute to the immune response to intracellular pathogens and viruses through complex cross-talk with other immune cells, such as DCs, lymphocytes, and NK cells. Much of this cross-talk is mediated by the ability of neutrophils to secrete a host of cytokines or express a large number of cell surface molecules that directly interact with other immune cells [35]. These findings are changing our traditional view of neutrophils from terminally differentiated effector cells to transcriptionally and functionally active partners in the entire immune response. Once recruited into inflamed tissues as well as in lymph nodes, neutrophils can interact with a number of different cell types such as macrophages, natural killer, dendritic and mesenchymal stem cells, B and T lymphocytes, or platelets. This cross-talk occurs either directly by contact-dependent mechanisms, or indirectly by cell-derived soluble factors, such as proteases, cytokines, and radical oxygen species. [53-55]. Neutrophils receive signals able to modulate their survival and effector functions on the one hand, whereas they initiate, amplify, and/or suppress innate or adaptive immune effector responses, on the other hand [35, 56].

AGING

Aging is a complex biological process that leads to several physiological changes. Among these changes, the most striking are those involving both the innate and the adaptive arms of the immune system, resulting in their deregulation [57, 58]. Furthermore, an impairment of the immune system functions may impact on pathogenesis of severe age-related diseases, such as Alzheimer disease, atherosclerosis, type-2 diabetes, sarcopenia, and osteoporosis [59, 60]. Age-associated decline of the immune system, referred to as *immunosenescence* [61], has been linked to poor responses to vaccines and higher incidence of infections (including influenza and bacterial pneumonia) [62], cancer and neurodegenerative and cardiovascular diseases, that ultimately contribute to increased morbidity and mortality in the elderly [63, 64]. During aging, many cells acquire the senescence-associated secretory phenotype (SASP) characterized by the increased expression of IL-1α and other inflammatory cytokines such as IL-6 and CXCL8, which alter and remodel the tissue microenvironment [65]. SASP is able to alter the function of nearby cells and generates a sort of low-level chronic inflammation [66]. Components such as IL-6, CXCL8, and MMPs can promote tissue repair, but also cancer progression. Senescent cells indeed accumulate in tissues of humans with age, as well as at sites of tissue injury. Furthermore, cells with senescent cell properties can be found in affected tissues of patients with age-related diseases such as osteoarthritis, pulmonary fibrosis, atherosclerosis, and Alzheimer's disease, in turn suggesting that attracting immune cells and inducing local inflammation are common properties of senescent cells [66, 67].

A large part of the aging phenotype, including *immunosenescence*, is explained by an imbalance between the proinflammatory and anti-inflammatory network, which results in the low grade chronic inflammatory status that C. Franceschi proposed to call *inflammaging* [68]. This state is characterized by increased levels of inflammatory mediators, e.g. TNFα, IL-6, IL-1β and acute phase proteins including CRP [69]. Moreover, evaluation of several cohorts has shown an association between elevated serum levels of IL-6 and mortality and disability in the elderly, leading to consider the high levels of IL-6 in the serum as a reliable marker/predictive index of *inflammaging* [65, 68, 70-72]. Studies in

large elderly cohorts show that serum levels of IL-6 are markedly higher in randomly selected elderly humans compared with strictly selected healthy elderly humans (SENIEUR protocol [73]). Taken together, these findings indicate that it is important the way in which elderly subjects are chosen for the analysis. Using a protocol which strictly selects healthy cohorts, such as the SENIEUR protocol, is crucial in order to exclude the incidence of diseases or pharmacological interferences.

Low-level, chronic, "sterile" inflammation is a hallmark of aging that initiates or promotes most, if not all, major age-related diseases [71]. Accordingly, *immunosenescence*, concomitantly with *inflammaging*, is considered as the leading cause of age-related diseases including cardiovascular, neurodegenerative and metabolic diseases, and cancer. Studies have suggested that immune senescence and *inflammaging* are due to epigenetic regulation of the aging immune system [74-76]. The idea is that epigenetic modifications contribute to aging by compromising the ability of the genome to code optimally for the maintenance of a youthful state [77]. Previous studies mainly focused on age-related changes in adaptive immunity have shown changes in T cell redistribution. Commonly observed in the elderly are: decreased T cell memory, exhaustion of the naïve T cell population with involution of the thymus [58], decline in B cell production reflected in defective humoral immunity [78]. Cumulative evidence indicates that aging exerts significant effects also on various cells of the innate immune system, such as dendritic cells, NK, NKT, neutrophils and monocytes [8].

Aging and neutrophils

All immune cells are subjected to the aging process, thus contributing to the elevated susceptibility to infections as well as to the decreased response to vaccination and, in turn, increased mortality observed in the elderly, due to viral and bacterial infections [68, 74]. The impact of aging on immunity has been mainly addressed within the context of the acquired arm of the immune system, generally referred to the altered functions of $CD4^+$ and $CD8^+$ T lymphocytes [79]. In contrast, knowledge concerning age-related defects of the innate immune

system, particularly of neutrophils, is scarce. Because neutrophils are the leukocytes that more rapidly respond to invasion by pathogens, it is plausible that an age-related decline in their function may be partially responsible for the increased susceptibility to infections seen in the elderly [74, 80, 81]. Moreover, there are clinical data showing that age is an independent risk factor for the development of chronic inflammatory diseases characterized by a partial dysfunction of neutrophil activities [82].

The literature presents several studies appearing contradictory, some of them reporting discrete functional changes related to neutrophils in elderly individuals, that other studies do not. Many of these studies have some weaknesses, in particular concerning the levels of neutrophil purity and the lack of systematic approaches. Whatever the case is, while there is consistency about the fact that the number of circulating neutrophils remains constant throughout aging [83, 84], several studies propose that some of their functions, such as the ability to infiltrate tissues, adhere, move towards chemotactic gradients, phagocytose, rescue from apoptosis and production of superoxide anion or other reactive oxygen species, might be compromised in the elderly, even though it has not been observed always unanimously [85-87].

The regulation of apoptosis of neutrophils is important either to maintain longer survival in inflamed tissues, or for the resolution of inflammation (neutrophils display a fast-apoptotic rate in vitro as well as in vivo). For this reason, apoptosis has to be well-balanced to ensure neutrophil survival and production; if the balance is shifted, the risk of chronic inflammatory diseases is enhanced. Without stimulation, the susceptibility of neutrophils to apoptosis has been reported to be either slightly increased or unaffected by aging [19, 88]. In contrast, there are some studies reporting that rescue from apoptosis is disturbed. It has been shown that the functions and the rescue from apoptosis by survival factors of neutrophils, such as G-CSF, GM-CSF, TNF-α or LPS, which usually can extend their life-span, can diminish as we age [19, 89, 90]. An altered signal transduction mechanism was demonstrated by Fortin and collegues which reported that the protective effect of GM-CSF did not occur in neutrophils isolated from elderly subjects [90]. They found that Jak/STAT and MAPK pathways

become altered with aging in PMN upon GM-CSF stimulation. Overall, hypothesizing that the increase of the apoptotic rates of neutrophils at the site of infection in the elderly might cause decreased bactericidal functions. Moreover, an impaired apoptosis may lead to the accumulation of neutrophils with altered functions in the elderly.

Some investigators reported that chemotaxis remains largely unaltered in the elderly [91, 92] or at least display a normal reaction after stimulation with fMLP [93, 94]. Other research groups, instead, found impaired chemotaxis when using other chemotactic substances, GM-CSF, LPS, or complement [87, 95]. The consequence of the latter is that a fast recruitment to sites of infection is functionally restricted. In addition, an unchanged chemotaxis is described in centenarians [96], suggesting that the loss of this function could indeed be partly responsible for the failure of a proper immune reaction in the elderly. That might explain the occurrence of severe wound infections by elderly persons since small numbers of pathogens cannot be efficiently eliminated.

After receiving a chemotactic signal, the rolling neutrophil adheres via integrin molecules to endothelial cells and migrates through the endothelium (diapedesis) towards the site of infection. Adhesion is reported to be unchanged [91, 95] or slightly increased in old donors. Several studies demonstrated that neutrophils from young and old donors, stimulated with PMA, zymosan, or fMLF, show the same ability to adhere to endothelium, gelatin, plastic, or nylon surfaces [91]. Regarding surface expression of activation markers, contrasting data are present in the literature [86, 97-99], probably due to differences in the timing and type of stimulation or the measurement in whole blood instead of isolated neutrophils. The expression of CD11a and CD11c by neutrophils, necessary for their extravasation [94], as well as the expressions of CD11a/CD18, CD11b/CD18, and CD14, are not altered or even slightly increased. The slightly increased expression of CD11b and CD15 could be, thus, the result of the enhanced adhesion of neutrophils from aged subjects to endothelial cell monolayers after the stimulation with fMLF or PMA [92, 96]. On the contrary, no increase of CD11b and CD15 but a decrease of CD62L was observed by others in neutrophils from elderly donors [98, 100]. It should be here recalled that CD62L

is usually shed by neutrophils upon activation. The changes found in decreased chemotactic functions may be associated with the loss of CD62L. In fact, CD62L-mediated migration might be hampered, and this might lead to increased infection. As postulated by other groups [100], the shedding of CD62L from the cell surface of neutrophils is also a sign of pre-activation which might contribute to the inflammatory status in the elderly. Moreover, one has to be cautious with regards to the purification process of neutrophils since some substances may cause a decrease in CD62L expression. Together, the enhanced adhesion and the impaired chemotaxis could be correlated with the higher susceptibility to infections. These changes result in the reduced ability to eliminate bacteria and fungi, but also inhibit the interaction with and influence on the adaptive immune system.

Whether there are age-related decreases in the phagocytic ability of neutrophils, is still controversially discussed. Some studies show that phagocytosis is unimpaired in the elderly [96, 98], in contrast to others reporting that is normal [93]. Reasons for the decreased phagocytotic ability could be the changes in actin polymerization [101], or the significant age-related reduction of expression of Fcγ III receptor CD16, which is necessary for phagocytosis [98].

Additional studies reveal that neutrophils from elderly have a pre-activated basal state confirmed by an increased ROS production compared to neutrophils from young subjects [86]. However, there are controversial findings concerning changes in oxidative burst by neutrophils from aged individuals. Some *in vitro* studies using neutrophils from old donors describe a normal O_2^- production after stimulation with fMLF or gram-negative *Escherichia coli* [93, 94]. While other groups report that after stimulation with GM-CSF, LPS, TREM-1, and fMLF, or the direct injection of gram-positive bacteria such as *Staphylococcus aureus*, oxidation burst of is impaired in neutrophils of old individuals [88, 91, 102, 103]. Moreover, other studies using SENIEUR protocol selected donors could not detect a difference in respiratory burst compared to younger persons even after stimulation with fMLP [93, 94]. An early report by Fulop et al. [95] showed that signal pathways may be impaired: after stimulation with fMLF or GM-CSF for 24 h, a reduced superoxide anion production is reported for neutrophils from aged donors. These data [94] suggest that the different outcomes of the studies are

partly due to differences in timing of the analyses. Since the oxidative burst is a complex reaction and can be detected by the measurement of various parameters, the difference of results can also be due to different measuring techniques.

Recent publications indicate a decline in signal transduction as being responsible for receptor-mediated responses and apoptotic rescue mechanisms, in reference to this, there is growing evidence that altered plasma membrane content and fluidity of neutrophils in the elderly appear to influence signal transduction [7, 91, 93, 95]. In contrast to other cells the fluidity of the neutrophil membrane increases with age, caused by alterations in the cholesterol/phospholipid content of the membrane [95]. These modulations result in changed function of lipid rafts, which might directly influence TLRs and GM-CSF signaling. Additionally actin, which may play a role in cell-surface receptor movement and expression, has been indicated to contribute to the changed ROS production [104].

The age-related changes occurring in the elderly include a gradual increase in the production and circulation of proinflammatory cytokines such as IL-6 and TNFα, which might contribute to a systemic chronic low-grade state of inflammation in the elderly. However, since there are few reports showing age-dependent changes in cytokine production by neutrophils, and since the few studies to date published are heavily influenced by monocyte contamination [105], this is, certainly, an important issue to investigate. It should be pointed out that different neutrophil isolation techniques and monocyte contaminations cannot be excluded as a possible explanation for the controversial results published by distinct groups of investigators [7].

EPIGENETICS

Interest in epigenetics, as well as in the usage of the term *epigenetic*, has increased significantly since the definition of the field by Conrad Waddington in the early 1940s. By definition, epigenetics represents "*the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail change in DNA sequence*" [106, 107]. The word "epigenetic" literally means "in addition to changes in genetic sequence". The term has evolved to

include any process that alters gene activity without changing the DNA sequence and leads to modifications that can be transmitted to daughter cells. Although the chromosomes in our genome carry the genetic information, the epigenome is responsible for its functional use and stability. Epigenetic changes can be either spontaneous or driven by external or internal influences. Epigenetic plays a critical role in the regulation of cellular processes, including gene expression and is a crucial element for the developmental process, cell differentiation, stress response and pathological state. Epigenetic regulatory processes include DNA methylation, post-transcriptional histone modification including methylation, acetylation, ubiquitylation and phosphorylation, chromatin remodeling and regulation by small and long non-coding RNAs. Epigenetic modifications can be very stable and passed on to generations, but, in some cases, they can change in response to cellular conditions or environmental stimuli. When this happens, the result is the mis-regulation of these mechanisms, which can be detrimental for health. They are, therefore, emerging as important diagnostic/prognostic biomarkers in medicine.

Epigenetic and gene expression

The modifications of DNA and histone proteins can impact gene expression by altering chromatin structure or recruiting histone modifiers. This is because the local chromatin environment of a given gene strongly influences its expression.

A histone modification is a covalent post-translational modification to histone proteins, which includes methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation. Histone modifications act in diverse biological processes such as transcriptional activation/inactivation, chromosome packaging, and DNA damage/repair. The basic unit of chromatin is the nucleosome, which consists of approximately 146 base pairs (bp) of DNA wrapped around an octamer of proteins called histones, in order to form chromosomes (**Figure 7**). This octamer is composed of two copies of each H2A, H2B, H3, and H4, or specialized

variants of these proteins. The histone H1 and its isoforms behave as a linker and are involved in chromatin compaction [108].

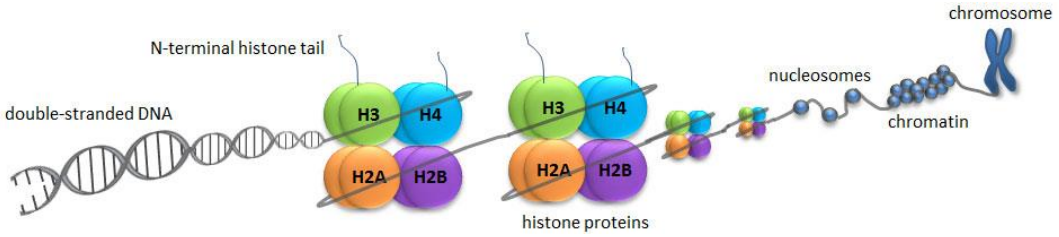

Figure 7 | Organization and packaging of genetic material nucleosomes. Nucleosomes are represented by DNA (grey), wrapped around eight histone proteins, H2A, H2B, H3 and H4 (colored circles). N-terminal histone tails (blue) are shown protruding from H3 and H4.

Generally, chromatin can be functionally classified into two forms: heterochromatin and euchromatin. Heterochromatin is tightly compacted and associated with transcriptionally silent genomic regions, whereas euchromatin has a more open conformation and is permissive for transcription. Despite these basic distinctions between euchromatin and heterochromatin, it is increasingly recognized that chromatin is not a fixed entity but is instead a highly dynamic system. The disruption and reassembly of chromatin structures is necessary for many essential processes such as gene transcription, DNA replication, and repair.

Histone acetylation and methylation, for instance, are the most-studied post-transcriptional histone modifications. Histone acetylation occurs by the enzymatic addition of an acetyl group ($COCH_3$) from acetyl coenzyme A. The modifying enzymes involved in histone acetylation are called histone acetyltransferases (HATs), and they play a critical role in controlling histone H3 and H4 acetylation. Acetylation occurs on the lysine residues in the N-terminal tails of histone proteins, and it is able to neutralize positive charges, causing the weakening of charge-based interactions between histones and DNA. It thereby facilitates chromatin de-condensation and increases the access of RNA polymerase and transcription factors to gene promoters. Acetylated lysine is recognized and targeted by specific factors such as transcriptional regulators and remodeling enzymes that contain specific protein domains called bromodomains.

These histone proteins are modified by the addition or removal of acetyl groups and can loosen or tighten the packaging of DNA. This makes the DNA more or less available to transcription, impacting the expression of particular genes. In most cases, histone acetylation enhances transcription, while histone deacetylation represses transcription. Histone acetylation is catalyzed by histone acetyltransferases (HATs), while deacetylation is catalyzed by histone deacetylases (HDACs). Several different families of HATs and HDACs have been identified. CREB-binding protein (CBP) and p300 are probably the best studied HATs [109]. Detecting whether histone H3 is acetylated at its lysine residues would provide useful information for further characterization of acetylation patterns or sites, thereby leading to a better understanding of epigenetic regulation of gene activation.

Histone methylation is defined as the transfer of one, two, or three methyl groups from S-adenosyl-L-methionine to lysine or arginine residues of histone proteins by histone methyltransferases (HMTs). HMTs control or regulate DNA methylation through chromatin-dependent transcriptional repression or activation. Several different histone methyltransferases exist that are specific for the lysine or arginine residue, which they modify. In the cell nucleus, when histone methylation occurs, specific genes within the DNA complexed with the histone may be activated or silenced. Methyl groups typically/mostly affect DNA accessibility by turning genes off. These methyl groups are attached to the backbone of DNA molecules at cytosine rings by the enzyme DNA methyltransferases (DNMT3a, b or DNMT1) [110]. Both H3-K9 and H3-K27 methylation mediates heterochromatin formation and also participates in silencing gene expression at euchromatic sites [111]. Increased global H3-K27 methylation is also found to be involved in some pathological processes such as cancer progression. On the other hand, arginine methylation of histones H3 and H4 promotes transcriptional activation and is mediated by a family of protein arginine methyltransferases (PRMTs). There are 9 types of PRMTs found in humans but only 7 members are reported to methylate histones. They can mediate mono or di-methylation of arginine residues. Histone demethylation is the removal of methyl groups in modified histone proteins via histone demethylases. These demethylases

have been found to have potential oncogenic functions and involvement in other pathological processes. The discovery of histone demethylases demonstrates that histone methylation is not a permanent modification but rather a more dynamic process.

Epigenetic mechanisms in human neutrophils

The genome of neutrophils is highly compacted, mostly heterochromatic and, consequently, poorly permissive for transcription [112]. The limited transcriptional capacity of neutrophils has been always considered an intrinsic constraint. Nonetheless, neutrophils can broadly and actively change their transcriptome during all functional phases of their life cycle, from bone marrow maturation to homeostasis and activation. For instance, during maturation, neutrophils are able to progressively silence genes involved in biosynthetic, metabolic and proliferative processes, but concomitantly induce genes encoding for granule components, antimicrobial factors and immune responses [4]. The process occurs through the action of transcription factors working in specific developmental stages, such as myeloid lineage-determining TFs (e.g. PU.1, C/EBPα/β) and maturation TFs (e.g. KLF5 [113], C/EBPε), which have the ability to dictate the epigenomic organization in neutrophils [48].

Terminal neutrophil maturation is in fact associated with active gene transcription, as mature circulating neutrophils express genes involved in effector functions and immune defense, as opposed to their bone marrow precursors [114].

Even after reaching terminal maturation, neutrophils can sense and adapt to stimuli by mechanisms also involving their epigenome through which they are able to fine-tune their gene expression programs [48]. For instance, neutrophils exposed to microbial components, cytokines and growth factors can dynamically upregulate hundreds of genes, with high coordination. Some examples are illustrated in **Figure 8**. For instance, there are proinflammatory related genes, exemplified by CXCL8 (encoding for CXCL8/IL-8), that can be induced in neutrophils with fast kinetics. This category of genes has a pre-poised local chromatin organization able to support immediate transcription without the need

for nucleosome remodeling [48]. Quite the opposite occurs with the induction of another proinflammatory gene such as IL-6, which requires previous chromatin remodeling and deposition of histone marks at regulatory elements, in order to permit transcription [27]. Moreover, in human neutrophils, there are chromatin-dependent mechanisms which are able to prevent the induction of genes at specific loci, for instance IL-10 [115] (**Figure 8**).

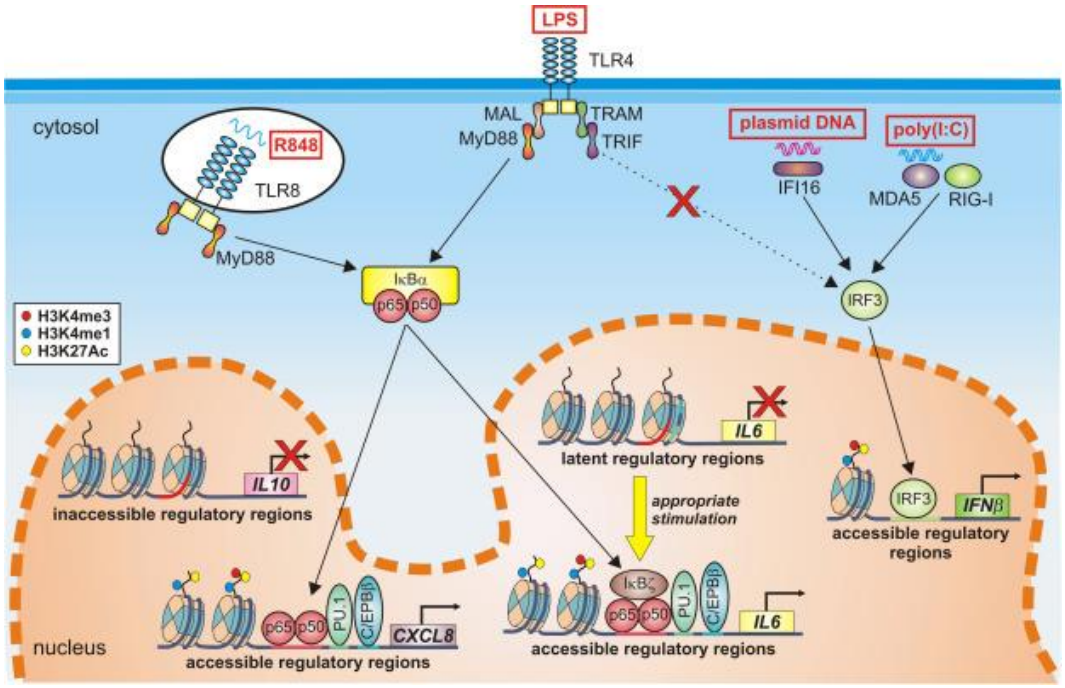

Figure 8 | Examples of epigenetic mechanisms controlling gene expression in human neutrophils.
IL10 locus of neutrophils display inaccessible regulatory regions, as evidenced by the absence of histone marks associated with active transcription. Such a chromatin conformation prevents IL-10 mRNA transcription, precluding the binding of TFs, also after activation with stimuli (e.g.e TLR ligands or other PAMPs). By contrast, the *CXCL8* locus has an accessible conformation that is ensured by the constitutive binding of both TFs (PU.1 and C/EBPβ). Upon neutrophil activation, TF (e.g. NF-κB) promotes the transcription of CXCL8 mRNA once it's recruited to its corresponding binding sites. *IL6* locus of neutrophil is not accessible under basal state. However, upon appropriate stimulation, pioneer TFs, such as PU.1 and C/EBPβ, start to open the chromatin, favoring in this manner the binding of activated TFs and the activation of IL-6 mRNA transcription. Moreover, the cartoon clarifies that, even if the *IFN*β genomic locus is not in a closed conformation, there is no IFNβ mRNA transcription in TLR-stimulated neutrophils; this is due to the inability to activate IRF3 by TLR4-dependent signals [48].

A recent analysis made in primary human immune cells shows that there is a variability of the basal neutrophil transcriptome of healthy donors [116]. This variability between different donors is higher in neutrophils than in monocytes or in lymphocytes [117]. Genes with hypervariable expression in neutrophils were enriched in specific immune functions such as inflammasome activation and antiviral responses. These studies reveal that genetic factors are the responsible of the inter-individuals variability in neutrophil gene expression. However, some genes with high variance in neutrophils were not associated with genetic factors, but rather correlated with epigenetic control. For example, studies of genome-wide DNA methylation show that human neutrophils display high levels of inter-individual epigenetic variability [118].

EPIGENETIC OF AGING

Over the past decades, numerous studies have shown that the molecular and cellular deterioration of aging can be efficiently categorized into several evolutionarily conserved *hallmarks* or *pillars* of aging. These consist of: epigenetic alterations, genomic instability, telomere attrition, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication [77], as depicted in **Figure 9** [77, 119].

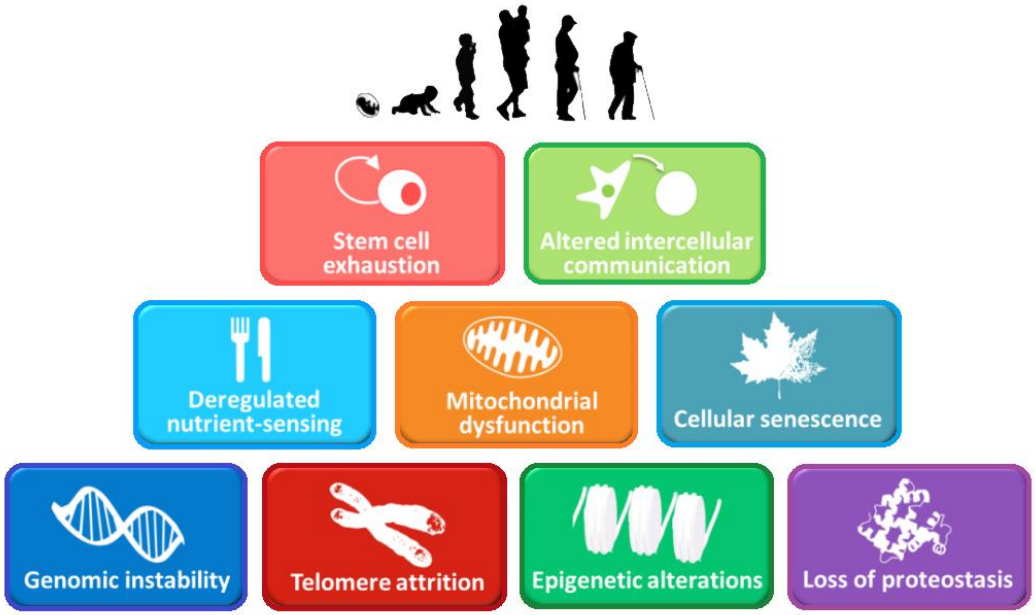

Figure 9 | The hallmarks of aging.
The scheme enumerates the nine hallmarks of aging: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. Adapted from Lopez Otin et al., 2013 [77].

Among these hallmarks, epigenetic alterations represent one crucial mechanism behind the deteriorated cellular functions observed during aging and in age-related disorders. These epigenetic changes can either be spontaneous or driven by external or internal influences, and occur at various levels, including altered patterns of histone post-translational modifications and DNA methylation,

replacement of canonical histones with histone variants, and altered noncoding RNA expression (**Figure 10**). The result is an altered local accessibility to the genetic material, leading to aberrant gene expression, reactivation of transposable elements, and genomic instability [120].

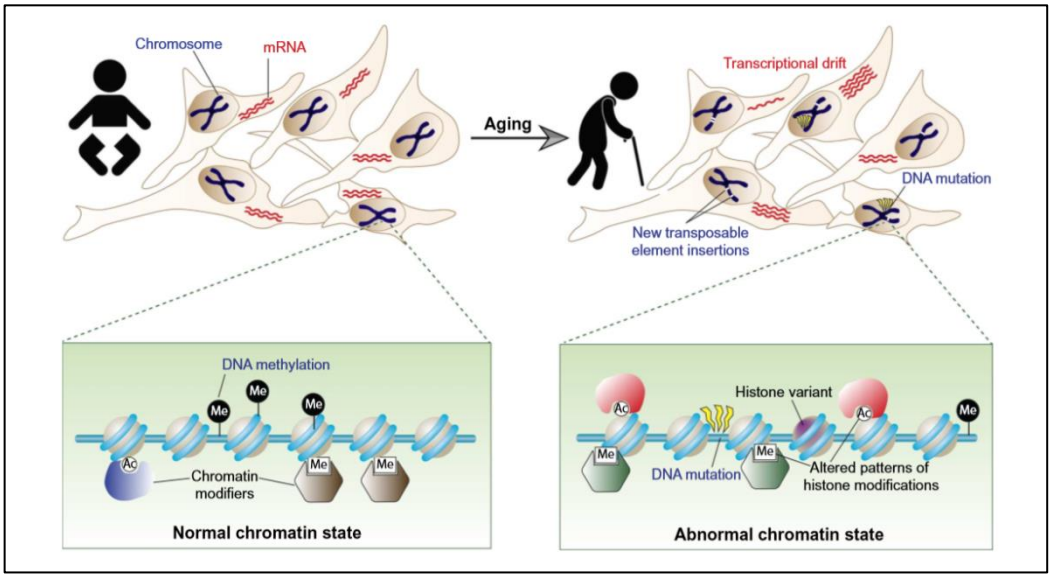

Figure 10 | Overview of epigenetic changes during aging.
In young individuals, the cells within each cell type have a similar pattern of gene expression, determined in large part by each cell having similar epigenetic information. During aging, the epigenetic information changes sporadically in response to exogenous and endogenous factors. The resulting abnormal chromatin state is characterized by different histone variants being incorporated, altered DNA methylation patterns, and altered histone modification patterns, resulting in the recruitment of different chromatin modifiers. The abnormal chromatin state in old cells includes altered transcription patterns and transcriptional drift within the population. The abnormal chromatin state in old cells also leads to new transposable elements being inserted into the genome and genomic instability, including DNA mutations [120].

The epigenome continues to change throughout life. This age-related "epigenetic drift" is associated with impaired maintenance of epigenetic marks and the loss of phenotypic plasticity. It is becoming evident that environmental factors encountered over the lifespan strongly influence the epigenome. Moreover, these changes are believed to launch the development of inflammatory and autoimmune diseases.

Epigenetic modifications that are altered during aging include, but are not limited to, DNA methylation, histone methylation, and histone acetylation.

Epigenetics potentially operates as the missing link to explain why the pattern of aging is different between two genetically identical individuals, such as identical twins [121] [122]. Studies of genetically-identical (monozygotic) twins have illustrated the influence of non-genetic factors in autoimmune and inflammatory diseases. Longevity studies on the human population have shown that genetic factors could explain only a small percentage, about 20-30 %, of the differences observed in life spans of monozygotic twins, the majority of the others being thought to have arisen through epigenetic modifications during their lifetime [122]. Accordingly, delineating and understanding the epigenetic changes that happen during aging may potentially lead the way to the development of novel therapeutic approaches to delay aging and age-related diseases.

AIM

During aging, the mechanisms that normally maintain health and stress resistance functionally decline, resulting in frailty and high susceptibility to infections. Exactly when and how this decline occurs is unknown. Changes in transcriptional networks and chromatin state could be crucial for the age-dependent decline. It is proposed that the dysregulation of transcriptional and chromatin networks is a crucial component of aging. Understanding age-dependent epigenomic changes will yield key insights into how aging begins and progresses and should lead to the development of new therapeutics that delay or even reverse aging and age-related diseases.

In this context, a growing body of evidence suggests that a key role in aging is played by neutrophils, whose functions and activities seem to be impaired in aged people. However, it is unclear why neutrophils from old donors have functional deficits. It has been hypothesized that the low-grade inflammatory systemic environment seen with aging may lead to epigenetic changes in immune cells, which in turn may impact on cellular phenotype and function. Whether this occurs in neutrophils too is not known.

In order to characterize the genes and pathways specifically involved in the process of aging, we recruited two groups of healthy donors, including both males and females. The first group consisted of young participants between 25 and 35 years and the second group was comprised of elderly more than 65 years old. This study aims at verifying whether the functional properties of neutrophils from healthy aged individuals are different from those of healthy young participants, as well as their transcriptomes. Data are expected to identify genetic regulatory circuits and, possibly, transcription factors, which can eventually explain functional defects of neutrophils in the elderly.

MATERIALS & METHODS

Cohort selection/ subject recruitment

A total of 40 healthy subjects were enrolled from Borgo Roma and Borgo Trento hospitals (Department of Medicine, Geriatric Section, Verona), and divided in two groups: young (aged 20-35 years, n = 20) and elderly (aged ≥ 65 years, n = 20) individuals. Characteristics of the donors are summarized in **Table 1**.

Table 1 | Summary of the characteristics of the subjects enrolled in the study

donor groups	age (years)	number	sex (M / F ratio)	sBP (mmHg)	dBP (mmHg)	smokers
Young	20 - 35	20	7 / 13	118,6 ± 8,7	68,9 ± 9,2	1
Elderly	> 65	20	8 / 12	126,2 ± 6,3	76,2 ± 7,4	

Data referred to systolic blood pressure (sBP) and diastolic blood pressure (dBP) are expressed as mean ± SD.

Using a screening questionnaire, participants were asked about lifestyle, clinical history, and usage of medications. In order to separate age-related changes from alterations due to nutrition, lifestyle, medications or underlying diseases, participants have been be selected according to the SENIEUR protocol [73], which discriminates between healthy and frail subjects.

Subjects who self-reported comorbid conditions, including cancer (within the last 5 years), immunocompromising disorders, rheumatoid arthritis or any other inflammatory conditions, administration of immunomodulating medications including steroid, acute illness 2 weeks before recruitment, were excluded. Some exclusion criteria are listed in **Table 2**. On the other hand, inclusion criteria comprised controlled hypertension, occasional/tolerable aching joint, as well as not taking daily nonsteroidal anti-inflammatory drugs. All study participants gave their written informed consent prior to the enrolment in the study. All experimental protocols were approved by the Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona, Italy. The methods were carried out in accordance with the approved guidelines.

Table 2 | Exclusion criteria used to select healthy young and old cohorts

Immuno-compromising disorders	Medications (Anti-inflammatory drugs, hormones, analgesics, antiplatelet drugs, steroids, statins)
Comorbid conditions	Dementia
Parkinson's disease	Pregnancy
Alzheimer disease	Malnutrition
Stroke with residual hemiparesis	Alcoholism
Cardiac insufficiency	Drug abuse
Atherosclerosis	Diabetes
Hypertension	Vaccinations < 3 months

Blood tests

To assess their health status, each participants performed a series of blood tests including the following parameters: hematocrit, hemoglobin, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelets, mean platelet volume, white blood cell count and related formula, CRP, urea, creatinine, e GFR, uric acid, glucose, cholesterol (HDL, LDL, non-HDL), triglycerides, proteins, AST, ALT, ALP, pancreatic amylase.

Cell isolation and culture

For cell isolation and leukocyte count, a total of 15 ml of blood from each donor was drawn in collection tube (VACUTEST® KIMA srl, Arzergrande, Italy) containing as anticoagulant K_2EDTA. For biochemical blood test, 3 ml of blood were collected in tube containing as anticoagulant Lithium-Heparin. Blood was generally processed within one hour from the withdrawal.

Neutrophils and autologous $CD14^+$-monocytes were isolated from young and old healthy donors using Ficoll-Paque (GE Healthcare) gradient centrifugation (30 min at RT, 400 × *g*) and manipulated under endotoxin-free conditions [123].

The granulocytic fraction was subjected to dextran sedimentation followed by erythrocyte hypotonic lysis. Neutrophils were isolated using the EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, Canada), achieving 99.7 ± 0.2 % purity after positively removing all contaminating cells [124]. $CD14^+$-monocytes were obtained from the Peripheral Blood Mononuclear Cells (PBMCs) fraction. PBMCs were washed three times with PBS in order to remove platelets (centrifugation at 300 × *g* for 5 min) and incubated for 15 min at 4° with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD14^+$-monocytes were then isolated using autoMACS automated magnetic separation unit (Miltenyi Biotec) and were found to be consistently > 98 % pure. After purification, neutrophils and $CD14^+$-monocytes were suspended at 5×10^6/ml and 2.5×10^6 /ml, respectively, in RPMI 1640 medium (Sigma) supplemented with 10 % low endotoxin (< 0.5 EU/ml) FBS (BioWhittaker-Lonza, Basel, Switzerland). Neutrophils were then incubated with or without 5 μM R848, 1 μg/ml ultrapure LPS (from *E. coli* 0111:B4 strain, Alexis, Enzo Life Sciences, Farmingdale, NY, USA), 1000 U/ml G-CSF (Myelostim, Italfarmaco Spa, Milano, Italy), 20 ng/ml GM-CSF (Miltenyi Biotec), 10 ng/ml TNFα (Peprotech, Rocky Hill, NJ, USA), 10-100 nM N-formyl-methionyl-leucyl-phenylanine (fMLF) (Sigma Chemicals, Deisenhofen, Germany), 20 ng/ml phorbol myristate acetate (PMA) (Sigma) for different times, depending on the assay. Cells were then plated either in 24/48/96-well tissue culture plates (from Greiner Bio-One, Kremsmünster, Austria) for culture at 37° in 5 % CO_2 atmosphere and, after the desired incubation period, were either processed for chromatin immunoprecipitation (ChIP) experiments, or collected and spun at 300 × g for 5 min, for other types of assays, as listed in the flow chart of **Figure 11**. In the latter case, cell-free supernatants were immediately frozen and stored at – 20°, while the corresponding cell pellets were extracted for total RNA.

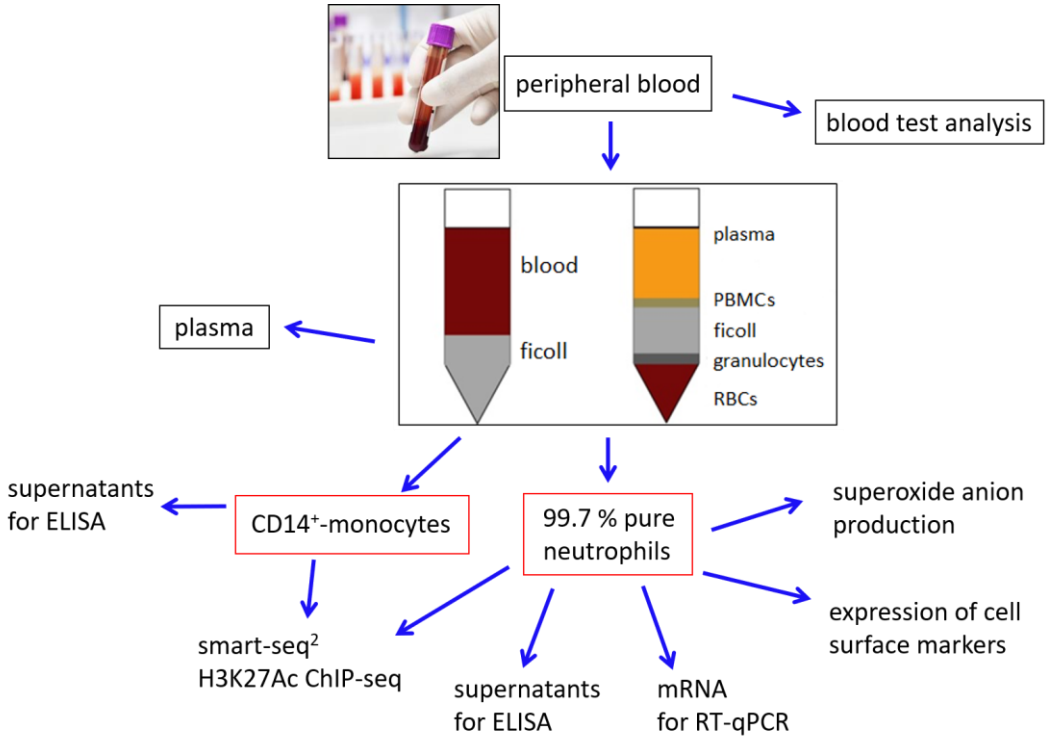

Figure 11 | Project flow-chart.
18 ml of blood were withdrawn from each donor. A small amount was used for some blood tests (complete blood count and biochemical analysis of diverse parameters), while the majority was subjected to Ficoll-Paque PLUS gradient density centrifugation from which neutrophils (purity of 99.7 ± 0.2 %) and autologous $CD14^+$-monocytes were isolated. A number of functional and molecular assays were then performed to evaluate: the production of superoxide anion, the expression of surface markers (by flow cytometry) and gene expression (by RT-qPCR) by neutrophils. Cell-free supernatants were also collected to measure their content of cytokines, by ELISA. Pelleted neutrophils and $CD14^+$-monocytes, both unstimulated (time 0) or stimulated for 20 h with 5 µM R848, were also used for Smart-seq^2 and H3K27Ac Chip-seq experiments.

Flow cytometry

The expression of cell surface markers has been evaluated by the use of fluorochrome-conjugated antibodies. Phenotypic studies have been performed in neutrophils either freshly isolated or incubated for 20 h with stimuli (see above). 1 × 10^5 neutrophils were centrifuged and suspended in 100 µL PBS containing 5 % complemented-inactivated human serum for FcγR blocking. Neutrophils were then stained for 15 min at RT, or 30 min at 4°C, with the following antibodies: CD35 VioblueBV421 anti-human (BD Horizon clone E11), Brilliant Violet anti-

human CD45 (Biolegend clone HI30), FITC anti-human CD66b (Biolegend clone GI0F5), PE anti-human CD10 (Biolegend clone HI10a), CD83 PE-human (Miltenyi clone HB15), PE-vio770 anti-human CD11b (Biolegend clone ICRF44), APC anti-human CD62L (clone 145/15 Miltenyi Biotec) and APC-Cy7 anti-human CD16 (Biolegend clone 3G8). Fluorochrome-conjugated antibodies were used at working dilutions as specified in the corresponding datasheets. Sample fluorescence was then measured by a seven-color MACSQuant Analyzer (Miltenyi Biotec), while data analysis was performed using FlowJo software Version 10 from Tree Star (Ashland, OR, USA).

For whole blood staining, 100 µl whole blood were stained with: VioBlue anti-human CD14 (Miltenyi clone TÜK4 e REA 599), VioBlue anti-human CLEC9A/CD370 VioBlue human (Miltenyi clone 8F9), Brilliant Violet anti-human CD45 (Biolegend clone HI30), anti-slan MDC8 FITC human (Miltenyi clone DD-1), CD303 (BDCA-2)-FITC human (Miltenyi clone AC144), PE anti-human CD56 (Miltenyi clone AF12-7H3 and REA196), CD1c (BDCA-1)-PE human (Miltenyi clone AD5-8E7), PerCP-Cy5.5 mouse anti-human CD16 (Biolegend clone 3G8), PE-Vio770 anti-human CD3 (Miltenyi clone BW264/56), PE-Vio770 anti-human CD123 (Miltenyi clone AC145), APC anti-human CD19 (Miltenyi clone LT19), CD141(BDCA-3)-APC human (Miltenyi AD5-14H12), APC-Cy7 anti-human HLA-DR (Biolegend clone L243). After red cells lysis using ammonium chloride buffer, sample fluorescence was immediately measured as previously described.

Neutrophil Viability

Neutrophil viability has been assessed by VYBRANT/SYTOX staining. Briefly, after an overnight treatment with the agonists indicated above, 1×10^5 neutrophils were centrifuged at $300 \times g$ for 5 min, medium removed and ultimately suspended in 100 µl HBSS buffer containing 10 nM Vybrant DyeCycleTM Violet stain (Life Technologies) and 5 µM SYTOX AADvanced (Life Technologies). Cells were then put on ice for 30 min, protected from light. Cells were washed with HBSS, suspended in 100 µl HBSS buffer. Finally, sample fluorescence was measured by

MACSQuant Analyzer (Miltenyi Biotec). Cell viability was defined as the percentage of cells that were double negative for both stains (Vybrant/Sytox, respectively).

RNA extraction and quantification by Ribogreen® assay

Total RNA was extracted from neutrophils (1×10^6 cells) and monocytes (0.1×10^6 cells), by using the RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands) according to the manufacturer's instructions. An on-column DNase digestion with the RNase-free DNase set (Qiagen), was performed to remove any possible contaminating DNA during total RNA isolation.

Extracted RNA was then quantified using the Quant-iT™ RiboGreen™ RNA Assay Kit (Invitrogen, Cat. no. R11490). This kit enables quantitation of as little as 1 ng/mL total RNA. Following the manufacturer's instructions, it has been created a standard RNA curve with ribosomal RNA standard provided in the kit and then 1 µl sample RNA and 99 µl TE were added to each well. Subsequently a solution of TE with the appropriate dilution (1:2000) of Ribogreen reagent was added to each well to reach a final volume of 200 µl. Samples were mixed and incubated for 2 to 5 minutes at RT, protected from light. Finally, fluorescence was measured using a multilabel plate reader (VICTOR Multilabel Plate Reader, Perkin Elmer) at these wavelengths: excitation ~480 nm, emission ~520 nm. The fluorescence value of the reagent blank was then subtracted from that of each of the samples. RNA concentration of the samples was calculated by four parameters logistic regression (4PL) from RNA standard curve using GraphPad Prism 6 software.

Reverse transcription quantitative real-time PCR (RT-qPCR)

Purified RNA was then reverse-transcribed into cDNA using Superscript III (Life Technologies, Carlsbad, CA, USA) and random hexamer primers (Life Technologies). Quantitative PCR (qPCR) was then carried out using Fast SYBR® Green Master Mix (Life Technologies). Sequences of gene-specific primer pairs (Life Technologies) are listed in **Table 3**.

Table 3 | Sequences of human gene-specific primer pairs (Life Technologies) used in RT-qPCR

Gene	Sequence	
	forward primers	**reverse primers**
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
SOCS3	GGCCACTCTTCAGCATCTC	ATCGTACTGGTCCAGGAACTC
IL-1ra	TTCCTGTTCCATTCAGAGACGAT	AATTGACATTTGGTCCTTGCAA
IL-6	GGCACTGGCAGAAAACAACC	GCAAGTCTCCTCATTGAATCC
TNFα	GAGCACTGAAAGCATGATCC	CGAGAAGATGATCTGACTGCC
CXCL8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT

Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression (MNE) units after GAPDH and RPL32 normalization.

Production of superoxide anion (O_2^-)

After isolation, neutrophils were suspended in HBSS buffer containing 0.5 mM $CaCl_2$ and 1 mg/ml glucose, and then distributed in a 96-well plate (0.1×10^6 cells/100 µl/well) to be incubated for 10 min at 37º prior to the addition of and the indicated stimuli, including 10-100 nM fMLF, 20 ng/ml GM-CSF and 20 ng/ml PMA (Sigma). Plates were then incubated at 37º in an automated ELx808IU microplate reader (BioTek Instruments, Inc., Winooski, VT) to record cytochrome C (from bovine heart, C-7752, Sigma) reduction, measuring at intervals of 5 min for 90 min the Δ O.D. 550 nm / 465 nm. O_2^- production was finally calculated using an extinction coefficient of 24.5 mM [125].

Smart-seq2 RNA assay

Libraries for transcriptome analysis were prepared using the Smart-seq2 protocol [126] which allows to obtain a library from less than 500 pg of total RNA. The generation of the sequencing libraries occurs through the use of the Tn5 transposase, an enzyme that catalyzes in vitro integration of selected oligonucleotides into target DNA. A flowchart of the protocol is provided in **Figure 12**.

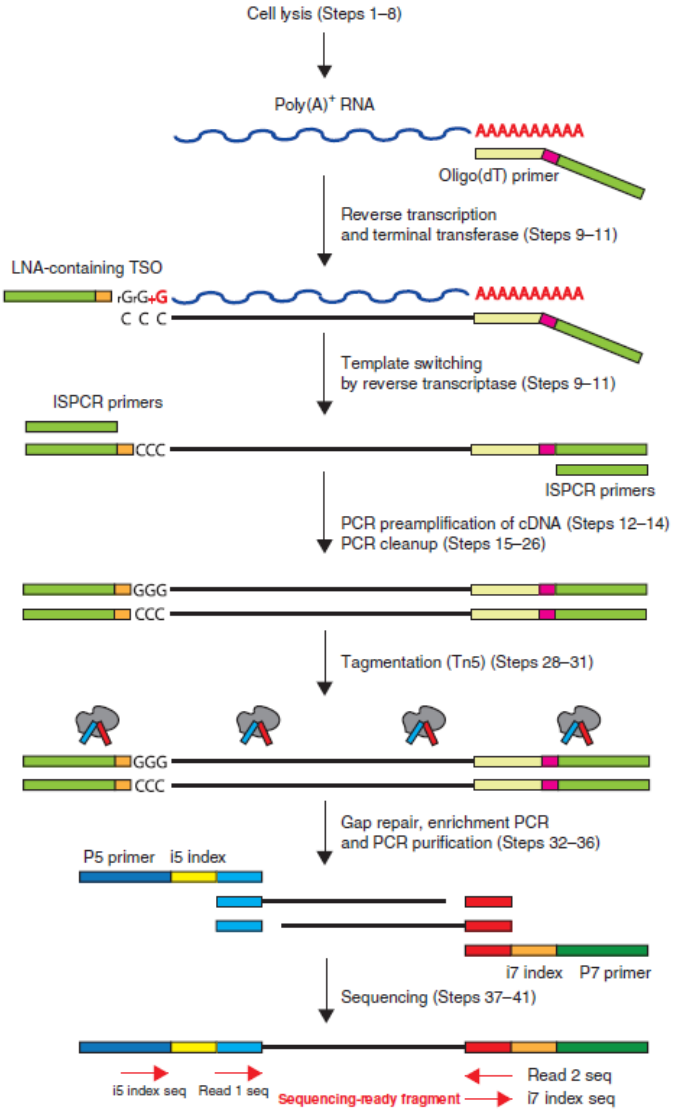

Figure 12| Flowchart for Smart-seq2 protocol for library preparation with the corresponding procedure steps
[126].

Quality control of the total RNA was performed using Agilent 2100 Bioanalyzer (Agilent Technologies). RNA integrity (RIN) was routinely found to be optimal (RIN ≥ 7.0). To proceed with library preparation, 2 ng of total RNA were incubated with dNTPs and oligo(dT)-tailed oligonucleotides containing a universal 5′-anchor sequence necessary for primer annealing of subsequent steps. Next, samples were incubated at 72 °C for 3 min and then immediately put on ice to allow annealing of oligo(dT) oligonucleotides to the poly(A) tail of all the mRNA molecules.

Smart-seq2 exploits two intrinsic properties of the Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase: Reverse Transcription (RT) and Template Switching. Template switching is the ability of the MMLV reverse transcriptase to introduce a few untemplated nucleotides, predominantly 2-5 cytosines, when it reaches the 5′-end of the RNA template, corresponding to the 3′-end of the newly synthesized cDNA strand. These extra nucleotides work as a docking site for a helper oligonucleotide defined Template Switching Oligonucleotide (TSO) carrying two riboguanosines in the third- and second-last positions and a modified guanosine to produce a locked nucleic acid (LNA) as the last base at the 3′ end. These locked nucleotides enhance TSO thermal stability and their annealing ability. The reverse transcriptase is then able to switch the template (from mRNA to the DNA of the TSO) and synthesize a complementary DNA strand using the helper oligonucleotide as template. Thus, Template Switching makes possible the introduction of an arbitrary sequence at the end of the transcript and, along with the known sequence located at the 5′-end of the oligo-dT primer, allows, in the following PCR steps, and efficient amplification of all the transcripts of the sample.

Tagmentation, a reaction where the DNA is simultaneously tagged and fragmented, is then used to quickly and efficiently construct sequencing libraries from the amplified double strand cDNA [126]. Tagmentation is performed using a hyperactive variant of a Tn5 transposase (kind gift of Prof. G. Natoli from Humanitas, MI) that carries out the fragmentation of double-stranded DNA and ligates synthetic oligonucleotides (“tags”) at both ends.

The fragments in the tagmented DNA library have an average size that usually ranges from 200 to 600 bp and are ready for enrichment PCR. We followed the protocol developed by Illumina that allows the pooling of up to 96 samples through the use of a dual-index strategy, referred to as index 1 (i7) and index 2 (i5) (**Figure 12**). Samples were pooled after enrichment PCR and sequenced together on the same flow cell of a NextSeq 500 sequencer (Illumina).

Computational analysis of Smart-seq2 data

Computational analysis of the 80 transcriptome datasets generated by Smart-seq2 has been performed using the following bioinformatic pipeline.

After quality filtering, according to the Illumina pipeline, reads were mapped onto the Ensembl human transcriptome (version 75) and quantified at the transcript level using Salmon software [127]. Salmon is a method of quantifying transcript abundance from RNA-seq reads, as it quantifies the sum of bases in all reads that map to a single gene across known exons, newly-defined exonic regions and all transcript isoforms. Then, transcripts were combined to gene level using tximport packages [127]. Gene counts were normalized among various samples using DESeq2 [128], and adjusted for batch effects using the sva (surrogate variable analysis) packages [129]. DESeq2 [126] was then used to generate the expression metric, fragment per kilobase of exon model per million mapped reads (FPKM). FPKM normalization divides the read count for each gene by the length of the transcript for that gene, and then scales all read counts per million reads in each data file. This normalization step allows comparison of expression levels between two genes in the same sample, or of the same gene between different samples. Differentially expressed genes were identified using DESeq2 [128], by using as selection parameter a p-values lower than 0.05. Tracks for the snapshots of the Integrative Genome Viewer (IGV) [130] were generated by mapping the reads to the human genome (Genome Reference Consortium GRCh38) by TopHat [131], with –r 250 setting and filtered for uniquely mapped reads. Tracks were linearly rescaled to the same sequencing depth (10 million of mapped reads), by using HOMER analysis package [132].

Gene Ontology

Gene Ontology analysis was performed using GOrilla [133]. GOrilla is a web-based application that identifies enriched GO terms in ranked lists of genes, without requiring the user to provide explicit target and background sets. This is particularly useful in many typical cases where genomic data may be naturally represented as a ranked list of genes (e.g. by level of expression or of differential expression). GOrilla employs a flexible threshold statistical approach to discover GO terms that are significantly enriched at the top of a ranked gene list. Building on a complete theoretical characterization of the underlying distribution, called mHG, GOrilla computes an exact p-value for the observed enrichment, taking threshold multiple testing into account without the need for simulations [133]. This enables rigorous statistical analysis of thousand of genes and thousands of GO terms in order of seconds. The output of the enrichment analysis is visualized as a hierarchical structure, providing a clear view of the relations between enriched GO terms.

Statistical Analysis

Data are represented as mean ± SEM. Statistical evaluation was performed by using, depending on the experiment type, Student's t test or 2-way ANOVA followed by Bonferroni's post hoc test. P values < 0.05 were considered as statistically significant. Data were analyzed, and figures generated, using GraphPad Prism 6 software.

RESULTS

1. Blood parameter differences between young and elderly healthy donors

One major challenge in immune-gerontologic studies is to separate the influence of medical disorders from the physiologic process of aging. Therefore, we decide to conform to the SENIEUR protocol, which establishes strict admission criteria for these types of studies, based on the analysis of haematological parameters. Hence, each of our participants performed blood tests (as listed in M&M) to assess their health status, and only those ones conforming to the criteria of the SENIEUR protocol were included in my study, while the others were excluded. Furthermore, all donors with diseases related to the immune system were excluded from the study, to ultimately consider "aging" as the only parameter of diversity between the healthy elderly and the young groups.

At this stage, comparative analysis of the hematological parameters obtained from the healthy young versus the elderly groups highlighted various elements modified by aging (**Figure 13**). In particular, I found statistically significant increased CRP levels in the healthy elderly group, which is suggestive of systemic inflammation. And in fact, CRP has been proposed as a marker~~s~~ of *immunosenescence* and frailty [134]. Other hematological values that I found significantly increased in the healthy elderly group were those concerning the concentration of urea, triglycerides and cholesterol. The latter was measured as total cholesterol, LDL and non-HDL, but overall these values were significantly higher in the healthy elderly than in the young group. By contrast, hematological parameters found to significantly decrease with age were the number of platelets, the total proteins and the eGFR.

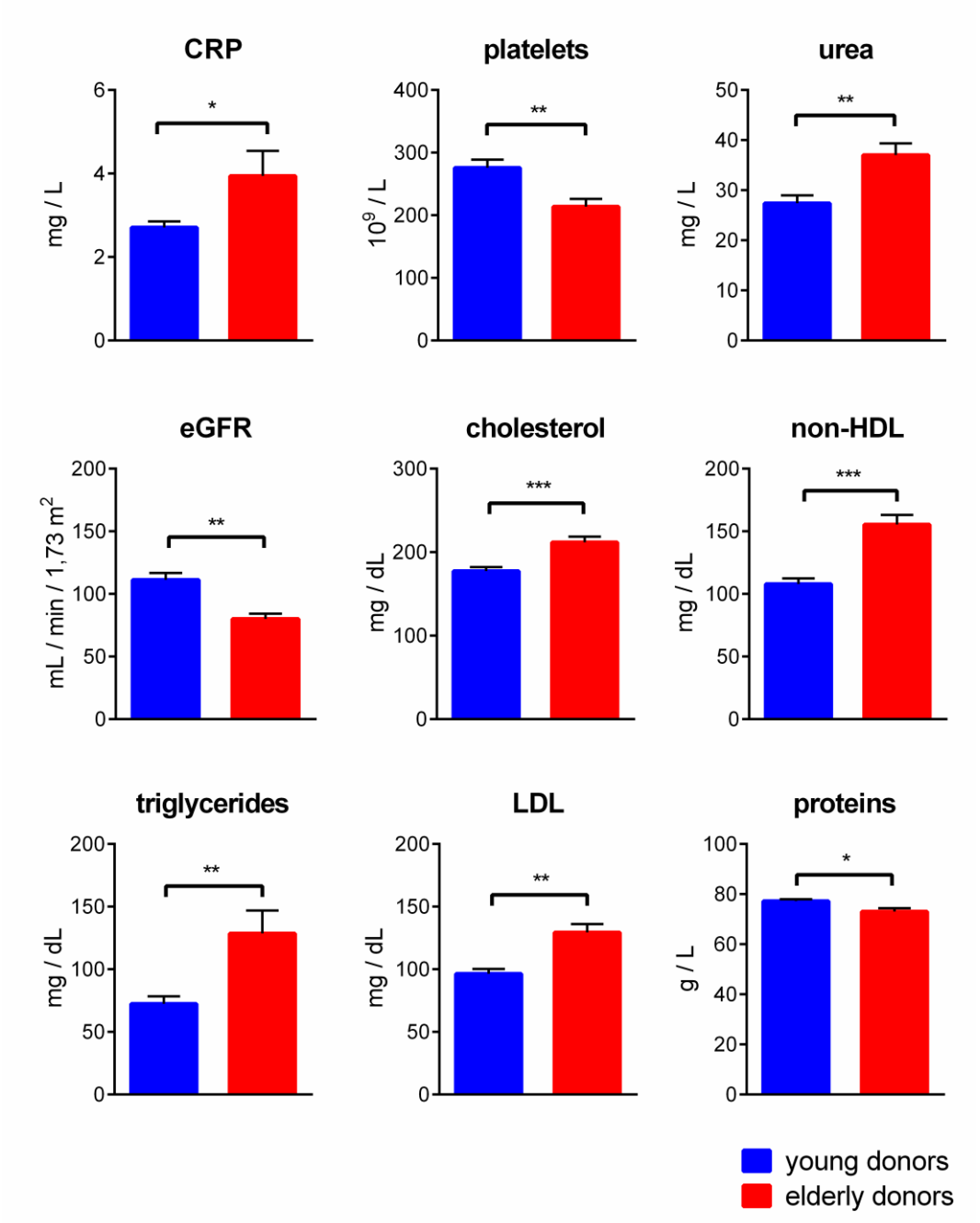

Figure 13 | Differences in blood test parameters between healthy young and elderly donors.
Parameters such as CRP, platelets, UREA, eGFR, cholesterol (total, non-HDL and LDL) triglycerides and total proteins result to be significantly different between the two groups. Values are presented as mean ± SEM (n=20 young, n=18 elderly). *P<0.05, **P<0.01, ***P<0.001 by unpaired t-test.

2. Leukocyte frequencies in healthy young and elderly donors

According to the current literature, whether the total number of neutrophils in blood changes with age is still debated. Whilst recent studies associated an age-related increase in the number of neutrophils with frailty [135] to a raised mortality of hospitalized subjects (during the following 2 years) [136], other groups claim that in healthy aging the number of neutrophils remains stable [87]. Thus, I checked the absolute number of various leukocyte types, including neutrophils, in the blood of our two cohorts (**Figure 14**). To do so, after red cells lysis by ammonium chloride buffer, I stained 100 µl whole blood with cell-type specific fluorescently conjugated antibodies and then counted the cells by an eight color cytofluorimeter.

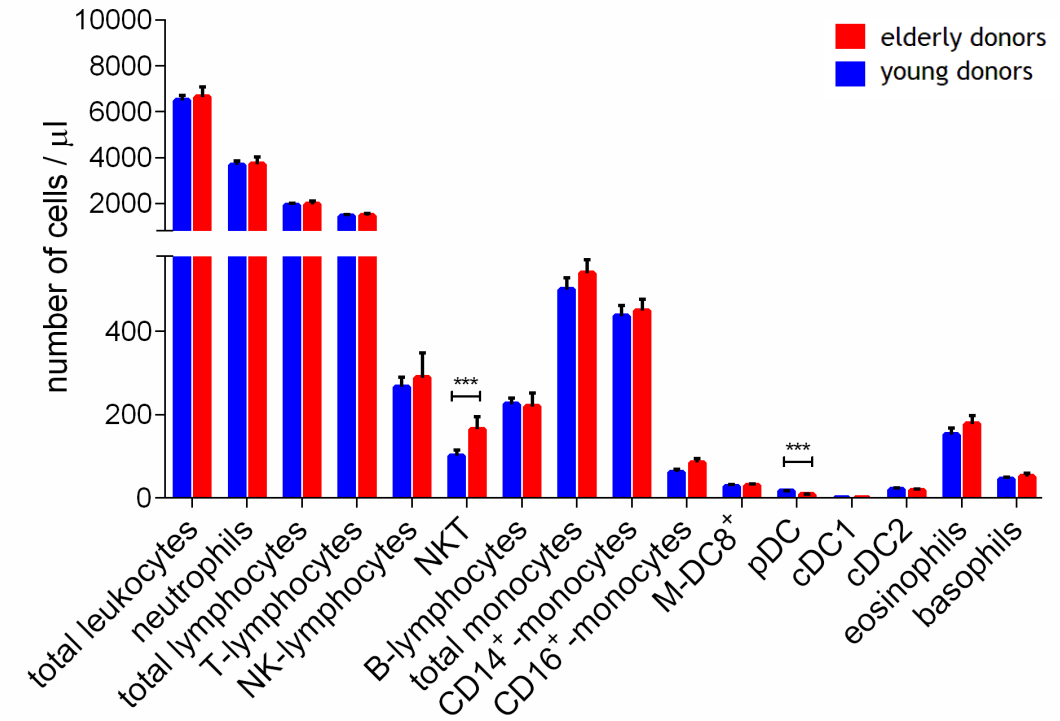

Figure 14 | Leukocyte count in healthy young and elderly donors.
Leukocytes were measured in whole blood by flow cytometry and expressed as number of cells/µl. *P < 0.05 by unpaired t-test.

Analysis of the numbers of immune cells calculated by flow cytometry revealed that they remain substantially unchanged with aging. In fact, as shown in **Figure 14**, the total number of leukocytes, or granulocytes (including neutrophils,

basophils and eosinophils), lymphocytes (including total lymphocytes, or the T, B and NK cells) and monocytes (including the total, or the $CD14^+$- and $CD16^+$-monocytes) is not changing between the two cohorts.

Significant differences between young and old individuals were found in the case of NKT cells (defined as cells expressing both CD3 and CD56 by flow cytometry), which were considerably increased in the healthy elderly group (**Figure 14**). Concerning DCs, while the absolute numbers of the cDC1 ($CD141^+$ DCs) and the cDC2 ($CD1c^+$ DCs) do not change with age, the number of pDCs was slightly, but significantly, decreased with age as compared to the values found in young donors. Altogether, these data indicate that the frequencies of the most represented leukocyte populations do not vary with ageing, while for less abundant cell populations some significant differences can be observed.

3. Viability of neutrophils isolated from elderly subjects

Since the ability of neutrophils to delay apoptosis in response to survival signals at the site of inflammation is reported to be impaired during aging [90], I decided to investigate it in my cohorts. I measured the viability of neutrophils by flow cytometry, either immediately after their isolation (T_0), or after their incubation for 20 h with or without R848, LPS, GM-CSF, G-CSF, or TNFα. Analysis of viability was performed by staining neutrophils with vybrant and sytox, which are two dyes that permit to exclude apoptotic and necrotic cells, respectively. Cells double negative for vybrant/sytox were considered as alive (**Figure 15a**).

In this example is reported the viability of neutrophils belonging to young participants untreated or treated with G-CSF for 20h. G-CSF increased neutrophils viability from17.6 % to 43.9 %.

As shown in **Figure 15b**, I found no difference in the viability of neutrophils belonging to healthy young and elderly subjects, either if freshly isolated (T_0 in **Figure 15b**), or after 20 h in culture without stimulation.

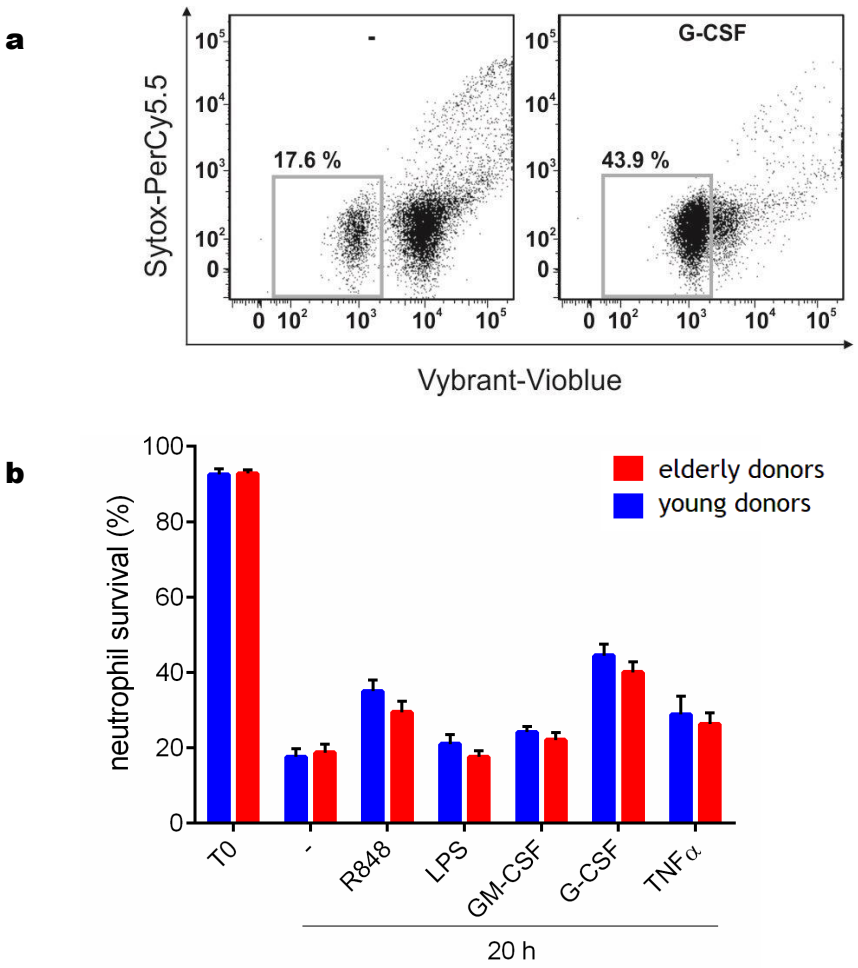

Figure 15 | Viability of neutrophils isolated from healthy young and elderly donors.

(**a**) Flow cytometry dot plots showing neutrophils viability by Vybrant/Sytox staining, in cells untreated (on the left) or treated with G-CSF (on the right) for 20h. Cells double negative for vybrant/sytox were considered as alive; (**b**) Histogram shows alive cells, expressed as percentage. Neutrophil viability was measured either immediately after cell isolation (T_0), or after 20 h of cell incubation with or without 5 µM R848, 1 µg/ml ultrapure LPS, 20 ng/ml GM-CSF, 1000 U/ml G-CSF or 10 ng/ml TNFα.

Incubation of neutrophils for 20 h with stimuli showed a general anti-apoptotic action, which was more marked for R848, G-CSF and TNFα (**Figure 15b**). Interestingly, the effects of all agonists appeared to be slightly less potent in neutrophils from healthy elderly subjects than from young subjects, although being not statistically significant (**Figure 15**).

4. Neutrophils from healthy elderly and young individuals express similar levels of CD11b, CD62L, CD16 and CD83

Then, I investigated the surface levels of some activation markers of neutrophils, namely CD11b and CD62L, as well as of markers important for phagocytosis (CD16) or cross-talk with lymphocytes (CD83). Neutrophils were thus isolated from healthy young and elderly donors, treated for 20 h with or without R848, LPS, GM-CSF, G-CSF or TNFα, and then evaluated for CD11b, CD62L, CD16 and CD83 expression by flow cytometry.

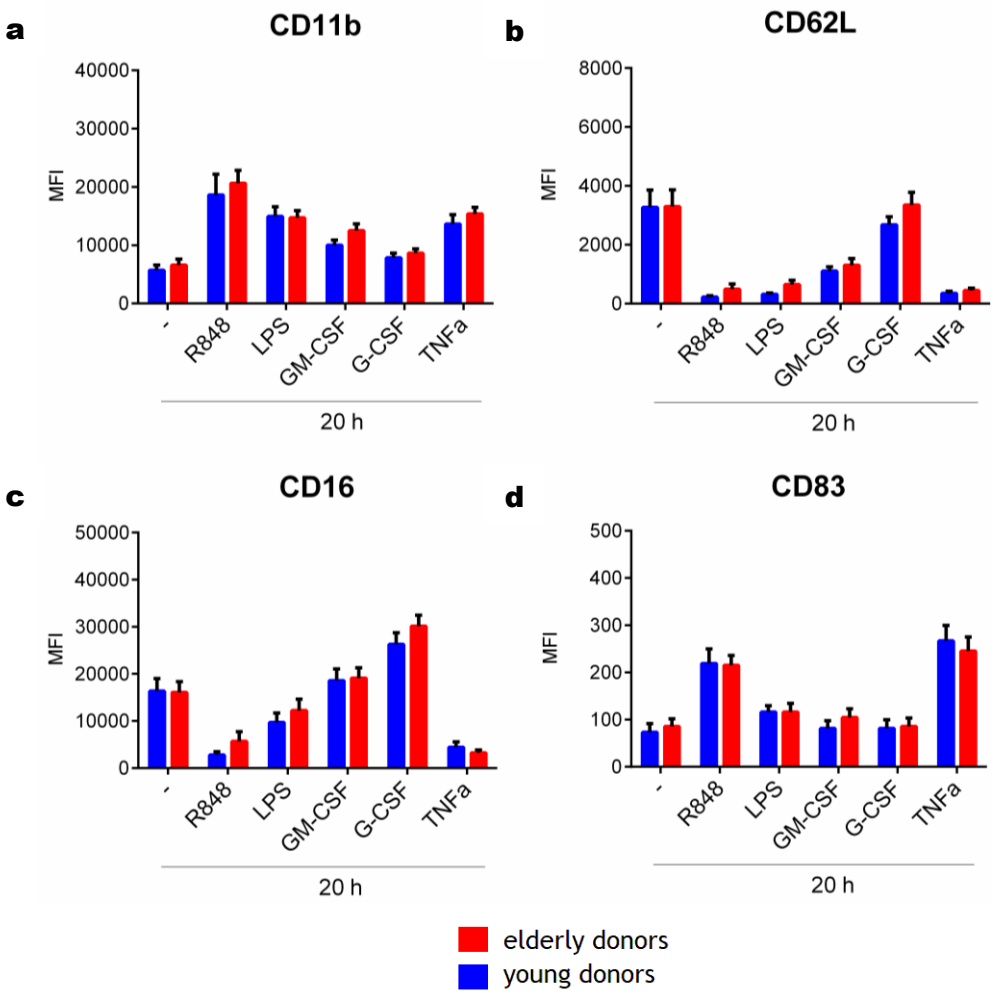

Figure 16 | CD11b, CD62L, CD16 and CD83 expression levels in neutrophils from elderly and young individuals.
Graphs show the expression levels, measured by flow cytometry, of each indicated marker in viable neutrophils after 20 h of incubation with 5 μM R848, 1 μg/ml ultrapure LPS, 20 ng/ml GM-CSF, 1000 U/ml G-CSF, or 10 ng/ml TNFα. Values are expressed as mean fluorescence intensity (MFI) (n=20 young and n=20 elderly).

Neutrophil activation is commonly associated with a modulation of surface adhesion molecules, more specifically with a decreased or an increased expression of, respectively, L-selectin (CD62L) and CD11b/CD18. The decrease expression of CD62L occurs through stimulus-induced shedding, while the increase of CD11b is due to its translocation to the membrane from intracellular granules [137]. Treatment of neutrophils for 20 h with the stimuli listed above triggered a general downregulation of CD62L, and an upregulation of CD11b (that were more marked in response to R848, LPS, GM-CSF and TNFα), as expected. Nevertheless, the actions of R848, LPS, GM-CSF and TNFα were less effective in neutrophils from elderly than in young subjects, although being not statistically significant (**Figure 16 a, b**).

CD16 (FcγRIII) is expressed at high levels on the surface of resting neutrophils. When neutrophils undergo apoptosis, they decrease the expression of CD16 by shedding it. Thus, the levels of surface CD16 could be a surrogate indicator of apoptotic vs non-apoptotic neutrophils. On the other hand, stimulation actually accelerates the rate of CD16 shedding by neutrophils [138]. CD16 shedding, occurring both during activation and apoptosis, relies on the activity of a metalloproteinase that cleaves the receptor from the plasma membrane [139]. Since CD16 levels are reported as reduced in neutrophils from aged individuals [98] (in this manner impacting on FcγR-mediated phagocytosis), I investigated CD16 expression under my experimental conditions. As shown in **Figure 16c**, I found that CD16 surface expression results strongly downregulated in neutrophils incubated for 20 h with R848 and TNFα, but slightly upregulated in cells incubated with GM-CSF and G-CSF. However, I did not find statistical changes in CD16 surface expression between the neutrophils belonging to the two groups under investigation.

With regard to CD83, which is a surface molecule endowed with immune stimulatory capacity for T cells, I found that it is expressed at very low levels in resting neutrophils, but it is strongly induced upon their incubation with R848 and TNFα. However, no differences in CD83 surface expression emerged between neutrophils from healthy elderly relative to young subjects (**Figure 16d**).

In sum, in our cohorts of study of healthy aged individuals, no differences were found for any of the other neutrophil surface molecules assessed in this study (CD11b, CD62L, CD16 and CD83) when compared to young subjects, demonstrating, to an extent, the absence of a basal neutrophils hyperactivation state.

5. O_2^- production by neutrophils from healthy elderly and young donors

The respiratory burst has been shown to be have different responses depending on experimental conditions [86, 91, 94, 95, 97, 140]. Therefore, I measured the capacity to release O_2^- by neutrophils stimulated with or without 100 nM fMLF or 20 ng/ml PMA for 90 min, by using the Cytochrome C assay (**Figure 17 a, b, c**).

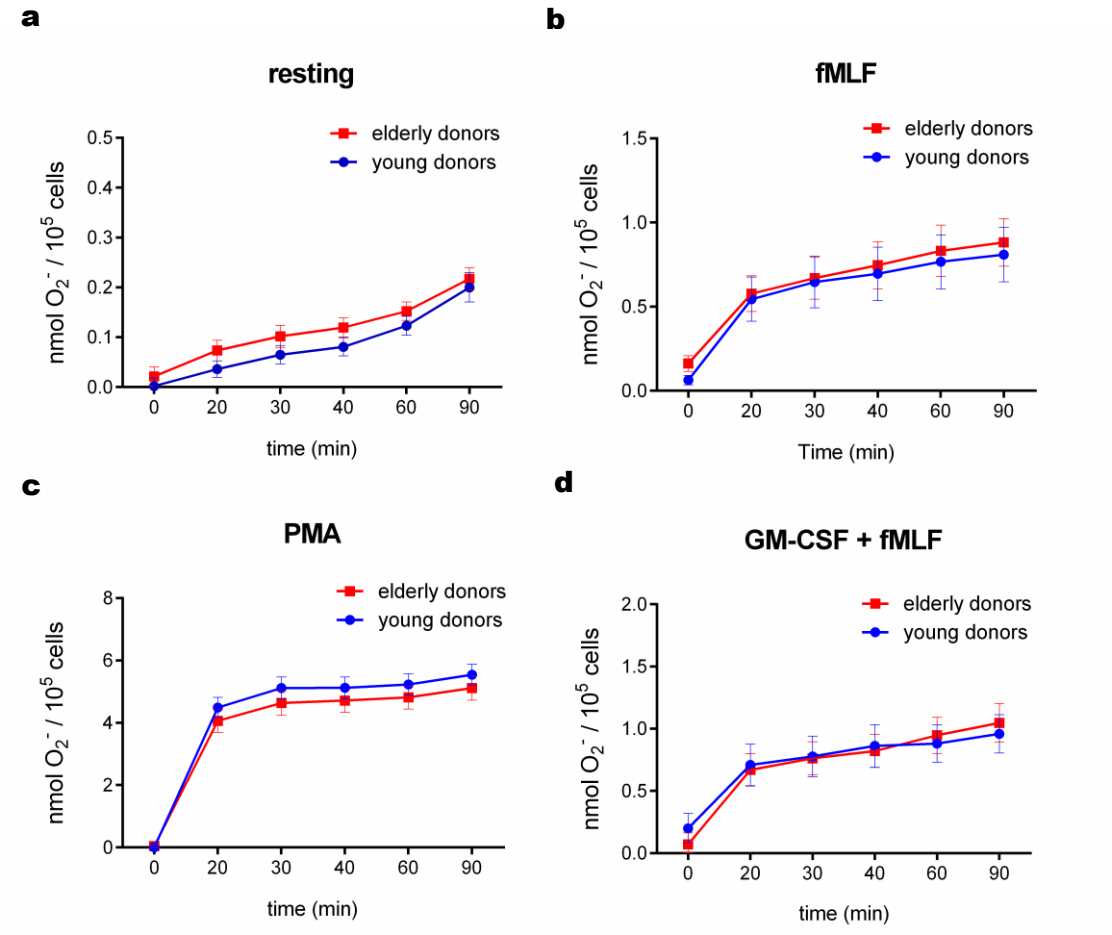

Figure 17 | O_2^- production by neutrophils isolated from healthy elderly and young donors.
Neutrophils were either stimulated with or without 100 nM fMLF or 20 ng/ml PMA for 90 min (**a**, **b**, **c**). In addition, neutrophils were pretreated with 20 ng/ml GM-CSF for 90 min prior to the addition of 10 nM fMLF for additional 90 min (**d**). O_2^- production was measured by the Cytochrome C-assay. Data are expressed as nmol of O_2^- for 1 x 10^5 cells/well ± SEM.

As a further experimental condition, I also measured the O_2^- production by neutrophils pretreated for 90 min with 20 ng/ml GM-CSF and then incubated for additional 90 min with 10 nM fMLF (**Figure 17d**). Results obtained in the two cohorts were then compared (**Figure 17**).

As shown in **Figure 17 b, c** , I found no differences in the capacity to produce superoxide anion following stimulation with either fMLF or PMA by neutrophils from the two groups under investigation. Similarly, no significant differences in the O_2^- release by neutrophils from both cohorts emerged after the priming experiments (**Figure 17d**). However, a slight, but not significant, increase of the constitutive O_2^- production by neutrophils from the healthy elderly group was observed without stimulation (**Figure 17a**).

Taken together, our results reported a normal production of superoxide anion by neutrophils of old subjects after stimulation, showing no differences in production in comparison to the youngest group. While, concerning resting neutrophils, our work suggests that neutrophils from elderly individuals may produce more superoxide anion constitutively.

6. mRNA expression of proinflammatory genes in neutrophils from healthy elderly and young donors activated *in vitro* by various stimuli

As mentioned in the introduction, our group has discovered that highly purified human neutrophils are able to produce a number of cytokines, including IL-6, IL-12p40, IL-23, G-CSF and CCL23 when incubated with R848 [47]. Interestingly, the highest mRNA expression of most of these cytokines in response to R848 is observed after 12-20 h, while in autologous monocytes is observed at earlier time-points [27]. It was then demonstrated that the delayed expression of IL-6, IL-12p40 and IL-23 mRNA in neutrophils derives from the necessity to remodel the chromatin, a process occurring slower than in monocytes [27].

The interest in eventual changes of gene expression in immune cells during aging is supported by the idea that age-dependent changes in immune function may contribute to the aging process of the whole body. Considering that,

according to the literature, there is an impairment of multiple functions of neutrophils in healthy elderly individuals, I decided to perform a preliminary analysis of age-related changes of cytokine expression in neutrophils. This was to evaluate whether alterations of expression of proinflammatory genes, for instance IL-6, CXCL8 or TNFα might be detected. For such a purpose, I performed RT-qPCR experiments using highly purified populations of neutrophils, isolated from healthy young and old donors, and then incubated for up to 20 h with or without various agonists, including R848, LPS, GM-CSF, G-CSF and TNFα.

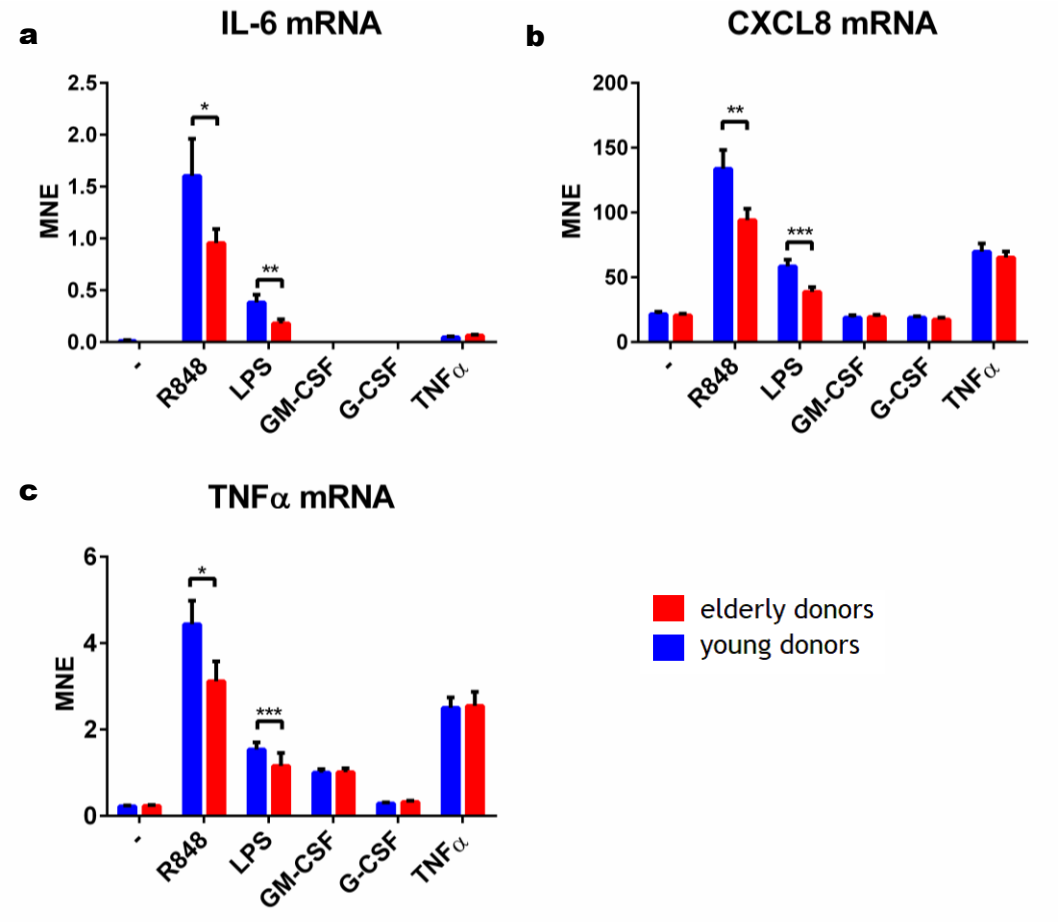

Figure 18 | mRNA expression of proinflammatory cytokines in stimulated neutrophils.
IL-6, CXCL8 and TNFα mRNA expression in ultra-purified neutrophils after 20 h of incubation with or without 5µM R848, 1µg/ml LPS, 20ng/ml GM-CSF, 1000 U/ml G-CSF, 10ng/ml TNFα by RT-qPCR. Neutrophils from young and old donors are reported in blue and red, respectively. Gene expression data are depicted as mean normalized expression (MNE) units (mean ± SEM, n young=20, n elderly=20) after RPL32 mRNA normalization. Asterisks indicate significant

differences between young and elderly subjects: *P<0.05, **P<0.01, ***P<0.001, by two-way ANOVA followed by Bonferroni's posttest.

As shown in **Figure 18a**, R848- or LPS-treated neutrophils of healthy elderly donors expressed lower levels of IL-6 mRNA than neutrophils from young subjects. Similarly, neutrophils of healthy elderly donors accumulated significantly lower levels of CXCL8 mRNA than neutrophils from young donors when incubated with R848 and LPS (**Figure 18b**). As expected [27], GM-CSF, G-CSF and TNFα had no effect in triggering the expression of IL-6 mRNA in neutrophils from both groups. Similarly, not even GM-CSF and G-CSF affected the levels of CXCL8 mRNA in neutrophils from both groups (**Figure 18b**). By contrast, CXCL8 mRNA was found to be upregulated by TNFα, but no differences in CXCL8 mRNA expression levels were found between neutrophils from healthy elderly and young donors.

Finally, also the expression levels of TNFα mRNA were significantly lower in healthy elderly donors than in young donors after treatment with R848 or LPS (**Figure 18c)**. Furthermore, while G-CSF was found unable to trigger the expression of TNFα mRNA in both neutrophil cohorts, GM-CSF and TNFα did so, yet with no variation between the neutrophils from the elderly cohort as compared to that from young donors. Overall, these data suggest that neutrophils deriving from the elderly group display an impaired capacity to express IL-6, CXCL8 and TNFα mRNA in response to R848 and LPS, but not to TNFα.

7. mRNA expression of anti-inflammatory genes in neutrophils from healthy elderly and young donors activated in vitro by various stimuli

Subsequently, I decided to investigate if, in neutrophils, age-related changes may also affect the mRNA expression of anti-inflammatory genes, such as SOCS3 and IL-1ra (**Figure 19**).

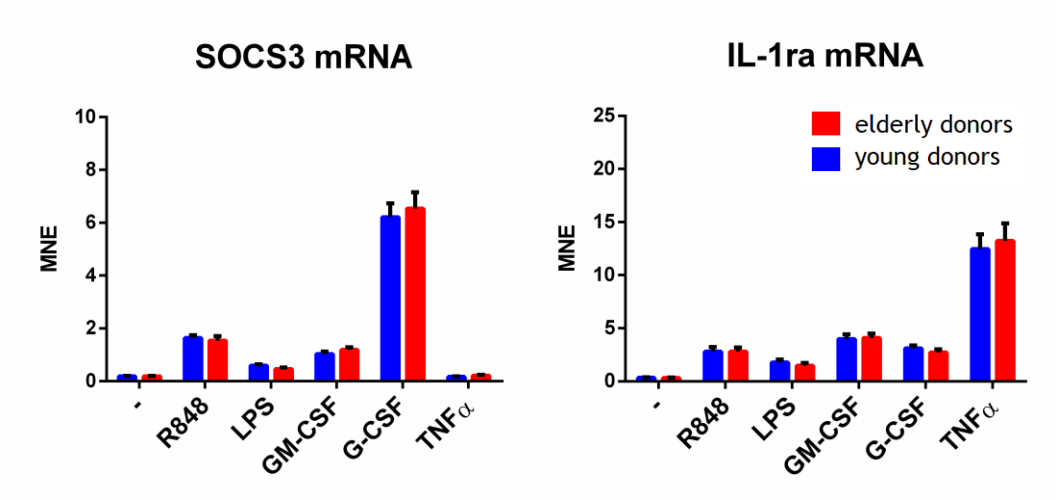

Figure 19 | mRNA expression of anti-inflammatory genes in stimulated neutrophils.
mRNA expression of SOCS3 and IL-1ra, in ultra-purified neutrophils after 20 h of incubation with or without 5μM R848, 1μg/ml LPS, 20ng/ml GM-CSF, 1000 U/ml G-CSF, 10ng/ml TNFα by RT-qPCR. Gene expression data are depicted as mean normalized expression (MNE) units (mean ± SEM, n young=18, n elderly=17) after RPL32 mRNA normalization. Asterisks indicate significant differences between young and elderly: *P<0.05, **P<0.01, ***P<0.001, by two-way ANOVA followed by Bonferroni's posttest.

However, contrary to what found in the case of IL-6, CXCL8 and TNFα (**Figure 18**), the expression levels of SOCS3 and IL-1ra mRNA in neutrophils from healthy elderly and young donors activated in vitro by R848, LPS, GM-CSF, G-CSF and TNFα did not significantly change (**Figure 19**). The fact that transcription of proinflammatory but not anti-inflammatory genes is reduced in TLR-stimulated neutrophils isolated from the elderly indicates that the regulation of these genes does not occur at the signaling pathway level but is rather gene specific and therefore possibly controlled at the epigenetic level.

8. Gene expression profiling in untreated and R848-treated neutrophils from healthy young and elderly donors

One of the main objectives of this thesis was to identify, at the whole transcriptome level, those genes that may have an altered expression in neutrophils or monocytes of elderly subjects. Using next generation sequencing techniques, I performed transcriptome analysis in neutrophils and, by comparison, autologous monocytes, isolated at the highest degree of purity from 20 elderly donors, to compare the data with the transcriptome analysis of neutrophils and monocytes from 20 young donors. mRNA experiments were performed using cells right after isolation or after stimulation with R848 for 20 h. Because of the very low numbers of highly purified neutrophils recoverable from a single healthy donor, and given that neutrophils possess low amounts of total RNA compared to other cell types (approximately 1 µg of total RNA per 10 million cells), I took advantage of the Smart-seq^2 technology [126], a recently developed method which allows to obtain a library for transcriptome analysis from little amounts of RNA, for instance, less than 500 picograms. Moreover, this method of library preparation is cheaper than conventional RNA-seq techniques, thus reducing the sample preparation costs (in our case, consisting of 160 samples). All libraries were thus sequenced by the Illumina next-seq 500 sequencing system, in turn obtaining a mean of 10 million reads per sample.

Spearman rank correlation analysis

Spearman rank correlation analysis was used to compare whole transcriptomes obtained by smart-seq^2 from neutrophils and CD14$^+$-monocytes isolated from old and young individuals. Spearman correlation is a non-parametric rank-based metric that makes it well suited for non-normal distributions and it is used to measure the degree of association between two variables. Using non-parametric methods when integrating datasets with different levels of variability is favorable. Correlation is a bivariate analysis that measures the strength of association between two variables and the direction of the relationship. This test does not carry any assumptions about the distribution of the data and therefore is

unbiased. A quick way to discover relationships between pairs of quantitative variables in a dataset is a heatmap based on pair-wise correlations.

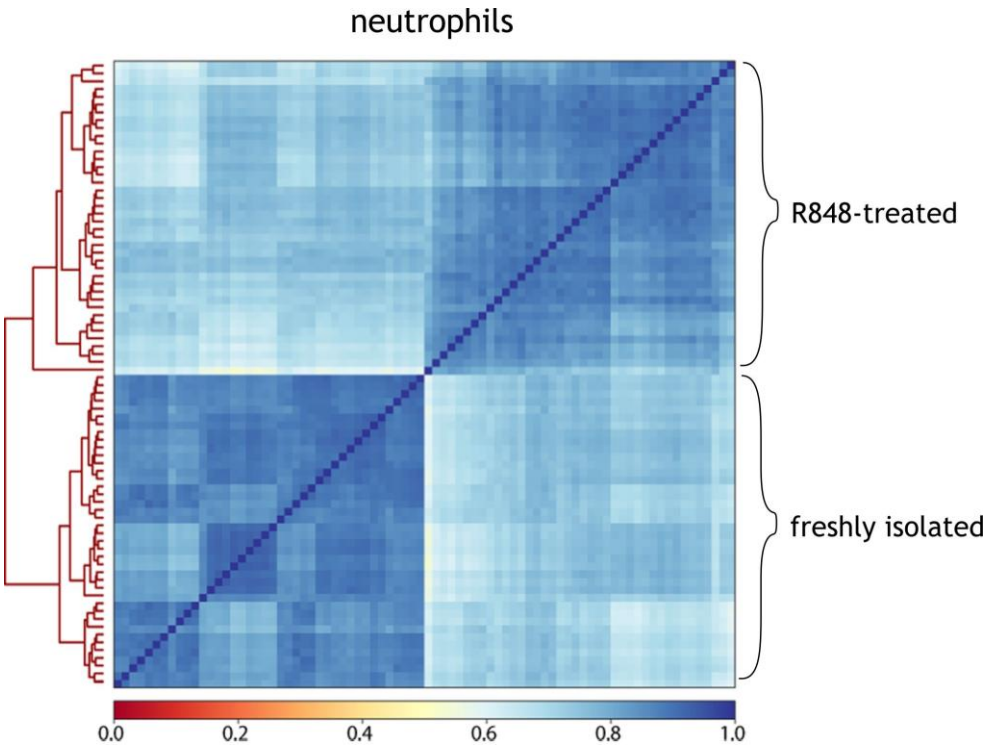

Figure 20 | Heat map of Spearman correlations between transcriptional profiles of neutrophils from healthy elderly and young donors freshly isolated or treated with R848 for 20 h
Heatmap displaying Spearman rank correlations between all pairwise comparisons of neutrophils Smart-seq2 data from healthy young and elderly donors. On the right of the graph it is indicated if the sample derives from R848-stimulated or the freshly isolated cells. The distance between the groups ranged from zero to one (Spearman rank), respectively colored by red and blue. The scale is reported in the lower part of the heatmap. A value of +1 (blue) indicates a perfect association of ranks, a value of zero (red) indicates no association between ranks. Nodes are shown on the left side of the heatmap, where the dendrogram is depicted in dark red.

In **Figure 20** it is depicted a heat map-based unsupervised hierarchical clustering of neutrophils, freshly isolated or stimulated for 20 h with R848, in which both the datasets of healthy young and elderly donors are present. The heatmap delineated that neutrophils stimulated with R848 clearly segregate from that of freshly isolated neutrophils, based on Spearman rank correlation.

Similar results were also observed in autologous CD14^{+}-monocytes. In fact, looking at the heatmap which represent the transcriptional profile of all CD14^{+}-monocytes of both the cohorts, stimulation represents the main variable that permits to cluster samples also for this cell type (**Figure 21**).

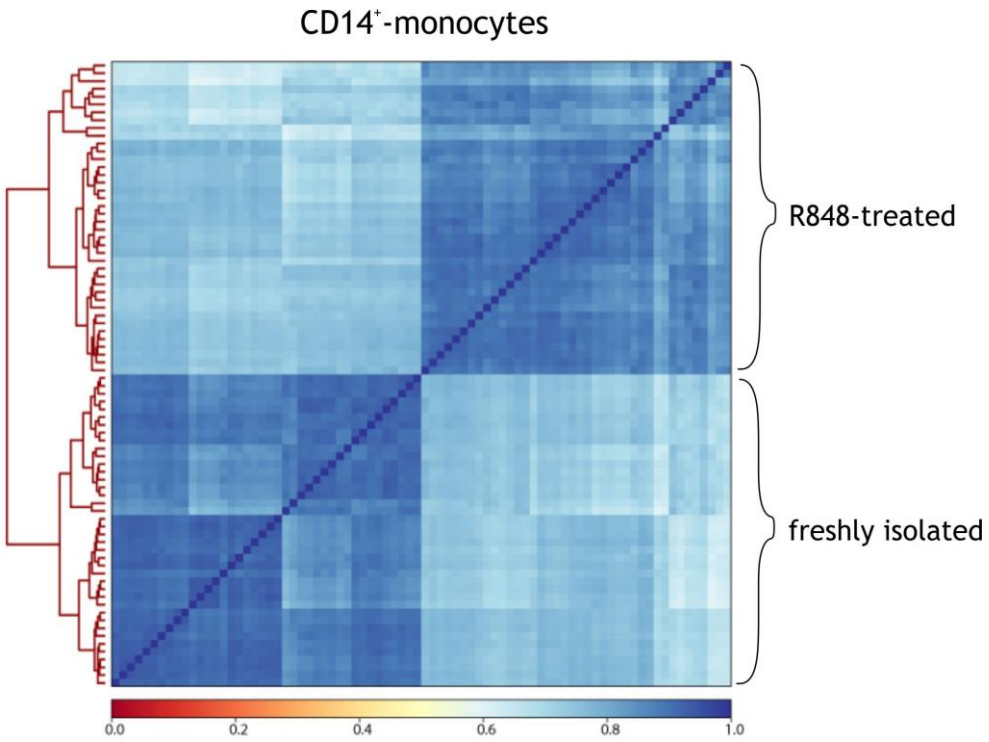

Figure 21 | Heat map of Spearman correlations between transcriptional profiles of CD14^{+}-monocytes from healthy elderly and young donors freshly isolated or treated with R848 for 20 h
Heatmap displaying Spearman rank correlations between all pair-wise comparisons for CD14^{+}-monocytes Smart-seq2 data from healthy young and elderly donors. On the right of the graph it is indicated if the sample derives from R848-stimulated or the freshly isolated cells. The distance between the groups ranged from zero to one (Spearman rank), respectively colored by red and blue. The scale is reported in the lower part of the heatmap A value of +1 (blue) indicates a perfect association of ranks, a value of zero (red) indicates no association between ranks. Nodes are shown on the left side of the heatmap, where the dendrogram is depicted in dark red.

These data point out that the main factor causing differences between our samples, at the whole transcriptome level, is the stimulation with R848. On the one hand, these results indicate that the samples have been correctly prepared and that experiments are reproducible, even considering the high variability of response that is usually found between different human donors. On the other hand, combining the datasets of young and old donors in both resting and stimulated conditions, it is not possible to identify differences related to age, so far.
Therefore, I choose to analyse again neutrophils and $CD14^+$-monocytes keeping the R848-stimulated samples separated from those freshly isolated.

In **Figure 22** is reported the heatmap related to neutrophils, freshly isolated (**a**) and R848-stimulated (**b**) while **Figure 23** represents $CD14^+$-monocytes, freshly isolated (**a**) and R848-stimulated (**b**).

Unbiased hierarchical clustering of the different transcriptomes revealed that datasets from young and elderly individuals do not segregate among them, in any of the subgroups analyzed. In fact, as depicted in **Figure 22** and **Figure 23**, even if the dendrograms showed some clusters, it was not possible to identify, macroscopically, the variables responsible of this clustering. Interestingly, since the gender of the donors was known, it was also possible to exclude that the various datasets could cluster according to the gender. Spearman rank correlation of the datasets produced in this study indicates that analyzing all transcripts at a global level does not allow the identification of any statistically significant differences related to age.

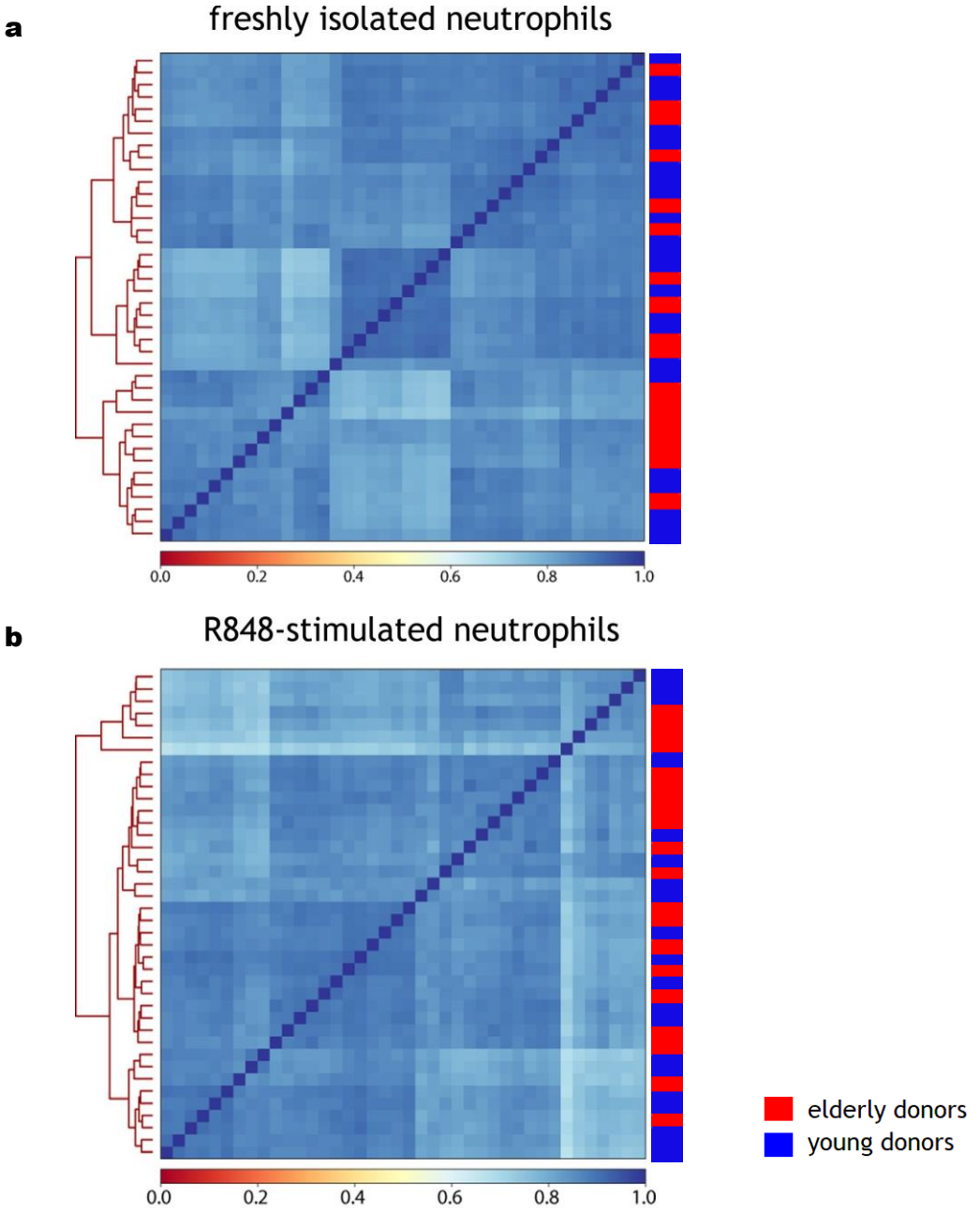

Figure 22 | Heat map of Spearman correlations between transcriptional profiles of neutrophils from healthy elderly and young donors freshly isolated or treated with R848 for 20 h

The heatmap displays a correlation matrix based on Spearman rank correlation of read counts in neutrophils from young and elderly donors. The correlation has been done separately between each group of freshly isolated neutrophils (**a**) or R848-treated (**b**). On the lower part of each heatmaps, there's the scale used: the distance between the groups ($\mathbf{r_s}$ = spearman rank) ranged from zero to one, respectively grading from red to blue. A value of +1 (blue) indicates a perfect association of ranks, a value of zero (red) indicates no association between ranks. Nodes are shown on the left side of the heatmap in which the dendrogram is depicted in dark red. On the right of the heatmap it is reported if the sample arises from a young (bright blue square) or an elderly (bright red square) donor.

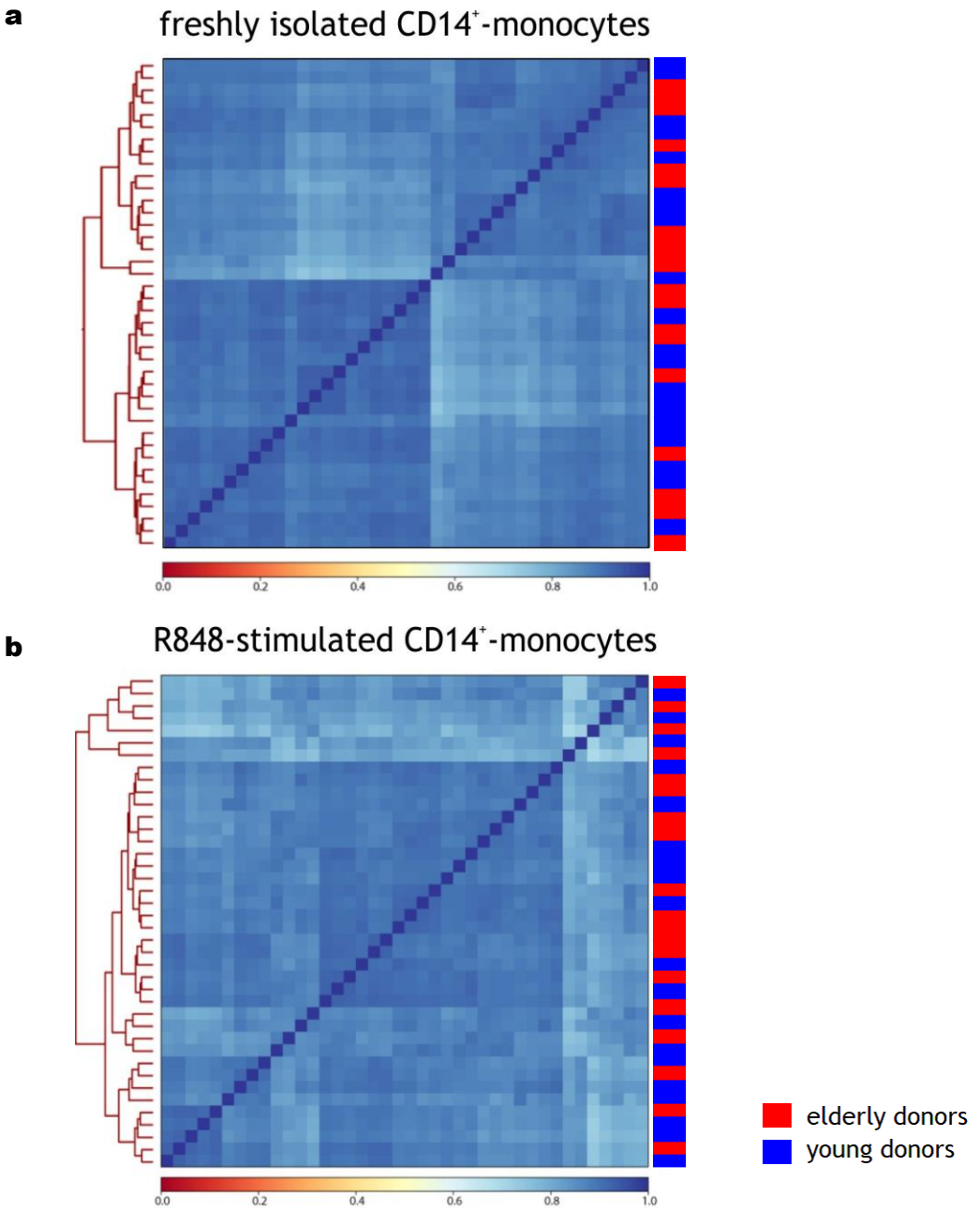

Figure 23 | Heat map of Spearman correlations between transcriptional profiles of CD14$^+$-monocytes from healthy elderly and young donors freshly isolated or treated with R848 for 20 h

The heatmap displays a correlation matrix based on Spearman rank correlation of read counts in CD14$^+$-monocytes from young and elderly donors. The correlation has been done separately between each group of freshly isolated (**a**) or R848-treated (**b**). On the lower part of each heatmaps, there's the scale used: the distance between the groups ($\mathbf{r_s}$ = spearman rank) ranged from zero to one, respectively grading from red to blue. A value of +1 (blue) indicates a perfect association of ranks, a value of zero (red) indicates no association between ranks. Nodes are shown on the left side of the heatmap in which the dendrogram is depicted in dark red. On the right of the heatmap it is reported if the sample arises from a young (bright blue square) or an elderly (bright red square) donor.

Identification of differentially expressed genes between healthy elderly and young donors

Since Spearman rank correlation did not reveal substantial differences between the healthy young and the elderly groups, I decided to concentrate my analysis to the differentially expressed genes (DEGs), rather than to the totality of the expressed genes. DEGs between healthy elderly and young donors were identified using DESeq2 [128], both in neutrophils and monocytes, maintaining separated freshly isolated and R848-stimulated cells. In this context, DEGs with higher expression levels in elderly than in young donors were designated as 'up-regulated', while those with lower expression levels in elderly were labeled as 'down-regulated'. The results are shown as volcano plots, in which the red dots represent the significantly ($p<0.05$) more expressed transcripts in aged donors, while the blue dots represent transcripts whose expression is significantly ($p<0.05$) lower in aged donors. For freshly isolated neutrophils, 285 DEGs between elderly and young subjects were identified, of which 197 were more expressed and 88 less expressed (**Figure 24**).

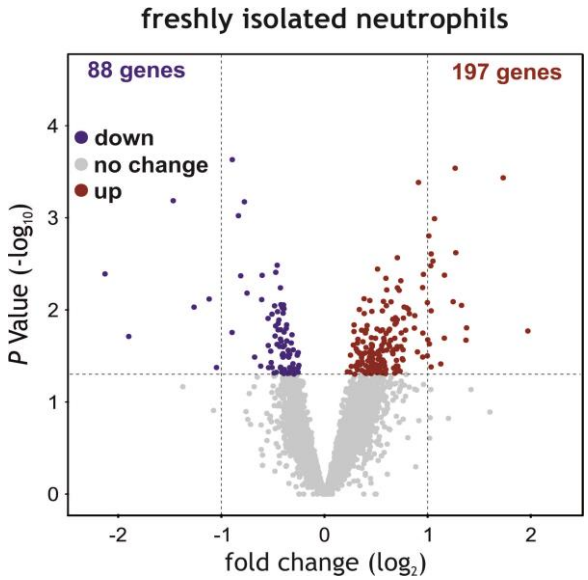

Figure 24 | Differential gene expression in freshly isolated neutrophils of elderly vs young donors.
Differential gene expression from smart-seq2 data is depicted in a Volcano plot. The $\log_2$ fold change difference between the samples from elderly and young donors is represented on the x-axis, and the negative $\log_{10}$ of *P*-values is represented on the y-axis. Significantly increased or decreased expressed genes in elderly are marked by red and blue dots, respectively. Non-significant genes are shown as gray dots.

To get more insights into the biological behavior of neutrophils in aging, it was necessary to understand the functional distribution of these DEGs. For this purpose, I decided to perform a Gene Ontology analysis using GOrilla [133]. The results of enrichment analysis by Gene Ontology terms obtained from the list of downregulated genes in freshly isolated neutrophils is depicted in **Figure 25a**.

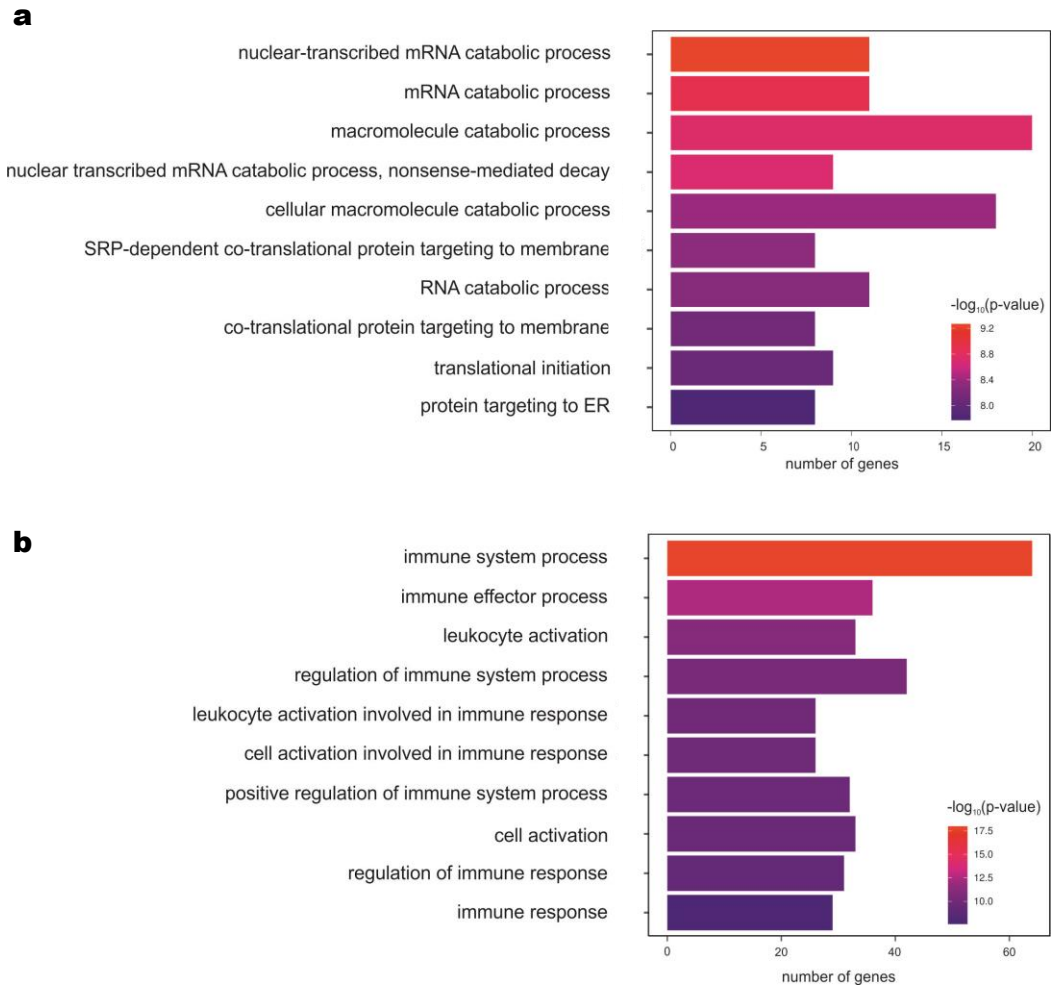

Figure 25 | Enrichment analysis of Gene Ontology terms of differentially expressed genes between freshly isolated neutrophils from Elderly vs those from young donors.
The results of enrichment analysis of Gene Ontology terms obtained from the list of genes of freshly isolated neutrophils less (**a**) or more (**b**) expressed in elderly are shown. The top 10 enriched terms, based on a hypergeometric distribution *P*-value, are represented in a bar plot, listed from the most to the less significant one. The number of genes belonging to a specific process is indicated in the x-axis.

Interestingly, the most enriched GO terms of genes downregulated in the elderly were related to mRNA catabolic process, that consists in the chemical reactions and pathways resulting in the breakdown of mRNA. Among the genes associated to mRNA catabolic process the most interesting were EIF4A3 (eukaryotic translation initiation factor 4a3), a gene involved in pre-mRNA splicing as component of the spliceosome [141], as well as many ribosomal proteins including RPL35, RPS21, RPS12, RPLP1, RPL4, RPL36 and RPS20. These data indicate that maybe a reduced capacity to translate mRNA is present in neutrophils from elderly donors.

Instead, the most enriched GO terms identified in genes more expressed in freshly isolated neutrophils from elderly were related to the immune system process (**Figure 25b**). Genes belonging to this group were: IL18R1 (interleukin 18 receptor 1), XBP1 (x-box binding protein 1), IFNAR2 (interferon (alpha, beta and omega) receptor 2), CR1 (complement component (3b/4b) receptor 1), CD14, CD83, CD40, MMP9 (matrix metallopeptidase 9), CX3CR1 (chemokine (c-x3-c motif) receptor 1), TNFAIP3/A20 (tumor necrosis factor, alpha-induced protein 3), NFKBID (nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, delta), NFKBIZ (nuclear factor of kappa light polypeptide gene enhancer in b-cells inhibitor, zeta), EGR1 (early growth response 1), EDN1 (endothelin 1), CD177, FUT7 (fucosyltransferase 7 (alpha (1,3) fucosyltransferase)), LAIR1 (leukocyte-associated immunoglobulin-like receptor 1), LILRA4 (leukocyte immunoglobulin-like receptor, subfamily a, member 4), LILRB5 (leukocyte immunoglobulin-like receptor, subfamily b, member 5), LILRB4 (leukocyte immunoglobulin-like receptor, subfamily b, member 4) and many others. Interestingly, most of the genes mentioned above are localized into the membrane.

The number of DEGs in R848-stimulated neutrophils from the healthy elderly (**Figure 26**), compared to the respective freshly isolated neutrophils (**Figure 24**), increased to 583, of which 452 were more expressed and 131 less expressed in the elderly cohort.

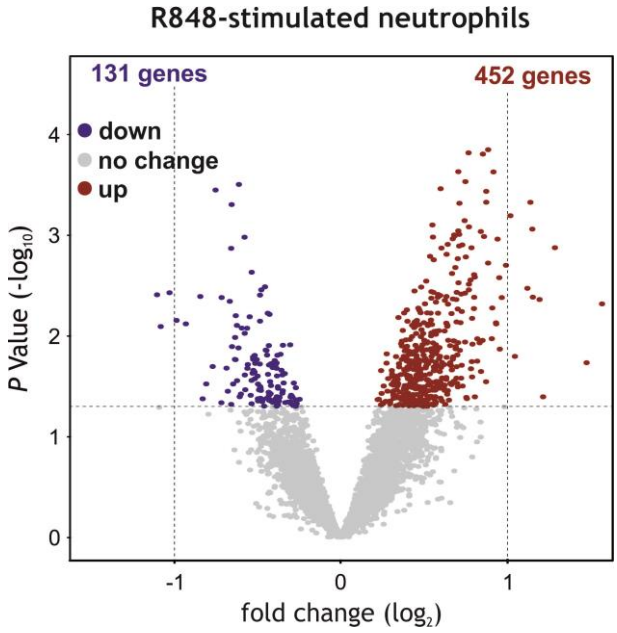

Figure 26 | Differential gene expression in neutrophils treated for 20 h with R848 from elderly vs young donors.
Differential gene expression from smart-seq2 data is depicted in a Volcano plot. The $\log_2$ fold change difference between the samples from elderly and young donors is represented on the x-axis, and the negative $\log_{10}$ of *P*-values is represented on the y-axis. Significantly increased or decreased expressed genes in elderly are marked by red and blue dots, respectively. Non-significant genes are shown as gray dots.

GO analysis of the genes less expressed did not identify any relevant GO term. However, in the R848-stimulatory condition, the list of the genes less expressed in the neutrophils from elderly donor, was enriched in many cytokine genes, such as IL12B, encoding for the p40 subunit of IL-12, CCL18, IL-16 and TNFSF8 (CD30 Ligand). Similarly to what observed in freshly isolated neutrophils **Figure 25a**, EIF4A3 was less expressed in the neutrophils from elderly also upon R848-stimulation.

In **Figure 27** is reported a snapshot taken from the Integrated Genomics Viewer showing IL-6 gene expression level in neutrophils from young and elderly donors, freshly isolated or treated with R848 for 20 h. For each horizontals line are reported the tracks of the samples. Even if having a *p-value* higher than 0.05, due to the high interindividual variability of gene expression observed among different human donors, the mRNA expression of IL-6 in R848-stimulated cells was lower in neutrophils from the elderly than young donors.

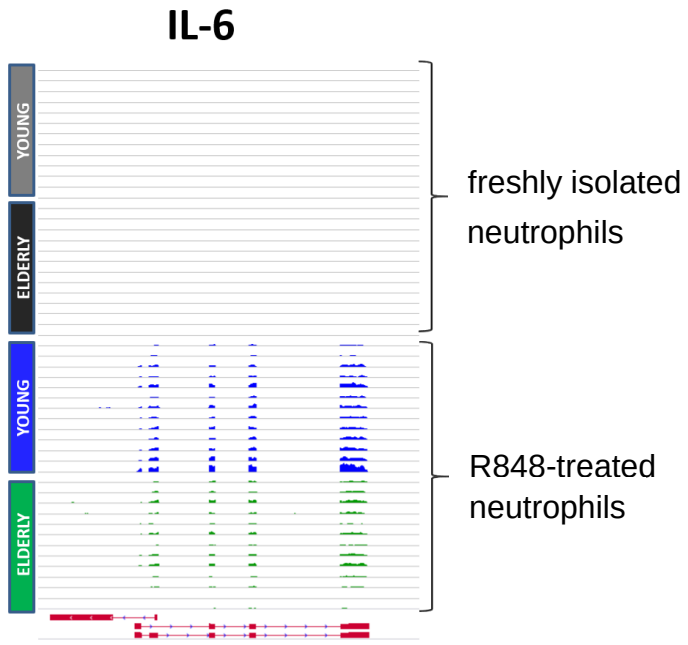

Figure 27 | Integrated Genomics Viewer snapshot showing IL-6 gene expression levels in neutrophils from young and elderly donors, freshly isolated or incubated with R848 for 20 h.
In the figure every horizontal line correspond to a sample track and the height of the signal for every single exon is proportional to the number of reads obtained from sequencing. Grey and black colors refer to freshly isolated neutrophils belonging to the young and to the elderly group, respectively, while blue and green are stimulated neutrophils from young (blue) and elderly (green). In red, at the bottom of the figure, are shown the exons which compose IL-6 transcripts.

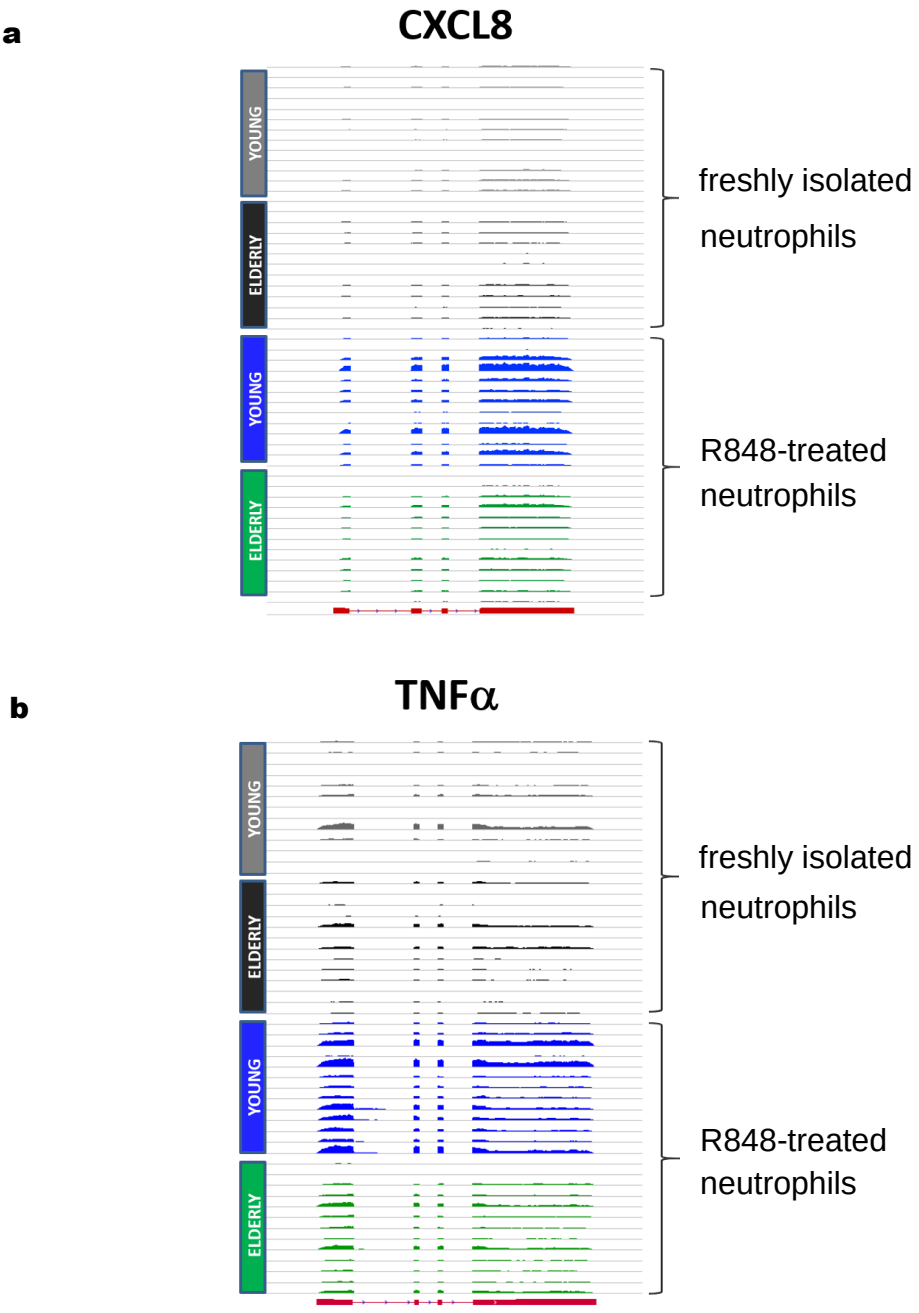

Figure 28 | Integrated Genomics Viewer snapshot showing CXCL8 and TNFα gene expression level in neutrophils from young and elderly donors, freshly isolated or incubated with R848 for 20 h.
(a) IGV snapshot of CXCL8 gene expression levels; (b) IGV snapshot of TNFα gene expression levels. In the figure, every horizontal line correspond to a sample track and the height of the signal for every single exon is proportional to the number of reads obtained from sequencing, . Grey and black colors refer to freshly isolated neutrophils belonging to the young and to the elderly group, respectively, while blue and green are stimulated neutrophils from young (blue) and elderly (green). In red, at the bottom of each panel, are shown the exons which compose CXCL8 and TNFα transcripts, respectively.

In **Figure 28a** is depicted an Integrated Genomics Viewer snapshot of CXCL8 gene expression levels in neutrophils from young and elderly donors, freshly isolated or incubated with R848 for 20 h. As displayed in the IGV snapshot, R848-treated neutrophils of elderly donors expressed lower mRNA CXCL8 expression. Similar results were obtained also for TNFα mRNA, as reported in **Figure 28b**.

Overall, data represented in **Figure 27** and **Figure 28** point out that mRNA expression of proinflammatory cytokines, for instance IL-6, CXCL8 and TNFα, in R848-stimulated cells was lower in neutrophils from the elderly than young donors. These data, despite having a *p-value* higher than 0.05, due to the high interindividual variability of gene expression observed among different human donors, confirmed the data obtained by RT-qPCR (**Figure 18**).

In the R848-stimulatory condition, the most enriched GO terms identified from the list of up-regulated genes in neutrophils from elderly were related to immune system process (**Figure 29**) similarly to what observed in freshly isolated neutrophils **Figure 25b**. Even if the process identified is the same, only part of these genes, such as CR1, LILRB4 and FUT7 are more expressed in the elderly in both freshly isolated and stimulatory conditions. Interestingly, other GO terms particularly enriched in R848-stimulated neutrophils from elderly were related to the granule proteins (**Figure 29**). Among the genes more enriched in these GO terms, the most interesting were: CST3 (cystatin c), GRN (granulin), CNN2 (calponin 2), CAT (catalase), GCA (grancalcin, ef-hand calcium binding protein), GSDMD (gasdermin d) and TMEM63A (transmembrane protein 63a).

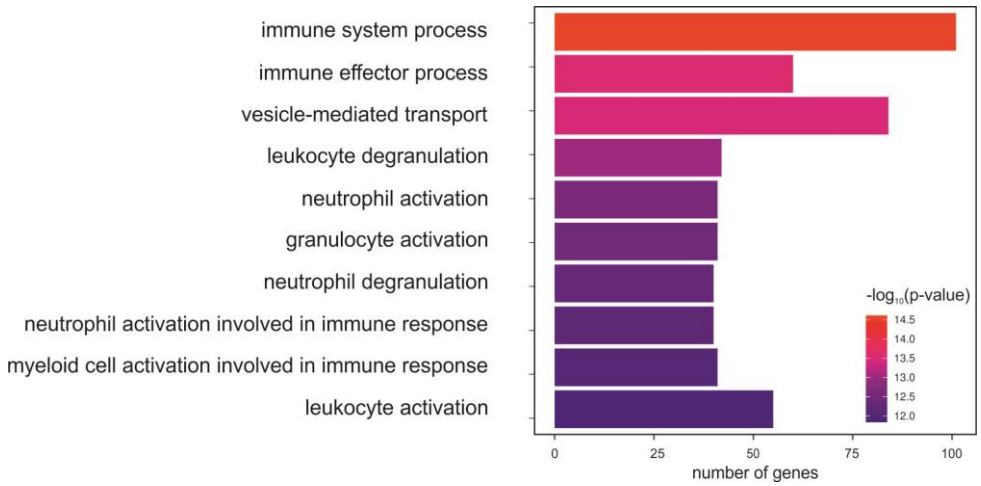

Figure 29 | Enrichment analysis of Gene Ontology terms of differentially expressed genes between R848-treated neutrophils from Elderly vs those from young donors.
The results of enrichment analysis of Gene Ontology terms obtained from the list of genes of R848-treated neutrophils from elderly are shown. The top 10 enriched terms, based on a hypergeometric distribution *P*-value, are represented in a bar plot, listed from the most to the less significant one. The number of genes belonging to a specific process is indicated in the x-axis.

Next, we examined the DEGs expressed by the autologous CD14$^+$-monocytes isolated from young and elderly individuals. With regards to resting, freshly isolated, CD14$^+$-monocytes, we found 603 DEGs, 334 of which were up-regulated and 269 down-regulated (**Figure 30**).

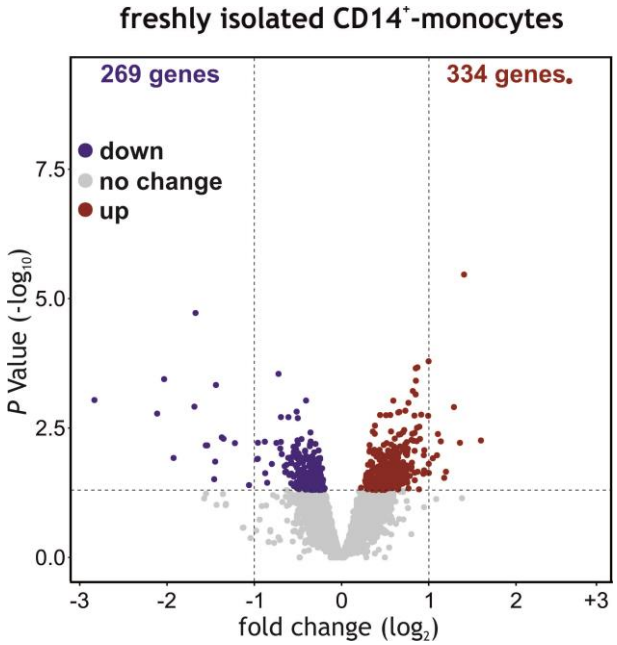

Figure 30 | Differential gene expression in freshly isolated CD14$^+$-monocytes of elderly vs young donors.
Differential gene expression from smart-seq2 data is depicted in a Volcano plot. The $\log_2$ fold change difference between the samples from elderly and young donors is represented on the x-axis, and the negative $\log_{10}$ of *P*-values is represented on the y-axis. Significantly increased or decreased expressed genes in elderly are marked by red and blue dots, respectively. Non-significant genes are shown as gray dots.

The most enriched GO terms identified in genes down-regulated in freshly isolated CD14$^+$-monocytes from elderly were related to "cotranslational protein targeting to membrane" and "translational initiation" (**Figure 31a**). Many ribosomal proteins belong to these groups, which, interestingly, can be identified also in the group of genes less expressed in freshly isolated neutrophils from elderly. Moreover, similarly to what observed for neutrophils, the eukaryotic translation initiation factor EIF4A3 is one of the more down-regulated gene also

in monocytes isolated from elderly. Among the GO terms that instead were enriched in the up-regulated genes in freshly isolated monocytes from elderly, I could identify general terms related to protein transport and localization (**Figure 31b**).

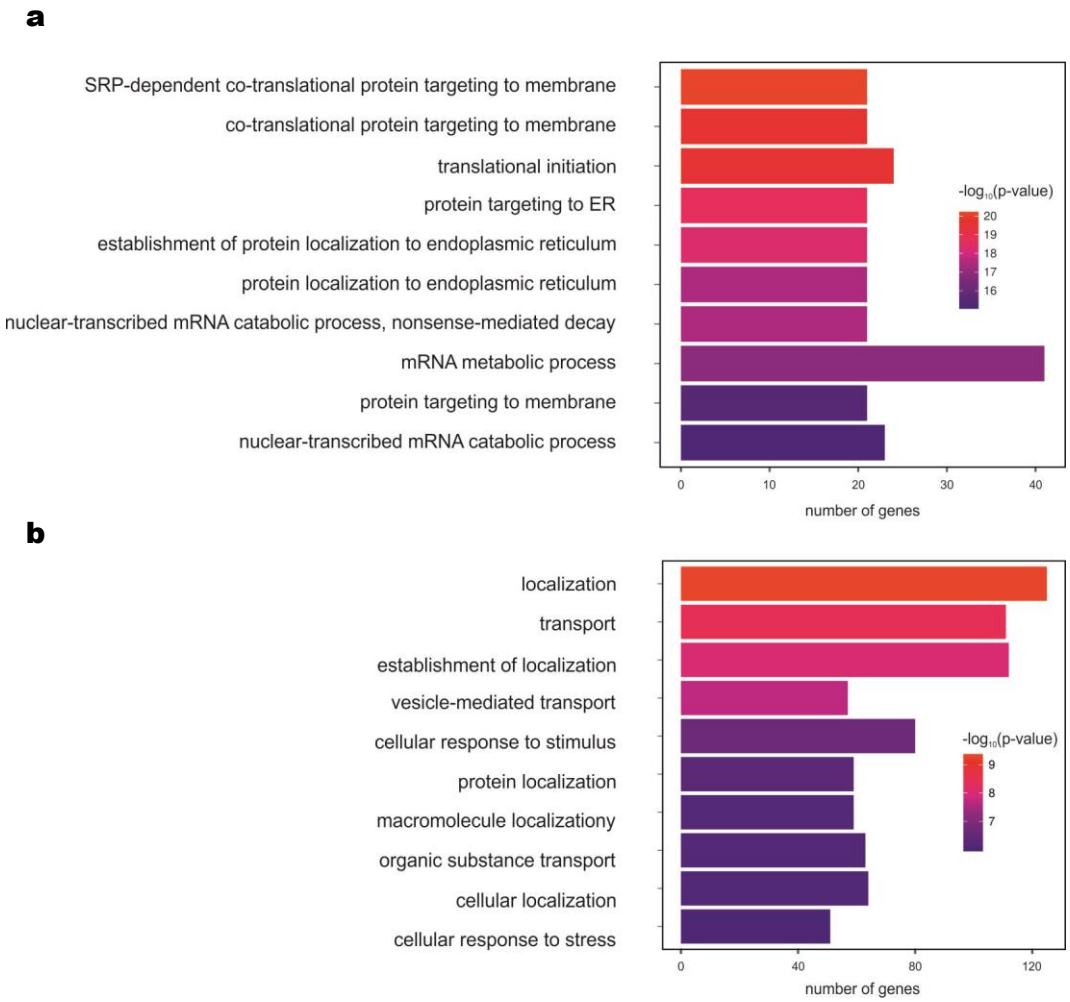

Figure 31 | Enrichment analysis of Gene Ontology terms of differentially expressed genes between freshly isolated CD14$^+$-monocytes from Elderly vs those from young donors.
The results of enrichment analysis of Gene Ontology terms obtained from the list of genes of freshly isolated CD14$^+$-monocytes less (**a**) or more (**b**) expressed in elderly are shown. The top 10 enriched terms, based on a hypergeometric distribution *P*-value, are represented in a bar plot, listed from the most to the less significant one. The number of genes belonging to a specific process is indicated in the x-axis.

Figure 32 depicts, in the R848-stimulatory condition, the number of significantly modified genes more or less expressed in CD14^{+}-monocytes from elderly or young donors. We found a total of 706 DEGs, of which 300 were up-regulated in the elderly, while the other 406 genes showed lower expression.

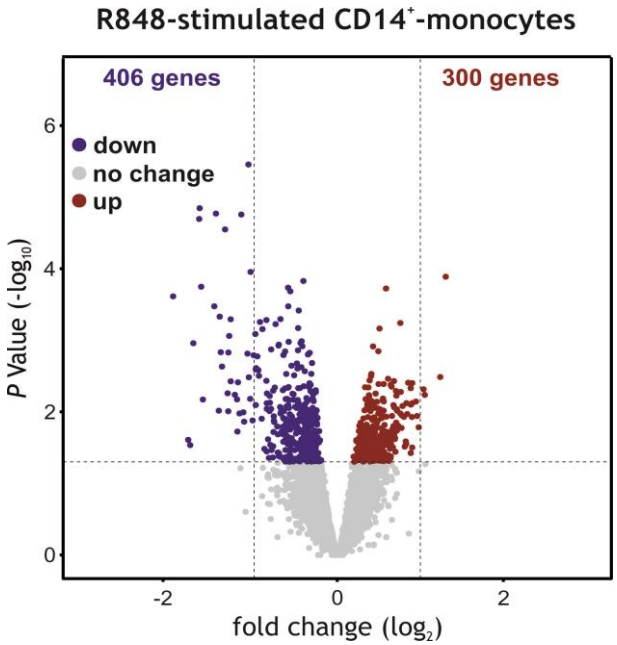

Figure 32 | Differential gene expression in CD14^{+}-monocytes treated for 20 h with R848 of elderly vs young donors.
Differential gene expression from smart-seq2 data is depicted in a Volcano plot. The $\log_2$ fold change difference between the samples from elderly and young donors is represented on the x-axis, and the negative $\log_{10}$ of *P*-values is represented on the y-axis. Significantly increased or decreased expressed genes in elderly are marked by red and blue dots, respectively. Non-significant genes are shown as gray dots.

The results of enrichment analysis of Gene Ontology terms obtained from the list of down-regulated genes in R848-stimulated monocytes from elderly are shown in **Figure 33a**. The GO term "immune effector process" was the most enriched, and different cytokines and chemokines (such as CCL18, CCL19, IL-10, CCL5 and VEGFA), as well as macrophage-associated genes (such as CD163, MARCO (macrophage receptor with collagenous structure), FCGR1A/CD64 (Fc

fragment of IgG receptor Ia), SLAMF1 (Signaling Lymphocyte Activation Molecule), were present in this list. Of note, in R848-stimulatory condition, many interferon stimulated genes, for instance IFTM1 (interferon induced transmembrane protein 1), IFTM2, IFTM3, ISG20, CCL5 and IRF2, were significantly less expressed in monocytes isolated from elderly compared to young donors.

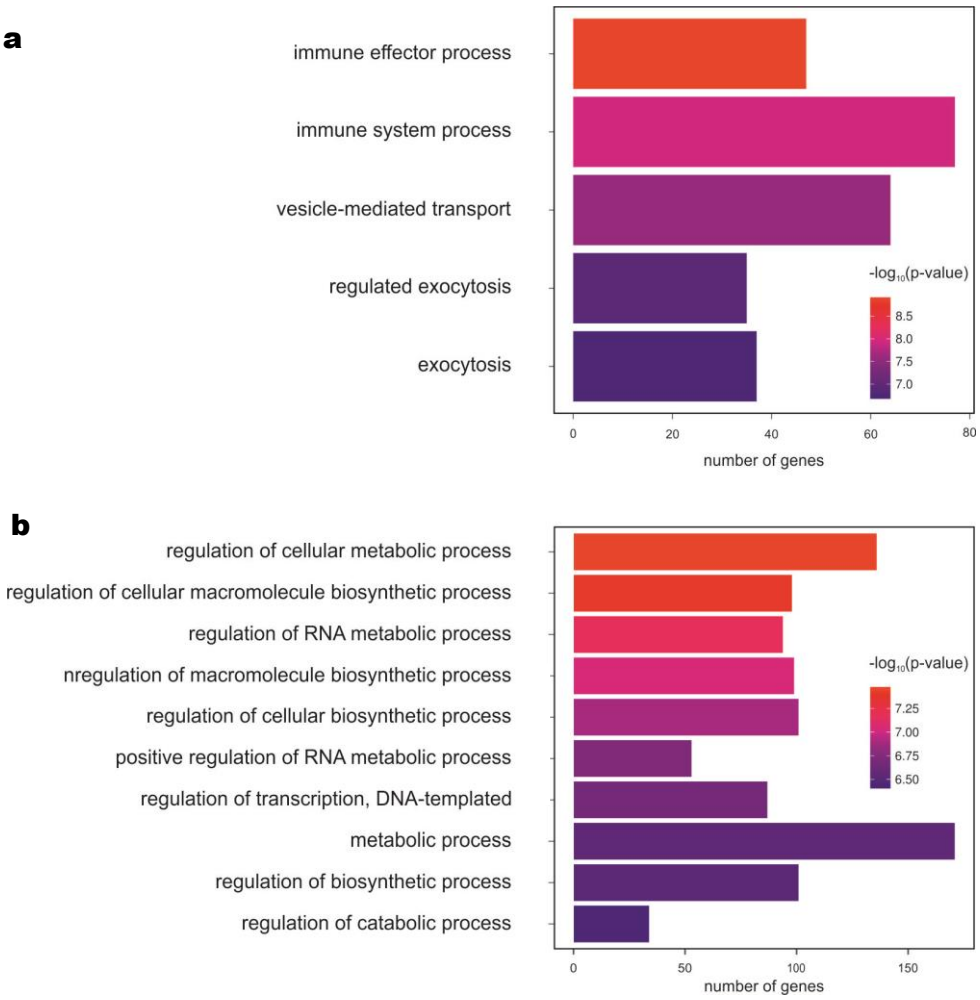

Figure 33 | Enrichment analysis of Gene Ontology terms of differentially expressed genes between R848-treated CD14^{+}-monocytes from Elderly vs those from young donors.
The results of enrichment analysis of Gene Ontology terms obtained from the list of genes of R848-treated CD14^{+}-monocytes less (**a**) or more (**b**) expressed in elderly are shown. The top 10 enriched terms, based on a hypergeometric *P*-value, are represented in a bar plot, listed from the most to the less significant one. The number of genes belonging to a specific process is indicated in the x-axis.

In R848-stimulatory condition, the most enriched GO terms identified in genes up-regulated in R848-stimulated monocytes from elderly were related to regulation of cellular metabolic process (**Figure 33b)**. Among the enriched genes, we recognised genes with chromatin remodelling functions such as: BRD3 (bromodomain containing 3), BRWD3 (bromodomain and wd repeat domain containing 3), SMARCA4 (swi/snf related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4), ARID1B (at rich interactive domain 1b), KAT6A (k(lysine) acetyltransferase 6a), KMT2A (lysine (k)-specific methyltransferase 2a), CHAF1A (chromatin assembly factor 1 subunit A), CHD3 (chromodomain helicase DNA binding protein 3) and CHD8 (chromodomain helicase DNA binding protein 8). In the group of genes were present also many transcription factors such as: ETV5 (ets variant 5), IRF8 (interferon regulatory factor 8), IKZF1 (ikaros family zinc finger 1), PPARA (peroxisome proliferator-activated receptor alpha), CEBPD (ccaat/enhancer binding protein (c/ebp), delta), UBTF (upstream binding transcription factor, rna polymerase i), POU2F2 (pou class 2 homeobox 2), KLF13 (kruppel-like factor 13) and USF2 (upstream transcription factor 2, c-fos interacting).

In reference to the top ten of the most enriched GO terms in $CD14^{+}$-monocytes treated with R848, we found processes mostly related to metabolism and biosynthesis and, interestingly, one of the process found in this list was related to regulation of transcription.

DISCUSSION

In this study, I have investigated whether human neutrophils from elderly have impaired functions and/or differentially expressed genes compared to those of young donors. For such a purpose, I used multiple methodological approaches, including RT-qPCR, smart-seq^2, flow cytometry and respiratory burst assays.

A major challenge in immune-gerontologic studies is to separate the influence of medical disorders from the physiologic process of aging [142]. In such regard, neutrophils and monocytes were isolated from healthy subjects selected using an extensive screening procedure, including personal history questionnaire and testing of blood for various biochemical parameters, following the inclusion criteria of the SENIEUR protocol [73]. For this study, enrolment criteria for older subjects included nondementia and independent living with control hypertension and arthritis, and were excluded older individuals with comorbidities and with the usage of medication that could interfere with immune system and other conditions listed in the protocol [73]. This screening procedure reduces the impact of diseases or other conditions that may influence final results, thus, providing accurate representative of healthy cohorts.

In the current study, CRP plasmatic concentration was used as inflammatory marker. CRP is an acute phase protein produced mainly by hepatocytes in response to proinflammatory diseases. Expected values for young population go up to 3 mg/L. Small elevations in CRP may indicate chronic inflammation, whereas a consistent increase of CRP plasmatic concentration correlates with a wide range of age-associated diseases. [143, 144]. In accordance with other investigations [86, 100], I found increased plasma levels of CRP in healthy elderly as compared to young donors, which may indicate a sort of proinflammatory state in the group of elderly, consistent with the eventual presence of a basal inflammatory state, known as *inflammaging* [68, 69]. Moreover, similarly to what is reported in clinical studies, I found other blood parameters that were significantly different in healthy elderly and young individuals, such as the concentration of cholesterol, lipoproteins, urea, the number of platelets and the estimated glomerular filtration rate. Among the

parameters listed before, the number of platelets and the eGFR were significantly decreased in old subjects, while the others increased. Concerning the reduction of platelets it has been suggested from other studies that it may reflect a decrease in the hematopoietic stem cell reserve during aging [145]. The decrease of eGFR in healthy old subjects confirms what is reported in the literature, namely that this decline is part of the normal physiologic process of cellular and organ senescence, and is associated with structural changes in the kidney [146]. Moreover, some studies report an increase in plasma urea in healthy elderly individuals, which I found too, hypothesizing that this is due to an impaired urea clearance [147]. The number of neutrophils, similarly to monocytes and other populations, was not altered in my cohort of subjects in line with previous observations [87]. Surprisingly, I found differences in the numbers of only the less abundant cell populations, for instance pDCs and NKT cells: pDCs were found decreased in the elderly, while NKT cells minimally increased. As a whole, it appears that differences in leukocyte numbers between elderly and young do not justify different responses to pathogens and predisposition to diseases. The anomaly must therefore be investigated at the level of functional defects of the different type of cells. Under this view, I therefore investigated whether various functions of neutrophils, for instance, rescue from apoptosis, surface markers expression, respiratory burst, and gene expression could be altered in old donors.

Neutrophils are known to have a limited life span *in vitro* and *in vivo*, ultimately undergoing apoptosis. In this way, neutrophils may be prevented from harming normal tissue through abnormal release of toxic substances, such as proteolytic enzymes. On the other hand, survival of neutrophils may be beneficial for the host in case of Gram-negative bacterial infections, since they survive long enough to phagocytose and kill bacteria. Although the apoptosis process of neutrophils is believed altered in the elderly [19, 90], the data on the effects of stimuli known to increase the survival of neutrophils are controversial. In my study, apoptosis was measured in highly pure population of neutrophils by vital dye exclusion using vibrant/sytox evaluation by flow cytometry. By doing so, I observed that agents such as R848, LPS, GM-CSF, G-CSF and TNFα, which are known to prevent apoptosis in neutrophils, have a similar effect on neutrophils

obtained from either elderly or young subjects. While there are studies reporting that the rate of spontaneous neutrophil apoptosis was either not altered with age or was slightly increased [19, 88], others showed that the ability of factors such as G-CSF, GM-CSF, TNF-α or LPS, to delay apoptosis was significantly reduced in neutrophils from old subjects [19, 89, 90]. Potential explanations for different results might be related to the use of different isolation techniques of neutrophils populations and/or to the different methods use to evaluate apoptosis. There are, in fact, some studies in which the reported purity of neutrophil population is not optimal [19, 98, 99, 148], as well as other ones in which the methods to evaluate neutrophil apoptosis were not comparable among themselves, since they included the morphologic analysis [19] or trypan blue staining [19] or annexin/PI [148, 149].

Usually, studies that investigate leukocyte functions in the elderly focus on possible alterations in their ability to respond when facing an inflammatory challenge [150, 151]. The hypothesis is that as there is a persistent inflammatory scenario in aged organisms, in addition to an unbalanced pro and anti-oxidant status, and for this reason neutrophils could exhibit a somewhat pre-activated state in response to their original microenvironment. This could lead to altered neutrophil response to certain stimuli.

Different results have been shown for surface activation markers levels in aged subjects, compared to young subjects [86, 97-99]. In my work, I evaluated the expression levels of some important surface markers, such as CD11b, CD16, CD62L and CD83 in neutrophils freshly isolated or incubated for 20 h with various stimuli (see M&M). As a result, I observed no statistically significant differences of CD11b expression between the neutrophils isolated from the two cohorts, confirming what already found by some researchers [94, 97, 99]. As stated before, different results have been shown for activation markers, for instance, CD11b was found lower in neutrophils isolated from nursing home residents when compared to community dwellers [152], conversely, in a cohort study with healthy aged individuals, no differences in neutrophil CD11b expression were found when compared to young subjects, which is in line with

our results [98]. Moreover, increased CD11b levels have been associated with hypertension, a condition of high prevalence in the elderly population [153]. Overall these results suggested that also cohort selection criteria are important, in order to exclude the incidence that some "pathological conditions" could have in the study and that might be the cause of the contrasting results shown in the literature. In my hands, also the expression of CD16/FcγRIII (a receptor important for phagocytosis) was not differentially regulated in the two cell populations considered. To date, there is only one publication concerning neutrophil apoptosis in the elderly, which is in contrast with our results, showing a significant decline in CD16 levels with age [98]. In the case of selectin CD62L, a molecule that is generally shed by neutrophils upon their activation, I have found that its expression, under resting conditions, is not altered in the cohorts of elderly and the younger group, similarly to what observed by Sauce et al. [97]. After neutrophil activation, down-regulation of CD62L was less pronounced in neutrophils from elderly than in young subjects, although at not statistically significant levels. By contrast, other studies have shown a statistically significant decreased in CD62L expression in neutrophils from elderly donors after stimulation [98-100], but these experiments were performed after incubation of whole blood with TNFα and LPS for 15 min [99]. Finally, I analysed the levels of CD83, which are not constitutively expressed by neutrophils, and that are critical for the cross-talk of neutrophils with lymphocytes. In such regard, I found that although CD83 was up-regulated in response to R848 and TNFα, its enhancement in the two groups was comparable. In the present work, the analysis of the main surface molecules involved in cell adhesion, cross-talk and phagocytosis, revealed no statistically significant differences between the two cohorts of elderly and young donors, whereas data present in literature are contrasting [86, 97-99]. This is likely due to the different experimental conditions used to measure surface markers, for instance related to the timing and type of stimulation, or to the measurement in whole blood rather than in isolated neutrophils, moreover the criteria used for cohorts selection might be another key factor able to explain these differences.

Superoxide anion and its related reactive oxygen species (ROS) are the principal defensive pro-oxidants generated by the respiratory burst of neutrophils. Our work suggest that neutrophils from elderly individuals may produce more superoxide anions constitutively and that this production may contribute to vascular damages in aging organisms as reported by literature [86]. It may also reflect neutrophils intracellular disrupted redox balance, that may influence the redox-sensitive cell processes, altering neutrophils function in aging. Of note that oxidative stress has an important role in aging and in the regulation of immune responses, probably playing a role in the development of age-related diseases. However, there has been a number of controversies related to some neutrophil functions from elderly, especially ROS production. In fact, some authors reported a decreased ROS production with aging, whereas others demonstrated normal or increased oxidative burst in old individuals [88, 91, 93, 94, 102, 103]. Data concerning changes in oxidative burst of neutrophils from aged individuals are variable in the literature, likely because of the different experimental conditions used. For instance, some research groups used the Cytochrome-C reduction assay, others used flow cytometry methods. Similarly, the stimuli used in the various studies are not the same, as well as the kinetics of stimulation [91]. While some researchers have measured the oxidative burst during phagocytosis, finding a decreased ROS production by neutrophils from the elderly [91, 102], other groups found that ROS production after E.Coli and PMA stimulation was not altered with age [87].

During my experiments, I investigated the generation of O_2^- by neutrophils using the cytochrome C assay, and found that, under resting conditions, the respiratory burst of neutrophils from the elderly was slightly higher (although not statistically significant) than in the young group, confirming some of the data presents in the literature [91]. However, I also found that upon stimulation with fMLF, neutrophils from the elderly and young group produced identical amount of O_2^-. Together, my data suggest that, at steady state, neutrophils produce higher amount of ROS during aging, that might contribute the establishment of the aging-associated inflammatory condition consistent with *inflammaging*. [86].

In additional studies performed by RT-qPCR, I found that IL-6, CXCL8 and TNFα, but not SOCS3 and IL-1ra, mRNA induction by LPS and R848 was slightly impaired in neutrophils from the elderly. These data have been further supported by preliminary results obtained by next-generation sequencing techniques. In fact, by analysing the neutrophil transcriptome made by smart-seq^2 experiments, I noticed a trend related to IL-6, CXCL8 and TNFα mRNA that mirrors the impaired response to TLR8 and TLR4 ligands of neutrophils isolated from elderly donors.

There are some groups [148, 154] sustaining that the alterations in neutrophil functions in the elderly might be correlated to alterations in the signal transduction pathways of the various receptors, for instance TLR4. They hypothesize that these alterations could be related to changes in the physicochemical properties of neutrophil membrane occurring with aging, which alter its fluidity. Changes in membrane fluidity affect the function of lipid rafts, which are membrane microdomains specialized for intracellular signaling, playing an important role in cellular functions. However, from my results I cannot sustain this speculation. I observed a decrease accumulation of transcripts related to the proinflammatory genes analyzed (IL-6, CXCL8, TNFα) in the elderly, after the use of TLR8 and TLR4 agonists, but not related to anti-inflammatory genes analyzed (IL1ra and SOCS3). Therefore, if the hypothesis of the lipid raft is true, the signal transduction pathways related to TLR8 and TLR4 should be impaired in the elderly because of its altered membrane fluidity, but in both proinflammatory and anti-inflammatory pathways. By contrast, my data show that only the pro-inflammatory and not the anti-inflammatory pathways appear to be altered, thus disproving that theory. I can only hypothesize that, in neutrophils from elderly, there might be defective mechanisms related to a single gene or to something in common to the pathways of the genes analyzed: IL-6, IL-8 and TNFα. Altogether, my results predict that, compared with their counterparts in younger subjects, neutrophils from elderly donors are not fully responsive in terms of proinflammatory cytokine expression to infectious stimuli upon recruitment to the site of infection.

One of the main goal of this PhD thesis was to identify genes or regulatory circuits that could be correlated with the defective functions occurring in innate immune cells during aging [155]. To achieve this aim, I performed Smart-seq^2 experiments using neutrophils and autologous CD14$^+$-monocytes. Smart-seq2 is a next generation sequencing method, recently developed [126], that allows to perform transcriptome experiments starting from small amounts of total RNA, such as few nanograms. To avoid inter-individual variability that can be expected in a population of healthy humans, I utilized 20 donors per each group of donors. Studies on neutrophil transcriptome profiles from patients affected by various diseases, such as tuberculosis, asthma, cystic fibrosis, COPD, sepsis and autoimmune diseases, provided an opportunity to identify candidate pathways involved in the pathogenesis of these different pathologies [156-159]. However, differences involved in transcriptional and epigenetic control of neutrophil functions in healthy young and old individuals, either under resting or stimulated conditions, have not been systematically investigated. By contrast, age-associated changes in cytokine, chemokine and interferon production, as well as expression of co-stimulatory proteins, have been identified by transcriptomic analysis of PRR-stimulated peripheral mononuclear cells (PBMCs) from healthy non-frail adults and older subjects [159].

During my research, a preliminary analysis of the whole transcriptome of freshly isolated and R848-stimulated neutrophils and, in parallel, CD14$^+$-monocytes, isolated from old and young individuals, by the use of Spearman Rank test, did not identify any differences related to age. Using non-parametric methods, such as Spearman's correlation, when integrating datasets with different levels of variability is, usually, favorable. Spearman rank correlation can discriminate only macroscopic differences between the samples and, in fact, a clear clusterization of the Smart-seq2 data based on the stimulation with R848 was perfectly identified in both neutrophils and monocytes. It is known, in fact, that stimulation with TLR ligands induces in neutrophils and monocytes a drastic change at the transcriptome level, with the up- and down- modulation of thousands of genes [160]. The effect of age on neutrophil or monocyte transcriptome was not appreciated using Spearman rank correlation, probably,

because the differences caused by ageing were lower in respect to the ones caused by stimulation. This result is presumably due to a careful screening of our donor cohorts, that were selected to be healthy, so without major diseases that could have increased transcriptional differences.

We decided therefore to perform a more focused and deep analysis for the identification of differentially expressed genes in neutrophil and monocyte datasets derived from elderly and young individuals.

We first compared the freshly isolated cells in order to determine whether there was an intrinsic difference between elderly and young donors, independent of stimulation. Most of the literature is composed by studies performed in whole blood [161, 162] or PBMC [159, 163]. In addition, there are other few studies focusing the analysis more in details on the three subsets of monocytes [164] or specifically focused on $CD14^+$-monocytes upon stimulation with LPS [165], while studies of neutrophils in healthy aging are missing.

We observed differences in gene expression of freshly isolated neutrophils with a total of 285 genes differentially expressed at higher levels in elderly relative to young individuals, of which 88 genes were less expressed in the elderly and 197 genes more expressed. In particular, one of the most under-represented gene sets in the elderly group was “initiation of translation” and “mRNA catabolic process”. In these categories we found several transcripts coding for numerous ribosomal proteins and, in particular, the transcript of the subunit A3 of the eukaryotic translation initiation factor 4, EIF4A3. EIF4A3 is a spliceosome component [166] and encodes for a member of the DEAD box protein family, which are putative RNA helicases. These genes are implicated in a number of cellular processes involving alteration of RNA secondary structure, such as translation initiation, nuclear and mitochondrial splicing, and ribosome and spliceosome assembly. EIF4A3 was found to be less expressed in both neutrophils and $CD14^+$-monocytes freshly isolated from elderly donors. The fact that this gene is less expressed in the elderly may reflect a general age-related impairment of neutrophils and monocytes translation processes.

Another study based on a microarray analysis of whole blood samples of adults and old donors, reported several biological pathways down-regulated in the group of elderly [161]. Among these pathways there were genes related to the biosynthetic capacity and metabolism, indicators of a reduced protein synthesis in blood cells of the elderly. In this study a large number of genes coding for ribosomal proteins and factors involved in the phases of initiation and elongation of translation were found to be down-regulated in the elderly, including subunits of the eukaryotic translation initiation factor 3 (EIF3). In literature there are several subunits of EIF3 which have been found to be down-regulated in the elderly. In mammalians, for example, eIF3 plays a crucial role in several steps of the initiation of mRNA translation and its deregulation may affect cell growth as well as causing cancer [167]. The down-regulation of these genes in old subjects suggests that binding of the mRNA and the interactions with the 40S ribosomal subunit could become progressively impaired or less efficient with aging. Another study in rats liver showed a decrease in the activity of eukaryotic initiation factor 2 (eIF-2), suggesting that the decline in protein synthesis that occurred in rat liver and brain during development and aging is associated with this decrease [168].

In our datasets, we observed a down-regulation of many ribosomal proteins with aging, some of which are component of the 60S subunit (RPL35, RPLP1, RPL4, RPL36) and others are component of the 40S subunit (RPS21, RPS12 and RPS20). The fact that many ribosomal proteins are less expressed in the freshly isolated neutrophils of the elderly could suggest that protein synthesis may slows down during aging. Notably, the association between ribosomal proteins and ageing in freshly isolated $CD14^+$-monocytes has been previously reported in other different studies [169, 170], while in neutrophils has been never explored.

Reynolds et al. [169] performed a study in which they analyzed the transcriptomic profiles of $CD14^+$-monocytes using microarrays and they identified genes belonging to the ribonucleoprotein complex, mitochondrial ribosome, and oxidative phosphorylation pathway enriched among age-associated genes. In another study, in which was performed a large whole-blood gene expression meta-analysis, it was revealed that among the genes down-regulated with age there were

genes encoding cytosolic ribosomal subunits such as RPL-genes (RPL8, RPL11, RPL18, RPL28, RPL30, RPL35 and RPL36), RPS-genes (RPS14, RPS16 and RPS29) and UBA52 (ribosomal protein L40) supporting the hypothesis that age-dependent protein synthesis dysfunction plays a causal role in human aging [171]. In fact, changes in the regulation of protein synthesis, posttranslational modifications, and protein turnover are crucial determinants of age-related decline in the maintenance, repair, and survival of the organism.

Slowing down of protein synthesis is one of the most commonly observed biochemical changes during aging [172]. The implications and consequences of slower rates of protein synthesis could be many, including a decrease in the availability of enzymes for the maintenance, repair, and normal metabolic functioning of the cell, an inefficient removal of inactive, abnormal, and damaged macromolecules in the cell, the inefficiency of the intra- cellular and intercellular signaling pathways. Age-related changes in the activity, specificity, and stability of a large number of proteins have been reported in a wide variety of cells, tissues, organs and organisms and confirmed by a number of studies where protein synthesis was monitored [173, 174]. In one of these studies protein synthesis, measured in liver tissues of rodents [175], was shown to decrease significantly (from 20 to 75%) proportionally with age. Moreover, it was found that the activity for formation of the initiation complex of 40S ribosomal subunits in the livers of old mice was found to be 15–20% lower compared to young ones [172, 176]. The amount and activity of translation factors is known to decline with age, which also contributes to the reduction of protein synthesis rates [168].

Furthermore, inhibition of global protein synthesis has been associated to lifespan increases across species, including yeast, nematodes and the fruit fly [173]. A number of mechanisms could explain how protein synthesis rate might influence the process of aging [173]. On the one hand, a rapid protein biosynthesis is accompanied by the production of damaged proteins, due to transcriptional or translational errors and co-translational misfolding. As a result, erroneous proteins are not able to maintain their native function and are accumulated in the cell, with the risk of creating potentially toxic aggregates. The accumulation of cellular damage is considered to be one of the main indications and cause of cellular aging

and aging-associated diseases [77]. Attenuation of global protein synthesis induced by the depletion of translation machinery components, can induce a decrease in the protein synthesis of normal as well as damaged proteins, and, thus, it might reduce the risk of toxic proteins accumulation [177].

Another hypothesis that explains why reduction of global protein synthesis can increase organism lifespan consists in taking into account that protein synthesis is one of the most energy-consuming cellular processes. It indeed requires ∼75 % of the total cell energy [178], and, consequently, a reduction of mRNA translation can increase, overall, energy availability. It may allow re-allocation of critical resources that can be mobilized towards cellular maintenance and repair processes, thus promoting organism longevity [179]. This theory is supported by studies which showed that the depletion of ribosomal proteins and translational factors in several model organisms decreases protein synthesis, increases stress resistance and, at the end, extends lifespan [180].

Other transcripts were found to be age-dependent in our study, for instance, transcripts of genes in the management of cellular oxidative stress and detoxification were decreased in neutrophils of elderly subjects. GSTP1, Glutathione S-transferases P1, is one of these genes that we found to be less expressed in freshly isolated neutrophils of elderly. It belongs to the Glutathione S-transferases (GSTs) family of enzymes that play an important role in detoxification of reactive oxygen species by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Soluble GSTs are categorized into 4 main classes: alpha, mu, pi, and theta, based on their biochemical, immunologic, and structural properties. This GST family member is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases. The down-regulation of this gene may lead to an impairment of successfully managing oxidative stress, even if, in our hands, we didn’t notice differences in ROS production in the cell tested with the cytochrome-C assay.

The most striking aspect in the changes associated with the process of aging is the dysregulation of inflammatory response, with an imbalance between inflammatory and anti-inflammatory mechanisms. Interestingly, and conforming to the *inflammaging* concept, that refers to the gradual deterioration of the immune system [68], I observed an increased expression of genes related to the immune system process in freshly isolated neutrophils. In literature it is reported that higher levels of pro-inflammatory cytokines and chemokines, in particular interleukin-1β (IL-1β), IL-6, IL-8, and TNFα are measurable in serum from elderly individuals. In particular, IL-6 is the most relevant cytokine associated to aging and considered as a biomarker of aging to predict age-relate diseases together with CRP. For instance, according to data obtained so far in almost 4,000 individuals enrolled in the PolSenior and Framingham Heart studies, IL-6 serum levels resulted to be good predictor of mortality in elderly adults [181]. The presence of high levels of these cytokines in the blood may be responsible of the basal activated state observed in freshly isolated neutrophils of old subjects. Moreover, this finding is in accordance with the increased production of ROS by steady state neutrophils during aging [86], and indicate that neutrophils potentially partecipate to the chronic, low-grade inflammatory state that has been observed in elderly [155]. On the contrary, in resting, freshly isolated $CD14^+$-monocytes, no increase in inflammatory genes was observed, as also reported by other studies [159, 164].

Focusing our attention on classical monocytes, we observed an increase expression of CX3CR1 in freshly isolated $CD14^+$-monocytes of elderly. CX3CR1 is the receptor for fractalkine, a transmembrane protein and chemokine involved in the adhesion and migration of leukocytes. It has been shown that an increased expression of CX3CR1 on monocytes from old individuals could be related to the increased incidents of chronic inflammatory diseases observed in this population. An increased expression of CX3CR1 has been identified, both at transcriptional and at protein level, in classical and non-classical monocytes from old subjects compared with adults [164]. This strongly suggests that higher expression of CX3CR1 in monocytes of old subjects could lead to enhanced recruitment of monocytes to inflammatory sites and might contribute to pathogenic effects in the

heart and other organs observed in the elderly. Moreover, we found a reduced levels of CCL20 in freshly isolated $CD14^+$-monocytes of old subjects. The reduced expression of this mediator, which works in the recruitment of immature DCs and lymphocytes into mucosal-associated tissues, may result in a reduced migration of immune cells to sites of infection such as lung and gastric mucosa. This effect could explain why infections such as influenza and bacterial pneumonia have worse outcomes in old subjects.

We then determined whether aging could impact the immune system in response to agonist by comparing the transcriptional profiles of young and old individuals.

Conversely to what we found in freshly isolated $CD14^+$-monocytes, upon R848 stimulation, genes related to inflammation were less expressed in elderly donors and, likewise, this occurred with neutrophils. In fact, confirming RT-qPCR results, many genes encoding for proinflammatory cytokines were less expressed in neutrophils from elderly. However, no statistically significant difference was observed at the level of proinflammatory cytokine mRNA expression in R848-stimulated $CD14^+$-monocytes (data not shown), differently from what observed in R848-stimulated neutrophils, in which there was lesser expression of CXCL8, TNFα and IL-6 mRNA in the elderly.

Metcalf and colleagues [159, 164], by the analysis of TLR8-stimulated $CD14^+$-monocytes isolated from elderly, were able to identify a clear reduction in TNFα, IL-1β and IL-6 protein release but not in the mRNA expression, similarly to what it is shown in this thesis. The different result concerning monocytes could be explained by the different kinetics of mRNA and protein induction observed after stimulation in the two cell types. In fact, as reported in a previous study by our group, the peaks of mRNA induction for cytokines such as IL-6 and TNFα, upon R848 stimulation occurs later in neutrophils than in $CD14^+$-monocytes (12-20 h for neutrophils and 3-6 h for $CD14^+$-monocytes) [27]. For this reason, the differences observed in cytokines release in the supernatants of TLR8-activated $CD14^+$-monocytes at 20 h, might be the results of a decreased transcription occurred at 3-6 h. The results obtained from R848-stimulated cells indicate a

general decreased capacity of neutrophils and $CD14^{+}$-monocytes from elderly to respond to external insults, in accordance with the theory of immunosenescence [155].

According to the original schedule, I also prepared samples to perform Chromatin Immunoprecipitation for next generation sequencing (ChIP-seq) for the histone 3 acetylated Lysine 27 (H3K27Ac), which is a histone modification specifically associated with active regulatory genomic regions. I have generated initial ChIP-seq data using both highly purified neutrophils and autologous monocytes from normal adult donors to determine the overall feasibility of the project. I obtained high quality datasets indicating that while monocytes and neutrophils use a common set of active and poised genomic regulatory elements, they also use specific sets of enhancers and promoters that are not shared with the other cell lineage. These data will be instrumental for the identification of the transcriptional circuits that with age undergo alterations in neutrophils and in monocytes. The basic idea is to take advantage of the genome-wide identification of footprints (chromatin modifications) generated by TF binding to chromatin to identify those TFs whose differential activity determines an alteration of neutrophil and monocyte transcriptional output, and eventually function, with age.

CONCLUSION

The data published so far on the decline of neutrophil function in the elderly are conflicting in many instances and lacking in information on the mechanisms of functional decline with age. However, the precise contribution of these age-related changes in neutrophil functions remains to be confirmed. This confusing scenario is the fuel for new and even more exciting research. Moreover, elucidation with more rigorous and sophisticated assays of age-changes in neutrophil functions is urgently required as they could have a great impact on the immune response in the elderly.

In this study, I outlined the features of neutrophils during aging, showing that many neutrophil functions in old subjects, including apoptosis, surface expression of molecular markers, superoxide anion production after stimulation do not differ from young people, while I find interesting results suggesting that the response to stimuli by neutrophils from elderly donors might be impaired for proinflammatory cytokine expression. These data have been further supported by preliminary results obtained by transcriptome analysis.

In conclusion, data shown in this study are consistent with the notion that human neutrophils undergo changes with age, indicating that they can potentially participate to the chronic, low-grade inflammatory state that has been observed in elderly.

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APPENDICES

During my PhD, besides my **main project** concerning **aging**, I took an active part in **other research projects focused on neutrophils** in different areas ranged from regulation of neutrophil derived cytokines to cancer.

Some of the projects are already published whereas others are submitted or ongoing.

One of the studies (*Cytokine production by human neutrophils: Revisiting the "dark side of the moon"*) is an **overview of all the cytokines** known to be produced by human neutrophils. Human neutrophils are known to express to produce many pro- inflammatory cytokines, chemokines, colony stimulating and angiogenic factors, TNF family members and growth factors, especially upon appropriate stimulation. It's becoming clear that neutrophils have an active role in the resolution of inflammation and the fact that when they're activated, they can produce cytokines de novo it's becoming widely accepted. However, knowledge of the molecular mechanism controlling cytokine expression it's not complete, thus, it's important to delve deep into the world of cytokine production, in order to acquire effective therapies which may target neutrophils derived cytokines.

Another study (*A Reappraisal on the Potential Ability of Human Neutrophils to Express and Produce IL-17 Family Members In Vitro: Failure to Reproducibly Detect It*) linked to the first one mentioned, is essentially an evaluation of the ability of neutrophils to produce specific interleukins belonging to the IL-17 family. It's well known that neutrophils perform various effector functions important for both innate and adaptive immune responses, for instance the synthesis and secretion of different cytokines. Nevertheless, there are controversial data in the literature, one of this was related to IL-17 production by human neutrophils *in vitro*. We were finally able to reject this hypothesis, confirming, also at epigenetic level, other previous findings on the **inability** of highly purified populations of human neutrophils **to express/produce IL-17**A, IL-17B, and IL-17F mRNAs/proteins *in vitro*.

In addition, this study (*Human neutrophils activated via TLR8 promote Th17 polarization through IL-23*) shows that human neutrophils incubated with TLR8 agonists are able to produce IL-23 in a TNFα-dependent fashion. It is known that human neutrophils contribute to the regulation of inflammation via the generation of a range of cytokines. In this study it has been investigated their ability to express members of the IL-12 family after incubation with TLR8 agonists, measuring the expression levels of transcripts and proteins for IL-12 family member subunits. We report that **highly pure human neutrophils express and produce IL-23**, further supporting the key roles played by these cells in the important IL-17/IL-23 network and Th17 responses.

The last study submitted (*Multisystem autoimmune disease caused by gain of function STAT3 mutation leading to constitutive STAT3 phosphorylation)* deal with **STAT3 signaling**. Mutations in STAT3 are associated with a broad spectrum of manifestations including immunodeficiency, and autoimmunity. However, these mutations can have dissimilar impact on STAT3 signaling, leading to reduced response or increased activation depending on the effects on phosphorylation, nuclear translocation and/or gene transcription. I this study we report a 7-year-old boy with multisystem autoimmune disease due to a novel heterozygous STAT3 **mutation** (p.M329R), and detectable **plasmatic levels of IL-6, IL-17 and IL-10.**

Moreover, in the first year of my PhD, in another laboratory in the University of Verona, I took part in the study of BCR-ABL and in the role of **PTPRG** (Protein Tyrosine Phosphatase, Receptor Type, G) in **chronic myeloid leukemia**. The research *"Assaying BCR-ABL1 kinase activity in Chronic Myeloid Leukemia using a peptide biosensor"* was focused on the development of a method able to evaluate the kinase activity of BCR-ABL1 in cell lysate of leukemic cells, with the purpose of predicting the outcome of Tyrosine Kinases treatments in CML within 1-2 days from first-line treatments.

sum, our data confirm and extend, also at epigenetic level, previous findings on the inability of highly purified populations of human neutrophils to express/produce IL-17A, IL-17B, and IL-17F mRNAs/proteins *in vitro*, at least under the experimental conditions herein tested. Data also provide a number of justifications explaining, in part, why it is possible to false positively detect IL-17A$^+$-neutrophils.

Keywords: neutrophils, IL-17 members, IL-17A, IL-17B, IL-17F

INTRODUCTION

The IL-17 family of cytokines consists of six members, namely IL-17A (usually referred to as IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F (1). After the discovery of a subtype of CD4$^+$ T helper, expressing IL-17A and IL-17F (currently known as Th17 cells), plenty of studies have been published correlating Th17 cells with a wide range of physiological and pathological processes. IL-17A and IL-17F are not only the most studied but also the most closely related, since they share 50% of amino acid sequence identity, adjacent gene localization (2) and binding to the same IL-17R, in this case composed by the IL-17RA and IL-17RC subunits (1). The IL-17R group comprises, in fact, five receptor subunits, IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE (3). IL-17RA was the first to be described, is ubiquitously expressed (particularly in hematopoietic cells), and functions as a common receptor subunit used by at least four ligands, namely IL-17A, IL-17C, IL-17E, and IL-17F (3). IL-17F is often coproduced with IL-17A, so that together they can also form an IL-17F/IL-17A heterodimer (4) binding to the IL-17RA/IL17RC complex as either homodimers or heterodimers (3). IL-17A and IL-17F are proinflammatory cytokines that play key regulatory roles in host defense and inflammatory diseases. They mainly mediate immune regulatory functions by promoting the generation of proinflammatory cytokines/growth factors (including G-CSF, GM-CSF, and IL-6) and chemokines (such as CXCL8, CXCL6, and CXCL1) by epithelial and other stromal cells, which ultimately lead to the attraction and activation of neutrophils and macrophages into the inflammatory site (5), as well as to granulopoeisis (6). Although crucial in protecting the host from invasion by many types of pathogens, including bacteria and fungi (7), dysregulated IL-17A and IL-17F production can lead to the development of autoimmune diseases, such as psoriasis, multiple sclerosis, and rheumatoid arthritis (RA), as well as cancer progression (5, 8). The latter observations hence make IL-17A/F as a very important target for the development of new therapies (1, 8).

As mentioned, Th17 cells are considered the main sources of IL-17A and IL-17F. However, other innate immune cells produce these cytokines, including γδ T cells, natural killer T cells, invariant natural killer cells, Paneth cells, TCRβ$^+$ natural Th17 cells, lymphoid-tissue inducer-like cells, IL-17-expressing type 3 innate lymphoid cells, and mast cells (8, 9). By contrast, it is still questionable whether human polymorphonuclear neutrophils represent sources of IL-17A or IL-17F. It is currently well established that neutrophils are crucial players in innate immune responses, not only for their capacity to perform defensive functions (10) but also for their ability to produce a large variety of cytokines (11). Concerning IL-17A and/or IL-17F, in 2010, we reported that highly purified populations of human neutrophils (>99.7%), incubated for up to 20 h with IFNγ and/or LPS *in vitro*, do not produce IL-17A (12). While a few papers substantially confirm our findings (13–17), the majority of the subsequent studies report that human neutrophils may represent sources of IL-17A (18–53). Experimental evidence proving that human neutrophils express IL-17A mostly, but not only, derives by immunohistochemistry (IHC) and/or immunofluorescence (IF) studies documenting IL-17A$^+$-neutrophils in tissue specimens from a variety of pathological conditions (18, 19, 21, 22, 25, 27, 28, 30–32, 34, 36–39, 41–43, 45, 47–49, 51, 53). Interestingly, many of these studies focus on psoriasis (20, 25, 30, 32, 35, 49), a disease characterized by an early accumulation of neutrophils in skin lesions in which neutrophil-derived mediators (such as reactive oxygen species, granule proteins, and cytokines) may alter the homeostatic state of keratinocytes and endothelial cells (54). At the end of 2014, however, Tamarozzi et al. (13) not only reported the absence of IL-17A mRNA expression and production by highly pure (99.9%) populations of resting or activated neutrophils but also demonstrated that some of the commercial polyclonal anti-IL-17A antibodies (Abs) stain neutrophils for their non-specific recognition of various intracellular proteins different from antigenic IL-17A. Nevertheless, reports describing either IL-17A-positive neutrophils in tissue samples from diseases or *in vitro*-stimulated neutrophils as sources of IL-17A, continue to be published (20, 23, 24, 26, 29, 33, 35, 40, 44, 46, 50, 52). Based on these premises, we decided to more accurately analyze the issue of whether human neutrophils produce IL-17A, as well as other IL-17 members *in vitro*.

MATERIALS AND METHODS

Cell Purification and Culture

Neutrophils were isolated from buffy coats of healthy donors (HDs) and manipulated under endotoxin-free conditions (12). In selected experiments, neutrophils were also isolated from peripheral blood of patients with severe psoriasis, as defined by either >10% body surface area involved, or Psoriasis Area and Severity Index score >10, or Dermatology Life Quality Index score >10 (55). After Ficoll-Paque gradient centrifugation of buffy coats or peripheral blood, followed by dextran sedimentation of granulocytes and hypotonic lysis of erythrocytes, neutrophils were isolated to reach 99.7 ± 0.2% purity by positively removing all contaminating cells using the EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, BC, Canada) (56). Neutrophils were then suspended at 5 × 10^6/ml in RPMI 1640

medium supplemented with 10% low (<0.5 EU/ml) endotoxin FBS (BioWhittaker-Lonza, Basel, Switzerland), incubated with or without 5 μM R848, 500 μg/ml particulate β-glucan (Invivogen, San Diego, CA, USA), 100 ng/ml ultrapure LPS (from *E. coli* 0111:B4 strain, Alexis, Enzo Life Sciences, Farmingdale, NY, USA), 1 μg/ml Pam3CSK4 (Invivogen), 50 μg/ml poly(I:C) (Invivogen), 1,000 U/ml G-CSF (Myelostim, Italfarmaco Spa, Milano, Italy), 100 U/ml IFNγ (R&D Systems, Minneapolis, MN, USA), 10 ng/ml GM-CSF (Miltenyi Biotec), 5 ng/ml TNFα (Peprotech, Rocky Hill, NJ, USA), 2–20 μg/ml IL-6 (R&D Systems), 0.2–2 μg/ml IL-23 (R&D Systems), 100–500 ng/ml IL-17A (R&D Systems), 10 μg/ml anti-IL-17A neutralizing Abs (secukinumab, Novartis, Basel, Switzerland), 100 nM fMLF, 500 μg/ml curdlan (Sigma, Saint Louis, MO, USA), 20 ng/ml phorbol mysistate acetate (PMA) (Sigma), 1 μg/ml Ionomycin (Sigma), 100 μg/ml CpG oligodeoxynucleotides (ODN) (Invivogen), and 1,000 U/ml PEGylated IFNα-2a (Pegasys, Roche, Basel, Switzerland). Inactivated conidia and hyphae from *Aspergillus fumigatus* were kindly provided by prof. Luigina Romani (University of Perugia, Italy), and used at a neutrophil-fungi ratio of 1:5 for *A. fumigatus* conidia and 1:1 for *A. fumigatus* hyphae, as previously described (57). Neutrophils were plated either in 6/24-well tissue culture plates or in polystyrene flasks (from Greiner Bio-One, Kremsmünster, Austria) for culture at 37°C, 5% CO_2 atmosphere. After the desired incubation period, neutrophils were either processed for chromatin immunoprecipitation (ChIP) experiments or collected and spun at 300 × *g* for 5 min for other types of assays. In the latter case, cell-free supernatants were immediately frozen in liquid nitrogen and stored at −80°C, while the corresponding cell pellets were either extracted for total RNA or lysed for protein analysis. Th1 and Th17 clones (58) were kindly provided by prof. Francesco Annunziato (University of Firenze). $CD4^+$ T cells were isolated by $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) and stimulated for up 72 h with anti-CD3 and anti-CD28 mAbs (5 μg/ml, BD Biosciences).

Flow Cytometry Experiments

For flow cytometry, 10^5 neutrophils were harvested after the desired treatment, centrifuged, and suspended in 100 μl PBS containing 10% complement-inactivated human serum for FcγR blocking. Neutrophils were then stained for 15 min at T room with: APC anti-human IL-17RA/CD217 (clone 424LTS) and APC mouse IgG1κ, as isotype control (clone P3.6.2.8.1) from eBioscience (San Diego, CA, USA); PE anti-human IL-17RC (clone 309822) and mouse PE IgG2B isotype control from R&D systems; PE-vio770 anti-human CD11b (clone ICRF44), FITC anti-human CD66b (clone G10F5), and PerCP-Cy5.6 anti-human CD16 (clone 3G8) from BioLegend (San Diego, CA, USA); APC anti-human CD62L (clone 145/15 Miltenyi Biotec), all at working dilutions specified in the corresponding datasheets. Sample fluorescence was then measured by MACSQuant Analyzer (Miltenyi Biotec), while data analysis performed using FlowJo software version 10 from Tree Star (Ashland, OR, USA) (59). For neutrophils of psoriatic patients, 100 μl whole blood were stained with APC anti-human IL-17RA and PE anti-human IL-17RC Abs in combination with the following mAbs: VioBlue anti-human CD14 (clone TÜK4), PE anti-human CD56 (clone AF12-7H3), PE-Vio770 anti-human CD3 (clone BW264/56), APC anti-human CD19 (clone LT19) from Miltenyi; Brilliant Violet anti-human CD45 (clone 2D1), PerCP-Cy5.5 anti-human CD16 (clone 3G8), and APC-Cy7 anti-human HLA-DR (clone L243) from BioLegend. After red cells lysis by the ammonium chloride buffer, sample fluorescence was immediately measured as previously described.

Superoxide Anion Measurement

After isolation, neutrophils were suspended at the concentration of 2×10^6 cells/ml in HBSS buffer containing 0.5 mM $CaCl_2$ and 1 mg/ml glucose. Neutrophils (100 μl/well) were then distributed in a 96-well plate and incubated for 10 min at 37°C prior to the addition of 80 μM cytochrome *c*, 2 mM NaN_3 (Sigma) and the indicated stimuli, including 20 ng/ml PMA as control. Plates were then incubated at 37°C in an automated ELx808IU microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) to record cytochrome *c* reduction (*via* absorbance at 550 and 468 nm, at intervals of 5 min for 90 min). O_2^- production was finally calculated using an extinction coefficient of 24.5 mM (60).

Immunocitochemistry, IHC and IF

Cytospin preparations of neutrophils (61) previously cultured with the indicated stimuli were stained by ematoxylin and eosin for morphological evaluation. After coverslip removal, specimens were rehydrated through a scale of alcohols, with endogenous peroxidase activity blocked by treatment with 0.3% H_2O_2 in methanol for 20 min. Anti-human IL-17A (AF-317-NA), IL-17B (AF1248), and CXCL8 (AF-208) goat IgG pAbs from R&D Systems were 1:50 diluted, added to specimens for 60 min and then revealed using the goat-on-Rodent HRP-polymer (Biocare Medical, Pacheco, CA, USA) followed by diaminobenzidine. Omission of the primary antibody, as well as isotype control staining, was also performed as negative controls. For IL-17A and IL-17B tissue immunostaining, 4-μm tissue sections from two FFPE cases of pustular psoriasis were deparaffinized and rehydrated through a scale of alcohols. Endogenous peroxidase activity was then blocked by treatment with 0.3% H_2O_2 in methanol for 20 min. Epitope retrieval was performed using a microwave oven in 1.0 mM EDTA buffer (pH 8.0), for 3 cycles of 5 min at 750 W. IL-17A and IL-17B were diluted 1:50 and revealed using the goat HRP-polymer (IHC) or the horse anti-goat IgG biotinylated (Vector Laboratories, Peterborough, UK) followed by streptavidin-FITC (Southern Biotech, Birmingham, AL, USA). DAPI was used for counterstaining. For double IHC, anti-IL-17A and IL-17B Abs were diluted 1:500, and after revelation (as detailed above), anti-CD66b Abs (diluted 1:80 from BioLegend) were added to the sections. Mach4 AP polymer was used as secondary antibody followed by Ferangi Blue as chromogen. Ematoxylin was used for counterstaining.

Cytokine Production

Cytokine concentrations in cell-free supernatants and cell lysates were measured by commercial enzyme-linked immunosorbent (ELISA) kits, specific for: IL-17A (DY317 from R&D systems and 88-7176 from eBioscience), IL-17A/F (88-7117, eBioscience), IL-17B [ABKA2223 from Abnova (Taipei, Taiwan) and ab171344 from Abcam (Cambridge, United Kingdom)], IL-17F (887478, eBioscience), and CXCL8 (Mabtech, Nacka Strand, Sweden). ELISA detection limits were 4 pg/ml (eBioscience) and

15.6 pg/ml (R&D) for IL-17A, 30 pg/ml for IL-17A/F, 24 pg/ml (Abnova) and 10 pg/ml (Abcam) for IL-17B, 16 pg/ml for IL-17F, and 8 pg/ml for CXCL8.

Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from neutrophils by the RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands), as previously detailed (62). To completely remove any possible contaminating DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation. Total RNA was then reverse-transcribed into cDNA using Superscript III (Life Technologies, Carlsbad, CA, USA) and random hexamer primers (Life Technologies), while qPCR was carried out using Fast SYBR® Green Master Mix (Life Technologies). Sequences of gene-specific primer pairs (Life Technologies) are listed in Table S1 in Supplementary Material. Data were calculated by Q-Gene software[1] and expressed as mean normalized expression units after GAPDH normalization (63).

Immunoblotting Experiments

Total neutrophil proteins were recovered from protein-rich flow-through solutions after the first centrifugation step of the RNeasy mini kit (Qiagen) procedure used for total RNA extraction, as previously described (62). Protein-rich flow-through from neutrophils were then immunoblotted by standard procedures using the anti-human IL-17A (AF-317-NA) and IL-17B (AF1248) goat IgG pAbs from R&D Systems; anti-human phospho-STAT3 (Tyr705) rabbit pAbs (#9131, Cell Signaling, Beverly, MA, USA); anti-human STAT3 rabbit pAbs (sc-482, Santa Cruz Biotechnology, Dallas, TX, USA), and anti-human β-actin mAbs (A5060 from Sigma). Blotted proteins were detected by using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA) (62).

ChIP Assays

Chromatin immunoprecipitation experiments were performed exactly as previously described (62). Briefly, nuclear extracts from 2×10^6 neutrophils or Th17 cell lines were immunoprecipitated using 1 µl anti-H3K4me1 (ab8895) and anti-H3K27Ac (ab4729) pAbs (both from Abcam, Cambridge, United Kingdom). Coimmunoprecipitated material was subjected to qPCR analysis using the specific promoter primers (purchased from Life Technologies) listed in Table S2 in Supplementary Material. Data from qPCR were expressed as percentage over input DNA and are displayed as mean ± SEM.

ChIP-seq

Purified DNA from H3K27Ac and H3K4me1 ChIP assays (performed as described in the previous paragraph) was adapter-ligated and PCR-amplified for sequencing on HiSeq2000 platform (Illumina, Cambridge, UK) using TruSeq DNA Library Prep Kit (Illumina). After sequencing, reads were quality-filtered according to the Illumina pipeline. Single end (51 bp) reads were then mapped to the human genome (Genome Reference Consortium GRCh37, Feb/2009) using BOWTIE v1.0.0 (64). Only reads with no more than two mismatches (when compared to the reference genome) were converted to tag directories using HOMER's module known as "makeTagDirectory," and then converted to BedGraph format using HOMER's module known as "makeUCSCfile," to be finally normalized to 10^7 total tag counts. ChIP-seq signals were visualized using Integrative Genomics Viewer. For H3K4me1 and H3K27Ac ChIP-seqs of Th17 cells, 36 bp reads, already filtered and mapped, were downloaded from database of the "roadmap epigenomics project"[2] (NIH Epigenomics Roadmap Initiative). Aligned reads were then converted to BedGraph format and normalized to 10^7 total tag counts.

Gene Expression Data Set of Normal Hematopoietic Stem and Progenitor Cells

Gene expression profiles of cells from normal bone marrow at different stages of human granulopoiesis were downloaded from Gene Expression Omnibus Database (GEO number: GSE42519) (65). Gene expression means and SEs were calculated from the values of the biological replicates present in the GEO database.

Statistical Analysis

Data are expressed as mean ± SEM or mean ± SD. Statistical evaluation was performed by using, depending on the experiment type, Student's *t*-test or two-way ANOVA followed by Bonferroni's *post hoc* test. *P* values <0.05 were considered as statistically significant.

Study Approval

Human samples were obtained following informed written consent by both HDs and psoriatic patients. This study was carried out in accordance with the recommendations of Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy). All the experimental protocols were approved by the Ethic Committee and all subjects gave written informed consent in accordance with the Declaration of Helsinki.

RESULTS

Human Neutrophils Incubated With a Variety of Agonists *In Vitro* Do Not Express IL-17 Members at Both mRNA and Protein Levels

We have previously shown that human neutrophils (>99.7% purity), incubated with 100 U/ml IFNγ and/or 100 ng/ml ultrapure LPS for up to 20 h *in vitro*, do not produce IL-17A protein (12). Additional RT-qPCR experiments not only confirmed our previous data (**Figure 1A**) but also revealed that other agonists, including 5 µM R848 and/or 1,000 U/ml IFNα (**Figure 1B**), 10 ng/ml GM-CSF, 100 nM fMLF (**Figure 1C**),

[1] http://www.gene-quantification.de/download.html (Accessed: February 10, 2018).

[2] http://egg2.wustl.edu/roadmap/web_portal/processed_data.html (Accessed: February 10, 2018)

1,000 U/ml G-CSF, and 5 ng/ml TNFα (**Figure 1D**), similarly fail to induce an accumulation of transcripts encoding IL-17A (**Figures 1A–D**, left panels), IL-17F (**Figures 1A–D**, middle panels), IL-17B, IL-17C, IL-17D, and IL-17E (data not shown) in neutrophils. LPS and/or IFNγ, R848 and/or IFNα, GM-CSF or fMLF, however, were found to modulate the expression of CXCL8 mRNA (**Figures 1A–C**, right panels), while G-CSF or TNFα modulated that of IL-1ra mRNA (**Figure 1D**, right panel), as expected (62, 66, 67). Consistent with the gene expression data, neither IL-17A, IL-17F (**Table 1**) nor IL-17A/F and IL-17B (data not shown) proteins could be detected in supernatants harvested from neutrophils incubated for 20 h with the stimuli used for the experiments shown in **Figure 1**, as well as with 500 μg/ml β-glucan, 500 μg/ml curdlan, 1 μg/ml Pam3CSK4,

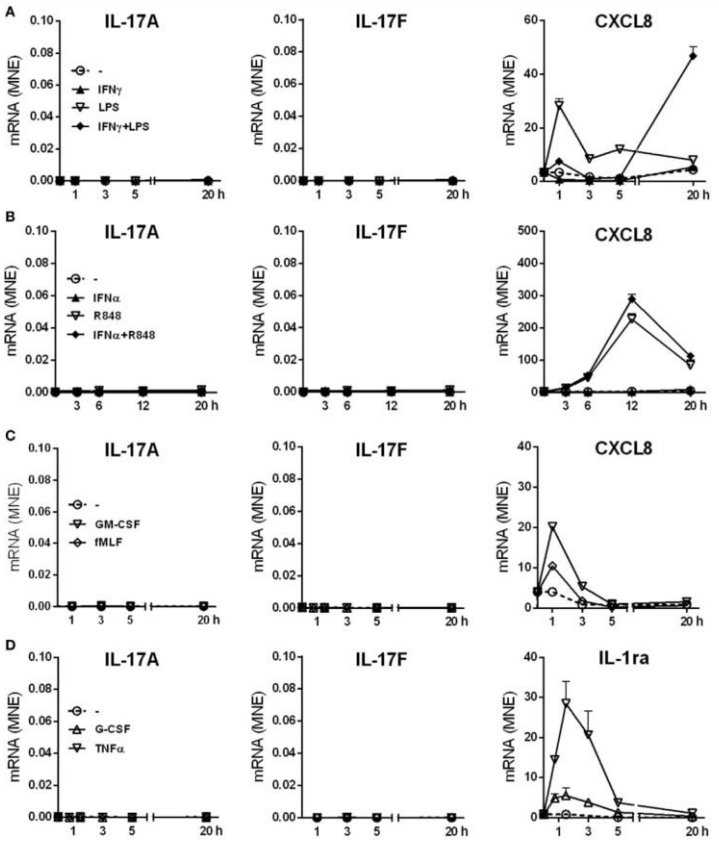

FIGURE 1 | IL-17A, IL-17F, CXCL8, and IL-1ra mRNA expression levels in human neutrophils activated by a variety of stimuli. Human neutrophils were cultured at 5 × 10^6/ml for up to 20 h with **(A)** 100 U/ml IFNγ and/or 100 ng/ml LPS; **(B)** 1,000 U/ml IFNα and/or 5 μM R848; **(C)** 10 ng/ml GM-CSF or 100 nM fMLF; **(D)** 1,000 U/ml G-CSF or 5 ng/ml TNFα. IL-17A, IL-17F, CXCL8, and IL-1ra mRNA expression was evaluated by reverse transcription quantitative real-time PCR (RT-qPCR) and data depicted as mean normalized expression (MNE) units after GAPDH mRNA normalization. The experiments depicted in each panels **(A–D)** are representative of at least three ones with similar results. Error bars stand for SEs calculated from triplicate qPCR reactions.

50 μg/ml poly(IC), and 100 μg/ml CpG ODN. Noteworthy, we used ELISA kits from two different commercial sources (see Materials and Methods) for either IL-17A or IL-17B, in both cases giving equivalent information. On the other hand, stimulus-dependent levels of CXCL8 could be measured in supernatants from our stimulated neutrophils, indicating that agonists were effective and cells fully responsive (**Table 1**). In any case, validity of both IL-17 primers and ELISA kits was demonstrated by the detection of either IL-17A, IL-17D, IL-17E, and IL-17F transcripts in human Th17, but not Th1, cell lines (Figure S1 in Supplementary Material), or IL-17A and IL-17F proteins in supernatants from $CD4^+$ T cells activated with anti-CD3/anti-CD28 mAbs (**Table 1**). We could also detect intracellular IL-17B in lysates of human cerebral cortex (data not shown), as expected (68).

In other experiments, neutrophils were incubated for 3 h with 20 μg/ml IL-6 plus 2 μg/ml IL-23, in the presence or the absence of inactivated conidia, or hyphae, from *A. fumigatus*. These experiments were done with the purpose to mimic, as much as possible, recently described experimental conditions shown to induce not only IL-17A and IL-17F but also IL-17RC, mRNA expression (23, 24, 29, 39, 40, 44). Neutrophils were also incubated with 100–500 ng/ml IL-17A to reinvestigate (12) whether they respond to IL-17A or not. As shown in **Figure 2**, neutrophils treated with either IL-17A or IL-6 plus IL-23 (in the presence or the absence of inactivated *A. fumigatus* conidia/hyphae), showed neither induction of IL-17A (**Figure 2A**), IL-17F (**Figure 2B**), and IL-17RC (**Figure 2C**) mRNAs nor upregulation of the constitutively expressed IL-17RA transcript levels (**Figure 2D**). Similar results were obtained when incubation was prolonged up to 6 h (data not shown), or when neutrophils were stimulated with PMA/ionomycin after pretreatment for 1 h with IL-6 plus IL-23 (Figure S2 in Supplementary Material). Elevated levels of IL-17RC mRNAs were, however, detected in HBECs (data not shown), used as control cells (12), thus confirming that our primers were correctly designed. Importantly, the capacity of IL-6 plus IL-23 to stimulate neutrophils was evidenced by their ability to time-dependently promote STAT3 phosphorylation (**Figure 2E**), as well as to upregulate SOCS3 mRNA expression (**Figure 2F**), such an effect being potentiated by inactivated *A. fumigatus* conidia/hyphae (**Figure 2F**). By contrast, IL-17A-treatment influenced neither SOCS3 (**Figure 2F**) nor CXCL8 (data not shown) mRNA levels in neutrophils. Furthermore, no IL-17A (**Figure 3A**), IL-17F, or IL-17AF (data not shown) proteins were detected by ELISA either intracellularly or in supernatants harvested from neutrophils incubated with IL-6 plus IL-23, in the presence or the absence of inactivated *A. fumigatus* conidia/hyphae. Under the same experimental conditions, CXCL8 protein was newly synthesized and released by neutrophils incubated with IL-6 plus IL-23 in the presence of inactivated *A. fumigatus* conidia/hyphae, but not in their absence (**Figure 3B**). Finally, no IL-17A was detected in IL-6 plus IL-23-stimulated neutrophils by intracellular staining experiments (data not shown), using the anti-human IL-17A eBio64DEC17 mouse IgG1 (from eBioscience) previously shown to function under identical experimental conditions by Taylor et al. (39). We have no clues explaining why we did not reproduce the positive effects on IL-17 expression by IL-6 plus IL-23 (23, 24, 29, 39, 40), with or without inactivated *A. fumigatus* conidia/hyphae. One possibility is that the hyphal extracts from *A. fumigatus* used by Taylor and colleagues (39), but not our inactivated conidia/hyphae, contain some undefined PAMP(s) that effectively promote(s) IL-17A production/IL-17RC expression by human neutrophils.

Taken together, our data extend previous findings on the inability of human neutrophils to express IL-17 members at the mRNA and protein levels under various activating conditions (13–16). Data also confirm and extend our previous findings (12) on the inability of IL-17A to directly modify IL-17A, IL-17F, IL-17RA, IL-17RC, SOCS3, and CXCL8 gene expression in human neutrophils.

TABLE 1 | Lack of IL-17A and IL-17F production by activated human neutrophils.

Stimuli	IL-17A (pg/ml)	IL-17F (pg/ml)	CXCL8 (ng/ml)
Neutrophils			
–	nd	nd	0.07 ± 0.05
500 μg/ml β-glucan	nd	nd	0.41 ± 0.16*
500 μg/ml curdlan	nd	nd	0.49 ± 0.02***
10 ng/ml GM-CSF	nd	nd	0.30 ± 0.13*
100 nM fMLF	nd	nd	0.33 ± 0.12*
5 ng/ml TNFα	nd	nd	1.22 ± 0.90
1 μg/ml Pam3CyS	nd	nd	10.31 ± 3.85**
50 μg/ml poly(I:C)	nd	nd	0.02 ± 0.02
100 ng/ml LPS	nd	nd	0.89 ± 0.22**
5 μM R848	nd	nd	9.47 ± 3.35**
100 μg/ml CpG ODN	nd	nd	5.57 ± 1.1***
100 U/ml IFNγ	nd	nd	0.10 ± 0.04
100 U/ml IFNγ + 100 ng/ml LPS	nd	nd	2.51 ± 1.1**
$CD4^+$ T cells			
–	nd	nd	2.51 ± 1.1
5 μg/ml anti-CD3/CD28	739.6 ± 56.6***	948.9 ± 95.4***	172.6 ± 25.1***

Human neutrophils (5 × 10^6/ml) were cultured for 20 h with the indicated stimuli. $CD4^+$ T cells were stimulated for up to 72 h with anti-CD3 and anti-CD28 mAbs. Cell-free supernatants were then harvested and IL-17A, IL-17F, and CXCL8 content measured by specific ELISA. Values represent the mean ± SD (n = 3).

*Asterisks stand for significant increases as compared to untreated cells: *P < 0.05, **P < 0.01, ***P < 0.001, by Student's t-test.*

nd, not detected; ODN, oligodeoxynucleotides.

Human Neutrophils Incubated With IL-6 Plus IL-23, in the Presence or the Absence of Inactivated *A. fumigatus* Hyphae/Conidia, Do Not Express IL-17RC

Flow cyometry experiments confirmed (12) that neutrophils, either freshly isolated, or incubated for 3 h in the absence, or the presence of IFNγ plus LPS (**Figure 4A**), display only surface IL-17RA, but not IL-17RC. No IL-17RC surface levels were also observed in neutrophils incubated with either R848 (**Figure 4A**), or IL-6 plus IL-23, in the latter case in the absence, or in the presence of either inactivated *A. fumigatus* conidia/hyphae, or IL-17A (**Figure 4B**). IL-17RA surface levels were downregulated in neutrophils treated with IFNγ plus LPS, R848 (**Figure 4A**) and IL-6 plus IL-23 with IL-17A (**Figure 4B**). In these experiments, HBEC were, again, used as positive control

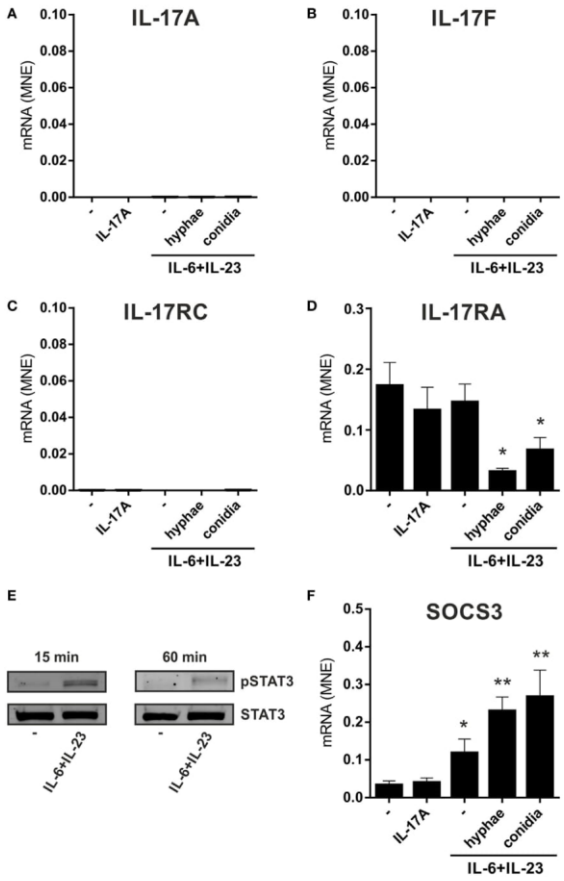

FIGURE 2 | No induction of IL-17A, IL-17F, and IL-17RC mRNA expression in neutrophils incubated with IL-6 plus IL-23, in combination with inactivated *Aspergillus fumigatus* hyphae or conidia. Neutrophils (5×10^6/ml) were incubated either with 100 ng/ml rIL-17A for 2 h or with or without 20 μg/ml IL-6 plus 2 μg/ml IL-23 for 1 h, prior to adding, or not, inactivated *A. fumigatus* conidia (1:5 neutrophils/conidia ratio) and hyphae (1:1 neutrophils/hyphae ratio) for additional 1 h. Neutrophils were then harvested for RNA extraction to evaluate IL-17A **(A)**, IL-17F **(B)**, IL-17RC **(C)**, IL-17RA **(D)**, and SOCS3 **(F)** mRNA expression by reverse transcription quantitative real-time PCR. Gene expression data are depicted as mean normalized expression (MNE) units after GAPDH mRNA normalization (mean ± SEM, $n = 4$). Asterisks stand for significant differences as compared to untreated cells: $^*P < 0.05$, $^{**}P < 0.01$, by Student's *t*-test. **(E)** Immunoblot displaying STAT3 tyrosine phosphorylation in neutrophils, either untreated or cultured for 15 or 60 min with 20 μg/ml IL-6 plus 2 μg/ml IL-23 (representative experiment, $n = 2$).

for both IL-17RA and IL-17RC surface expression (data not shown) (12). It should be pointed out that, for the investigation of surface IL-17RC, we have been using the same anti-IL-17RC, directly PE-conjugated, Abs used in Taylor et al.'s study (39), other than the anti-IL-17RC biotin-conjugated Abs that necessitate PE-conjugated streptavidin for detection (12), without noticing any difference between them. By the way, IFNγ plus LPS and R848 (Figure S3A in Supplementary Material), as well as IL-6 plus IL-23 in the presence of inactivated *A. fumigatus* conidia/hyphae (Figure S3B in Supplementary Material),

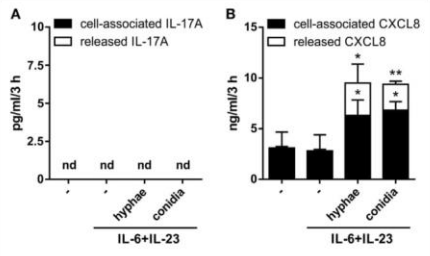

FIGURE 3 | Lack of IL-17A and IL-17F production by human neutrophils activated by IL-6 plus IL-23 in combination with inactivated *Aspergillus fumigatus* hyphae or conidia. Neutrophils (5×10^6/ml) were incubated with or without 20 μg/ml IL-6 plus 2 μg/ml IL-23 and then cultured for three more hours in the presence or not of inactivated *A. fumigatus* conidia and hyphae (used at 1:5 and 1:1, respectively). After incubation, IL-17A **(A)** and CXCL8 **(B)** levels were determined in cell-free supernatants and in corresponding cell pellets by specific ELISA. Values are depicted as the mean ± SD or as not detected (nd) when values were under the detection limit ($n = 3$). Asterisks stand for significant differences as compared to untreated cells: $^*P < 0.05$, $^{**}P < 0.01$, by Student's *t*-test.

variably modulated both CD11b and CD62L expression. All in all, data illustrate that IL-6 plus IL-23, regardless of their combination with inactivated *A. fumigatus* conidia/hyphae, and despite their capacity to upregulate SOCS3 mRNA expression (**Figure 2F**), do not induce the expression of IL-17RC in our hands, contradicting some studies (39, 44).

O_2^- Production by Neutrophils Stimulated With Inactivated *A. fumigatus* Hyphae After Preincubation With IL-6 plus IL-23 Is Not Modified by Either Exogenous IL-17A or IL-17A Inhibitors

We then measured the capacity to release O_2^- by neutrophils preincubated with or without IL-6 plus IL-23 for 1 h, and then treated for one additional hour with inactivated *A. fumigatus* hyphae, in the presence or the absence of either IL-17A or anti-IL-17A neutralizing Abs (Figure S4 in Supplementary Material). As control, neutrophils were also stimulated with either inactivated *A. fumigatus* hyphae alone or 20 ng/ml PMA. As shown in Figure S4 in Supplementary Material, inactivated *A. fumigatus* hyphae were found to trigger a remarkable O_2^- production by neutrophils, even though lower than PMA. However, *A. fumigatus* hyphae-stimulated O_2^- release was not potentiated by the preincubation of neutrophils with IL-6 plus IL-23 (which, by themselves, did not trigger any O_2^- production) (Figure S4 in Supplementary Material). Under the latter experimental conditions, addition of either IL-17A or anti-IL-17A neutralizing Abs (αIL-17A Abs) did not influence the effect of inactivated *A. fumigatus* hyphae on neutrophil-derived O_2^- (Figure S4 in Supplementary Material), supporting the lack of induction of surface IL-17RC expression and endogenous IL-17A, respectively.

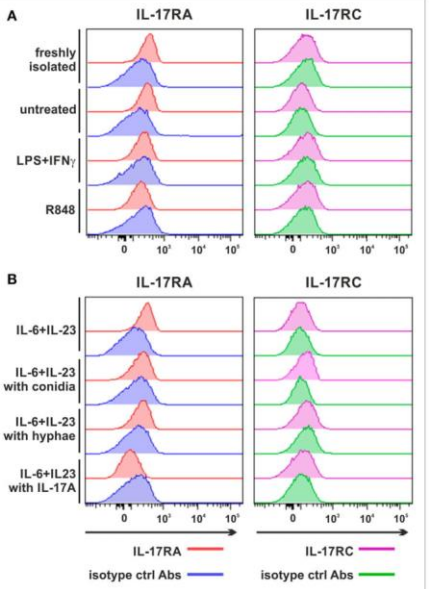

FIGURE 4 | Expression of surface IL-17RA and IL-17RC in neutrophils activated under various experimental conditions. Expression of surface IL-17RA (left panel) and IL-17RC (right panel) was evaluated by flow cytometry in neutrophils either freshly isolated or cultured for 3 h without or with 100 U/ml IFNγ plus 100 ng/ml LPS, 5 μM R848 **(A)**, 20 μg/ml IL-6 plus 2 μg/ml IL-23 alone or in the presence of inactivated *Aspergillus fumigatus* conidia, hyphae or 500 ng/ml rIL-17A **(B)**. Graphs depict a representative experiment out of three independent ones with similar results. Histograms show staining by specific and isotype control Abs, respectively, for each stimulatory condition.

Chromatin Organization at the IL-17A and IL-17F Genomic Loci of Human Neutrophils

Signatures of histone posttranslational modifications at a specific gene locus provide indicative elements to predict whether such a gene can be transcribed or not (69, 70). Therefore, we evaluated the presence of histone marks associated to active (e.g., H3K27Ac) and poised (e.g., H3K4me1) genomic regulatory elements (71) at the *IL17A* and *IL17F* loci of human neutrophils. Genome-wide ChIP-seq assays demonstrated that, in freshly isolated neutrophils, the entire genomic region containing *IL17A* and *IL17F* loci is completely devoid of H3K27Ac and H3K4me1 (**Figure 5**). By contrast, based on data available from the NIH Epigenomics Roadmap Initiative (72), multiple H3K4me1 peaks are present in the same genomic regions of PMA/ionomcyin-stimulated Th17 cells, while H3K27Ac peaks

localize at the *IL17A* locus only (**Figure 5**). To validate the previous data, we performed H3K27Ac and H3K4me1 qPCR ChIPs using neutrophils incubated for 1 h either with or without 20 µg/ml IL-6 plus 2 µg/ml IL-23, as well as Th17 cell lines (in which IL-17A and IL-17F mRNA is constitutively transcribed), used as positive controls (**Figure 6**). Based on the H3K4me1 peaks from the ChIP-seqs of Th17 cell lines (72) (**Figure 5**), we designed specific primers amplifying potential regulatory regions at the *IL17A* and *IL17F* genomic loci, namely IL-17A#1 and IL-17F#1 for enhancers, and IL-17A#2, IL-17A#3, and IL-17F#2 for promoters (**Figures 6A,B**). As expected, Th17 cell lines displayed constitutively bound H3K4me1 at their IL-17A and IL-17F promoters and enhancers (**Figures 6A,B**, left panels). We also detected high levels of H3K27Ac at the IL-17A and IL-17F promoters and enhancers of Th17 cell lines (**Figures 6A,B**, right panels), in line with their constitutive expression of both IL-17A and IL-17F mRNA (data not shown). By contrast, we did not observe any H3K4me1 or H3K27Ac at the *IL17A* and *IL17F* loci of neutrophils, either under resting conditions (thus confirming the ChIP-seq data shown in **Figure 5**) or after incubation with IL-6 plus IL-23 (**Figures 6A,B**). In fact, the H3K4me1 and H3K27Ac levels at the IL-17A and IL-17F enhancers in neutrophils were similar to those ones present at the PRL promoter, a genomic region with a closed chromatin conformation in myeloid cells, herein used to determine the signal background (**Figures 6A,B**). Notably, measurable amounts of H3K4me1 and H3K27Ac were found at the SOCS3 promoter of neutrophils under resting conditions, as well as in Th17 cell lines (**Figure 6C**). Interestingly, H3K27Ac levels tended to increase in neutrophils incubated with IL-6 plus IL-23 (**Figure 6C**), in accordance with a supposed STAT3-dependent induction of SOCS3 mRNA (73). Taken together, data indicate that the organization of the *IL17A* and *IL17F* loci in human neutrophils is characterized by the absence of poised chromatin marks, unlike that of IL-17A- and IL-17F-producing Th17 cell lines. Data also indicate that human neutrophils do not reorganize the chromatin of the *IL17A* and *IL17F* loci in response to IL-6 plus IL-23, consistent with their inability to *de novo* accumulate IL-17A and IL-17F mRNA.

Human Neutrophils From Patients With Psoriasis Do Not Express IL-17A and/or IL-17F mRNA

We subsequently investigated whether neutrophils isolated from patients with active psoriasis could express/produce IL-17A, IL-17F, and/or IL-17RC mRNA, either constitutively or upon incubation for 20 h with IFNγ plus LPS, R848, or IL-17A. As shown in **Figure 7A**, the latter was not the case, as psoriatic neutrophils behaved similarly to neutrophils from HDs. Psoriatic neutrophils did not also respond to IL-17A (**Figure 7A**), due to their lack of surface IL-17RC expression (**Figure 7B**). Nonetheless, psoriatic neutrophils fully responded to either R848 or IFNγ plus LPS, as they accumulated CXCL8, TNFα, and SOCS3 transcripts at levels comparable to those in HD neutrophils (**Figure 7A**).

Commercial Anti-IL-17A Abs (AF-317-NA) Positively Stain Cytospins of Resting and Activated Neutrophils due to Their Non-Specific Recognition of Neutrophil Intracellular Proteins Different From IL-17A

In additional experiments, cytospin slides of resting and R848-stimulated neutrophils were incubated with goat anti-human IL-17A AF-317-NA Abs, previously shown to stain neutrophils in pathological tissues (18–22, 25, 27, 28, 30–33, 35, 36, 41–43, 45, 47–50), as also confirmed by our IHC/IF staining of inflamed psoriatic tissue (**Figure 8A**). Consistently, neutrophil cytospin slides became strongly positive upon incubation with AF-317-NA, yet with no difference between resting or R848-activated neutrophils (**Figure 8B**). By contrast, immunostaining of the same cytospins slides with anti-human CXCL8 Abs showed a strong positivity only in R848-treated neutrophils (**Figure 8B**), thus excluding methodological artifacts. Not surprisingly, neutrophils from the same experiments were found totally negative for both IL-17A mRNA expression and IL-17A production once processed for RT-qPCR analysis and ELISA. The detection of IL-17A-positive neutrophils by IHC, in the absence of IL-17A mRNA, could

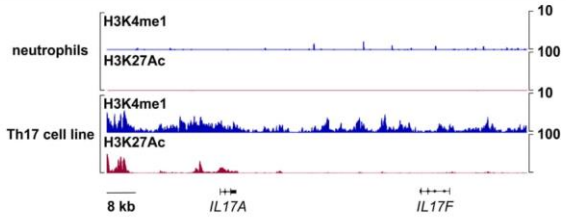

FIGURE 5 | Chromatin immunoprecipitation (ChIP)-Seq profiles of H3K4me1 and H3K27Ac at the *IL17A* and *IL17F* loci in human neutrophils and Th17 cell lines. Representative snapshots depicting H3K4me1 and H3K27Ac ChIP-seqs at the *IL17A* and *IL17F* genomic loci in freshly isolated human neutrophils or, as retrieved from NIH Epigenomics Roadmap Initiative (72), in phorbol mysistate acetate/ionomycin-stimulated Th17 cell lines.

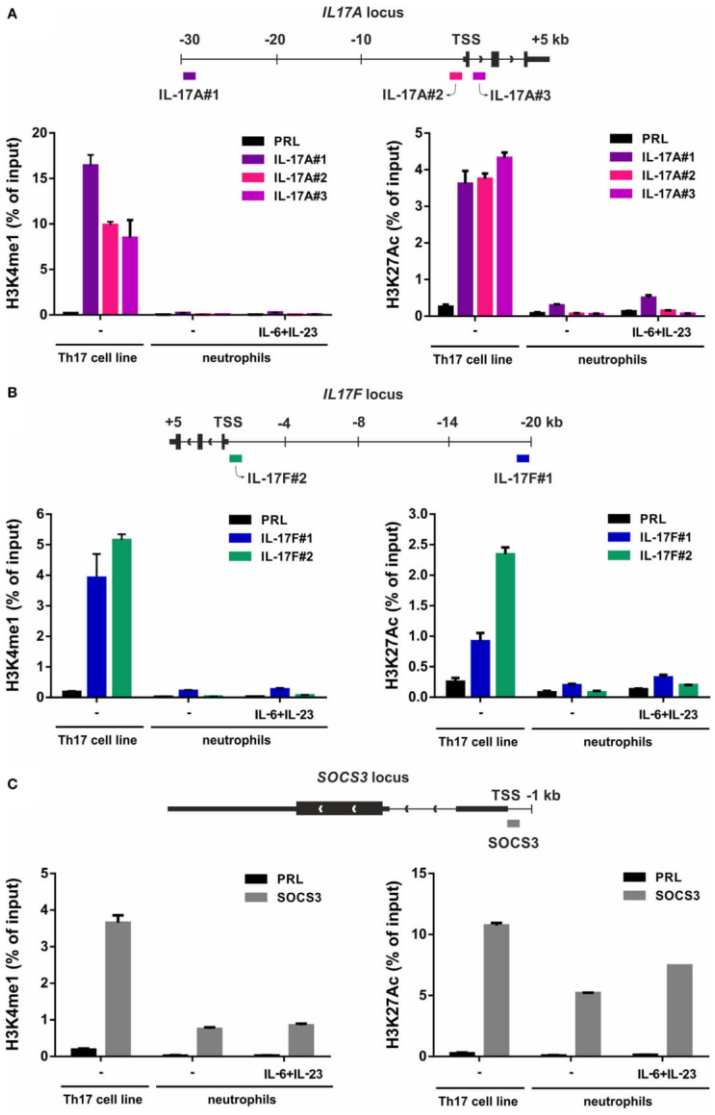

FIGURE 6 | Continued

FIGURE 6 | H3K4me1 or H3K27Ac levels at the IL-17A, IL-17F, and SOCS3 genomic loci of Th17 cell lines and resting/IL-6 plus IL-23-activated neutrophils. Enrichment levels of H3K4me1 (left panels) and H3K27Ac (right panels) at the IL-17A **(A)**, IL-17F **(B)**, and SOCS3 **(C)** genomic loci by chromatin immunoprecipitation (ChIP) analysis in human Th17 cell lines and neutrophils incubated for 1 h with or without 20 μg/ml IL-6 plus 2 μg/ml IL-23. **(A–C)** Schemes illustrating the positions of the designed primer pairs amplifying promoter and potential enhancer regions of IL-17A, IL-17F, and SOCS3 for ChIP analysis are depicted at the top of each panel. Coimmunoprecipitated DNA samples were expressed as percent of the total input. Panels in **(A–C)** depict a representative experiment out of two independent ones with similar results. Error bars represent SEs calculated from triplicate qPCR reactions.

be explained by the fact that the cytokine may be synthesized in bone marrow neutrophil precursors, at stages during which granule proteins, such as myeloperoxidase (MPO), elastase, and azurocidin 1, are formed (74). However, in transcriptomes made by Rapin et al., generated from cells isolated at different stages during granulopoiesis (65), we did not identify any IL-17A mRNA accumulation (**Figure 9**). In the same database, we not even detected IL-17RC and IL-10 mRNA (**Figure 9**), consistent with the inability of mature neutrophils to express them (12, 70). By contrast, we did find MPO, elastase, and azurocidin 1 mRNA expression only in transcriptomes of neutrophil precursors, as expected (74), thus validating the reliability of the database (65) (**Figure 9**). In any case, consistent with the absence of intracellular IL-17 (**Figure 3A**), immunoblots performed with AF-317-NA revealed that whole neutrophil lysates did not show any positive signal in correspondence of recombinant human IL-17A (rhIL-17A) molecular weight (MW) (**Figure 8C**). In these experiments, neutrophils were either freshly isolated (D1 and D2 in **Figure 8C**), or cultured for 3 h with or without R848, 2 μg/ml IL-6 plus 0.2 μg/ml IL-23 (low IL-6 plus IL-23 in **Figure 8C**), or 20 μg/ml IL-6 plus 2 μg/ml IL-23 (high IL-6 plus IL-23 in **Figure 8C**). By contrast, AF-317-NA strongly reacted in correspondence of neutrophil proteins displaying higher MW than that of rIL-17A, with no difference in signals among freshly isolated, stimulated, or untreated neutrophils (**Figure 8C**). While these data confirm the observations reported by Tamarozzi et al. (13), who also used mouse anti-IL-17A mAbs (#41802, from R&D) in addition to AF-317-NA, they are in contrast with Lin et al.'s findings (30) illustrating a constitutive IL-17A (but not IL-17F) expression in neutrophil lysates, as revealed by immunoblotting with #41802. Halwani et al. (23) too found constitutive IL-17A amounts in lysates of neutrophils from asthmatic patients, even increasing upon cell incubation with IL-21 and/or IL-23 for 18 h, as revealed by immunoblotting with unspecified Abs from R&D. However, since only portions of the immunoblots are shown in Halwani et al. (23) and Lin et al. (30) paper, it is not known whether additional proteins were recognized by Abs used. Whatever the case is, our experiments suggest that the positive staining of neutrophils detected by IHC and IF using AF-317-NA on cytospins and, possibly, tissue slides, stands for an IL-17A-unrelated binding(s) to neutrophils.

Human Neutrophils Do Not Express/Produce IL-17B

In a separate set of experiments, we also tested goat anti-IL-17B (AF1248) Abs that, in recent publications, have been shown to positively stain, by IHC and IF, neutrophils present in tissue samples from RA (75) and colon carcinoma (CCR) (76) patients. Consistently, we found that also neutrophils present in inflamed psoriatic tissue were strongly detectable by IHC and IF stainings with AF1248 (**Figure 10A**). On cytospin slides, AF1248 stained neutrophils isolated from the blood of HDs and incubated for 3 h with or without 5 μM R848 in an equivalent manner (**Figure 10B**). However, by immunoblotting of whole lysates prepared from neutrophils treated with R848 or IL-6 plus IL-23, AF1248 did not recognize any protein corresponding to the rhIL-17B MW (**Figure 10C**). These negative observations were also confirmed by measurement of intracellular, as well as, released IL-17B by two commercial ELISA (see Materials and Methods). Accordingly, no antigenic IL-17B could be measured in supernatants and whole lysates from neutrophils incubated with 5 μM R848 with or without 1,000 U/ml IFNα, 100 μg/ml LPS with or without 100 U/ml IFNγ, 2/20 μg/ml IL-6 plus 2 μg/ml IL-23 (data not shown), in agreement with the lack of IL-17B mRNA induction. Detectable IL-17B levels were, however, measured in lysates of human cerebral cortex (68), demonstrating that our two IL-17B ELISA kits were sensitive enough. Altogether, our data indicate that, similarly to the case of AF-317-NA, the positive stainings of neutrophils in cytospin slides and, possibly, tissue samples by AF1248, likely stand for an IL-17B-unrelated, non-specific, recognition occurring in human neutrophils.

DISCUSSION

In this study, we have reinvestigated in-depth whether human neutrophils produce IL-17A, IL-17B, IL-17F, and IL-17A/F *in vitro*. According to the literature, in fact, information on such an issue appears discordant, as the majority of papers sustain that human neutrophils do express/produce IL-17A (18–53), while a minority fail to detect it (12–17). This issue is even more critical if one takes into account that also the capacity of murine neutrophils to produce IL-17A, shown in a variety of mouse models of infectious and autoimmune inflammation (24, 39, 40, 77–81), has been recently questioned (82, 83). Preclinical models evidencing neutrophil-derived IL-17 as pathogenic in diseases might be, in fact, prematurely taken as proof-of-concept for immediate translational applications in humans.

Herein, by using multiple methodological approaches (RT-qPCR, ChIP, ChIP-seq, ELISA, intracellular staining, and immunoblotting), we confirm and greatly extend our previous findings (12) showing that highly purified (>99.7%) populations of human neutrophils, either resting or activated by a variety of stimulatory conditions, including TLR and dectin ligands, fungal PAMPs and cytokines, used singly or in combinations, neither express IL-17A, IL-17F, IL-17B, IL-17C, IL-17D, and IL-17E mRNA nor produce IL-17A, IL-17F, IL-17A/F, and IL-17B *in vitro*. Similarly, we show that also neutrophils isolated from

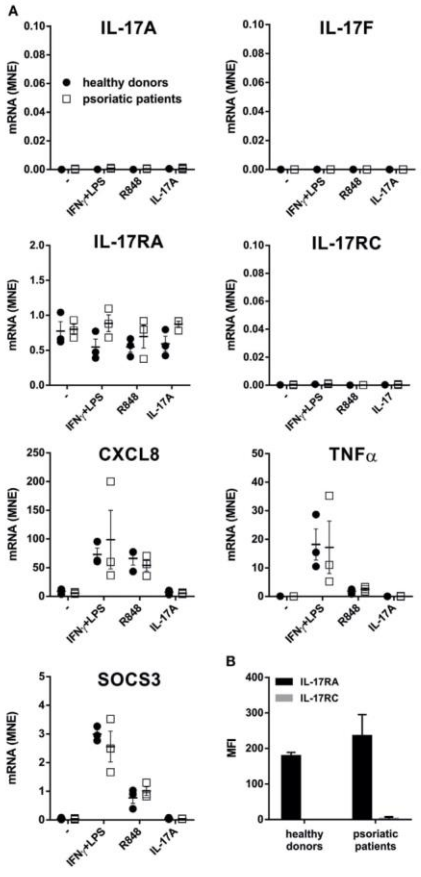

FIGURE 7 | IL-17A, IL-17F, IL-17RA, IL-17RC, CXCL8, TNFα, and SOCS3 mRNA expression, as well as IL-17R surface expression, in neutrophils from patients with psoriasis. **(A)** Neutrophils isolated from healthy donors (HDs) ($n = 3$) or psoriatic patients ($n = 3$) were cultured for 20 h with 100 U/ml IFNγ plus 100 ng/ml LPS, 5 μM R848, or 500 ng/ml IL-17A to evaluate IL-17A, IL-17F, IL-17RA, IL-17RC, CXCL8, TNFα, and SOCS3 mRNA expression by reverse transcription quantitative real-time PCR. Gene expression data are depicted as mean normalized expression (MNE) units after GAPDH mRNA normalization. **(B)** Surface IL-17RA and IL-17RC expression evaluated by flow cytometry in human neutrophils from HDs or psoriatic patients. Values represent the mean ± SEM ($n = 3$). For the data of panels **(A,B)** no significant differences between HDs or psoriatic patients were observed by two-way ANOVA followed by Bonferroni's post-test.

patients with active psoriasis do not express IL-17F, IL-17B, IL-17C, IL-17D, and IL-17E as well as IL-17RC mRNA when activated by R848, IFNγ plus LPS, and IL-17A *in vitro*. In such regard, RNA-Seq experiments made by Tamarozzi et al. (13), using neutrophils isolated by negative-selection (>99.9% pure) from HDs or RA patients (as we do), then treated for 1 h with a range of inflammatory cytokines (TNFα, GM-CSF, G-CSF, IL-6, IL-1β, CXCL8, IFNα, and IFNγ), also failed to detect any of the mRNA for IL-17 cytokine family. By contrast, Yamanaka et al. (15) have been recently reported the presence of constitutive IL-17A transcripts in neutrophils from HDs and psoriasis patients isolated by density gradient cell separation (92% purity). However, when the same cell populations were further purified by magnetic sorting (reaching a 99% purity), they were found totally devoid of IL-17A mRNA (15), indicating that contaminating monocytes/lymphocytes were actually responsible for the IL-17A mRNA expression in unsorted "neutrophils." Needless to say that Yamanaka et al.'s observations (15) are example of a notion that we have been always recommending in our studies (56, 59, 84), namely the requirement of using highly purified cell populations if one wants to obtain correct results when examining neutrophil gene expression or neutrophil-derived cytokines.

Interestingly, other studies confirm that human neutrophils do not constitutively contain IL-17A transcripts (13, 24, 29, 30, 35, 39, 40, 44), including those ultimately showing a concurrent positivity for IL-17A protein, as revealed by intracellular flow cytometry (24, 39, 40), ELISA (24, 39), confocal microscopy (39), or IHC (29). Some authors (30, 35) speculated that the absence of IL-17A mRNA in mature neutrophils indicates that the cytokine is synthesized in bone marrow neutrophil precursors, at the stages when granule proteins are formed (74). However, we would exclude such a hypothesis, as our analysis of transcriptomes generated from all types of bone marrow cell populations (65) failed to identify an IL-17A mRNA accumulation at any stage of neutrophil maturation.

We were unable to detect IL-17A and IL-17F mRNA/production/release even by human neutrophils incubated with IL-6 plus IL-23, in contrast to what repeatedly found (23, 24, 29, 39, 40). In our experiments, neutrophils did, however, respond to IL-6 plus IL-23 in terms of STAT3 phosphorylation and SOCS3 mRNA induction, indicating that the two cytokines are effectively stimulatory for neutrophils. It is intriguing that, apart from Halwani et al. (23), who found that either 20 ng/ml IL-6 or 20 ng/ml IL-23, singly used, directly induced IL-17A mRNA and protein in a fraction of neutrophils from asthmatic patients, other groups highlighted the necessity to use at least 20 μg/ml IL-6 plus 2 μg/ml IL-23 (29, 39, 40) (as we did). In this context, the paper by Hu et al. (24), based on the use of neutralizing Abs and pharmacological inhibitors, identified endogenous IL-6 and IL-23 as indirect inducers of IL-17A expression in a fraction of neutrophils either infected with *Mycobacterium tuberculosis* (MTB), or stimulated with LPS or Pam3CSK4. In this latter study, however, IL-6 and IL-23 levels corresponded to 1 ng/ml at the best. Herein, we failed to detect IL-17A mRNA expression and production in neutrophils incubated with either LPS

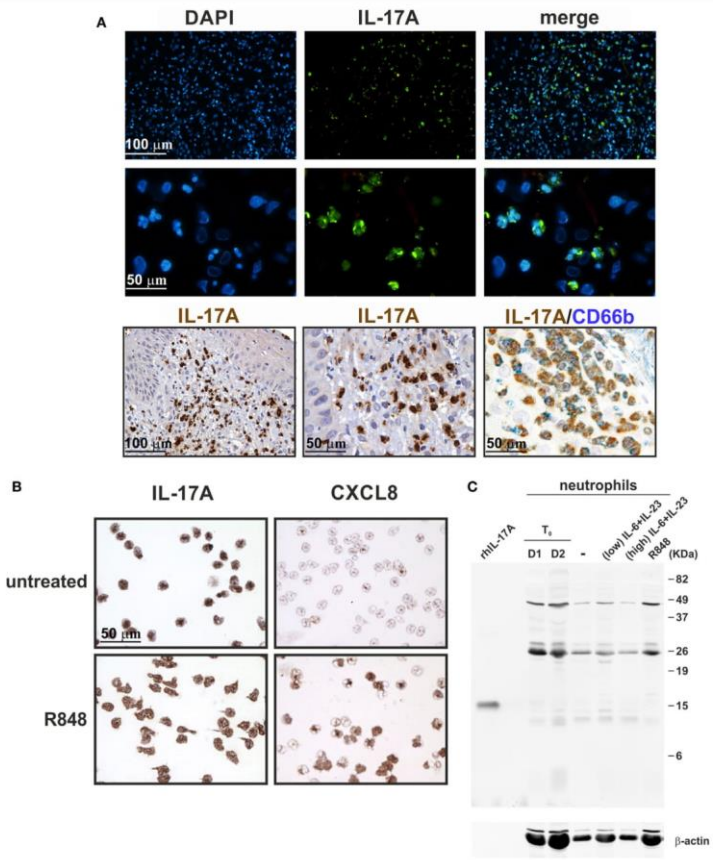

FIGURE 8 | Staining human neutrophils by anti-IL-17A (AF-317-NA) polyclonal antibodies (Abs). **(A)** Immunofluorescence (top panels) and immunohistochemistry (lower panels) stainings of two FFPE cases of human pustular psoriasis using anti-IL-17A (AF-317-NA) and anti-CD66b Abs (as labeled). Top panels show DAPI, FITC channel, and merge to recognize neutrophil shape; lower panels show different magnification of IHC and double IHC to characterize IL-17A$^+$ cells with the neutrophil marker CD66b. **(B)** Cytospins of neutrophils, either untreated (top panels) or treated with 5 μM R848 (bottom panels) for 3 h, were stained with anti-IL-17A (AF-317-NA, left panels) and anti-CXCL8 (right panels) Abs. Original magnification 200× [first row in **(A)** and left image in third row, scale bar 100 μm] and 400× [second row in **(A)**, center/right images in third row in **(A)**, as well as in **(B)**, scale bar 50 μm]. Images of the second row in **(A)** represent magnifications of images in first row. **(C)** AF-317-NA immunoblot of lysates from neutrophils either freshly isolated (T_0, from two donors) or incubated for 3 h with or without 2 μg/ml IL-6 plus 0.2 μg/ml IL-23 (low), 20 μg/ml IL-6 plus 2 μg/ml IL-23 (high), or 5 μM R848. Recombinant human IL-17A (rhIL-17A) was used as positive control. Panels **(B,C)** display representative experiments out of two independent ones with similar results.

or Pam3CSK4, even if it is true that they produce IL-6 (62) and IL-23 (our unpublished observations). Whether stimulation of neutrophils with MTB effectively promotes IL-17A expression *via* endogenous IL-6 and IL-23 remains to be verified. However, no IL-17A, IL-17B, IL-17C, or IFNγ secretion from *Mycobacterium bovis* Bacille-Calmette Guerin (BCG)-stimulated neutrophils was recently reported (14). It should be also remarked that the purity of neutrophils in studies showing an IL-17 production in

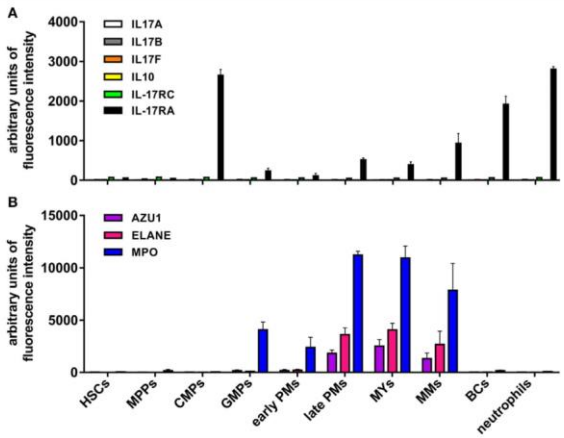

FIGURE 9 | Levels of IL-17A, IL-17B, IL-17F, IL-10, IL-17RC, IL-17RA, azurocidin, neutrophil elastase, and myeloperoxidase (MPO) mRNA expression in neutrophils at different stages of maturation. mRNA expression data derive from Gene Expression Omnibus database (accession number GSE42519) (65). **(A)** IL-17A, IL-17B, IL-17F, IL-10, IL-17RC, and IL-17RA or **(B)** azurocidin (AZU1), neutrophil elastase (ELANE), and MPO mRNA expression levels were measured in the following cell types: hematopoietic stem cells (HSCs), multipotent progenitors (MPPs), common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), early and late promyelocytes (PMs), myelocytes (MYs), metamyelocytes (MMs), band cells (BCs), and bone marrow polymorphonuclear neutrophil granulocytes. Values represent the mean ± SEM as calculated from data of the biological replicates present in the database.

response to IL-6 plus IL-23 (23, 29, 39, 40), reported to be >96% at the best (29), does not sufficiently secure fully genuine results at least in our opinion.

Nevertheless, we investigated potential mechanisms helping to clarify whether human neutrophils respond to IL-6 plus IL-23 in terms of IL-17A expression or not. ChIP assays revealed that, in resting, as well as in IL-6 plus IL-23-stimulated, neutrophils, but not in Th17 cell lines, the *IL17A* locus does not contain any H3K4me1 and H3K27Ac, which are two histone marks that are usually present in those genomic regions that act as active enhancers (85). On the other hand, the levels of H3K27Ac were found increased at the SOCS3 promoter of neutrophils incubated with IL-6 plus IL-23, consistent with the potentially inducible SOCS3 mRNA transcription. Notably, the complete absence of H3K4me1 at the *IL17A* locus of neutrophils is particularly informative, since such a histone modification is known to precede very early, but time-consuming (86), events necessary for the assembly of the transcriptional machinery, including nucleosomal depletion, H3K27Ac deposition, and enhancer activation (85). Based on our data, it appears that the chromatin at the *IL17A* locus of human neutrophils likely displays a closed conformation, inaccessible to transcription factors and, consequently, RNA polymerase, ultimately preventing IL-17A mRNA transcription in resting as well as stimulated neutrophils. It is thus very unlikely that H3K4me1 modification could be induced within 1 h, e.g., the time-point at which IL-17 mRNA expression in IL-6 plus IL23-stimulated neutrophils has been observed (29, 39, 40). Obviously, this does not exclude that there could exist some stimulatory conditions able to modify the chromatin at the *IL17A* or *IL17F* loci of human neutrophils.

A variety of studies report the presence of IL-17A$^+$-neutrophils in sample tissues from many diseases, including psoriasis (20, 25, 30, 32, 35, 49), skin inflammation (27), bullous pemphigoid (28), hidradenitis suppurativa (50), fungal keratitis (26), RA (31, 75), ankylosing spondylitis (18), systemic lupus erythematosus (41, 52), human ANCA-associated glomerulonephritis (47), cystic fibrosis (19, 36, 44), nasal polyps (53), chronic obstructive pulmonary disease (22), lung tissues during bacterial pneumonia (46), alcoholic liver diseases (48), acute renal allograft rejection (42), atherosclerotic plaques (21), cutaneous T cell lymphoma lesions (45), gastric cancer (29), cervical cancer (33), and prostate cancer (51), as revealed by IHC, IF, or intracellular flow cytometry using various commercial anti-IL-17A Abs. Not surprisingly, results occasionally appear discordant. For example, while Moran et al. (31) reported IL-17A-positive synovial tissue neutrophils using the AF-317-NA, van Baarsen et al. (16) show that synovial tissue neutrophils from arthritis patients are not stained by another antibody, namely #41802. By IHC experiments using AF-317-NA, we too detected IL-17A$^+$-neutrophils not only in skin sections of psoriasis patients but also in cytospin slides of neutrophils isolated from HDs and incubated for 3 h with or without R848, at similar levels. By contrast, we found that whole lysates of the same neutrophil populations displayed major signals at levels of proteins having MW not corresponding to that of IL-17A when

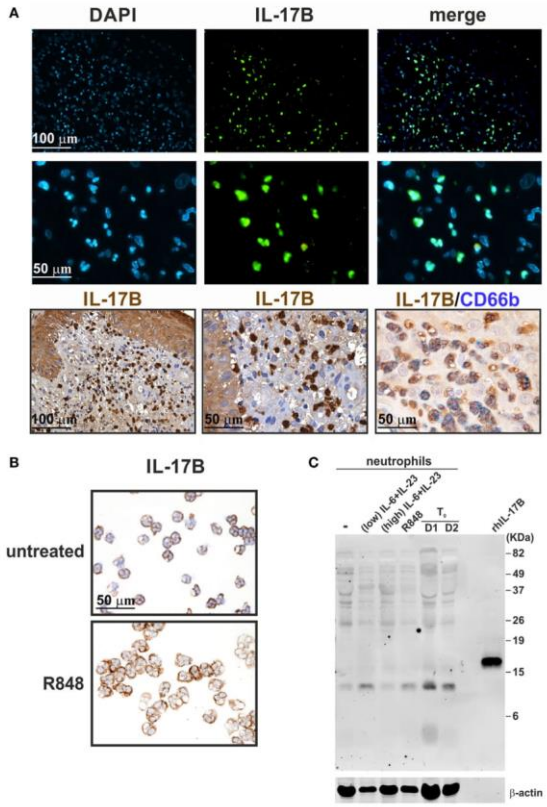

FIGURE 10 | Staining human neutrophils by anti-IL-17B (AF1248) antibodies (Abs). **(A)** Immunofluorescence (top panels) and immunohistochemistry (lower panels) staining of two FFPE cases of human pustular psoriasis using anti-IL-17B (AF1248) and CD66b Abs (as labeled). Top panels show DAPI, FITC channel, and merge to recognize neutrophil shape; lower panels show different magnification of IHC and double IHC to characterize IL-17A$^+$ cells with the neutrophil marker CD66b. **(B)** Cytospins of neutrophils incubated without (top panel) or with 5 μM R848 (bottom panel) for 3 h. Original magnification 200× [first row in **(A)** and left image in third row, scale bar 100 μm] and 400× [second row in **(A)**, center/right images in third row in **(A)**, as well as in **(B)**, scale bar 50 μm]. Images of the second row in **(A)** represent magnifications of images in first row. **(C)** AF1248 immunoblot of lysates from neutrophils either freshly isolated (T_0, from two donors) or incubated for 3 h with or without 2 μg/ml IL-6 plus 0.2 μg/ml IL-23 (low), 20 μg/ml IL-6 plus 2 μg/ml IL-23 (high), or 5 μM R848. Recombinant human IL-17B (rhIL-17B) was used as positive control. Panels **(B,C)** display representative experiments out of two independent ones with similar results.

immunoblotted with AF-317-NA. Our findings substantially confirm the observations previously made by Tamarozzi et al. (13) who also did not detect any IL-17A expression in highly pure populations of neutrophils (99.9%) by using a variety of assays including RT-qPCR, RNA-seq, western blot and ELISA, despite of finding IL-17A$^+$-neutrophils in *Wolbachia Onchocerca volvulus*-positive nodules by IHC using AF-317-NA. Notably, by immunoprecipitation experiments followed by mass spectrometry, Tamarozzi et al. (13) also uncovered that both AF-317-NA and #41802 bind to several proteins expressed in granules (including MPO, lactoferroxin, and lysozyme C) and cytoskeleton (such as keratin and profilin) of neutrophils, while other anti-human IL-17A Abs (sc-6077 from Santa Cruz, and PRS4877 from Sigma) were found to recognize multiple non-specific bands

in neutrophil immunoblots (13). All in all, data suggest that the IL-17A-positivity of human neutrophils detected by AF-317-NA and #41802 is, at least *in vitro*, likely an artifact. Whether these or other anti-IL-17A Abs, including sc-7927 (from Santa Cruz) (33, 43), ab9565 (from Abcam) (37), ab136668 (from Abcam) (46), 500-P07 and 500-P07G (from Peprotech) (43), and eBio64Dec17 (from eBioscience) (20, 26, 43), are instead reliable in specifically detecting IL-17A$^+$-neutrophils in tissue samples should be more convincingly established. For instance, in models of skin inflammation resembling psoriasis (27), accumulated neutrophils stained by AF-317-NA were shown to express IL-17 mRNA transcripts. In other studies, tissue neutrophil staining by AF-317-NA was blocked after antibody pre-adsorption with rIL-17A (18, 47), or confirmed by costaining of the same section by eBio64DEC17 (47). It is worth recalling that neutrophils express high levels of IL-17RA (12) that could in theory bind exogenously derived IL-17A, consequently leading to a positive signal in IHC or IF experiments without actual intracellular IL-17 production (87), as observed in the case of mast cells (88). Whatever the case is, we would recommend to always validate by multiple investigation methods an eventual detection of IL-17A-positive neutrophils exclusively by IHC, or IF or intracellular flow cytometry (18, 19, 21, 22, 28, 32–34, 36–38, 42, 43, 48–52).

Similar concerns can be made for the, to date, reported IL-17B expression by human neutrophils. Accordingly, IL-17B has been detected in neutrophils infiltrating the synovial membrane of RA patients (75) and the stroma of CCR cancer (76) by IHC/IF, as well as in freshly isolated neutrophils by immunoblotting (75), in all cases using #AF1248 Abs. We also detected IL-17B-positive neutrophils in psoriasis plaques and cytospin slides of freshly isolated neutrophils by IHC using #AF1248. However, we could not measure any IL-17B in lysates of freshly isolated/activated neutrophils either by using two different commercial ELISA or by #AF1248 immunoblotting. In the latter experiments, many proteins with MW different from that of rIL-17B were recognized by #AF1248, thus invalidating at least the cytospin results. Intriguingly, Kouri et al. (75) did detect IL-17B protein in lysates of neutrophils (95% pure), by both ELISA and immunoblotting using #AF1248. However, these authors showed only a portion of the western blot (75), thus rendering impossible to know whether additional major proteins were recognized by #AF1248. Curiously, we, Tamarozzi et al. (13) and Kouri et al. (75), all found that human neutrophils do not transcribe IL-17B mRNA under resting or activating condition. Furthermore, no IL-17B secretion from BCG-stimulated neutrophils was recently shown (14). In such regard, Koury et al. (75) suggested that IL-17B is synthesized only at the promyelocyte and myelocyte stage in the bone marrow, disappearing in mature neutrophils. However, our analysis of transcriptomes generated from all types of bone marrow cell populations (65) revealed that, similarly to IL-17A, also IL-17B is never transcribed during the different stages of neutrophil maturation. Altogether, data suggest that human neutrophils do not express IL-17B *in vitro*. They also suggest that the positive staining of neutrophils by IHC using AF1248 is likely due to a non-specific, IL-17B independent, binding of these Abs.

In conclusion, data shown in this study are consistent with the notion that human neutrophils are unable to express and produce IL-17A, IL-17B, or IL-17F *in vitro*.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy). All the experimental protocols were approved by the Ethic Committee and all subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

All authors were involved in discussing and drafting the article, approved the final version to be published, and had full access to all data, taking responsibility for their integrity and analysis accuracy. In particular, NT, FA-S, SG, EG, SL, LG, and FC performed the experiments; FA-S, FB-A, NT, SL, WV, and MC analyzed the results; GG provided patients; and FS, NT, AM, WV, and MC conceived the experiments and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.00795/full#supplementary-material.

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REVIEW

WILEY

Cytokine production by human neutrophils: Revisiting the "dark side of the moon"

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Abstract

Polymorphonuclear neutrophils are the most numerous leucocytes present in human blood, and function as crucial players in innate immune responses. Neutrophils are indispensable for the defence towards microbes, as they effectively counter them by releasing toxic enzymes, by synthetizing reactive oxygen species and by producing inflammatory mediators. Interestingly, recent findings have highlighted an important role of neutrophils also as promoters of the resolution of inflammation process, indicating that their biological functions go well beyond simple pathogen killing. Consistently, data from the last decades have highlighted that neutrophils may even contribute to the development of adaptive immunity by performing previously unanticipated functions, including the capacity to extend their survival, directly interact with other leucocytes or cell types, and produce and release a variety of cytokines. In this article, we will summarize the main features of, as well as emphasize some important concepts on, the production of cytokines by human neutrophils.

KEYWORDS
CCL23, human neutrophils, IL-10, IL-17, IL-6, TLRs

1 | INTRODUCTION

Human neutrophils have been shown to express and produce either in vitro or in vivo, particularly upon appropriate stimulation, many pro- and anti-inflammatory cytokines (including TNFα, IL-1β, IL-1ra, IL-6), chemokines (including CXCL1, CXCL8, CXCL10, CCL2, CCL3, CCL4 and, as more recently described, CCL23), colony-stimulating and angiogenic factors (including G-CSF and VEGF), TNF family members (including TRAIL, FasL and BAFF) and growth factors (HB-EGF)[1-3] (Figure 1). These findings, in the face of multifold evidence[1-5] are making it clear that neutrophils are functionally involved in a variety of physiological and/or pathological processes, such as hematopoiesis, angiogenesis, wound healing, autoimmune and neoplastic diseases, obviously in addition to acute inflammatory diseases.

It should be recalled that neutrophil-derived cytokines are potentially detectable by a variety of methods, including enzyme-linked immunosorbent assays (ELISA), cytokine secretion assays, immunoblotting and/or immunoprecipitation in cell-free supernatants or cell lysates, immunohistochemistry (IHC) and/immunofluorescence (IF) in tissue samples, intracellular staining by flow cytometry or confocal microscopy in intact cells, and RT-qPCR and RNA-seq for mRNA expression.[1,3,6] In our opinion, designating neutrophils as sources of a given cytokine, based on findings made only by IHC, or IF, or intracellular staining, does not guarantee a 100% certainty of being correct. In fact, if one is unaware, data from such methodologies might be affected by technical artefacts, which must be known and carefully excluded. For example, human neutrophils display an elevated auto-fluorescence that can be erroneously confused with a positive cytokine signal.[7,8] On the same line,

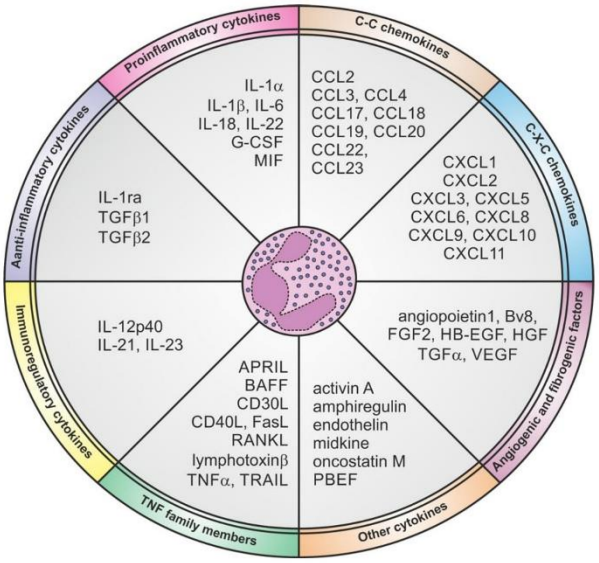

FIGURE 1 Cytokines that human neutrophils can potentially produce. The cartoon lists the cytokines reproducibly shown to be produced by human neutrophils

anti-cytokine antibodies might give false positive signals because they may either cross-react with intracellular proteins,[7-9] or non-specifically bind to FcγRII/CD32 and FcγIII/CD16. Consequently, in human neutrophils it would be more appropriate using IHC, IF or intracellular staining only to support findings obtained by other, more trustable, cytokine detection methods.

Currently, the notion that activated neutrophils may synthesize cytokines de novo has become widely accepted. This function can be modulated either positively by IFNγ[10] or negatively by IL-10.[11] Doubts on the importance of such a neutrophil function in in vivo settings[12] have no reason to exist at the light of multiple evidence.[1-3] For example, a recent work has clearly shown that abrogation of CARD9 in murine neutrophils inhibits inflammatory reactions during autoantibody-induced sterile inflammation, since it specifically suppresses the in vivo production and release of neutrophil-derived chemokines and cytokines, but not LTB_4.[13] Nevertheless, it does not hurt to recall 2 fundamental aspects that must be always kept in mind when studying neutrophil-derived cytokines. One is that human neutrophils typically possess 10-20 times less total RNA than other leucocytes.[14] Consequently, on a *per cell* basis neutrophils generally produce exceedingly lower cytokine amounts than monocytes/macrophages, lymphocytes or dendritic cells (DCs) do,[15,16] although with some exceptions (eg BAFF, IL-1ra, VEGF, CCL19 and CCL23).[3,17-19] It follows that, for a correct and reliable evaluation and quantification of the profiles of either the cytokines produced, or (more broadly) the gene expressed by resting or activated neutrophils, it is mandatory to work with highly pure cell populations.[1,3] A study recently conducted in our lab [20] perfectly illustrates what kind of false positive results one could obtain if she/he is unaware of the requirement to use pure neutrophil populations for cytokine production/gene expression studies, and/or of the literature data in the specific field. Accordingly, we isolated peripheral neutrophils from the same donors using either our standard method (guaranteeing a neutrophil purity of more than 99.6/99.7%),[21] or 2 distinct commercial 1-step kits, specifically designed to guarantee a rapid purification of highly pure, "unstressed" peripheral neutrophils, by skipping density gradient centrifugation of blood. We could confirm that the neutrophils isolated by the 2 commercial 1-step kits reached purities in the range reported in their datasheets (98.9% and 98%, respectively).[20] However, when those neutrophils were analysed for cytokine mRNA expression/production in response to R848 (a synthetic mimic of viral ssRNA recognized by TLR8), many "false" results were found, the most resounding ones being a production of IL-10 and, after incubation for 4 hours, an expression of IL-6 mRNA, which were ultimately attributed to the very small percentages (0.2%-0.6%) of contaminating $slan^+CD16^+$-monocytes.[20] These experiments taught us that it is

essential to perform a round of blood density gradient centrifugation (including when using commercial kits), as it ensures the elimination of most PBMCs from the recovered granulocytes. Notably, neutrophils isolated by the 1-step kits, incubated with 10 ng/mL TNFα, did not differ from the neutrophils isolated by our method in terms of CXCL8 mRNA accumulation and production, or TNFα mRNA expression, indicating that the "neutrophil contamination issue" is very subtle, becoming evident only under discrete stimulatory conditions. This concept is not trivial, particularly if referred to a recent work in which neutrophils isolated either by a single-step centrifugation of whole blood onto Polymorphprep (a reagent mimicking Ficoll-Paque) (95% pure), or by the EasySep Human Neutrophil enrichment kit (without Ficoll-Paque centrifugation) (99% pure), were stimulated for a 1 hour with either 10 ng/mL TNFα or 5 ng/mL GM-CSF.[22] Analysis of transcriptome profiles identified only 25 genes as significantly differentially expressed between Polymorphoprep and negatively selected neutrophils across all 3 treatment groups (untreated, GM-CSF, TNFα),[22] leading the authors to conclude that "low numbers of contaminating leucocytes (<5%) in neutrophil preparations contribute very little to their overall gene expression profile." In light of the findings described above,[20] it is plausible to assume that, if these neutrophil populations had been stimulated *via* TLR agonists, the authors would have likely found dramatic changes in gene expression and, in turn, drawn a very different conclusion.

This being said, the fact that a single neutrophil, *per se*, generally produces very little amounts of cytokines does not mean that neutrophil-derived cytokines are not relevant. In fact, we should not forget that neutrophils usually greatly outnumber mononuclear leucocytes in acute infection/inflammatory sites by 1-2 orders of magnitude, implying that they could certainly dictate the evolution of the subsequent stages based on their secreted cytokine profiles.

2 | MOLECULAR MECHANISMS CONTROLLING CYTOKINE EXPRESSION IN HUMAN NEUTROPHILS

At molecular level, data in the literature clearly show that the production of cytokines by human neutrophils, as in other leucocytes, can be controlled by regulatory mechanisms that occur at the level of mRNA transcription (examples are CXCL8 and CCL3 mRNAs in LPS-treated neutrophils), mRNA stability (an example is IL-1ra mRNA in IL-13-treated neutrophils), mRNA translation (an example is TNFα mRNA in GM-CSF-treated neutrophils), or protein secretion (an example is CXCL1 in neutrophils phagocytosing opsonized yeasts).[1] Epigenetic mechanisms are also involved in the control of cytokine expression by human neutrophils.[23] Moreover, cytokines might be stored in intracellular compartments following de novo synthesis, either by mature cells[18,24] or during their bone marrow maturation,[17,25] ready to be promptly secreted in response to appropriate secretagogues. Notably, studies from our group[26] and other researchers[27] have shown that human neutrophils and autologous monocytes may display common, but also very distinct, gene and cytokine expression programmes in response to the same agonist. For instance, upon stimulation with LPS (a TLR4 ligand), only monocytes, but not neutrophils, express and produce IFNβ.[26] This is explained by the fact that, in human neutrophils, but not in monocytes, the activation of TLR4 by LPS, for some unknown reasons, fails to trigger the so-called TRIF-signalling pathway, which is known to downstream signal for the activation of interferon regulatory factor 3 (IRF3), a transcription factor (TF) crucial for the transcriptional induction of IFNβ mRNA. Moreover, because endogenous IFNβ is known to trigger the expression of a large set of interferon-stimulated genes (ISG), including CXCL9/CXCL10/CXCL11, the inability of human neutrophils to promote the TRIF-signalling pathway also explains why, unlike autologous monocytes, neutrophils do not express/produce CXCL9/CXCL10/CXCL11.[28] By contrast, human neutrophils display a fully functional MyD88-dependent pathway leading to an early wave of NF-κB and mitogen-activated protein kinase (MAPK) activation, which is the other signalling cascade triggered by LPS *via* TLR4. Consistently, human neutrophils incubated with LPS do produce most of the pro-inflammatory cytokines that one would expect from the triggering of the MyD88-dependent pathway.[26]

Other studies have uncovered that, beyond cell-specific TF expression/activation, chromatin-dependent mechanisms may represent another critical factor conditioning the transcriptional output of human neutrophils vs monocytes.[23] A regulatory role of the chromatin has been highlighted by studies investigating whether human neutrophils express IL-6 and IL-10.[16,29] For the latter cytokine, a comparative analysis of histone modifications associated to "permissive" chromatin, precisely H3K4me3 (localized at the transcription start site of active genes), H3K27Ac and H4Ac (both associated to activated regulatory elements), and H3K4me1 (mainly associated to active and poised enhancers), revealed that these histone marks are detectable at the *IL10* genomic locus of monocytes, but not neutrophils, freshly isolated from the blood.[29] Furthermore, H3K4me3 and H3K27Ac marks were shown to further increase in monocytes but not neutrophils, stimulated with LPS, Pam3CYS4 (a TLR2 ligand) or serum amyloid A (SAA).[29] In accordance with the observations made on histone modifications,

chromatin immunoprecipitation (ChIP) of those TFs previously proposed to bind to, and/or transactivate, the IL-10 gene in various cells of human or mouse origin,[30] revealed no binding of C/EBPβ, c-FOS, SP1 and NF-κBp50 to the IL-10 promoter of human neutrophils, either under resting conditions or upon simulation. Altogether, these data have provided a molecular mechanism explaining why human neutrophils are unable to produce IL-10,[6] in turn solving a controversial issue raised by several studies reporting the contrary.[29] In our opinion, either a scarce purity of the neutrophil preparations, or a very low threshold of the ELISA standard curve used to evaluate the production of IL-10,[31] may lead researchers to draw incorrect conclusions. Therefore, although it cannot be excluded that specific stimulatory conditions may revert the nonpermissive chromatin status of the *IL10* locus of human neutrophils, cautious interpretation must be taken about the studies reporting that human neutrophils express IL-10 under inappropriately controlled experimental conditions.[32]

Conflicting results concerning the ability of human neutrophils to express IL-6 have been also published.[1] Accordingly, several studies have reported a remarkable production of IL-6 by human neutrophils stimulated with LPS, GM-CSF or TNFα,[1,33] that, however, has not been reproduced by other groups,[1,33] including us.[14] Even though the culture conditions, the methods and the timepoints to detect IL-6 mRNA expression/production used in these various studies are not always comparable, it should be remarked that, in general, the publications pointing human neutrophils as IL-6 producers have rarely specified that they were free of contaminating monocytes. Human monocytes are indeed well known to represent major sources of IL-6, being 2/3 orders of magnitude more efficient than neutrophils, as well as releasing the cytokine much earlier.[16] Hence, it is obvious that a minimal percentage of accidentally contaminating monocytes may greatly contribute to the IL-6 levels measured in supernatants from neutrophils, either resting or activated for short periods. In any case, our own studies have recently clarified that human neutrophils may certainly produce IL-6 in vitro, but only under experimental conditions able to reorganize the chromatin at their *IL6* locus.[16] Chromatin reorganization in neutrophils has been shown to occur in response to R848, CL075 (another TLR8 specific ligand), or very elevated concentrations of ultrapure LPS (not less than 1-10 μg/mL).[16] Chromatin remodelling has been shown to require time and, consistently, neutrophil-derived IL-6 was found to be optimally measurable after an overnight incubation of the cells, but absent within 4 hours. Consistently, ChIP experiments have revealed that the *IL6* locus of highly purified, freshly isolated, neutrophils does not contain any H3K4me3, H3K27Ac, H4Ac and H3K4me1 histone marks, indicating that the underlying chromatin stands in a closed conformation[16] (Figure 2). Incubation of neutrophils with R848 was found to slowly, but steadily promote the deposition of H3K4me3, H3K27Ac, H4Ac and H3K4me1 at the *IL6* locus, at maximal levels after 20 hours.[16] In other words, we demonstrated that TLR engagement signals to open the chromatin at the *IL6* locus of neutrophils, in turn rendering it accessible to those R848-activated TFs able to transactivate IL-6 transcription, as shown for PU.1, C/EBPβ, NF-κB and IκBζ by ChIP assays[16] (Figure 2). Notably, we could also uncover that such TLR-dependent reorganization of the *IL6* chromatin renders neutrophils responsive to endogenous TNFα in terms of IL-6 gene expression induction.[16,34] In fact, no IL-6 transcription could be observed in human neutrophils incubated with exogenous TNFα, indicating that TNFα is unable to modify the inaccessible *IL6* locus.[16]

Another example of the differential cytokine expression between human neutrophils and monocytes concerns CCL23, which is a chemokine that binds to CCR1 and thus exerts chemotactic activities on monocytes, DCs and resting T lymphocytes. We found that while neutrophils and monocytes produce similar levels of CCL23 in response to R848, only monocytes, but not neutrophils, manufacture CCL23 upon incubation with IL-4.[3] Under the latter experimental conditions, however, neutrophils promptly accumulate IL-1ra mRNA, indicating that they do respond to IL-4. Our unpublished genome-wide map of both PU.1 (an Ets-family transcription factor marking the majority of regulatory elements in myeloid cells) [35] and H3K4me1, to explore at molecular level potential explanations for the inability of IL-4-treated neutrophils to express CCL23 mRNA, revealed that monocytes contain 2 regulatory regions at the *CCL23* locus (1 and 25 kb upstream from *CCL23* TSS), while human neutrophils contain only one of them (1 kb upstream from *CCL23* TSS). Although not formally proven, we believe that the "closed" chromatin conformation in neutrophils likely prevents the binding of IL-4-activated transcription factor(s) to it, and consequently the transcription of CCL23 mRNA.

3 | THE CASE OF IL-17 EXPRESSION IN HUMAN NEUTROPHILS

As discussed for IL-10 and IL-6, also other cytokines, including IFNα and IL-17A, are debated in relation to their potential expression/production by activated human neutrophils. For details on the knowledge about IFNα, the reader could refer to our recent publication.[34] In brief, we failed to detect any IFNα production by human neutrophils incubated with either R848, or various CpG preparations (CpG-ODN 2006 and 2216)—known to activate TLR9

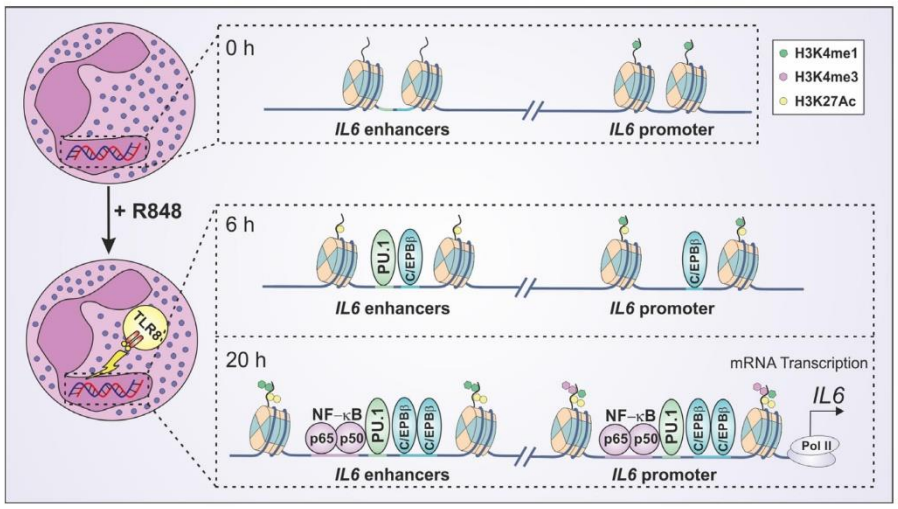

FIGURE 2 R848 induces a reorganization of the chromatin at the *IL6* locus of human neutrophils. Scheme illustrating the occupancy of PU.1, NF-κB, C/EBPβ and histone modifications (H3K4me1, H3H4me3 and H3K27Ac) at the regulatory regions of the *IL6* locus of human neutrophils. *IL6* locus in resting neutrophils is inactive based on the absence of histone modifications (top panel). Upon treatment with R848, a recruitment of PU.1, C/EBPβ and NF-κB, as well as a deposition of significant levels of histone marks (H3K4me1, H3H4me3 and H3K27Ac) occur at various *IL6* locus regions, indicating an induction of latent regulatory sites (such as enhancers), ultimately leading to IL-6 gene transcription

[36]—previously shown to induce neutrophil-derived IFNα at high levels.[37] It is hard to demonstrate, but it might have been possible that, by chance, we caught some of the so-called "non-responder donors".[37] Unfortunately, we did not have the chance to stimulate neutrophils with chromatin preparations, which were actually shown to be the most potent IFNα inducers.[37]

Concerning IL-17A (usually referred to as IL-17), we originally published that human neutrophils activated in vitro by LPS and/or IFNγ do not produce it.[21] Since then, the majority of the studies have instead reported that human neutrophils represent potential sources of IL-17A, for instance in response to PMA and fMLF,[38] ionomicin plus PMA,[39] *Mycobacterium,* LPS and Pam3CSK4,[40] or *L. pneumophila.*[41] Similarly, 4 different groups[40,42-45] have shown that IL-6 plus IL-23 efficiently induce IL-17A, IL-17F and IL-17RC mRNA expression and protein production by human neutrophils. Curiously, 2 of these groups have specified that concentrations lesser than 20 μg/mL IL-6 plus 2 μg/mL for IL-23 are not effective in terms of IL-17A induction,[42-44] while another group found 20 ng/mL IL-6 and/or 20 ng/mL IL-23 as perfectly functional in neutrophils from asthmatic subjects,[45] as well as up to 100 ng/mL in control neutrophils. Whatever the case is, the most numerous evidence for the expression of IL-17 by human neutrophils derives from IHC and/or IF studies documenting IL-17A$^+$-neutrophils in tissue specimens from a variety of pathological conditions (see Table 1). In this context, some of the commercial anti-IL-17A antibodies often used for IHC and/or IF studies (for instance, AF-317-NA and #41802) were shown to detect proteins that did not display the MW of IL-17A when tested in Western blotting experiments using neutrophil and PBMC lysates.[7] Moreover, immunoprecipitation experiments followed by mass spectrometry have also uncovered that both AF-317-NA and #41802 antibodies actually recognize several proteins expressed in the granules and cytoskeleton of neutrophils, while other anti-human IL-17A antibodies (sc-6077) were found to non-specifically recognize multiple proteins in immunoblotting experiments of neutrophil lysates.[7]

Since studies reporting neutrophil positivity to IL-17A continue to be published,[40,41,43-53] ignoring either the previously mentioned information, or the few works showing the contrary,[7,54-57] recently we decided to perform additional investigations.[9] Using multiple methodological approaches (RT-qPCR, ChIP-seq, ELISA, intracellular

TABLE 1 Expression of IL-17A by human neutrophils as detected by immunohistochemistry (IHC) and/or immunofluorescence (IF) studies

Type of biological sample	Detection method(s)	Reagents	Notes	Reference
Liver biopsies from patients with alcoholic liver disease	IHC and IF	Goat polyclonal anti-human IL-17A antibody (AF-317-NA, R&D Systems)		Lemmers et al. (2009)[60]
Atherosclerotic plaque tissue	IHC	AF-317-NA		de Boer et al. (2010)[61]
Biopsies from lesional psoriatic skin	IHC	AF-317-NA		Res et al. (2010)[62]
Facet joints of patients with ankylosing spondylitis	IF	AF-317-NA	Positive staining by anti-IL-17 antibody blocked by recombinant IL-17	Appel et al. (2011)[63]
Tissue of explanted lung from patients with cystic fibrosis	IHC	AF-317-NA		Brodlie et al. (2011)[64]
Lung tissue and sputum from COPD patients	IHC and IF	AF-317-NA		Eustace et al. (2011)[65]
Biopsies of inflamed synovial membrane	IHC	AF-317-NA		Moran et al. (2011)[66]
Endobronchial biopsies and BALF cytospins from patients with cystis fibrosis.	IF	AF-317-NA		Tan et al. (2011)[67]
Skin biopsies from patients with SLE	IHC	AF-317-NA		Villanueva et al. (2011)[68]
Prostatectomy specimens	IHC	Anti-IL17A antibody (R&D systems)		Vykhovanets et al. (2011)[69]
Acute renal allograft rejection biopsies	IHC	AF-317-NA		Yapici et al. (2011)[70]
Tissue from adult nasal polyposis or cystis fibrosis	IHC	Anti-IL17A antibody (R&D systems)		Derycke et al. (2012)[71]
	Intracellular flow cytometry	Anti-IL17A antibody (ebioscience)		
Skin biopsy of patient affected by mycosis fungoides	IHC	AF-317-NA		Fontao et al. (2012)[72]
Renal biopsy from patients with ANCA-associated glomerulonephritis	IHC and IF	AF-317-NA, validated by mouse monoclonal anti-human IL-17 antibody (eBio64DEC17 from eBioscience)	Positive staining by anti-IL-17 antibody blocked by recombinant IL-17	Velden et al. (2012)[38]
Tonsillar tissue	IHC	AF-317-NA	Antibodies from Peprotech and eBioscience did not show any interpretable staining.	Yapici et al. (2012)[73]
		eBio64Dec17		
		Polyclonal rabbit anti-human IL-17A antibody (sc-7927, Santa Cruz)		
		Polyclonal rabbit and goat anti human IL-17 antibodies (500-P07 and 500-P07G, respectively, Peprotech)	Santa Cruz and R&D Systems IL-17 antibodies produced qualitatively conflicting results.	

(Continues)

TABLE 1 (Continued)

Type of biological sample	Detection method(s)	Reagents	Notes	Reference
Skin biopsies from psoriatic tissue	IHC	AF-317-NA		Patel et al. (2013)[74]
Skin of patients with pustular psoriasis	IHC and IF in situ hybridization	AF-317-NA		Kakeda et al. (2014)[75]
In vivo models of inflamed skin	IHC in situ hybridization Intracellular flow cytometry	AF-317-NA IL-17A Alexa Fluor 488 (eBioscience)		Keijsers et al. (2014)[76]
Skin biopsies from bullous pemphigoid (BP) patients	IHC	AF-317-NA		Le Jan et al. (2014)[77]
Biopsies from patients undergoing peritoneal dialysis	IHC and IF	polyclonal rabbit anti-human IL-17A antibody (ab9565 Abcam)		Rodrigues-Diez et al. (2014)[78]
Sections of nodules from patients infected with *O. volvulus*	IHC and IF	AF-317-NA monoclonal mouse anti-human IL-17A antibody (41802, R&D Systems) polyclonal goat anti-human IL-17A antibody (sc-6077, Santa Cruz) polyclonal rabbit anti-human IL-17A antibody (PRS4877, Sigma)		Tamarozzi et al. (2014)[7]
Squamous cervical cancer specimens	IHC and IF	sc-7927		Punt et al. (2015)[48]
Skin biopsies from psoriatic lesions	IHC and IF	AF-317-NA	no detection of IL-17A mRNA in granulocytes freshly isolated from blood or psoriatic plaques	Reich et al. (2015)[47]
Infected lung and normal lung sections	IF Intracellular flow cytometry	Polyclonal rabbit anti-human IL-17A antibody (ab136668, Abcam)		Cai et al. (2016)[41]
Skin biopsies from patients with hidradenitis suppurativa	IHC	AF-317-NA		Lima et al. (2016)[52]

staining and immunoblotting), we could confirm that highly purified populations of human neutrophils, either resting, or activated by a variety of stimulatory conditions, such as TLR and dectin ligands, fungal PAMPs and cytokines, including 20 μg/mL IL-6 plus 2 μg/mL IL-23, neither express IL-17A, IL-17F, IL-17B, IL-17C, IL-17D and IL-17E mRNA, nor produce IL-17A, IL-17F, IL-17A/F and IL-17B in vitro.[9] Moreover, by performing ChIP assays for H3Kme1 and H3K27Ac, we found that the *IL17A* and *IL17F* loci of resting, as well as IL-6 plus IL-23-stimulated, neutrophils, do not contain any of these 2 histone marks.[9] By contrast, the levels H3K27Ac were found increased at the *SOCS3* promoter of human neutrophils incubated with IL-6 plus IL-23, an observation consistent with the capacity of the 2 cytokines to be effectively stimulatory for neutrophils.[9] These data indicate that the chromatin at the *IL17A* and *IL17F* loci of neutrophils likely display a closed conformation, consequently preventing IL-17A and IL-17F mRNA transcription either under resting or IL-6 plus IL-23 stimulatory conditions.

We could also fully confirm and extend the observations concerning the AF-317-NA antibody.[7] Accordingly, by IHC staining using AF-317-NA antibody we detected IL-17A$^+$-neutrophils not only in skin sections of psoriasis

patients, but also in cytospin slides of peripheral cells isolated from healthy donors and incubated for 3 hours with or without R848.[9] However, whole lysates of the same neutrophil populations displayed major signals at levels of proteins having MW not corresponding to that of IL-17A when immunoblotted with AF-317-NA antibody.[9] It is evident that the IL-17-positivity of human neutrophils detected by the AF-317-NA antibody is, at least in vitro, an artefact. Whether AF-317-NA or other anti-IL-17A antibodies are reliable in specifically detecting IL-17A$^+$-neutrophils in pathological tissues must be established with absolute certainty.

Interestingly, we made similar findings in relation to the expression of IL-17B by human neutrophils.[9] Again, we tested a commercial anti-IL-17B antibody (#AF1248) previously shown to detect IL-17B$^+$-neutrophils in synovial membranes of RA patients[58] and in the stroma of colon carcinoma cancer[59] by IHC/IF, as well as by immunoblotting of freshly isolated cells.[58] As expected, by IHC experiments using #AF1248 antibody we could detect IL-17B-positive neutrophils in psoriasis plaques and cytospin slides of freshly isolated neutrophils.[9] By contrast, we could not measure any IL-17B in lysates of freshly isolated/activated neutrophils, either by immunoblot using #AF1248 antibody or using 2 different commercial ELISA.[9] In the former experiments, many proteins with MW different from that of rIL-17B were recognized by #AF1248 antibody, thus suggesting that the positive staining of neutrophils in cytospin slides was likely due to its nonspecific, IL-17B-independent, cell binding.[9]

4 | CONCLUSIONS

Our understanding of cytokine production by human neutrophils is far from being complete. Clearly, its full appreciation will likely provide new insights into the biology of these cells. We know very little on the repertoire of cytokines produced in vivo by neutrophils residing in various tissues and how it may influence the homoeostasis or the pathogenesis of those diseases in which neutrophils are the first actors. Similarly, knowledge of the molecular mechanisms controlling cytokine expression in human neutrophils is only at its beginning. The challenge is to rapidly move the field and, in turn, translate the acquired knowledge into more effective therapies eventually targeting neutrophil-derived cytokines.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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ARTICLE

Human neutrophils activated via TLR8 promote Th17 polarization through IL-23

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Abstract

Human neutrophils contribute to the regulation of inflammation via the generation of a range of cytokines that affect all elements of the immune system. Here, we investigated their ability to express some of the members of the IL-12 family after incubation with TLR8 agonists. Highly pure human neutrophils were thus incubated for up to 48 h with or without R848, or other TLR8 agonists, to then measure the expression levels of transcripts and proteins for IL-12 family member subunits by RNA-seq, reverse transcription quantitative PCR, and ELISA. We show a TLR8-mediated inducible expression of IL-12B and IL-23A, but not IL-12A, mRNA, which occurs via chromatin remodeling (as assessed by ChIP-seq), and subsequent production of IL-23 and IL-12B, but no IL-12, proteins. Induction of IL-23 requires endogenous TNF-α, as both mRNA and protein levels were blocked in TLR8-activated neutrophils via a TNF-α-neutralizing Ab. We also show that supernatants from TLR8-activated neutrophils, but not autologous monocytes, induce the differentiation of Th17 cells from naïve T cells in an IL-23-dependent fashion. This study unequivocally demonstrates that highly pure human neutrophils express and produce IL-23, further supporting the key roles played by these cells in the important IL-17/IL-23 network and Th17 responses.

KEYWORDS
IL-23, neutrophils, Th17 cells, TLR8, TNF-α

1 | INTRODUCTION

Neutrophils are currently recognized as potential sources of cytokines, including chemokines and growth factors.[1] Neutrophil-derived cytokines not only regulate inflammation and immunity, but also orchestrate a variety of physiologic processes such as hematopoiesis, angiogenesis, and fibrogenesis, as well as pathologic conditions such as infectious, inflammatory, autoimmune, or neoplastic diseases.[2–4] Circulating blood neutrophils from healthy individuals do not normally express cytokines, but can generate them in response to stimulus-specific environmental signals.[1] Many ligands can activate cytokine expression by human neutrophils, for instance microbial factors such as pathogen-associated molecular patterns (PAMPs) binding to pattern recognition receptors (PRR), including TLRs,[5] RIG-I[6,7] and DNA sensors,[7–9] or host-generated cytokines. In addition, neutrophil-derived factors can themselves enhance/generate additional cytokine expression via autocrine feedback loops.[10–12]

We recently identified R848 and CL075 as very powerful agonists able to trigger a remarkable extracellular production of cytokines, including TNF-α, IL-6, G-CSF, and CCL23.[11,13,14] R848 and CL075 are synthetic compounds that in human neutrophils specifically act via TLR8, because TLR7, their other receptor, is absent and not inducible following cell activation.[5,11,14] Interestingly, by investigating the molecular pathways leading to the expression of IL-6 mRNA—an IκBζ-dependent gene—we identified a previously undescribed mechanism of cytokine induction in human neutrophils, resulting from

Abbreviations: CD, cluster of differentiation; ChIP, chromatin immunoprecipitation; ChIP-seq, ChIP followed by high throughput sequencing; FPKM, fragments per kilobase of transcript per million mapped reads; H3K27Ac, histone 3 lysine 27 acetylation; HOMER, Hypergeometric Optimization of Motif EnRichment; PAMPs, pathogen associated molecular patterns; PRR, pattern recognition receptor; RT-qPCR, reverse transcription quantitative PCR; TSS, transcriptional start site; UCB, umbilical cord blood.

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chromatin remodeling.[11] Specifically, we observed that the induction of IL-6 transcription by R848 depended on an inducible remodeling of chromatin at the IL-6 genomic locus, turning it from an "inactive" to an "active" configuration.[11] We also observed that, among the IκBζ-dependent genes, transcripts for IL-12B (corresponding to the p40 subunit of the IL-12 cytokine family) were greatly induced in TLR8-treated neutrophils.[11] However, we did not further investigate whether IL-12B mRNA was converted into protein, and/or whether other members of the IL-12 cytokine family to which p40 associates are expressed/produced by TLR8-activated neutrophils. IL-12B is a β-chain of the IL-12 family of heterodimeric cytokines that can assemble with some α-chains of the same family, such as IL-23A (the p19 subunit), and IL-12A (the p35 subunit), to generate the heterodimers IL-12 (IL-12A + IL-12B, which form the p70 complex) and IL-23 (IL-23A + IL-12B).[15] In addition, human IL-12B can also form homodimers that bind to the IL-12 receptor but these do not mediate any biologic activity.[16]

Because of the critical roles of the IL-12 family members in innate and adaptive immunity,[17,18] we specifically investigated whether human neutrophils activated via TLR8 express/produce IL-12 and/or IL-23. We report that TLR8-treated neutrophils, unlike circulating blood neutrophils, express and produce IL-23, but not IL-12. We also report that supernatants from TLR8-activated neutrophils promote the differentiation of Th17 cells from naïve T cells, a finding that adds a new dimension to the ability of these cells to regulate immune functions during infections and inflammation.

2 | MATERIALS AND METHODS

2.1 | Cell purification and culture

Highly purified neutrophils were isolated from the venous blood or from buffy coats from healthy individuals using a combined method consisting of Ficoll-Paque gradient centrifugation, dextran or Hetasep (StemCell Technologies, Vancouver, Canada) sedimentation of granulocytes, and hypotonic lysis of erythrocytes, followed by removal of contaminating immune cells using the EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, Canada) as described previously.[19,20] This procedure yields neutrophils of ~99.7% purity. Human monocytes were isolated from PBMCs, after Ficoll-Paque gradient centrifugation, by anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to reach >98% purity. Neutrophils and monocytes were then suspended at 5×10^6/ml and 2.5×10^6/ml, respectively, in RPMI 1640 medium containing 10% (<0.5 EU/ml endotoxin) FBS (BioWhittaker-Lonza, Basel, Switzerland). Cells were incubated for periods of up to 48 h (as indicated in the text) in the absence (control) or presence of 0.25–10 μM R848 (InvivoGen, San Diego, CA, USA), 0.25–10 μM CL075 (InvivoGen), 0.25–10 μM VTX-2337 (Selleck Chem, Boston, MA, USA), 1 μg/ml LPS (ultrapure, *Escherichia coli* 0111:B4 strain, InvivoGen), 10 ng/ml TNF-α (R&D Systems, Minneapolis, MN, USA) or 10 μg/ml adalimumab (Humira, Abbott Biotechnology Limited, Barceloneta, Puerto Rico).

2.2 | Cell viability

Cell viability was assessed by flow cytometry using Vybrant DyeCycleTM Violet (Thermo Fisher Scientific, Waltham, MA, USA) and SYTOX AADvanced (Thermo Fisher Scientific) stain, as previously described.[14]

2.3 | RNA isolation

After incubation as described in the text, neutrophils were pelleted by centrifugation, and total RNA was extracted with either Trizol or RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands). To completely remove any possible contaminating DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation.

2.4 | Reverse transcription quantitative PCR

Total RNA was reverse-transcribed into cDNA using Superscript III (Thermo Fisher Scientific) and random hexamer primers (Thermo Fisher Scientific). Transcript levels of individual genes were measured by reverse transcription quantitative PCR (RT-qPCR) using specific primer pairs (Thermo Fisher Scientific or Bio-Rad, Hertfordshire, UK) listed in Table 1. Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression units after RPL32 normalization.

2.5 | RNA sequencing

Prior to RNA-seq, total RNA was enriched for mRNA using poly(A) selection. Standard Illumina (San Diego, CA, USA) protocols were used to generate 50 bp single-end read libraries. In brief, mRNA was fragmented, reverse transcribed, adapted with sequencing primers and sample barcodes, size selected, and PCR-enriched. Libraries were sequenced on the Illumina HiSeq 2000 platform. Reads were mapped to the reference human genome (hg19) using TopHat version 2.0.14

TABLE 1 List of human primer sets utilized for the RT-qPCR experiments

	Sequences	
RT-qPCR primers	Forward primers	Reverse primers
IL-12A	CTGGACCACCTCAGTTTGG	TTTGTCGGCCTTCTGGAG
IL-12B	GGACATCATCAAACCTGACC	AGGGAGAAGTAGGAATGTGG
IL-23A	GGACACATGGATCTAAGAGAAGAG	CTATCAGGGAGCAGAGAAGG
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC

TABLE 2 List of human primer sets utilized for qPCR of ChIP assay

		Sequence	
ChIP primer name	Location (relative to the gene TSS)	Forward primers	Reverse primers
IL-12B #1	−308 to −64	CCCTCCTCGTTATTGATACACAC	GCTTGGGAAGTGCTTACCTTG
IL-12B #2	−12163 to −12070	GCAGAGGCAACACCTAAAGC	GCCCTTGATGAAGAAATGAGTG
IL-12B #3	−29315 to −29125	CCACTTCCCTTTTGACTTTAGG	CCCTGGGTTAGTACAGATTCG
IL-23A #1	−2166 to −2055	AGTTGTAGCCCTGGATGTAGTTC	CTCTGCCTCTTTGTTTCACTTC
IL-23A #2	−5233 to −5072	GATAGGGCAAGGGTCAGATG	GGAGAACTGGGGAAACTGG
PRL	+386 to +506	AGGGAAACGAATGCCTGATT	GCAGGAAACACACTTCACCA

and Bowtie 2 version 2. Gene expression values (fragments per kilobase of transcript per million mapped reads [FPKM]) were calculated using Cufflinks version 2.02. A minimum FPKM threshold of expression of ≥0.3 was applied to the expression data to minimize the risk of including false positives against discarding true positives from the datasets.

2.6 | Immunoblotting experiments

Total proteins from neutrophils and monocytes were recovered from protein-rich flow-through solutions obtained after the first centrifugation step of the RNeasy mini kit procedure (Qiagen, used for total RNA extraction), as previously described.[11] Proteins were then immunoblotted by standard procedures using the anti-human IRF8 pAbs (kindly provided by Prof. G Natoli from Humanitas University, Milan), and anti-human β-actin mAbs (A5060 from Sigma, Saint Louis, MO, USA). Blotted proteins were detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).[11]

2.7 | Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) experiments were performed exactly as previously described.[11] Briefly, nuclear extracts from formaldehyde-fixed 2.5–10 × 10^6 neutrophils were immunoprecipitated using 4 μl anti-PU.1 (sc-352) or 1 μl anti-H3K27Ac (histone 3 lysine 27 acetylation; ab4729) pAbs (Abcam, Cambridge, United Kingdom). Coimmunoprecipitated material was subjected to qPCR analysis using the specific primers listed in Table 2 (purchased from Thermo Fisher Scientific). Data from qPCR are expressed as percentage over input DNA and are displayed as mean ± SEM.

2.8 | ChIP sequecing

Purified DNA from PU.1 and H3K27Ac ChIP assays (performed as described in the previous paragraph) was adapter-ligated and PCR-amplified for sequencing on HiSeq2000 platform (Illumina, Cambridge, UK) using TruSeq DNA Library Prep Kit (Illumina). After sequencing, reads were quality-filtered according to the Illumina pipeline. Single end (51 bp) reads were then mapped to the human genome (Genome Reference Consortium GRCh37, Feb/2009) using BOWTIE v1.0.0. Only reads with no more than 2 mismatches (when compared to the reference genome) were converted to tag directories using Hypergeometric Optimization of Motif EnRichment (HOMER)'s module known as "makeTagDirectory," and then converted to BedGraph format using HOMER's module known as "makeUCSCfile," to be finally normalized to 10^7 total tag counts. ChIP-seq signals were visualized using Integrative Genomics Viewer.

2.9 | Cytokine production

Cytokine concentrations in cell-free supernatants were measured by commercially available ELISA kits, specific for human: IL-1β (eBioscience, San Diego, CA, USA), IL-23 (Mabtech, Nacka Strand, Sweden), IL-23A/IL-23p19 (Abcam, Cambridge, United Kingdom), TGFβ1 (R&D Systems), IL-6, IL-12/IL-12p70, and IL-12B/IL-12p40 (Mabtech). Lower detection limits of these ELISA were: 4 pg/ml for IL-1β, 4 pg/ml for IL-23, 20 pg/ml for IL-23A, 31 pg/ml for TGF-β1, 8 pg/ml for IL-6, 6 pg/ml for IL-12, and 10 pg/ml for IL-12B.

2.10 | Functional assays

Isolation of $CD4^+$ T cells from PBMCs of umbilical cord blood (UCB) was performed by using the CD4 isolation kit II (Miltenyi Biotec). After washing, cells were separated by immunomagnetic cell sorting (Miltenyi Biotec). Purified UCB-derived $CD4^+$ T cells were incubated for 1 week with 5 μg/ml of anti-CD3 + 5 μg/ml anti-CD28 mAbs, in the absence or the presence of either 10 ng/ml IL-1β + 20 ng/ml IL-23 (R&D Systems) or the indicated conditioned media from neutrophils and monocytes. In some experiments, antihuman IL-23p19 Abs (R&D Systems) were used in culture at the final concentration of 10 μg/ml. On day 7, T cells were stimulated with 10 ng/ml PMA + 1 μM ionomycin for 6 h, the last 4 h in the presence of 5 μg/ml brefeldin A, and then analyzed for intracellular IL-17, in association with CD161 membrane expression, by BD LSRII flow cytometry with FACS Diva software (BD Biosciences, Franklin Lakes, NJ, USA), as previously described.[21,22]

2.11 | Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation was performed by using, depending on the experimental data, Student's *t*-test, 1-way ANOVA followed by Tukey's post hoc test or 2-way ANOVA followed by Bonferroni's post hoc test. *P*-values < 0.05 were considered as statistically significant.

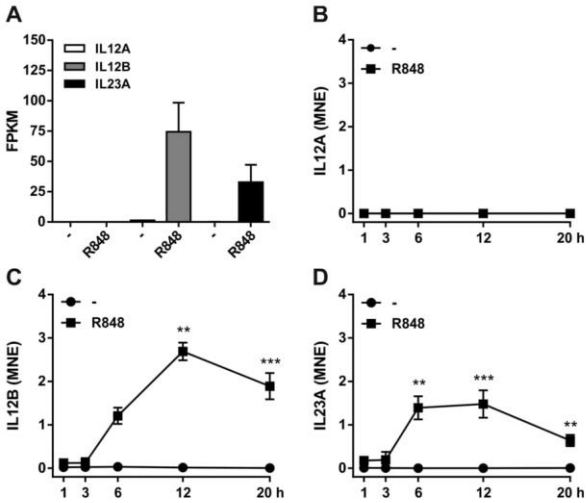

FIGURE 1 **Expression of IL-12A, IL-12B, and IL-23A mRNA in neutrophils incubated with R848.** In (**A**), highly pure neutrophils were incubated for 20 h in the absence (–) or the presence of 5 μM R848. Expression levels of transcripts for IL12A (white bars), IL12B (grey bars), and IL23A (black bars) were measured by RNA-seq ($n = 2$). Similar results were observed from RNA-seq of neutrophils incubated with R848 for 7 h ($n = 2$, not shown). In (**B**), highly pure neutrophils were incubated for up to 20 h in the presence or the absence of 5 μM R848, and then IL12A (B), IL12B (**C**), and IL23A (**D**) mRNA expression was measured by RT-qPCR. Gene expression is depicted as mean normalized expression (MNE) units after normalization to GAPDH mRNA (mean ± SEM, $n = 3$–13). Asterisks indicate significant differences: $^{**}P < 0.01$; $^{***}P < 0.001$, by 2-way ANOVA followed by Bonferroni's post-test

2.12 | Study approval

Human samples were obtained following informed, written consent by healthy donors in accordance with the Declaration of Helsinki. This study was carried out in accordance with the recommendations of Committee on Research Ethics (CORE, University of Liverpool), Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy), and the Regional Committee on Human Experimentation (Florence, Italy).

3 | RESULTS

3.1 | Human neutrophils incubated with R848 and other TLR8 agonists are induced to express IL-12B and IL-23A, but not IL-12A, mRNA

In initial experiments, we incubated highly pure human neutrophils in the absence (control) and the presence of 5 μM R848—in line with our previous studies[11,13]—and then measured changes in global gene expression by RNA-seq at 7 and 20 h. Under these experimental conditions, survival rate of neutrophils was higher than 75 and 20% after, respectively, 20 and 44 h of incubation.[14] Notably, in R848-treated neutrophils, mRNAs for IL-23A and IL-12B, the constituent components of IL-23,[23] were expressed at remarkably high levels at both timepoints (Fig. 1A, and data not shown). By contrast, mRNA levels of IL12A, the chain associating with IL12B to form IL-12,[24] were at, or below, the detection limit and cutoff threshold FPKM values (<0.3) in both control and R848-treated neutrophils (Fig. 1A). We then performed kinetic experiments by RT-qPCR (Fig. 1B), which not only confirmed the RNA-seq data, but also indicated that the optimal incubation time with R848 to induce maximal expression of IL12B and IL23A mRNAs in human neutrophils is 12 h. Consistent with the RNA-seq data (Fig. 1A), IL-12A remained substantially undetectable at all timepoints investigated (Fig. 1B). Interestingly, induction of IL-12B and IL-23A mRNAs by R848 was only detected after 3 h of incubation (Fig. 1B), which, at least in the case of IL12B, is consistent with the necessity to preliminarily activate the synthesis of IκBζ.[25] Incubation of human neutrophils with other TLR8 ligands, such as 1 μM CL075[26] or 2 μM Motolimod/VTX-2337[27] showed similar results (data not shown). Collectively, these data demonstrate that highly pure human neutrophils incubated with R848 express the mRNAs encoding the chains composing IL-23, but not IL-12.

3.2 | The *IL23A* and *IL12B* genomic loci of human neutrophils are characterized by latent enhancers

To identify and characterize, at a whole genome level, changes in the genomic regulatory regions of neutrophils following incubation with R848, we performed ChIP-seq experiments (NT et al., manuscript in preparation) for the genome-wide mapping of both H3K27Ac (marking active *cis*-regulatory elements) and PU.1 (a myeloid lineage determining TF). Based on the results shown in Fig. 1, we analyzed the levels of the H3K27Ac and PU.1 peaks at the *IL12A*, *IL12B*, and *IL23A* genomic loci, to infer precise information of their chromatin status in neutrophils. As shown in Fig. 2A, no PU.1 or H3K27Ac peaks were detectable at the *IL12A* locus, regardless of the neutrophil-treatment with R848. This finding indicates an inactive genomic region, in line with the absence of *IL12A* transcription as shown in Fig. 1A and B, as well as Fig. 2A. By contrast, the levels of H3K27Ac and PU.1 at the genomic regulatory regions of both *IL12B* and *IL23A* were found in a latent state,[28] being very low in untreated neutrophils (Fig. 2B and

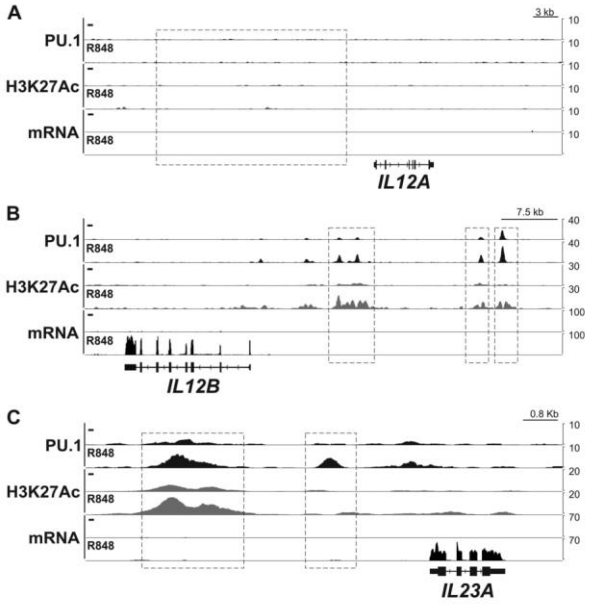

FIGURE 2 ChiP-seq profiles of H3K27Ac and PU.1 at the *IL12A*, *IL12B*, and *IL23A* genomic loci in human neutrophils incubated with or without R848. Genomic snapshots showing PU.1 and H3K27Ac peaks, as well as mRNA expression levels for *IL12A* (**A**), *IL12B* (**B**), and *IL23A* (**C**), in neutrophils incubated for 20 h in the absence (–) or the presence of R848. Changes in the regulatory regions (boxed in **A**–**C**) of the *IL23A* and *IL12B* genes, but not the *IL12A* gene, can be observed in human neutrophils following treatment with R848

C), but dramatically increased following neutrophil incubation with R848. This was especially evident at the genomic regions located at 12, 29, and 31 kb from the *IL12B* TSS (Fig. 2B), and at 2.5 and 5.5 kb from the *IL23A* TSS (Fig. 2C). Altogether, data from these ChIP-seq experiments not only are in line with the results shown in Fig. 1, but also support the notion that, in human neutrophils, chromatin re-modeling regulates IL-12B and IL-23A mRNA induction by R848, similarly to that previously observed for IL-6.[11]

3.3 | Neutrophils incubated with R848 produce IL-23, but not IL-12

We then prepared cell-free supernatants harvested from neutrophils and (as control) autologous CD14$^+$ monocytes to measure the levels of IL-12B, IL-12, and IL-23 by specific ELISA kits (Fig. 3). In these experiments, cells were also incubated with 1 μg/ml ultrapure LPS, to compare its activity to that of R848. Neutrophils incubated for 20 h with R848 were found to produce and release both IL-23 and IL-12B, but not IL-12 (Fig. 3A), with IL-23 and IL-12B levels further increasing after 44 h incubation (Fig. 3A). A similar qualitative pattern of neutrophil-derived cytokines was observed in response to LPS, but at lower levels (Fig. 3A). Measurements of the IL-23A monomer in supernatants from unstimulated or LPS- and R848-stimulated neutrophils for 20 h (1.7 $\pm$ 0.6, 17.4 $\pm$ 6.1, and 23.6 $\pm$ 5.5 pg/ml, respectively) did not substantially differ from that of the IL-23 heterodimer. Additional experiments confirmed that 5 μM R848 represent the optimal concentrations to induce the production of IL-23 and IL-12B by neutrophils (data not shown), and that other TLR8 agonists, including 1 μM CL075[26] or 2 μM VTX-2337,[27] also potently induce the release of these cytokines (data not shown). Not surprisingly,[26,29] CD14$^+$ monocytes incubated with R848 and LPS produced much higher amounts of both IL-23 and IL-12B than neutrophils (Fig. 3B). However, in contrast to neutrophils, activated CD14$^+$ monocytes also produced IL-12, particularly in response to R848 (Fig. 3B), confirming previous observations.[26] Accordingly, we found that monocytes do accumulate IL-12A mRNA upon TLR8 activation (data not shown), consistent with similar observations made by other groups.[30,31] We also observed that CD14$^+$ monocytes, unlike neutrophils (Fig. 3B), express high levels of IRF8, a transcription factor that, by interacting with IRF1 at the *IL12A* genomic locus, is essential for IL-12A transcription.[32] Altogether, these data show that human neutrophils treated with R848 produce IL-23, but not IL-12, protein.

3.4 | Biologic activity of neutrophil-derived supernatants

Because IL-23, along with IL-1β, is involved in promoting the differentiation of Th17 cells,[33] we tested whether supernatants harvested from neutrophils and, for comparison, autologous CD14$^+$ monocytes, could induce UCB-derived "naïve" CD4 T cells to differentiate into Th17 cells.[22] For these experiments, UCB-derived "naïve" CD4 T cells were polyclonally stimulated with anti-CD3 and anti-CD28 mAbs

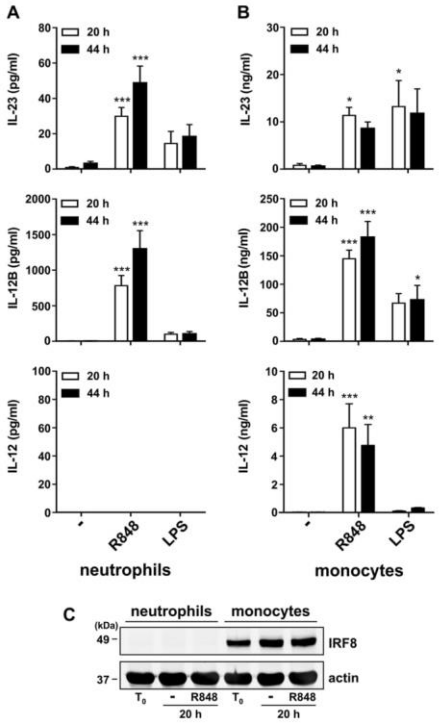

FIGURE 3 **Human neutrophils produce and release IL-23, but not IL-12, in response to either R848 or LPS.** Highly pure neutrophils (**A**) and autologous CD14$^+$ monocytes (**B**) were incubated for 20 and 44 h in the absence (–) or the presence of either 5 μM R848 or 1 μg/ml LPS. Cell-free supernatants were then collected and the levels of IL-23, IL-12B, and IL-12 proteins measured by ELISA ($n = 6$). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, by 2-way ANOVA followed by Bonferroni's post-test. (C) Immunoblot displaying IRF8 and actin protein expression in neutrophils and autologous CD14$^+$ monocytes, either freshly isolated or cultured for up to 20 h with or without 5 μM R848 (representative experiment, $n = 2$)

and cultured in medium in the absence, or the presence, of IL-1β + IL-23 as a positive control (Fig. 4A, top panels), as well as in supernatants derived from either neutrophils or CD14$^+$ monocytes incubated for 24 h with or without R848 (Fig. 4A, bottom panels). Previous measurement of IL-1β in supernatants from R848-treated neutrophils and monocytes revealed concentrations of 11.6 pg/ml and 1.3 ng/ml, respectively. We also found IL-6 levels corresponding to 180 pg/ml and 200 ng/ml in R848-stimulated neutrophils and monocytes, respectively, while TGF-β1 levels were below the threshold limit of the ELISA used. As shown in Fig. 4A, only supernatants from R848-treated neutrophils were capable of inducing the appearance of cord blood-derived Th17 cells (from 0.1 to 0.6%, $n = 4$), as determined by the induction of intracellular IL-17 associated with an increased expression of CD161 (a Th17 marker).[22] By contrast, supernatants from either untreated neutrophils or CD14$^+$monocytes, regardless of their treatment with R848, had no effects on Th17 induction (Fig. 4A). Similar results were observed by using neutrophil-derived supernatants harvested after 48 h of incubation with R848 (data not shown). Finally, the addition of anti-IL-23p19-neutralizing Abs to supernatants from R848-treated neutrophils drastically reduced the frequency of cord blood-derived Th17 cells (Fig. 4B). Altogether, these data indicate that supernatants from R848-treated neutrophils, but not CD14$^+$ monocytes, are able to promote the differentiation of Th17 cells, mainly in an IL-23-dependent fashion.

3.5 | Effect of endogenous and exogenous TNF-α on TLR8-induced IL-23 mRNA and protein expression

Previous work has uncovered a role for endogenous TNF-α in amplifying R848-induced expression of IL-6.[11] Therefore, to verify whether endogenous TNF-α also regulates the production of IL-23, we incubated neutrophils with R848 for 20 h, in the presence or the absence of 10 μg/ml adalimumab, a TNF-α-neutralizing Ab.[34] Figure 5A shows that the expression of IL-23 and IL-12B proteins, induced by R848, is largely dependent on endogenous TNF-α (IL-23 and IL-12B protein decreased by 79 and 70%, respectively in the presence of adalimumab). Figure 5B shows that such adalimumab-mediated inhibitory effect also occurs at the level of mRNA expression for both IL-23A and IL-12B (IL23A and IL12B mRNA on average decreased by 80 and 73%, respectively, by adalimumab). We subsequently incubated neutrophils with or without R848, in the presence or the absence of 10 ng/ml TNF-α, to further investigate the direct ability of TNF-α to trigger the expression/production of IL-12 family members. As shown in Fig. 5C, exogenous TNF-α alone was able to trigger both IL-23 and IL-12B expression, albeit at lower levels than R848. However, the combination of R848 + TNF-α produced a synergistic/additive effect on the production/release of both IL-23 and IL-12B (Fig. 5C), and these effects were mirrored at the mRNA level (Fig. 5D). Nonetheless, in the in vitro model of Th17 differentiation from UCB-derived CD4$^+$ T cells, supernatants from R848 + TNF-α-treated neutrophils did not increase the frequency of IL-17 producing cells compared to supernatants derived from neutrophils treated with R848 only (Fig. 4B). Once again, neither expression of IL12A mRNA nor production of IL-12 was detectable by neutrophils incubated with R848 + TNF-α (Fig. 5C and D). In parallel experiments, we incubated neutrophils with or without R848, in the presence or the absence of 10 ng/ml GM-CSF. However, GM-CSF by itself was unable to trigger the expression/production of IL-12A, IL-12B, and IL-23A, and it also did not potentiate the effects of R848 on the same genes/proteins (data not shown).

To get more insights into the molecular mechanisms underlying the synergistic induction of both IL12B and IL23A mRNA transcription by R848 + TNF-α, we performed H3K27Ac and PU.1 ChIP-qPCR experiments targeting selected regulatory regions of the neutrophil *IL12B* (indicated by the black, white, and grey boxes in Fig. 6A) and *IL23A* (indicated by the black and white boxes in Fig. 6C) loci. These regions were chosen on the basis of the high deposition of PU.1 and H3K27Ac

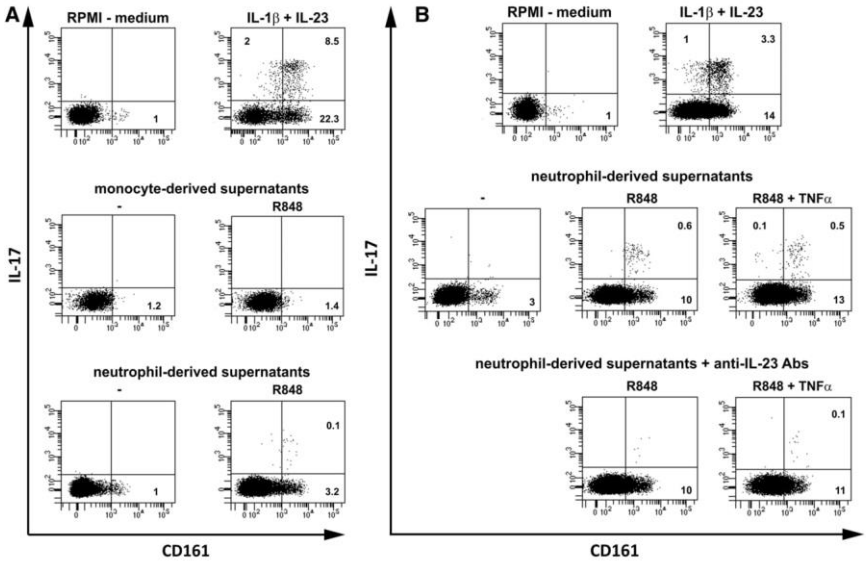

FIGURE 4 **Biological activity of neutrophil-derived supernatants**. Umbilical cord blood (UCB)-derived $CD4^+$ T cells were incubated for 1 week with 5 μg/ml of anti-CD3 + 5 μg/ml anti-CD28 mAbs, (**A**) in the absence or the presence of 10 ng/ml IL-1β + 20 ng/ml IL-23, or the indicated conditioned supernatants derived from neutrophils or $CD14^+$ monocytes incubated with or without R848 for 20 h; (**B**) in the absence or the presence of 10 ng/ml IL-1β + 20 ng/ml IL-23, or the indicated conditioned supernatants derived from neutrophils incubated with or without R848, or R848 + TNF-α for 20 h and used in culture in the presence or absence of 10 μg/ml neutralizing human anti-IL-23p19 Ab. Intracellular IL-17 production and CD161 expression were evaluated by flow cytometry after stimulation with PMA + ionomycin (see Materials and Methods). Representative experiments out of 4 and 2 are shown in panels (A) and (B), respectively

observed in our ChIP-seqs (Fig. 2). As shown in Fig. 6, we found that either TNF-α or (more efficiently) R848 alone triggers an increase of the levels of H3K27Ac and PU.1 at all regulatory regions of both IL-12B (Fig. 6B) and IL-23A (Fig. 6D). Costimulation of neutrophils with R848 + TNF-α further raised the levels of H3K27Ac and PU.1 already induced by either R848 or TNF-α alone, resulting in an additive effect.

4 | DISCUSSION

We have recently discovered that activation of highly pure human neutrophils with TLR8 triggers the production of remarkable quantities of cytokines, including TNF-α, IL-6, G-CSF, and CCL23.[11,13,14] In this study, we demonstrate that, under the same experimental conditions, human neutrophils also express and produce IL-23, one of the members of the IL-12 family that is generated from the association of the IL-23A/IL-23p19 and IL-12B/IL-12p40 subunits. By contrast, human neutrophils were found not to express IL-12/IL-12p70, which is derived from the association of the IL-12A/IL-12p35 and IL-12B/IL-12p40 subunits. We show that TLR8-activated neutrophils are induced to time-dependently express transcripts for IL-12B (encoding IL-12p40) and IL-23A (encoding IL-23p19), but not IL-12A (encoding IL-12p35), as determined by RNA-seq and RT-qPCR experiments. We also show that they produce and release the corresponding proteins, IL-12B, IL-23A, and IL-23, but not IL-12, as determined by ELISA. Incubation of highly pure human neutrophils with LPS (a TLR4 agonist) also induced similar patterns of expression, although the amounts of IL-12B, IL-23A, and IL-23 recovered in neutrophil-derived supernatants were lower than those measured after stimulation with TLR8 agonists. In contrast, autologous $CD14^+$ monocytes incubated with either R848 or LPS were found to produce IL-23A, IL-12B, and IL-23 at much higher levels than neutrophils. However, while in neutrophils, R848 was a more potent trigger of IL-23 production than LPS and in monocytes both agonists had near equal potency. Moreover, $CD14^+$ monocytes could also produce IL-12, but, consistent with the literature,[30,31,35] only if incubated with TLR8 agonists. These differences in cellular responses to different agonists clearly demonstrate that the results obtained with our highly pure neutrophil populations are not due to their potential contamination with $CD14^+$ monocytes. That neutrophils express IL-12B and IL-23A, but not IL-12A, mRNA, in response to either TLR8 or TLR4 activation implying intracellular signaling cascades triggered by the MyD88-dependent pathway.[36] Signaling via this pathway leads in fact to the activation of NF-κB and MAPKs, which ultimately regulate the transcription of IL-12p40, IL-23p19.[37–39] In

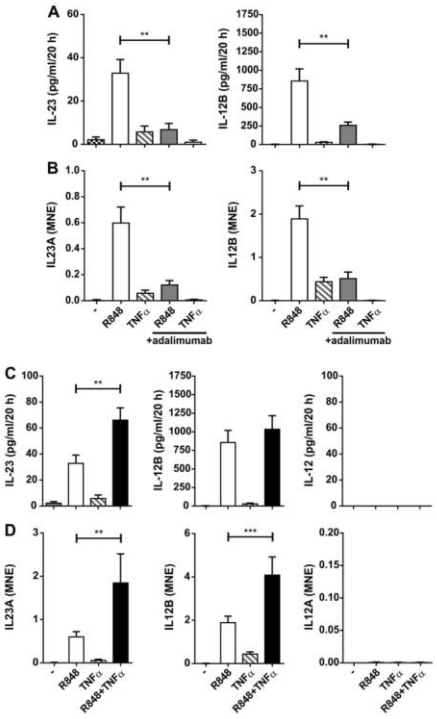

FIGURE 5 **R848-induced production of IL-23 by neutrophils is partially dependent on, and amplified by, TNF-α.** Highly pure neutrophils (**A** and **B**) were pre-incubated for 30 min with 10 μg/ml adalimumab and then cultured for 20 h in the presence of either 5 μM R848 or 10 ng/ml TNF-α. In (**C** and **D**), neutrophils were incubated for 20 h in the absence (–) or the presence of R848, 10 ng/ml TNF-α, or both agonists in combination. The levels of IL-23 (**A** and **C**), IL-12B (**A** and **C**), and IL-12 (**C**) proteins were measured by ELISA ($n = 6$), while (**B**) IL-23A (**B** and **D**), IL-12B (**B** and **D**), and IL-12A (**D**) mRNA expression was determined by RT-qPCR ($n = 6$). Gene expression is depicted as mean normalized expression units after normalization with GAPDH mRNA (mean ± SEM). $^{**}P < 0.01$; $^{***}P < 0.001$, by (**A** and **B**) 1-way ANOVA followed by Tukey's post-test or (**C** and **D**) 2-way ANOVA followed by Bonferroni's post-test

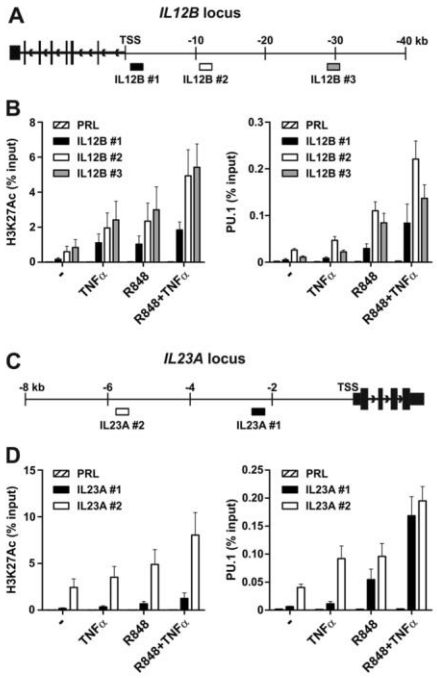

FIGURE 6 H3K27Ac and PU.1 levels at the *IL12B* and *IL23A* genomic loci of neutrophils incubated with R848, alone or in combination with TNF-α. (**A** and **C**) Schemes illustrating the positions of the designed primer pairs amplifying promoter and potential enhancer regions of *IL12B* (indicated by the black, white, and grey boxes) and *IL23A* (indicated by the black and white boxes) for ChIP analysis. Panels (**B**) and (**D**) show the enrichment levels of H3K27Ac (left panels) and PU.1 (right panels) at the *IL12B* (**A**) and *IL23A* (**C**) genomic loci by ChIP analysis in human neutrophils incubated for 20 h with or without 5 μM R848 and/or 10 ng/ml TNF-α. Coimmunoprecipitated DNA samples were expressed as percent of the total input. Panels in (**B**) and (**D**) depict a representative experiment out of 3 independent ones with similar results. Error bars represent SE calculated from triplicate qPCR reactions

contrast, the induction of IL-12A gene expression by TLR activation in myeloid cells is usually dependent on endogenous type I IFN,[40] whose production, at least in the case of TLR4 activation, results from the stimulation of the TRIF-dependent pathway.[41] For reasons that are not yet explained at the molecular level, LPS-treated neutrophils are unable to trigger the TRIF-dependent cascade and consequently do not produce type I IFN.[42,43] Similarly, while monocytes stimulated by TLR8 agonists produce type I IFN via IRF5 activation,[44] it is not known whether this pathway is somewhat defective in human neutrophils. In any case, the lack of type I IFN production would contribute to the explanation as to why human neutrophils do not express IL12A mRNA in response to either TLR8 or TLR4 activation. Another potential explanation for the inability of neutrophils to accumulate IL12A mRNA is that they do not express IRF8, a transcription factor that, in association with IRF1 at the *IL12A* locus, is essential for IL12A transcription in monocytes.[32]

We performed ChIP-seq experiments to characterize the H3K27Ac and PU.1 levels at the *IL12A*, *IL12B*, and *IL23A* genomic loci, in order to identify their genomic regulatory regions.[28] Our experiments revealed that the *IL12B* and *IL23A* loci are in a latent state in unstimulated

neutrophils, and that, upon TLR8 activation, they become not only accessible to lineage determining transcription factors, such as PU.1, but also become marked by histone modifications characteristic of active genomic regions (H3K27Ac). Therefore, as in the case of IL-6 mRNA expression,[11] changes in the chromatin landscape induced by R848 appear to regulate IL12B and IL23A mRNA induction in human neutrophils. By contrast, we found that the *IL12A* locus remained inactive regardless of neutrophil-treatment with R848, and therefore preventing IL12A mRNA transcription, consistent with our RNA-seq and RT-qPCR data. In this respect, the chromatin status of the *IL12A* locus in human neutrophils resembles that of IL-10, which we have previously shown to be in a closed/inactive conformation, preventing its transcription.[45] Moreover, because of the lack of IL12A gene expression, it is plausible to speculate that TLR4/TLR8-activated neutrophils do not even produce IL-35, which is another heterodimeric cytokine of the IL-12 family composed by the association of the IL-12A and EBI3 subunits.

In additional experiments, we incubated neutrophils with GM-CSF, alone, or in combination with R848, and found that GM-CSF neither induced the expression of *IL12A*, *IL12B*, and *IL23A* mRNA nor influenced the effects of R848 on the same genes. In contrast, the use of a TNF-α-neutralizing Ab, adalimumab,[34] demonstrated that the production of IL-23 by TLR8-activated neutrophils is partially dependent on the endogenous expression and release of TNF-α. Adalimumab decreased the production of IL-23 protein by ~70%, and also decreased levels of IL12B and IL23A transcripts. The effect of adalimumab on IL-23 production is reminiscent of that on IL-6, for which endogenous TNF-α was also shown to play an amplifying role.[11,14] However, while TNF-α exogenously added to neutrophils was unable by itself to trigger the production of IL-6,[11] it was able to induce IL-23 expression, albeit at much lower levels than R848. Moreover, when used in combination with R848, exogenous TNF-α resulted in a synergistic effect on the production and release of IL-23 by enhancing the expression of both IL12B and IL23A transcripts. Such a different capacity of exogenous TNF-α to trigger low levels of IL-23, but not IL-6, mRNA expression/production, might be explained by the fact that in resting human neutrophils, the chromatin at the *IL6* genomic locus is inaccessible,[11] while that at the *IL12B* and *IL23A* genomic loci is more opened and thus accessible to low levels of transcription via signal dependent transcription factors, such as NF-κB. Accordingly, we observed that TNF-α is able to activate the regulatory regions at both *IL12B* and *IL23A* genomic loci, by recruiting PU.1 and by favoring the deposition of H3K27Ac. Notably, we also observed that TNF-α further enhanced the effects triggered by R848 at the same regions, explaining the enhanced transcription in the presence of both agonists. On the other hand, the stimulation of neutrophils with TNF-α together with R848 did not induce either the expression of IL12A mRNA or the IL-12 production, indicating that even the combination of these 2 potent stimuli is not sufficient to provoke a chromatin remodeling at the *IL12A* locus.

Since IL-23 represents one of the cytokines responsible for the differentiation of Th17 cells,[33] along with IL-1β, IL-6, and TGF-β1, we tested whether supernatants harvested from R848-treated neutrophils could promote this process. Of note, in our system of Th17 induction,[22] we detected the appearance of low but reproducible levels of UCB-derived Th17 cells from "naïve" $CD4^+$ T cells incubated for 1 week with supernatants from neutrophils cultured with R848, but not in medium from unstimulated cells. However, we did not observe the same effects on Th17 induction using supernatants from $CD14^+$ monocytes cultured with or without R848. The findings that supernatants derived from neutrophils stimulated with R848 + TNF-α did not increase the frequency of Th17 UCB-derived $CD4^+$ T cells compared to the R848-stimulated only supernatants, despite the higher levels of IL-23, might be due to the fact that they contain additional cytokines negatively interfering with Th17 polarization. In fact, the Th17 differentiation obtained after 1 week of in vitro culture derives from many polarizing signals on naïve $CD4^+$ T cells and depends on the balance of different cytokines in the culture medium. Moreover, the experiments performed in the presence of anti-IL-23p19-neutralizing Ab clearly demonstrate that IL-23 present in supernatants from R848- (and R848 + TNF-α)-treated neutrophils is mainly responsible for their Th17-promoting effects, thus emphasizing previous findings on the crosstalk between neutrophils and Th17 cells.[46,47] Accordingly, human neutrophils and Th17 cells have been previously shown to reciprocally chemoattract each other via the production of various chemokines, including CCL20 from neutrophils and CXCL8 from Th17 cells.[48] Moreover, GM-CSF and/or IFN-γ derived from Th17 cells were shown to promote neutrophil survival as well as enhance various neutrophil effector functions.[48] More recently, neutrophil-derived elastase has been shown to process dendritic cell-derived CXCL8 into a truncated, potent Th17 cell-inducing form.[49] Our current data on the ability of TLR8-activated neutrophils to also drive via IL-23 the differentiation of Th17 cells, is consistent with another study showing that Group B *Streptococcus* (GBS)-stimulated neonatal neutrophils induce robust Th1- and Th17-type responses in neonatal $CD4^+$ T cell and Treg populations through mechanisms involving cell-cell contact and soluble mediators,[50] adding new knowledge on the regulation and activation of the neutrophil/Th17 cell crosstalk. It is now necessary to determine if such processes occur in vivo during inflammation or infections.

Our data greatly extend previous observations on the ability of human neutrophils to express/produce IL-23. For example, it was previously shown that, while *Helicobacter pylori* Neutrophil Activating Factor triggers the expression of IL-23p19 mRNA by human neutrophils,[51] *Borrelia burgdorferi* NapA, but not OspA, induces IL-23 at both gene expression and protein level.[52] In another study, both blood neutrophils and neutrophils infiltrating colon tissue of pediatric patients with inflammatory bowel disease were found to express IL-23p19 (as revealed by immunohistochemistry and immunofluorescence),[53] using an Ab detecting only the IL-23p19 monomer, which is secreted by many cell types but is biologically inactive. More recently, human neutrophils (whose purity was not stated) were shown to express IL-23 (presumably IL-23p19) mRNA, as well as to produce very elevated levels of IL-23 protein, when infected with *Mycobacterium tuberculosis* H37Rv or when incubated with either LPS or Pam3CSK4 (a TLR2 agonist).[54] However, in these experiments the infected neutrophils were also reported to express IL-17,[54] which, according to our previous work,[55] does not occur. Finally, immunofluorescence and flow

cytometry analyses revealed that, in patients with castration-resistant prostate cancer (CRPC), tumor-infiltrating myeloid-derived suppressor cells with the neutrophil phenotype ($CD11b^+CD33^+CD15^+$ cells, polymorphonuclear cell-myeloid-derived suppressor cells) express IL-23.[56]

In summary, we show that highly pure neutrophils express IL-23, which is capable of driving the differentiation of Th17 cells. The expression of this key cytokine by human neutrophils requires chromatin remodeling at the normally transcriptionally silent IL-23 gene loci by agents such as TLR8 agonists. These data clearly support the role for neutrophils in the regulation of inflammation via the important IL-17/IL-23 network.

AUTHORSHIP

N.T., M.A.C., S.W.E., R.J.M., L.M., and F.A. were associated with experimental design. N.T., F.A.S., E.G., F.B.A., S.G., F.M., and M.C. were associated with experimental work. N.T., F.A.S., F.B.A., F.M., H.L.W., L.M., and F.A. were associated with data analysis. M.A.C., S.W.E., N.T., and L.M. were associated with manuscript preparation.

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DISCLOSURE

The authors declare no conflicts of interest

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