UNIVERSITÀ DEGLI STUDI DI VERONA

DEPARTMENT OF MEDICINE
SECTION OF GENERAL PATHOLOGY

PhD SCHOOL OF LIFE AND HEALTH SCIENCES

DOCTORAL PROGRAM IN
INFLAMMATION, IMMUNITY AND CANCER

CYCLE XXX/2014

MOLECULAR MECHANISMS REGULATING CYTOKINE PRODUCTION BY HUMAN NEUTROPHILS

S.S.D. MED/04

Coordinator: Prof. GABRIELA CONSTANTIN

Tutors: Prof. MARCO A. CASSATELLA
Dr. NICOLA TAMASSIA

PhD Student: FABIO ARRUDA E SILVA

ACCADEMIC YEARS 2014-2017

Sommario

I neutrofili, i leucociti più abbondanti nel sangue umano, sono noti per svolgere funzioni effettrici cruciali per la risposta immunitaria innata e adattativa contro le infezioni. Inoltre, i neutrofili sono in grado di rispondere a diverse componenti di origine microbica inducendo la sintesi e la secrezione di svariate citochine. In questo contesto, l'obiettivo principale di questo studio è stato quello di verificare la capacità dei neutrofili umani di esprimere e produrre citochine della famiglia di IL-17, tra cui IL-17A, IL-1B, IL-17F e IL-17AF, dato che attualmente la letteratura è in disaccordo su questo argomento. Attraverso metodiche quali RT-qPCR, immunoistochimica (IHC), immunoblotting, misurazione di proteine mediante ELISA, immunoprecipitazione della cromatina (ChIP) e ChIP-seq, abbiamo valutato la regolazione epigenetica e trascrizionale, così come la produzione di citochine utilizzando popolazioni di neutrofili isolati ad un elevato grado di purezza (> 99,7%). In accordo con alcuni dati precedentemente pubblicati sia dal nostro che da altri gruppi, abbiamo osservato che i neutrofili incubati con stimoli di diversa natura non esprimono ne producono, a livello di mRNA e proteina, nessuna citochina della famiglia di IL-17, comprese IL-17A, IL-17F, IL-17B o IL-17AF. Risultati analoghi sono stati ottenuti anche utilizzando neutrofili isolati da pazienti con psoriasi attiva. Inoltre, contrariamente a quanto osservato in studi pubblicati recentemente, anche in neutrofili incubati con concentrazioni molto elevate di IL-6 e IL-23, in combinazione con ife o conidi inattivati ottenuti da *Aspergillus fumigatus,* non è stata misurata nessuna espressione di mRNA o sintesi di proteina per IL-17A o IL-17F. In accordo con questi dati, sul locus genomico di IL-17A/F, in neutrofili stimolati o meno con IL-6 più IL-23, non è stata rilevata alcuna presenza di H3K27Ac e H3K4me1, due modificazioni istoniche post-traduzionali che contrassegnano elementi regolatori genomici attivi o "*poised*". Tale risultato è quindi coerente con l'incapacità dei neutrofili umani di esprimere l'mRNA di IL-17A o IL-17F. Un altro dato importante emerso in questo studio è stato la conferma che in immunoistochimica, anticorpi anti-IL-17A e IL-17B utilizzati in svariati lavori, danno un segnale positivo in citocentrifugati di neutrofili stimolati o meno con IL-6 più IL-23. In immunoblotting però gli stessi anticorpi non riconoscono proteine intracellulari del peso molecolare corretto per IL-17A e IL-17B ma altre proteine con peso molecolare molto più alto. Si può concludere quindi che il

segnale positivo dato da questi anticorpi in IHC è frutto di una reazione aspecifica ed è indipendente dalla presenza di IL-17A o IL-17B. In conclusione, i risultati esposti in questo studio non solo confermano e ampliano nostre osservazioni precedenti a riguardo dell'incapacità dei neutrofili umani di produrre IL-17A, IL-17B e IL-17F, ma forniscono anche una spiegazione del motivo per cui in altre pubblicazioni queste citochine sono rilevate nei neutrofili.

Abstract

Neutrophils are known to perform a series of effector functions that are crucial for the innate and adaptive responses towards infections. Furthermore, neutrophils respond to various stimuli, including microbial components, by synthetizing and secreting a variety of cytokines. In this context, the main objective of this study was to re-evaluate the capacity of human neutrophils to express and produce cytokines of the IL-17 family, including IL-17A, IL-17B, IL-17F and IL-17AF since the current literature on this topic is discordant. By performing RT-qPCR, immunohistochemistry (IHC), immunoblotting, protein measurement via commercial ELISA, chromatin immunoprecipitation (ChIP) and ChIP-seq, we evaluated transcriptional and epigenetic regulation, as well as production of the latter cytokines by highly pure (> 99.7 %) populations of human neutrophils. In agreement with some published data, we found that neutrophils do not express/produce IL-17A, IL-17F, IL-17AF or IL-17B mRNA/protein upon incubation with a variety of agonists. Similar findings were observed by analyzing neutrophils obtained from active psoriatic patients. No IL-17A and IL-17F mRNA expression/production was found even when human neutrophils from healthy donors were incubated with IL-6 plus IL-23 at very elevated concentrations in combination with inactivated hyphae or conidia from *Aspergillus fumigatus*, unlike shown in multiple studies. Moreover, consistent with the inability of human neutrophils to express IL-17A and IL-17F mRNA, no deposition of H3K27Ac and H3K4me1, which are histone marks of, respectively, active and poised genomic regulatory elements, was detected at the IL-17A/F genomic locus in resting or IL-6 plus IL-23-stimulated neutrophils. In addition, although we found that anti-IL-17A and anti-IL-17B commercial antibodies positively stained cytospin preparations of resting and activated neutrophils by IHC, these antibodies do not recognize any intracellular protein having the correct MW of either IL-17A or IL-17B in corresponding lysates of the same neutrophil preparations by immunoblotting. Since the same antibodies were found to strongly stain other intracellular proteins of neutrophils, we conclude that their ability to positively stain neutrophils derives from IL-17A- or IL-17B-independent unspecific binding. In conclusion, our data not only confirm and further support our previous original findings on the inability of human

neutrophils to express/produce IL-17A, IL-17B and IL-17F mRNAs/proteins, but also attempt to explain why other published studies continue to report the opposite.

Table of contents

ABBREVATIONS

ANCA	anti-neutrophil cytoplasm antibody-related
APC	allophycocyanin
BATF	basic leucine zipper ATF-like transcription factor
BLIMP	PR domain zin finger protein 1
C/EBP	CCAAT/enhancer binding protein
CCL	chemokine CC motif ligand
CCR	CC chemokine receptor
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP followed by high troughput sequencing
CLEC	C-type lectin domain family
CMP	common myeloid progenitor
COPD	chronic obstructive pulmonary disease
CpG	5'- cytosineguanine-3' dinucleotides
CXCL	chemokine CXC motif ligand
CXCR	CXC-chemokine receptor
DNA	Deoxyribonucleic acid
ECAM	endothelial cell adhesion molecule
EDTA	ethylenediamine tetraacetic acid
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
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA-DR	major histocompatibility complex class II
HOMER	Hypergeometric Optimization of Motif EnRichment
HT	high-throughput
ICAM	Intercellular Adhesion Molecule
IF	immunofluorescence
IHC	immunohistochemistry
iNKT	invariant natural killer T cells
IL-17	Interleukin-17

IL-17R	Interleukin-17 receptor
ILC	innate lymphoid cell
IFN	Interferon
IGV	Integrative Genome Viewer
IP	Immunoprecipitation
IRF	interferon regulatory factor
ISG	interferon stimulated gene
JAM	junctional adhesion molecule
kbp	kilo base pairs
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAL	MyD88-adaptor-like
MAPK	mitogen-activated protein kinase
MDA5	Melanoma Differentiation-Associated protein 5
MDL	myeloid DAP12-associating lectin
MMP	matrix metalloproteinase
MNE	mean normalized expression
MPO	myeloperoxidase
mRNA	messenger RNA
MTB	*Mycobacterium tuberculosis*
mTOR	mammalian target of rapamycin
MW	molecular weight
MyD88	myeloid differentiation factor 88
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NETs	neutrophil extracellular traps
NKT	natural killer T cells
NLR	nucleotide-binding oligomerization domain (NOD)-like receptor
NLRC	NLR with a CARD domain
NLRP	NLR with a pyrin domain
nt	nucleotide
PAMPs	pathogen-associated molecular patterns
PE	phycoerythrin
PECAM	platelet endothelial cell adhesion molecule
PerCP	peridinin chlorophyll protein
PCR	Polymerase Chain Reaction

PMA	phorbol myristate acetate
PMN	polymorphonuclear neutrophils
PRR	pattern recognition receptors
PSA	psoriatic arthritis
PSGL-1	P-selectin glycoprotein ligand 1
RA	rheumatoid arthritis
RIG-I	Retinoic acid-inducible gene I
RLH	retinoic acid-inducible gene (RIG)-like helicase
RNAi	RNA interference
ROI	reactive oxygen intermediates
RORγt	RAR-related orphan receptor gamma
ROS	reactive oxygen species
RT-qPCR	reverse transcription quantitative PCR
SAA	serum amyloid A
SLE	systemic lupus erythematosus
SP1	specific protein 1
STAT	signal transducer and activator of transcription
TF	Transcription factor
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing interferon β protein
TSS	transcription start site
TREM	triggering receptor expressed on myeloid cells
VCAM	vascular cell adhesion molecule

LIST OF FIGURES AND TABLES

1. INTRODUCTION

1.1 POLYMORPHONUCLEAR NEUTROPHILS

Among leukocytes, the polymorphonuclear neutrophils are the most numerous cells present in human bloodstream. These cells are crucial players in innate immune response. Nonetheless, during the last years, research has demonstrated that neutrophils also act as important regulators of adaptive immunity. Neutrophils are indispensable for defence against pathogens, since they are the first cells to reach the inflammatory sites where they rapidly exert their effector functions. In an injury or microbial infection site, neutrophils are able to effectively counter the cause of the injury by, for example, releasing enzymes, synthetizing reactive oxygen species and producing inflammatory mediators. Neutrophils display a wide range of actions and are believed to participate in protection against intracellular pathogens such as viruses and mycobacteria, to interact with the adaptive immune system and to be involved in the resolution of inflammation. However, neutrophils have a dark side in that if they are improperly activated they lead to tissue damage, thus contributing to the development of autoimmune diseases such as psoriasis, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and anti-neutrophil cytoplasmic antibody-related (ANCA) vasculitis, or exaggerated inflammatory reactions such as inflammatory bowel diseases [1].

Prior to reaching the blood circulation, neutrophils pass through a differentiation process in the bone marrow called myelopoiesis, arising from the pluripotent stem cells in a generation rate of 1 to 2×10^{11} mature neutrophils/day [2]. Once these cells are released into the bloodstream, they do not stay there for long, due to their very short lifespan, that usually ranges between 6-20 hours under healthy conditions. However, neutrophil life-span is increased when they migrate into the infection site and enter in contact with cytokines such as tumour necrosis factor alpha (TNFα), type I and II interferons (IFN), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage stimulating factor (GM-CSF) [3, 4]. Furthermore, bacterial products such as LPS and viral ssRNA have also been shown to prolong neutrophil survival [3, 5]. In the absence of infection or inflammation, neutrophils

undergo through the apoptosis process and are in turn, removed by macrophages. Apoptosis is an important homeostatic mechanism, as its alteration in neutrophils would have serious consequences for the inflammatory response and resolution of inflammation. Furthermore, the regulation of neutrophil turnover represents a critical checkpoint because neutrophils constitute the majority of leukocytes in humans, predominate at the infection sites and contain cytotoxic molecules that can damage host tissues.

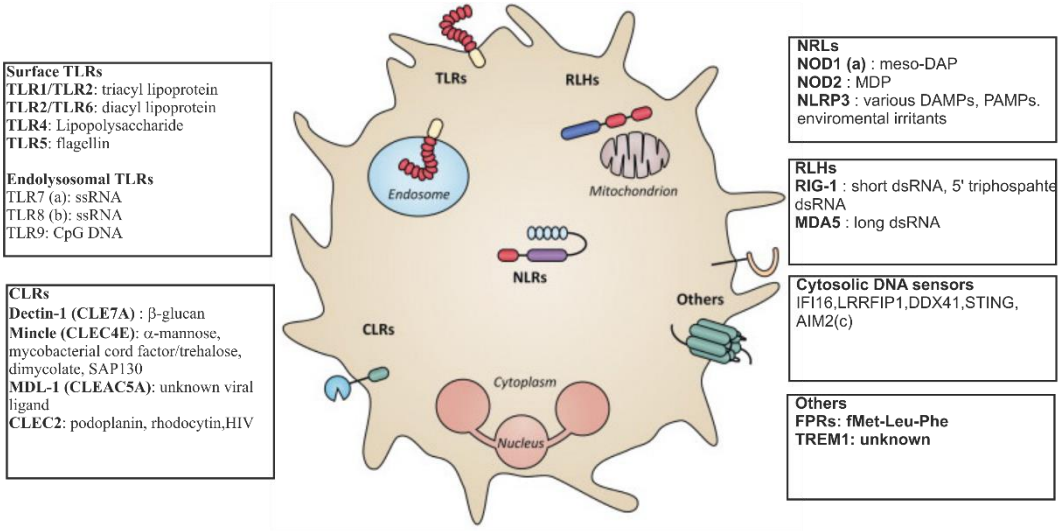
1.1.1 Effector functions of neutrophils

While circulating in the vasculature, neutrophils can rapidly migrate into the infection sites in response to chemokines such as CXCL8, which is the most potent neutrophil chemoattractant. CXCL8 is produced in response to pro-inflammatory molecules by macrophages, epithelial cells, mast cells, endothelial cells, keratinocytes, fibroblasts and neutrophils themselves [6, 7]. Moreover, products from bacteria such as fMLF and peptidoglycan can also contribute to the recruitment of neutrophils [6]. The process of neutrophil migration starts with a slow roll on the surface of the endothelial cells mediated by constitutive expression of adhesion molecules of the selectin family, which establish a low-affinity binding between L-selectin expressed by neutrophils to P- and E-selectins present on the plasma membrane of activated endothelial cells[6]. A high-affinity interaction can be then established, in the presence of chemotactic factors, between β2-integrins present on the neutrophils surface and endothelial cell intracellular adhesion molecule (ICAM)-1 and ICAM-2 on the endothelial cells. Neutrophils start to crawl on the vasculature surface and ultimately transmigrate through the endothelium into tissues without damaging these structures, following a gradient that is believed to be set up by chemotactic factors [8, 9]. Transmigration is facilitated by several surface molecules, including integrins and CAMs (ICAM1, ICAM2 and vascular cell adhesion protein 1 (VCAM1)), junctional proteins such as platelet/endothelial cell adhesion molecule 1 (PECAM1; also known as CD31), CD99, junctional adhesion molecules (JAMs) and epithelial cell adhesion molecule (ECAM) [8]. Once in the interstitial tissue, neutrophils follow chemotactic gradients in order to move in an oriented manner, locate and kill microorganisms using

their antimicrobial arsenal, including reactive oxygen species (ROS) and release of peptides, proteins and enzymes contained in granules and phagocytosis. Phagocytosis is the process whereby neutrophils bind and ingest invading microorganisms. Neutrophils recognize microbes via their pattern recognition receptors (PRRs) that bind specific structures called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA) and flagellin. These PAMPs generally trigger signal transduction pathways leading to the expression of adhesion molecules, promotion of phagocytosis, release of cytokines, chemokines, ROS and degranulation. Moreover, neutrophil phagocytosis can be efficiently enhanced by antibodies and complement products that opsonize microbes. Phagocytosis leads to the formation of a phagosome that, in turn, fuses with azurophilic granules (peroxidase-positive granules) creating a vacuole lumen with antimicrobial molecules including α-defensins, cathepsins, proteinase-3, elastase, azurodicin and lysozyme.

1.1.2 Pattern recognition receptors by neutrophils

One of the most important discovery regarding the innate immunity has been the identification of pattern recognition receptors, whose importance was firstly highlighted by Charles Janeway in 1989 [10]. Today, PRRs include members of Toll-like receptor (TLR) family, the C-type lectin receptors dectin 1 (also known as CLEC7A), and CLEC2 (also known as CLEC1B), as well as cytoplasmic RNA sensors (RIG-I and MDA5), cytoplasmic DNA sensors and cytoplasmic NACHT-leucine-rich repeat receptors (NLRs), some of them being components of inflammasomes [11, 12]. Among them, neutrophils express a broad range of PRRs, as illustrated in **Figure 1.**



Adapted from Thomas CJ and Schroder K. 2013. Trends Immunol. 34:317 [13].
Based on the data from Tamassia N and Cassatella MA. 2013. Current Opinion in Pharmacology. 13:547 [12].

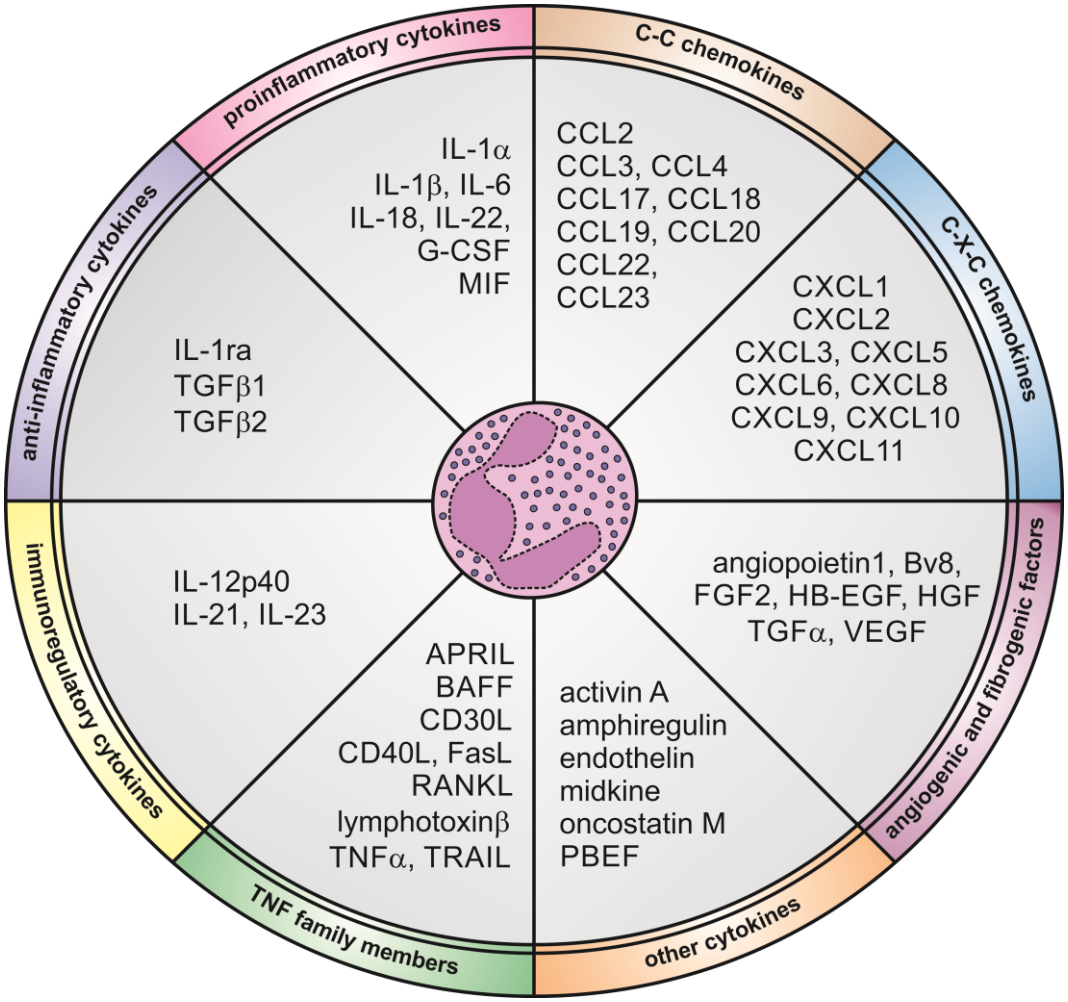
Figure 1 | Pattern recognition receptors expressed by neutrophils
Abbreviations: CLEC, C-type lectin domain family; CLR, C-type lectin receptor; DAMP, danger-associated molecular pattern; meso-DAP, meso diaminopimelic acid; FPR, formyl peptide receptor; MDL, myeloid DAPs12-associating lectin; NLR, nucleotide-binding oligomerization domain (NOD)-like receptor; NLRC, NLR with a CARD domain; NLRP, NLR with a pyrin domain; PAMP, pathogen-associated molecular pattern; RLH, retinoic acid-inducible gene (RIG)-like helicase; SAP, spliceosome-associated protein; TLR, Toll- like receptor; TREM, triggering receptor expressed on myeloid cells. (a) Described in mouse but not human neutrophils; (b) Described in human but not mouse neutrophils; (c) Described in mouse neutrophils, no data available in humans.

TLRs, which to date comprises 10 members in humans (TLR1-10), were the first PRRs to be discovered and are the most studied PRRs in neutrophils. Almost all TLRs are expressed and functional in human neutrophils, with the exception of TLR3 and TLR7. TLR4, which recognizes LPS, has special features in its activation pathway. In fact, while immune cells such as monocytes, macrophages and dendritic cells activate two signalling pathways downstream of TLR4, respectively coordinated by signal adaptors myeloid differentiation factor 88 (MyD88)/MyD88 adaptor-like (Mal) and TIR domain-containing adaptor protein inducing interferon β (TRIF)/TRIF-related adaptor molecule (TRAM), neutrophils mobilizes only the MyD88-dependent pathway [14]. Such a cell-specific activation by LPS influences the expression profile, strength and kinetics of cytokine production by neutrophils.

1.1.3 Cytokine expression by neutrophils

Neutrophils have been shown to express and produce a large number of pro- and anti-inflammatory cytokines, chemokines, colony-stimulating and angiogenic factors, either constitutively or following appropriate stimulation [11] (**Figure 2**). The production of cytokines by neutrophils is controlled by regulatory mechanisms occurring at the level of mRNA transcription [15], stability or translation, as well as at the level of protein secretion. Moreover, cytokines are eventually stored in intracellular pools following synthesis, ready to be promptly secreted when neutrophils are stimulated with secretagogue-like molecules [16].

It is important to mention two important issues for those people who want to study neutrophil-derived cytokines: 1) neutrophils possess 10/20 times less RNA than other leukocytes and consequently; 2) with few exceptions, they usually produce much lesser cytokine amounts than monocytes/macrophages or lymphocytes on a *per cell* basis. Hence, highly pure populations of neutrophils should be used for the evaluation of their cytokine gene expression/production profiles. Accordingly, in a study conducted in our lab, different published procedures to isolate neutrophils directly from human blood were found to generate percentages of neutrophil purity very diverse, in some cases causing artefacts [17]. By the way, the fact that a single neutrophil, *per se*, produce little amounts of cytokine does not mean that neutrophil-derived cytokines are not important, as neutrophils usually outnumber mononuclear leukocytes in infection/inflammatory sites by one to two orders of magnitude [15, 18, 19]. Neutrophil-derived cytokines can be measured in cell-free supernatants or cell lysates by using a variety of methods, including enzyme-linked immunoadsorbent assays (ELISA), immunoprecipitation, immunohistochemistry, intracellular staining by flow cytometry or confocal microscopy. The latter two techniques should be not only carefully interpreted, but also used only to support other cytokine detection methods, since elevated neutrophil autofluorescence, antibody cross-reactivity or aspecific binding of antibody to Fcγ receptors (especially CD16), could generate artefacts.

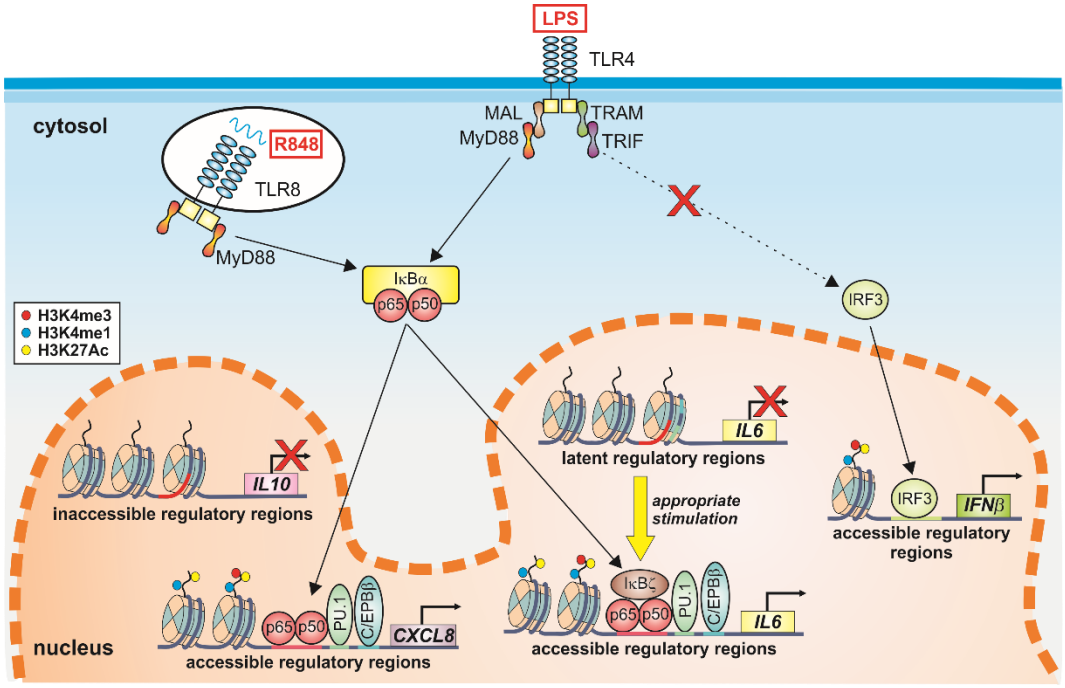


Adapted from: Tamassia, N., et al. (2018). *"Cytokine production by human neutrophils: Revisiting the "dark side of the moon"*. Eur J Clin Invest, 2018 [20].

Figure 2 | Cytokines that human neutrophils can potentially express and produce. 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.

Interestingly, activation of the same PRR in autologous neutrophils and monocytes may trigger distinct gene as well as cytokine expression programs [14, 21, 22], reflecting cell type-specific mechanisms of transcriptional regulation. For instance, upon stimulation with LPS, IFNβ, an antiviral and immunomodulatory cytokine [23], is induced in human monocytes, but not in neutrophils. As explained on page 18, the activation of TLR4 by LPS in neutrophils fails to trigger the TRIF-signalling pathway, that is indeed crucial for the activation of the transcription factor interferon regulatory factor 3 (IRF3) (**Figure 3**). On the contrary, the activation of TLR4 by LPS in

monocytes leads to the triggering of both the MyD88-signalling pathway (and, in turn, NF-κB) and the TRIF-signalling pathway (mainly activating IRF3). Activation of both NF-κB and IRF3 is essential for the activation of IFNβ transcription [24, 25] which, in an autocrine manner, stimulates the expression of a large set of interferon-stimulated genes (ISG) displaying antiviral and immunomodulatory functions[23].



Adapted from: Ostuni, R., et al. *Epigenetic regulation of neutrophil development and function.* Semin Immunol 28(2): 83-93, 2016 [26].

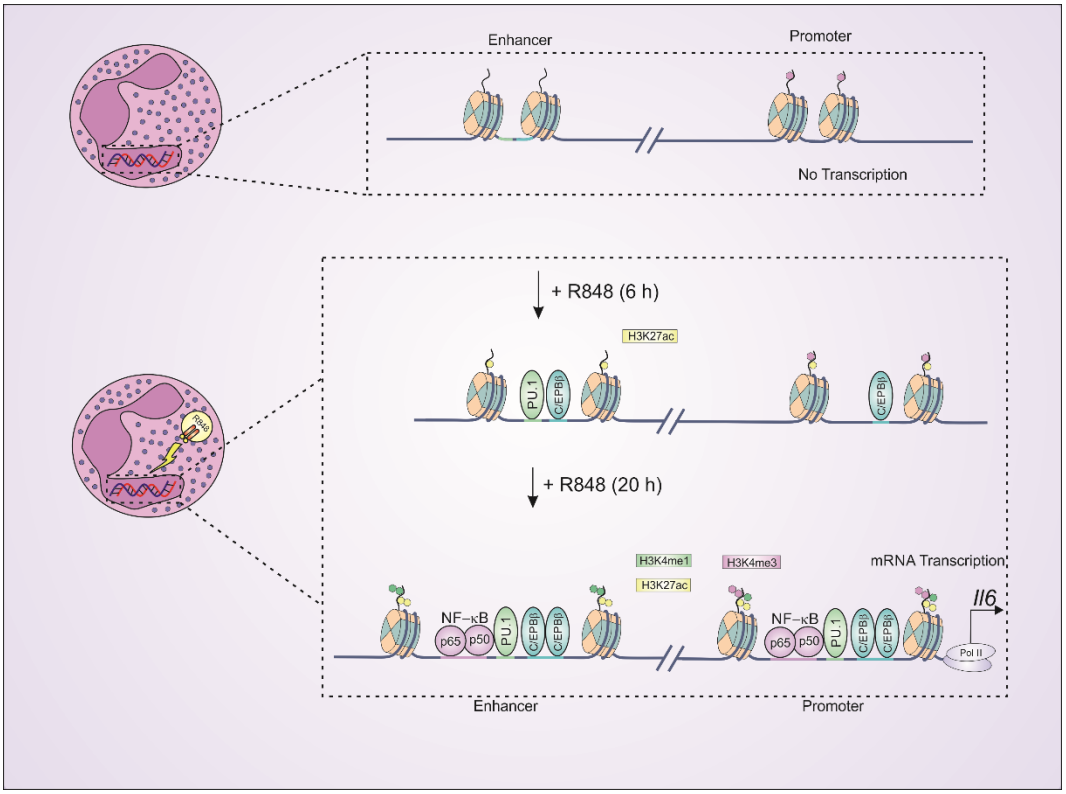
Figure 3 | Examples of epigenetic mechanisms controlling gene expression in human neutrophils.
The cartoon illustrates that the *IL10* locus of neutrophils display inaccessible regulatory regions, as evidenced by the absence of histone marks associated with active transcription. Such a chromatin conformation prevents the binding of transcription factors (TFs) activated by TLR ligands or other PAMPs, therefore preventing IL-10 mRNA transcription. By contrast, the CXCL8 locus has an accessible conformation that is ensured by the constitutive binding of both PU.1 and C/EBPβ. Upon neutrophil activation, NF-κB is recruited to its corresponding binding sites and thus promotes transcription of CXCL8 mRNA. Figure also shows that the *IL6* locus of neutrophil is not accessible under basal state. However, upon appropriate stimulation, pioneer TFs such as PU.1 and C/EBPβ initiate chromatin opening, in turn favoring the binding of activated TFs and ultimately activating IL-6 mRNA transcription. Finally, even if the IFNβ genomic locus is not in a closed conformation, no IFNβ mRNA transcription occurs in TLR-stimulated neutrophils because of the inability to activate IRF3 by TLR-dependent signals.

Beyond discrete transcription factor (TF) activation, chromatin organization may represent another critical factor at the basis of the transcriptional differences observed in neutrophils and monocytes. Indeed, pro-inflammatory TFs activated by external stimuli such as TLR ligands largely act within a pre-established chromatin landscape [27]. On the other hand, recent studies in neutrophils and monocytes have shown that chromatin-dependent mechanisms control their expression of interleukin-10 (IL-10), a potent anti-inflammatory cytokine that prevents inflammatory and autoimmune diseases[28]. Specifically, a comparative analysis at the *IL10* genomic *locus* of histone modifications associated with permissive chromatin, precisely H3K4me3 (localized at the transcription start site of active genes), H3K27Ac and H4Ac (both associated with activated regulatory elements), and H3K4me1 (a histone mark mainly associated with active and poised enhancers), revealed that these histone modifications are detectable in autologous monocytes, but not in autologous neutrophils, already at the steady-state. Furthermore, H3K4me3 and H3K27Ac marks were shown to further increase in monocytes stimulated with TLR2 and TLR4 ligands (Pam3CYS4 and LPS respectively) or serum amyloid A (SAA), while remaining undetectable in neutrophils [21, 29]. In accordance with what observed for histone modifications, chromatin immunoprecipitation (ChIP) of a number of transcription factors, 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 neutrophils, either constitutively or upon activation with LPS. These data, generated using highly purified human neutrophils isolated by antibody conjugated magnetic beads, provided a mechanism explaining why human neutrophils are unable to produce IL-10 [31-34]. Such an issue has been in fact controversial for many years, as a number of studies were reporting a production of IL-10 by human neutrophils, under resting or stimulatory conditions [35-39]. In our opinion, controversial reports are explained by the scarce purity of neutrophil preparations used to evaluate the production of IL-10, therefore avoiding the essential exclusion of contaminant cells, such as lymphocytes or monocytes, which can strongly affect the final results [17, 21]. Confirming this hypothesis, studies conducted by our laboratory with a preparation of highly purified neutrophils (> 99.7 ± 0.2 %) have never detected mRNA expression/production of IL-10 in response to plenty of inflammation-associated stimuli, such as PRR agonists (LPS, R848, Pam3CSK4, poly(I:C), curdlan), SAA,

cytokines (IFNγ, TNFα, GM-CSF or G-CSF), chemoattractants (fMLF) or insoluble immunocomplexes [21]. By contrast, autologous monocytes promptly produce detectable amounts of IL-10 when stimulated with SAA, TLR ligands or curdlan [21]. Although it cannot be excluded that specific stimulatory conditions may revert the non-permissive state of the chromatin in their *IL10* locus, human neutrophils are unable to express IL-10 upon stimulation with TLR ligands or SAA. The bottom line is that caution must be taken with studies that report IL-10 expression by human neutrophils stimulated with TLR-ligands, especially when contamination with other leukocytes is not excluded. The latter precaution is, anyway, mandatory when cytokine production and/or gene expression by neutrophils need to be investigated [18, 40].

Not only the expression of IL-10 in human neutrophils has been a target of debate for a long time, but also of other cytokines such as IL-6 and IL-17. IL-6 is a pleiotropic cytokine displaying both pro- and anti-inflammatory activities, playing a crucial role in host defence against pathogens and acute stress [41]. However, uncontrolled IL-6 regulation can contribute to the pathogenesis of inflammatory and autoimmune diseases [41], as well as cancer [42]. Conflicting results have been published on the ability of neutrophils to accumulate IL-6 mRNA, either constitutively or after stimulation [43, 44]. In addition, several studies have reported a remarkable production of IL-6 by neutrophils stimulated with LPS, GM-CSF or TNFα [43], while other studies did not reproduce the same data [45, 46]. It is important to note that different culture conditions, time points of analysis and methods to detect IL-6 production were used in these various studies. Still, these controversial publications rarely met the employment of neutrophil preparations free of contaminant monocytes, which are major producers of IL-6 [45]. As illustrated in **Figure 4**, the regulation of expression/production of IL-6 by neutrophils was recently found to be regulated at chromatin reorganization level. Specifically, data have shown the absence of H3K4me3, H3K27Ac, H4Ac and H3K4me1 histone marks in highly purified resting neutrophils [47]. However, incubation of neutrophils with either R848, a synthetic mimic viral ssRNA recognized by TLR8, or very high concentrations (10 μg/ml) of LPS increased the presence of these histone marks at the *IL6* locus, in accordance with the ability of TLR ligands to promote chromatin reorganization and *de novo* formation of latent enhancers [48-50]. Interestingly, histone modifications were prominently detected after an overnight incubation, while PU.1 and C/EBPβ were recruited at the

297 *IL6* locus as early as 6 h after R848 stimulation, suggesting a "pioneer" activity of these
298 TF also in human neutrophils [51, 52]. On the other hand, in autologous monocytes
299 the *IL6* locus was found to be in a "poised" conformation, which is a chromatin status
300 given by the presence of H3K4me1, PU.1 and C/EBPβ already under resting
301 conditions. Such a chromatin organization influences the kinetics of IL-6 transcription,
302 which in monocytes is much more accelerated than in neutrophils [47]. The different
303 kinetics of IL-6 gene expression observed between neutrophils and monocytes is
304 controlled also by other factors, including: 1) expression of IκBζ, a transcriptional
305 coactivator that is required for the IL-6 transcription [53], which in monocytes is more
306 rapidly induced by TLR stimulation than in neutrophils; 2) endogenous production of
307 IL-10, which occurs only in monocytes [21] and that turns off IL-6 transcription at
308 delayed time-points[54]; 3) induction of miR187 by endogenous IL-10, occurring only
309 in monocytes, which regulate IκBζ expression[55]; and 4) the presence of distinct cell-
310 specific enhancers, located at -14 kb and at -49 and -64 kb from the IL-6 TSS in
311 neutrophils and monocytes, respectively [47].
312 The importance of epigenetic in regulating cytokine gene expression in human
313 neutrophils is exemplified by the fact that R848 induces an increase of the chromatin
314 accessibility at their *IL6* locus, and by doing so confers responsiveness to TNFα, which
315 by itself is not able to trigger IL-6 transcription in resting neutrophils [47]. When the
316 IL6 locus is accessible, as in TLR8-activated neutrophils, TNFα induces the
317 recruitment of C/EBPβ and histone acetylation, in turn promoting IL-6 transcription
318 [47]. Indeed, in the presence of adalimumab and etanercept [56], two TNFα inhibitors,
319 the mRNA expression and production of IL-6 by TLR8-activated neutrophils
320 dramatically decreases, indicating that endogenous TNFα plays a crucial role in the
321 amplification of IL-6 expression [47]. Furthermore, an important role of endogenous
322 TNFα in amplifying neutrophil-derived IL-6 was recently confirmed by studies using
323 TLR8-stimulated neutrophils in the presence of IFNα, hence mimicking a potential
324 situation occurring in systemic lupus erythematosus (SLE) patients [57]. These findings
325 indicate that TLR8 ligands, IFNα and TNFα, three players often coexisting in many
326 diseases of viral or autoimmune origin, promote a strong production of IL-6 by human
327 neutrophils, placing this cell type among potential targets for immunotherapeutic
328 interventions.
329



Adapted from: Zimmermann, M., et al., *Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils.* Nat Commun, 6: p. 6061, 2015 [47].

Figure 4 | R848 induces a reorganization of the chromatin at the *IL-6* 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 *IL-6* locus of neutrophils. *IL-6* locus in neutrophils is inactive based on the absence of histone modifications and TF binding. Upon neutrophils treatment with R848, recruitment of PU.1, C/EBPβ, NF-κB and deposition of significant levels of histone marks (H3K4me1, H3H4me3 and H3K27Ac) occur at various *IL-6* locus positions, suggesting an induction of latent regulatory sites (such as enhancers) leading to IL-6 gene transcription.

1.1.4 Chemokine expression by neutrophils

Among the cytokines produced by neutrophils, chemokines represent interesting ones given their ability to recruit selective leukocytes into the site of inflammation. In this manner, chemokines regulate leukocyte trafficking and immune system responses, other than influencing B and T cell development and modulating angiogenesis. As displayed in **Figure 2**, human neutrophils are a source of many chemokines, including CXCL1, CXCL8, CXCL10, CCL2, CCL3, CCL4 [58] and, as more recently described, CCL23 [59]. Interestingly, some of the chemokines released by neutrophils potently amplify their own recruitment [60]. Furthermore, by releasing

chemokines, neutrophils orchestrate sequential recruitment and/or activation of distinct leukocyte types into the inflamed tissue, such as monocytes, dendritic cells (DCs), natural killer (NK) cells, and T-helper type 1 (Th1) and type 17 (Th17) cells [58, 61].

Neutrophils express many chemokine receptors as well, including CXCR2, CXCR4 and CCR1, and are the first cell types targeted by chemokines during inflammation. CXCR4 expression, for example, progressively decreases during the maturation of neutrophils in the bone marrow, allowing the release of mature neutrophils into the bloodstream [62]. In this context, a decrease of CXCL12 (the ligand of CXCR4) and CXCR4 expression by neutrophils can be promoted by G-CSF, that in this manner further decreases the number of neutrophils that are retained in the bone marrow. On the contrary, neutrophils increase their CXCR4 expression with senescence, which favours their return to the bone marrow where they undergo apoptosis [63]. As already explained on page 16, circulating neutrophils promptly respond to activated endothelium by binding selectin and integrin ligands, thus rolling and spreading along the endothelium to finally, transmigrate in response to chemokines, including CXCL1, CXCL2 and CXCL8. Some of these chemokines are produced by endothelial cells or other cell types at the infection site, including neutrophils themselves. Once transmigrated, neutrophils follow chemoattractant gradients created by chemokines/chemotactic factors to migrate to the inflammation site and exert their various effector functions.

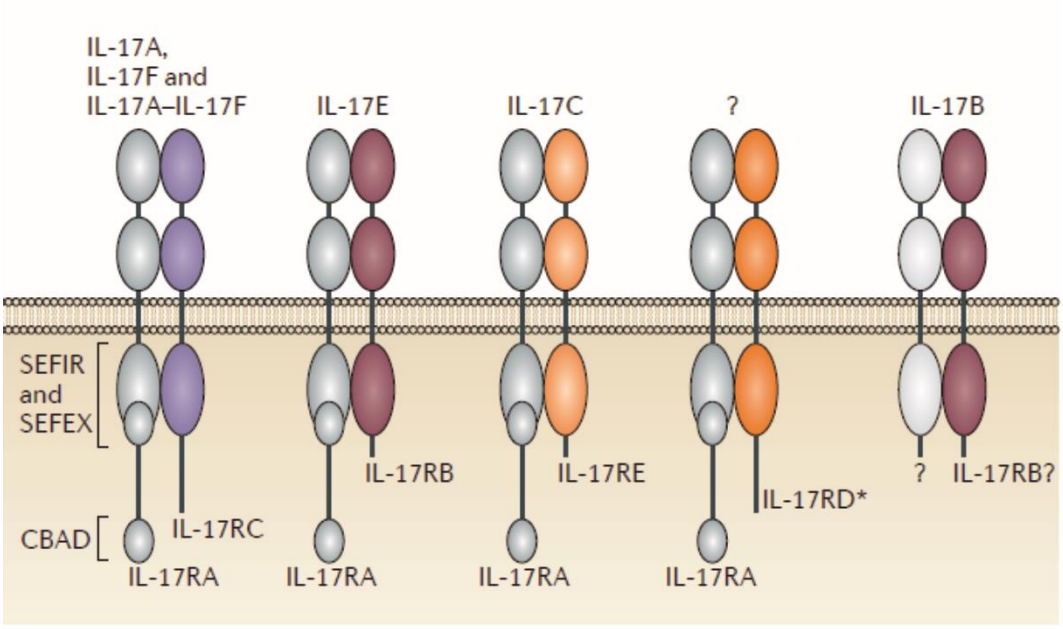
1.2 INTERLEUKIN 17

1.2.1 IL-17 and receptors

The IL-17 family consists of six cytokines, IL-17A (IL-17), IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F (**Figure 5**). IL-17A and IL-17F are the most closely related, since IL-17F has 50% sequence identity with IL-17A and its gene is localized adjacent to the IL-17A gene on human chromosome 6 [64]. IL-17F is often coproduced with IL-17A and together they can form an IL-17F/IL-17A heterodimer [65]. Being the most potent one, IL-17A induces the expression of proinflammatory

mediators, including IL-1, IL-6, TNFα, CXCL8, G-CSF and GM-CSF by epithelial cells and stromal cells, which together lead to the recruitment and activation of neutrophils. IL-17B is expressed in various tissues, such as pancreas, small intestine and brain [66], and has been shown to induce inflammatory cytokines such as IL-1β and TNFα by monocyte cell line THP-1 [67] and IL-1α, IL-6 and IL-23 by murine fibroblast cell line 3T3 [68]. IL-17B is present in the rheumatoid arthritis synovium and was shown to enhance the TNFα-induced production of G-CSF and IL-6 in fibroblasts [69]. Moreover, IL-17B can play an important role in lymphoid tissues by regulating the trafficking of germinal center B cells by downregulation of the expression of RGS16 protein [70]. Furthermore, IL-17B has been shown to have potential roles in the pathogenesis of inflammatory and autoimmune diseases and tumor progression [66]. Less is known regarding the other IL-17 members. IL-17C has been shown to bind to IL-17RE and induce the activation of nuclear IkappaB family member (IκBζ) in Th17 cells, hence leading to a potentiation of IL-17 production [71], while IL-17E (also known as IL-25) has been correlated with the augmentation of allergic responses, by enhancing Th2 memory cell function and suppressing the Th17 responses [72].

The IL-17R group comprises five receptor subunits, IL-17RA, IL-17RB, IL-17RC, IL-17RD and IL-17RE. IL-17RA was the first to be described and is ubiquitously expressed, particularly in hematopoietic cells (**Figure 5**). It is by far the largest member of the family and functions as a common receptor subunit used by at least four ligands, namely IL-17A, IL-17C, IL-17E and IL-17F[73]. For example, both IL-17A and IL-17F, as either homodimers or heterodimers, induce signals through the IL-17RA/IL17RC complex. It is important to note that IL-17RA is unique among the interleukin receptors because it recruits an adaptor protein known as ACT1 [74, 75]. Binding of IL-17A to IL-17R leads to the activation of canonical nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which requires the recruitment of ACT1 and the presence of TNF receptor associated factor 6 (TRAF6)[76]. Furthermore, IL-17A triggers the activation of the CCAAT/enhancer binding proteins (C/EBPs) for the transcription of IL-17-target genes [77].



Gaffen, S.L., et al., *The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing.* Nat Rev Immunol, 2014. 14(9): p. 585-600 [78].

Figure 5 | Members and related receptors of the IL-17 family.
CBAD, C/EBPβ activation domain; SEFEX, SEFIR extension; SEFIR, SEF/IL-17R. *IL-17RD is also known as SEF.

1.2.2 IL-17 and Th17 cells

IL-17A is a cytokine cloned in 1993 that is important in host defence and inflammation [79]. After the discovery of a subtype of CD4+ T helper (Th) expressing IL-17A (currently known as Th17 cells), plenty of studies have been published correlating Th17 with a wide range of physiological and pathological processes. Although Th17 cells are considered the main sources of IL-17A and IL-17F, other innate immune cells produce these cytokines, including γδ T cells, natural killer T cells (NKT), invariant natural killer cells (iNKT), Paneth cells, $TCR\beta^{+}$ natural Th17 cells, lymphoid-tissue inducer-like cells (LTi), IL-17-expressing type 3 innate lymphoid cells (ILC3s) and mast cells [80, 81]. Nonetheless, an exaggerated IL-17A production can lead to the development of autoimmune diseases, such as psoriasis, multiple sclerosis and rheumatoid arthritis, as well as cancer progression, which hence makes IL-17 as a very important target for the development of new therapies [78].

The expression of IL-17 by Th17 cells is regulated by specific transcription factors and chromatin status. Specifically, the activation of the T cell receptor (TCR)

signalling pathway leads to the recruitment of pioneering transcription factors, namely basic leucine zipper transcription factor ATF-like (BATF) and interferon-regulatory factor 4 (IRF4), which cooperatively make accessible target genes on the chromatin. When an inflammatory stimuli is present, such as IL-6 or activation of mTOR during hypoxia, transcription factor signal transducer activator of transcription 3 (STAT3) is recruited to the accessible chromatin, in turn promoting the transcription of genes encoding retinoic acid receptor-related orphan receptor-γt (RORγt; encoded by Rorc) and hypoxia-inducible factor 1 alpha (HIF1α; encoded by Hif1a) leading to lineage specification of T helper 17 (Th17) cells. A further activation by IL-23 promotes the recruitment of B lymphocyte-induced maturation protein 1 (BLIMP1) to the RORγt-STAT3 transcriptional complex enhancing the expression of Th17 induced-genes, such as IL-17A, IL-17F, IL-23R, CSF2 and IL-2 [78].

Although IL-17A and IL-17F are modest activators of signaling, a notable feature of these cytokines is their strong synergistic effect with other pro-inflammatory molecules, in particular with TNF, but also with IFNγ, IL-22, lymphotoxin, IL-1β and LPS[82]. Such synergistic effect could occur at the level of promoter (for example, *Il6* and *Lnc2*) and/or mRNA stability (for example, CXCL1 and CXCL2) [83, 84]. Moreover, IκBζ is also upregulated upon IL-17 activation and in turn, promotes the expression of some target genes [85].

1.2.3 Neutrophils and IL-17

The relationship between human neutrophils and the IL-17 cytokines indirectly begins with the demonstration that *in vitro* activated neutrophils release chemoattractant molecules, including CCL2 and CCL20, that efficiently recruit Th17 cells to the inflamed tissue [86]. Th17 cells further produce IL17A and IL17F on one side and CXCL8 on the other, which promote, respectively, the release of neutrophil chemoattractant molecules by epithelial cells and the direct recruitment of neutrophils. Moreover, activated Th17 cells release cytokines, such as TNFα, GM-CSF and IFNγ, that positively modulate survival and expression of activation markers by human neutrophils [40, 87]. However, whether human neutrophils respond to IL-17 or not appears to be still controversial. In fact, despite of our original data showing that neutrophils cannot be directly activated by IL-17A and/or IL-17F due to their lack of

IL-17RC expression[87], confirming previous studies reporting the inability of IL-17 to directly affect neutrophil apoptosis[88], subsequent studies have claimed that IL-17A is instead capable to attenuate the anti-apoptotic effect of GM-CSF [89], as well as increase the killing rate of pneumococcal when IL-17A is used at high concentrations (> 1 μg/ml) [90].

While Th17 cells are considered the major producers of IL-17 by far, whether human neutrophils represent an IL-17 source again appears to be currently an unclear issue, based on the controversial data reported in the literature. In such regard, our group reported no detection of IL-17A or IL-17F mRNA/protein expression/production by highly purified populations (> 99.7 %) of human neutrophils incubated for up to 20 h with 100 U/ml IFNγ and/or 100 ng/ml LPS [87, 91-93]. By contrast, diverse studies have reported that neutrophils are capable of transcribing/producing IL-17A. For instance, in a recent study, Taylor et al. [94] have reported that a putative subset of human neutrophils express IL-17A mRNA after 1 h stimulation with 20 μg/mL IL-6 and 2 μg/mL IL-23, as well as produce IL-17A after 3 h [94]. Moreover, neutrophils were shown to express RORγt, which upon incubation with supernatants containing IL-6 and IL-23 that were obtained from PBMCs treated with *Aspergillus fumigatus* hyphae that, was found translocated into the nucleus and, consequently, retained as responsible for the activation of IL-17A gene. When neutrophils treated with PBMC supernatants were incubated with antibodies against IL-6 or IL-23, their ability to express IL-17A mRNA vanished [94]. It is important to note that Taylor et al. [94] have also reported that, in the presence of IL-6, IL-23 and *A. fumigatus* hyphae, neutrophils are induced to express surface IL17RC, in turn proposing that neutrophils can respond to IL-17A in an autocrine fashion, for instance by producing ROS [94]. The same group has also shown that the JAK/STAT signalling pathway activated by IL-6 plus IL-23 plays a very important role in the ROS production and hyphal killing by neutrophils [95]. In a further study [96], the plasma of both healthy controls and patients with fungal keratitis was found to contain IL-17A, IL-6 and IL-23, showing no significant differences between them. Peripheral neutrophils from patients with fungal keratitis were analysed by intracellular flow cytometry, immunofluorescence and RT-qPCR and found to express IL-17A, suggesting a possible effect in the corneal inflammation and cytokine production in response to growing hyphae [96]. Another group has instead recognized a subset of

peripheral human neutrophils expressing IL-17 during *Mycobacterium tuberculosis* (MTB) infection [97]. According to the authors, such IL-17 production by neutrophils is due to an autocrine activity of the IL-6 and IL-23 production during MTB infection [97]. Furthermore, IL-6 and IL-23 have also been found in the microenvironment of human gastric cancer, where neutrophils were, again, found to be IL-17A-, as well as MMP9-, positive by IHC and IF [98]. In the same study, neutrophils (> 96 % pure) isolated from the whole blood of gastric cancer patients were shown to express IL-17A and RORγt mRNAs when stimulated with 20 μg/ml IL-6 and 2 μg/ml IL-23 [98].

IL-17-producing neutrophils have been also found to be related with chronic diseases, such as asthma, psoriasis and rheumatoid arthritis (RA). For instance, neutrophils (> 95 % pure) isolated from the blood of severe asthma patients stimulated with IL-21, IL-23, IL-21 plus IL-23 and IL-6 (all used at 20 ng/ml) were found to express IL-17A and IL-17F mRNA after 4 h by RT-qPCR and after 12 h by intracellular flow cytometry. In addition, the same authors observed a basal IL-17A expression in resting neutrophils by western blot, which was further increased upon stimulation with IL-21, IL-23 and IL-21 plus IL-23 for 18 h [99]. Consistently, a subset of IL-17-positive neutrophils was found in the whole blood of patients with allergic asthma, those one being allergic to fungi having a higher percentage of them [100].

IL-17 has become a very interesting target for the development of therapies towards autoimmune diseases, especially psoriasis, which displays a high infiltration of neutrophils in the damaged tissue. Immunofluorescence and immunohistochemistry experiments detected IL-17A in neutrophils present in biopsies from moderate-to-severe psoriasis patients [92]. Since no mRNA expression was observed in neutrophils isolated from active plaques, the authors suggested that IL-17 might be stored (pre-formed) by neutrophils [92]. Furthermore, because treatment of these patients with secukinumab, a human monoclonal anti-IL-17A antibody, was found to promote a clearance of neutrophils in the psoriasis plaques and decrease CXCL8 mRNA expression, authors proposed that IL-17A may have a role in the cross-talk between neutrophils and keratinocytes leading to the release of CXCL8 and further neutrophils recruitment [92]. A different study identified IL-17A-positive neutrophils and mast cells in psoriasis biopsies by immunofluorescence, leading the authors to state that both cell types represent the major sources of IL-17 in human skin [81]. Moreover, as detected by immunofluorescence of psoriasis biopsies, neutrophils and mast cells were

found to release IL-17A during extrusion of extracellular traps, even though authors reported that *"mRNA from human neutrophils from several subjects showed either low or undetectable levels of IL-17 using real time RT-PCR and Affymetrix gene array experiments (M.J.Kaplan, data not shown)",* suggesting, similarly to Reich et al (ref), that IL-17A is pre-formed and stored intracellularly by human neutrophils and mast cells[81]. However, a recent report by Yamanaka et al. [91] contradicts the notion that human neutrophils represent the major source of IL-17A in psoriasis. Accordingly, authors show no evident IL-17A mRNA expression in highly purified populations of neutrophils isolated from peripheral blood of psoriasis patients [91]. However, authors show that IL-17A expression become evident if neutrophils are isolated by gradient centrifugation only since they are contaminated by $CD3^+$ lymphocytes, which generate false-positive results for IL-17A mRNA expression[91]. In the case of RA and psoriatic arthritis (PsA), a study demonstrated the presence of IL-17A expressing–neutrophils, -mast cells and –T cells within the inflamed synovial membrane from these patients [101]. Using immunohistochemistry for IL-17A and dual-immunofluorescence staining for CD15 and IL-17A, authors found that the percentage positivity of IL-17A was highest in neutrophils[101]. Opposite results were however reported by van Baarsen et al. [93], who failed to detect a co-localisation of IL-17A with CD15 by dual-immunofluorescence staining of synovial biopsies from RA, PsA and inflammatory osteoarthritis (OA) patients [93]. The contrasting results between the two latter studies are likely explained by the different antibodies used, as Moran et al [101] utilized polyclonal anti-IL-17A (polyclonal goat, R&D systems), while van Baarsen et al. [93, 101] performed the dual-immunofluorescence using a monoclonal antibody from the same manufacturer. That is not trivial, taking into account in fact Tamarozzi et al.'s paper [102]. These authors, in fact, initially reported the presence of IL-17A-positive neutrophils in Wolbachia-positive *Onchocerca volvulus* nodules by immunohistochemistry (IHC) [102]. Subsequently, they found that IL-17A protein is not detectable in highly pure population of neutrophils (99.9 %) using alternative assays, including western blot and ELISA or RT-qPCR for mRNA expression [102].

Based on these premises, this study has the objective to clarify whether human neutrophils express and/or produce IL-17. To do so, we have used highly pure populations of human neutrophils (> 99.7 %) to evaluate the mRNA expression and

567 production of IL-17 members and related receptors, in response to various stimuli,
568 including those supposed to be very effective according to the literature.
569

570

2 MATERIALS AND METHODS

2.1 Cell purification and culture

Neutrophils were isolated from buffy coats of healthy donors and manipulated under endotoxin-free conditions. In selected experiments, neutrophils were also isolated from peripheral blood of patients with severe. 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, Canada) [17]. 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 0.2-50 μM R848, 5-50 μM R837, 500 μg/ml particulate β-glucan (Invivogen, San Diego, CA, USA), 0.1-100 μg/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), 1000 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 μg/ml phorbol mysistate acetate (PMA) (Sigma), 1 μg/ml Ionomycin (Sigma), 100 μg/ml CpG oligodeoxynucleotides (ODN) (Invivogen), 1000 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), and used at a neutrophil-fungi ratio of 1:5 for *A. fumigatus* conidia and 1:1 for *A. fumigatus* hyphae, as previously described [103]. 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°, 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°, while the corresponding cell pellets were either extracted for total RNA or lysed for protein analysis. Th17 clones, kindly provided by prof. Francesco Annunziato (University of Firenze), were stimulated for up 72 h with anti-CD3 and anti-CD28 mAbs (5μg/ml, BD Biosciences) essentially as described [104].

2.2 **Flow Cytometry**

For flow cytometry, 10^5 neutrophils were harvested after the desired treatment, centrifuged and suspended in 100 μL PBS containing 10 % complemented-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, California, USA); APC anti-human CD62L (clone 145/15 Miltenyi Biotec), all at working dilutions specified in the corresponding datasheets. For intracellular staining, neutrophils were incubated for 20 min in intracellular (IC) fixation buffer (eBioscience), followed by permeabilization buffer (eBioscience) and 1h incubation with antibody in the presence of Fc block. 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) [18]. For neutrophils within the blood 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.

2.3 **Respiratory burst activity**

After isolation, neutrophils were suspended at the concentration of 2 x 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 then incubated for 10 min at 37º prior to the addition of 80 µM cytochrome C, 2 mM NaN3 (Sigma) and the indicated stimuli, including 20 ng/ml phorbol mysistate acetate (PMA) as control. Plates were then incubated at 37º in an automated ELx808IU microplate reader (BioTek Instruments, Inc., Winooski, VT) 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 [105].

2.4 **Immunohistochemistry**

Cytospin preparations of neutrophils [106] 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_20_2 in methanol for 20 min. Anti-human IL-17A (AF-317-NA) and IL-17B (AF1248) goat IgG pAbs from R&D Systems were 1:50 diluted, added to specimens for 60 min and then revealed using the goat HRP-polymer (Biocare Medical, Pacheco, CA, USA) followed by diaminobenzidine (DAB). Omission of the primary antibody was also performed as negative control. For IL-17A and IL-17B tissue immunostaining, four-micron tissue sections from two FFPE cases of pustular psoriasis were deparaffinised and rehydrated through a scale of alcohols. Endogenous peroxidase activity was then blocked by treatment with 0.3 % H_20_2 in methanol for 20 min. Epitope retrieval in sections was performed using a slide steamer in 1.0 mM ethylene diamine EDTA buffer (pH 8.0), for 40 min at 98º C [107]. IL-17A and IL-17B antibodies were diluted 1:50 and revealed using goat HRP-polymer (IHC) or horse anti-goat IgG biotinylated antibody (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.

2.5 **Enzyme-linked immunosorbent assay**

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, 8 pg/ml for CXCL8.

2.6 **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 [47]. 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 1**. Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression (MNE) units after GAPDH normalization [108].

695

696

697 **Table 1 | List of human primer sets utilized for RT-qPCR assays**

Gene	Sequence	
	forward primers	**reverse primers**
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
SOCS3	GGCCACTCTTCAGCATCTC	ATCGTACTGGTCCAGGAACTC
IL-1ra	TTCCTGTTCCATTCAGAGACGAT	AATTGACATTTGGTCCTTGCAA
IL-17A	CTCATTGGTGTCACTGCTACTG	CCTGGATTTCGTGGGATTGTG
IL-17B	ACAACCTGCTGTTTCTTCTTACC	ACCATCTCCTCGATGTTCCTC
IL-17C	GCTACTCGGCTGAGGAACTG	GTGTCCACACGGTATCTCCA
IL-17D	CTACTGGAGCAGCTGTACG	GTCGTAGGAGATTCTGTAGGC
IL-17E	TGGAGATATGAGTTGGACAGAG	GCTAAGGAAACACGGTACAG
IL-17F	CTGGAATTACACTGTCACTTGG	GAGATGTCTTCCTTTCCTTGAG
IL-17RA	AGACACTCCAGAACCAATTCC	TCTTAGAGTTGCTCTCCACCA
IL-17RC	GTCACTGTGGACAAGGTTCTC	CTCCAACAGTAGCACATCGTC
TNFα	GAGCACTGAAAGCATGATCC	CGAGAAGATGATCTGACTGCC
CXCL8	CTGGCCGTGGCTCTCTTG	CCTTGGCAAAACTGCACCTT

698

699

700 ### 2.7 **Immunoblotting experiments**

701 Total neutrophil proteins were recovered from protein-rich flow-through
702 solutions after the first centrifugation step of the RNeasy mini kit (Qiagen) procedure

used for total RNA extraction, as previously described [47]. Lysates of human cerebral cortex were kindly provided by Prof. Bruno Bonetti (Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona) and suspended in RIPA buffer (100 mM Tris pH 7.5, 600 mM NaCl, 4 % Triton X-100, 4 % sodium deoxycholate and 0.4 % SDS) supplemented with inhibitors of proteases (5 μg/ml leupeptin, 5 μg/ml pepstatin and 1 mM PMSF) and phosphatases (1 mM Na_3VO_4, 20 μM PAO and 50 mM NaF). Protein-rich flow-through from neutrophils and extracts of cerebral cortex 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, the anti-human phospho-STAT3 (Tyr705) rabbit pAbs (#9131, Cell Signaling, Beverly, MA, USA); the 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) [47].

2.8 **Chromatin Immunoprecipitation (ChIP) assays**

Protein-DNA cross-linking was achieved by incubating 5×10^6 neutrophils with 1% formaldehyde for 10 minutes at room T under gentle agitation. Cross-linking reaction was stopped by adding glycine to a final concentration of 125 mM and incubating at room T for another 5 minutes. After fixation, cells were washed with ice-cold PBS, collected by scraping, and finally pelleted by centrifugation (5 min, 300 x g, 4 °C). Pellet was resuspended in 900 μl of lysis buffer L1 (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% IGEPAL, 10% glycerol) supplemented with proteases (5 μg/ml leupeptin, 5 μg/ml pepstatin, and 1 mM PMSF) and phosphatases (1 mM Na_3VO_4, 20 μM PAO, and 50 mM NaF) inhibitors. Resulting nuclei were pelleted at 1000 x g at 4 °C and resuspended in 300 μl lysis L2 buffer (50 mM Tris, pH 8.0, 1% SDS, 5 mM EDTA) including protease inhibitors. Chromatin was sheared to an average DNA size of 300-400 bp by sonication [6 pulses of 15 seconds at 50% maximum potency with 15 seconds pause on wet ice using a BANDELIN SONOPLUS ultrasonic homogenizers HD 2070 (Bandelin, Berlin, Germany). The lysate was then cleared by centrifugation (10 min, 13,000 x g, 12 °C) to remove debris, and diluted 10 times in

dilution buffer (50 mM Tris, 5 mM EDTA, 200 mM, 0.5 % IGEPAL). Immunoprecipitations were carried out overnight at 4°C using 1 µl anti-H3K4me1 (ab8895) and anti-H3K27Ac (ab4729) (Abcam, Cambridge, United Kingdom). Then, immune complexes were collected by adding 15 µl of protein A sepharose-coupled magnetics beads (GE Healthcare, Piscataway, NJ, USA) for 1h at 4°C in gentle rotation. Beads were then immobilized on a magnetic support and, after keeping 5 % of the supernatant [unbound DNA used to normalize the amount of immunoprecipitated DNA (specified in the text as "input" DNA)] and washed three times in washing buffer (20 mM Tris, pH 8.0, 0.1% SDS, 2 mM EDTA, 1% IGEPAL, 500 mM NaCl) and one in TE. The resulting protein complexes were then eluted in TE containing 2 % SDS and reversed crosslinked by incubation overnight at 65 °C. The DNA was purified by QiaQuick PCR purification kit (Qiagen) according to the manufacturer's instructions and eluted in 50-100 µl. 2 µl of the immunoprecipitated DNA, which was used in each quantitative PCR (qPCR) reaction, was carried out using Fast SYBR® Green Master Mix (Life Technologies). To establish the background levels of ChIP experiments, the precipitation signal was quantified also at the promoter of prolactin (PRL) since it is completely silent in myeloid cells[109]. The coimmunoprecipitated material was subjected to qPCR analysis using the following promoter specific primers (purchased from Life Technologies) listed in the **Table 2**.

2.9 **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 [110]. 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 (IGV). 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" (http://egg2.wustl.edu/roadmap/web_portal/processed_data.html) (NIH Epigenomics Roadmap Initiative). Aligned reads were then converted to BedGraph format and normalized to 10^7 total tag counts.

Table 2 | List of human primer sets utilized for qPCR of ChIP assays

ChIP primer name	Location (relative to the gene TSS)	Sequence	
		forward primers	reverse primers
IL-17A#1	-30260 to -30119	CACAAAACCGCAGGTACTCAG	TCACCACAAAGCCCACAAAG
IL-17A#2	-177 to -53	TGCCCTTCCCATTTTCCTTC	TCCTTCTGTGGTCACTTACG
IL-17A#3	+704 to +898	TAGCACCAACAGCACTTCTAGC	CAGCACATGCATCATTGTCAG
IL-17F#1	-19472 to -19371	AAGACATGACCCCCAGAGATC	GTTTTCCTTGAGAGCAATCGTG
IL-17F#2	-223 to -132	CAATGGGGGTGGAAGTAGG	CCGAAGGGGAACAAAAGGG
IL-17F#3	+1411 to +1532	CCATTGTTATCCCCACCATAC	GGCATAGGCTTTGATGTCAG
SOCS3	-113 to -45	TCTCTGCTGCGAGTAGTGAC	CCGCCCCCCGATTCCTGGA
PRL	+386 to +506	AGGGAAACGAATGCCTGATT	GCAGGAAACACACTTCACCA

2.10 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)[111]. Gene expression means and standard errors were calculated from the values of the biological replicates present in the GEO database.

2.11 Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation was performed by using, depending on the experiment type, Student's t test, 1-way ANOVA followed by

789 Tukey's post hoc test or 2-way ANOVA followed by Bonferroni's post hoc test. *P*
790 values < 0.05 were considered as statistically significant.
791
792
793 2.12 **Study approval**
794 Human samples were obtained following informed written consent by both
795 healthy donors and psoriatic patients. All experimental protocols were approved by
796 the Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona
797 (Italy). The methods were carried out in accordance with the approved guidelines.
798

799

3 RESULTS

3.1 Human neutrophils incubated with various agonists *in vitro* do not express IL-17 members, either at mRNA or at protein levels

We have previously shown that human neutrophils (> 99.7 % purity), *in vitro* incubated with 100 U/ml IFNγ and/or 100 ng/ml LPS for up to 20 h, neither express IL-17A or IL-17F mRNA, nor produce IL-17A or IL-17F protein [87]. Additional RT-qPCR experiments not only confirmed our previous data (**Figure 6A**, **left panel**), but also revealed that other agonists, including 5 μM R848 and/or 1000 U/ml IFNα (**Figure 6B**, **left panel**), 10 ng/ml GM-CSF, 100 nM fMLF (**Figure 6C, left panel**), 1000 U/ml G-CSF and 5 ng/ml TNFα (**Figure 6D**, **left panel**), similarly fail to induce an accumulation of transcripts encoding IL-17A (**Figure 6A-D**, **left panels)**, IL-17F (**Figure 6A-D**, **central 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 (**Figure 6A-C**, **right panels**), while G-CSF or TNFα modulated that of IL-1ra mRNA **(Figure 6D, right panel**), as expected [47, 57, 112]. Consistent with the gene expression data, neither IL-17A, IL17F (**Table 3**) nor IL-17A/F and IL-17B (**data not shown**) proteins could be detected in supernatants harvested from neutrophils incubated for 20 h with either the stimuli used for the experiments shown in Figure 6, or with 500 μg/ml β-glucan, 500 μg/ml curdlan, 1μg/ml Pam3CSK4, 50 μg/ml poly(IC) and 100 μg/ml CpG ODN. Noteworthy, ELISA kits from two different commercial sources (see M&M) were used 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 the same supernatants, indicating that stimuli were effective and neutrophils fully responsive (**Table 3**). 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, clones (**data not shown**), or IL-17A and IL-17F proteins in supernatants from $CD4^+$ T cells activated with anti-CD3/anti-

CD28 mAbs (**data not shown**). We could also detect intracellular IL-17B in lysates of human cerebral cortex (**data not shown**), as expected [113].

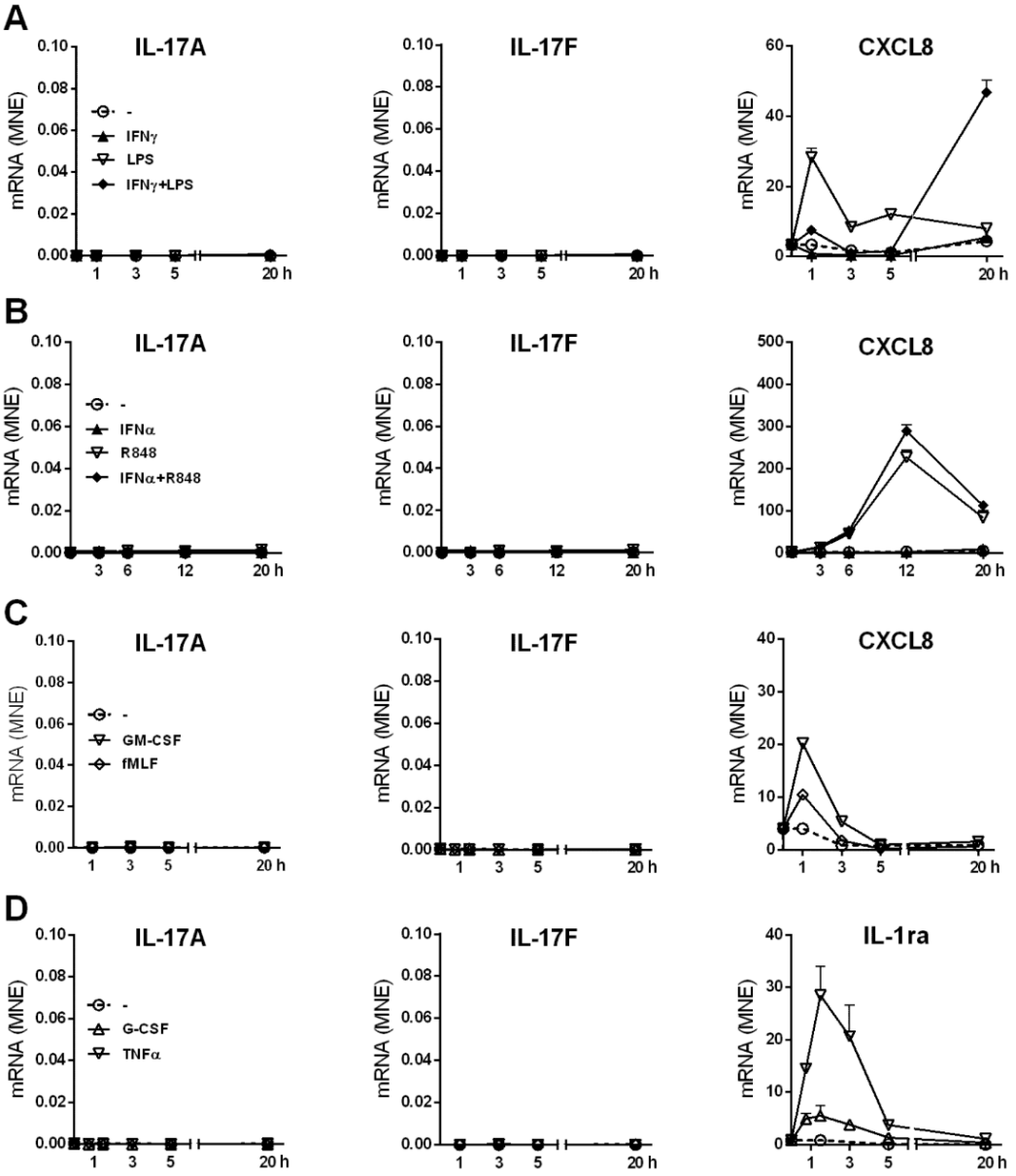


Figure 6 | IL-17A, IL-17F, CXCL8 and IL-1ra mRNA expression levels in neutrophils activated by a variety of stimuli.

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**, 1000 U/ml IFNα and/or 5 μM R848; **C**, 10 ng/ml GM-CSF or 100 nM fMLF; **D**, 1000 U/ml G-CSF or 5 ng/ml TNFα. IL-17A, IL-17F, CXCL8 and IL-1ra mRNA expression was evaluated by 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 two ones with similar results. Error bars stand for standard errors calculated from triplicate qPCR reactions.

Table 3 | Lack of IL-17A production by neutrophils activated under various experimental conditions.

neutrophils			
stimuli	IL-17A (pg/ml)	IL-17F (pg/ml)	CXCL8 (ng/ml)
-	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 antiCD3/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 are represented as the mean ± SD or as not detected (nd) when the values were under the detection limit ($n = 3$). Asterisks stand for significant increases as compared to untreated cells: *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, by Student's t-test.

In other experiments, neutrophils were incubated for 2 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 [94, 95, 97-99]. Neutrophils were also incubated with 100-500 ng/ml IL-17A to reinvestigate their eventual

responsiveness. As shown in **Figure 7,** 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 7A**), IL-17F (**Figure 7B**) and IL-17RC (**Figure 7C**) mRNAs, nor upregulation of the constitutively expressed IL-17RA transcript levels (**Figure 7D**). Similar results were obtained when incubation under identical conditions was prolonged up to 6 h (**data not shown**). Elevated levels of IL-17RC mRNAs were however detected in HBECs (**data not shown**), used as control cells [87], 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 7E**), as well as to upregulate SOCS3 mRNA expression (**Figure 7F**), such an effect being potentiated by the presence of inactivated *A.fumigatus* conidia/hyphae (**Figure 7F**). By contrast, IL-17A-treatment influenced neither SOCS3 (**Figure 7F**), nor CXCL8 (**data not shown**) mRNA levels in neutrophils. Finally, no IL-17A (**Figure 8A**), IL-17F (**Figure 8B**) or IL-17AF (**data not shown**) proteins were detected either intracellularly in, 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 8C**). Also using intracellular flow cytometry, consistent with previous findings, IL-17A expression was not detected in human neutrophils untreated or stimulated with R848 or IL-6 plus IL-23 (**Figure 9**). In fact, in all the conditions tested, the fluorescence signal obtained using the isotype control antibody was higher than that achieved with specific anti-IL-17A antibodies (**Figure 9**). Taken together, data extend our previous findings on the inability of human neutrophils to express IL-17 members at mRNA and protein levels under various activating conditions. Data also confirm and extend the inability of IL-17A to directly modify IL-17A, IL-17F, IL-17RA, IL-17RC, SOCS3 and CXCL8 gene expression in human neutrophils [87].

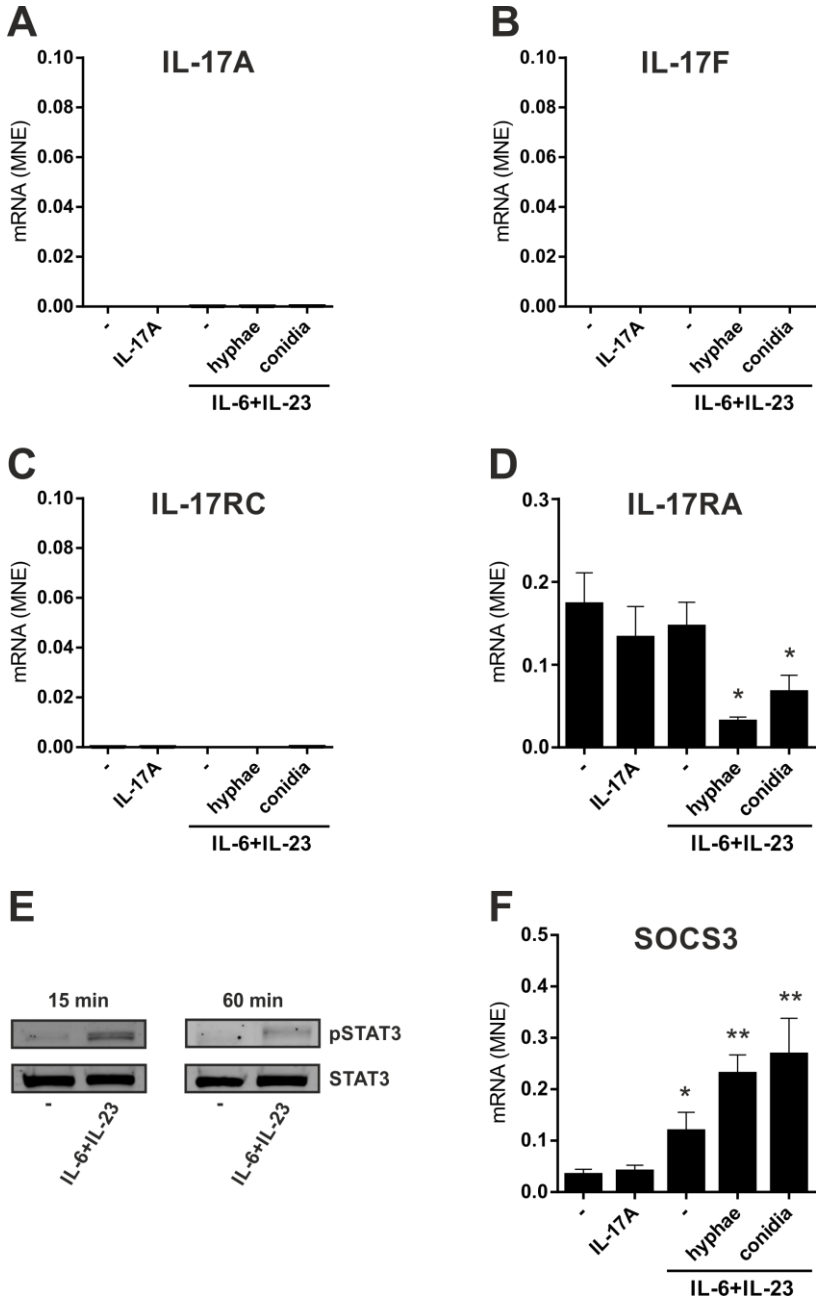


Figure 7| No induction of IL-17A, IL-17F and IL-17RC mRNA expression in neutrophils incubated with IL-6 plus IL-23 in combination with inactivated *A.fumigatus* hyphae or conidia

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. Cells were then harvested for mRNA extraction to evaluate IL-17A **(A)**, IL-17F **(B)**, IL-17RC **(C)**, IL-17RA **(D)** and SOCS3 **(F)** mRNA expression by RT-qPCR. Gene expression data are depicted as mean normalized expression (MNE) units after GAPDH mRNA normalization (means ± 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, $n = 2$).

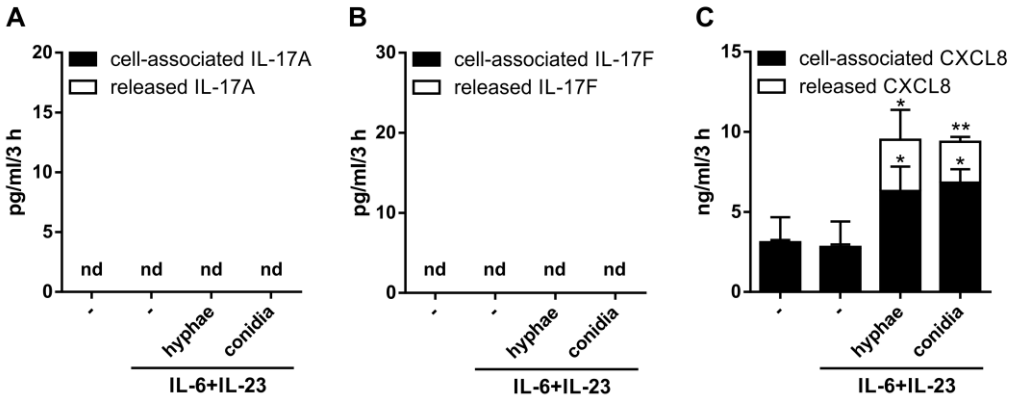


Figure 8 | Lack of IL-17A and IL-17F production by human neutrophils activated by IL-6 plus IL-23 in a combination with inactivated *A.fumigatus* hyphae or conidia
Neutrophils (5×10^6/ml) were incubated with or without 20 μg/ml IL-6 plus 2 μg/ml IL-23 and then cultured for 3 more hours in the presence or not of inactivated *A. fumigatus* conidia and hyphae (used 1:5 and 1:1 respectively). After incubation, IL-17A (**A**), IL-17F (**B**) and CXCL8 (**C**) 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.

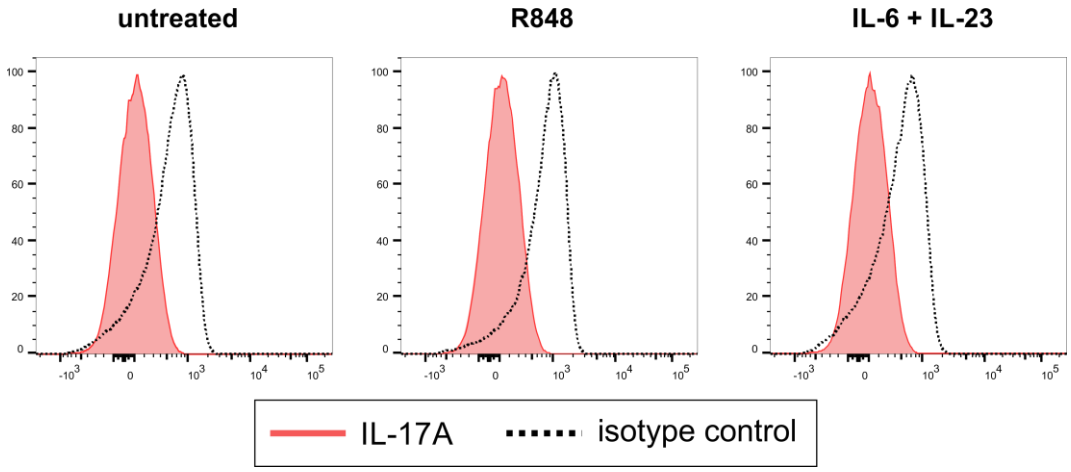


Figure 9| No induction of intracellular IL-17A expression in CD66b+ neutrophils
Expression of intracellular IL-17A by flow cytometry in CD66b+ neutrophils cultured for 1 h without or with 5 μM R848 or 20 μg/ml IL-6 plus 2 μg/ml IL-23. Graphs depict a representative experiment out of three independent ones with similar results. . Filled and empty histograms show staining with IL-17A specific Abs and isotype control Abs, respectively.

3.2 **Human neutrophils incubated with IL-6 plus IL-23, in the presence or the absence of inactivated *A.fumigatus* hyphae or conidia, do not express IL-17RC.**

Flow cyometry experiments confirmed [87] that neutrophils, either freshly isolated (**Figure 10A**), or incubated for 3 h in the absence (**Figure 10B**) or the presence of IFNγ plus LPS (**Figure 10C**), display only surface IL-17RA, but not IL-17RC. No IL-17RC surface levels were also observed in neutrophils incubated with either R848 (**Figure 10D**), or IL-6 plus IL-23 (**Figure 10E-H**), in the latter case either in the absence (**Figure 10E**) or in the presence of inactivated *A.fumigatus* conidia/hyphae (**Figure 10F** and **G**), or of IL-17A (**Figure 10H**). IL-17RA surface levels were downregulated in neutrophils treated with IFNγ plus LPS (**Figure 10C**), R848 (**Figure 10D**) and IL-6 plus IL-23 with IL-17A (**Figure 10H**). In these experiments, HBEC were used again as positive control for both IL-17RA and IL-17RC surface expression (**data not shown**) [87]. It should be pointed out that, for the investigation of surface IL-17RC, we have been using the same anti-IL-17RC antibody used in Taylor *et al.*'s study [94] directly PE-conjugated, other than the anti-IL-17RC biotin-conjugated antibodies that necessitate PE-conjugated streptavidin for detection [87], without noticing any difference between them. By the way, IFNγ plus LPS (**Figure 10C**) and R848 (**Figure 10D**), as well as IL-6 plus IL-23, either alone (**Figure 10E**), or in the presence of either inactivated *A.fumigatus* conidia/hyphae (**Figure 10F** and **G**), or of IL-17A (**Figure 10H**), variably modulated the expression of both CD11b and CD62L. All in all, data illustrate that, in our hands, 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 7F**), are unable to induce the expression of IL-17RC, thus leaving human neutrophils unresponsive to IL-17.

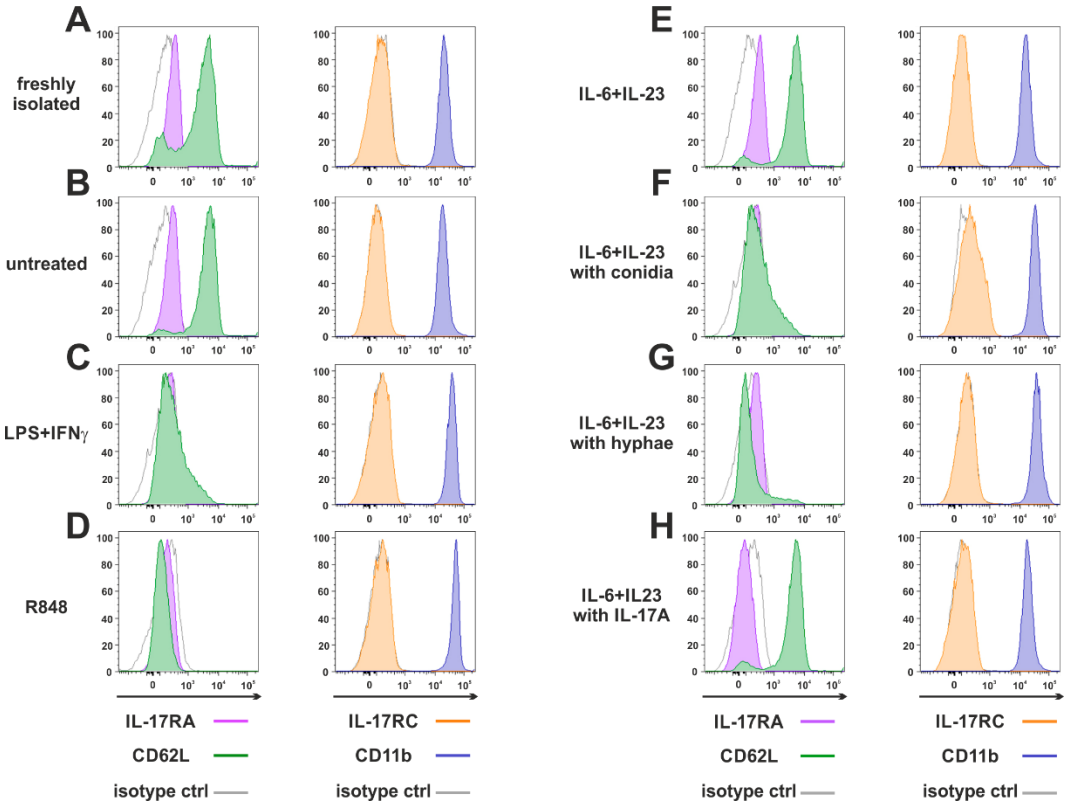


Figure 10 | IL-17RA, IL-17RC, CD62L and CD11b surface expression in neutrophils activated under various experimental conditions.
Expression of surface IL-17RA, IL-17RC, CD62L and CD11b was evaluated by flow cytometry in neutrophils either freshly isolated (**A**), or cultured for 3 h without (**B**) or with 100 U/ml IFNγ plus 100 ng/ml LPS (**C**), 5 μM R848 (**D**), 20 μg/ml IL-6 plus 2 μg/ml IL-23 alone (**E**) or in the presence of inactivated *A. fumigatus* conidia (**F**), *A. fumigatus* hyphae (**G**) and 500 ng/ml rIL-17A (**H**). Graphs depict a representative experiment out of three independent ones with similar results. Filled and empty histograms show staining with specific and isotype control Abs, respectively.

3.3 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 IL17A or IL-17A inhibitors

We then measured the capacity to release O_2^- by neutrophils pretreated with or without IL-6 plus IL-23 for one h, and then incubated for one more h with inactivated *A.fumigatus* hyphae, in the presence or the absence of either IL-17A or anti-IL-17A neutralizing Abs (**Figure 11**). As control, neutrophils were also stimulated with either inactivated *A.fumigatus* hyphae alone or 20 ng/ml PMA (**Figure 11**). As shown in **Figure 11**, inactivated *A.fumigatus* hyphae were found to trigger a remarkable O_2^-

production by neutrophils, even though lower than PMA. However, such O_2^- release was not potentiated by the preincubation with IL-6 plus IL-23 (which, by themselves, did not trigger any O_2^- production by neutrophils) (**Figure 11**). 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 11**).

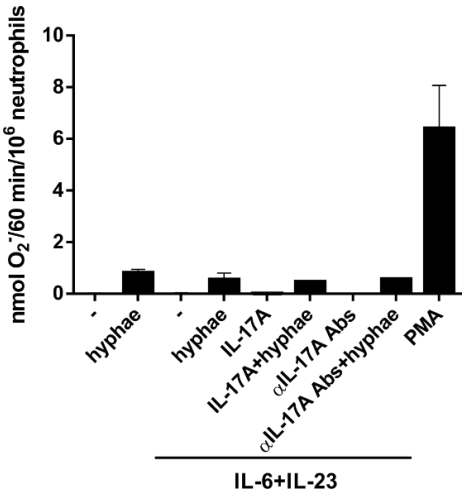


Figure 11 | Superoxide anion production by neutrophils stimulated by inactivated hyphae from *A.fumigatus* in combination with IL-6 plus IL-23, IL-17A or secukinumab.
Neutrophils (5×10^6/ml) were preincubated with or without 20 μg/ml IL-6 plus 2 μg IL-23 for 1 h and then treated for 1 more hour with inactivated hyphae from *A. fumigatus*, in combination or not with 500 ng/ml IL-17A or 10 μg/ml anti-IL-17A neutralizing mAb (secukinumab). As control, neutrophils were also stimulated with 20 ng/ml PMA for 1 h. Graphs depict a representative experiment out of three independent ones with similar results.

3.4 Chromatin organization at the IL-17A and IL-17F promoters and enhancers in 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 [29, 114]. Therefore, we evaluated the presence of histone marks associated to active (e.g. H3K27Ac) and poised (e.g. H3K4me1) genomic regulatory elements [115] 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 IL-17A and IL-17F loci is completely devoid of H3K27Ac and H3K4me1 (**Figure 12**). By contrast, based on data available from the NIH Epigenomics Roadmap Initiative [116], multiple H3K4me1 peaks are present in the same genomic regions of PMA/Ionomcyin-stimulated Th17 cells, while H3K27Ac peaks localize at the IL-17A locus only (**Figure 12**). 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 clones (**Figure 13**). Based on the H3K4me1 peaks from the ChIP-seqs of Th17 cells [116] (**Figure 12**), we designed specific primers amplifying potential regulatory regions at the IL-17A and IL-17F 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 (**Figure 13A** and **B**). As expected, Th17 clones, used as positive controls, displayed constitutively bound H3K4me1 at their IL-17A and IL-17F promoters and enhancers (**Figure 13A** and **B**, left panels). We also detected high levels of H3K27Ac at the IL-17A and IL-17F promoters and enhancer of Th17 clones (**Figure 13A** and **B**, right panels), in line with their constitutive expression of both IL-17A and IL-17F mRNA (**data not show**n). 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 12**), or after incubation with IL-6 plus IL-23 (**Figure 13A and B**). In fact, the H3K3me1 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 (**Figure 13A** and **B**). Notably, measurable amounts of H3K3me1 and H3K27Ac were found at the SOCS3 promoter of neutrophils under resting conditions, as well as of Th17 clones (**Figure 13C**). Interestingly, H3K27Ac levels tended to increase in neutrophils incubated with IL-6 plus IL-23 (**Figure 13C**), in accordance with a supposed STAT3-dependent induction of SOCS3 mRNA [117]. 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 IL17A- and IL17F-producing Th17 clones. 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.

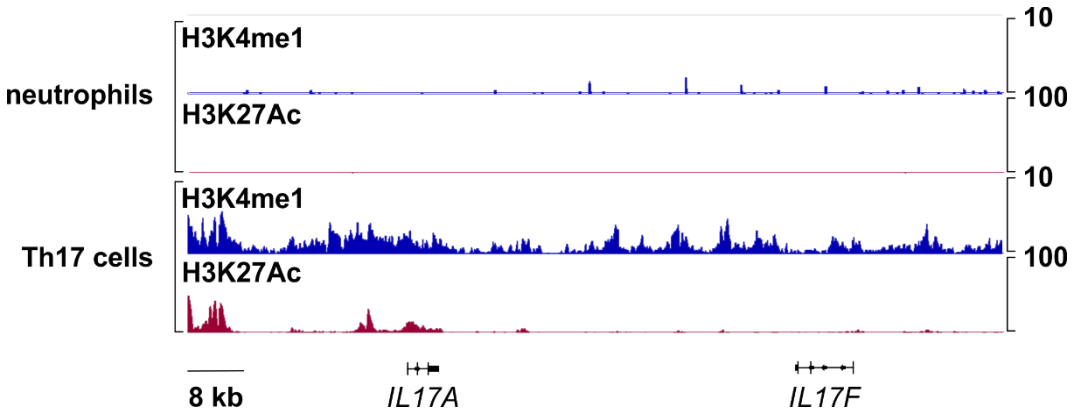


Figure 12 | ChIP-Seq profiles of H3K4me1 and H3K27Ac at the IL17A and IL17F loci in human neutrophils and Th17 cells.
Representative snapshots depicting H3K4me1 and H3K27Ac ChIP-seqs at the *IL17A* and *IL17F* genomic loci in freshly isolated human neutrophils or, as retrieved from NIH Epigenomics Roadmap Initiative [116], in PMA/ionomcyin-stimulated Th17 cells.

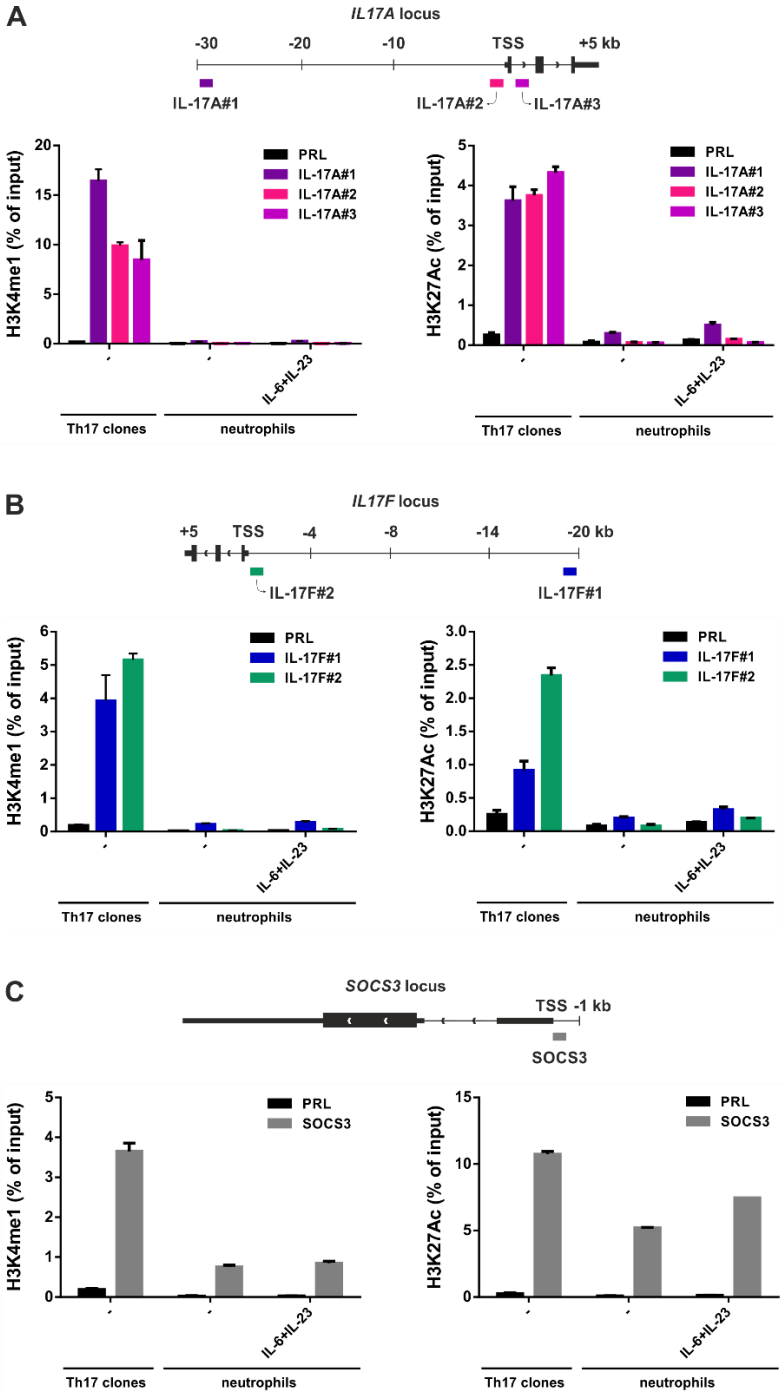


Figure 13 | H3K4me1 or H3K27Ac levels at the IL-17A, IL-17F and SOCS3 genomic loci of Th17 cells and resting/ IL-6 plus IL-23-activated neutrophils.
Enrichment levels of H3K4me1 (**left panels**) and H3K27Ac (**right panels**) at the IL-17A (**A**), IL-17F (**B**) and SOCS3 (**C**) genomic loci by ChIP analysis in human Th17 clones and neutrophils incubated for 1 h with or without 20 μg/ml IL-6 plus 2 μg/ml IL-23. (**A-C**) A scheme 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. Co-immunoprecipitated 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 standard errors calculated from triplicate qPCR reactions.

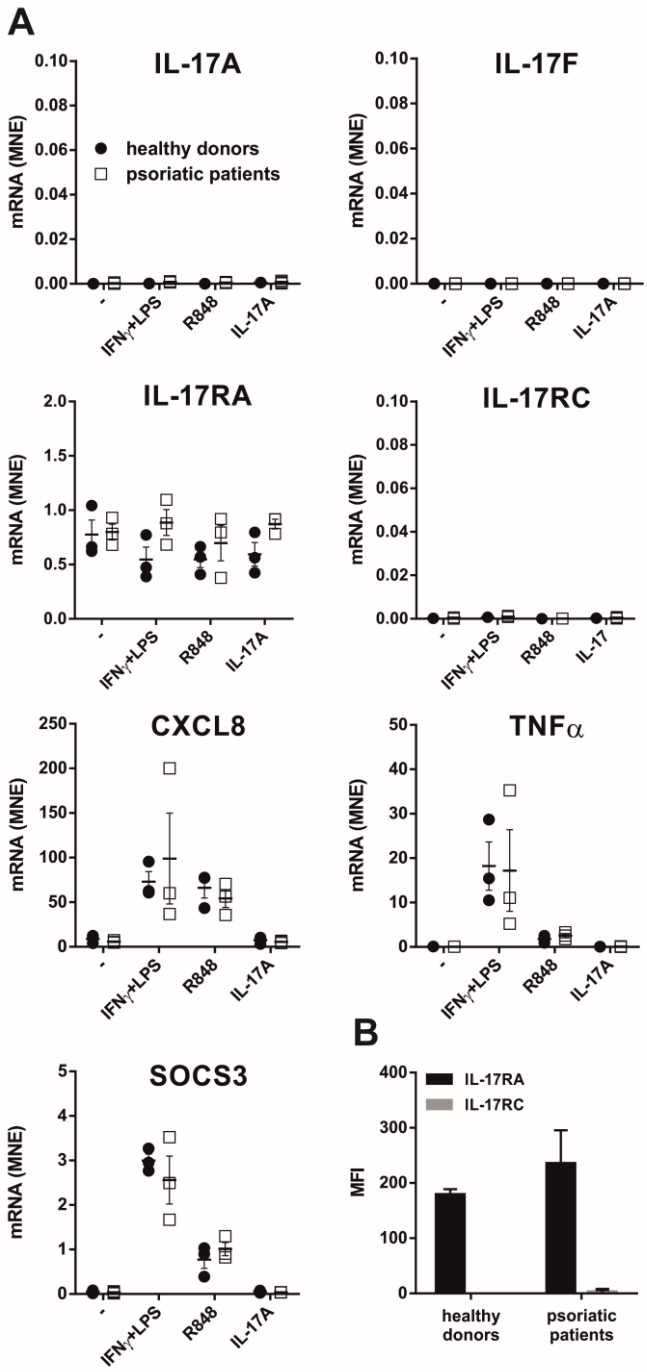


Figure 14 | IL-17A, CXCL8, TNFα and SOCS3 mRNA expression, as well as IL-17R surface expression, in neutrophils from patients with psoriasis

(**A**) Neutrophils isolated from healthy donors (n = 3) or psoriatic patients (n = 3) were cultured for 20 h with 100 U/ml IFNγ plus 1 μg/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 RT-qPCR. 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 healthy donors or psoriatic patients Values represent the mean ± SEM (n = 3). For the data of panels **A** and **B**, no significant differences between healthy donors or psoriatic patients were observed by 2-way ANOVA followed by Bonferroni's post-test.

3.5 Human neutrophils from patients with psoriasis do not express/produce IL-17A and/or IL-17F

We subsequently investigated whether neutrophils isolated from patients with active psoriasis could express IL-17A, IL-17F and/or IL-17RC mRNA, either constitutively or upon incubation for 20 h with either IFNγ plus LPS, R848 or IL-17A. As shown in **Figure 14A**, the latter was not the case, as they behaved similarly to neutrophils from healthy donors. Psoriatic neutrophils did not also respond to IL-17A (**Figure 14A**), due to their lack of surface IL-17RC expression (**Figure 14B**). 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 of healthy donors neutrophils (**Figure 14A**).

3.6 Commercial anti-IL-17A antibodies stain cytospins of resting and activated neutrophils but not their corresponding lysates

In additional experiments, cytospins of resting and R848-stimulated neutrophils were incubated with anti-human IL-17A goat antibodies (AF-317-NA) previously shown to stain neutrophils pathological tissues [81, 92, 101, 118-136], as also confirmed by our IHC/IF staining of inflamed psoriatic tissue (**Figure 15A**). Consistently, neutrophils became strongly IL-17A-positive, yet with no difference between the two experimental conditions (**Figure 15B**). By contrast, immunostaining of the same cytospins with anti-human CXCL8 Abs showed a positivity only in R848-treated neutrophils (**Figure 15B**), thus excluding methodological arctifacts. Not surprisingly, once processed for RT-qPCR analysis and ELISA, the correspondingly neutrophils samples were found totally negative for both IL-17A mRNA expression and IL-17A production (**data not shown**). The detection by IHC of IL-17A positive neutrophils, in the absence of IL-17A mRNA, could be explained by the fact that the cytokine is synthesized in bone marrow neutrophil precursors, at the stages when granule proteins, such as myeloperoxidase (MPO), elastase and azurocidin 1, are formed [137]. However in transcriptomes generated from cells isolated at different stages during granulopoiesis [111], we do not identified IL-17A mRNA accumulation

(**Figure 16**). In the same database, we not even detected IL-17RC and IL-10 mRNA (**Figure 16**), consistent with the inability of mature neutrophils to express them [29, 87]. By contrast, we did find MPO, elastase and azurocidin 1 mRNA expression only in transcriptomes of neutrophil precursors, as expected [137], thus validating the reliability of the database [111] (**Figure 16**).

In line with the latter data, immunoblots performed with AF-317-NA revealed that whole lysates prepared from neutrophils do not show any positive signal in correspondence of rhIL-17A molecular weight (**Figure 15C**). In these experiments, neutrophils were either freshly isolated (D1 and D2 in **Figure 15C**), 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 15C**), or 20 μg/ml IL-6 plus 2 μg/ml IL-23 (high IL-6 plus IL-23 in **Figure 15C**). 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 fresh isolated, stimulated or untreated neutrophils (**Figure 15C**). While these data confirm the observations reported by Tamarozzi et al [102], 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 [81] illustrating a constitutive IL-17A (but not IL-17F) expression in neutrophil lysates, as revealed by immunoblotting with #41802. Halwani *et al* [99] 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 antibodies from R&D. However, since only portions of the immunoblots are shown in Halwani *et al* [99] and Lin *et al* [81] paper, it is not known whether additional proteins were recognized by antibodies 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.

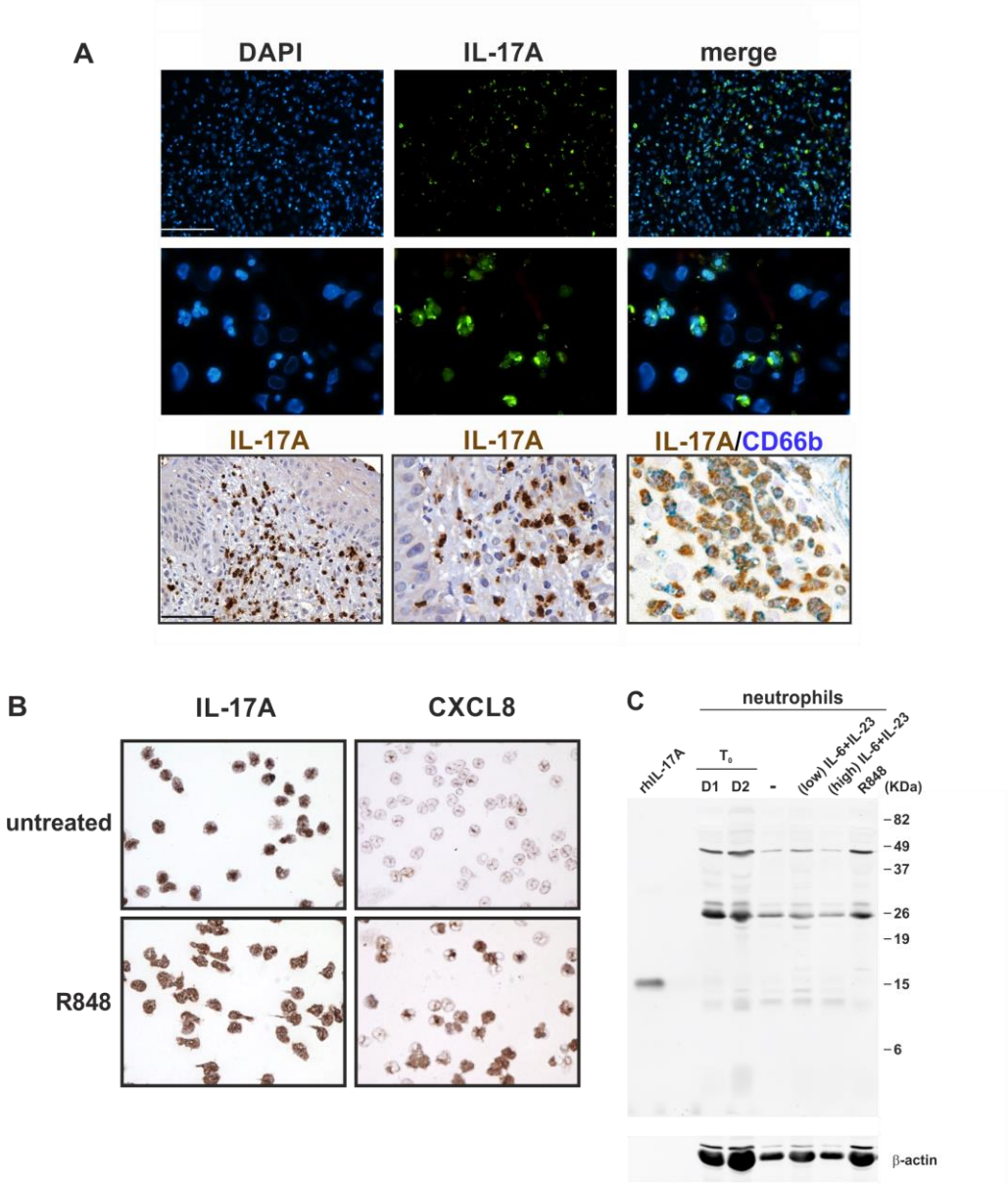


Figure 15 | Staining human neutrophils by anti-IL-17A (AF-317-NA) polyclonal antibodies

(A) Immunofluorescence (**top panels**) and immunohistochemistry (**lower panels**) staining of two FFPE cases of human pustular psoriasis using anti-IL-17A (AF-317-NA) and anti-CD66b antibodies (as labeled). Top panels show DAPI, FITC channel and merge in order 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 200x (first row in **A**, and left image in third row, scale bar 100 μm) and 400x (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 (T0, from 2 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** and **C** display representative experiments out of two independent ones with similar results.

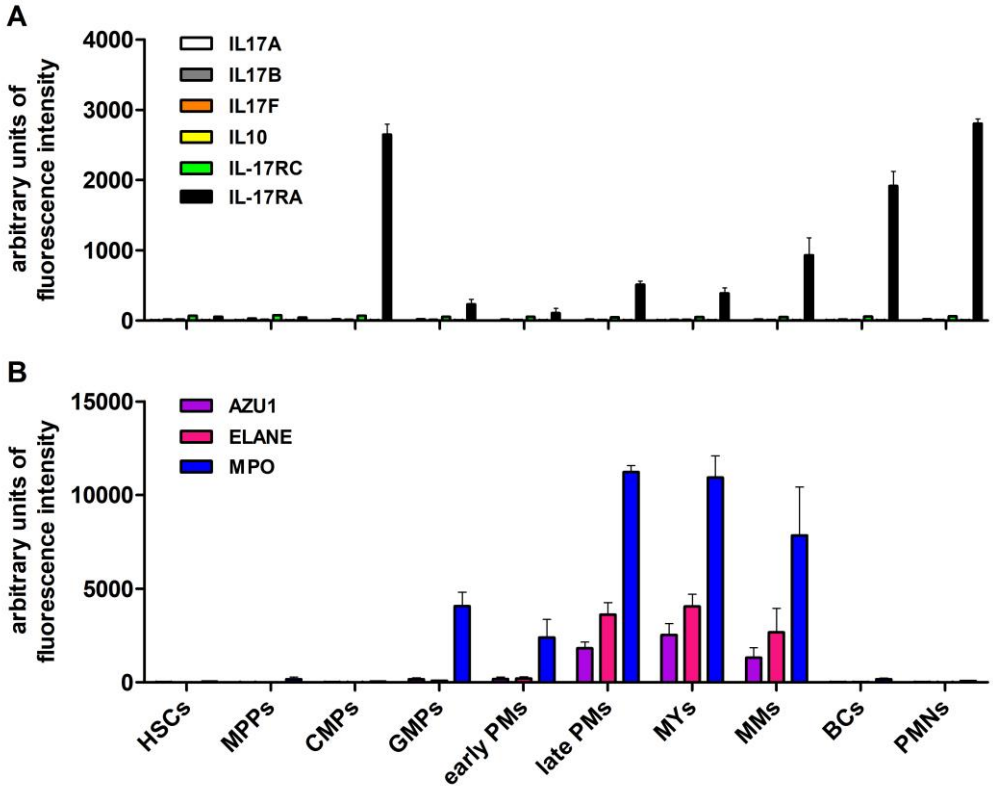


Figure 16 | IL-17A, IL-17B, IL-17F, IL-10, IL-17RC, IL-17RA azurocidin, neutrophil elastase and MPO mRNA expression levels at different stages of neutrophil maturation.

mRNA expression data derive from Gene Expression Omnibus database (accession number GSE42519) [111]. (**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 (PMNs). Values represent the mean ± SEM as calculated from data of the biological replicates present in the database.

3.7 **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, has been shown to positively stain, by IHC and IF, neutrophils present in tissue samples from rheumatoid arthritis (RA) [69] and colon carcinoma (CCR) [138] patients. Consistently, we found that also neutrophils present in inflamed psoriatic tissue were strongly detectable by IHC and IF staining with AF1248 (**Figure 17A**). AF1248 stained neutrophils isolated from the blood of healthy donors and incubated for 3 h with or without 5 μM R848 in an equivalent manner (**Figure 17B**). However, by immunoblotting, AF1248 did not recognize any protein corresponding to the MW of rhIL-17B in whole lysates prepared from neutrophils treated with R848 or IL-6 plus IL-23, as previously described (**Figure 17C**). These negative observations were also confirmed by measurement of intracellular as well as released IL-17B by two commercial ELISA (see M&M). Accordingly, no antigenic IL-17B could be measured in supernatants and whole lysates from neutrophils incubated with 5 μM R848 with or without 1000 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 [113], 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 staining 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.

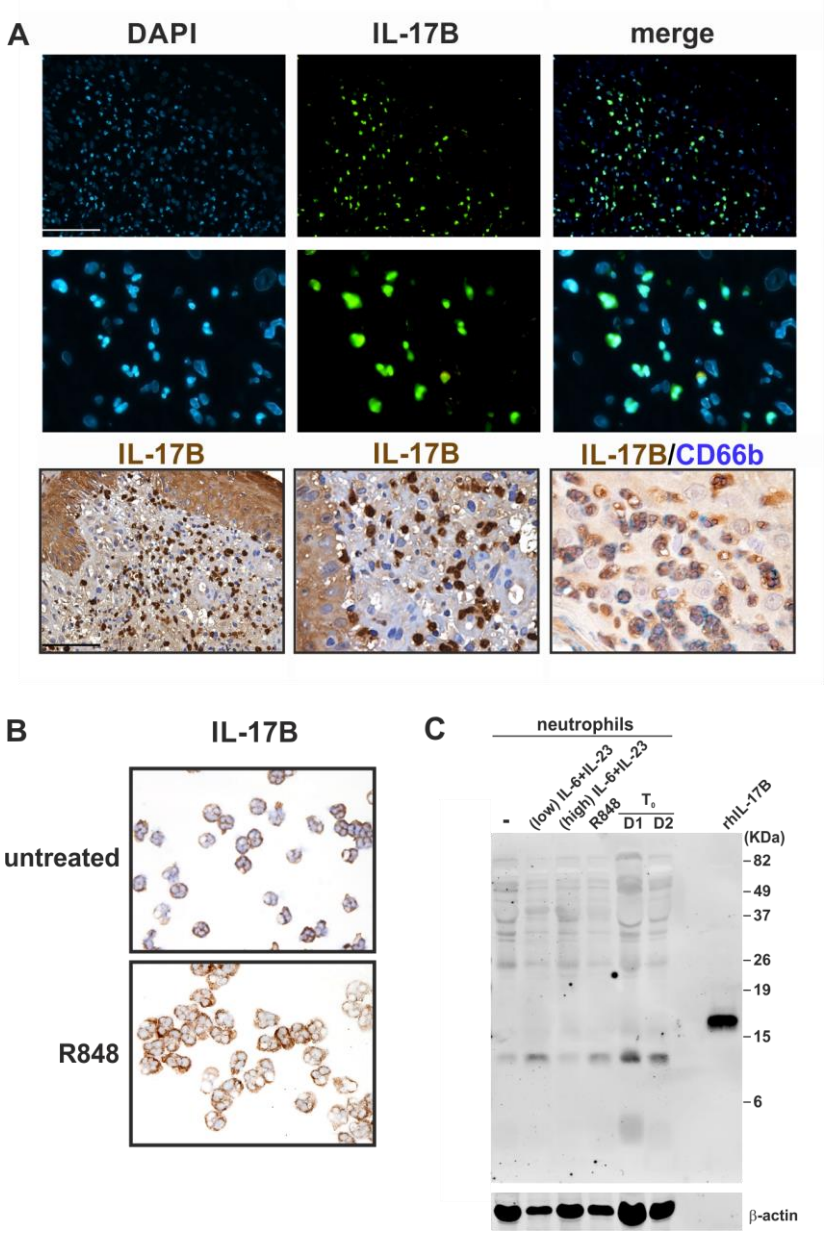


Figure 17 | Staining human neutrophils by anti-IL-17B (AF1248) antibodies
(A) Immunofluorescence (top panels) and immunohistochemistry (lower panels) staining of two FFPE cases of human pustular psoriasis using anti-IL-17B (AF1248) and CD66b antibodies (as labeled). Top panels show DAPI, FITC channel and merge in order 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 200x (first row in **A,** and left image in third row, scale bar 100 μm) and 400x (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 2 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** and **C** display representative experiments out of two independent ones with similar results.

1212
1213

4 DISCUSSION AND CONCLUSION

In this study, we have reinvestigated in-depth whether human neutrophils produce IL-17A or 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 [81, 92, 94-101, 118-136, 139-145], while a minority fail to detect it [87, 91, 93, 102, 146, 147]. 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 [94, 95, 97, 148-152], has been recently questioned [153, 154]. 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 2010 findings [87] 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 patients with active psoriasis do not express IL-17A, 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* [102], using neutrophils isolated by negative-selection (> 99.9 % pure) from healthy donors 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, IFNγ), also failed to detect any of the mRNA for IL-17 cytokine family. By contrast, Yamanaka *et al* [91] have been recently reported the presence of constitutive IL-17A transcripts in neutrophils from healthy donors 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 [91], 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 [91] are example of a notion that we have been always recommending in our studies [17, 18, 21], 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 [81, 92, 94, 95, 97, 98, 102, 141], including those ultimately showing a concurrent IL-17A protein positivity, as revealed by intracellular flow cytometry [94, 95, 97], ELISA [94, 97], confocal microscopy [94] or IHC [98]. Some authors [81, 92] 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 proteinase formed [137]. However, we would exclude such a hypothesis as our analysis of transcriptomes generated from all types of bone marrow cell populations [111] 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 [94, 95, 97-99]. 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. [99], 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 [94, 95, 98] (as we did). In this context, the paper by Hu *et al* [97], based on the use of neutralizing antibodies 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 or Pam3CSK4, even if it is true that they produce IL-6 [47] 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 [146]. In addition, we failed to detect intracellular IL-17A expression by intracellular flow cytometry using the same protocol and the same reagents reported by Taylor 2014 [94]. The fact that isotype control give higher fluorescence signal respect to specific anti-IL-17A Abs indicates that results obtained by other groups using this method could be due to non-specific binding of the antibody to intracellular protein. It should be also remarked that the purity of neutrophils in the studies showing their IL-17 production in response to IL-6 plus IL-23 [94, 95, 98, 99], reported to be > 96 % at the best [98], 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 IL-17A locus does not contain any H3Kme1 and H3K27Ac, which are two histone marks that are usually present in those genomic regions that act as active enhancers [155]. On the other hand, the levels 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 IL-17A locus of neutrophils is particularly informative, since such a histone modification is known to precede very early, but time-consuming [48], events necessary for the assembly of the transcriptional machinery, including nucleosomal depletion, H3K27Ac deposition and enhancer activation [155]. Based on our data, it appears that the chromatin at the IL-17A 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 one hour, e.g., the time-point at which IL-17 mRNA expression in IL-6 plus IL23-stimulated neutrophils has been observed [94, 95, 98]. Obviously, this does not exclude that there could exist given 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 [81, 92, 120, 123, 126, 135], skin inflammation [124], bullous pemphigoid [125], hidradenitis suppurativa [136], fungal keratitis [96], RA [69, 101], ankylosing spondylitis [118], systemic lupus erythematosus (SLE) [129, 144], human ANCA-associated glomerulonephritis [133], cystic fibrosis [119, 128, 141], nasal polips [145], chronic obstructive pulmonary disease (COPD) [122], lung tissues during bacterial pneumonia [142], alcoholic liver diseases [134], acute renal allograft rejection [130], atherosclerotic plaques [121], cutaneous T cell lymphoma lesions [132], gastric cancer [98], cervical cancer [127] and prostate cancer [143], as revealed by IHC, IF or intracellular flow cytometry using various commercial anti-IL-17A antibodies. Not surprisingly, results occasionally appear discordant. For example, while Moran *et al* [101] reported IL-17A-positive synovial tissue neutrophils using the AF-317-NA, van Baarsen *et al* [93] show that synovial tissue neutrophils from arthritis patients are not stained by #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 healthy donors 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 immunoblotted with AF-317-NA. Our findings substantially confirm the observations previously made by Tamarozzi *et al* [102] 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. Notably, by immunoprecipitation experiments followed by mass spectrometry, Tamarozzi *et al* [102] also uncovered that both AF-317-NA and #41802 bind to several proteins expressed in granules (including myeloperoxidase, lactoferroxin and lysozyme C) and cytoskeleton (such as keratin and profilin) of neutrophils, while other anti-human IL-17A antibodies (sc-6077 from Santa Cruz, and PRS4877 from Sigma) were found to recognize multiple non-specific bands in neutrophil immunoblots [102]. 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 antibodies, including sc-7927 (from Santa Cruz) [127, 131], ab9565 (from abcam) [139], ab136668 (from Abcam)

[142], 500-P07 and 500-P07G (from Peprotech) [131] and eBio64Dec17 (from eBioscience) [96, 120, 131], 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 [124], 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 [118, 133], or confirmed by costaining of the same section by eBio64DEC17 [133]. It is worth recalling that neutrophils express high levels of IL-17RA [87] 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 [156], as observed in the case of mast cells [157]. Whatever the case is, we would recommend to always validate by multiple IL-17A-investigation methods an eventual detection of IL-17A-positive neutrophils by exclusively IHC, or IF or intracellular flow cytometry [100, 118, 119, 121, 122, 125-128, 130, 131, 134-136, 139, 140, 143, 144].

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 [69] and the stroma of colon carcinoma cancer [138] by IHC/IF, as well as in freshly isolated neutrophils by immunoblotting [69], in all cases using #AF1248 antibodies. 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* [69] 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 [69], thus rendering impossible to know whether additional major proteins were recognized by #AF1248. Curiously, we, Tamarozzi *et al* [102] and Kouri *et al.* [69], 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 [146]. In such regard, Koury *et al* [69] 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 [111] 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 nonspecific, IL-17B-independent, binding of these antibodies.

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*.

5 REFERENCES

1. Nathan, C., *Neutrophils and immunity: challenges and opportunities.* Nat Rev Immunol, 2006. **6**(3): p. 173-82.

2. Borregaard, N., *Neutrophils, from marrow to microbes.* Immunity, 2010. **33**(5): p. 657-70.

3. Colotta, F., et al., *Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products.* Blood, 1992. **80**(8): p. 2012-20.

4. Kobayashi, S.D., et al., *Spontaneous neutrophil apoptosis and regulation of cell survival by granulocyte macrophage-colony stimulating factor.* J Leukoc Biol, 2005. **78**(6): p. 1408-18.

5. Lindemans, C.A., et al., *Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism.* J Immunol, 2006. **176**(9): p. 5529-37.

6. Kobayashi, S.D. and F.R. DeLeo, *Towards a comprehensive understanding of the role of neutrophils in innate immunity: a systems biology-level approach.* Wiley Interdiscip Rev Syst Biol Med, 2009. **1**(3): p. 309-33.

7. Yoshimura, T., et al., *Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines.* Proc Natl Acad Sci U S A, 1987. **84**(24): p. 9233-7.

8. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.

9. McDonald, B., et al., *Intravascular danger signals guide neutrophils to sites of sterile inflammation.* Science, 2010. **330**(6002): p. 362-6.

10. Janeway, C.A., Jr., *Approaching the asymptote? Evolution and revolution in immunology.* Cold Spring Harb Symp Quant Biol, 1989. **54 Pt 1**: p. 1-13.

11. Mantovani, A., et al., *Neutrophils in the activation and regulation of innate and adaptive immunity.* Nat Rev Immunol, 2011. **11**(8): p. 519-31.

12. Tamassia, N. and M.A. Cassatella, *Cytoplasmic receptors recognizing nucleic acids and mediating immune functions in neutrophils.* Curr Opin Pharmacol, 2013. **13**(4): p. 547-54.

13. Thomas, C.J. and K. Schroder, *Pattern recognition receptor function in neutrophils.* Trends Immunol, 2013. **34**(7): p. 317-28.

14. Tamassia, N., et al., *The MyD88-independent pathway is not mobilized in human neutrophils stimulated via TLR4.* J Immunol, 2007. **178**(11): p. 7344-56.

15. Cassatella, M.A., *Neutrophil-derived proteins: selling cytokines by the pound.* Adv Immunol, 1999. **73**: p. 369-509.

16. Scapini, P., F. Bazzoni, and M.A. Cassatella, *Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils.* Immunol Lett, 2008. **116**(1): p. 1-6.

17. Calzetti, F., et al., *The importance of being "pure" neutrophils.* J Allergy Clin Immunol, 2017. **139**(1): p. 352-355.e6.

18. Scapini, P., F. Calzetti, and M.A. Cassatella, *On the detection of neutrophil-derived vascular endothelial growth factor (VEGF).* J Immunol Methods, 1999. **232**(1-2): p. 121-9.

19. Tecchio, C., A. Micheletti, and M.A. Cassatella, *Neutrophil-Derived Cytokines: Facts Beyond Expression.* Front Immunol, 2014. **5**.

20. Tamassia, N., et al., *Cytokine production by human neutrophils: Revisiting the "dark side of the moon".* 2018: p. e12952.

21. Davey, M.S., et al., *Failure to detect production of IL-10 by activated human neutrophils.* Nat Immunol, 2011. **12**(11): p. 1017-8; author reply 1018-20.

22. Davidson, D., et al., *Differential effect of exogenous interleukin-10 versus glucocorticoids on gene expression and pro-inflammatory cytokine release by polymorphonuclear leukocytes and monocytes of the newly born.* Am J Transl Res, 2013. **5**(1): p. 103-15.

23. Ivashkiv, L.B. and L.T. Donlin, *Regulation of type I interferon responses.* Nat Rev Immunol, 2014. **14**(1): p. 36-49.

24. Panne, D., T. Maniatis, and S.C. Harrison, *An atomic model of the interferon-beta enhanceosome.* Cell, 2007. **129**(6): p. 1111-23.

25. Ramirez-Carrozzi, V.R., et al., *A unifying model for the selective regulation of inducible transcription by CpG islands and nucleosome remodeling.* Cell, 2009. **138**(1): p. 114-28.

26. Ostuni, R., et al., *Epigenetic regulation of neutrophil development and function.* Semin Immunol, 2016. **28**(2): p. 83-93.

27. Glass, C.K. and G. Natoli, *Molecular control of activation and priming in macrophages.* Nat Immunol, 2016. **17**(1): p. 26-33.

28. Iyer, S.S. and G. Cheng, *Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease.* Crit Rev Immunol, 2012. **32**(1): p. 23-63.

29. Tamassia, N., et al., *Cutting edge: An inactive chromatin configuration at the IL-10 locus in human neutrophils.* J Immunol, 2013. **190**(5): p. 1921-5.

30. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nat Rev Immunol, 2010. **10**(3): p. 170-81.

31. Reglier, H., et al., *Lack of IL-10 and IL-13 production by human polymorphonuclear neutrophils.* Cytokine, 1998. **10**(3): p. 192-8.

32. McColl, S.R., et al., *Immunomodulatory impact of the A2A adenosine receptor on the profile of chemokines produced by neutrophils.* Faseb j, 2006. **20**(1): p. 187-9.

33. Morel, C., et al., *Mycobacterium bovis BCG-infected neutrophils and dendritic cells cooperate to induce specific T cell responses in humans and mice.* Eur J Immunol, 2008. **38**(2): p. 437-47.

34. Smedman, C., et al., *ELISpot analysis of LPS-stimulated leukocytes: human granulocytes selectively secrete IL-8, MIP-1beta and TNF-alpha.* J Immunol Methods, 2009. **346**(1-2): p. 1-8.

35. Acorci-Valerio, M.J., et al., *Role of TLR2 and TLR4 in human neutrophil functions against Paracoccidioides brasiliensis.* Scand J Immunol, 2010. **71**(2): p. 99-108.

36. Alvarez-Arellano, L., et al., *Activation of human neutrophils with Helicobacter pylori and the role of Toll-like receptors 2 and 4 in the response.* FEMS Immunol Med Microbiol, 2007. **51**(3): p. 473-9.

37. Glowacka, E., et al., *IL-8, IL-12 and IL-10 cytokines generation by neutrophils, fibroblasts and neutrophils- fibroblasts interaction in psoriasis.* Adv Med Sci, 2010. **55**(2): p. 254-60.

38. Lewkowicz, N., et al., *Neutrophil--CD4+CD25+ T regulatory cell interactions: a possible new mechanism of infectious tolerance.* Immunobiology, 2013. **218**(4): p. 455-64.

39. De Santo, C., et al., *Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A.* Nat Immunol, 2010. **11**(11): p. 1039-46.

40. Pelletier, M., A. Micheletti, and M.A. Cassatella, *Modulation of human neutrophil survival and antigen expression by activated CD4+ and CD8+ T cells.* J Leukoc Biol, 2010. **88**(6): p. 1163-70.

41. Hunter, C.A. and S.A. Jones, *IL-6 as a keystone cytokine in health and disease.* Nat Immunol, 2015. **16**(5): p. 448-57.

42. Guo, Y., et al., *Interleukin-6 signaling pathway in targeted therapy for cancer.* Cancer Treat Rev, 2012. **38**(7): p. 904-10.

43. Cicco, N.A., et al., *Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha.* Blood, 1990. **75**(10): p. 2049-52.

44. Tamassia, N., M.A. Cassatella, and F. Bazzoni, *Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils.* Methods Mol Biol, 2014. **1124**: p. 451-67.

45. Takeichi, O., et al., *Human polymorphonuclear leukocytes derived from chronically inflamed tissue express inflammatory cytokines in vivo.* Cell Immunol, 1994. **156**(2): p. 296-309.

46. Wang, P., et al., *Interleukin-10 inhibits interleukin-8 production in human neutrophils.* Blood, 1994. **83**(9): p. 2678-83.

47. Zimmermann, M., et al., *Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils.* Nat Commun, 2015. **6**: p. 6061.

48. Ostuni, R., et al., *Latent enhancers activated by stimulation in differentiated cells.* Cell, 2013. **152**(1-2): p. 157-71.

49. Kaikkonen, M.U., et al., *Remodeling of the enhancer landscape during macrophage activation is coupled to enhancer transcription.* Mol Cell, 2013. **51**(3): p. 310-25.

50. Foster, S.L., D.C. Hargreaves, and R. Medzhitov, *Gene-specific control of inflammation by TLR-induced chromatin modifications.* Nature, 2007. **447**(7147): p. 972-8.

51. Heinz, S., et al., *Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities.* Mol Cell, 2010. **38**(4): p. 576-89.

52. Ghisletti, S., et al., *Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages.* Immunity, 2010. **32**(3): p. 317-28.

53. Yamamoto, M., et al., *Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta.* Nature, 2004. **430**(6996): p. 218-22.

54. de Waal Malefyt, R., et al., *Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes.* J Exp Med, 1991. **174**(5): p. 1209-20.

55. Rossato, M., et al., *IL-10-induced microRNA-187 negatively regulates TNF-alpha, IL-6, and IL-12p40 production in TLR4-stimulated monocytes.* Proc Natl Acad Sci U S A, 2012. **109**(45): p. E3101-10.

56. Tracey, D., et al., *Tumor necrosis factor antagonist mechanisms of action: a comprehensive review.* Pharmacol Ther, 2008. **117**(2): p. 244-79.

57. Zimmermann, M., et al., *IFNalpha enhances the production of IL-6 by human neutrophils activated via TLR8.* Sci Rep, 2016. **6**: p. 19674.

58. Scapini, P., et al., *The neutrophil as a cellular source of chemokines.* Immunol Rev, 2000. **177**: p. 195-203.

59. Arruda-Silva, F., et al., *Human Neutrophils Produce CCL23 in Response to Various TLR-Agonists and TNFalpha.* Front Cell Infect Microbiol, 2017. **7**: p. 176.

60. Sadik, C.D., N.D. Kim, and A.D. Luster, *Neutrophils cascading their way to inflammation.* Trends Immunol, 2011. **32**(10): p. 452-60.

61. Scapini, P. and M.A. Cassatella, *Social networking of human neutrophils within the immune system.* Blood, 2014. **124**(5): p. 710-9.

62. Griffith, J.W., C.L. Sokol, and A.D. Luster, *Chemokines and chemokine receptors: positioning cells for host defense and immunity.* Annu Rev Immunol, 2014. **32**: p. 659-702.

63. Martin, C., et al., *Chemokines acting via CXCR2 and CXCR4 control the release of neutrophils from the bone marrow and their return following senescence.* Immunity, 2003. **19**(4): p. 583-93.

64. Hymowitz, S.G., et al., *IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding.* Embo j, 2001. **20**(19): p. 5332-41.

65. Wright, J.F., et al., *Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells.* J Biol Chem, 2007. **282**(18): p. 13447-55.

66. Bie, Q., et al., *IL-17B: A new area of study in the IL-17 family.* Mol Immunol, 2017. **90**: p. 50-56.

67. Li, H., et al., *Cloning and characterization of IL-17B and IL-17C, two new members of the IL-17 cytokine family.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 773-8.

68. Yamaguchi, Y., et al., *IL-17B and IL-17C are associated with TNF-alpha production and contribute to the exacerbation of inflammatory arthritis.* J Immunol, 2007. **179**(10): p. 7128-36.

69. Kouri, V.P., et al., *Neutrophils produce interleukin-17B in rheumatoid synovial tissue.* Rheumatology (Oxford), 2014. **53**(1): p. 39-47.

70. Ferretti, E., et al., *IL-17 superfamily cytokines modulate normal germinal center B cell migration.* J Leukoc Biol, 2016. **100**(5): p. 913-918.

71. Chang, S.H., et al., *Interleukin-17C promotes Th17 cell responses and autoimmune disease via interleukin-17 receptor E.* Immunity, 2011. **35**(4): p. 611-21.

72. Kleinschek, M.A., et al., *IL-25 regulates Th17 function in autoimmune inflammation.* J Exp Med, 2007. **204**(1): p. 161-70.

73. Gaffen, S.L., *Structure and signalling in the IL-17 receptor family.* Nat Rev Immunol, 2009. **9**(8): p. 556-67.

74. Chang, S.H., H. Park, and C. Dong, *Act1 adaptor protein is an immediate and essential signaling component of interleukin-17 receptor.* J Biol Chem, 2006. **281**(47): p. 35603-7.

75. Qian, Y., et al., *The adaptor Act1 is required for interleukin 17-dependent signaling associated with autoimmune and inflammatory disease.* Nat Immunol, 2007. **8**(3): p. 247-56.

76. Miossec, P. and J.K. Kolls, *Targeting IL-17 and TH17 cells in chronic inflammation.* Nat Rev Drug Discov, 2012. **11**(10): p. 763-76.

77. Shen, F., et al., *Identification of common transcriptional regulatory elements in interleukin-17 target genes.* J Biol Chem, 2006. **281**(34): p. 24138-48.

78. Gaffen, S.L., et al., *The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing.* Nat Rev Immunol, 2014. **14**(9): p. 585-600.

79. Yao, Z., et al., *Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor.* Immunity, 1995. **3**(6): p. 811-21.

80. Cua, D.J. and C.M. Tato, *Innate IL-17-producing cells: the sentinels of the immune system.* Nat Rev Immunol, 2010. **10**(7): p. 479-89.

81. Lin, A.M., et al., *Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis.* J Immunol, 2011. **187**(1): p. 490-500.

82. Onishi, R.M. and S.L. Gaffen, *Interleukin-17 and its target genes: mechanisms of interleukin-17 function in disease.* Immunology, 2010. **129**(3): p. 311-21.

83. Shen, F. and S.L. Gaffen, *Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy.* Cytokine, 2008. **41**(2): p. 92-104.

84. Hartupee, J., et al., *IL-17 enhances chemokine gene expression through mRNA stabilization.* J Immunol, 2007. **179**(6): p. 4135-41.

85. Karlsen, J.R., N. Borregaard, and J.B. Cowland, *Induction of neutrophil gelatinase-associated lipocalin expression by co-stimulation with interleukin-17 and tumor necrosis factor-alpha is controlled by IkappaB-zeta but neither by C/EBP-beta nor C/EBP-delta.* J Biol Chem, 2010. **285**(19): p. 14088-100.

86. Ouyang, W., J.K. Kolls, and Y. Zheng, *The biological functions of T helper 17 cell effector cytokines in inflammation.* Immunity, 2008. **28**(4): p. 454-67.

87. Pelletier, M., et al., *Evidence for a cross-talk between human neutrophils and Th17 cells.* Blood, 2010. **115**(2): p. 335-43.

88. Laan, M., et al., *A role of GM-CSF in the accumulation of neutrophils in the airways caused by IL-17 and TNF-alpha.* Eur Respir J, 2003. **21**(3): p. 387-93.

89. Dragon, S., et al., *IL-17 attenuates the anti-apoptotic effects of GM-CSF in human neutrophils.* Mol Immunol, 2008. **45**(1): p. 160-8.

90. Lu, Y.J., et al., *Interleukin-17A mediates acquired immunity to pneumococcal colonization.* PLoS Pathog, 2008. **4**(9): p. e1000159.

91. Yamanaka, K., et al., *Neutrophils are not the dominant interleukin-17 producer in psoriasis.* J Dermatol, 2017. **44**(7): p. e170-e171.

92. Reich, K., et al., *Evidence that a neutrophil-keratinocyte crosstalk is an early target of IL-17A inhibition in psoriasis.* Exp Dermatol, 2015. **24**(7): p. 529-35.

93. van Baarsen, L.G.M., et al., *Heterogeneous expression pattern of interleukin 17A (IL-17A), IL-17F and their receptors in synovium of rheumatoid arthritis, psoriatic arthritis and osteoarthritis: possible explanation for nonresponse to anti-IL-17 therapy?* Arthritis Res Ther, 2014. **16**(4).

94. Taylor, P.R., et al., *Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORgammat and dectin-2.* Nat Immunol, 2014. **15**(2): p. 143-51.

95. Taylor, P.R., et al., *JAK/STAT regulation of Aspergillus fumigatus corneal infections and IL-6/23-stimulated neutrophil, IL-17, elastase, and MMP9 activity.* J Leukoc Biol, 2016. **100**(1): p. 213-22.

96. Karthikeyan, R.S., et al., *Interleukin 17 expression in peripheral blood neutrophils from fungal keratitis patients and healthy cohorts in southern India.* J Infect Dis, 2015. **211**(1): p. 130-4.

97. Hu, S., et al., *IL-17 Production of Neutrophils Enhances Antibacteria Ability but Promotes Arthritis Development During Mycobacterium tuberculosis Infection.* EBioMedicine, 2017.

98. Li, T.J., et al., *Interleukin-17-Producing Neutrophils Link Inflammatory Stimuli to Disease Progression by Promoting Angiogenesis in Gastric Cancer.* Clin Cancer Res, 2017. **23**(6): p. 1575-1585.

99. Halwani, R., et al., *Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma.* J Asthma, 2017: p. 1-12.

100. Ramirez-Velazquez, C., et al., *IL-17-producing peripheral blood CD177+ neutrophils increase in allergic asthmatic subjects.* Allergy Asthma Clin Immunol, 2013. **9**(1): p. 23.

101. Moran, E.M., et al., *IL-17A expression is localised to both mononuclear and polymorphonuclear synovial cell infiltrates.* PLoS One, 2011. **6**(8): p. e24048.

102. Tamarozzi, F., et al., *A lack of confirmation with alternative assays questions the validity of IL-17A expression in human neutrophils using immunohistochemistry.* Immunol Lett, 2014. **162**(2 Pt B): p. 194-8.

103. Bozza, S., et al., *Dendritic cells transport conidia and hyphae of Aspergillus fumigatus from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus.* J Immunol, 2002. **168**(3): p. 1362-71.

104. Annunziato, F., et al., *Phenotypic and functional features of human Th17 cells.* J Exp Med, 2007. **204**(8): p. 1849-61.

105. Cassatella, M.A., et al., *Interferon-gamma inhibits interleukin-8 production by human polymorphonuclear leucocytes.* Immunology, 1993. **78**(2): p. 177-84.

106. Scapini, P., et al., *Myeloid cells, BAFF, and IFN-gamma establish an inflammatory loop that exacerbates autoimmunity in Lyn-deficient mice.* J Exp Med, 2010. **207**(8): p. 1757-73.

107. Vermi, W., et al., *slanDCs selectively accumulate in carcinoma-draining lymph nodes and marginate metastatic cells.* Nat Commun, 2014. **5**: p. 3029.

108. Muller, P.Y., et al., *Processing of gene expression data generated by quantitative real-time RT-PCR.* Biotechniques, 2002. **32**(6): p. 1372-4, 1376, 1378-9.

109. Tamassia, N., et al., *Uncovering an IL-10-dependent NF-kappaB recruitment to the IL-1ra promoter that is impaired in STAT3 functionally defective patients.* Faseb j, 2010. **24**(5): p. 1365-75.

110. Langmead, B., et al., *Ultrafast and memory-efficient alignment of short DNA sequences to the human genome.* Genome Biol, 2009. **10**(3): p. R25.

111. Rapin, N., et al., *Comparing cancer vs normal gene expression profiles identifies new disease entities and common transcriptional programs in AML patients.* Blood, 2014. **123**(6): p. 894-904.

112. Crepaldi, L., et al., *Molecular basis of the synergistic production of IL-1 receptor antagonist by human neutrophils stimulated with IL-4 and IL-10.* Int Immunol, 2002. **14**(10): p. 1145-53.

113. Moore, E.E., et al., *Expression of IL-17B in neurons and evaluation of its possible role in the chromosome 5q-linked form of Charcot-Marie-Tooth disease.* Neuromuscul Disord, 2002. **12**(2): p. 141-50.

114. Heintzman, N.D., et al., *Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome.* Nat Genet, 2007. **39**(3): p. 311-8.

115. Northrup, D.L. and K. Zhao, *Application of ChIP-Seq and related techniques to the study of immune function.* Immunity, 2011. **34**(6): p. 830-42.

116. Bernstein, B.E., et al., *The NIH Roadmap Epigenomics Mapping Consortium.* Nat Biotechnol, 2010. **28**(10): p. 1045-8.

117. Zhang, L., et al., *IL-6 signaling via the STAT3/SOCS3 pathway: functional analysis of the conserved STAT3 N-domain.* Mol Cell Biochem, 2006. **288**(1-2): p. 179-89.

118. Appel, H., et al., *Analysis of IL-17(+) cells in facet joints of patients with spondyloarthritis suggests that the innate immune pathway might be of greater relevance than the Th17-mediated adaptive immune response.* Arthritis Res Ther, 2011. **13**(3): p. R95.

119. Brodlie, M., et al., *Raised interleukin-17 is immunolocalised to neutrophils in cystic fibrosis lung disease.* Eur Respir J, 2011. **37**(6): p. 1378-85.

120. Dyring-Andersen, B., et al., *Interleukin (IL)-17A and IL-22-producing neutrophils in psoriatic skin.* Br J Dermatol, 2017. **177**(6): p. e321-e322.

121. de Boer, O.J., et al., *Differential expression of interleukin-17 family cytokines in intact and complicated human atherosclerotic plaques.* J Pathol, 2010. **220**(4): p. 499-508.

122. Eustace, A., et al., *Identification of cells expressing IL-17A and IL-17F in the lungs of patients with COPD.* Chest, 2011. **139**(5): p. 1089-1100.

123. Kakeda, M., et al., *Innate immune cells express IL-17A/F in acute generalized exanthematous pustulosis and generalized pustular psoriasis.* Arch Dermatol Res, 2014. **306**(10): p. 933-8.

124. Keijsers, R., et al., *In vivo induction of cutaneous inflammation results in the accumulation of extracellular trap-forming neutrophils expressing RORgammat and IL-17.* J Invest Dermatol, 2014. **134**(5): p. 1276-1284.

125. Le Jan, S., et al., *Innate immune cell-produced IL-17 sustains inflammation in bullous pemphigoid.* J Invest Dermatol, 2014. **134**(12): p. 2908-2917.

126. Patel, D.D., et al., *Effect of IL-17A blockade with secukinumab in autoimmune diseases.* Ann Rheum Dis, 2013. **72 Suppl 2**: p. ii116-23.

127. Punt, S., et al., *Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer.* Oncoimmunology, 2015. **4**(1): p. e984539.

128. Tan, H.L., et al., *The Th17 pathway in cystic fibrosis lung disease.* Am J Respir Crit Care Med, 2011. **184**(2): p. 252-8.

129. Villanueva, E., et al., *Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus.* J Immunol, 2011. **187**(1): p. 538-52.

130. Yapici, U., et al., *Interleukin-17 positive cells accumulate in renal allografts during acute rejection and are independent predictors of worse graft outcome.* Transpl Int, 2011. **24**(10): p. 1008-17.

131. Yapici, U., J.J. Roelofs, and S. Florquin, *The importance of testing anti-IL-17 antibodies from different suppliers.* Am J Transplant, 2012. **12**(2): p. 504-5; author reply 506.

132. Fontao, L., et al., *Interleukin-17 expression in neutrophils and Th17 cells in cutaneous T-cell lymphoma associated with neutrophilic infiltrate of the skin.* Br J Dermatol, 2012. **166**(3): p. 687-9.

133. Velden, J., et al., *Renal IL-17 expression in human ANCA-associated glomerulonephritis.* Am J Physiol Renal Physiol, 2012. **302**(12): p. F1663-73.

134. Lemmers, A., et al., *The interleukin-17 pathway is involved in human alcoholic liver disease.* Hepatology, 2009. **49**(2): p. 646-57.

135. Res, P.C., et al., *Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis.* PLoS One, 2010. **5**(11): p. e14108.

136. Lima, A.L., et al., *Keratinocytes and neutrophils are important sources of proinflammatory molecules in hidradenitis suppurativa.* Br J Dermatol, 2016. **174**(3): p. 514-21.

137. Theilgaard-Monch, K., et al., *The transcriptional program of terminal granulocytic differentiation.* Blood, 2005. **105**(4): p. 1785-96.

138. Al-Samadi, A., et al., *Distinctive expression pattern of interleukin-17 cytokine family members in colorectal cancer.* Tumour Biol, 2016. **37**(2): p. 1609-15.

139. Rodrigues-Diez, R., et al., *IL-17A is a novel player in dialysis-induced peritoneal damage.* Kidney Int, 2014. **86**(2): p. 303-15.

140. Tan, Z., et al., *RORgammat+IL-17+ neutrophils play a critical role in hepatic ischemia-reperfusion injury.* J Mol Cell Biol, 2013. **5**(2): p. 143-6.

141. Taylor, P.R., et al., *Neutrophils from F508del cystic fibrosis patients produce IL-17A and express IL-23 - dependent IL-17RC.* Clin Immunol, 2016. **170**: p. 53-60.

142. Cai, S., et al., *IFN-gamma induction by neutrophil-derived IL-17A homodimer augments pulmonary antibacterial defense.* Mucosal Immunol, 2016. **9**(3): p. 718-29.

143. Vykhovanets, E.V., et al., *IL-17 Expression by macrophages is associated with proliferative inflammatory atrophy lesions in prostate cancer patients.* Int J Clin Exp Pathol, 2011. **4**(6): p. 552-65.

144. Lopez, P., et al., *A pathogenic IFNalpha, BLyS and IL-17 axis in Systemic Lupus Erythematosus patients.* Sci Rep, 2016. **6**: p. 20651.

145. Derycke, L., et al., *IL-17A as a regulator of neutrophil survival in nasal polyp disease of patients with and without cystic fibrosis.* J Cyst Fibros, 2012. **11**(3): p. 193-200.

146. Tenland, E., et al., *Innate Immune Responses after Airway Epithelial Stimulation with Mycobacterium bovis Bacille-Calmette Guerin.* PLoS One, 2016. **11**(10): p. e0164431.

147. Nadkarni, S., et al., *Neutrophils induce proangiogenic T cells with a regulatory phenotype in pregnancy.* Proc Natl Acad Sci U S A, 2016. **113**(52): p. E8415-24.

148. Ferretti, S., et al., *IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger.* J Immunol, 2003. **170**(4): p. 2106-12.

149. Werner, J.L., et al., *Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection.* Infect Immun, 2011. **79**(10): p. 3966-77.

150. Katayama, M., et al., *Neutrophils are essential as a source of IL-17 in the effector phase of arthritis.* PLoS One, 2013. **8**(5): p. e62231.

151. Li, L., et al., *IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury.* J Clin Invest, 2010. **120**(1): p. 331-42.

152. Hoshino, A., et al., *MPO-ANCA induces IL-17 production by activated neutrophils in vitro via classical complement pathway-dependent manner.* J Autoimmun, 2008. **31**(1): p. 79-89.

153. Huppler, A.R., et al., *Neutrophils Do Not Express IL-17A in the Context of Acute Oropharyngeal Candidiasis.* Pathogens, 2015. **4**(3): p. 559-72.

154. Price, A.E., et al., *Marking and quantifying IL-17A-producing cells in vivo.* PLoS One, 2012. **7**(6): p. e39750.

155. Calo, E. and J. Wysocka, *Modification of enhancer chromatin: what, how, and why?* Mol Cell, 2013. **49**(5): p. 825-37.

156. Huppler, A.R. and S.L. Gaffen, *Editorial: Fake it 'til you make it: mast cells acquire IL-17 exogenously.* J Leukoc Biol, 2016. **100**(3): p. 445-6.

157. Noordenbos, T., et al., *Human mast cells capture, store, and release bioactive, exogenous IL-17A.* J Leukoc Biol, 2016. **100**(3): p. 453-62.

158. Tamassia, N., et al., *A Reappraisal on the Potential Ability of Human Neutrophils to Express and Produce IL-17 Family Members In Vitro: Failure to Reproducibly Detect It.* Front Immunol, 2018. **9**: p. 795.

6 APPENDICES

During my PhD studies, I have been involved in some research projects ongoing in Prof. M.A. Cassatella's lab, focused on the regulation of neutrophil-derived cytokines under different stimulatory conditions. One of them is presented in this thesis and was recently published in Frontiers of Immunology Journal[158]. The other projects are described below followed by the respective publications.

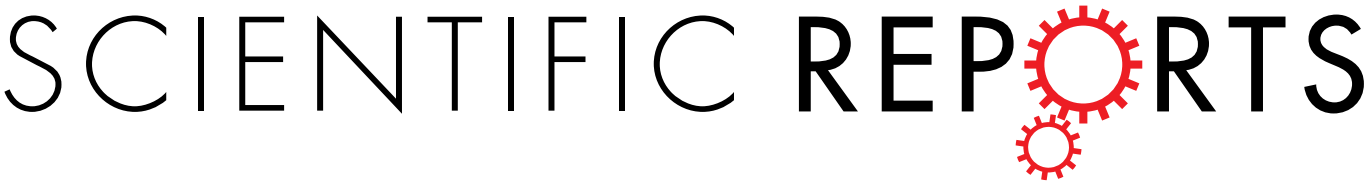
In the first study, entitled **"IFNα enhances the production of IL-6 by human neutrophils activated via TLR8"[57]**, we have demonstrated that interferon alfa (IFNα), a cytokine that is crucial for innate antiviral defences, strongly upregulates IL-6 production in neutrophils incubated with TLR8 ligands, which mimic infection by RNA viruses. We have proved that the increased IL-6 production is independent from a presumed TLR7 induction by IFNα, but rather mediated by an upregulated synthesis and release of TNFα acting autocrinally. By performing chromatin immunoprecipitation assays (ChIPs), we have also shown that the molecular mechanisms underlying the activation of IL-6 mRNA expression in IFNα plus R848-treated neutrophils occur through an increased recruitment of C/EBPβ, but not STAT1 or IRF1, to the IL-6 promoter. Finally, we have shown that highly purified neutrophils from systemic lupus erythematosus (SLE) patients, displaying an IFN-induced gene expression signature (hence with active disease state), produce increased amounts of both IL-6 and TNFα in response to R848 as compared to healthy donors, thus corroborating our in vitro observations. Data from this study have uncovered a novel effects that type I IFN exerts in activated neutrophils, which enlarge our knowledge on the various biological actions that type I IFN orchestrates during infectious and autoimmune diseases.

In the second study, entitled **"The importance of being "pure" neutrophils"[17]**, we performed experiments with the purpose to compare different

isolation methods of human neutrophils. Since one-step isolation kits were recently designed for isolation of neutrophils from whole blood, we decided to compare them with our usual isolation procedure, which allows the isolation of > 99.6 % pure neutrophils from either whole blood or buffy coats. We investigated the precise purity of wbNeuM (MACSexpress® Neutrophil isolation kit) wbNeuE (EasySep™ direct human neutrophil isolation kit) and Neu (our conventional procedure) by flow cytometric analysis focusing on thirteen different cell markers. While Neu confirmed to be 99.7 % pure, both wbNeuM and wbNeuE, although 98.9 ± 0.5 % and 97.5 ± 1 % pure, respectively, were found to be reproducibly contaminated by eosinophils (0.29 ± 0.37 % for wbNeuM and 0.53 ± 0.61 % for wbNeuE) and slan+CD16+-monocytes (0.33 ± 0.16 % for wbNeuM and 0.22 ± 0.19 % for wbNeuE). Because slan+CD16+-monocytes display a well-known capacity to produce elevated amounts of cytokines when stimulated by TLR ligands, we set up a protocol to remove them from wbNeuM and wbNeuE. Notably, wbNeuM completely lost the capacity to produce IL-10, as well as to express IFIT1 and ISG15 mRNA in response to either R848 or poly(I:C) after slan+CD16+-monocyte removal. In addition, both wbNeuM and wbNeuE lost the capacity to express or produce IL-6 when stimulated with R848 for 4 h, again after slan+CD16+-monocyte depletion. On the other hand, slan+CD16+-monocyte-depletion was less effective for wbNeuE, particularly for IFIT1 and ISG15 transcription, indicating that other contaminating cells are responsible for the mRNA expression of IFN stimulated genes. These findings support the notion that is absolutely mandatory to use highly purified populations of neutrophils when gene expression and/or neutrophil-derived cytokines are investigated

In the third study, entitled **"Human neutrophils produce CCL23 in response to various TLR-agonists and TNFα"[59]**, we showed that human neutrophils in addition to release CCL2, CCL3 and CCL4, are able to produce another CCR1-binding chemokine, namely CCL23, which selectively recruits resting T lymphocytes and monocytes, inhibits proliferation of myeloid progenitor cells and promotes angiogenesis. Other CCR1-binding chemokines, including CCL5, CCL7, CCL13, CCL14, CCL15 and CCL16 were found as not induced at the mRNA level in neutrophils, at least after R848-treatment. Surprisingly, the amounts of CCL23

produced by neutrophils incubated with R848 for 24 h were comparable to those made by the same number of autologous R848-treated monocytes. This finding is quite unusual because, on a per cell basis, activated monocytes usually produce higher cytokine/chemokine levels than autologous neutrophils, although some other exceptions exist, for instance CCL19/MIP3β. Moreover, activated neutrophils may represent major sources of CCL23, even in consideration of the fact that, during bacterial or viral infections, neutrophils often outnumber mononuclear leukocytes by one to two orders of magnitude. Furthermore, we also report that the production of CCR1-binding chemokines by R848-activated neutrophils is differentially modulated by IFNα. In fact, while production of CCL3 and CCL4 was not affected by the addition of IFNα to neutrophils, that of CCL23 was strongly inhibited. By contrast, CCL2 production was strongly enhanced in neutrophils incubated with R848 in the presence of IFNα. Given the potent effect of TLR8 agonists in inducing neutrophil-derived CCL23, and its negative regulation by IFNα, data also contribute to extend our knowledge on the complex role of neutrophils to both host defense and disease in response to viral infections.



OPEN

IFNα enhances the production of IL-6 by human neutrophils activated *via* TLR8

Received: 27 August 2015
Accepted: 04 December 2015
Published: 21 January 2016

Maili Zimmermann[1], Fabio Arruda-Silva[1], Francisco Bianchetto-Aguilera[1], Giulia Finotti[1], Federica Calzetti[1], Patrizia Scapini[1], Claudio Lunardi[2], Marco A. Cassatella[1] & Nicola Tamassia[1]

Recently, we reported that human neutrophils produce biologically active amounts of IL-6 when incubated with agonists activating TLR8, a receptor recognizing viral single strand RNA. In this study, we demonstrate that IFNα, a cytokine that modulates the early innate immune responses toward viral and bacterial infections, potently enhances the production of IL-6 in neutrophils stimulated with R848, a TLR8 agonist. We also show that such an effect is not caused by an IFNα-dependent induction of TLR7 and its consequent co-activation with TLR8 in response to R848, but, rather, it is substantially mediated by an increased production and release of endogenous TNFα. The latter cytokine, in an autocrine manner, leads to an augmented synthesis of the IkBζ co-activator and an enhanced recruitment of the C/EBPβ transcription factor to the *IL-6* promoter. Moreover, we show that neutrophils from SLE patients with active disease state, hence displaying an IFN-induced gene expression signature, produce increased amounts of both IL-6 and TNFα in response to R848 as compared to healthy donors. Altogether, data uncover novel effects that type I IFN exerts in TLR8-activated neutrophils, which therefore enlarge our knowledge on the various biological actions which type I IFN orchestrates during infectious and autoimmune diseases.

Neutrophils are the first and the most numerous innate immune cells recruited to the sites of infection, where they play a crucial role in destroying and eliminating invading pathogens[1] . Because of their powerful microbicidal equipment, neutrophils are often depicted as harmful cells that can cause damage to the surrounding tissues during acute inflammation[1]. Nonetheless, extensive research performed in the last decades has recognized neutrophils as highly versatile and sophisticated cells displaying an important role in linking the innate and adaptive arms of the immune response, as well as a significant synthetic capacity[2,3]. For instance, neutrophils produce and release a wide range of cytokines having pro-inflammatory, anti-inflammatory and immunoregulatory actions as a result of their interactions with microbes and other environmental substances[3]. Ligands for Toll-like receptors (TLR) or other pattern recognition receptors (PRR) function, in fact, as very powerful stimuli for cytokine expression in neutrophils[4–7]. In this context, we have recently reported that human neutrophils can *de novo* express and produce biologically active amounts of IL-6[8]; however, they do so only after an overnight incubation, and more significantly in response to engagement of TLR8– which commonly recognizes single strand RNA (ssRNA) of viral origin[9]. Accordingly, we have shown that the induction of IL-6 production by neutrophils primarily occurs because TLR8 agonists are able to trigger a sequence of time-dependent molecular events, which, ultimately, remodel the chromatin at the *IL-6* genomic locus from an "inactive" to an "active" configuration[8]. Most of these events were found to be sustained and amplified by endogenous TNFα (also produced in abundant amounts in response to TLR8 activation) and include, among others: the *de novo* expression of the co-activator IκBζ, which is required to drive IL-6 transcription[10]; the induction of a latent enhancer located 14 kb upstream of the *IL-6* transcriptional start site (TSS); the CCAAT/enhancer binding protein-β (C/EBPβ) recruitment to, as well as histone acetylation induction at, *IL-6* regulatory regions[8]. Notably, the identification of neutrophils as potential source of IL-6, as well as the molecular mechanisms specifically regulating such a function, has definitively clarified controversial literature in the field[11]. Moreover, in light of findings demonstrating that neutrophils produce IL-6 upon incubation with respiratory syncytial virus (a single-stranded RNA virus)[12], recognize HIV-1 *via* TLR8[13], and express a broad repertoire of functional PRR involved in the recognition of nucleic acids of viral origin[6,7],

[1]Department of Medicine, Division of General Pathology, University of Verona, Verona, Italy. [2]Department of Medicine, University of Verona, Verona, Italy. Correspondence and requests for materials should be addressed to M.A.C. (email: marco.cassatella@univr.it) or N.T. (email: nicola.tamassia@univr.it)

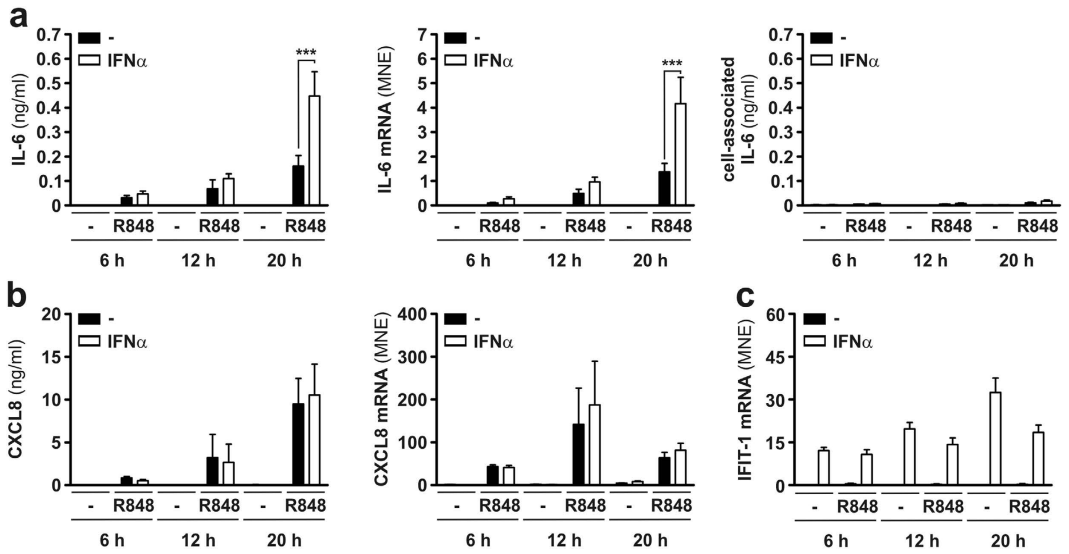


Figure 1. IFNα enhances the production of IL-6 in R848-treated neutrophils. Neutrophils (5×10^6/ml), isolated from the peripheral blood of healthy donors, were cultured with or without 5 μM R848, 1000 U/ml IFNα or IFNα plus R848 for 6, 12 and 20 h to evaluate: released (**a**, left panel) and cell-associated (**a**, right panel) IL-6, as well as released CXCL8 (**b**, left panel), by ELISA; IL-6 (**a**, central panel), CXCL8 (**b**, right panel), and IFIT1 (**c**) mRNA expression, by RT-qPCR. ELISA values stand for the mean ± SEM (n = 3–11). Gene expression data (mean ± SEM, n = 3–9) are depicted as mean normalized expression (MNE) units after GAPDH mRNA normalization. Asterisks indicate a significant enhancement by IFNα: ***$p < 0.001$, by 2-way ANOVA followed by Bonferroni's post-test.

our data have further corroborated the notion that neutrophils should be included among the cells responding to viral infections[14].

Type I interferon (IFN) is known to mediate the early innate immune responses to viral infections, acting either directly, by inhibiting viral replication, or indirectly, by activating and potentiating effector functions exerted by immune cells[15]. Interestingly, type I IFN targets also human neutrophils, for instance by prolonging their survival[16], or by inducing the expression of CXCL10 mRNA[17], and the production of biologically active TNF-related apoptosis-inducing ligand (TRAIL)[18]. There is also evidence that type I IFN potentiates the expression of IL-6, for example in double strand RNA (dsRNA)-stimulated HeLa cells[19], or in circulating PBMCs of chronic hepatitis C patients[20]. Besides, an uncontrolled production of type I IFN is involved in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE) or rheumatoid arthritis[21].

Based on these premises, herein we investigated whether type I IFN modulates the production of IL-6 by TLR8-activated neutrophils and, if so, at which molecular level. To validate the biological significance of *in vitro* results, we also investigated whether neutrophils isolated from patients with active SLE, hence displaying the "IFN-signature", produce altered levels of IL-6 in response to activation *via* TLR8.

Results

IFNα potentiates the production of IL-6 by human neutrophils incubated with R848. To investigate the effect of type I IFN on the production of IL-6 by TLR8-stimulated neutrophils, we incubated highly purified neutrophils (99.7 ± 0.2 % purity)[22] with or without 5 μM R848[8], in the presence or absence of 1000 U/ml IFNα[18], for up to 20 h. We found that IFNα, while not triggering *per se* any IL-6 production (Fig. 1a, left panel) or mRNA expression (Fig. 1a, middle panel), enhanced the yields of IL-6 recovered in supernatants from neutrophils incubated with R848, as well as the accumulation of the related mRNA (Fig. 1a, middle panel). Such a potentiation was significantly evident only after 20 h of cell incubation (Fig. 1a, left and middle panels), and not due to an enhanced secretion of an intracellularly stored pool of IL-6 (Fig. 1a, right panel). Under the same experimental conditions, IFNα neither triggered the expression of CXCL8, nor influenced the stimulatory effect of R848 on the CXCL8 production and mRNA accumulation (Fig. 1b), while it potently induced the accumulation of IFIT1 mRNA (Fig. 1c), a classical interferon-dependent gene[23]. Taken together, these data demonstrate that IFNα greatly enhances the production of IL-6 by neutrophils stimulated with R848.

IFNα does not induce the expression of TLR7 in human neutrophils. To identify the mechanisms whereby IFNα augments the production of IL-6 in R848-treated neutrophils, we initially investigated whether an IFNα-mediated *de novo* induction of TLR7 and its consequent co-activation with TLR8 could occur. In this regard, we have recently shown that resting mature neutrophils do not express TLR7[8]. However, earlier observations have reported that SLE-serum treated neutrophils express TLR7 and respond to its specific ligand, R837[24], by producing augmented levels of CXCL8[25]. Such an acquisition to express TLR7 mRNA and, in turn, respond to R837, was suggested to be caused by the presence of type I IFN in SLE serum, also because, *in vitro*, 1000 U/ml IFNα were shown to induce TLR7 mRNA expression in neutrophils from healthy donors[25].

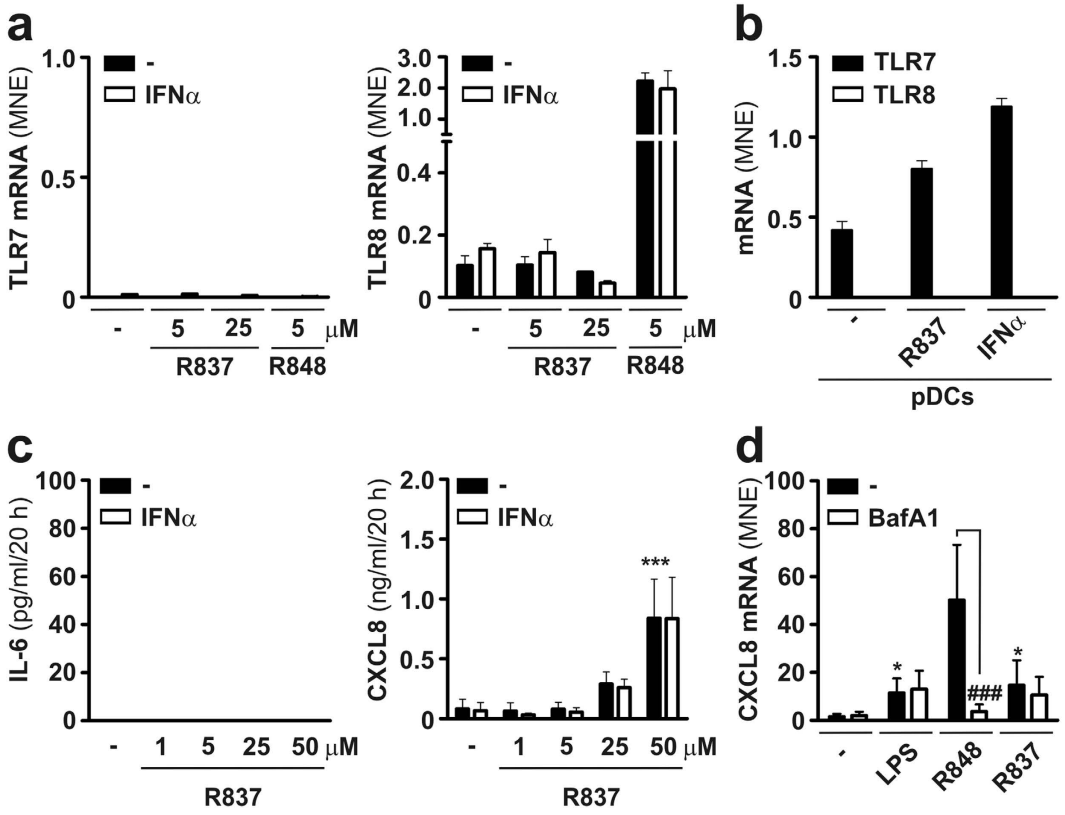


Figure 2. TLR7 is not expressed in human neutrophils incubated with IFNα. (**a**) TLR7 (left panel) and TLR8 (right panel) mRNA expression either in (**a**), neutrophils cultured with or without 5 μM R848, 5 and 25 μM R837, in the presence or absence of 1000 U/ml IFNα for 20 h, or (**b**), pDCs cultured for 5 h with or without 5 μM R837 or 1000 U/ml IFNα. In (**a**), no significant effect by IFNα was found by 2-way ANOVA followed by Bonferroni's post-test. (**c**) IL-6 and CXCL8 levels detectable in supernatants from neutrophils treated for 20 h with or without 1–50 μM R837, in the presence or absence of 1000 U/ml IFNα. (**d**) CXCL8 mRNA expression in neutrophils pretreated with 25 nM Bafilomycin A1 (BafA1) for 30 min and then incubated for 5 h with 100 ng/ml LPS, 5 μM R848 or 50 μM R837. Values in panels (**a,c,d**) stand for the mean ± SEM (n = 5), while panel (**b**) depicts a representative experiment out of three independent ones with similar results. Asterisks in panel (**c,d**) indicate a significant increase with respect to untreated cells, while # symbols in panel (**d**) indicate a significant inhibition exerted by BafA1: $^{*}p < 0.05$, *** and $^{###}p < 0.001$, by 2-way ANOVA followed by Bonferroni's post-test.

In the same study, IRS-661 (a specific TLR7 inhibitor) was found to inhibit the upregulation of CD83 in plasmacytoid dendritic cells (pDCs) treated with supernatants harvested from SLE neutrophils previously incubated with anti-ribonucleoprotein antibodies, the latter being shown to trigger neutrophil extracellular trap release *via* TLR7[25]. In our populations of highly purified neutrophils incubated for 20 h with 1000 U/ml IFNα, either alone or in combination with either R848 or R837, no induction of TLR7 mRNA was, however, detected (Fig. 2a, left panel). Similarly, IFNα did not influence the expression of TLR8 mRNA (Fig. 2a, right panel), which, instead, was found to be remarkably upregulated by R848 (Fig. 2a, right panel). Control experiments confirmed[26] that TLR7, but not TLR8, mRNA is expressed in human pDCs under resting conditions, TLR7 being upregulated upon pDC incubation with R837 or IFNα (Fig. 2b).

Consistent with the lack of TLR7 mRNA expression, no IL-6 production was observed in neutrophils stimulated for 20 h by up to 50 μM R837, either in the absence or in the presence of IFNα (Fig. 2c, left panel). On the other hand, we could confirm that R837 stimulates the production of CXCL8 by neutrophils (Fig. 2c, right panel), but only at elevated concentrations (e.g., 25–50 μM) and, once again, without being influenced by the IFNα co-addition (Fig. 2c, right panel). Such R837-induced CXCL8 production occurred in a TLR7-independent manner (Fig. 2d), as it was not abrogated by the pretreatment of neutrophils with BafilomycinA1 (BafA1), a potent inhibitor of endosomal acidification that is a required condition for efficient TLR7 and TLR8, but not TLR4, signaling[27] (Fig. 2d). Altogether, data prove that the enhanced IL-6 production by neutrophils co-treated with IFNα and R848 is not mediated by the co-activation of TLR8 with newly induced TLR7. Data also indicate that neutrophil-derived CXCL8 in response to R837 is independent of endosomal TLR7 (as well as TLR8), and, likely, occurs *via* other, not yet identified mechanisms.

Endogenous TNFα partially mediates the upregulatory effect of IFNα on the R848-stimulated IL-6 production. Additional experiments uncovered that also the production of TNFα is significantly

augmented by IFNα in neutrophils incubated with R848 for 20 h (by approximately three-fold, Fig. 3a, left panel). Interestingly, no significant changes of the TNFα transcript accumulation were observed in IFNα plus R848-treated neutrophils as compared to cells treated with R848 only (Fig. 3a, right panel), indicating that IFNα enhances the production of TNFα likely by acting at the translational level. Therefore, at the light of our recently published observations, briefly described in the introduction[8], we investigated the contribution of endogenous TNFα in mediating the enhancement of IL-6 expression in neutrophils treated with IFNα plus R848. To do so, we used two TNFα neutralizing drugs, namely adalimumab (ADA) and etanercept (ETA)[28], and compared the grade of their inhibitory effects on the production of IL-6 by neutrophils incubated for 20 h with R848 only *versus* neutrophils incubated with IFNα and R848. As shown in left panel of Fig. 3b, the release of IL-6 by neutrophils treated with IFNα and R848 was inhibited to a slightly higher extent than in neutrophils treated with R848 only (61.1 ± 3.7 % *versus* 55.7 ± 2.1 in the case of ADA; by 58.4 ± 3.9 % *versus* 50.5 ± 2.0 % in the case of ETA, $n = 5$), indicating that endogenous TNFα is crucial for the IFNα-dependent IL-6 enhancement. Yet, neither ADA, nor ETA, reduced the production of IL-6 by neutrophils co-treated with IFNα and R848 to the levels detected in supernatants harvested from neutrophils treated with R848 only. Nonetheless, in IFNα plus R848-treated neutrophils, ADA reduced the accumulation of IL-6 transcripts only after 20, but not 6, h of incubation (Fig. 3c). Furthermore, that endogenous TNFα greatly contributes to mediate the upregulatory effect of IFNα on the production of IL-6 was further supported by the western blot experiment displayed in Fig. 3d. The latter, in fact, shows that ADA inhibits the enhanced expression of IκBζ protein that is detectable in neutrophils treated with IFNα plus R848 as compared to cells treated with R848 only. Notably, results shown in right panel of Fig. 3b also show that TNFα neutralizing drugs do not suppress all cytokines produced by neutrophils treated with R848 and/or IFNα. In fact, blocking the activity of autocrine TNFα did not significantly influence the release of IL-1ra by neutrophils incubated with R848, IFNα, or IFNα plus R848, the latter combination triggering a synergistic IL-1ra production. All in all, data indicate that the increased production of TNFα occurring in neutrophils co-treated with IFNα and R848, with respect to neutrophils incubated with R848 only, largely mediates their enhanced production of IL-6.

IFNα potentiates the R848-induced recruitment of C/EBPβ to the IL-6 genomic locus. Results from primary transcript (PT) experiments (Fig. 4a), as well as from ChIP of Polymerase II (Pol II) recruitment to the *IL-6* TSS (Fig. 4b, left panel), indicated that the potentiation of IL-6 expression in neutrophils co-treated with IFNα and R848 for 20 h occurred at the transcriptional level. At the light of these data, we subsequently investigated whether IFNα, directly or indirectly, activates transcription factors (TFs) able to transactivate IL-6 gene expression, including C/EBPβ[29]. In this context, it has been recently shown that type II IFN/IFNγ, known to potently upregulate cytokine production in neutrophils[30], also increases the transcription of IL-6 in human and murine macrophages stimulated with TLR ligands, *via* induction of a sustained signal transducer and activator of transcription 1 (STAT1) and IRF-1 occupancy at the *IL-6* locus[31]. However, no recruitment of either STAT1 (Fig. 4c) or IRF-1 (**data not shown**) to the promoter (Fig. 4c) or enhancers (**data not shown**) of the *IL-6* locus was detected in neutrophils treated with IFNα, either alone or in combination with R848. Conversely, an evident Pol II (Fig. 4b, right panel) and STAT1 (Fig. 4c, right panel) recruitment at the *IFIT1* promoter occurred in response to IFNα, at both the 6 and 20 h time-points. Instead, in neutrophils co-treated with IFNα plus R848 we detected an increased recruitment of C/EBPβ to the *IL-6* promoter with respect to neutrophils treated with R848 only (Fig. 4d), which, interestingly, was measured already after 6 h of incubation. Notably, the increased recruitment of C/EBPβ to the *IL-6* promoter of neutrophils treated for 20 h with IFNα plus R848 was partially, but not completely, reduced by ADA to the levels reached in neutrophils treated with R848 only, similarly to what observed in the case of IL-6 release (Fig. 3b) and IL-6 mRNA (Fig. 3c). Taken together, data demonstrate that, in R848-treated neutrophils, IFNα increases IL-6 transcription in a STAT1/IRF1-independent manner. Data also demonstrate that, under the same experimental conditions, IFNα augments the recruitment of C/EBPβ to the *IL-6* promoter induced by R848 in a manner partially dependent on endogenous TNFα.

IFNα does not increase the prosurvival effect that R848 exerts on neutrophils. Given the well-known tendency of neutrophils to undergo apoptosis once cultured *in vitro*[32], the observation that both IL-6 and TNFα are produced at maximal levels after an overnight incubation with IFNα plus R848 might appear intriguing. It should be, however, mentioned that TLR8 ligands have been already shown to delay neutrophil apoptosis[33–35], as also confirmed by our flow cytometric analysis by Vybrant DyeCycle violet/Sytox stain (Fig. 5a). Because also IFNα delays neutrophil apoptosis[36,37], we next investigated whether potential factors whereby R848 delays neutrophil apoptosis also favor the production of IL-6, and whether IFNα had a positive effect on them, to ultimately enhance IL-6 expression. Since, R848-treated neutrophils produce, in addition to TNFα, also high amounts of G-CSF (Fig. 5b), and given that both G-CSF and TNFα delay neutrophil apoptosis[32,38], we initially asked whether endogenous G-CSF and/or TNFα could play a role in mediating the prosurvival effect of R848. In the case of G-CSF, we found that G-CSF-blocking antibodies did not change the viability of neutrophils observed after 20 h of culture in the presence of R848 (Fig. 5c), even though they significantly decreased the prosurvival effect of exogenous G-CSF (Fig. 5c). Consistently, G-CSF neutralization did not have any effect on the induced IL-6 or CXCL8 mRNA accumulation in R848-treated neutrophils (Fig. 5d), while it almost completely abolished the induction of SOCS3 mRNA in response to exogenous G-CSF (Fig. 5d). Notably, neutrophil-derived G-CSF was biologically active, as supernatants harvested from R848-activated neutrophils (R848-SN in Fig. 5e) triggered, in 60 min, a G-CSF-dependent STAT3 tyrosine phosphorylation in freshly isolated heterologous neutrophils (Fig. 5e). Interestingly, the inability of R848-treated neutrophils to respond to endogenous G-CSF was found to likely rely on a complete downregulation of G-CSFR (Fig. 5f). Conversely, ADA, as well as ETA (**data not shown**), significantly decreased the prosurvival effect exerted by R848 in neutrophils (by 25 ± 4% at 20 h, $n = 6$) (Fig. 5g). Nonetheless, despite of the fact that it potently upregulates the production of TNFα in R848-treated neutrophils

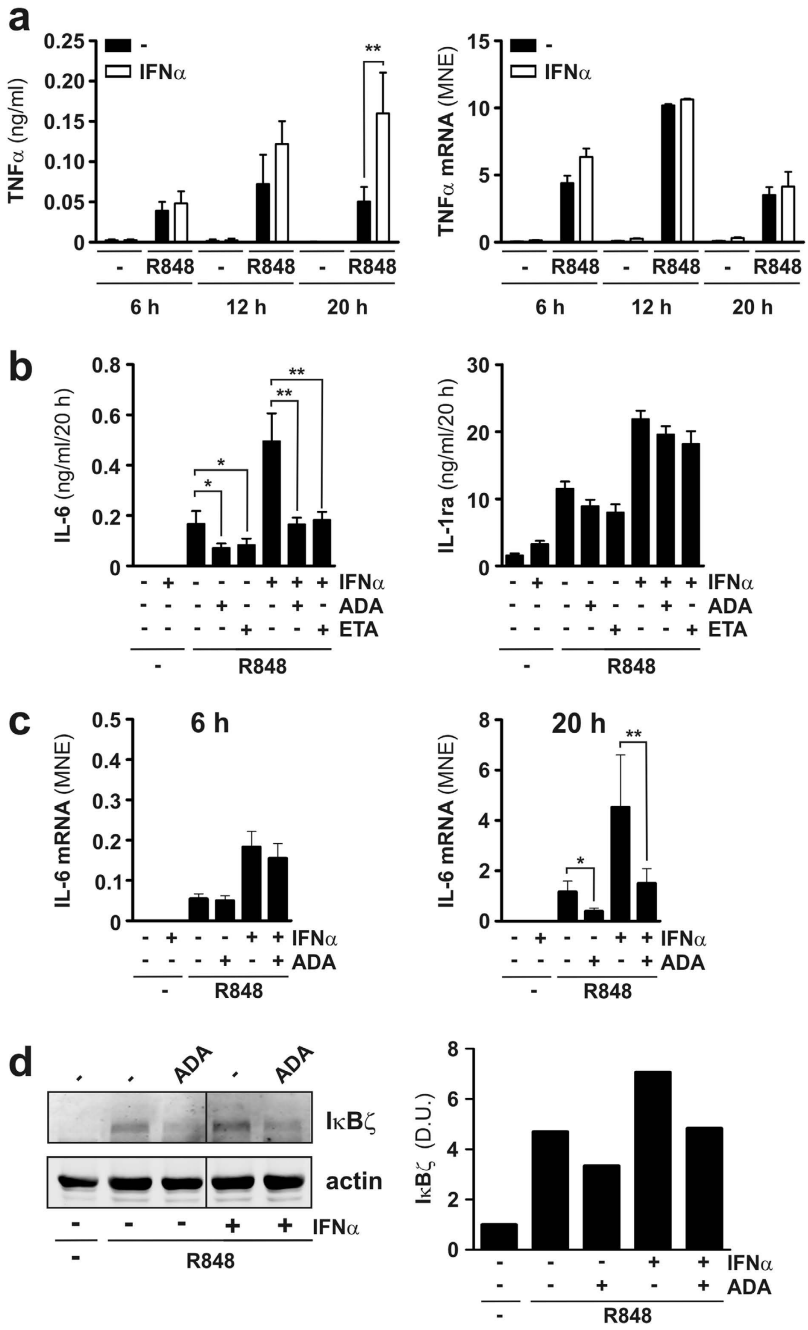


Figure 3. Role of endogenous TNFα in mediating the enhancing effect of IFNα on the production of IL-6 by R848-treated neutrophils. (a) Neutrophils (5×10^6/ml), isolated from the peripheral blood of healthy donors, were cultured with or without 5 μM R848, 1000 U/ml IFNα or IFNα plus R848 for 6, 12 and 20 h to evaluate: released (n = 3–11) (left panel), TNFα, as measured by ELISA, and TNFα mRNA expression (right panel), by RT-qPCR (n = 3–8). Asterisks indicate significant increase: $^{**}p < 0.01$, by 2-way ANOVA followed by Bonferroni's post-test. **(b)** IL-6 (left panel) and IL-1ra (right panel) levels in supernatants from neutrophils pretreated for 30 min with or without 10 μg/ml ADA or 10 μg/ml ETA and then incubated for further 20 h with 1000 U/ml IFNα and/or 5 μM R848. Values represent the mean ± SEM (n = 5). **(c)** IL-6 mRNA expression in neutrophils pretreated for 30 min with or without 10 μg/ml ADA and then incubated for further 6 (left panel) or 20 h (right panel) with 1000 U/ml IFNα and/or 5 μM R848. Values represent the means ± SEM (n = 5). In (**b,c**), asterisks indicate significant inhibition by ADA: $^{*}p < 0.05$, $^{**}p < 0.01$, by 1-way ANOVA followed by Tukey's post-test. (**d**) IκBζ antigen expression (by western blot analysis) in neutrophils pretreated for 30 min with or without 10 μg/ml ADA and then incubated for further 20 h with 1000 U/ml IFNα and/or 5 μM R848. Samples were run on the same gel, but lanes were noncontiguous. A representative experiment out of two independent ones with similar results is shown (left panel). The graph (right panel) illustrates the relative densitometric quantification of IκBζ levels (normalized by actin).

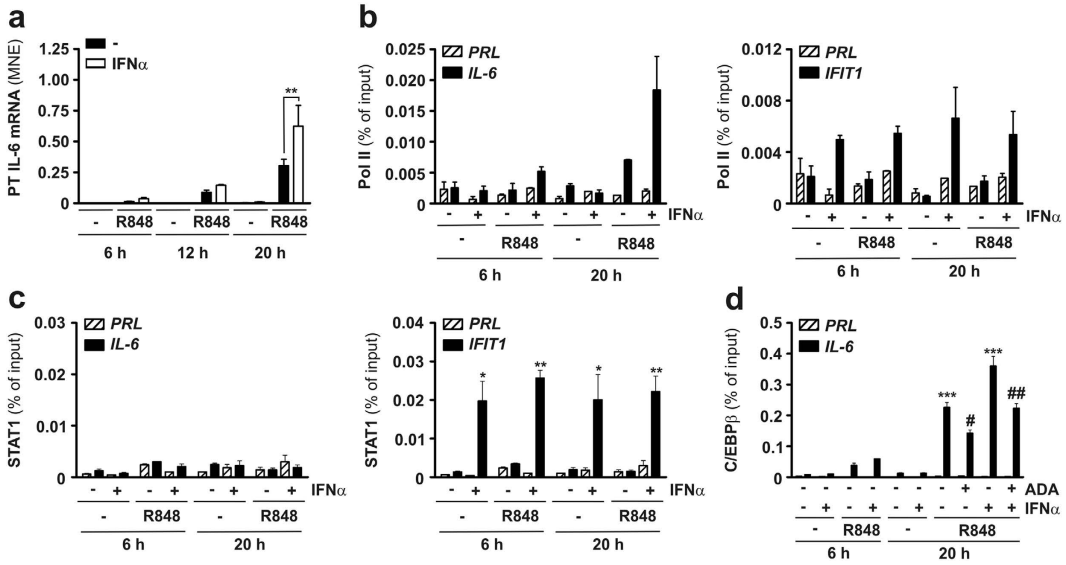


Figure 4. Effect of IFNα on Pol II, STAT1 and C/EBPβ recruitment to the IL-6 promoter in R848-treated neutrophils. (a) Neutrophils (5×10^6/ml), isolated from the peripheral blood of healthy donors, were cultured with or without 5 μM R848, 1000 U/ml IFNα or IFNα plus R848 for 6, 12 and 20 h to evaluate IL-6 primary transcript (PT) expression by RT-qPCR (n = 3–8). Asterisks indicate a significant increase: $^{**}p < 0.01$, by 2-way ANOVA followed by Bonferroni's post-test. Evaluation of Pol II (**b**) and STAT1 (**c**) binding to the *IL-6* (**b,c,** left panel) or to the *IFIT1* (**b,c,** right panel) promoters by ChIP analysis in neutrophils incubated for 6 h and 20 h with or without 1000 U/ml IFNα and/or 5 μM R848. **(d)** Neutrophils were pretreated for 30 min with or without 10 μg/ml ADA and then incubated for further 6 or 20 h with 1000 U/ml IFNα and/or 5 μM R848 to be processed for ChIP analysis using C/EBPβ Abs. In panels **b–d**, the co-immunoprecipitated DNA samples were amplified using specific primer pairs and expressed as percent of the total input. Panel **b** depicts a representative experiment out of two ones with similar results while values in panels **c,d** stand for the mean ± SEM (n = 3). Asterisks in panel (**c,d**) indicate a significant increase with respect to untreated cells while $^{\#}$ symbols indicate a significant inhibition exerted by ADA: * and $^{\#}p < 0.05$, ** and $^{\#\#}p < 0.01$ and $^{***}p < 0.001$, by 1-way ANOVA followed by Tukey's post-test.

(Fig. 3a), IFNα did not exert any additional prosurvival effect on top of that promoted by R848 alone (Fig. 5h). All in all, data demonstrate that R848-induced viability is partially dependent on endogenously produced TNFα. Data, however, exclude that IFNα amplifies the production of IL-6 by R848-treated neutrophils simply because it enhances neutrophil survival.

R848-treated neutrophils do not express type I IFNs but produce increased levels of IL-6 when coincubated with type II IFN. Next, we ruled out any autocrine action of potential endogenous type I IFN in regulating IL-6 production by TLR8-activated neutrophils. RT-qPCR experiments, in fact, failed to detect any mRNA accumulation for both IFNβ (Fig. 6a, left panel) and IFNα, in the latter case as measured using primers recognizing all IFNα transcripts ($IFN\alpha_{1-13}$, Fig. 6a, central panel), or specifically $IFN\alpha_2$ (Fig. 6a, right panel). Lack of IFNα production was also confirmed at the protein level, as revealed by ELISA testing supernatants harvested from neutrophils treated for 20h, not only with R848, but also with various CpG preparations (CpG-ODN 2006 and 2216) (Fig. 6b, left panel), which are known to activate TLR9[39]. As expected[39], pDCs incubated for 20 h with either CpG-ODN 2216, or R848, were found to release significant amounts of IFNα (**data not shown**). On the other hand, neutrophils released significant amounts of CXCL8 in response to CpG-ODN 2006 and R848 (Fig. 6b, right panel), indicating that they were properly activated.

In other experiments, and supporting previous findings obtained in murine macrophages[31], we observed that also type II IFN/IFNγ remarkably enhances the production of IL-6 by neutrophils incubated with R848. The latter phenomenon was observed to occur after 20 h of neutrophil incubation (Fig. 6c, left panel), concomitantly with an upregulation of IL-6 transcripts (Fig. 6d, left panel). Similarly to IFNα, while IFNγ did not exhibit any effect on CXCL8 mRNA expression (Fig. 6c, right panel) and protein production in R848-treated neutrophils (Fig. 6d, right panel), its effect on the increased IL-6 mRNA expression was accompanied by an enhanced C/EBPβ recruitment to the IL-6 promoter (Fig. 6e).

Production of IL-6 in response to TLR7 and/or TLR8 agonists by SLE neutrophils. In a final series of experiments, we investigated whether highly purified neutrophils from SLE patients with active disease (SLEDAI > 5, see Table 1 for patient characterization), thus constitutively displaying remarkably elevated levels of IFN-dependent genes, such as IFIT1, LGP2, IGS15, OASL and MDA5 (Fig. 7a), produce more IL-6 than neutrophils from healthy donors (HD) in response to TLR8 and/or TLR7 agonists. We found that SLE neutrophils treated with 5 μM R848 produced twice as much IL-6 than control neutrophils (Fig. 7b). SLE neutrophils

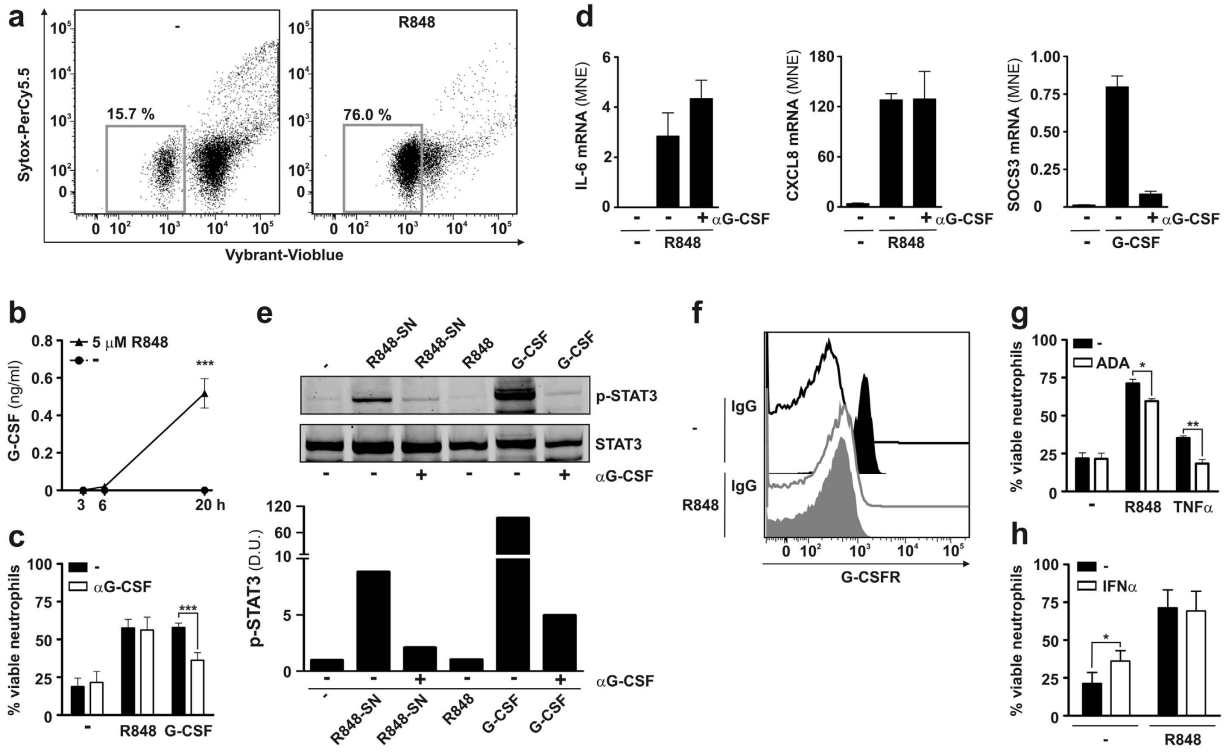


Figure 5. The enhanced viability of R848-treated neutrophils is partially dependent on endogenous TNFα, but not G-CSF. (**a**) Neutrophils were cultured with or without 5 μM R848 for up to 20 h, to be processed for viability evaluation by flow cytometry analysis. The percentage of alive cells was defined as Vybrant/Sytox double negative cell population (grey boxes). **(b)** G-CSF levels in supernatants from neutrophils cultured with or without 5 μM R848 for up to 20 h. (**c,g,h**), viability of neutrophils after culture for 20 h with 5 μM R848 (**c,g,h**), 1000 U/ml G-CSF (**c**), 10 ng/ml TNFα (**g**), in the presence or not of, respectively, 10 μg/ml mAbs neutralizing G-CSF (**c**), 10 μg/ml ADA (**g**), or 1000 U/ml IFNα (**h**). Data are expressed as mean ± SEM (n = 3 for panel **c**, n = 6 for panels **g** and **h**), while asterisks indicate a significant inhibition (for panels **c,g**) or increase (for panel **h**), as it follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by 2-way ANOVA followed by Bonferroni's post-test. **(d)** IL-6, CXCL8 and SOCS3 mRNA expression in neutrophils pretreated for 30 min with or without 10 μg/ml neutralizing G-CSF mAbs and then incubated for further 20 h with 5 μM R848 or 1000 U/ml G-CSF. Error bars represent the SEM calculated from triplicate qPCR reactions. A representative experiment out of three independent ones with similar results is shown. **(e)** STAT3 tyrosine phosphorylation in freshly isolated neutrophils, either untreated or cultured for 60 min with 5 μM R848, 1000 U/ml G-CSF or supernatants from allogenic neutrophils incubated for 20 h with 5 μM R848 (R848-SN), in the presence or absence of 10 μg/ml neutralizing G-CSF mAbs (representative experiment, n = 2). The graph in the lower panel displays the relative densitometric quantification of p-STAT3 levels (normalized by total STAT3). **(f)** Neutrophils cultured for 20 h with or without 5 μM R848 were analysed by flow cytometry for G-CSFR membrane expression using an anti-G-CSFR (filled histogram) or matched isotype control (empty histogram) mAbs.

also released significantly more TNFα in response to R848 (Fig. 7b), and, interestingly, showed a tendency to release more CXCL8 than HD neutrophils (Fig. 7b). However, no increase of IL-1ra could be observed under the same experimental conditions (Fig. 7b), in line with the proinflammatory status of these SLE neutrophils[40]. By contrast, SLE neutrophils neither produced IL-6 or TNFα, nor significantly upregulated their IL-1ra production in response to 5–50 μM R837 (Fig. 7b), in accordance with their lack of TLR7 mRNA expression (Fig. 7a). Nonetheless, CXCL8 was released in a dose-dependent manner by both control and SLE neutrophils, yet at similar levels (Fig. 7b). Finally, no IFNα expression/production was observed under R848 or R837 stimulation (**data not shown**). The fact that neutrophils isolated from patients with active SLE – thus constitutively displaying the so-called IFN signature (Fig. 7a) – produce higher levels of both IL-6 and TNFα in response to R848 than HD neutrophils is consistent with the *in vitro* effects of IFNα on the same cytokines (Figs 1a and 3a). It is also plausible that factors other than type I IFN might control the cytokine-producing capacity of neutrophils in SLE patients, as suggested by their different behavior to release CXCL8 and IL-1ra in response to R848 as compared to HD neutrophils incubated with IFNα and R848.

Discussion

IL-6 is a multifunctional cytokine involved in regulation of the immune system. As a potent pro-inflammatory cytokine, IL-6 plays a pivotal role in host defense against pathogens and acute stress[41]. Nonetheless, IL-6 plays

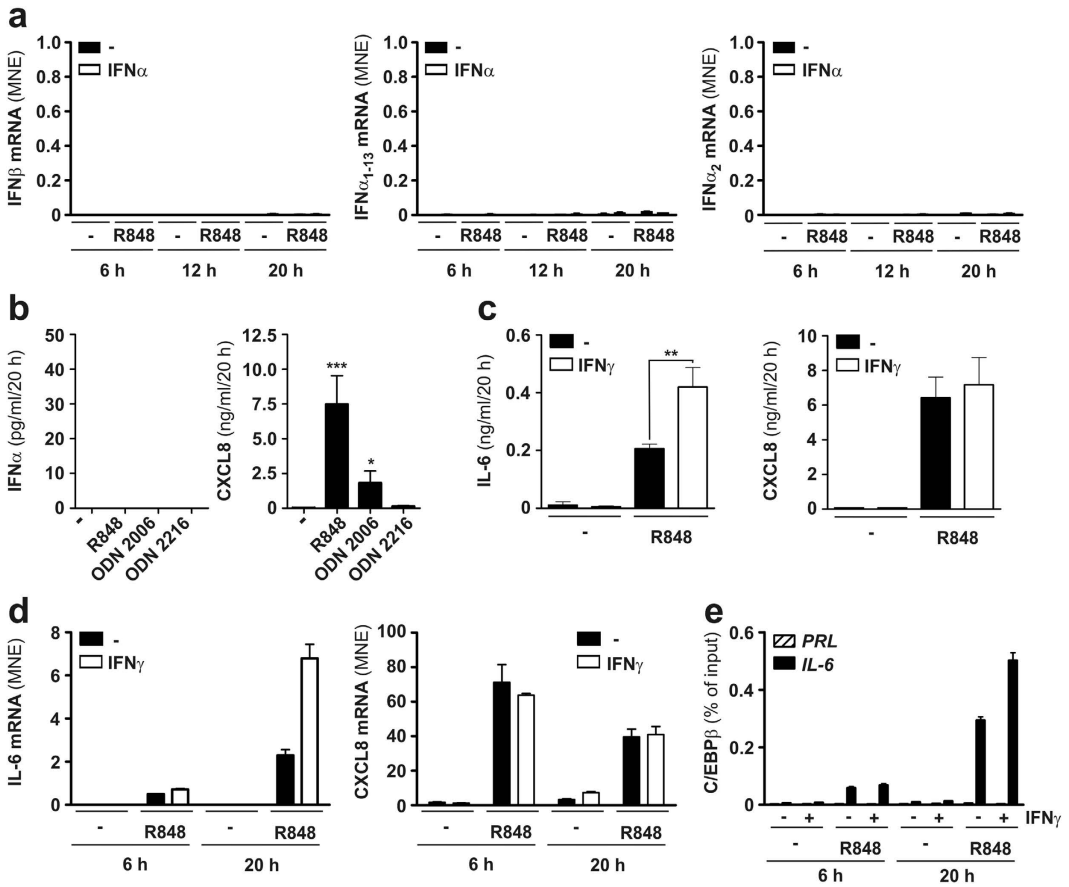


Figure 6. R848-treated neutrophils do not express type I IFN but produce higher levels of IL-6 when coincubated with IFNγ. (**a**) Neutrophils were cultured with or without 5 μM R848, 1000 U/ml IFNα or IFNα plus R848 for 6, 12 and 20 h to evaluate IFNβ, IFNα_{1-13} and IFNα_2 mRNA expression by RT-qPCR (n = 3–8). In panel (**b**) neutrophils were cultured with or without 5 μ μM R848, 2 μM CpG ODN 2006 and 2 μM CpG ODN 2216 for 20 h and then IFNα (left panel) and CXCL8 (right panel) release were measured by specific ELISA. In panel (**c**–**e**) neutrophils were cultured with or without 5 μM R848, 100 U/ml IFNγ or IFNγ plus R848 for 20 h (**c**), or 6 and 20 h (**d**,**e**), to evaluate: i) IL-6 (**c**, left panel) and CXCL8 (**c**, right panel) production by ELISA; ii) IL-6 (**d**, left panel) and CXCL8 (**d**, right panel) mRNA expression by RT-qPCR; iii) C/EBPβ binding to the IL-6 and PRL promoters by ChIP analysis (**e**). Panels (**d**,**e**) depict a representative experiment out of two ones with similar results. ELISA values in panel (**b**,**c**) stand for the mean ± SEM (n = 5). Asterisks indicate a significant increase over control (**b**), or enhancement by IFNγ (**c**): *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by 1-way ANOVA (**b**) or 2-way ANOVA (**c**).

Patient code	Age	Ethnicity[a]	Gender	Disease state (SLEDAI)	Symbol[b]
SLE # **1**	36	C	F	6	Δ
SLE # **2**	68	C	F	8	o
SLE # **3**	23	C	F	5	□
SLE # **4**	27	H	F	14	◊
SLE # **5**	48	C	F	12	∇
SLE # **6**	42	C	F	11	*

Table 1. Characteristics of SLE patients. [a]H = Hispanic; C = Caucasian. [b]Symbols refer to Fig. 7a.

also a role in the pathogenesis of inflammatory and autoimmune diseases[41]. Recently, we demonstrated that upon activation of TLR8 by specific imidazoquinolines exerting antiviral activities, including R848 and CL075, neutrophils display the capacities to produce IL-6 in biologically active amounts[8]. Since neutrophils outnumber other immune cells under diverse inflammatory conditions, a detailed knowledge on how their production of IL-6 is regulated is of notable interest. In such regard, very crucial cytokines that, amongst others, modulate cytokine expression of immune cell and consequently also innate immune responses, are type I IFNs[15]. While being protective during acute viral infections, type I IFNs can also have deleterious roles in bacterial infections and

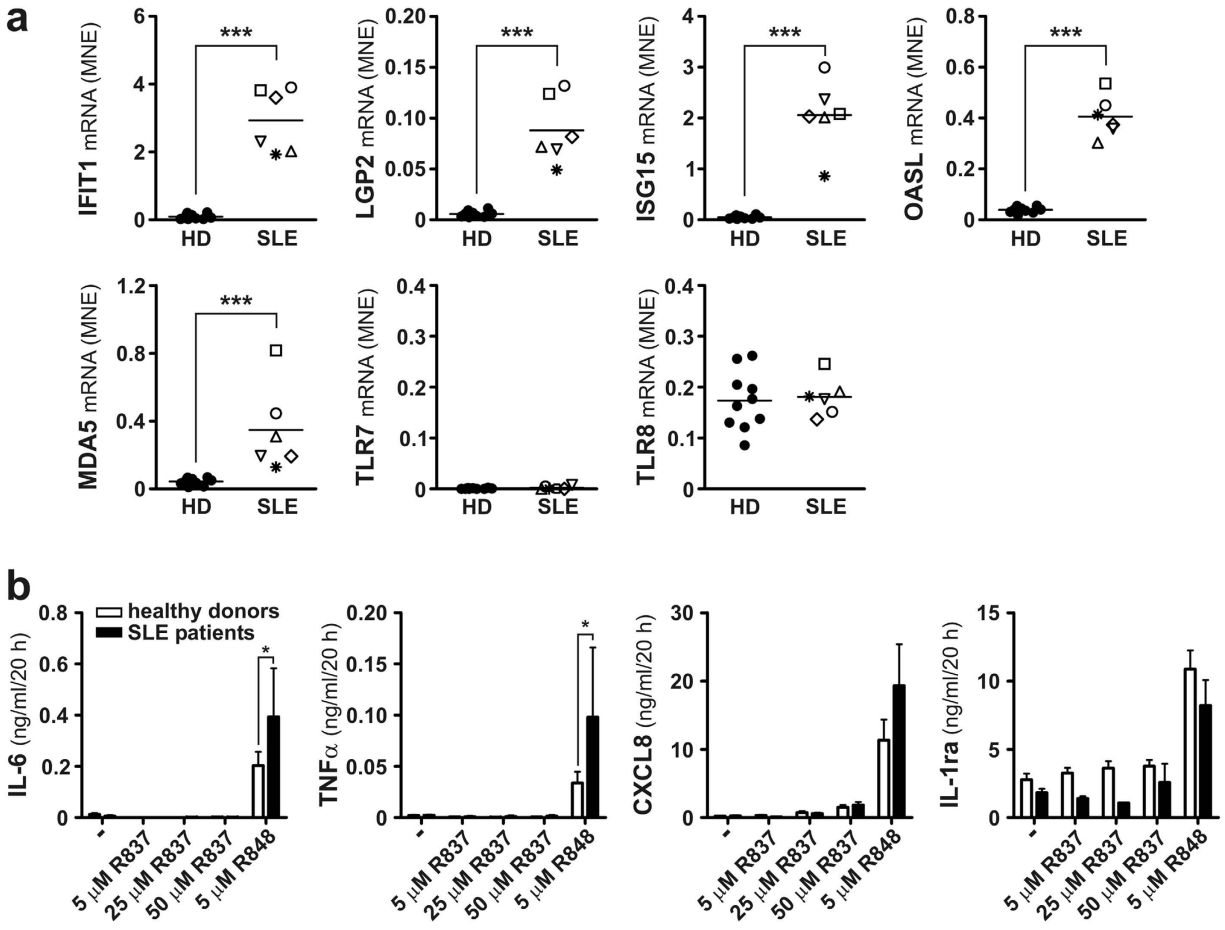


Figure 7. Gene expression profile and IL-6, TNFα, CXCL8 and IL-1ra production by neutrophils isolated from SLE patients. (**a**) IFIT1, LGP2, ISG15, OASL, MDA5, TLR7 and TLR8 mRNA expression in neutrophils freshly isolated from the peripheral blood of SLE patients with high SLEDAI (n = 6, each symbol identifying a different patient, see Table 1) and healthy donors (n = 10). Asterisks (***$p < 0.001$, by Student's t test) indicate a significant difference between the two groups. (**b**) Amounts of IL-6, TNFα, CXCL8 and IL-1ra produced by peripheral neutrophils isolated from SLE patients (n = 6) and healthy donors (n = 6) cultured for 20 h in the presence of 5 μM R848 and 5–50 μM R837. Asterisks indicate a significant increase: *$p < 0.05$, by 2-way ANOVA followed by Bonferroni's post-test.

autoimmune diseases[42], including pathologies in which neutrophils are involved, such as sepsis[43], pediatric SLE[25] and rheumatoid arthritis[44]. In this study, we show that TLR8-activated neutrophils produce approximately three times more IL-6 when cultured for 20 h in the presence of IFNα than in its absence, a phenomenon controlled at the level of both mRNA transcription and accumulation. Similarly, we report that neutrophils isolated from SLE patients with active disease produce significantly higher levels of IL-6 than neutrophils from healthy donors, when stimulated *in vitro* with R848 for 20 h. Because neutrophils from SLE patients displayed a strong "IFN signature", it is tempting to speculate that their increased capacity to produce IL-6 likely depends on previous *in vivo* exposures to circulating type I IFN, thus consistent with *in vitro* experiments. Instead, no direct effect of IFNα on IL-6 gene expression could be detected. Also type II IFN/IFNγ was found to remarkably enhance the production of IL-6 by neutrophils treated with R848, further highlighting the capacity of these cells to fully respond to the interferon-induced signals during viral and autoimmune diseases.

In the attempt to clarify the molecular bases of such an IFNα-dependent enhancement of neutrophil-derived IL-6 we could exclude that IFNα does so simply by increasing the viability of neutrophils. In fact, even though we confirmed that TLR8 activation potently prolongs the survival of neutrophils[12,33–35], viability of neutrophils incubated in the presence of both IFNα and R848 did not differ from that measured in neutrophils incubated in the presence of R848 only. Notably, under the latter experimental conditions, neutrophils were found to release remarkable amounts of biologically active G-CSF, as demonstrated by the capacity of supernatants harvested from R848-treated neutrophils to trigger a G-CSF-dependent STAT3 phosphorylation in heterologous neutrophils. However, contrary to our expectations, endogenous G-CSF was ineffective in R848-treated neutrophils, as surface G-CSFRs were completely downregulated. The biological meaning of such a G-CSFR disappearance in R848-treated neutrophils is unknown, but it has been observed to occur also in LPS-, TNFα-, fMLF- and C5a-treated cells[45] as well as *in vivo,* after intravenous injection of LPS[46]. By contrast, we uncovered that the

extended survival of R848-treated neutrophils partially depends on endogenous TNFα. Whether the enhanced viability mediated by endogenous TNFα also helps to sustain IL-6 production in R848-treated neutrophils remains to be demonstrated.

We also excluded that the IFNα-dependent enhancement of neutrophil-derived IL-6 is caused by an induction of TLR7 and its consequent co-activation with TLR8 in response to R848. Under our experimental conditions, neither neutrophils from healthy donors incubated with IFNα, nor SLE neutrophils, were found to express TLR7 or respond to the TLR7-specific agonist R837 in terms of IL-6 production. These findings are in partial contradiction with the results of a previous publication reporting that neutrophils isolated from juvenile SLE patients express TLR7 mRNA, as well as that, *in vitro*, 1000 U/ml IFNα could induce TLR7 mRNA expression in neutrophils from HDs[25]. While it should be kept in mind that juvenile and adult SLE are two clinically distinct diseases[47], in which circulating neutrophils may be likely exposed to different mediators and, eventually, function diversely, other factors might explain the differences between our results and those by Garcia-Romo and collegues[25]. For instance, knowing that PBMCs express discrete levels of TLR7[48], it is plausible to hypothesize that potential contaminating PBMCs might have influenced the data outcome in the work by Garcia-Romo and collegues[25]; in fact, while neutrophils are isolated at a purity level greater than 99.7% in our hands[8,49], in the study by Garcia-Romo and collegues[25] neutrophils were stated greater than 98 % pure. On the same line, other studies, in which no precaution for completely removing all possible contaminating leukocytes were undertaken, have reported that resting neutrophils isolated from the blood could express low levels of TLR7 mRNA[33,50,51]. By contrast, no TLR7 mRNA expression was detected when neutrophils were isolated to high levels of purity, in particular in terms of contaminating eosinophils[52,53]. How critical is the purity of neutrophils to obtain genuine and reliable results in the context of gene expression studies has been already evidenced[3,54].

Interestingly, we found that both neutrophils from healthy donors and SLE patients similarly respond to R837 in terms of CXCL8 production, but only if the TLR7 agonist was used at 25–50 μM. In this latter case, our observations confirm the results by Garcia-Romo *et al.*[25], who also showed that juvenile SLE neutrophils produce CXCL8 in response to 36 μM R837[25]. However, since in our experiments CXCL8 produced by R837-treated neutrophils was not abrogated by the pretreatment of neutrophils with BafilomycinA1, it is our opinion that it likely occurs *via* other, not yet identified, TLR7-independent mechanism. Our observations are, in any case, consistent with previous findings demonstrating that chemotaxis and H_2O_2 release induced by R837 in human neutrophils occur in an IRAK4-independent manner, thus without activating a canonical, TLR-activated MyD88-dependent signaling pathway[55]. In this study, we also demonstrate that the augmentation of IL-6 production by IFNα in R848-treated neutrophils largely coincides with an enhanced production and release of TNFα, which, in turn, appears to substantially mediate it. Even neutrophils from SLE patients with active disease were found to produce greater TNFα amounts than healthy controls in response to R848, further supporting our *in vitro* results on the effects of IFNα on TNFα expression as well. Consistent with the role of endogenous TNFα in mediating the effects of IFNα on IL-6 gene expression, we demonstrated an enhanced recruitment of C/EBPβ to the IL-6 promoter in IFNα plus R848-treated neutrophils[8]. Although the notion that endogenous TNFα may mediate the enhanced IL-6 production exerted by IFNα in TLR8-activated neutrophils is not so surprising at the light of our recently published data[8], it nonetheless emphasizes how important is TNFα for neutrophil physiopathology. This is further highlighted in studies showing that the interferon gene expression signature in neutrophils from rheumatoid arthritis patients correlates with a good response to anti-TNF therapy[44], once again indicating that IFN activity is mediated *via* TNFα induction.

Concomitantly, we failed to detect any expression of type I IFN in neutrophils incubated with IFNα and/or R848, thus excluding an autocrine action by endogenous type I IFN in regulating the production of IL-6. In our hands, lack of IFNα production was also observed in neutrophils treated with CpG-ODN 2006 and 2216, namely under experimental conditions previously shown by Lindau and colleagues[56] to induce neutrophil-derived IFNα at greater levels than R848. In the latter study[56], neutrophils were stated to be approximately 99.8 % pure, thus excluding the presence of contaminating cells likely producing IFNα. Moreover, that our neutrophil preparations were properly activated was demonstrated by the fact that they released significant amounts of CXCL8 in response to CpG-ODN 2006. Therefore, we do not know how to explain why we failed to detect type I IFN in neutrophils. One possible explanation is that we might have found, by chance, "non-responder donors", as also occurred to Lindau and colleagues themselves[56]. In summary, our data uncover that TLR8 ligands, IFNα and TNFα, three players often coexisting in many diseases of viral or autoimmune origin, promote a strong production of IL-6 in human neutrophils placing this cell type among potential targets for immunotherapeutic interventions.

Materials and Methods

Cell purification and culture. Granulocytes were isolated from buffy coats of healthy donors or, for selected experiments, from peripheral blood of SLE patients (see below) and as previously described, manipulated under endotoxin-free conditions[57]. Briefly, buffy coats or peripheral blood was centrifuged on Ficoll-Paque PLUS gradient (1078 g/ml density, GE Healthcare, Little Chalfont, United Kingdom) at 400 × *g* for 30 min, at room T, at a 1:1 ratio. Then, granulocytes were obtained by dextran sedimentation followed by hypotonic lysis of erythrocytes. Finally, 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, Canada)[8,22]. Neutrophils were then suspended at 5×10^6/ml in RPMI 1640 medium supplemented with 10 % low endotoxin FBS (<0.5 EU/ml; from BioWhittaker-Lonza, Basel, Switzerland), treated or not with 1000 U/ml pegylated IFNα-2a (Pegasys, Roche, Basel, Switzerland), 100 U/ml IFNγ (R&D Systems, Minneapolis, MN, USA) 5 μM R848, 5–50 μM R837, 2 μM CpG ODN 2006 and 2 μM CpG ODN 2216 (Invivogen, San Diego, CA, USA), 10 ng/ml TNFα (Peprotech, Rocky Hill, NJ, USA), 100 ng/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), and then 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. In selected experiments, neutrophils were preincubated for 30 min with 10 μg/ml adalimumab (Humira, Abbott Biotechnology Limited, Barceloneta, Puerto Rico), 10 μg/ml etanercept (Enbrel, Amgen, Thousand Oaks, CA, USA), or 5 μg/ml anti-human G-CSF (Clone 3316, R&D Systems), prior to stimulation. 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 assay types. 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. pDCs were isolated as previously described[58] using the BDCA-4 Diamond Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

ELISA. Cytokine concentrations in cell-free supernatants and cell-lysates were measured by commercially available ELISA kits, specific for human IL-6 (eBioscience, San Diego, CA, USA), TNFα (eBioscience), G-CSF (R&D Systems), IL-1ra (R&D Systems), CXCL8 (Mabtech, Nacka Strand, Sweden) and IFNα (Mabtech). Detection limits of these ELISA were: 8 pg/ml for IL-6 and IFNα, 4 pg/ml for TNFα, 40 pg/ml for IL-1ra, 16 pg/ml for G-CSF and CXCL8.

Flow cytometry. Phenotypic studies were performed exactly as previously described[8]. For G-CSFR staining, 10^5 neutrophils, incubated with or without 5 μM R848 for the indicated times, were harvested and stained for 15 min at room T with 1:20 PE anti-human CD114 (G-CSF-R) mAb (clone LMM741, Biolegend, San Diego, CA, USA), or with 1:20 PE control mouse IgG1 (BD Biosciences, San Jose, CA, USA). Then, cells were washed and stained for Vybrant DyeCycleTM (Life Technologies, Carlsbad, CA, USA) to discriminate cells that were alive, as described below. Sample fluorescence was measured by a seven-color MACSQuant Analyzer (Miltenyi Biotec), while data analysis was performed by using FlowJo software Version 8.8.6 (Tree Star, Ashland, OR, USA).

Neutrophil viability. After an overnight treatment with the agonists indicated above, 10^5 neutrophils were centrifuged at 300 × *g* for 5 min, medium removed and ultimately suspended in 100 μl HBSS buffer containing 10 nM Vybrant DyeCycleTM Violet stain (Life Technologies) and 5μM SYTOX AADvanced (Life Technologies). Cells were then put on ice for 30 min, protected from light. Cell viability was defined as the percentage of cells that were double negative for both stains (Vybrant/Sytox, respectively).

Reverse transcription quantitative real-time PCR (RT-qPCR). Total RNA was extracted from neutrophils after lysis by RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands), according to the manufacturer's instructions. 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. Purified total RNA was then reverse-transcribed into cDNA using Superscript III (Life Technologies) and random hexamer primers (Life Technologies) while qPCR was carried out using Fast SYBR® Green Master Mix (Life Technologies)[59]. Sequences of gene-specific primer pairs (Life Technologies) are available in the public database RTPrimerDB (www.rtprimerdb.org) under the following entry codes: GAPDH (3539), TNFα (3551), CXCL8 (3553), IL-6 (3545), PT-IL-6 (8687), $IFN\alpha_{1-13}$ (3541), $IFN\alpha_2$ (8955), IFN-β (3542), G-CSF (8615), SOCS3 (3828), TLR7 (8684), TLR8 (8685), IFIT1 (3540), MDA5 (3917), ISG15 (3547), LGP2 (3918) and OASL (3550). Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression (MNE) units after GAPDH normalization.

Immunoblots. Whole-cell proteins were recovered from protein-rich flow-through solutions after the first centrifugation step of the RNeasy Mini Kit (Qiagen) used for total RNA extraction, as previously described[8]. Proteins were then immunoblotted by standard procedures using the following antibodies: anti-IκBζ rabbit pAb (#9244) and anti-phospho-STAT3 (Tyr705) rabbit pAb (#9131) from Cell Signaling (Beverly, MA, USA); rabbit pAb anti-STAT3 (sc-482, Santa Cruz Biotechnology, Dallas, TX, USA); rabbit pAb anti-actin (A5060) from Sigma. Blotted proteins were detected and quantified by using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Densitometry values were calculated by subtracting, for each lane, the respective background levels.

Chromatin Immunoprecipitation (ChIP) experiments. ChIP experiments were performed exactly as previously described[8]. Briefly, nuclear extracts from 5×10^6 or 10^7 neutrophils (for ChIP targeting, respectively, H3K27Ac or STAT1, IRF1, C/EBPβ and PolII) were immunoprecipitated with 1 μl anti-H3K27Ac (ab4729) (Abcam, Cambridge, United Kingdom), 25 μl anti-STAT1 (sc-346), 25 μl anti-IRF1 (sc-497), 20 μl anti- C/EBPβ (sc-150), 20 μl anti-PolII (sc-899) (Santa Cruz Biotechnology). To establish the background levels of ChIP experiments, the precipitation signal was quantified also at the promoter of prolactin (*PRL*) since it is completely silent in myeloid cells. The coimmunoprecipitated material was subjected to qPCR analysis using the following promoter specific primers (purchased from Life Technologies): IL-6 forward: 5′-TAGCCTCAATGACGACCTAAG-3′; IL-6 reverse: 5′-GTGGGGCTGATTGGAAACCT-3′; IFIT1 forward: 5′-GGCAGCAATGGACTGATGTTC-3′; IFIT1 reverse: 5′-GGAAACCGAAAGGGGAAAGTG-3′; and PRL forward: 5′-AGGGAAACGAATGCCTGATT-3′; PRL reverse: 5′-GCAGGAAACACACTTCACCA-3′. Data from qPCR were expressed as percentage over input DNA and are displayed as means ± SEM.

Statistical analysis. Data are expressed as mean ± SE. Statistical evaluation was performed using 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. Values of $P < 0.05$ were considered as statistically significant.

Study approval. Human samples were obtained following informed written consent by both healthy donors and SLE patients. Clinical evaluation of SLE diseases activity was assessed by the SLEDAI[60] at the moment of the venipuncture. As reported in Table 1, only patients with moderate or severe flare (e.g., SLEDAI higher than 5) were recruited for our analysis. 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.

References

1. Scapini, P., Tamassia, N., Pucillo, C. & Cassatella, M. A. Granulocytes and Mast Cells. In: *Fundamental immunology* (eds Paul W. E.). 7th edn. Wolters Kluwer Health/Lippincott Williams & Wilkins (2013).
2. Scapini, P. & Cassatella, M. A. Social networking of human neutrophils within the immune system. *Blood* **124,** 710–719 (2014).
3. Tecchio, C., Micheletti, A. & Cassatella, M. A. Neutrophil-derived cytokines: facts beyond expression. *Front. Immunol.* **5,** 508 (2014).
4. Tamassia, N. *et al.* Activation of an immunoregulatory and antiviral gene expression program in poly(I:C)-transfected human neutrophils. *J. Immunol.* **181,** 6563–6573 (2008).
5. Tamassia, N. *et al.* IFN-beta Expression Is Directly Activated in Human Neutrophils Transfected with Plasmid DNA and Is Further Increased *via* TLR-4-Mediated Signaling. *J. Immunol.* **189,** 1500–1509 (2012).
6. Thomas, C. J. & Schroder, K. Pattern recognition receptor function in neutrophils. *Trends Immunol.* **34,** 317–328 (2013).
7. Tamassia, N. & Cassatella, M. A. Cytoplasmic receptors recognizing nucleic acids and mediating immune functions in neutrophils. *Curr. Opin. Pharmacol.* **13,** 547–554 (2013).
8. Zimmermann, M. *et al.* Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils. *Nat. Commun.* **6,** 6061 (2015).
9. Heil, F. *et al.* Species-specific recognition of single-stranded RNA *via* toll-like receptor 7 and 8. *Science* **303,** 1526–1529 (2004).
10. Yamamoto, M. *et al.* Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* **430,** 218–222 (2004).
11. Cassatella, M. A. Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* **73,** 369–509 (1999).
12. Lindemans, C. A. *et al.* Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. *J. Immunol.* **176,** 5529–5537 (2006).
13. Saitoh, T. *et al.* Neutrophil extracellular traps mediate a host defense response to human immunodeficiency virus-1. *Cell Host Microbe* **12,** 109–116 (2012).
14. Drescher, B. & Bai, F. Neutrophil in viral infections, friend or foe? *Virus Res.* **171,** 1–7 (2013).
15. Trinchieri, G. Type I interferon: friend or foe? *J. Exp. Med.* **207,** 2053–2063 (2010).
16. Scheel-Toellner, D. *et al.* Cytokine-mediated inhibition of apoptosis in non-transformed T cells and neutrophils can be dissociated from protein kinase B activation. *Eur. J. Immunol.* **32,** 486–493 (2002).
17. Gasperini, S. *et al.* Gene expression and production of the monokine induced by IFN-gamma (MIG), IFN-inducible T cell alpha chemoattractant (I-TAC), and IFN-gamma-inducible protein-10 (IP-10) chemokines by human neutrophils. *J. Immunol.* **162,** 4928–4937 (1999).
18. Cassatella, M. A. *et al.* Interferon-activated neutrophils store a TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) intracellular pool that is readily mobilizable following exposure to proinflammatory mediators. *J. Leukoc. Biol.* **79,** 123–132 (2006).
19. Harcourt, J. L. & Offermann, M. K. Interferon-alpha synergistically enhances induction of interleukin-6 by double stranded RNA in HeLa cells. *Eur. J. Biochem.* **267,** 2768–2777 (2000).
20. Ito, N. *et al.* Induction of interleukin-6 by interferon alfa and its abrogation by a serine protease inhibitor in patients with chronic hepatitis C. *Hepatology* **23,** 669–675 (1996).
21. Hall, J. C. & Rosen, A. Type I interferons: crucial participants in disease amplification in autoimmunity. *Nat. Rev. Rheumatol.* **6,** 40–49 (2010).
22. Pelletier, M. *et al.* Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood* **115,** 335–343 (2010).
23. Chebath, J., Merlin, G., Metz, R., Benech, P. & Revel, M. Interferon-induced 56,000 Mr protein and its mRNA in human cells: molecular cloning and partial sequence of the cDNA. *Nucleic Acids Res.* **11,** 1213–1226 (1983).
24. Hemmi, H. *et al.* Small anti-viral compounds activate immune cells *via* the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* **3,** 196–200 (2002).
25. Garcia-Romo, G. S. *et al.* Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci. Transl. Med.* **3,** 73ra20 (2011).
26. Hornung, V. *et al.* Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J. Immunol.* **168,** 4531–4537 (2002).
27. Lund, J. M. *et al.* Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc. Natl. Acad. Sci. USA* **101,** 5598–5603 (2004).
28. Tracey, D., Klareskog, L., Sasso, E. H., Salfeld, J. G. & Tak, P. P. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol. Ther.* **117,** 244–279 (2008).
29. Matsusaka, T. *et al.* Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl. Acad. Sci. USA* **90,** 10193–10197 (1993).
30. Meda, L., Gasperini, S., Ceska, M. & Cassatella, M. A. Modulation of proinflammatory cytokine release from human polymorphonuclear leukocytes by gamma interferon. *Cell. Immunol.* **157,** 448–461 (1994).
31. Qiao, Y. *et al.* Synergistic activation of inflammatory cytokine genes by interferon-gamma-induced chromatin remodeling and toll-like receptor signaling. *Immunity* **39,** 454–469 (2013).
32. Witko-Sarsat, V., Pederzoli-Ribeil, M., Hirsch, E., Sozzani, S. & Cassatella, M. A. Regulating neutrophil apoptosis: new players enter the game. *Trends Immunol.* **32,** 117–124 (2011).
33. Nagase, H. *et al.* Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand. *J. Immunol.* **171,** 3977–3982 (2003).
34. Hoarau, C. *et al.* TLR9 activation induces normal neutrophil responses in a child with IRAK-4 deficiency: involvement of the direct PI3K pathway. *J. Immunol.* **179,** 4754–4765 (2007).
35. Radsak, M. P., Salih, H. R., Rammensee, H. G. & Schild, H. Triggering receptor expressed on myeloid cells-1 in neutrophil inflammatory responses: differential regulation of activation and survival. *J. Immunol.* **172,** 4956–4963 (2004).
36. Tecchio, C. *et al.* IFNalpha-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. *Blood* **103,** 3837–3844 (2004).
37. Sakamoto, E. *et al.* Type I and type II interferons delay human neutrophil apoptosis *via* activation of STAT3 and up-regulation of cellular inhibitor of apoptosis 2. *J. Leukoc. Biol.* **78,** 301–309 (2005).
38. Colotta, F., Re, F., Polentarutti, N., Sozzani, S. & Mantovani, A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80,** 2012–2020 (1992).
39. Kerkmann, M. *et al.* Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type IIFN synthesis in human plasmacytoid dendritic cells. *J. Immunol.* **170,** 4465–4474 (2003).

40. Bennett, L. *et al.* Interferon and granulopoiesis signatures in systemic lupus erythematosus blood. *J. Exp. Med.* **197,** 711–723 (2003).
41. Hunter, C. A. & Jones, S. A. IL-6 as a keystone cytokine in health and disease. *Nat. Immunol.* **16,** 448–457 (2015).
42. Ivashkiv, L. B. & Donlin, L. T. Regulation of type I interferon responses. *Nat. Rev. Immunol.* **14,** 36–49 (2014).
43. Kelly-Scumpia, K. M. *et al.* Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10. *J. Exp. Med.* **207,** 319–326 (2010).
44. Wright, H. L., Moots, R. J. & Edwards, S. W. The multifactorial role of neutrophils in rheumatoid arthritis. *Nat. Rev. Rheumatol.* **10,** 593–601 (2014).
45. Khwaja, A., Carver, J., Jones, H. M., Paterson, D. & Linch, D. C. Expression and dynamic modulation of the human granulocyte colony-stimulating factor receptor in immature and differentiated myeloid cells. *Br. J. Haematol.* **85,** 254–259 (1993).
46. Dekkers, P. E. *et al.* Granulocyte colony-stimulating factor receptors on granulocytes are down-regulated after endotoxin administration to healthy humans. *J. Infect. Dis.* **181,** 2067–2070 (2000).
47. Hoffman, I. E. *et al.* Juvenile-onset systemic lupus erythematosus: different clinical and serological pattern than adult-onset systemic lupus erythematosus. *Ann. Rheum. Dis.* **68,** 412–415 (2009).
48. Gorden, K. B. *et al.* Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J. Immunol.* **174,** 1259–1268 (2005).
49. Davey, M. S. *et al.* Failure to detect production of IL-10 by activated human neutrophils. *Nat. Immunol.* **12,** 1017–1018 (2011).
50. Hayashi, F., Means, T. K. & Luster, A. D. Toll-like receptors stimulate human neutrophil function. *Blood* **102,** 2660–2669 (2003).
51. Gaudreault, E. & Gosselin, J. Leukotriene B4 potentiates CpG signaling for enhanced cytokine secretion by human leukocytes. *J. Immunol.* **183,** 2650–2658 (2009).
52. Berger, M. *et al.* Neutrophils express distinct RNA receptors in a non-canonical way. *J. Biol. Chem.* **287,** 19409–19417 (2012).
53. Janke, M. *et al.* Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. *J. Allergy Clin. Immunol.* **123,** 1026–1033 (2009).
54. Scapini, P., Calzetti, F. & Cassatella, M. A. On the detection of neutrophil-derived vascular endothelial growth factor (VEGF). *J. Immunol. Methods* **232,** 121–129 (1999).
55. van Bruggen, R. *et al.* Toll-like receptor responses in IRAK-4-deficient neutrophils. *J. Innate Immun.* **2,** 280–287 (2010).
56. Lindau, D. *et al.* TLR9 independent interferon alpha production by neutrophils on NETosis in response to circulating chromatin, a key lupus autoantigen. *Ann. Rheum. Dis.* **73,** 2199–2207 (2014).
57. Cassatella, M. A., Guasparri, I., Ceska, M., Bazzoni, F. & Rossi, F. Interferon-gamma inhibits interleukin-8 production by human polymorphonuclear leucocytes. *Immunology* **78,** 177–184 (1993).
58. Finotti, G., Tamassia, N., Calzetti, F., Fattovich, G. & Cassatella, M. A. Endogenously produced TNF-alpha contributes to the expression of CXCL10/IP-10 in IFN-lambda3-activated plasmacytoid dendritic cells. *J. Leukoc. Biol.* (2015).
59. Tamassia, N., Cassatella, M. A. & Bazzoni, F. Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils. *Methods Mol. Biol.* **1124,** 451–467 (2014).
60. Bencivelli, W. *et al.* Disease activity in systemic lupus erythematosus: report of the Consensus Study Group of the European Workshop for Rheumatology Research III. Development of a computerised clinical chart and its application to the comparison of different indices of disease activity. The European Consensus Study Group for Disease Activity in SLE. *Clin. Exp. Rheumatol.* **10,** 549–554 (1992).

Acknowledgements

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC, IG-1545) to M.A.C. and F.A.-S. is supported by Brazilian fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Author Contributions

M.Z., F.A.-S., F.B.-A., G.F., F.C. and N.T. performed the experiments, M.Z., P.S. and N.T. analysed the results, C.L. provided patient samples, M.Z., N.T. and M.A.C. conceived the experiments and wrote the paper.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zimmermann, M. *et al.* IFNα enhances the production of IL-6 by human neutrophils activated *via* TLR8. *Sci. Rep.* **6**, 19674; doi: 10.1038/srep19674 (2016).

Manuela Baronio, PhD[a]
Giacomo Tampella, PhD[a]
Jos W. M. van der Meer, MD[c]
Raffaele Badolato, MD, PhD[a]
Manfred Fliegauf, PhD[d]
Alessandro Plebani, MD, PhD[a]

From [a]the Department of Clinical and Experimental Sciences, Pediatrics Clinic and Institute for Molecular Medicine A. Nocivelli, University of Brescia, Spedali Civili di Brescia, Italy; [b]the Institute for Molecular Medicine A. Nocivelli, and Department of Pathology, Laboratory of Genetic Disorders of Childhood, Department of Molecular and Translational Medicine, University of Brescia, Spedali Civili di Brescia, Italy; [c]the Department of Internal Medicine, Radboud University Medical Centre, Nijmegen, The Netherlands; and [d]the Center for Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Freiburg, Germany. E-mail: vlougarisbs@yahoo.com.

The research leading to these results has received funding from the European Community's Seventh Framework Programme FP7/2007-2013 under grant agreement no. 201549 (EURO-PADnet HEALTH-F2-2008-201549) and from the Italian Ministerial Grant GR-2010-2315762. The research leading to these results also received funding from the "Fondazione C. Golgi," Brescia, Italy, and the Jeffrey Modell Foundation. This study was supported by the German Federal Ministry of Education and Research (grant no. BMBF 01EO1303) and by E-Rare EURO-CMC (grant no. 01GM1502).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Gerondakis S, Grumont R, Gugasyan R, Wong L, Isomura I, Ho W, et al. Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models. Oncogene 2006;25:6781-99.
2. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell 1995;80:321-30.
3. Snapper CM, Zelazowski P, Rosas FR, Kehry MR, Tian M, Baltimore D, et al. B cells fron p50/NF-kappa B knockout mice have selective defects in proliferation, differentiation, germ-line CH transcription and Ig class switching. J Immunol 1996;156:183-91.
4. Chen K, Coonrod EM, Kumánovics A, Franks ZF, Durtschi JD, Margraf RL, et al. Germline mutations in NFKB2 implicate the noncanonical NF-kB pathway in the pathogenesis of common variable immunodeficiency. Am J Hum Genet 2013;93:812-24.
5. Lougaris V, Tabellini G, Vitali M, Baronio M, Patrizi O, Tampella G, et al. Defective natural killer-cell cytotoxic activity in NFKB2 mutated CVID-like disease. J Allergy Clin Immunol 2015;135:1641-3.
6. Fliegauf M, Bryant VL, Frede N, Slade C, Woon ST, Lehnert K, et al. Haploinsufficiency of the NF-kB1 subunit p50 in common variable immunodeficiency. Am J Hum Genet 2015;97:389-403.
7. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood 2008;111:77-85.
8. Wehr C, Eibel H, Masilamani M, Illges H, Schlesier M, Peter HH, et al. A new CD21low B cell population in the peripheral blood of patients with SLE. Clin Immunol 2004;113:161-71.
9. Claudio E, Saret S, Wang H, Siebenlist U. Cell-autonomous role for NF-kappa B in immature bone marrow B cells. J Immunol 2009;182:3406-13.
10. Gerondakis S, Siebenlist U. Roles of the NF-kappaB pathway in lymphocyte development and function. Cold Spring Harb Perspect Biol 2010;2:a000182.

Available online July 16, 2016.
http://dx.doi.org/10.1016/j.jaci.2016.05.045

The importance of being "pure" neutrophils

To the Editor:

The identification of polymorphonuclear neutrophils as cellular source of cytokines prompts researchers in the field to investigate whether the production of neutrophil-derived cytokines can be altered in human diseases.[1] For such a purpose, we developed a simple procedure that allows the isolation of more than 99.6% pure neutrophils (here defined as *Neu*) from either whole blood or buffy coats.[2] Our procedure includes a density gradient centrifugation step of whole blood/buffy coats over Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, United Kingdom), dextran sedimentation, and red cell osmotic lysis of the granulocyte pellet, and a final immunomagnetic negative selection step using the "EasySep Human Neutrophil Enrichment Kit"[2] (StemCell Technologies, Vancouver, British Columbia, Canada). By doing so, we have recently reported that *Neu* incubated with 5 μM R848 (a TLR8 ligand) produce IL-6 and TNF-α starting from 6 hours, and at maximal levels after an overnight incubation (up to 200 pg/mL/5×10^6 cells).[2] Under the same experimental conditions, however, *Neu* neither express mRNA encoding IL-10[2] or interferon-stimulated genes (ISGs) such as interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and ISG15,[E1] nor produce IL-10.[3]

Recently, 1-step kits specifically designed to guarantee a rapid isolation of "unstressed," highly pure, neutrophils from the blood, skipping any density gradient centrifugation step, have been released into the market. These kits include the "MACSxpress Neutrophil isolation kit" (Miltenyi Biotec, Bergisch Gladbach, Germany) and the "EasySep direct Human Neutrophil Isolation Kit," the resulting neutrophils defined here as *wbNeuM* and *wbNeuE*, respectively. To our surprise, both *wbNeuM* and *wbNeuE* incubated with R848 for 20 hours were found to express remarkable levels of IL-10, IFIT1, and ISG15 mRNA (Fig 1, *A*), as well as to release detectable amounts of IL-10 (Fig 1, *B*). Furthermore, although both *wbNeuM* and *wbNeuE* accumulated IFIT1 and ISG15 transcripts also upon incubation with 50 μg/mL poly(I:C) (a TLR3 ligand) for 20 hours (Fig 1, *A*), *Neu* did not, consistent with the notion that human neutrophils lack TLR3.[4,E2] R848-treated *wbNeuM* and *wbNeuE* were also induced to accumulate and release elevated levels of IL-6 mRNA (Fig 1, *A*) and IL-6 protein (Fig 1, *B*), respectively, already after 4 hours of incubation, unlike *Neu*.[2] Similarly, TNF-α and CXCL8 mRNA expression (Fig 1, *A*) and production (Fig 1, *B*) were much higher in *wbNeuM* and *wbNeuE* than in *Neu*. In contrast, *wbNeuM*, *wbNeuE*, and *Neu* did not show remarkable differences upon incubation with 10 ng/mL TNF-α, in terms of either CXCL8 mRNA accumulation and production, or other cytokine expression (Fig 1).

Given these unexpected results, we evaluated the precise purity of *wbNeuM*, *wbNeuE*, and *Neu* by flow cytometric analysis, using a panel of 13 markers allowing the identification of the most representative leukocyte types in the blood. *Neu* was confirmed to be 99.77% ± 0.15% pure,[2] whereas both *wbNeuM* and *wbNeuE*, although reaching a purity of 98.86% ± 0.25% and 97.46% ± 1.06%, respectively (confirming what is declared in their related kit datasheets), were found to be reproducibly contaminated by eosinophils and slan$^+$CD16$^+$-monocytes[5] (also known as slanDCs[E3]), the latter cells representing the majority of the *wbNeuM*- and *wbNeuE*-copurified CD16$^+$-monocytes (see Table E1 in this article's Online Repository at www.jacionline.org). However, eosinophil contamination was found irrelevant because neutrophils isolated by Ficoll-Paque followed by dextran sedimentation and erythrocyte lysis (here defined as *NeuF*), although containing 1.52% ± 1.12% eosinophils, did not differ from *Neu* in terms of CXCL8 production, IL-6 and TNF-α mRNA expression in response to R848, or IFIT1 and ISG15 mRNA expression in response to either R848 or poly(:C) (see Fig E1 in this article's Online Repository at

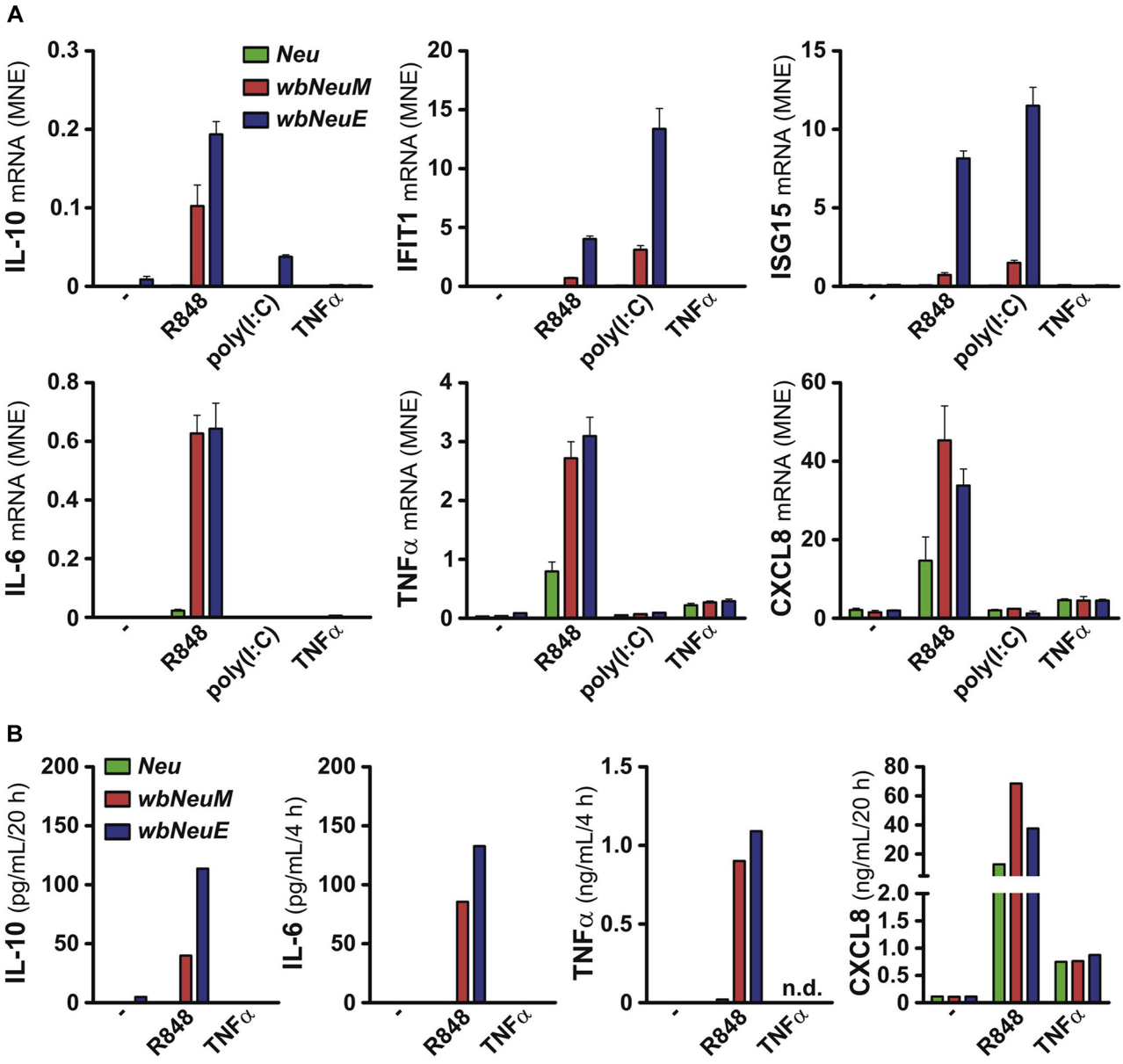


FIG 1. Patterns of gene expression and cytokine production by *Neu*, *wbNeuM*, and *wbNeuE* incubated with R848, poly(I:C), or TNF-α. *Neu, wbNeuM*, and *wbNeuE* were incubated with 5 μM R848, 50 μg/mL poly(I:C), or 10 ng/mL TNF-α for mRNA expression analysis by RT-quantitative PCR **(A)** and cytokine production by ELISA **(B)**. Analysis of IL-6 and TNF-α was performed after 4 hours of incubation, whereas that of CXCL8, IL-10, IFIT1, and ISG15 was performed after 20 hours. Panels *A* and *B* display representative experiments out of 3 performed with similar results. *MNE*, Mean normalized units; *n.d.*, not done.

www.jacionline.org). slan^{+}CD16^{+}-monocytes were consequently hypothesized as most likely responsible for the abnormal cytokine-producing response by *wbNeuM* and *wbNeuE*, given their well-known capacity to produce remarkably elevated amounts of cytokines when stimulated by R848 or other TLR ligands.[E4] Our prediction appeared to be correct as, after depletion of slan^{+}CD16^{+}-monocytes by saturating amounts of αM-DC8–linked microbeads (see Fig E2 in this article's Online Repository at www.jacionline.org), *wbNeuM* completely lost the capacity to produce IL-10 (Fig 2, *A*), as well to express IFIT1 and ISG15 mRNA in response to either R848 or poly(I:C) (Fig 2, *B*). The effect of slan^{+}CD16^{+}-monocyte-depletion was less effective in the case of *wbNeuE*, particularly for IFIT1 and ISG15 mRNA expression (Fig 2, *B*). Nonetheless, both *wbNeuM* and *wbNeuE* depleted of slan^{+}CD16^{+}-monocytes lost the capacity to express IL-6, and produced TNF-α at much lesser levels than did untouched *wbNeuM* and *wbNeuE*, after incubation with R848 for 4 hours (Fig 2, *A* and *B*). Notably, that slan^{+}CD16^{+}-monocytes, but not neutrophils, were the cells actually producing IL-10 in response to R848 was clearly evidenced by a specific "IL-10 secretion assay" showing that only slan^{+}CD16^{+}-monocytes stained positive for IL-10 within *wbNeuE* (see Fig E3 in this article's Online Repository at www.jacionline.org) or *wbNeuM* (data not shown).

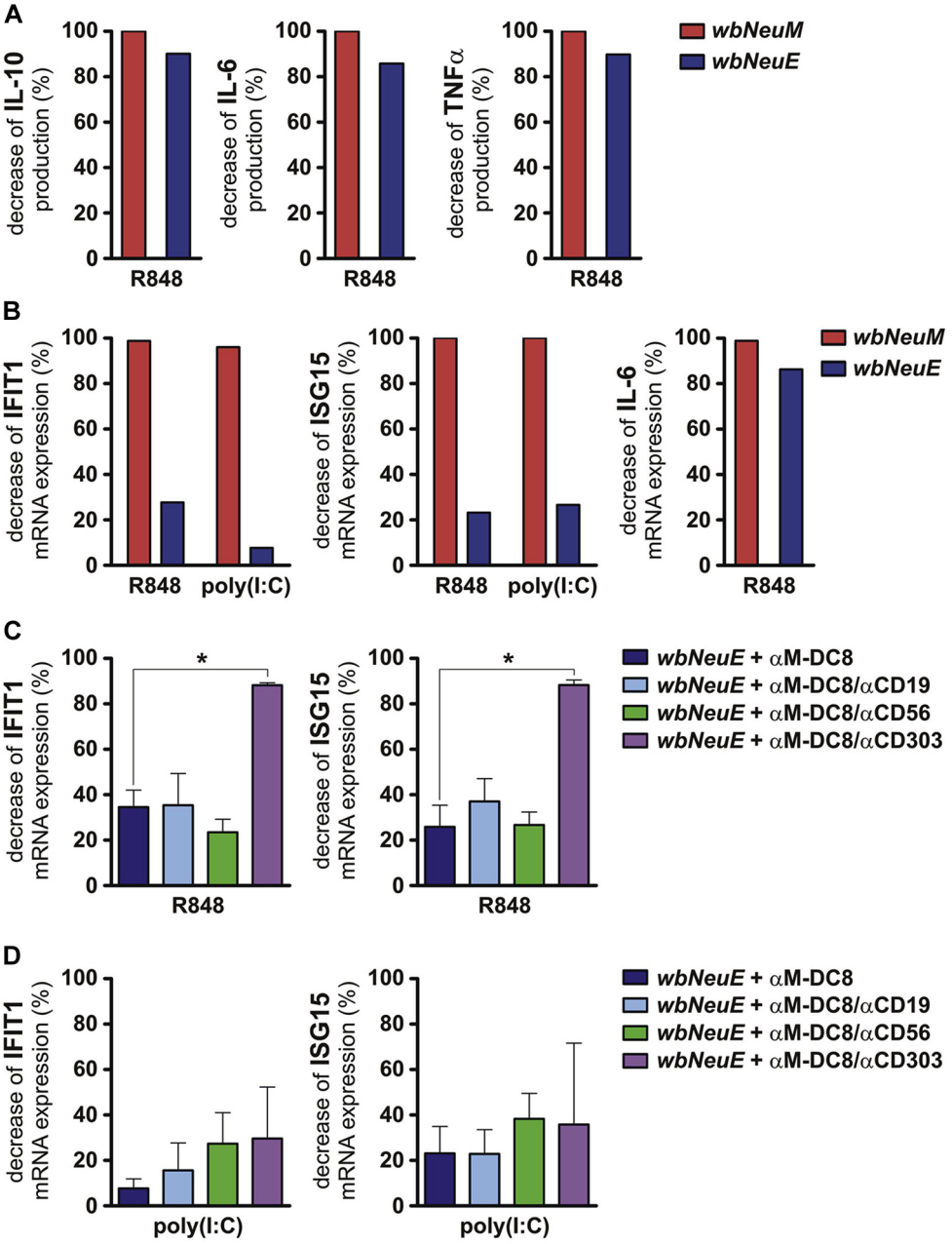


FIG 2. Effects of the removal of various contaminating leukocyte subtypes from *wbNeuM* and *wbNeuE*. Percentages of decrease in cytokine production **(A)** and gene expression **(B-D)**, observed in *wbNeuM* (Fig 2, *A* and *B*) and *wbNeuE* (Fig 2, *A-D*) depleted of slan$^+$CD16$^+$-monocytes, alone (Fig 2, *A* and *B*) (n = 2-5), or together with B cells, natural killer cells, or pDCs (Fig 2, *C* and *D*) (n = 3). Analysis of IL-6 and TNF-α mRNA expression/production was performed after 4 hours, whereas that of IL-10, IFIT1, and ISG15 was performed after 20 hours of incubation (Fig 2, *C* and *D*). *$P < .05$ by 1-way ANOVA followed by Tukey posttest.

To identify which leukocyte type(s), in addition to slan$^+$CD16$^+$-monocytes, could be responsible for IFIT1 and ISG15 mRNA expression in *wbNeuE* treated with either R848 or poly(I:C), we performed additional depletion experiments also targeting B cells, or natural killer cells, or plasmacytoid dendritic cells (pDCs) (see Fig E4 in this article's Online Repository at www.jacionline.org). As shown in Fig 2, *C*, depletion of both slan$^+$CD16$^+$-monocytes and pDCs from *wbNeuE* treated with R848 almost completely abolished the expression of IFIT1 and ISG15 mRNAs, consistent with the capacity of pDCs to produce IFN-α in response to R848 stimulation and, in turn, express ISGs.[E5] In the case of poly(I:C) stimulation, removal of slan$^+$CD16$^+$-monocytes together with either natural killer cells or pDCs slightly, but not significantly, decreased the expression of IFIT1 and ISG15 mRNAs in *wbNeuE* (Fig 2, *D*), whereas removal of slan$^+$CD16$^+$-monocytes together with B cells was irrelevant. The latter findings indicate that other leukocyte subtypes, or combination of them, are responsible

for IFIT1 and ISG15 mRNA induction in poly(I:C)-treated *wbNeuE*.

Taken together, our data demonstrate that the profile of cytokines derived from the stimulation of *wbNeuM* and *wbNeuE* may appear profoundly altered as a consequence of their contamination with very small percentages of slan^{+}CD16^{+}-monocytes (0.1% to 0.6%), alone or in combination with pDCs or other leukocyte subtypes. Such an inconvenience becomes, in fact, evident if *wbNeuM* and *wbNeuE* are stimulated with TLR ligands, but not with TNF-α. These findings support what we have been always recommending,[1,6] namely, that it is absolutely mandatory to use highly purified populations of neutrophils when gene expression and/or neutrophil-derived cytokines are investigated. Hence, based on the data presented here, the use of commercial kits aimed at rapidly purifying neutrophils is certainly advisable, provided that these kits are used after a round of blood density gradient centrifugation. Without this step, which ensures the elimination of most PBMCs from granulocytes, in turn favoring an optimal negative selection of contaminating cells by these specific kits, researchers may inadvertently obtain false-positive results: these may include the detection of either cytokines/genes made by contaminating cells,[7] or unusually high amounts of neutrophil-derived cytokines, as shown here or elsewhere.[8,9,E6] Moreover, although it has been unequivocally demonstrated that, in human neutrophils, the IL-10 locus is in an inactive state and cannot be remodeled in response to TLR ligands,[3] articles reporting the detection of IL-10 in supernatants from TLR-activated neutrophils[7] continue to be, since then, published. Given that *wbNeuM* or neutrophils with unascertained purity were used in these reports,[7] caution should be undertaken on their reliability based on the data presented here.

Federica Calzetti, BS
Nicola Tamassia, PhD
Fabio Arruda-Silva, MS
Sara Gasperini, PhD
Marco A. Cassatella, MD, PhD

From the Department of Medicine, Section of General Pathology, University of Verona, Verona, Italy. E-mail: marco.cassatella@univr.it.

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (grant no. IG-15454) to M.A.C. F.A.-S. is supported by a Brazilian fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior.

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Scapini P, Cassatella MA. Social networking of human neutrophils within the immune system. Blood 2014;124:710-9.
2. Zimmermann M, Aguilera FB, Castellucci M, Rossato M, Costa S, Lunardi C, et al. Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils. Nat Commun 2015;6:6061.
3. Tamassia N, Zimmermann M, Castellucci M, Ostuni R, Bruderek K, Schilling B, et al. Cutting edge: an inactive chromatin configuration at the IL-10 locus in human neutrophils. J Immunol 2013;190:1921-5.
4. Tamassia N, Le Moigne V, Rossato M, Donini M, McCartney S, Calzetti F, et al. Activation of an immunoregulatory and antiviral gene expression program in poly(I: C)-transfected human neutrophils. J Immunol 2008;181:6563-73.
5. Hofer TP, Zawada AM, Frankenberger M, Skokann K, Satzl AA, Gesierich W, et al. slan-defined subsets of CD16-positive monocytes: impact of granulomatous inflammation and M-CSF receptor mutation. Blood 2015;126:2601-10.
6. Scapini P, Calzetti F, Cassatella MA. On the detection of neutrophil-derived vascular endothelial growth factor (VEGF). J Immunol Methods 1999;232:121-9.
7. Lewkowicz N, Mycko MP, Przygodzka P, Cwiklinska H, Cichalewska M, Matysiak M, et al. Induction of human IL-10-producing neutrophils by LPS-stimulated Treg cells and IL-10. Mucosal Immunol 2016;9:364-78.
8. Naegelen I, Plançon S, Nicot N, Kaoma T, Muller A, Vallar L, et al. An essential role of syntaxin 3 protein for granule exocytosis and secretion of IL-1α, IL-1β, IL-12b, and CCL4 from differentiated HL-60 cells. J Leukoc Biol 2015;97:557-71.
9. Gaber T, Hahne M, Strehl C, Hoff P, Dorffel Y, Feist E, et al. Disentangling the effects of tocilizumab on neutrophil survival and function. Immunol Res 2016;64:665-76.

Available online July 25, 2016.
http://dx.doi.org/10.1016/j.jaci.2016.06.025

Toluene downregulates filaggrin expression via the extracellular signal-regulated kinase and signal transducer and activator of transcription–dependent pathways

To the Editor:

Volatile organic compounds (VOCs) can be an important environmental risk factor in the development and/or aggravation of atopic dermatitis (AD). A previous study reported that xylene and formaldehyde intensified allergic inflammation in an experimental mouse model.[1] In a prospective study to evaluate the clinical effects of air pollution including VOCs on skin symptoms in children with AD,[2] concentrations of total VOCs were higher on days when the patients had symptoms of AD than on days without symptoms. A significant increase in transepidermal water loss after exposure to VOCs was also reported.[3] Although these accumulated data show an association between exposure to VOCs and aggravation of AD, the molecular effects of VOCs on the aggravation of AD are still unclear.

Filaggrin is a key regulator in epidermal barrier function. The pathogenesis of AD may be proposed as an epidermal barrier defect that leads to diminished epidermal defense mechanisms.[3] In a cohort study, filaggrin mutations were associated with the predilection sites of AD that are exposed areas of the body such as hands and cheeks, whereas there were no predilection sites in persons with the wild-type gene.[4] These sites are typically exposed to environmental factors including irritants, air pollution, and chemicals. This suggests that persons with mutations in genes encoding proteins of the skin barrier are susceptible to AD when exposed to environmental triggers. On the basis of these reports, we investigated the effects of toluene, a typical VOC that usually occurs in indoor air from the use of common household products (paints, paint thinners, adhesives, synthetic fragrances, and nail polish) and cigarette smoke, on filaggrin-mediated epidermal barrier dysfunction.

After toluene exposure, the expression of filaggrin mRNA and protein decreased (Fig 1, *A* and *B*). Furthermore, to determine whether toluene affects the expression of other keratinocyte differentiation-associated markers (eg, involucrin, loricrin, keratin 10, and keratin 5), we measured the mRNA levels of each of these molecules after toluene treatment. The present study demonstrated that toluene did not significantly affect the expression of involucrin, loricrin, keratin 10, and keratin 5 (data not shown). Fluorescence microscopy analysis also showed that filaggrin levels were lower in toluene-treated human skin equivalent models than in untreated human skin equivalent models (Fig 1, *C*). In addition, we evaluated the filaggrin breakdown products such as natural moisturizing factor (NMF) associated with transepidermal water loss. Pyrrolidone carboxylic acid and urocanic acid are known as the main components of NMF. As a result, levels of NMF components

METHODS

Cell purification and culture

Neutrophils from the same donors were isolated by different methods, as follows: (1) by density gradient centrifugation of whole blood over Ficoll-Paque PLUS (GE Healthcare), dextran sedimentation, and erythrocyte osmotic lysis (*NeuF*); (2) similarly to *NeuF*, but with an additional immunomagnetic negative selection step, using the "EasySep Human Neutrophil Enrichment Kit" (StemCell Technologies) (*Neu*); (3) directly isolated from whole blood, by the "MACSxpress Neutrophil isolation kit" (Miltenyi Biotec) (*wbNeuM*); and (4) directly isolated from whole blood by the "EasySep direct Human Neutrophil Isolation Kit" (StemCell Technologies) (*wbNeuE*). In selected experiments, 8×10^6 *wbNeuM* or *wbNeuE* were incubated with or without 30 μL of αM-DC8 microbeads (Miltenyi Biotec) for 20 minutes on ice, and then slan$^+$CD16$^+$-monocytes were removed by separation via MACS LD column (Miltenyi Biotec). In other experiments, *wbNeuE* were stained by a combination of 10 μL of αM-DC8-fluorescein isothiocyanate (FITC) (Miltenyi Biotec) with or without 20 μL of αCD19-FITC (Miltenyi Biotec) or 20 μL of αCD56-FITC (Miltenyi Biotec) or 10 μL of αCD303-FITC (Miltenyi Biotec) for 20 minutes on ice. Removal of stained cells was then achieved by incubation with 20 μL of anti-FITC microbeads for 20 minutes on ice and subsequent passage through MACS LD column. Neutrophils were suspended at 5×10^6/mL in RPMI 1640 medium supplemented with 10% low-endotoxin FBS (<0.5 EU/mL, from Sigma, St Louis, Mo), treated with 5 μM R848 (Invivogen, San Diego, Calif), 50 μg/mL poly(I:C) (Invivogen), and 10 ng/mL TNF-α (Peprotech, Rocky Hill, NJ), and finally plated in 48-well tissue culture plates (Greiner Bio-One, Kremsmünster, Austria) for culture at 37°C, 5% CO_2 atmosphere. After 4 or 20 hours, neutrophils were collected and spun at 300*g* for 5 minutes. Cell-free supernatants were immediately frozen in liquid nitrogen and stored at −80°C, while the corresponding pellets were extracted for total RNA.

ELISA

Cytokine concentrations in cell-free supernatants were measured by ELISA kits specific for IL-6 (eBioscience, San Diego, Calif), IL-10 (eBioscience), TNF-α (eBioscience), and CXCL8 (Mabtech, Nacka Strand, Sweden). Detection limits of these ELISAs were 8 pg/mL for IL-6, 4 pg/mL for IL-10 and TNF-α, and 8 pg/mL for CXCL8.

RT-quantitative real-time PCR

Total RNA (0.1 μg) extracted by the RNeasy mini kit (Qiagen, Venlo, Limburg, The Netherlands) from 1×10^6 neutrophils was reverse transcribed for RT-quantitative PCR using gene-specific primer pairs (Life Technologies, Carlsbad, Calif) available in the public database RTPrimerDB (www.rtprimerdb.org) under the following entry codes: glyceraldehyde-3-phosphate dehydrogenase (3539), TNF-α (3551), CXCL8 (3553), IL-6 (3545), IL-10 (8230), IFIT1 (3540), and ISG15 (3547). Data were calculated by Q-Gene software (http://www.genequantification.de/download.html) and expressed as mean normalized expression units after glyceraldehyde-3-phosphate dehydrogenase normalization.

Phenotype assessment by flow cytometry

A total of 10^5 freshly isolated neutrophil populations were suspended in 50 μL PBS containing 10% complement-inactivated human serum (for Fcγ receptor blocking). Neutrophils were then stained for 15 minutes at room temperature with different combinations of the following regents: αCD14-Vioblue (clone TÜK4), αCD3-PE-Vio770 (clone BW 264156), αCD19-APC (clone LT19), αCD56-PE (clone REA196), αM-DC8-FITC (clone DD1), αCD11c-Vioblue (clone MJ4-27G12), αCD303-FITC (clone AC144), αCD141-APC (clone AD5-14H12), αFCeR1a-PE (clone CRA1) antibodies from Miltenyi Biotec; αCD1c-Alexa488 (clone L161), αCD45 brilliant Violet 510 (clone H130), αCD16-PerCP-Cy5.5 (clone 3G8), and αHLA-DR-APC-CY7 (clone L243) antibodies from Biolegend (Sa Diego, Calif) and αCD123-PE (clone 9F5) antibodies from BD Bioscience (San Jose, Calif). After washings, sample fluorescence was measured by an 8-color MACSQuant Analyzer (Miltenyi Biotec), for which at least 40,000 cells were acquired. Data analysis was performed using FlowJo software Version 8.8.6 (Tree Star, Ashland, Ore).

IL-10 secretion assay

A total of 2×10^6 neutrophils were treated with or without 5 μM R848 or 10 ng/mL of TNF-α for 14 hours at 37°C, then washed, incubated for 5 minutes with IL-10–catching reagent (Miltenyi Biotec) on ice, immediately diluted with warm medium, and then left for 45 minutes at 37°C on constant rotation to let IL-10 secretion proceed. After extensive washings, cells were stained on ice with αIL-10PE, αM-DC8-FITC, and αHLA-DR APC-Cy7 Abs. Vybrant Violet (Life Technologies) was used as cell viability reagent. Neutrophils were gated as high SSChigh/Vybrant-negative cells, whereas slan$^+$CD16$^+$-monocytes were gated as M-DC8/HLA-DR$^+$, Vybrant-negative cells. A total of 800,000 cells per sample were acquired, as average, by MACSQuant Analyzer.

REFERENCES

E1. Zimmermann M, Arruda-Silva F, Bianchetto-Aguilera F, Finotti G, Calzetti F, Scapini P, et al. IFNalpha enhances the production of IL-6 by human neutrophils activated via TLR8. Sci Rep 2016;6:19674.
E2. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood 2003;102:2660-9.
E3. Schakel K, Kannagi R, Kniep B, Goto Y, Mitsuoka C, Zwirner J, et al. 6-Sulfo LacNAc, a novel carbohydrate modification of PSGL-1, defines an inflammatory type of human dendritic cells. Immunity 2002;17:289-301.
E4. Hansel A, Gunther C, Ingwersen J, Starke J, Schmitz M, Bachmann M, et al. Human slan (6-sulfo LacNAc) dendritic cells are inflammatory dermal dendritic cells in psoriasis and drive strong TH17/TH1 T-cell responses. J Allergy Clin Immunol 2011;127:787-94, e1-9.
E5. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, et al. Subsets of human dendritic cell precursors express different Toll-like receptors and respond to different microbial antigens. J Exp Med 2001;194:863-9.
E6. Dalboni TM, Abe AE, de Oliveira CE, Lara VS, Campanelli AP, Gasparoto CT, et al. Activation profile of CXCL8-stimulated neutrophils and aging. Cytokine 2013;61:716-9.

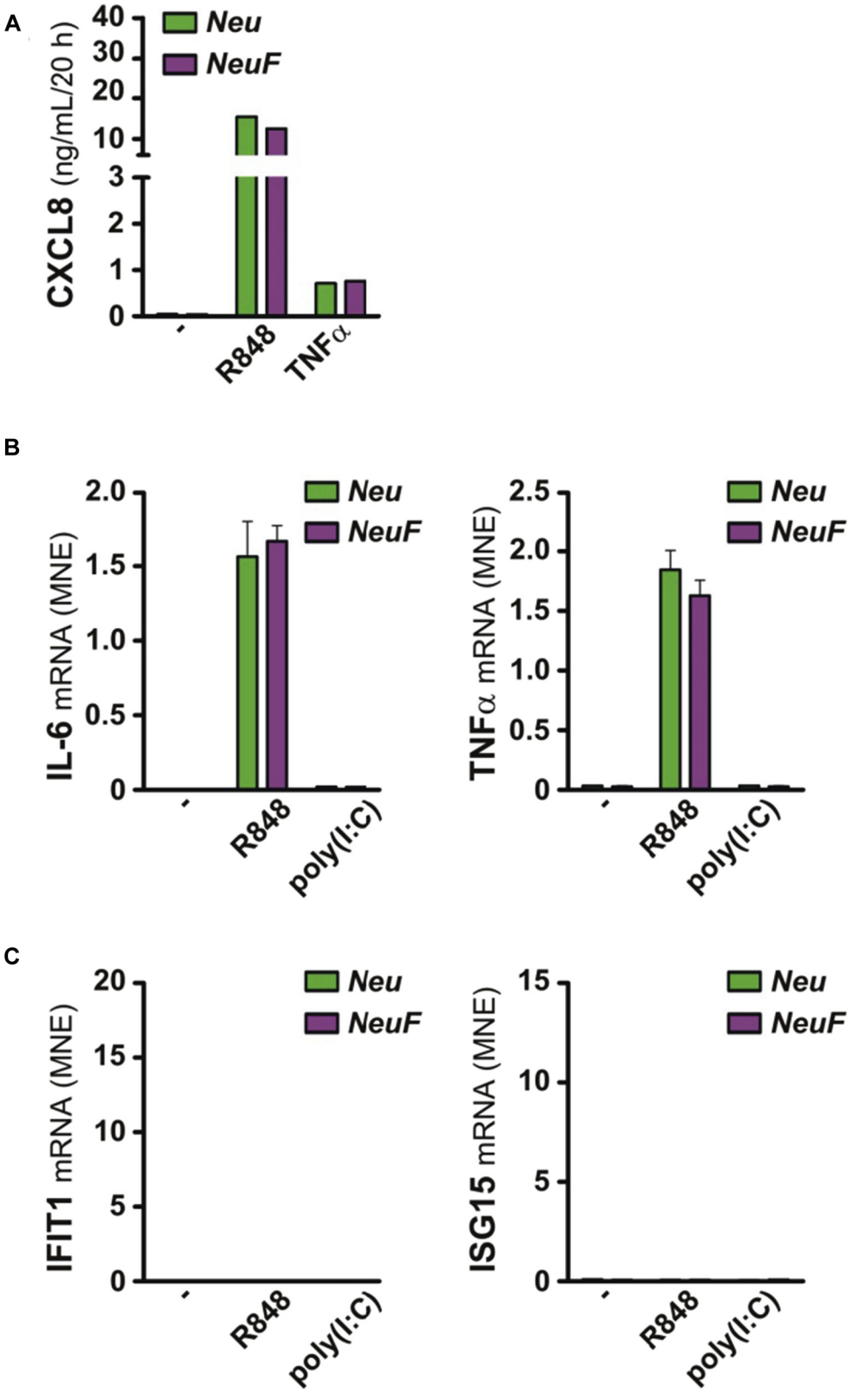


FIG E1. CXCL8 production and gene expression patterns in *Neu* and *NeuF* incubated with R848 or poly(I:C). *Neu* and *NeuF* were incubated for 20 h with either 5 μM R848 or 10 ng/ml TNFα for CXCL8 release **(A)**, or with either R848 or 50 μg/ml poly(I:C) for IL-6 and TNFα **(B)**, or IFIT1 and ISG15 **(C)**, mRNA expression analysis, by RT-qPCR, performed after 4 and 20 h of incubation, respectively. Panel **A** reports the mean of 2 experiments, while panels **B** and **C** display representative experiments out of 3 performed with similar results.

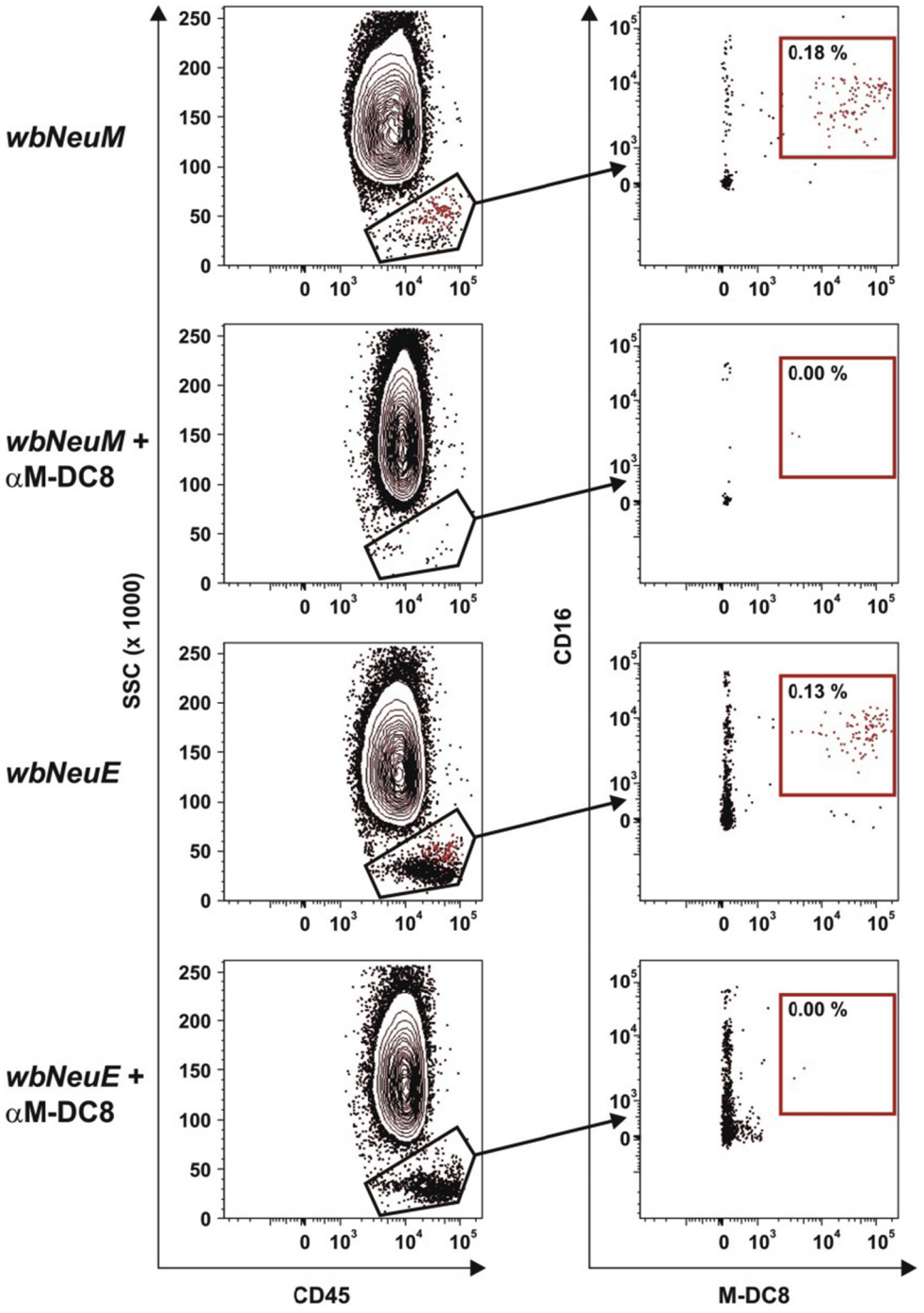


FIG E2. Assessment of slan^{+}CD16^{+}-monocyte removal from *wbNeuM* and *wbNeuE*. After *wbNeuM* and *wbNeuE* neutrophils isolation, slan^{+}CD16^{+}-monocytes were removed by separation using αM-DC8-linked microbeads *via* MACS column. Flow cytometry analysis was then used to assess the levels of contaminant slan^{+}CD16^{+}-monocytes present in neutrophils preparations. Accordingly, contaminating PBMCs were first identified by SSC^{low}/CD45 positivity (*left panels*, *black* gate), and then slan^{+}CD16^{+}-monocytes were selected among them, as M-DC8/CD16 double positive cells (*right panels*, *red* gate) and overlayed (*red dots*) on total CD45 cells.

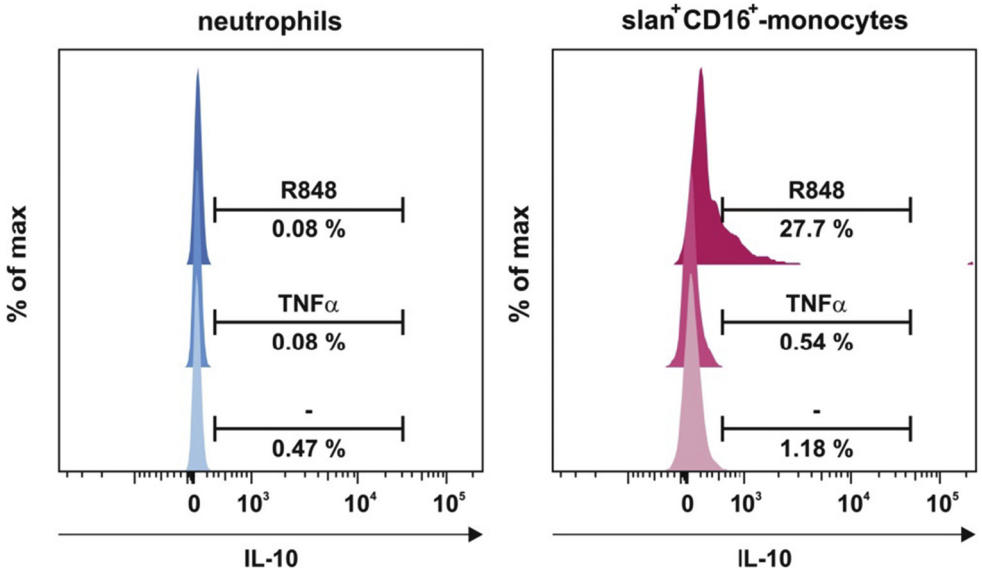


FIG E3. IL-10 secretion assay. 2 x 10^6 *WbNeuE* were treated with or without 5 μM R848 or 10 ng/mL TNFα for 14 h and then assayed for IL-10 secretion. Histograms, displaying 1 representative experiment out of 2 with similar results, show that only slan$^+$CD16$^+$-monocytes *(right panel)*, but not neutrophils *(left panel)*, secrete IL-10. Similar results were obtained using *WbNeuM*.

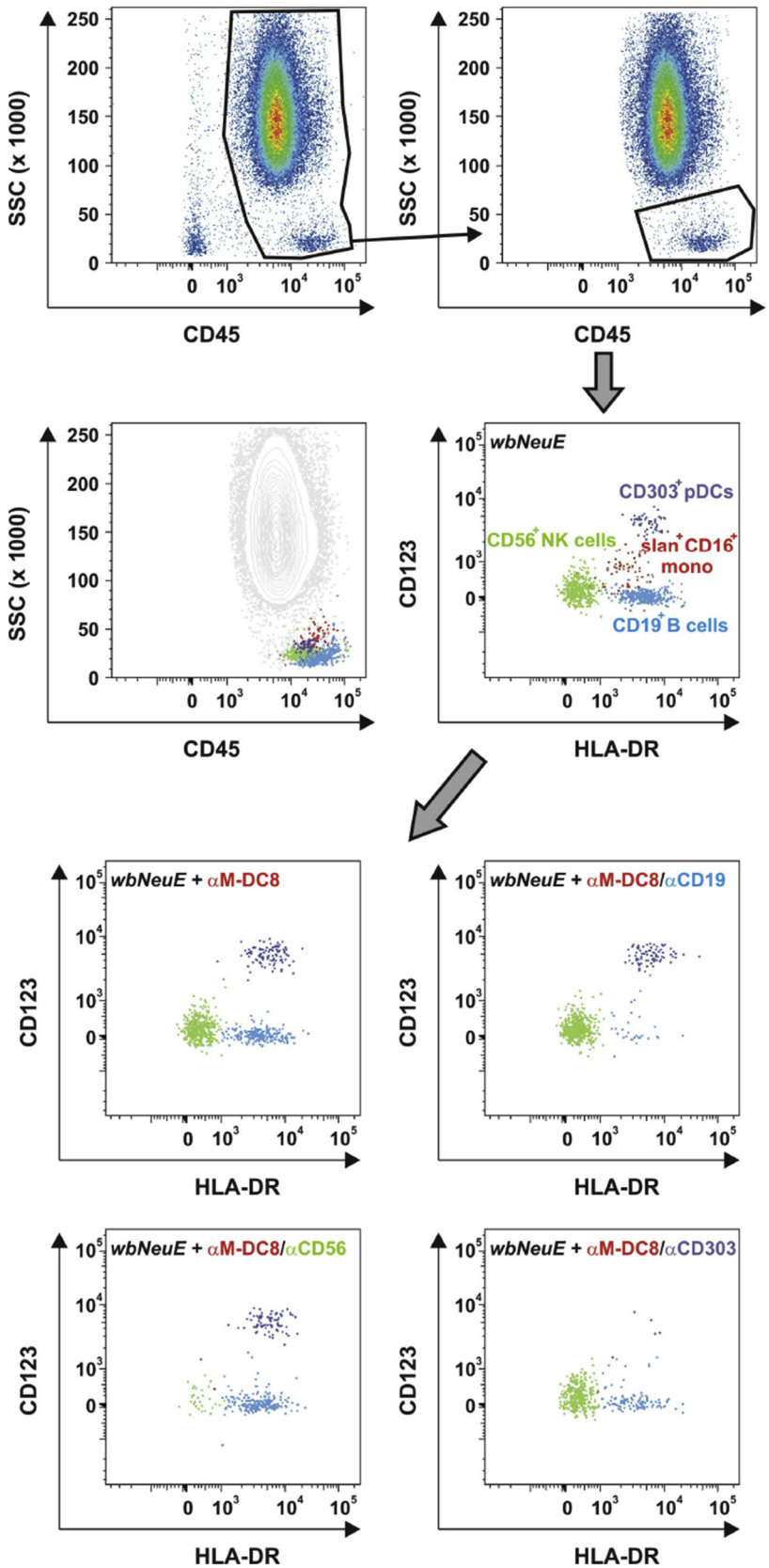


FIG E4. Assessment of slan^{+}CD16^{+}-monocyte, B cell, NK cell and pDC removal from *wbNeuE*. After isolation, *wbNeuE* were stained with FITC-conjugated antibodies towards M-DC8 (for slan^{+}CD16^{+}-monocytes) alone or in combination with CD19 (for B cells), CD56 (for NK cells) or CD303 (for pDCs). Panels show that removal of stained cells was efficiently achieved by anti-FITC microbeads via MACS column separation. In fact, contaminating PBMCs were first gated by SSC^{low}/CD45 positivity (upper panels, *black* gate), and then slan^{+}CD16^{+}-monocytes (*red* dots), B cells (light *blue* dots), NK cells (*green* dots) and pDCs (*purple* dots) were identified, using specific CD markers overlaid on HLA-DR/CD123 dot plots.

TABLE E1. Cellular composition of the neutrophil populations isolated from human blood by various procedures

Cell type	*Neu* (Ficoll and Easysep kit) (n = 5)	*WbNeuM* (MACSxpress kit) (n = 5)	*WbNeuE* (Easysep direct kit) (n = 7)	*NeuF* (Ficoll-Paque) (n = 3)
Neutrophils*	99.77 ± 0.15	98.86 ± 0.25	97.46 ± 1.06	96.17 ± 2.66
Eosinophils†	0.07 ± 0.06	0.29 ± 0.37	0.53 ± 0.61	1.52 ± 1.12
$CD14^+$-monocytes‡	0.00 ± 0.00	0.02 ± 0.02	0.04 ± 0.04	0.03 ± 0.03
Total $CD16^+$-monocytes§	0.00 ± 0.00	0.38 ± 0.20	0.25 ± 0.18	0.00 ± 0.00
$slan^+CD16^+$-monocytes¶	0.00 ± 0.00	0.33 ± 0.16	0.22 ± 0.19	0.00 ± 0.00
CD1c DCs‖	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CD141 DCs#	0.00 ± 0.00	0.01 ± 0.00	0.02 ± 0.02	0.00 ± 0.01
pDCs**	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.03	0.00 ± 0.00
T cells††	0.05 ± 0.09	0.09 ± 0.05	0.13 ± 0.30	1.95 ± 1.81
B cells‡‡	0.00 ± 0.00	0.02 ± 0.02	0.33 ± 0.16	0.00 ± 0.01
NK cells§§	0.01 ± 0.01	0.11 ± 0.08	0.25 ± 0.18	0.27 ± 0.46

Values are in %. Cell percentages were calculated on the basis of total $CD45^+$ cells. The various leukocyte populations were identified by flow cytometry analysis using the following criteria/surface markers: *$SSC^{hi}/CD16^+$ cells = neutrophils; †$SSC^{hi}/CD16^-$ cells = eosinophils; ‡$CD14^+/CD16^-$ cells = $CD14^+$-classical monocytes; §$CD16^+$/$HLA\text{-}DR^+/CD56^-$ cells = total $CD16^+$-monocytes; ¶$CD16^+/HLA\text{-}DR^+/M\text{-}DC8^+/CD14^{dim/neg}$ cells = $slan^+CD16^+$-monocytes; ‖$CD1c^+/Fc\varepsilon R1^+/HLA\text{-}DR^+$ cells = CD1c DCs; #$CD141^+/HLA\text{-}DR^+/CD11c^{dim}$ cells = CD141 DCs; **$CD303^+/CD123^+$ cells = pDCs; ††$CD3^+$ cells = T lymphocytes; ‡‡$CD19^+/HLA\text{-}DR^+$ cells = B lymphocytes; §§$CD56^+$ cells = NK cells.

DC, Dendritic cell; *NK*, natural killer.

frontiers in Cellular and Infection Microbiology

ORIGINAL RESEARCH
published: 12 May 2017
doi: 10.3389/fcimb.2017.00176

Human Neutrophils Produce CCL23 in Response to Various TLR-Agonists and TNFα

Fabio Arruda-Silva[1], Francisco Bianchetto-Aguilera[1], Sara Gasperini[1], Sara Polletti[2,3], Emanuela Cosentino[4], Nicola Tamassia[1] and Marco A. Cassatella[1]**

[1] Section of General Pathology, Department of Medicine, University of Verona, Verona, Italy, [2] Humanitas Clinical and Research Center, Milan, Italy, [3] Department of Biomedical Sciences, Humanitas University, Milan, Italy, [4] Functional Genomic Lab, Department of Biotechnology, University of Verona, Verona, Italy

CCL23, also known as myeloid progenitor inhibitory factor (MPIF)-1, macrophage inflammatory protein (MIP)-3, or CKβ8, is a member of the CC chemokine subfamily exerting its effects via CCR1 binding. By doing so, CCL23 selectively recruits resting T lymphocytes and monocytes, inhibits proliferation of myeloid progenitor cells and promotes angiogenesis. Previously, we and other groups have reported that human neutrophils are able to produce chemokines upon appropriate activation, including CCR1-binding CCL2, CCL3, and CCL4. Herein, we demonstrate that human neutrophils display the capacity to also express and release CCL23 when stimulated by R848 and, to a lesser extent, by other pro-inflammatory agonists, including LPS, Pam3CSK4, and TNFα. Notably, we show that, on a per cell basis, R848-activated neutrophils produce higher levels of CCL23 than autologous $CD14^+$-monocytes activated under similar experimental conditions. By contrast, we found that, unlike $CD14^+$-monocytes, neutrophils do not produce CCL23 in response to IL-4, thus indicating that they express CCL23 in a stimulus-specific fashion. Finally, we show that the production of CCL23 by R848-stimulated neutrophils is negatively modulated by IFNα, which instead enhances that of CCL2. Together, data extend our knowledge on the chemokines potentially produced by neutrophils. The ability of human neutrophils to produce CCL23 further supports the notion on the neutrophil capacity of orchestrating the recruitment of different cell types to the inflamed sites, in turn contributing to the control of the immune response.

Keywords: neutrophils, CCL23, R848, CCR1, IFN

OPEN ACCESS

Edited by:
Lee-Ann H. Allen,
University of Iowa, USA

Reviewed by:
Marcus Thelen,
Institute for Research in Biomedicine, Switzerland
Veronique Witko-Sarsat,
Institut National de la Santé et de la Recherche Médicale (INSERM), France

***Correspondence:**
Nicola Tamassia
nicola.tamassia@univr.it
Marco A. Cassatella
marco.cassatella@univr.it

Received: *27 February 2017*
Accepted: *24 April 2017*
Published: *12 May 2017*

Citation:
Arruda-Silva F, Bianchetto-Aguilera F, Gasperini S, Polletti S, Cosentino E, Tamassia N and Cassatella MA (2017) Human Neutrophils Produce CCL23 in Response to Various TLR-Agonists and TNFα. Front. Cell. Infect. Microbiol. 7:176. doi: 10.3389/fcimb.2017.00176

INTRODUCTION

Neutrophils are known to perform a series of effector functions, including phagocytosis, discharge of constitutively stored proinflammatory molecules, generation of massive amounts of superoxide anion, and active release of neutrophil extracellular traps (NETs), that are all crucial for innate immunity responses toward infections (Scapini et al., 2013). In addition to their defensive functions, neutrophils display the capacity to synthesize and secrete a variety of cytokines, including many chemokines (Tecchio and Cassatella, 2016). For instance, depending

on the stimulatory conditions, human neutrophils have been shown to produce both CXC chemokines, such as CXCL1 (GROα), CXCL3, CXCL5 (ENA78), CXCL6, CXCL8 (IL-8), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), and CC chemokines, including CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL17, CCL18, CCL19 (MIP-3β), and CCL20 (MIP-3α) (reviewed by Tecchio and Cassatella, 2016). Given that these chemokines recruit leukocytes associated to either the innate [including neutrophils themselves, monocytes, macrophages, dendritic cells (DCs), and natural killer (NK) cells] or the acquired (such as T cell subsets) immune responses (Viola and Luster, 2008; Griffith et al., 2014), it is plausible to speculate that neutrophils are in the position to orchestrate leukocyte trafficking during the entire course of specific responses to invading pathogens or other antigens (Tecchio and Cassatella, 2016).

In this context, CCL23, also known as myeloid progenitor inhibitory factor (MPIF)-1, macrophage inflammatory protein (MIP)-3, or CKβ8, is a recently identified CCL, which, similarly to CCL3, CCL5, CCL7, CCL13, CCL14, CCL15, and CCL16, binds CCR1 (Youn et al., 1998; Viola and Luster, 2008). Also CCL4 and CCL2 have been shown to bind CCR1: the former one, however, after natural truncation of its two NH2-terminal amino acids (Guan et al., 2002), the latter one with lower affinity as compared to its binding to CCR2 (Menten et al., 2002). Similarly to other CCR1 binding chemokines, CCL23 exerts chemotactic activities on monocytes, DCs and resting T lymphocytes (Patel et al., 1997; Youn et al., 1998; Nardelli et al., 1999), as well as on endothelial cells to ultimately induce tube formation (Hwang et al., 2005; Son et al., 2006). Moreover, CCL23 has been reported to play a role in bone formation, for its potent chemoattractant action on osteoclast precursors, but not for fully differentiated osteoclasts or osteoblasts (Votta et al., 2000). A more precise characterization of CCL23 function is however hindered by the fact that CCL23 is a gene with no mouse ortholog (Viola and Luster, 2008), which thus prevents the employment of loss-of-function strategies in experimental animal models. While CCL23/MPIF-1 cDNA was originally isolated from a human aortic endothelial library (Patel et al., 1997), subsequent analysis made on various cell lines has shown that CCL23 mRNA is readily detectable in myelomonocytic cell lines (Patel et al., 1997). Expression of CCL23 mRNA was then found in monocytes stimulated with IL-1β (Forssmann et al., 1997) and it is now established that IL-4-treated monocytes produce remarkable amounts of CCL23 (Nardelli et al., 1999; Novak et al., 2007). CCL23 can be detected in synovial fluids from rheumatoid arthritis patients (Berahovich et al., 2005), as well as in serum of systemic sclerosis patients (Yanaba et al., 2011). More recently, eosinophils freshly isolated from blood were found to express CCL23 mRNA and, in turn, secrete antigenic CCL23 upon incubation with IL-5 or GM-CSF (Matsumoto et al., 2011).

Since no information was present in the literature on an eventual expression/production of CCL23 by neutrophils, we decided to investigate such a possibility. As a result, we herein show that also human neutrophils produce CCL23 *in vitro*, yet upon incubation with selected stimuli and in a fashion regulated by endogenous TNFα and exogenous IFNα.

MATERIALS AND METHODS

Cell Purification and Culture

Granulocytes and autologous $CD14^+$-monocytes were isolated from buffy coats of healthy donors, under endotoxin-free conditions as previously described (Davey et al., 2011). Briefly, buffy coats were stratified on Ficoll-Paque PLUS gradient (GE Healthcare, Little Chalfont, United Kingdom) at a 1:1 ratio, and then centrifuged at 400 × g for 30 min at room T. Granulocytes were then collected and subjected to dextran sedimentation followed by hypotonic lysis to remove erythrocytes. Finally, neutrophils were isolated from granulocytes (to reach a 99.7 ± 0.2% purity) by positively removing all contaminating cells using the EasySep neutrophil enrichment kit (StemCell Technologies, Vancouver, Canada; Calzetti et al., 2017). $CD14^+$-monocytes were instead isolated from PBMCs, obtained after Ficoll-Paque gradient centrifugation, by anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) to reach a purity of ∼98%. Cells were then suspended at 5×10^6/ml in RPMI 1640 medium supplemented with 10% low endotoxin FBS (<0.5 EU/ml; from BioWhittaker-Lonza, Basel, Switzerland) and then 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, in the presence or the absence of: 0.2–50 μM R848, 5–50 μM R837, 1 μg/ml Pam3CSK4 (Invivogen, San Diego, CA, USA), 10 ng/ml TNFα (Peprotech, Rocky Hill, NJ, USA), 0.1–10 μg/ml ultrapure LPS from *E. coli* 0111:B4 strain (Alexis, Enzo Life Sciences, Farmingdale, NY, USA), 1,000 U/ml pegylated IFNα-2a (Pegasys, Roche, Basel, Switzerland), 100 U/ml IFNγ (R&D Systems, Minneapolis, MN, USA), 10 ng/ml GM-CSF (Miltenyi Biotec), 1,000 U/ml G-CSF (Myelostim, Italfarmaco Spa, Milano, Italy), 10–100 ng/ml IL-18 (MBL International, Nagoya, Japan), 10–100 ng/ml IL-33 (Peprotech), 10 nM fMLF (Sigma, Saint Louis, MO, USA), 500 μg/ml particulate β-glucan (InvivoGen), or 500 μg/ml curdlan (Sigma). In some experiments, neutrophils were preincubated for 30 min with either 10 μg/ml adalimumab (Humira, Abbott Biotechnology Limited, Barceloneta, Puerto Rico) or 10 μg/ml etanercept (Enbrel, Amgen, Thousand Oaks, CA, USA), prior to further incubation with stimuli. After the desired incubation period, neutrophils and monocytes were collected and spun at 300 × g for 5 min. 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.

ELISA

Cytokine concentrations in cell-free supernatants and cell-lysates were measured by commercially available ELISA kits, specific for human: CXCL8 (Mabtech, Nacka Strand, Sweden), CCL23 (R&D Systems, Minneapolis, MN, USA), CCL2 (R&D Systems), CCL3 (eBioscience, San Diego, CA, USA), and CCL4 (eBioscience). Detection limits of these ELISA were: 7.8 pg/ml for CCL23, 7.8 pg/ml for CCL2, 16 pg/ml for CCL3, 4 pg/ml for CCL4, and 16 pg/ml for CXCL8.

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

Total RNA was extracted from neutrophils and monocytes by 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 also performed during total RNA isolation to completely remove any possible contaminating DNA. Purified total RNA was then reverse-transcribed into cDNA using Superscript III (Life Technologies) and random hexamer primers (Life Technologies), while qPCR was carried out using Fast SYBR® Green Master Mix (Life Technologies) (Tamassia et al., 2014). Sequences of the gene-specific primers (Life Technologies) used in this study are the following: GAPDH, forward AACAGCCTCAAGATCATCAGC and reverse GGATGATGTTCTGGAGAGCC; CXCL8 forward CTGGCCGTGGCTCTCTTG and reverse CCTTGGCAAAAC TGCACCTT; IL-1ra forward TTCCTGTTCCATTCAGAGACG AT and reverse AATTGACATTTGGTCCTTGCAA; CCL2 forward GTCTCTGCCGCCCTTCTGT and reverse TTGCAT CTGGCTGAGCGAG; CCL3 forward AGCCCACATTCCGTC ACCTG and reverse CGTGTCAGCAGCAAGTGATG; CCL4 forward CGCCTGCTGCTTTTCTTACAC and reverse CAG ACTTGCTTGCTTCTTTTGG; and CCL23 forward GTACTT CTGGACATGCTCTGG and reverse CTGAACTTGCTTATC ACTGGG. Data were calculated by Q-Gene software (http://www.gene-quantification.de/download.html) and expressed as mean normalized expression (MNE) units after GAPDH normalization.

RNA Sequencing (RNA-seq)

Total RNA extracted from neutrophils (50×10^6/condition) was quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies). RNA integrity (RIN) was routinely found to be ≥7.0. RNA-seq libraries were prepared by oligo-dT selection using the TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina HiSeq 2000. After quality filtering according to the Illumina pipeline, 51-base-pair (bp) reads were aligned to the hg19 assembly (Genome Reference Consortium GRCh37) as well as the human transcriptome reference (UCSC annotation), using TopHat (Trapnell et al., 2013). We allowed up to two mismatches and specified a mean distance between pairs (–r) of 250 bp. The reference sequence and annotation files were downloaded from iGenomes repository at the following website: http://support.illumina.com/sequencing/sequencing_software/igenome.html. Read counts per gene were obtained from the aligned reads using "htseq-count" command from the HTSeq framework version

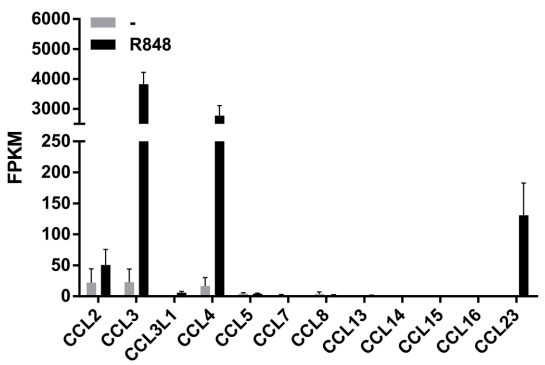


FIGURE 1 | Expression of CCR1-binding chemokine mRNAs in human neutrophils incubated with R848. Human neutrophils were incubated with or without 5 μM R848 for 24 h, and then total RNA was extracted and processed for RNA-seq experiments. Data are presented as FPKM (fragment per kilobase of exons per million fragments mapped) from two independent experiments (mean ± SEM).

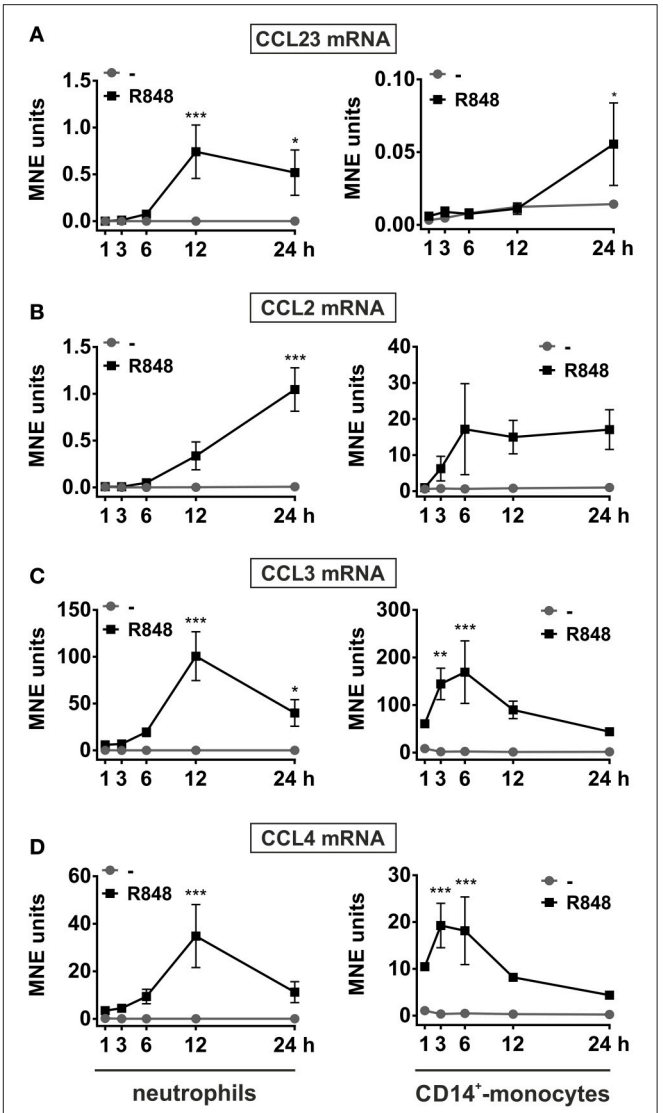


FIGURE 2 | Kinetics of CCL23, CCL2, CCL3, and CCL4 mRNA expression in neutrophils and autologousCD14$^+$-monocytes incubated with R848. Neutrophils and autologous CD14$^+$-monocytes were cultured with or without 5 μM R848 for up to 24 h to evaluate their CCL23 **(A)**, CCL2 **(B)**, CCL3 **(C)**, and CCL4 **(D)** mRNA expression by RT-qPCR. Gene expression is depicted as mean normalized expression (MNE) units after GAPDH mRNA normalization (mean ± SEM, $n = 5$). Asterisks stand for significant increase: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, by two-way ANOVA followed by Bonferroni's post-test.

0.6.1p1 (Anders et al., 2015), with the following parameters: "–f bam –r name –m union –s no –t exon –i gene id". Count normalization was performed using the Bioconductor/R packages DESeq2 (Love et al., 2014). Transcript abundance was indicated as Fragments Per Kilobase of transcript sequence per Million of mapped fragments (FPKM), using the "fpkm" function of DESeq2.

Statistical Analysis

Data are expressed as mean ± SEM. Statistical evaluation was performed by the Student's *t*-test, one-way ANOVA followed by Tukey's *post-hoc* test or two-way ANOVA followed by Bonferroni's *post-hoc* test. Values of $p < 0.05$ were considered as statistically significant.

Study Approval

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

RESULTS

Human Neutrophils Incubated with R848 Accumulate CCL23 Transcripts

We have recently reported that R848 represents a very powerful inducer of IL-6, TNFα, G-CSF, IL-12-p40, and CXCL8 production by human neutrophils (Zimmermann et al., 2015, 2016). We recall here that R848 is an imidazoquinoline compound that potently activates the signaling of both TLR7 and TLR8 (Jurk et al., 2002), but that in human neutrophils activates only TLR8 (Janke et al., 2009; Zimmermann et al., 2015) as these cells do not express TLR7 (Hayashi et al., 2003; Janke et al., 2009; Berger et al., 2012; Zimmermann et al., 2015). Moreover, results from RNA-seq experiments to discover unidentified molecules eventually generated by human neutrophils incubated for 24 h with 5 μM R848 indicated that, among the various CCR1-binding chemokines (Griffith et al., 2014), neutrophils express the mRNA for CCL2, CCL3, CCL4, and CCL23, but not CCL5, CCL7, CCL8, CCL13, CCL14, CCL15, and CCL16 (**Figure 1**). **Figure 1** further shows that, in R848-stimulated neutrophils, the levels of CCL3 and CCL4 mRNAs are remarkably higher than those of CCL2 and CCL23 mRNAs. While neutrophil expression of CCL2, CCL3, and CCL4 mRNA is well-established (Kasama et al., 1993, 1994; Cassatella, 1999; Yamashiro et al., 1999), that of CCL23 has been never described before.

To validate and extend these preliminary findings, neutrophils, as well as autologous $CD14^+$-monocytes, incubated with R848 were subjected to RT-qPCR experiments in which the kinetics of CCL23, CCL2, CCL3, and CCL4 mRNA expression were compared. As shown in **Figure 2**, CCL23 mRNA expression in neutrophils started to be detected after 3 h, peaked at the 12 h-time point and slowly declined thereafter (**Figure 2A**, left panel). Similarly to CCL23, also CCL2 mRNA started to be accumulated in neutrophils later than 3 h, but then steadily increased up to 24 h (**Figure 2B**, left panel). By contrast, CCL3 and CCL4 mRNAs were induced by R848 as early as after 1 h, but then followed a time-course pattern similar to CCL23 mRNA (**Figures 2C,D**, left panels). Interestingly, maximum expression levels of both CCL23 and CCL2 mRNAs in R848-treated neutrophils were similar (**Figures 2A,B**, left panels), but much lower than those of CCL3 and CCL4 mRNAs (**Figures 2C,D,** left panels). Notably, expression levels, as well as kinetics of CCL23 (**Figure 2A**, right panel), CCL2 (**Figure 2B**, right panel), CCL3 (**Figure 2C**, right panel), and CCL4 (**Figure 2D**, right panel) mRNAs in R848-treated monocytes strikingly differed from those in neutrophils. For instance, the peak of CCL2, CCL3, and CCL4 mRNA expression in R848-treated monocytes was found to occur earlier than in neutrophils (**Figures 2B,C**), unlike that of CCL23 mRNA, which was found to take place later, consistent with the increase of CCL23 transcripts starting not before 12 h of incubation (**Figure 2A**). Moreover, while CCL2 and CCL3 mRNAs accumulated in R848-treated monocytes at higher levels than in neutrophils (**Figures 2B,C**), the opposite was observed in the case of CCL23 and CCL4 transcripts (**Figures 2A,D**). Altogether, data demonstrate that neutrophils incubated with R848 genuinely express CCL23 mRNA, yet in a different fashion as compared to CCL2, CCL3, and CCL4

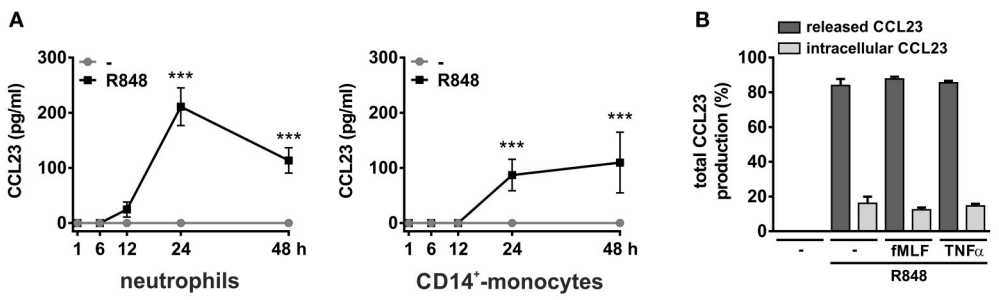


FIGURE 3 | Production of CCL23 by neutrophils and monocytes incubated with R848. Neutrophils and $CD14^+$-monocytes were cultured at 5×10^6 cells/ml with or without 5 μM R848 for up to 48 h to evaluate their capacity to produce and release CCL23, as detected by ELISA ($n = 4$–19) **(A)**. In **(B)**, neutrophils were cultured with or without 5 μM R848 for 24 h and then further incubated for 4 h with or without 10 nM fMLF or 10 ng/ml TNFα to evaluate released and cell-associated CCL23 ($n = 4$). Asterisks stand for significant increases: $^{***}P < 0.001$ by two-way ANOVA followed by Bonferroni's post-test.

genes, or to CCL23 mRNA expression induced in autologous monocytes.

Human Neutrophils Incubated with R848 Produce Discrete Levels of CCL23

Next, we analyzed whether R848-treated neutrophils are able to synthesize and release CCL23 and found that they actually do so. CCL23 protein was, in fact, detected in neutrophil-derived supernatants, but starting only after 12 h of cell incubation with R848, and with peak levels reached after 24 h (211.1 ± 34.2, $n = 4$–13) (**Figure 3A,** left panel). This is consistent with the delayed induction of CCL23 mRNA by R848, which, by the way, is also known to prolong neutrophil survival (Zimmermann et al., 2016). Moreover, previous dose-response experiments revealed that 5 μM R848 correspond to the most effective concentrations to induce the production of CCL23 (data not shown). We also observed that, of the total CCL23 synthesized by R848-treated neutrophils, ∼15% remains cell-associated (**Figure 3B**), indicating that CCL23 is almost completely released. And in fact, two potent secretagogues, namely fMLF or TNFα, were found unable to mobilize the small quote of intracellular CCL23 accumulated in R848-treated neutrophils (**Figure 3B**).

Monocytes too were found to release CCL23 in response to R848 (**Figure 3A,** right panel), surprisingly at lower levels (87.1 ± 28.5, $n = 11$) than neutrophils, but consistent with the gene expression data (**Figure 2A**). However, monocytes incubated with IL-4 for up to 48 h were found to accumulate and release remarkable levels of CCL23 mRNA and protein (308.6 ± 149.2 pg/ml, $n = 4$) (left panels in **Figure 4A** and **Figure 4C**, respectively), confirming previous data (Novak et al., 2007). By contrast, IL-4-treated neutrophils did not express/produce any CCL23 mRNA and protein (right panels in **Figure 4A** and **Figure 4C**, respectively), even though they accumulated IL-1ra mRNA (right panel in **Figure 4B**), as previously described (Crepaldi et al., 2002). Altogether, data prove that neutrophils genuinely produce CCL23 in response to R848 but not to IL-4.

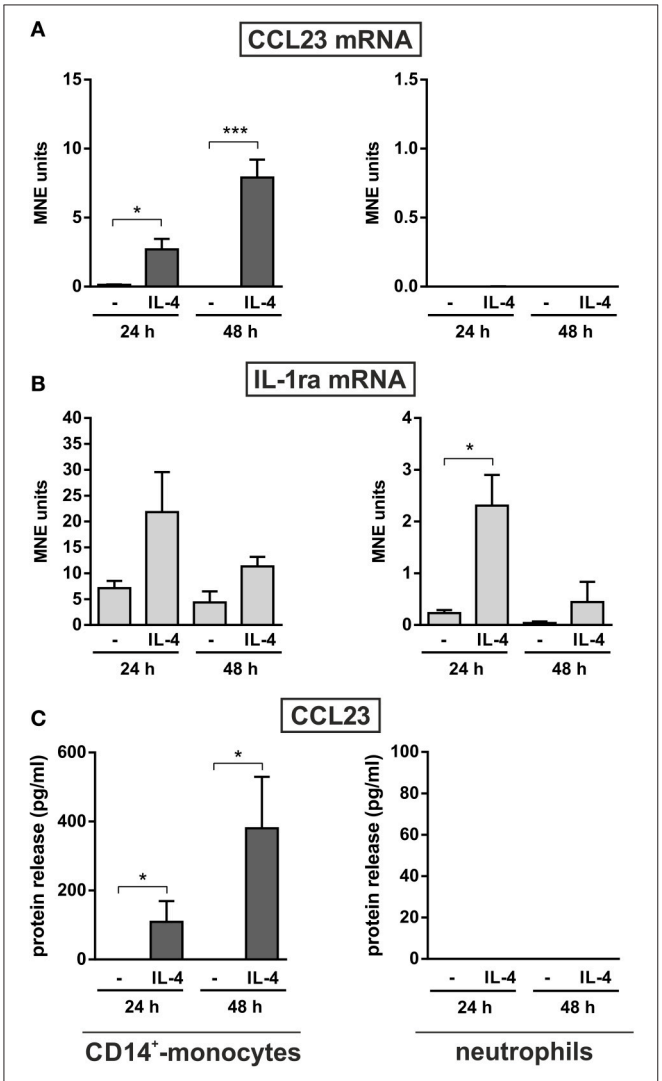


FIGURE 4 | Effect of IL-4 on the production of CCL23 by monocytes and neutrophils. $CD14^+$-monocytes and neutrophils were cultured at 5 × 10^6 cells/ml with or without 20 ng/ml IL-4 for 24 and 48 h to evaluate their CCL23 **(A)** and IL-1ra **(B)** mRNA expression by RT-qPCR, as well as their CCL23 release **(C)** by ELISA ($n = 4$). Gene expression data are depicted as MNE units after GAPDH mRNA normalization (mean ± SEM). Asterisks stand for significant increases: $^*P < 0.05$, $^{***}P < 0.001$ by two-way ANOVA followed by Bonferroni's post-test.

Effect of IFNα on CCL23 Production by R848-Treated Neutrophils

Given the recently reported capacity of IFNα to potently increase IL-6 and TNFα mRNA and protein expression in R848-treated

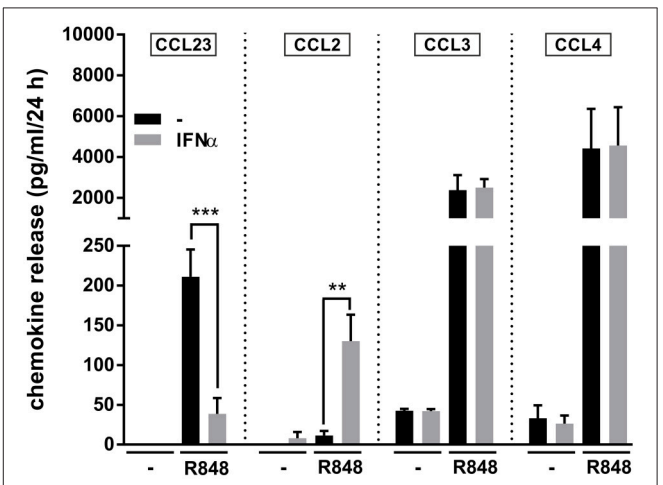


FIGURE 5 | IFNα downregulates the production of CCL23 by R848-treated neutrophils Neutrophils (5 × 10^6/ml) were cultured with or without 1,000 U/ml IFNα and/or 5 μM R848. After 24 h, supernatants were collected and subjected to CCL23, CCL2, CCL3, and CCL4 measurement by ELISA. Data are depicted as mean ± SEM ($n = 3$–19). Asterisks stand for significant increases: $^{**}P < 0.01$, $^{***}P < 0.001$, by two-way ANOVA followed by Bonferroni's post-test.

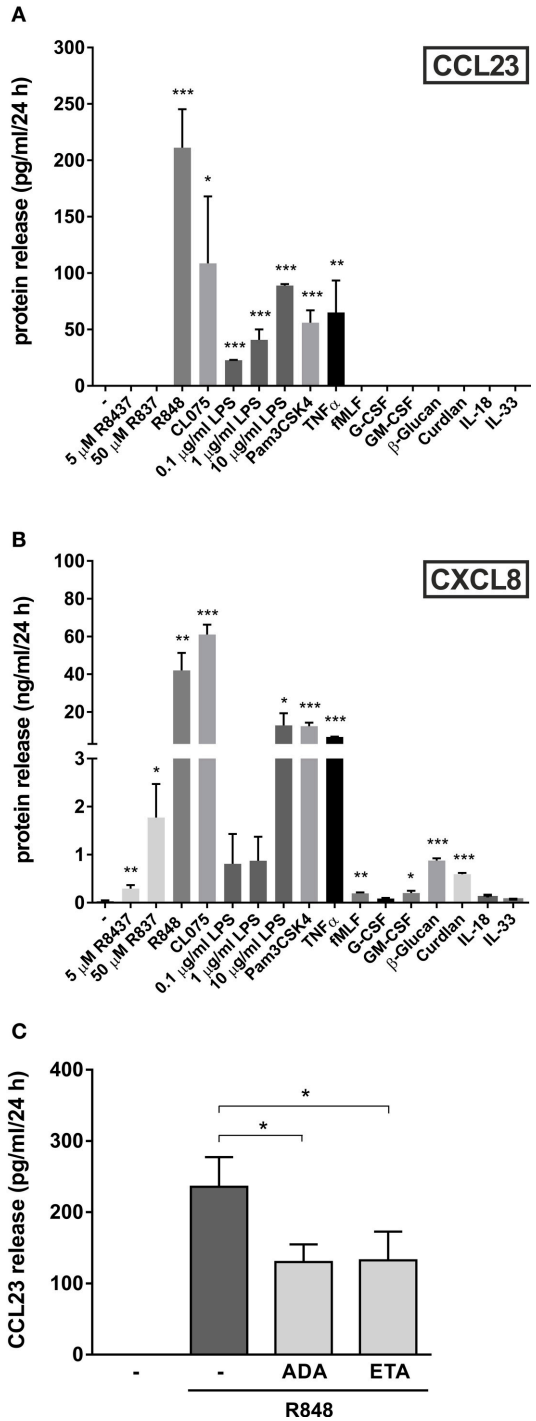


FIGURE 6 | Other agonists, besides R848, trigger the production of CCL23 by human neutrophils. Neutrophils (5×10^6/ml) were cultured with or without 5–50 μM R837, 5 μM R848, 5 μM CL075, 0.1–10 μg/ml LPS, 1 μg/ml Pam3CSK4, 10 ng/ml TNFα, 10 nM fMLF, 10 ng/ml G-CSF, 10 ng/ml GM-CSF, 500 μg/ml β-Glucan, 500 μg/ml Curdlan, 100 ng/ml IL-18, and 100 ng/ml IL-33 for 24 h. Then, extracellular supernatants were collected to evaluate CCL23 **(A)** and CXCL8 **(B)** production by ELISA (mean ± SEM, $n = 3$–5). Asterisks stand for significant increases as compared to untreated cells: $^*P < 0.05$,

(Continued)

FIGURE 6 | Continued

$^{**}P < 0.01$, $^{***}P < 0.001$, by Student's *t*-test. **(C)** Neutrophils (5×10^6/ml) were pretreated for 30 min with 10 μg/ml adalimumab (ADA) or 10 μg/ml etanercept (ETA), to be subsequently incubated for further 24 h with 5 μM R848. Cell-free supernatants were then collected to evaluate CCL23 production by ELISA (mean ± SEM, $n = 3$–5). Asterisks stand for significant inhibition: $^*P < 0.05$, by one-way ANOVA followed by Tukey's post-test.

neutrophils (Zimmermann et al., 2016), we investigated IFNα-mediated effects on neutrophil-derived CCL23 and other CCR1-binding chemokines. As displayed in **Figure 5**, we found that, while IFNα strongly downregulates the production of CCL23 by neutrophils incubated with R848 for 24 h, it significantly upregulates that of CCL2, but does not influence CCL3 and CCL4 release. Interestingly, **Figure 5** also illustrates that the amounts of CCL3 and CCL4 released by R848-stimulated neutrophils are higher than those of CCL2 and CCL23, in accordance with the gene expression data (**Figure 2**). Taken together, data provide evidence that IFNα negatively modulates CCL23, but not CCL2, CCL3, and CCL4 production by R848-treated neutrophils.

Other TLR Agonists, as well as TNFα, Trigger the Production of CCL23 by Human Neutrophils

In a final series of experiments, we investigated whether other agonists could induce the production of CCL23 by human neutrophils. We found that, in addition to R848 (the most potent one), also 5 μM CL075 (another ligand for TLR8), 1 μg/ml Pam3CSK4 (a ligand for TLR2), 10 μg/ml ultrapure LPS (a ligand for TLR4), and 10 ng/ml TNFα promoted the production of remarkable amounts of CCL23 by neutrophils (respectively, 108.6 ± 59.3, 61.8 ± 13.2, 88.8 ± 1.3, and 65.1 ± 28.2 pg/ml, $n = 3$–5) (**Figure 6A**). By contrast, 5–50 μM R837 (a ligand for TLR7), 500 μg/ml β-glucan and 500 μg/ml curdlan (both ligands for Dectin-1), 10 nM fMLF, 10 ng/ml GM-CSF, and 1,000 U/ml G-CSF, all failed to trigger any extracellular CCL23 production by neutrophils (**Figure 6A**), albeit able to stimulate the production of CXCL8 (**Figure 6B**). Finally, 100 ng/ml IL-18 and IL-33, two cytokines having potential roles in Th2-responses, resulted unable to induce CCL23 production in both human neutrophils (**Figure 6A**) and $CD14^+$-monocytes (data not shown). IL-18 and IL-33 also did not modulate the levels of CCL23 produced by R848-treated neutrophils (data not shown).

Given that R848-treated neutrophils are known to produce elevated amounts of TNFα (Zimmermann et al., 2015), we then investigated whether CCL23 production could be amplified by endogenous TNFα, as occurring in the case of IL-6 (Zimmermann et al., 2015). This was found to be the case, as adalimumab or etanercept, two potent TNFα-neutralizing drugs (Tracey et al., 2008), diminished the detection of extracellular CCL23 in supernatants from neutrophils treated with R848 for 24 h by, respectively, 44.7 ± 5.5% ($n = 3$) and 45.7 ± 11.4% ($n = 3$) (**Figure 6C**). Altogether, data uncover that neutrophils produce CCL23 in response to discrete stimuli. Data also

demonstrate that, at least in the case of R848-treated neutrophils, CCL23 production is amplified *via* endogenous TNFα.

DISCUSSION

Neutrophils are among the first cell types that infiltrate inflammatory sites. As such, they may play an important role in coordinating the subsequent recruitment of other leukocytes *via* the generation of a variety of chemoattractants, including chemokines (Tecchio and Cassatella, 2016). Among the latter molecules, neutrophils have been already shown to represent sources of some CCR1-binding chemokines, namely CCL3 and CCL4 (Kasama et al., 1994; Scapini et al., 2000), known to act on monocytes, DCs and resting T lymphocytes (Patel et al., 1997; Nardelli et al., 1999; Viola and Luster, 2008).

In this study, we show that human neutrophils are able to produce another CCR1-binding chemokine, namely CCL23, in response to ligands for TLR2 (Pam3CSK4), TLR4 (LPS), and much more efficiently, TLR8 (R848 and CL075). By contrast, a variety of inflammatory mediators previously identified as being effective stimuli for the production of chemokines by neutrophils, for instance G-CSF, GM-CSF, and fMLF (Cassatella, 1999), failed to trigger CCL23 production. Other CCR1-binding chemokines, including CCL5, CCL7, CCL13, CCL14, CCL15, and CCL16 were found as not induced in neutrophils, at least after R848-treatment. Surprisingly, the amounts of CCL23 produced by neutrophils incubated with R848 for 24 h were comparable to those made by the same number of autologous R848-treated monocytes. This finding is quite unusual because, on a per cell basis, activated monocytes usually produce higher cytokine/chemokine levels than autologous neutrophils (Cassatella, 1999; Dale et al., 2008), although some other exceptions exist, for instance CCL19/MIP3β (Scapini et al., 2001). Whatever the case is, our findings exclude that the observed CCL23 production by R848-treated neutrophils derives from potential contaminating monocytes. Moreover, they also imply that appropriately activated neutrophils may represent major sources of CCL23, even in consideration of the fact that, during bacterial or viral infections, neutrophils often outnumber mononuclear leukocytes by one to two orders of magnitude.

We also report that, differently from autologous monocytes (Novak et al., 2007), neutrophils do not produce CCL23 upon incubation with IL-4, a Th2 cytokine, even though they promptly accumulate IL-1ra mRNA (Re et al., 1993; Crepaldi et al., 2002). Although we do not have a formal molecular explanation for the inability of IL-4-treated neutrophils to express CCL23 mRNA yet, our preliminary genome-wide map of both histone H3 monomethylated at K4 (H3K4me1, which is associated with active or poised genomic regulatory elements) (Smale et al., 2014) and PU.1 (an Ets-family transcription factor marking the majority of regulatory elements in myeloid cells) (Smale et al., 2014) would reveal that neutrophils contain only one regulatory region at the *CCL23* locus (1 kb upstream from *CCL23* TSS), while monocytes contain two ones (1 and 25 kb upstream from *CCL23* TSS). Such a different chromatin conformation might be responsible for the differential ability of neutrophils and monocytes to express CCL23 mRNA in response to IL-4 stimulation, as the "closed" chromatin conformation in neutrophils would, in fact, prevent the binding of IL-4-activated transcription factor(s) to it, and consequently the transcription of CCL23 mRNA. Interestingly, other Th2 cytokines, namely IL-18 and IL-33, were found unable to trigger the production of CCL23 by human neutrophils. Therefore, along with the findings on IL-4, our data would exclude the generation of neutrophils conditioned by Th2 cytokines and associated to Th2-microenvironment.

According to previous findings (Youn et al., 1998), CCL23 may exist in multiple forms arising from both alternative splicing and post-translational processing. CCL23 cDNA encodes, in fact, a signal sequence of 21 aa followed by either a 99 aa (also known as CKβ8) or 116 aa (CKβ8-1) mature proteins, which both represent putative ligands for CCR1 (Youn et al., 1998). In such regard, our preliminary RT-qPCR experiments would indicate that R848-stimulated neutrophils express both mRNA isoforms, while monocytes express only CKβ8-1 mRNA (NT, FA, and MAC, unpublished results). As mentioned, eosinophils have been shown to express and release CCL23, and, similarly to R848-activated neutrophils, they accumulate both mRNA isoforms of CCL23 (Matsumoto et al., 2011). In any case, we exclude that the two CCL23 mRNAs detected in neutrophils derive from potential contaminating eosinophils, as our neutrophil purification method reduces eosinophil contamination below 0.1% (Calzetti et al., 2017). Moreover, GM-CSF, which triggers the release of CCL23 by eosinophils (Matsumoto et al., 2011), resulted ineffective in neutrophils.

In this work, we also report that the production of CCR1-binding chemokines by R848-activated neutrophils is differentially modulated by IFNα. In fact, while production of CCL3 and CCL4 was not affected by the addition of IFNα to neutrophils, that of CCL23 was strongly inhibited. By contrast, CCL2 production was strongly enhanced in neutrophils incubated with R848 in the presence of IFNα, similarly to what previously observed in neutrophils treated with IFNγ plus LPS (Yoshimura and Takahashi, 2007). Together, our data therefore reinforce the notion that type I interferons do not act as mere enhancers of inflammatory gene expression, but represent instead fine tuners regulating the transcription of a variety of mediators (González-Navajas et al., 2012; McNab et al., 2015), including neutrophil-derived chemokines. Given the potent effect of TLR8 agonists in inducing neutrophil-derived CCL23, and its negative regulation by IFNα, data also contribute to extend our knowledge on the complex role of neutrophils to both host defense and disease in response to viral infections (Tamassia and Cassatella, 2013; Galani and Andreakos, 2015).

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. FA, SG, SP, EC, and NT performed the experiments, FA, FB, NT, and MC analyzed the results, FA, NT, and MC conceived the experiments and wrote the paper.

FUNDING

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC, IG-15454) to MC and Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2015YYKPNN). FA is supported by Brazilian fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

ACKNOWLEDGMENTS

We thank Gioacchino Natoli (Department of Biomedical Sciences, Humanitas University, Rozzano, Milan, Italy) for his support and discussion on RNA-seq experiments.

REFERENCES

Anders, S., Pyl, P. T., and Huber, W. (2015). HTSeq–a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169. doi: 10.1093/bioinformatics/btu638

Berahovich, R. D., Miao, Z., Wang, Y., Premack, B., Howard, M. C., and Schall, T. J. (2005). Proteolytic activation of alternative CCR1 ligands in inflammation. *J. Immunol.* 174, 7341–7351. doi: 10.4049/jimmunol.174.11.7341

Berger, M., Hsieh, C. Y., Bakele, M., Marcos, V., Rieber, N., Kormann, M., et al. (2012). Neutrophils express distinct RNA receptors in a non-canonical way. *J. Biol. Chem.* 287, 19409–19417. doi: 10.1074/jbc.M112.353557

Calzetti, F., Tamassia, N., Arruda-Silva, F., Gasperini, S., and Cassatella, M. A. (2017). The importance of being "pure" neutrophils. *J. Allergy Clin. Immunol.* 139, 352.e356–355.e356. doi: 10.1016/j.jaci.2016.06.025

Cassatella, M. A. (1999). Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* 73, 369–509. doi: 10.1016/S0065-2776(08)60791-9

Crepaldi, L., Silveri, L., Calzetti, F., Pinardi, C., and Cassatella, M. A. (2002). Molecular basis of the synergistic production of IL-1 receptor antagonist by human neutrophils stimulated with IL-4 and IL-10. *Int. Immunol.* 14, 1145–1153. doi: 10.1093/intimm/dxf079

Dale, D. C., Boxer, L., and Liles, W. C. (2008). The phagocytes: neutrophils and monocytes. *Blood* 112, 935–945. doi: 10.1182/blood-2007-12-077917

Davey, M. S., Tamassia, N., Rossato, M., Bazzoni, F., Calzetti, F., Bruderek, K., et al. (2011). Failure to detect production of IL-10 by activated human neutrophils. *Nat. Immunol.* 12, 1017–1018. doi: 10.1038/ni.2111

Forssmann, U., Delgado, M. B., Uguccioni, M., Loetscher, P., Garotta, G., and Baggiolini, M. (1997). CKbeta8, a novel CC chemokine that predominantly acts on monocytes. *FEBS Lett.* 408, 211–216. doi: 10.1016/S0014-5793(97)00408-0

Galani, I. E., and Andreakos, E. (2015). Neutrophils in viral infections: current concepts and caveats. *J. Leukoc. Biol.* 98, 557–564. doi: 10.1189/jlb.4VMR1114-555R

González-Navajas, J. M., Lee, J., David, M., and Raz, E. (2012). Immunomodulatory functions of type I interferons. *Nat. Rev. Immunol.* 12, 125–135. doi: 10.1038/nri3133

Griffith, J. W., Sokol, C. L., and Luster, A. D. (2014). Chemokines and chemokine receptors: positioning cells for host defense and immunity. *Annu. Rev. Immunol.* 32, 659–702. doi: 10.1146/annurev-immunol-032713-120145

Guan, E., Wang, J., Roderiquez, G., and Norcross, M. A. (2002). Natural truncation of the chemokine MIP-1 beta /CCL4 affects receptor specificity but not anti-HIV-1 activity. *J. Biol. Chem.* 277, 32348–32352. doi: 10.1074/jbc.M203077200

Hayashi, F., Means, T. K., and Luster, A. D. (2003). Toll-like receptors stimulate human neutrophil function. *Blood* 102, 2660–2669. doi: 10.1182/blood-2003-04-1078

Hwang, J., Son, K. N., Kim, C. W., Ko, J., Na, D. S., Kwon, B. S., et al. (2005). Human CC chemokine CCL23, a ligand for CCR1, induces endothelial cell migration and promotes angiogenesis. *Cytokine* 30, 254–263. doi: 10.1016/j.cyto.2005.01.018

Janke, M., Poth, J., Wimmenauer, V., Giese, T., Coch, C., Barchet, W., et al. (2009). Selective and direct activation of human neutrophils but not eosinophils by Toll-like receptor 8. *J. Allergy Clin. Immunol.* 123, 1026–1033. doi: 10.1016/j.jaci.2009.02.015

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A. M., Wagner, H., et al. (2002). Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat. Immunol.* 3:499. doi: 10.1038/ni0602-499

Kasama, T., Strieter, R. M., Lukacs, N. W., Burdick, M. D., and Kunkel, S. L. (1994). Regulation of neutrophil-derived chemokine expression by IL-10. *J. Immunol.* 152, 3559–3569.

Kasama, T., Strieter, R. M., Standiford, T. J., Burdick, M. D., and Kunkel, S. L. (1993). Expression and regulation of human neutrophil-derived macrophage inflammatory protein 1 alpha. *J. Exp. Med.* 178, 63–72. doi: 10.1084/jem.178.1.63

Love, M. I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550. doi: 10.1186/s13059-014-0550-8

Matsumoto, K., Fukuda, S., Hashimoto, N., and Saito, H. (2011). Human eosinophils produce and release a novel chemokine, CCL23, *in vitro*. *Int. Arch. Allergy Immunol.* 155(Suppl. 1), 34–39. doi: 10.1159/000327263

McNab, F., Mayer-Barber, K., Sher, A., Wack, A., and O'Garra, A. (2015). Type I interferons in infectious disease. *Nat. Rev. Immunol.* 15, 87–103. doi: 10.1038/nri3787

Menten, P., Wuyts, A., and Van Damme, J. (2002). Macrophage inflammatory protein-1. *Cytokine Growth Factor Rev.* 13, 455–481. doi: 10.1016/S1359-6101(02)00045-X

Nardelli, B., Tiffany, H. L., Bong, G. W., Yourey, P. A., Morahan, D. K., Li, Y., et al. (1999). Characterization of the signal transduction pathway activated in human monocytes and dendritic cells by MPIF-1, a specific ligand for CC chemokine receptor 1. *J. Immunol.* 162, 435–444.

Novak, H., Müller, A., Harrer, N., Günther, C., Carballido, J. M., and Woisetschläger, M. (2007). CCL23 expression is induced by IL-4 in a STAT6-dependent fashion. *J. Immunol.* 178, 4335–4341. doi: 10.4049/jimmunol.178.7.4335

Patel, V. P., Kreider, B. L., Li, Y., Li, H., Leung, K., Salcedo, T., et al. (1997). Molecular and functional characterization of two novel human C-C chemokines as inhibitors of two distinct classes of myeloid progenitors. *J. Exp. Med.* 185, 1163–1172. doi: 10.1084/jem.185.7.1163

Re, F., Mengozzi, M., Muzio, M., Dinarello, C. A., Mantovani, A., and Colotta, F. (1993). Expression of interleukin-1 receptor antagonist (IL-1ra) by human circulating polymorphonuclear cells. *Eur. J. Immunol.* 23, 570–573. doi: 10.1002/eji.1830230242

Scapini, P., Lapinet-Vera, J. A., Gasperini, S., Calzetti, F., Bazzoni, F., and Cassatella, M. A. (2000). The neutrophil as a cellular source of chemokines. *Immunol. Rev.* 177, 195–203. doi: 10.1034/j.1600-065X.2000.17706.x

Scapini, P., Laudanna, C., Pinardi, C., Allavena, P., Mantovani, A., Sozzani, S., et al. (2001). Neutrophils produce biologically active macrophage inflammatory protein-3alpha (MIP-3alpha)/CCL20 and MIP-3beta/CCL19. *Eur. J. Immunol.* 31, 1981–1988. doi: 10.1002/1521-4141(200107)31:7<1981::AID-IMMU1981>3.0.CO;2-X

Scapini, P., Tamassia, N., Pucillo, C., and Cassatella, M. A. (2013). "Granulocytes and mast cells," in *Fundamental Immunology, 7th Edn.,* ed W. E. Paul (Philadelphia, PA: Wolters Kluwer Health; Lippincott Williams & Wilkins), 468–486.

Smale, S. T., Tarakhovsky, A., and Natoli, G. (2014). Chromatin contributions to the regulation of innate immunity. *Annu. Rev. Immunol.* 32, 489–511. doi: 10.1146/annurev-immunol-031210-101303

Son, K. N., Hwang, J., Kwon, B. S., and Kim, J. (2006). Human CC chemokine CCL23 enhances expression of matrix metalloproteinase-2 and invasion of vascular endothelial cells. *Biochem. Biophys. Res. Commun.* 340, 498–504. doi: 10.1016/j.bbrc.2005.12.037

Tamassia, N., and Cassatella, M. A. (2013). Cytoplasmic receptors recognizing nucleic acids and mediating immune functions in neutrophils. *Curr. Opin. Pharmacol.* 13, 547–554. doi: 10.1016/j.coph.2013.05.003

Tamassia, N., Cassatella, M. A., and Bazzoni, F. (2014). Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils. *Methods Mol. Biol.* 1124, 451–467. doi: 10.1007/978-1-62703-845-4_27

Tecchio, C., and Cassatella, M. A. (2016). Neutrophil-derived chemokines on the road to immunity. *Semin. Immunol.* 28, 119–128. doi: 10.1016/j.smim.2016.04.003

Tracey, D., Klareskog, L., Sasso, E. H., Salfeld, J. G., and Tak, P. P. (2008). Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol. Ther.* 117, 244–279. doi: 10.1016/j.pharmthera.2007.10.001

Trapnell, C., Hendrickson, D. G., Sauvageau, M., Goff, L., Rinn, J. L., and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* 31, 46–53. doi: 10.1038/nbt.2450

Viola, A., and Luster, A. D. (2008). Chemokines and their receptors: drug targets in immunity and inflammation. *Annu. Rev. Pharmacol. Toxicol.* 48, 171–197. doi: 10.1146/annurev.pharmtox.48.121806.154841

Votta, B. J., White, J. R., Dodds, R. A., James, I. E., Connor, J. R., Lee-Rykaczewski, E., et al. (2000). CKbeta-8 [CCL23], a novel CC chemokine, is chemotactic for human osteoclast precursors and is expressed in bone tissues. *J. Cell. Physiol.* 183, 196–207. doi: 10.1002/(sici)1097-4652(200005)183:2< 196::aid-jcp6>3.0.co;2-8

Yamashiro, S., Kamohara, H., and Yoshimura, T. (1999). MCP-1 is selectively expressed in the late phase by cytokine-stimulated human neutrophils: TNF-alpha plays a role in maximal MCP-1 mRNA expression. *J. Leukoc. Biol.* 65, 671–679.

Yanaba, K., Yoshizaki, A., Muroi, E., Ogawa, F., Asano, Y., Kadono, T., et al. (2011). Serum CCL23 levels are increased in patients with systemic sclerosis. *Arch. Dermatol. Res.* 303, 29–34. doi: 10.1007/s00403-010-1078-8

Yoshimura, T., and Takahashi, M. (2007). IFN-gamma-mediated survival enables human neutrophils to produce MCP-1/CCL2 in response to activation by TLR ligands. *J. Immunol.* 179, 1942–1949. doi: 10.4049/jimmunol.179.3.1942

Youn, B. S., Zhang, S. M., Broxmeyer, H. E., Cooper, S., Antol, K., Fraser, M. Jr., et al. (1998). Characterization of CKbeta8 and CKbeta8-1: two alternatively spliced forms of human beta-chemokine, chemoattractants for neutrophils, monocytes, and lymphocytes, and potent agonists at CC chemokine receptor 1. *Blood* 91, 3118–3126.

Zimmermann, M., Aguilera, F. B., Castellucci, M., Rossato, M., Costa, S., Lunardi, C., et al. (2015). Chromatin remodelling and autocrine TNFα are required for optimal interleukin-6 expression in activated human neutrophils. *Nat. Commun.* 6, 6061. doi: 10.1038/ncomms7061

Zimmermann, M., Arruda-Silva, F., Bianchetto-Aguilera, F., Finotti, G., Calzetti, F., Scapini, P., et al. (2016). IFNα enhances the production of IL-6 by human neutrophils activated via TLR8. *Sci. Rep.* 6:19674. doi: 10.1038/srep19674

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 17 April 2018
doi: 10.3389/fimmu.2018.00795

A Reappraisal on the Potential Ability of Human Neutrophils to Express and Produce IL-17 Family Members *In Vitro*: Failure to Reproducibly Detect It

*Nicola Tamassia[1†], Fabio Arruda-Silva[1,2†], Federica Calzetti[1], Silvia Lonardi[3], Sara Gasperini[1], Elisa Gardiman[1], Francisco Bianchetto-Aguilera[1], Luisa Benerini Gatta[3], Giampiero Girolomoni[4], Alberto Mantovani[5,6,7], William Vermi[3] and Marco A. Cassatella[1]**

[1] Department of Medicine, Section of General Pathology, University of Verona, Verona, Italy, [2] CAPES Foundation, Ministry of Education of Brazil, Brasilia, Brazil, [3] Department of Molecular and Translational Medicine, Section of Pathology, University of Brescia, Brescia, Italy, [4] Department of Medicine, Section of Dermatology and Venereology, University of Verona, Verona, Italy, [5] Humanitas Clinical and Research Center, Rozzano, Italy, [6] Humanitas University, Pieve Emanuele, Italy, [7] The William Harvey Research Institute, Queen Mary University of London, London, United Kingdom

OPEN ACCESS

Edited by:
Liwu Li,
Virginia Tech, United States

Reviewed by:
Min Wu,
University of North Dakota, United States
Petya Dimitrova,
Institute of Microbiology, Sofia, Bulgaria

***Correspondence:**
Marco A. Cassatella
marco.cassatella@univr.it

[†]These authors have contributed equally to this work.

Specialty section:
This article was submitted to Molecular Innate Immunity, a section of the journal Frontiers in Immunology

Received: 14 February 2018
Accepted: 03 April 2018
Published: 17 April 2018

Citation:
Tamassia N, Arruda-Silva F, Calzetti F, Lonardi S, Gasperini S, Gardiman E, Bianchetto-Aguilera F, Gatta LB, Girolomoni G, Mantovani A, Vermi W and Cassatella MA (2018) A Reappraisal on the Potential Ability of Human Neutrophils to Express and Produce IL-17 Family Members In Vitro: Failure to Reproducibly Detect It. Front. Immunol. 9:795. doi: 10.3389/fimmu.2018.00795

Neutrophils are known to perform a series of effector functions that are crucial for the innate and adaptive responses, including the synthesis and secretion of a variety of cytokines. In light of the controversial data in the literature, the main objective of this study was to more in-depth reevaluate the capacity of human neutrophils to express and produce cytokines of the IL-17 family *in vitro*. By reverse transcription quantitative real-time PCR, protein measurement *via* commercial ELISA, immunohistochemistry (IHC) and immunofluorescence (IF), flow cytometry, immunoblotting, chromatin immunoprecipitation (ChIP), and ChIP-seq experiments, we found that highly pure (>99.7%) populations of human neutrophils do not express/produce IL-17A, IL-17F, IL-17AF, or IL-17B mRNA/protein upon incubation with a variety of agonists. Similar findings were observed by analyzing neutrophils isolated from active psoriatic patients. In contrast with published studies, IL-17A and IL-17F mRNA expression/production was not even found when neutrophils were incubated with extremely high concentrations of IL-6 plus IL-23, regardless of their combination with inactivated hyphae or conidia from *Aspergillus fumigatus*. Consistently, no deposition of histone marks for active (H3K27Ac) and poised (H3K4me1) genomic regulatory elements was detected at the IL-17A and IL-17F locus of resting and IL-6 plus IL-23-stimulated neutrophils, indicating a closed chromatin conformation. Concurrent experiments revealed that some commercial anti-IL-17A and anti-IL-17B antibodies (Abs), although staining neutrophils either spotted on cytospin slides or present in inflamed tissue samples by IHC/IF, do not recognize intracellular protein having the molecular weight corresponding to IL-17A or IL-17B, respectively, in immunoblotting experiments of whole neutrophil lysates. By contrast, the same Abs were found to more specifically recognize other intracellular proteins of neutrophils, suggesting that their ability to positively stain neutrophils in cytospin preparations and, eventually, tissue samples derives from IL-17A- or IL-17B-independent detections. In

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,

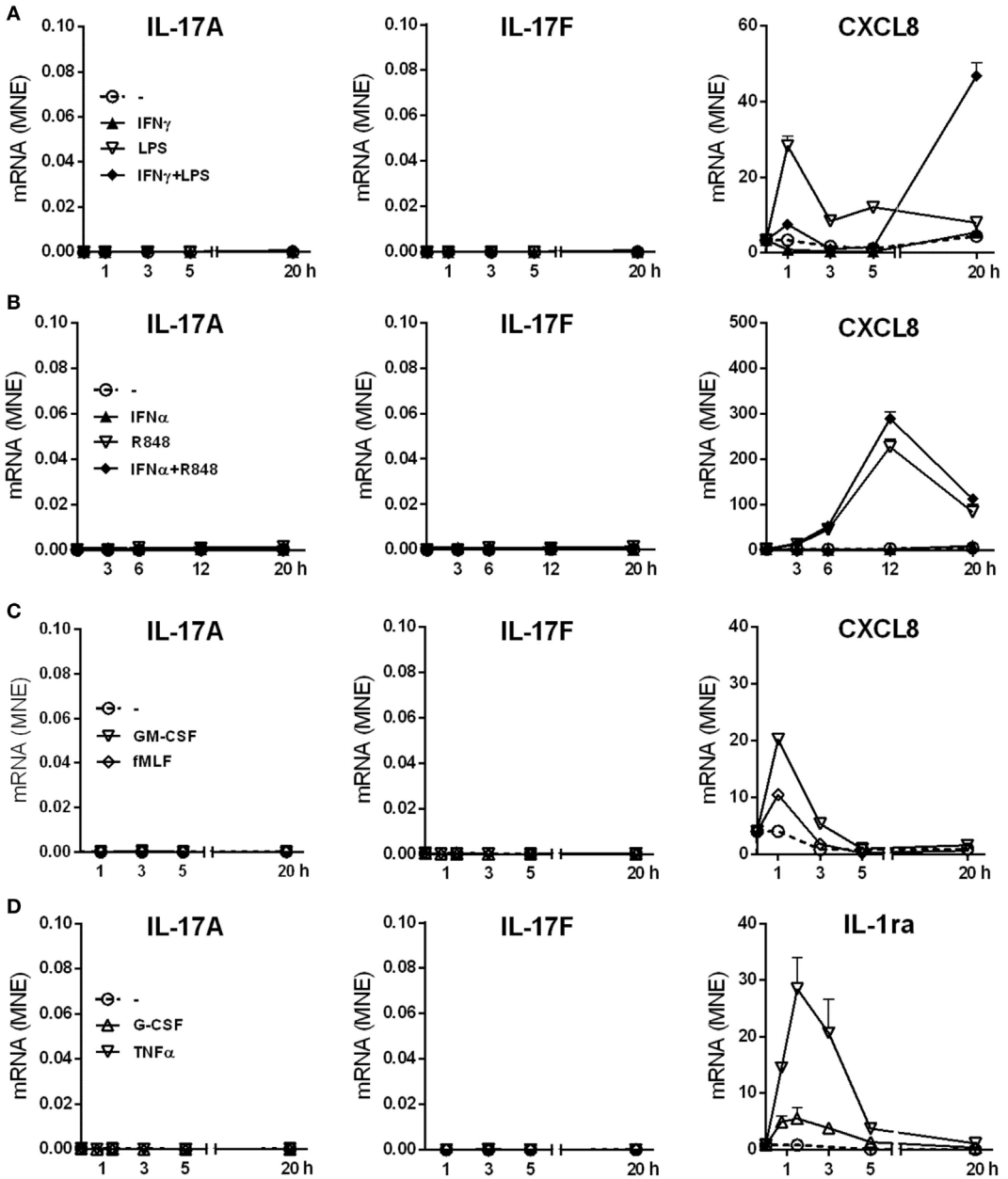


FIGURE 1 | IL-17A, IL-17F, CXCL8, and IL-1ra mRNA expression levels in human neutrophils activated by a variety of stimuli. Human neutrophils were cultured at 5 × 10^6/ml for up to 20 h with **(A)** 100 U/ml IFNγ and/or 100 ng/ml LPS; **(B)** 1,000 U/ml IFNα and/or 5 μM R848; **(C)** 10 ng/ml GM-CSF 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

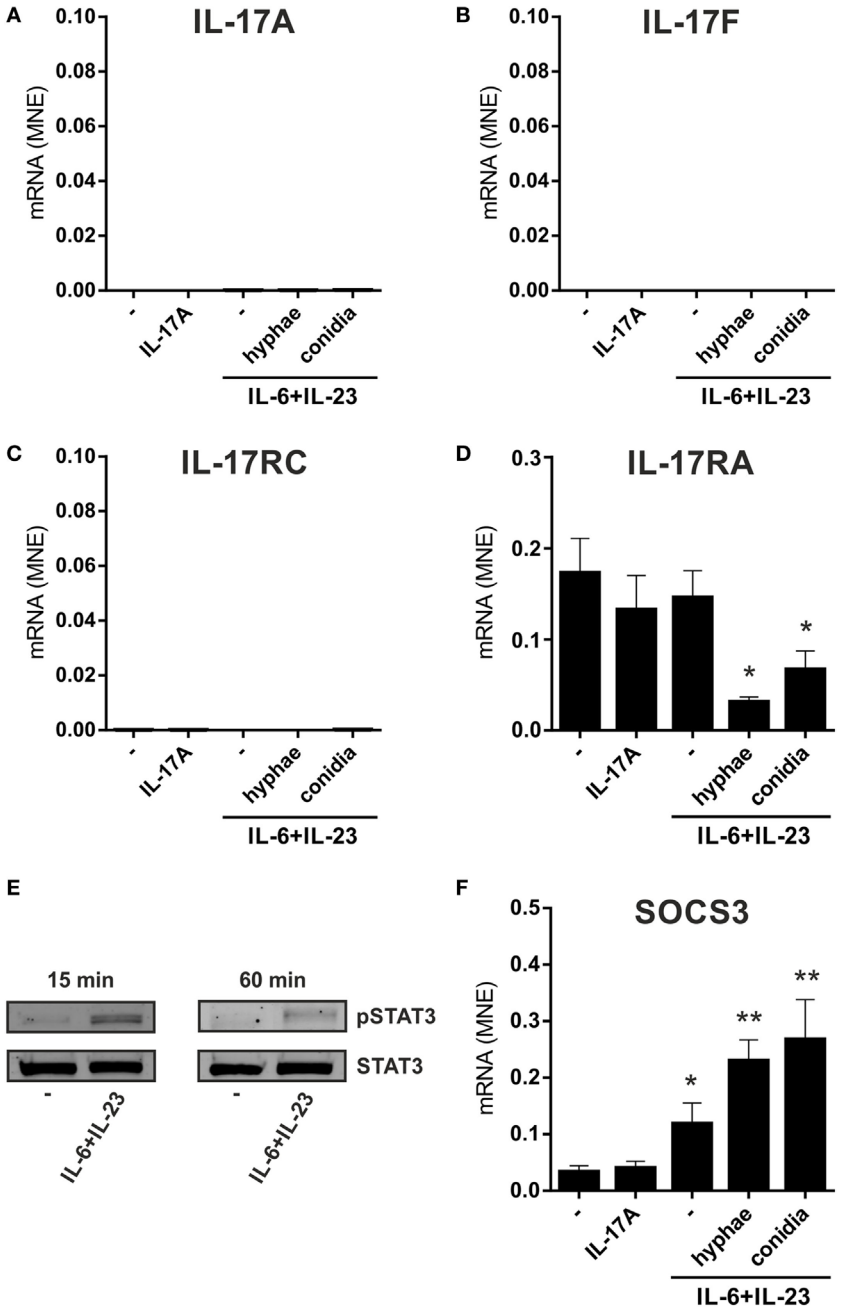


FIGURE 2 | No induction of IL-17A, IL-17F, and IL-17RC mRNA expression in neutrophils incubated with IL-6 plus IL-23, in combination with inactivated *Aspergillus fumigatus* hyphae or conidia. Neutrophils (5×10^6/ml) were incubated either with 100 ng/ml rIL-17A for 2 h or with or without 20 µg/ml IL-6 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),

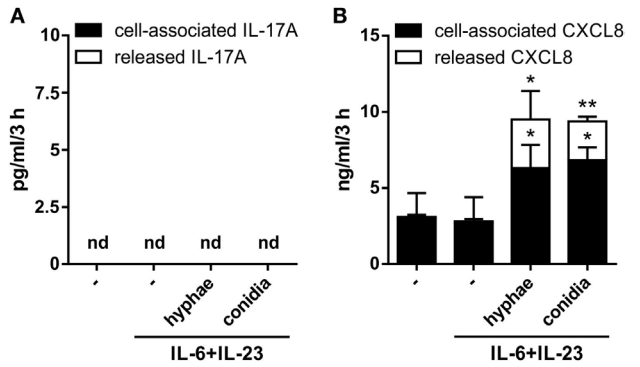


FIGURE 3 | Lack of IL-17A and IL-17F production by human neutrophils activated by IL-6 plus IL-23 in combination with inactivated *Aspergillus fumigatus* hyphae or conidia. Neutrophils (5×10^6/ml) were incubated with or without 20 μg/ml IL-6 plus 2 μg/ml IL-23 and then cultured for three more hours in 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.

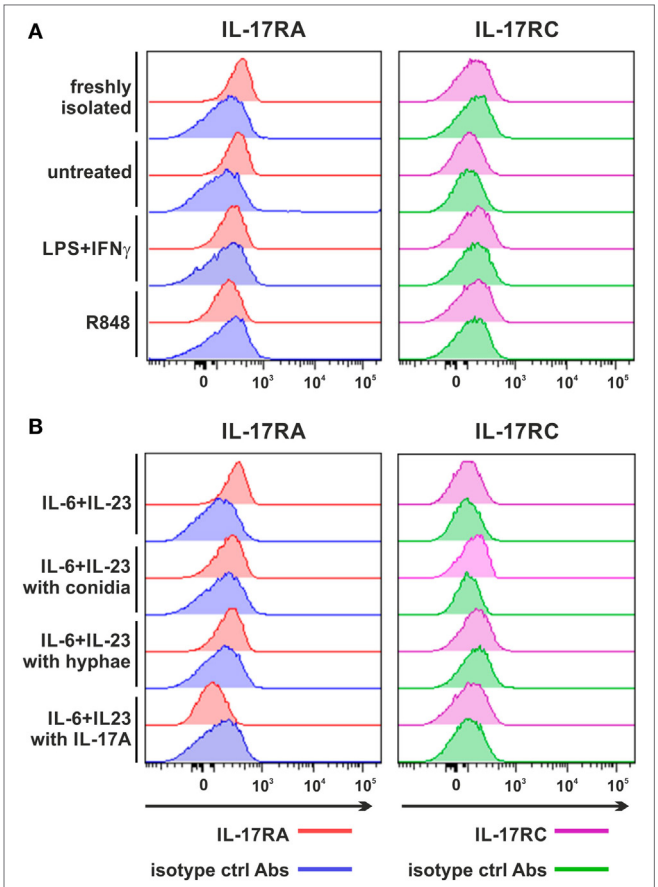


FIGURE 4 | Expression of surface IL-17RA and IL-17RC in neutrophils activated under various experimental conditions. Expression of surface IL-17RA (left panel) and IL-17RC (right panel) was evaluated by flow cytometry in neutrophils either freshly isolated or cultured for 3 h without or with 100 U/ml 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

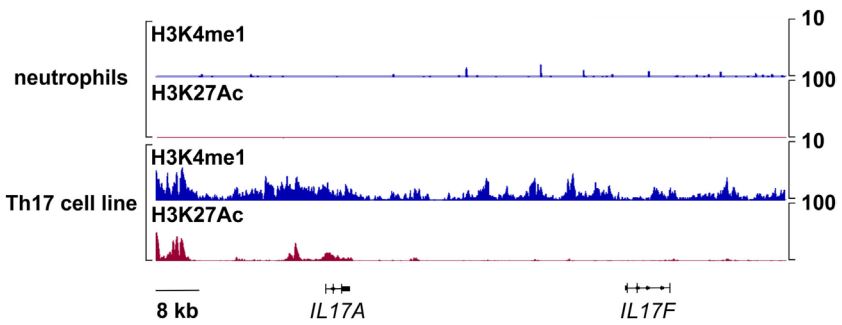


FIGURE 5 | Chromatin immunoprecipitation (ChIP)-Seq profiles of H3K4me1 and H3K27Ac at the *IL17A* and *IL17F* loci in human neutrophils and Th17 cell lines. Representative snapshots depicting H3K4me1 and H3K27Ac ChIP-seqs at the *IL17A* and *IL17F* genomic loci in freshly isolated human neutrophils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

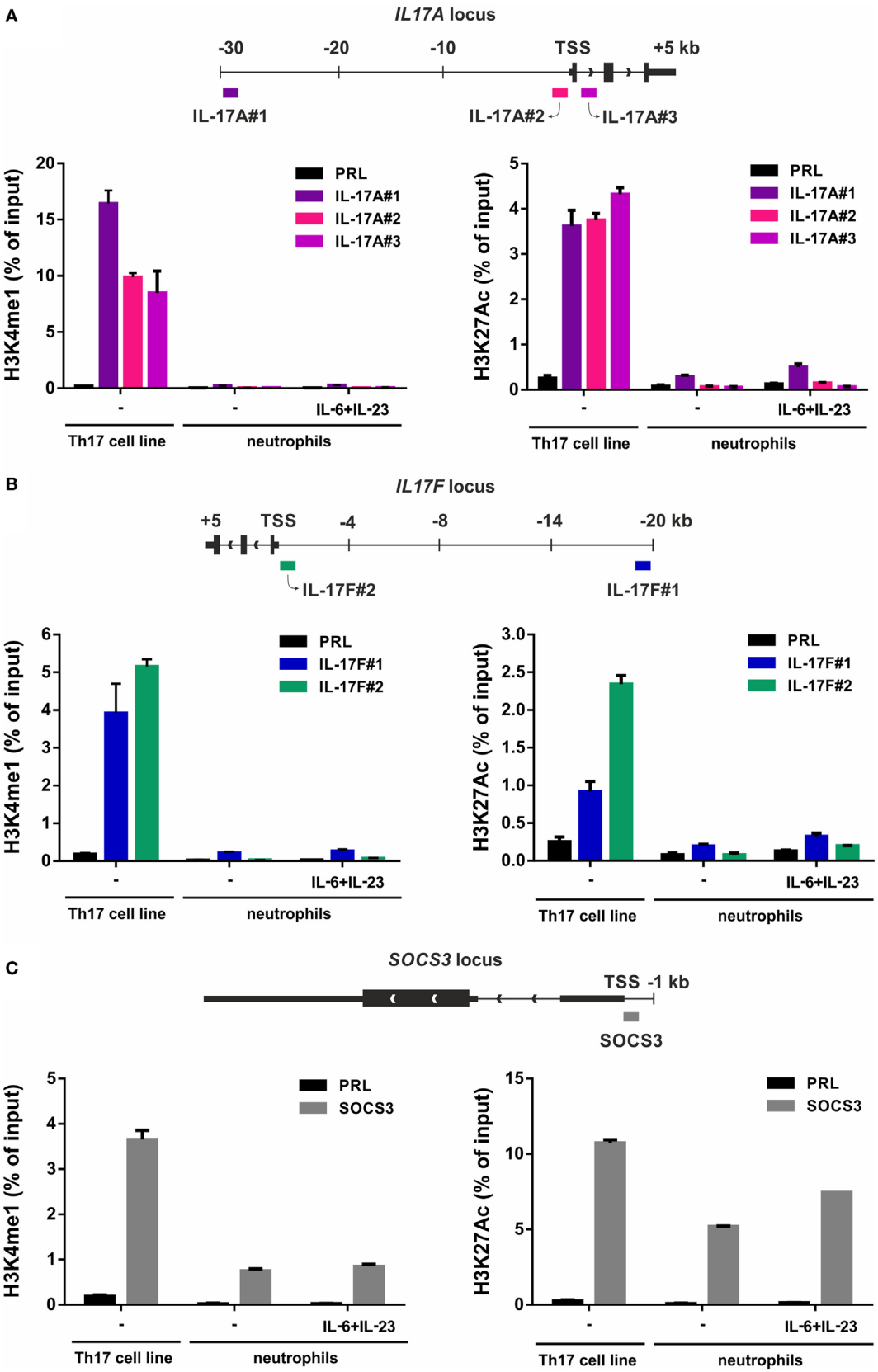


FIGURE 6 | Continued

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

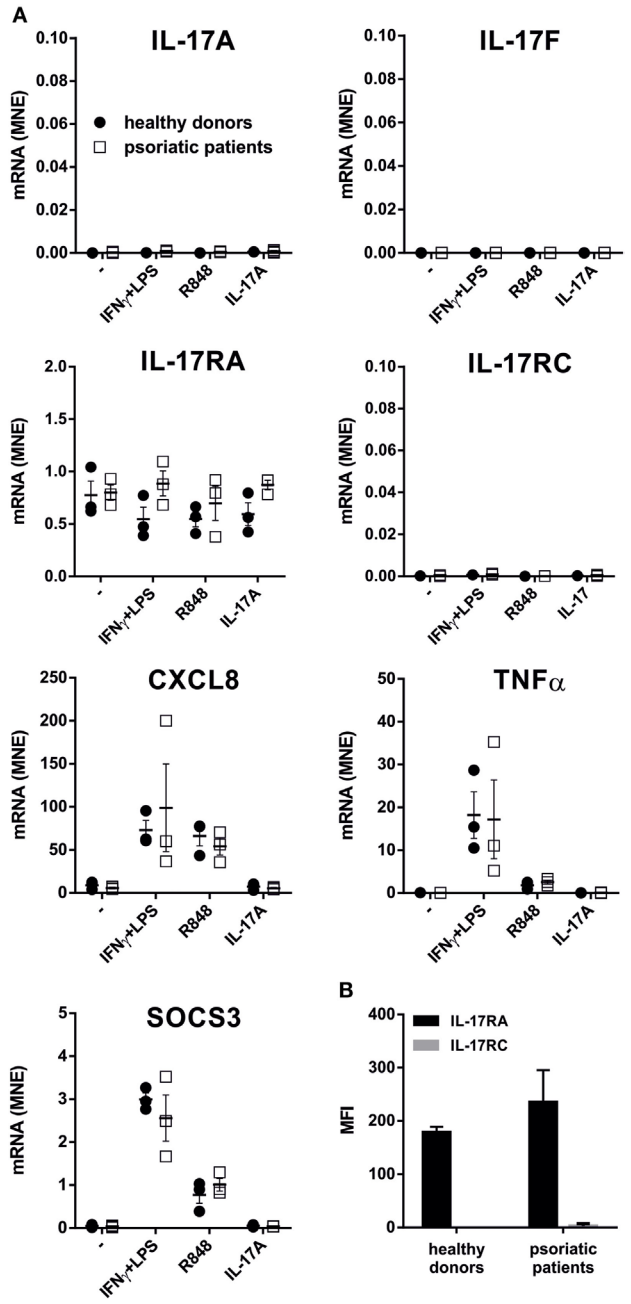


FIGURE 7 | IL-17A, IL-17F, IL-17RA, IL-17RC, CXCL8, TNFα, and SOCS3 mRNA expression, as well as IL-17R surface expression, in neutrophils from patients with psoriasis. **(A)** Neutrophils isolated from healthy donors (HDs) ($n = 3$) or psoriatic patients ($n = 3$) were cultured for 20 h with 100 U/ml 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

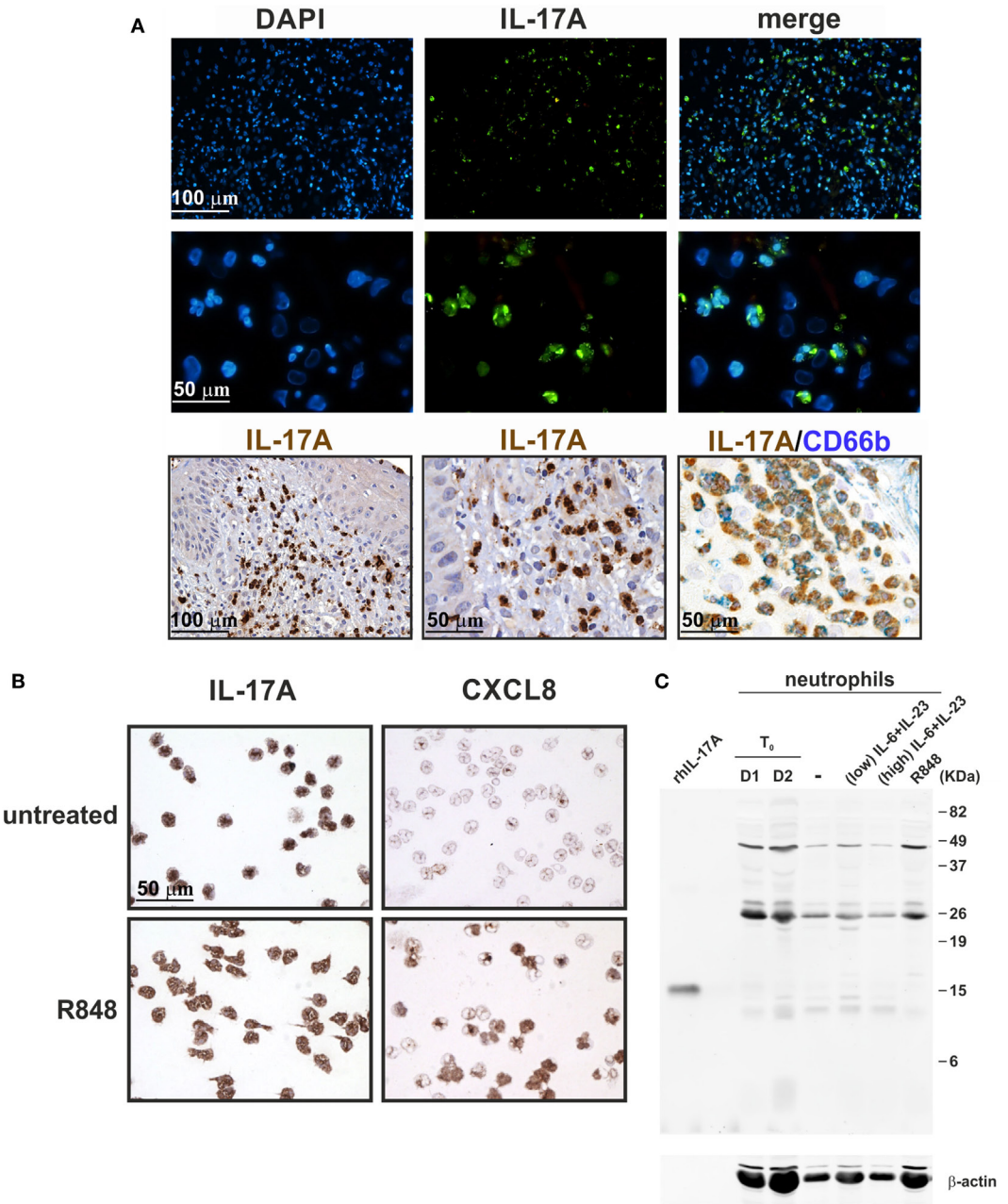


FIGURE 8 | Staining human neutrophils by anti-IL-17A (AF-317-NA) polyclonal antibodies (Abs). **(A)** Immunofluorescence (top panels) and immunohistochemistry (lower panels) stainings of two FFPE cases of human pustular psoriasis using anti-IL-17A (AF-317-NA) and anti-CD66b Abs (as labeled). Top panels 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

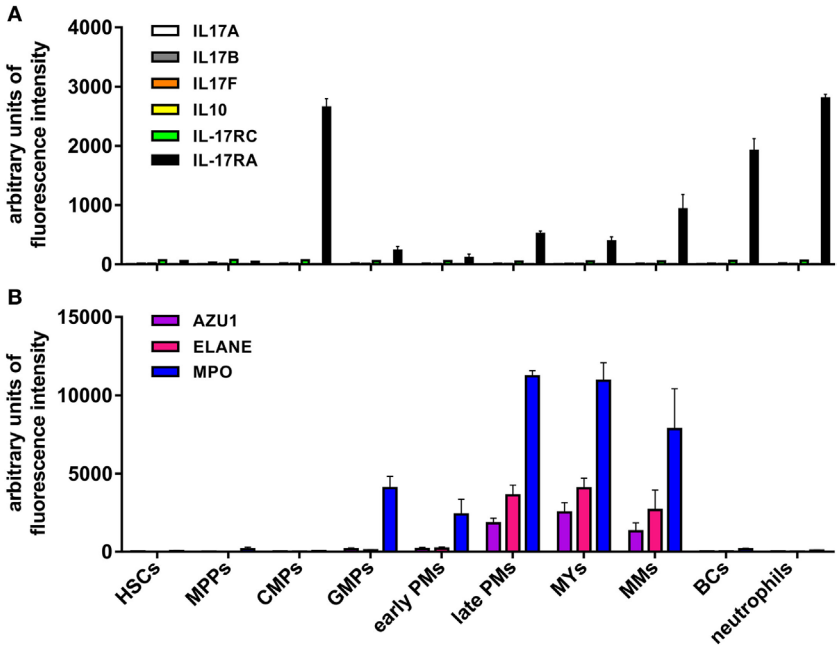


FIGURE 9 | Levels of IL-17A, IL-17B, IL-17F, IL-10, IL-17RC, IL-17RA, azurocidin, neutrophil elastase, and myeloperoxidase (MPO) mRNA expression in neutrophils at different stages of maturation. mRNA expression data derive from Gene Expression Omnibus database (accession number GSE42519) (65). **(A)** 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

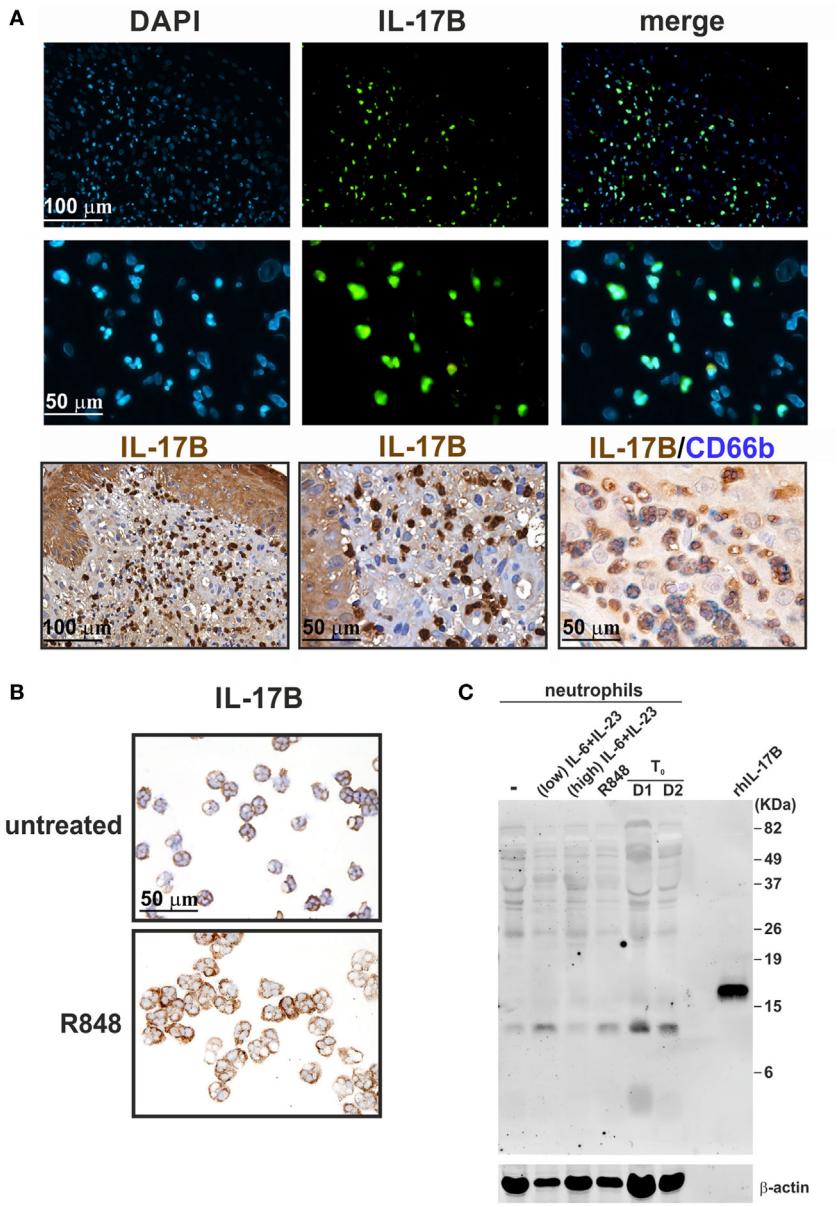


FIGURE 10 | Staining human neutrophils by anti-IL-17B (AF1248) antibodies (Abs). **(A)** Immunofluorescence (top panels) and immunohistochemistry (lower panels) staining of two FFPE cases of human pustular psoriasis using anti-IL-17B (AF1248) and CD66b Abs (as labeled). Top panels show DAPI, FITC channel, 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.

ACKNOWLEDGMENTS

We thank Luigina Romani (University of Perugia, Perugia, Italy) for inactivated *Aspergillus Fumigatus* hyphae and conidia, Alessio Mazzoni and Francesco Annunziato (University of Firenze, Firenze, Italy) for Th17 clones, Alessia Farinazzo and Bruno Bonetti (University of Verona, Verona, Italy) for protein lysate of human cerebral cortex and Gioacchino Natoli (Humanitas University, Rozzano, Milan, Italy) for his support and discussion on ChIP-seq experiments.

FUNDING

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC, IG-20339) and Ministero dell'Istruzione, dell'Università e della Ricerca (PRIN 2015YYKPNN) to MC, and from Novartis Farma SpA (Origgio, Italia) to AM. FA-S is supported by Brazilian fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, no Processo: 99999.013628/2013-05).

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.

REFERENCES

1. Amatya N, Garg AV, Gaffen SL. IL-17 signaling: the Yin and the Yang. *Trends Immunol* (2017) 38(5):310–22. doi:10.1016/j.it.2017.01.006

2. Hymowitz SG, Filvaroff EH, Yin JP, Lee J, Cai L, Risser P, et al. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J* (2001) 20(19):5332–41. doi:10.1093/emboj/20.19.5332

3. Gaffen SL, Jain R, Garg AV, Cua DJ. The IL-23-IL-17 immune axis: from mechanisms to therapeutic testing. *Nat Rev Immunol* (2014) 14(9):585–600. doi:10.1038/nri3707
4. Wright JF, Guo Y, Quazi A, Luxenberg DP, Bennett F, Ross JF, et al. Identification of an interleukin 17F/17A heterodimer in activated human CD4+ T cells. *J Biol Chem* (2007) 282(18):13447–55. doi:10.1074/jbc.M700499200
5. Veldhoen M. Interleukin 17 is a chief orchestrator of immunity. *Nat Immunol* (2017) 18(6):612–21. doi:10.1038/ni.3742
6. Schwarzenberger P, La Russa V, Miller A, Ye P, Huang W, Zieske A, et al. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J Immunol* (1998) 161(11):6383–9.
7. McAleer JP, Kolls JK. Mechanisms controlling Th17 cytokine expression and host defense. *J Leukoc Biol* (2011) 90(2):263–70. doi:10.1189/jlb.0211099
8. Miossec P, Kolls JK. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov* (2012) 11(10):763–76. doi:10.1038/nrd3794
9. Cua DJ, Tato CM. Innate IL-17-producing cells: the sentinels of the immune system. *Nat Rev Immunol* (2010) 10(7):479–89. doi:10.1038/nri2800
10. Scapini P, Tamassia N, Pucillo C, Cassatella MA. Granulocytes and mast cells. 7th ed. In: Paul WE, editor. *Fundamental Immunology*. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins (2013). p. 468–86.
11. Tecchio C, Scapini P, Pizzolo G, Cassatella MA. On the cytokines produced by human neutrophils in tumors. *Semin Cancer Biol* (2013) 23(3):159–70. doi:10.1016/j.semcancer.2013.02.004
12. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, Costantini C, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood* (2010) 115(2):335–43. doi:10.1182/blood-2009-04-216085
13. Tamarozzi F, Wright HL, Thomas HB, Edwards SW, Taylor MJ. A lack of confirmation with alternative assays questions the validity of IL-17A expression in human neutrophils using immunohistochemistry. *Immunol Lett* (2014) 162(2 Pt B):194–8. doi:10.1016/j.imlet.2014.10.025
14. Tenland E, Hakansson G, Alaridah N, Lutay N, Ronnholm A, Hallgren O, et al. Innate immune responses after airway epithelial stimulation with *Mycobacterium bovis* bacille-Calmette Guerin. *PLoS One* (2016) 11(10): e0164431. doi:10.1371/journal.pone.0164431
15. Yamanaka K, Yamagiwa A, Akeda T, Kondo M, Kakeda M, Habe K, et al. Neutrophils are not the dominant interleukin-17 producer in psoriasis. *J Dermatol* (2017) 44(7):e170–1. doi:10.1111/1346-8138.13807
16. van Baarsen LGM, Lebre MC, van der Coelen D, Aarrass S, Tang MW, Ramwadhdoebe TH, et al. Heterogeneous expression pattern of interleukin 17A (IL-17A), IL-17F and their receptors in synovium of rheumatoid arthritis, psoriatic arthritis and osteoarthritis: possible explanation for nonresponse to anti-IL-17 therapy? *Arthritis Res Ther* (2014) 16(4):426. doi:10.1186/s13075-014-0426-z
17. Nadkarni S, Smith J, Sferruzzi-Perri AN, Ledwozyw A, Kishore M, Haas R, et al. Neutrophils induce proangiogenic T cells with a regulatory phenotype in pregnancy. *Proc Natl Acad Sci U S A* (2016) 113(52):E8415–24. doi:10.1073/pnas.1611944114
18. Appel H, Maier R, Wu P, Scheer R, Hempfing A, Kayser R, et al. Analysis of IL-17(+) cells in facet joints of patients with spondyloarthritis suggests that the innate immune pathway might be of greater relevance than the Th17-mediated adaptive immune response. *Arthritis Res Ther* (2011) 13(3):R95. doi:10.1186/ar3370
19. Brodlie M, McKean MC, Johnson GE, Anderson AE, Hilkens CM, Fisher AJ, et al. Raised interleukin-17 is immunolocalised to neutrophils in cystic fibrosis lung disease. *Eur Respir J* (2011) 37(6):1378–85. doi:10.1183/09031936.00067110
20. Dyring-Andersen B, Honore TV, Madelung A, Bzorek M, Simonsen S, Clemmensen SN, et al. Interleukin (IL)-17A and IL-22-producing neutrophils in psoriatic skin. *Br J Dermatol* (2017) 177(6):e321–2. doi:10.1111/bjd.15533
21. de Boer OJ, van der Meer JJ, Teeling P, van der Loos CM, Idu MM, van Maldegem F, et al. Differential expression of interleukin-17 family cytokines in intact and complicated human atherosclerotic plaques. *J Pathol* (2010) 220(4):499–508. doi:10.1002/path.2667
22. Eustace A, Smyth LJC, Mitchell L, Williamson K, Plumb J, Singh D. Identification of cells expressing IL-17A and IL-17F in the lungs of patients with COPD. *Chest* (2011) 139(5):1089–100. doi:10.1378/chest.10-0779
23. Halwani R, Sultana A, Vazquez-Tello A, Jamhawi A, Al-Masri AA, Al-Muhsen S. Th-17 regulatory cytokines IL-21, IL-23, and IL-6 enhance neutrophil production of IL-17 cytokines during asthma. *J Asthma* (2017) 54(9):893–904. doi:10.1080/02770903.2017.1283696
24. Hu S, He W, Du X, Yang J, Wen Q, Zhong XP, et al. IL-17 production of neutrophils enhances antibacteria ability but promotes arthritis development during *Mycobacterium tuberculosis* infection. *EBioMed* (2017) 23:88–99. doi:10.1016/j.ebiom.2017.08.001
25. Kakeda M, Schlapbach C, Danelon G, Tang MM, Cecchinato V, Yawalkar N, et al. Innate immune cells express IL-17A/F in acute generalized exanthematous pustulosis and generalized pustular psoriasis. *Arch Dermatol Res* (2014) 306(10):933–8. doi:10.1007/s00403-014-1488-0
26. Karthikeyan RS, Vareechon C, Prajna NV, Dharmalingam K, Pearlman E, Lalitha P. Interleukin 17 expression in peripheral blood neutrophils from fungal keratitis patients and healthy cohorts in southern India. *J Infect Dis* (2015) 211(1):130–4. doi:10.1093/infdis/jiu381
27. Keijsers R, Hendriks AGM, van Erp PEJ, van Cranenbroek B, van de Kerkhof PCM, Koenen H, et al. In vivo induction of cutaneous inflammation results in the accumulation of extracellular trap-forming neutrophils expressing RORgammat and IL-17. *J Invest Dermatol* (2014) 134(5):1276–84. doi:10.1038/jid.2013.526
28. Le Jan S, Plee J, Vallerand D, Dupont A, Delanez E, Durlach A, et al. Innate immune cell-produced IL-17 sustains inflammation in bullous pemphigoid. *J Invest Dermatol* (2014) 134(12):2908–17. doi:10.1038/jid.2014.263
29. Li TJ, Jiang YM, Hu YF, Huang L, Yu J, Zhao LY, et al. Interleukin-17-producing neutrophils link inflammatory stimuli to disease progression by promoting angiogenesis in gastric cancer. *Clin Cancer Res* (2017) 23(6):1575–85. doi:10.1158/1078-0432.ccr-16-0617
30. Lin AM, Rubin CJ, Khandpur R, Wang JY, Riblett M, Yalavarthi S, et al. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. *J Immunol* (2011) 187(1):490–500. doi:10.4049/jimmunol.1100123
31. Moran EM, Heydrich R, Ng CT, Saber TP, McCormick J, Sieper J, et al. IL-17A expression is localised to both mononuclear and polymorphonuclear synovial cell infiltrates. *PLoS One* (2011) 6(8):e24048. doi:10.1371/journal.pone.0024048
32. Patel DD, Lee DM, Kolbinger F, Antoni C. Effect of IL-17A blockade with secukinumab in autoimmune diseases. *Ann Rheum Dis* (2013) 72(Suppl 2):ii116–23. doi:10.1136/annrheumdis-2012-202371
33. Punt S, Fleuren GJ, Kritikou E, Lubberts E, Trimbos JB, Jordanova ES, et al. Angels and demons: Th17 cells represent a beneficial response, while neutrophil IL-17 is associated with poor prognosis in squamous cervical cancer. *Oncoimmunology* (2015) 4(1):e984539. doi:10.4161/2162402x.2014.984539
34. Ramirez-Velazquez C, Castillo EC, Guido-Bayardo L, Ortiz-Navarrete V. IL-17-producing peripheral blood CD177+ neutrophils increase in allergic asthmatic subjects. *Allergy Asthma Clin Immunol* (2013) 9(1):23. doi:10.1186/1710-1492-9-23
35. Reich K, Papp KA, Matheson RT, Tu JH, Bissonnette R, Bourcier M, et al. Evidence that a neutrophil-keratinocyte crosstalk is an early target of IL-17A inhibition in psoriasis. *Exp Dermatol* (2015) 24(7):529–35. doi:10.1111/exd.12710
36. Tan HL, Regamey N, Brown S, Bush A, Lloyd CM, Davies JC. The Th17 pathway in cystic fibrosis lung disease. *Am J Respir Crit Care Med* (2011) 184(2):252–8. doi:10.1164/rccm.201102-0236OC
37. Rodrigues-Diez R, Aroeira LS, Orejudo M, Bajo MA, Heffernan JJ, Rodrigues-Diez RR, et al. IL-17A is a novel player in dialysis-induced peritoneal damage. *Kidney Int* (2014) 86(2):303–15. doi:10.1038/ki.2014.33
38. Tan Z, Jiang R, Wang X, Wang Y, Lu L, Liu Q, et al. RORgammat+IL-17+ neutrophils play a critical role in hepatic ischemia-reperfusion injury. *J Mol Cell Biol* (2013) 5(2):143–6. doi:10.1093/jmcb/mjs065
39. Taylor PR, Roy S, Leal SM Jr, Sun Y, Howell SJ, Cobb BA, et al. Activation of neutrophils by autocrine IL-17A-IL-17RC interactions during fungal infection is regulated by IL-6, IL-23, RORgammat and dectin-2. *Nat Immunol* (2014) 15(2):143–51. doi:10.1038/ni.2797
40. Taylor PR, Roy S, Meszaros EC, Sun Y, Howell SJ, Malemud CJ, et al. JAK/STAT regulation of *Aspergillus fumigatus* corneal infections and IL-6/23-stimulated neutrophil, IL-17, elastase, and MMP9 activity. *J Leukoc Biol* (2016) 100(1):213–22. doi:10.1189/jlb.4A1015-483R
41. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, et al. Netting neutrophils induce endothelial damage, infiltrate tissues, and

expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* (2011) 187(1):538–52. doi:10.4049/jimmunol.1100450
42. Yapici U, Kers J, Bemelman FJ, Roelofs JJ, Groothoff JW, van der Loos CM, et al. Interleukin-17 positive cells accumulate in renal allografts during acute rejection and are independent predictors of worse graft outcome. *Transpl Int* (2011) 24(10):1008–17. doi:10.1111/j.1432-2277.2011.01302.x
43. Yapici U, Roelofs JJ, Florquin S. The importance of testing anti-IL-17 antibodies from different suppliers. *Am J Transplant* (2012) 12(2):504–5. doi:10.1111/j.1600-6143.2011.03867.x
44. Taylor PR, Bonfield TL, Chmiel JF, Pearlman E. Neutrophils from F508del cystic fibrosis patients produce IL-17A and express IL-23-dependent IL-17RC. *Clin Immunol* (2016) 170:53–60. doi:10.1016/j.clim.2016.03.016
45. Fontao L, Brembilla NC, Masouye I, Kaya G, Prins C, Dupin N, et al. Interleukin-17 expression in neutrophils and Th17 cells in cutaneous T-cell lymphoma associated with neutrophilic infiltrate of the skin. *Br J Dermatol* (2012) 166(3):687–9. doi:10.1111/j.1365-2133.2011.10647.x
46. Cai S, Batra S, Langohr I, Iwakura Y, Jeyaseelan S. IFN-gamma induction by neutrophil-derived IL-17A homodimer augments pulmonary antibacterial defense. *Mucosal Immunol* (2016) 9(3):718–29. doi:10.1038/mi.2015.95
47. Velden J, Paust HJ, Hoxha E, Turner JE, Steinmetz OM, Wolf G, et al. Renal IL-17 expression in human ANCA-associated glomerulonephritis. *Am J Physiol Renal Physiol* (2012) 302(12):F1663–73. doi:10.1152/ajprenal.00683.2011
48. Lemmers A, Moreno C, Gustot T, Marechal R, Degre D, Demetter P, et al. The interleukin-17 pathway is involved in human alcoholic liver disease. *Hepatology* (2009) 49(2):646–57. doi:10.1002/hep.22680
49. Res PC, Piskin G, de Boer OJ, van der Loos CM, Teeling P, Bos JD, et al. Overrepresentation of IL-17A and IL-22 producing CD8 T cells in lesional skin suggests their involvement in the pathogenesis of psoriasis. *PLoS One* (2010) 5(11):e14108. doi:10.1371/journal.pone.0014108
50. Lima AL, Karl I, Giner T, Poppe H, Schmidt M, Presser D, et al. Keratinocytes and neutrophils are important sources of proinflammatory molecules in hidradenitis suppurativa. *Br J Dermatol* (2016) 174(3):514–21. doi:10.1111/bjd.14214
51. Vykhovanets EV, Maclennan GT, Vykhovanets OV, Gupta S. IL-17 expression by macrophages is associated with proliferative inflammatory atrophy lesions in prostate cancer patients. *Int J Clin Exp Pathol* (2011) 4(6):552–65.
52. Lopez P, Rodriguez-Carrio J, Caminal-Montero L, Mozo L, Suarez A. A pathogenic IFNalpha, BLyS and IL-17 axis in systemic lupus erythematosus patients. *Sci Rep* (2016) 6:20651. doi:10.1038/srep20651
53. Derycke L, Zhang N, Holtappels G, Dutre T, Bachert C. IL-17A as a regulator of neutrophil survival in nasal polyp disease of patients with and without cystic fibrosis. *J Cyst Fibros* (2012) 11(3):193–200. doi:10.1016/j.jcf.2011.11.007
54. Schon MP, Broekaert SM, Erpenbeck L. Sexy again: the renaissance of neutrophils in psoriasis. *Exp Dermatol* (2017) 26(4):305–11. doi:10.1111/exd.13067
55. Finlay AY. Current severe psoriasis and the rule of tens. *Br J Dermatol* (2005) 152(5):861–7. doi:10.1111/j.1365-2133.2005.06502.x
56. Calzetti F, Tamassia N, Arruda-Silva F, Gasperini S, Cassatella MA. The importance of being "pure" neutrophils. *J Allergy Clin Immunol* (2017) 139(1):352–5.e6. doi:10.1016/j.jaci.2016.06.025
57. Bozza S, Gaziano R, Spreca A, Bacci A, Montagnoli C, di Francesco P, et al. Dendritic cells transport conidia and hyphae of Aspergillus fumigatus from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *J Immunol* (2002) 168(3):1362–71. doi:10.4049/jimmunol.168.3.1362
58. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, Mazzinghi B, et al. Phenotypic and functional features of human Th17 cells. *J Exp Med* (2007) 204(8):1849–61. doi:10.1084/jem.20070663
59. Scapini P, Calzetti F, Cassatella MA. On the detection of neutrophil-derived vascular endothelial growth factor (VEGF). *J Immunol Methods* (1999) 232(1–2):121–9. doi:10.1016/S0022-1759(99)00170-2
60. Cassatella MA, Guasparri I, Ceska M, Bazzoni F, Rossi F. Interferon-gamma inhibits interleukin-8 production by human polymorphonuclear leucocytes. *Immunology* (1993) 78(2):177–84.
61. Scapini P, Nardelli B, Nadali G, Calzetti F, Pizzolo G, Montecucco C, et al. G-CSF-stimulated neutrophils are a prominent source of functional BLyS. *J Exp Med* (2003) 197(3):297–302. doi:10.1084/jem.20021343
62. Zimmermann M, Aguilera FB, Castellucci M, Rossato M, Costa S, Lunardi C, et al. Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun* (2015) 6:6061. doi:10.1038/ncomms7061
63. Muller PY, Janovjak H, Miserez AR, Dobbie Z. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* (2002) 32(6):1372–4.
64. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol* (2009) 10(3):R25. doi:10.1186/gb-2009-10-3-r25
65. Rapin N, Bagger FO, Jendholm J, Mora-Jensen H, Krogh A, Kohlmann A, et al. Comparing cancer vs normal gene expression profiles identifies new disease entities and common transcriptional programs in AML patients. *Blood* (2014) 123(6):894–904. doi:10.1182/blood-2013-02-485771
66. Zimmermann M, Arruda-Silva F, Bianchetto-Aguilera F, Finotti G, Calzetti F, Scapini P, et al. IFNalpha enhances the production of IL-6 by human neutrophils activated via TLR8. *Sci Rep* (2016) 6:19674. doi:10.1038/srep19674
67. Crepaldi L, Silveri L, Calzetti F, Pinardi C, Cassatella MA. Molecular basis of the synergistic production of IL-1 receptor antagonist by human neutrophils stimulated with IL-4 and IL-10. *Int Immunol* (2002) 14(10):1145–53. doi:10.1093/intimm/dxf079
68. Moore EE, Presnell S, Garrigues U, Guilbot A, LeGuern E, Smith D, et al. Expression of IL-17B in neurons and evaluation of its possible role in the chromosome 5q-linked form of Charcot-Marie-tooth disease. *Neuromuscul Disord* (2002) 12(2):141–50. doi:10.1016/S0960-8966(01)00250-4
69. Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet* (2007) 39(3):311–8. doi:10.1038/ng1966
70. Tamassia N, Zimmermann M, Castellucci M, Ostuni R, Bruderek K, Schilling B, et al. Cutting edge: an inactive chromatin configuration at the IL-10 locus in human neutrophils. *J Immunol* (2013) 190(5):1921–5. doi:10.4049/jimmunol.1203022
71. Northrup DL, Zhao K. Application of ChIP-Seq and related techniques to the study of immune function. *Immunity* (2011) 34(6):830–42. doi:10.1016/j.immuni.2011.06.002
72. Bernstein BE, Stamatoyannopoulos JA, Costello JF, Ren B, Milosavljevic A, Meissner A, et al. The NIH roadmap epigenomics mapping consortium. *Nat Biotechnol* (2010) 28(10):1045–8. doi:10.1038/nbt1010-1045
73. Zhang L, Badgwell DB, Bevers JJ III, Schlessinger K, Murray PJ, Levy DE, et al. IL-6 signaling via the STAT3/SOCS3 pathway: functional analysis of the conserved STAT3 N-domain. *Mol Cell Biochem* (2006) 288(1–2):179–89. doi:10.1007/s11010-006-9137-3
74. Theilgaard-Monch K, Jacobsen LC, Borup R, Rasmussen T, Bjerregaard MD, Nielsen FC, et al. The transcriptional program of terminal granulocytic differentiation. *Blood* (2005) 105(4):1785–96. doi:10.1182/blood-2004-08-3346
75. Kouri VP, Olkkonen J, Ainola M, Li TF, Bjorkman L, Konttinen YT, et al. Neutrophils produce interleukin-17B in rheumatoid synovial tissue. *Rheumatology (Oxford)* (2014) 53(1):39–47. doi:10.1093/rheumatology/ket309
76. Al-Samadi A, Moossavi S, Salem A, Sotoudeh M, Tuovinen SM, Konttinen YT, et al. Distinctive expression pattern of interleukin-17 cytokine family members in colorectal cancer. *Tumour Biol* (2016) 37(2):1609–15. doi:10.1007/s13277-015-3941-x
77. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. *J Immunol* (2003) 170(4):2106–12. doi:10.4049/jimmunol.170.4.2106
78. Werner JL, Gessner MA, Lilly LM, Nelson MP, Metz AE, Horn D, et al. Neutrophils produce interleukin 17A (IL-17A) in a dectin-1- and IL-23-dependent manner during invasive fungal infection. *Infect Immun* (2011) 79(10):3966–77. doi:10.1128/IAI.05493-11
79. Katayama M, Ohmura K, Yukawa N, Terao C, Hashimoto M, Yoshifuji H, et al. Neutrophils are essential as a source of IL-17 in the effector phase of arthritis. *PLoS One* (2013) 8(5):e62231. doi:10.1371/journal.pone.0062231
80. Li L, Huang L, Vergis AL, Ye H, Bajwa A, Narayan V, et al. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. *J Clin Invest* (2010) 120(1):331–42. doi:10.1172/jci38702
81. Hoshino A, Nagao T, Nagi-Miura N, Ohno N, Yasuhara M, Yamamoto K, et al. MPO-ANCA induces IL-17 production by activated neutrophils in vitro via classical complement pathway-dependent manner. *J Autoimmun* (2008) 31(1):79–89. doi:10.1016/j.jaut.2008.03.006

82. Huppler AR, Verma AH, Conti HR, Gaffen SL. Neutrophils do not express IL-17A in the context of acute oropharyngeal candidiasis. *Pathogens* (2015) 4(3):559–72. doi:10.3390/pathogens4030559
83. Price AE, Reinhardt RL, Liang HE, Locksley RM. Marking and quantifying IL-17A-producing cells in vivo. *PLoS One* (2012) 7(6):e39750. doi:10.1371/journal.pone.0039750
84. Davey MS, Tamassia N, Rossato M, Bazzoni F, Calzetti F, Bruderek K, et al. Failure to detect production of IL-10 by activated human neutrophils. *Nat Immunol* (2011) 12(11):1017–8. doi:10.1038/ni.2111
85. Calo E, Wysocka J. Modification of enhancer chromatin: what, how, and why? *Mol Cell* (2013) 49(5):825–37. doi:10.1016/j.molcel.2013.01.038
86. Ostuni R, Piccolo V, Barozzi I, Polletti S, Termanini A, Bonifacio S, et al. Latent enhancers activated by stimulation in differentiated cells. *Cell* (2013) 152(1–2):157–71. doi:10.1016/j.cell.2012.12.018
87. Huppler AR, Gaffen SL. Editorial: fake it 'til you make it: mast cells acquire IL-17 exogenously. *J Leukoc Biol* (2016) 100(3):445–6. doi:10.1189/jlb.3CE0216-071R
88. Noordenbos T, Blijdorp I, Chen S, Stap J, Mul E, Canete JD, et al. Human mast cells capture, store, and release bioactive, exogenous IL-17A. *J Leukoc Biol* (2016) 100(3):453–62. doi:10.1189/jlb.3HI1215-542R

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.