



+ 10:37 AM OCT 11, 2021
THE MOMENT A DIFFICULT
BIOLOGICAL QUESTION HAS
AN ANSWER_

THE DIFFERENCE OF BREAKTHROUGH DISCOVERIES ON YOUR TERMS



WITH AN APPROACHABLE, AFFORDABLE, AUTOMATED 4-WAY CELL SORTING SOLUTION IN YOUR LAB. Cell sorting may be complex but it doesn't need to feel complicated or out of reach. With intuitive software that requires minimal training, the BD FACSMelody™ Cell Sorter enables deep scientific insights with reliable results, cost savings and workflow efficiencies. Discover how better instrumentation can free up your time so you can focus your expertise where it matters most. **Discover the new BD.**

Learn how you can advance your research >



ARTICLE

Human neutrophils activated by TLR8 agonists, with or without IFN γ , synthesize and release EBI3, but not IL-12, IL-27, IL-35, or IL-39

Marco A. Cassatella¹  | Elisa Gardiman¹ | Fabio Arruda-Silva¹ |
 Francisco Bianchetto-Aguilera¹ | Sara Gasperini¹ | Mattia Bugatti² | William Vermi^{2,3} |
 Frederique Larousserie^{4,5} | Odile Devergne⁴ | Nicola Tamassia¹

¹Department of Medicine, Section of General Pathology, University of Verona, Verona, Italy

²Department of Molecular and Translational Medicine, Section of Pathology, University of Brescia, Brescia, Italy

³Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, Missouri, USA

⁴Sorbonne Université, INSERM, CNRS, Centre d'Immunologie et des Maladies Infectieuses, Paris, France

⁵Département de Pathologie, Hôpital Cochin, AP-HP, Université de Paris, Paris, France

Correspondence

Marco A. Cassatella, Department of Medicine, Section of General Pathology, University of Verona, Strada Le Grazie 8, 37134 Verona, Italy. Email: marco.cassatella@univr.it

Abstract

The IL-12 family of cytokines plays crucial functions in innate and adaptive immunity. These cytokines include heterodimers sharing distinct α (IL-12A, IL-23A, and IL-27A) with two β (IL-12B and Epstein-Barr virus induced gene 3 [EBI3]) chains, respectively, IL-12 (IL-12B plus IL-12A) and IL-23 (IL-12B plus IL-23A) sharing IL-12B, IL-27 (EBI3 plus IL-27A), IL-35 (EBI3 plus IL-12A), and IL-39 (EBI3 plus IL-23A) sharing EBI3. In this context, we have recently reported that highly pure neutrophils incubated with TLR8 agonists produce functional IL-23. Previously, we showed that neutrophils incubated with LPS plus IFN γ for 20 h produce IL-12. Herein, we investigated whether highly pure, TLR8-activated, neutrophils produce EBI3, and in turn IL-27, IL-35, and IL-39, the IL-12 members containing it. We report that neutrophils incubated with TLR8 ligands, TNF α and, to a lesser extent, LPS, produce and release remarkable amounts of EBI3, but not IL-27A, consequently excluding the possibility for an IL-27 production. We also report a series of unsuccessful experiments performed to investigate whether neutrophil-derived EBI3 associates with IL-23A to form IL-39. Furthermore, we show that neutrophils incubated with IFN γ in combination with either TLR8 or TLR4 ligands express/produce neither IL-12, nor IL-35, due to the inability of IFN γ , contrary to previous findings, to activate *IL12A* transcription. Even IL-27 was undetectable in supernatants harvested from IFN γ plus R848-treated neutrophils, although they were found to accumulate *IL27A* transcripts. Finally, by immunohistochemistry experiments, EBI3-positive neutrophils were found in discrete pathologies only, including diverticulitis, cholecystitis, Gorham disease, and *Bartonella Henselae* infection, implying a specific role of neutrophil-derived EBI3 in vivo.

KEYWORDS

EBI3, IL-12, IL-27, IL-35, Neutrophils, TLR8, TNF α

1 | INTRODUCTION

During the last 30 yr, accumulating evidence has uncovered that polymorphonuclear neutrophils, either in vitro under appropriate

experimental conditions, or in in vivo diseases/experimental models, can produce a variety of cytokines in a highly regulated fashion.¹ Accordingly, the list of neutrophil-derived cytokines currently includes chemokines, ILs, members of the TNF family, growth factors, and many others,¹ hence implying that neutrophils may be actively involved in physiopathologic processes that go beyond their well-recognized pro/anti-inflammatory functions.² Notably, the majority of the cytokines that neutrophils can potentially generate requires a stimulus-induced transcription of the related transcripts and, in turn, their de novo synthesis and release.^{1,3} Experimental

Abbreviations: BM, bone marrow; EBI3, Epstein-Barr virus induced gene 3; FPKM, fragments per kilobase of transcript per million mapped reads; IFIT, IFN-induced protein with tetratricopeptide repeats 1; IL-1ra, IL 1 receptor antagonist; MNE, mean normalized expression; pAbs, polyclonal antibodies; RNA-seq, RNA followed by high throughput sequencing; RPL, ribosomal protein L; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

conditions shown to promote cytokine gene expression and production by human neutrophils include ligands of pattern recognition receptors, cytokines themselves, damage-associated molecular patterns and phagocytosis.¹ In addition, neutrophil-derived cytokines can themselves enhance/generate additional cytokine expression via autocrine feedback loops.^{3,4} In this context, we recently identified TLR8 ligands, such as R848, CL075, and VTX-2337, as stimuli inducing neutrophils to produce cytokines at higher levels than other known agonists, as shown in the case of TNF α , IL-6, G-CSF, and CCL23 production.⁴⁻⁶ Nonetheless, when examining their potential capacity to express genes/produce a given protein/cytokine, one should never forget that, on a *per cell* basis, activated neutrophils accumulate at least 10- to 20-fold lower amounts of RNA, and/or produce exceedingly lower cytokine concentrations than other leukocyte types do.¹ Therefore, to avoid false positive results in terms of either patterns/amounts of cytokines produced, or cytokine mRNA profiles expressed, it is mandatory to isolate neutrophils at the maximal purity (>99.7% ideally).⁷

Cytokines belonging to the IL-12 family play critical functions in innate and adaptive immunity.^{8,9} They are composed of functional heterodimers that share distinct α - and β -subunits, namely, IL-12A/p35, IL-23A/p19, and IL-27A/p28 as α -chains, and IL-12B/p40 and Epstein-Barr virus-induced protein 3 (EBI3) as β -chains.^{8,10} A total of five IL-12 family cytokines are currently known, precisely IL-12 (IL-12A plus IL-12B), IL-23 (IL-23A plus IL-12B), IL-27 (IL-27A plus EBI3), IL-35 (IL-12A plus EBI3),⁹ as well as the recently reported murine IL-39 (IL-23A plus EBI3).¹¹ Interestingly, individual subunits have also been shown to have self-standing activities, as in the case of EBI3,¹² intracellular IL-23A,¹³ IL-12B homodimer (p80),¹⁴ and IL-27A (IL-30).¹⁵ IL-12 has been the first identified member of the family,¹⁶ which, similar to IL-23 and IL-27, is mainly produced by macrophages and dendritic cells.^{17,18} Differently, IL-35 is produced by activated B cells and forkhead box P3⁺ Treg cells,¹⁹ whereas IL-39 seems to be produced by murine activated B cells.¹¹ In this context, human neutrophils have been shown to produce IL-12,^{20,21} IL-23,²² and IL-27A²³ upon defined stimulatory conditions. For instance, TLR8-activated neutrophils have been shown to express and produce IL-12B, IL-23A, and IL-23, but not IL-12A,²⁴ consequently excluding the possibility that they could release IL-12 and IL-35.

In this study, we extended our analysis on the ability of TLR8-activated neutrophils to eventually express and produce EBI3, as well as the IL-12 cytokine members containing it, including IL-27 and IL-39. As a result, we report that neutrophils incubated with either TLR8 or TLR4 ligands express and produce elevated amounts of EBI3, but not IL-27A, consequently excluding the possibility of an IL-27 production under the same stimulatory conditions. We also report a series of unsuccessful experiments performed to investigate whether neutrophil-derived EBI3 associates with IL-23A to form IL-39. Finally, we show that neutrophils incubated with IFN γ in combination with either TLR8 or TLR4 ligands express/produce neither IL-12 nor IL-35, due to the inability of IFN γ to activate *IL12A* transcription, thus denying previous studies from our group.²⁰

2 | MATERIALS AND METHODS

2.1 | Cell purification and culture

Highly purified neutrophils (approximately 99.7% purity) were isolated from the venous blood or buffy coats from healthy individuals, as already described.⁷ Human CD14⁺-monocytes (approximately 98% purity) were isolated by anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from autologous PBMCs, previously obtained from blood after Ficoll-Paque gradient centrifugation.⁴ Neutrophils and monocytes were then suspended at 5×10^6 /ml and 2.5×10^6 /ml, respectively, in RPMI 1640 medium containing 10% FBS (with <0.5 EU/ml endotoxin, BioWhittaker-Lonza, Basel, Switzerland). Cells were incubated for up to 48 h in the absence (control) or the presence of: 0.2–10 μ M R848 (InvivoGen, San Diego, CA, USA); 1 μ g/ml ultrapure LPS (*E. coli* 0111:B4 strain, InvivoGen); 10 ng/ml human TNF α (R&D Systems, Minneapolis, MN, USA); 200 U/ml IFN γ (R&D Systems); 10 ng/ml GM-CSF (Miltenyi Biotec); 1000 U/ml G-CSF (Myelostim, Italfarmaco Spa, Milano, Italy); 0.2–10 μ M CL075 (InvivoGen); 0.2–10 μ M VTX-2337 (Selleck Chem, Boston, MA, USA); or 10 μ g/ml adalimumab (Humira, Abbott Biotechnology Limited, Barceloneta, Puerto Rico).

2.1.1 | Isolation of murine neutrophils

C57BL/6J mice, obtained from Jackson Laboratories (Bar Harbour, ME, USA) were housed under specific pathogen-free conditions at the University of Verona (Verona, Italy). Murine neutrophils were isolated either from bone marrow (BM), through discontinuous Percoll gradient centrifugation, according to standard procedures,²⁵ or from peritoneal exudates, harvested 16 h after i.p. injection of BioGel P Polyacrylamide Beads (BioRad Laboratories, Hercules, CA, USA).⁴ Neutrophils were then sorted by using a FACSaria II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).⁴ Briefly, cell suspensions were incubated with APC Cy7-conjugated anti-mouse CD11b (M1/70), APC-conjugated anti-mouse Ly6G (1A8), and Brilliant Violet 421-conjugated anti-mouse CD45 (30-F11) antibodies (all from BioLegend), and then suspended in PBS containing 2 mM EDTA, 1 mg/ml propidium iodide (PI, for viability staining; Sigma-Aldrich, St. Louis, MO, USA) and 2% FBS, before cell sorting. CD11b⁺Ly6G⁺ neutrophils (>99% pure) were then suspended at 5×10^6 /ml in RPMI 1640 medium, supplemented with 10% FBS, 1% ultraglutamine, and 1% penicillin/streptomycin (BioWhittaker-Lonza, Walkersville, MD, USA), and cultured (at 37°C, 5% CO₂), with or without 5 μ M R848 or 1 μ g/ml LPS or 10 ng/ml TNF α . All experiments were carried out in accordance with guidelines prescribed by the Ethics Committee for the use of laboratory animals for research purposes at the University of Verona, and by the Italian Ministry of Health.

2.2 | RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

After incubation, neutrophils and CD14⁺-monocytes were pelleted by centrifugation and then total RNA was extracted by either Trizol or RNeasy mini kit (Qiagen, Venlo, Limburg, Netherlands).²⁶

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

RT-qPCR primers	Sequences	
	Forward primers	Reverse primers
Human primer sets		
<i>IL12A</i>	CTGGACCACCTCAGTTTGG	TTTGTCCGGCCTTCTGGAG
<i>IL12B</i>	GGACATCATCAAACCTGACC	AGGGAGAAGTAGGAATGTGG
<i>IL23A</i>	GGACACATGGATCTAAGAGAAGAG	CTATCAGGGAGCAGAGAAGG
<i>RPL32</i>	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
<i>GAPDH</i>	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
<i>IL27A</i>	AGGGAGTTCACAGTCAGCCT	GGTGGAGATGAAGCAGAGACG
<i>EBI3</i>	ACAGAGCACATCATCAAGCC	CCACTTGGACGTAGTACTCTG
<i>IL1ra</i>	TTCCTGTTCCATTCAGAGACGAT	AATTGACATTTGGTCTTGCAA
Mouse primer sets		
<i>EBI3</i>	TACACTGAAACAGCTCTCGTG	CATTTAGCATGTAGGGCACC
<i>IL12B</i>	TCAGGGACATCATCAAACCAG	CGAACAAAGAAGCTTGAGGGAG
<i>GAPDH</i>	TGGCCTCCAAGGAGTAAGAA	GGTCTGGGATGGAAATTGTG

To remove any possible contaminant DNA, an on-column DNase digestion with the RNase-free DNase set (Qiagen) was performed during total RNA isolation.²⁶ Total RNA was reverse-transcribed into cDNA using Superscript III (Thermo Fisher Scientific, Waltham, MA, USA) and random hexamer primers (Thermo Fisher Scientific, Waltham, MA, USA).²⁶ Transcript levels of individual genes were measured by RT-qPCR performed using Fast SYBR Green Master Mix (Life Technologies, Carlsbad, CA, USA), using specific primer pairs (Thermo Fisher Scientific) 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.²⁶

2.3 | RNA-seq

Total RNA from activated neutrophils was enriched for mRNA using poly(A) selection. Standard Illumina (San Diego, CA, USA) protocols were used to generate 50 bp single-end read libraries. In brief, mRNA was fragmented, reverse transcribed, adapted with sequencing primers and sample barcodes, size selected, and finally PCR enriched. Libraries were then sequenced on the Illumina NextSeq 500 platform.²⁴ Reads were mapped to the reference human genome (hg19) using TopHat version 2.0.14 and Bowtie 2 version 2. Gene expression values (fragments per kilobase of transcript per million mapped reads) were calculated using Cufflinks version 2.0.2. RNA-seq signals were visualized using Integrative Genomics Viewer.²⁷

2.3.1 | Cytokine production

Cytokine concentrations in cell-free supernatants were measured by commercially available ELISA kits, specific for human EBI3 (LSBio, Seattle, WA, USA), IL-23A (Abcam, Cambridge, United Kingdom), IL-12, IL-12B, IL-23, IL-23A, IL-27, and CXCL8 (Mabtech, Nacka Strand, Sweden). The lowest detection limits of these ELISA were: 15 pg/ml for EBI3, 6 pg/ml for IL-12, 10 pg/ml for IL-12B, 4 pg/ml for IL-23,

20 pg/ml for IL-23A, 32 pg/ml for IL-27, and 4 pg/ml for CXCL8. To develop an ELISA for IL-39, we used either 1–10 µg/ml anti-human EBI3 mAbs (MAB6456 from R&D Systems) or 1–10 µg/ml anti-human EBI3 mAbs (LS-C314301 from LSBio) as coating antibodies, and 1–10 µg/ml biotinylated anti-human IL-23p19 mAbs (clone MT155, from Mabtech) as detection ones. To immunoprecipitate IL-39 from supernatants of human neutrophils and CD14⁺-monocytes incubated with IFN γ plus R848, we used anti-human EBI3 polyclonal antibodies (pAbs) and mAbs (H-110 and G-4, respectively, both from Santa Cruz, Biotech (Santa Cruz, CA, USA) and anti-human IL-23p19 pAbs and mAbs (H-113 and C-3, respectively, both from Santa Cruz, Biotech (Santa Cruz, CA, USA)). Recombinant human (rh) proteins used in these latter experiments were: rhEBI3 (ab123759 from Abcam), rhIL-23p19 (Origene, Rockville, MD, USA), rhIL-35 (Peprotech, Rocky Hill, NJ, USA), rhIL-27 (Miltenyi Biotec), and rhIL-39-Fc Chimera Protein (R&D Systems).

2.3.2 | ELISA for human IL-39

To try detecting IL-39, microtitration 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 µl of 1 to 10 µg/ml anti-human EBI3 mAbs (MAB6456 from R&D Systems), or alternatively, 1 to 10 µg/ml anti-human EBI3 mAbs (LS-C314301 from LSBio), then washed with PBS-0.05% Tween 20 (PBS-T), and blocked with 100 µl of PBS-5% BSA for 2 h. Samples (50 µl) were added to the wells and incubated for 2 h at room temperature. After washing, biotinylated anti-human IL-23A mAbs (clone MT155, from Mabtech) was added at 1 to 10 µg/ml and incubated for 2 h at room temperature. After washing, 50 µl of PBS containing streptavidin HRP (Sigma, St. Louis, MO, USA) diluted 1:2000 was added and the plates incubated for 30 min at room temperature. Wells were further washed with PBS-T, reactions developed by adding 50 µl of tetramethylbenzidine solution (Sigma) and stopped after 30 min by adding 50 µl of 1 M H₂SO₄. As done by Wang et al.¹¹ for murine IL-39, a mixture of 10 ng/ml rhEBI3 and rhIL-23A was used as IL-39 standard. Optical density was read at 450 nm.

2.3.3 | Immunoblotting experiments

Whole lysates from resting/activated neutrophils and CD14⁺-monocytes were recovered from protein-rich flow-through solutions after the first centrifugation step of the RNeasy mini kit procedure used for total RNA extraction, as previously described.⁶ Protein-rich flow-through from neutrophils were then immunoblotted by standard procedures⁴ using anti-human EB13 mAbs (clone 2G4H6)²⁸ and anti-human β -actin mAbs (A5060 from Sigma). Blotted proteins were detected by the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).⁶

2.3.4 | Immunohistochemistry

Human tissues, collected for diagnosis purposes, were retrieved from the tissue banks of the Department of Pathology of Cochin Hospital (Paris) and the Department of Pathology of Spedali Civili di Brescia (Brescia, Italy). Before staining, formalin-fixed paraffin-embedded tissues were subjected to antigen retrieval by heat pretreatment in citrate buffer (pH 6) or EDTA buffer (pH 8.0). Immunostaining was performed using either an indirect alkaline phosphatase kit (BioGenex, Fremont, CA, USA) and permanent red as chromogen, or using Novolink Polymer (Leica Microsystems, Wetzlar, Germany) followed by DAB (3,3'-diaminobenzidine). EB13 was detected by using the mouse monoclonal anti-EB13 Ab (clone 2G4H6)²⁸ at 2–4 μ g/ml. Sections were counterstained with Mayer hematoxylin.

2.4 | Statistical analysis

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

2.5 | Study approval

Human samples were obtained following informed, written, consent by healthy donors, in accordance with the Declaration of Helsinki. This study was carried out in accordance with the recommendations of Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy).

3 | RESULTS

3.1 | Expression and production of EB13 by human neutrophils treated with TLR8 and other agonists

Preliminary RNA-seq studies of highly pure neutrophils incubated in the absence and the presence of 5 μ M R848, for 6 and 20 h, revealed a remarkable induction of *EB13* mRNA under R848-activating conditions (Fig. 1A). Given that EB13 may form IL-27, IL-35, and IL-39, we performed RT-qPCR kinetic experiments of *EB13*, *IL27A*, and *IL12A* mRNA expression in neutrophils and autologous CD14⁺-

monocytes incubated not only with or without R848, but also with 1 μ g/ml LPS (Fig. 1B). We have in fact already shown that both R848- and LPS-treated neutrophils produce IL-23,²⁴ implying that they also express *IL23A* mRNA, as formally demonstrated in the case of R848-treatment.²⁴ Results shown in Figure 1B not only supported the RNA-seq data, but also indicated that the optimal incubation time to induce maximal expression of *EB13* mRNA in both neutrophils and CD14⁺-monocytes corresponds to 20 h. Similar to *IL12A*,²⁴ *IL27A* mRNA was undetectable in R848- and LPS-treated neutrophils, at all time points investigated (Fig. 1B). By contrast, *IL27A* and *IL12A* mRNAs were weakly induced by R848 in CD14⁺-monocytes, with maximum expression at 12 h. *EB13* mRNA in neutrophils and CD14⁺-monocytes, as well as *IL27A* and *IL12A* mRNA in CD14⁺-monocytes, were induced also by LPS, but at lower levels than R848 (Fig. 1B).

Consistent with the gene expression data, neutrophils and CD14⁺-monocytes were found to produce and release higher amounts of EB13 in response to R848 than to LPS, at statistically significant levels only in the case of neutrophils (Fig. 1C). Under the same stimulatory conditions, the pattern of CXCL8 production was found similar to that of EB13 (Fig. 1C), but it is evident that CD14⁺-monocytes release CXCL8 at levels at least 60-fold higher than neutrophils, as one would have expected.²⁹ Further demonstrations that activated neutrophils (and CD14⁺-monocytes) produce EB13 were obtained by Western blot experiments detecting the cytokine in their whole cell lysates (Fig. 2A), as well as by immunohistochemistry staining of diseased tissues, revealing EB13-positive neutrophils in Gorham disease (Fig. 2B), diverticulitis (Fig. 2C-E), cholecystitis (Fig. 2F), and two cases of *Bartonella Henselae* infection with suppurative lymphadenitis (Fig. 2G, H). By contrast, in primary carcinomas and related lymph node metastases, folliculitis, dermatitis, psoriasis, lichen planus, molluscum contagiosum, granulomatous lymphadenitis, appendicitis, Chron's disease, and COVID-19 pneumonia, neutrophils turned out as EB13-negative.

In another series of experiments, only TNF α (10 ng/ml), but not G-CSF (1000 U/ml) or GM-CSF (10 ng/ml), was found able to trigger the expression of *EB13* mRNA in neutrophils incubated for 20 h, at levels comparable to those induced by R848 (Fig. 3A). By contrast, *IL1RN* mRNA accumulation was found increased in both GM-CSF- and G-CSF-, other than in R848-, LPS-, and TNF α -treated neutrophils (Fig. 3A), in line with previous data,²⁹ whereas *IL27A* mRNA remained undetectable under all experimental conditions (data not shown). Because R848-treated neutrophils are known to release elevated amounts of TNF α ,⁴ we then examined whether endogenous TNF α could autocrinally regulate the expression of *EB13* mRNA, and found it to be the case. Indeed, incubation of neutrophils with R848 for 20 h, in the presence of 10 μ g/ml adalimumab (a TNF α neutralizing antibody)³⁰ revealed a 44.7 \pm 11.7% inhibition of *EB13* mRNA expression as compared to cells incubated in its absence (*n* = 4). Interestingly, we also found that, whereas the amounts of extracellular EB13 detected in supernatants from neutrophils incubated with R848 and/or TNF α for 20 h were substantially similar (Fig. 3B), at the same time point the *EB13* mRNA levels were additively increased (Fig. 3C). Finally, neutrophils incubated with other TLR8 ligands, namely, CL075,³¹ or Motolimod/VTX-233,³² displayed, similar to R848, up-regulatory

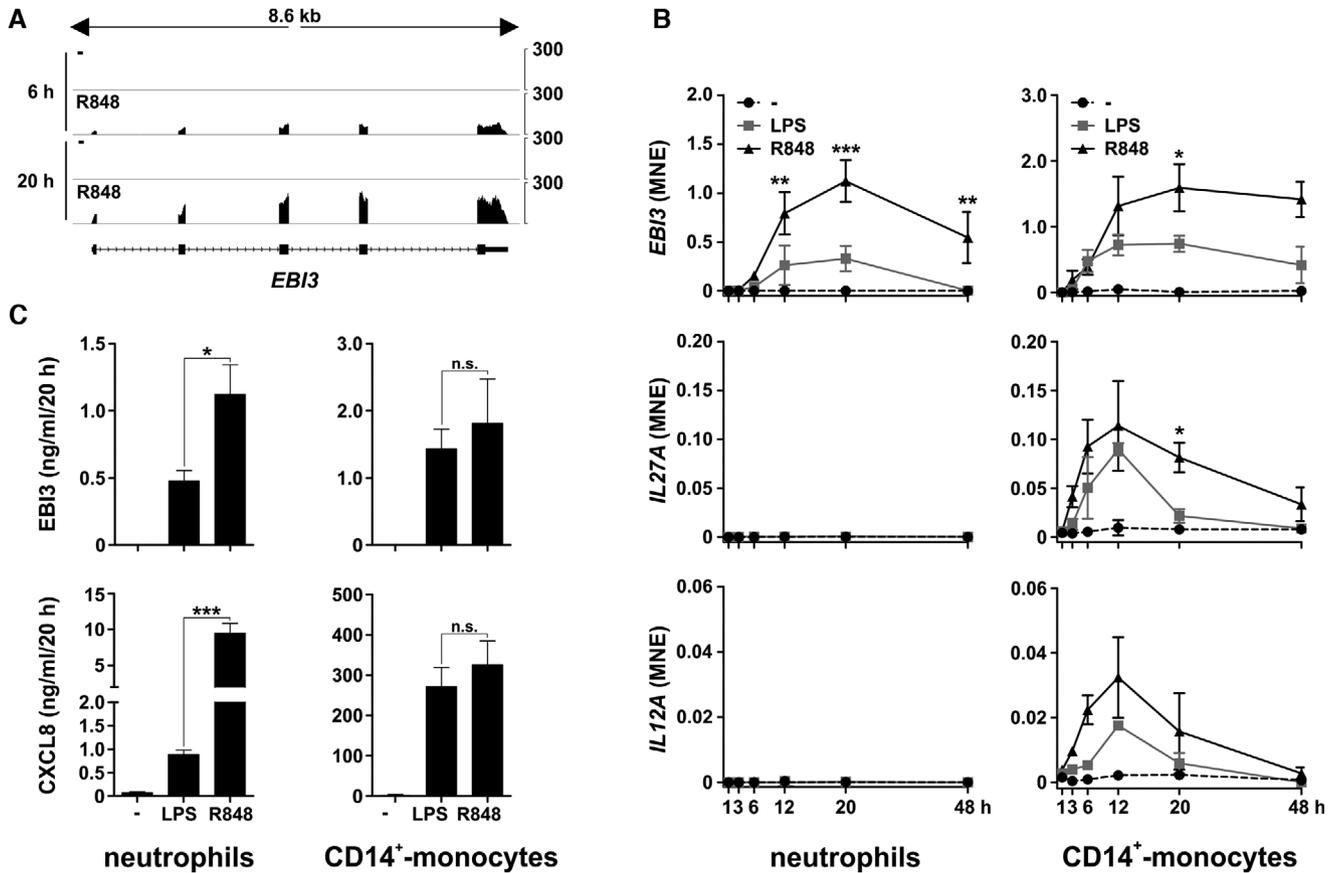


FIGURE 1 *EB13* mRNA and protein expression in neutrophils and CD14⁺-monocytes incubated with R848 or LPS. In (A), highly pure neutrophils (5×10^6 /ml) were incubated for 6 or 20 h in the absence (–) or the presence of 5 μ M R848. Expression levels of *EB13* transcripts were measured by RNA-seq (one representative experiment out of four is shown). In (B) 5×10^6 /ml highly pure neutrophils and 2.5×10^6 /ml CD14⁺-monocytes were incubated for up to 48 h in the presence or the absence of 5 μ M R848 or 1 μ g/ml LPS. Plots show kinetics of *EB13*, *IL27A*, and *IL12A* mRNA expression, as measured by RT-qPCR and depicted as mean normalized expression (MNE) units after normalization to *GAPDH* mRNA (mean \pm SEM, $n = 4-8$). Asterisks indicate significant differences between LPS and R848 in terms of *EB13*, *IL27A*, and *IL12A* mRNA induction: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, by 2-way ANOVA followed by Bonferroni's posttest. (C) Cell-free supernatants were collected from 5×10^6 /ml neutrophils and 2.5×10^6 /ml CD14⁺-monocytes incubated with R848 or LPS for 20 h, and the levels of *EB13* and *CXCL8* extracellular proteins measured by ELISA ($n = 4-6$). Asterisks indicate significant differences between R848 and LPS in terms of induced *EB13* and *CXCL8* production: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by 1-way ANOVA followed by Tukey's posttest. For (B) and (C), data are normalized per equal number of cells

effects on the mRNA levels of *EB13*, *IL12B*, *IL23A* (Fig. 3D), but not of *IL27A* and *IL12A* (data not shown), in a dose-dependent fashion. Collectively, these data prove that highly pure human neutrophils incubated with TLR8 ligands and TNF α genuinely express and produce *EB13*. Data also show that TLR8-activated neutrophils accumulate neither *IL12A*, nor *IL27A* mRNAs, implying that they cannot produce either IL-35 or IL-27.

3.2 | Expression of *EB13* transcripts in murine neutrophils

To evaluate whether *EB13* mRNA could be expressed also by murine neutrophils, the latter cells were isolated from the BM and inflamed peritoneum, and then subjected to an *in vitro* incubation with 5 μ M R848, 1 μ g/ml LPS and 10 ng/ml TNF α . After 6 and 20 h, neutrophils were harvested and lysed for total RNA purification. As shown in Figure 4, activated neutrophils were found to accumulate *EB13* tran-

scripts, but differently from their human counterpart, LPS resulted the strongest stimulus, regardless their source. Under the same experimental conditions, *IL12B* mRNA accumulation was detected only in BM, but not peritoneal, neutrophils, at higher levels in response to R848 than LPS (Fig. 4).

3.3 | Effect of IFN γ on the expression of *EB13* and other IL-12 family members by neutrophils coincubated with either R848 or LPS

We have previously published that neutrophils incubated for 20 h with LPS in combination with IFN γ produce IL-12, given the ability of LPS to induce the expression of *IL12B* (but not *IL12A*, mRNA) and of IFN γ to induce that of *IL12A* (but not *IL12B*) mRNA.²⁰ We therefore incubated highly pure neutrophils with IFN γ in the presence of either R848 or LPS, with the expectation of detecting,

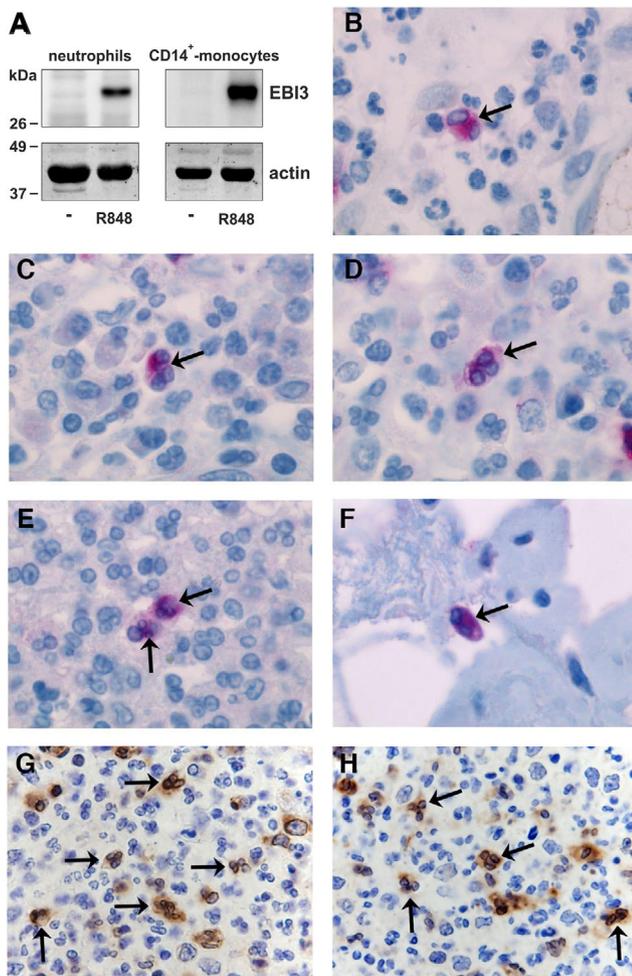


FIGURE 2 Antigenic EBI3 expression in human neutrophils. (A), Immunoblot displaying antigenic EBI3 and actin expression in lysates of neutrophils and autologous CD14⁺-monocytes cultured for 20 h with or without 5 μ M R848 (representative experiment, $n = 2$). (B–H), Sections from tissues of patients affected by Gorham Disease (B), diverticulitis (C–E), cholecystitis (F) and *Bartonella Henselae* infections showing suppurative lymphadenitis (G, H) were stained with anti-EBI3 mouse monoclonal antibodies and counterstained with Meyer's hematoxylin. Panels B–F were stained by using an indirect alkaline phosphatase kit and permanent red as chromogen, whereas panels G and H were stained by using Novolink polymer followed by DAB, as detailed in Materials and Methods. Original magnifications: 100 \times (B–F) and 60 \times (G, H)

concomitantly with *EBI3* mRNA accumulation, also *IL12A* transcripts. To our surprise, however, RT-qPCR experiments revealed that *IL12A* transcripts do not actually accumulate in neutrophils incubated with IFN γ , if either used alone, or in combination with either R848 or LPS (Fig. 5A), thus excluding the possibility for an IL-12 or an IL-35 production. Interestingly, although R848, LPS or IFN γ were found unable to induce *IL27A* mRNA expression in neutrophils, IFN γ plus either R848 or LPS triggered detectable levels of *IL27A* transcripts (Fig. 5A). In any case, the efficacy of IFN γ was confirmed by its direct, time-dependent, induction of IFN-induced protein with tetra-tricopeptide repeats 1 (*IFIT1*) mRNA expression (Fig. 5A), as well

as by its statistically significant, up-regulatory, actions on the R848- and LPS-mediated induction of *IL23A* and, as expected,²⁰ *IL12B* genes (Fig. 5A).

Measurement of cytokine levels in the corresponding cell-free supernatants uncovered that IFN γ increases the production of IL-12B, IL-23A, and IL-23, but not EBI3, by neutrophils coincubated with R848 (Fig. 5B), as well as that of IL-23 by neutrophils coincubated with LPS (Fig. 5B). Even in CD14⁺-monocytes, IFN γ was found unable to up-regulate the production of EBI3 in response to both R848 and LPS, whereas it modulated the production of IL-12B, IL-23A, and IL-23 almost similar (but not identically) to its actions on neutrophils (Fig. 5B). ELISA measurements also confirmed, at protein level, the lack of IL-12 production by activated neutrophils, while evidencing a remarkable potentiation by IFN γ of the scarce IL-12 release by CD14⁺-monocytes treated with either R848 or LPS⁷ (Fig. 5B). Even under "priming" conditions for CD14⁺ monocytes,³³ namely, after a 6 h preincubation with IFN γ followed by a 18 h stimulation with R848, neutrophils did not produce and release IL-12 (data not shown). Finally, IL-27 was not detected in cell-free supernatants harvested from neutrophils incubated under all culture conditions, in contrast to what observed with autologous CD14⁺-monocytes treated with R848 and cultured in the presence or the absence of IFN γ (Fig. 5B).

Altogether, data show that IFN γ does not modulate the expression/production of EBI3 either in TLR-activated neutrophils or in autologous CD14⁺-monocytes. Data also exclude that neutrophils could produce IL-35, even if incubated with IFN γ plus either R848 or LPS. Finally, data also negate previous publications on the in vitro production of IL-12 by human neutrophils incubated with IFN γ plus LPS,²⁰ extending such inability to the treatment with IFN γ plus R848.

3.4 | Investigation on the eventual ability of human neutrophils to produce IL-39

It has been recently described that EBI3 associates with IL-23A to form IL-39, at least in murine activated B cells.¹¹ However, although IL-39 has been shown to mediate inflammation in lupus-like mice,¹¹ no convincing evidence for its effective synthesis in humans actually exists.³⁴ Given the expression of antigenic EBI3 (this study) and IL-23A²⁴ by activated neutrophils,²⁴ we decided to check whether the latter cells could manufacture and secrete IL-39, even though no specific reagents recognizing or measuring human IL-39 are currently available. Initially, we tried to immunoprecipitate IL-39 putatively present in supernatants from IFN γ plus R848-treated neutrophils/monocytes, using either rabbit polyclonal or mouse monoclonal anti-IL-23A antibodies ([both from Santa Cruz, Biotech (Santa Cruz, CA, USA) and already shown to detect recombinant IL-23 by immunoblotting³⁵], followed by either rabbit polyclonal or mouse monoclonal anti-EBI3 antibodies³⁶ [Santa Cruz, Biotech (Santa Cruz, CA, USA)]), and vice versa, but our attempts were not successful (data not shown), even using a mixture of equal amounts (1 μ g) of rhIL-23A and rhEBI3 as control, as it was previously found to form a stable complex.¹¹ Then,

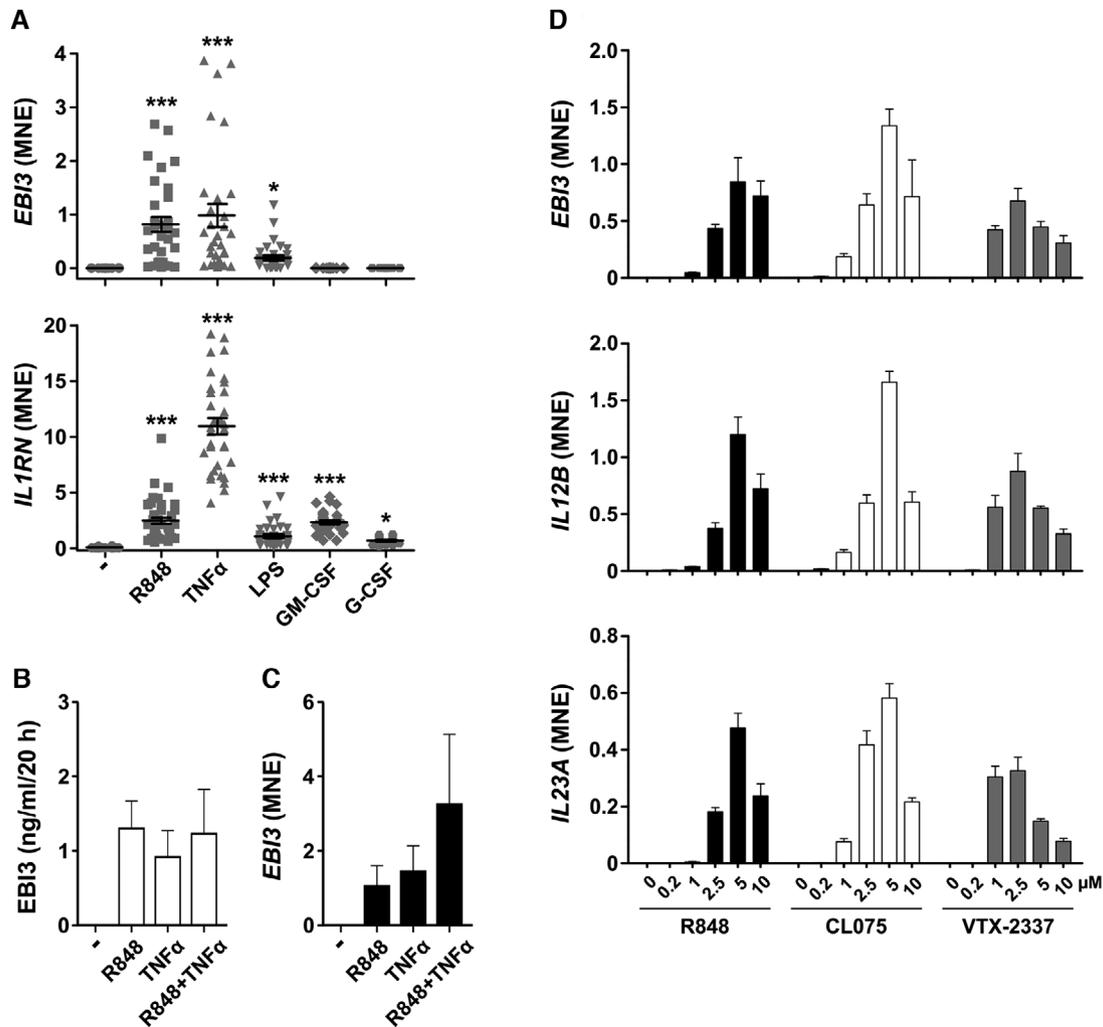


FIGURE 3 Effect of TLR8 ligands and other agonists on *EB13* mRNA expression by neutrophils. (A) 5×10^6 /ml neutrophils were cultured with or without $5 \mu\text{M}$ R848, 10 ng/ml TNF α , $1 \mu\text{g/ml}$ LPS, 1000 U/ml G-CSF and 10 ng/ml GM-CSF to evaluate *EB13* and *IL1ra* mRNA expression by RT-qPCR ($n = 32$). Asterisks stand for significant differences as compared to untreated cells: * $P < 0.05$, *** $P < 0.001$, by 1-way ANOVA followed by Tukey's posttest. (B–C) Neutrophils were cultured with or without $5 \mu\text{M}$ R848 and/or 10 ng/ml TNF α for 20 h, to evaluate their *EB13* release by ELISA (B) and *EB13* mRNA expression by RT-qPCR (C). Values represent the means \pm SEM ($n = 2$ –4). Asterisks indicate a significant increase exerted by R848 plus TNF α as compared with either R848 or TNF α singly used: * $P < 0.05$, *** $P < 0.001$, by Student's *t*-test. (D) Neutrophils were cultured with or without R848 (0.2 – $10 \mu\text{M}$), CL075 (0.2 – $10 \mu\text{M}$), or VTX-2337 (0.2 – $10 \mu\text{M}$), to evaluate *EB13*, *IL12B*, and *IL23A* mRNA accumulation by RT-qPCR. Results from a representative experiment out of two are depicted as mean normalized expression (MNE) units after normalization to *GAPDH* mRNA

we tried to develop an ELISA to detect human IL-39, as described in Materials and Methods. However, no increased absorbance over the blanks were obtained from supernatants harvested from IFN γ plus R848-treated neutrophils, as well as from IFN γ plus LPS-treated monocyte-derived DCs, which, in our hands, accumulate the highest levels of *EB13* and *IL23A* mRNA (data not shown).

In sum, our experiments on a presumed association of extracellular *EB13* with IL-23A released by activated neutrophils have collected negative results only. It is also important to remark, in this context, that the amount of IL-23A released by neutrophils incubated with R848 alone, or with IFN γ plus R848 (shown in Fig. 5B), stoichiometrically corresponds, more or less, to the IL-23 measured in the corresponding supernatants,²⁴ thus suggesting that IL-23A is unavailable for an eventual association with *EB13* to form IL-39.

4 | DISCUSSION

In the last years, our studies on the capacity of human neutrophils to produce cytokines have only in part focused on the members belonging to the IL-12 family. In 1995, we published that neutrophils express and release IL-12 in vitro, when co-treated with LPS in combination with IFN γ for at least 20 h, but not with the two agonists used alone.²⁰ At molecular level, such synergism was investigated by RNAse protection assay (RPA) experiments, which revealed that LPS induces the expression of *IL12B*, but not *IL12A*, mRNA, whereas IFN γ induces *IL12A*, but not *IL12B*, transcripts.²⁰ Our paper was then followed by other in vitro studies, in which the requirement of IFN γ in combination with another agonist to trigger heterodimeric IL-12 production was substantially confirmed.²¹ More recently, confirming previous notions,²²

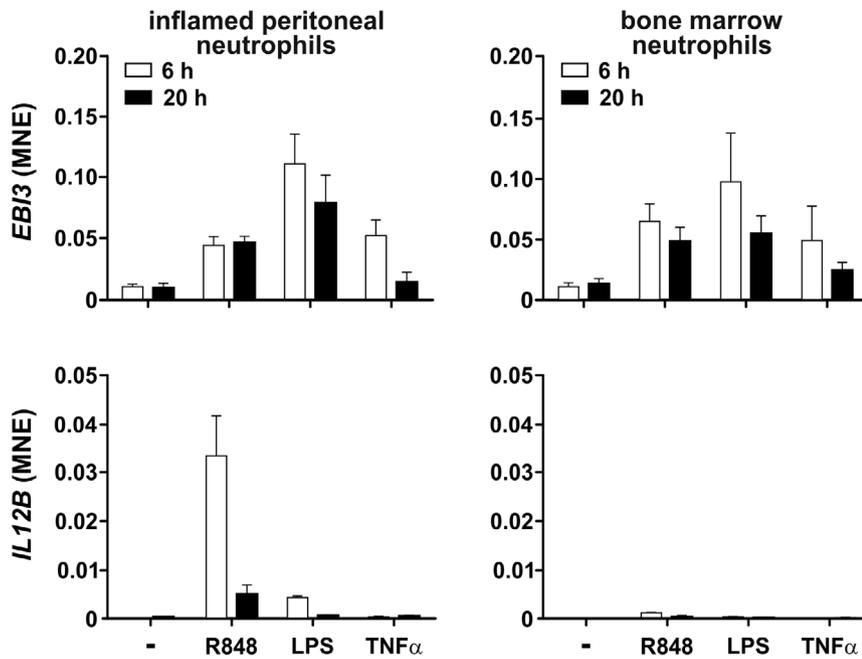


FIGURE 4 *EBI3* and *IL12B* mRNA expression in murine neutrophils incubated with R848, LPS, and TNF α . Neutrophils were isolated from peritoneal exudates and bone marrow of C57BL/6 mice, suspended at 5×10^6 /ml, and cultured with or without 5 μ M R848, 1 μ g/ml LPS, and 10 ng/ml TNF α for 6 and 20 h, to evaluate their *EBI3* and *IL12B* mRNA expression by RT-qPCR. Results from a representative experiment out of two are depicted as mean normalized expression (MNE) units after normalization to *GAPDH* mRNA

we have discovered that highly pure neutrophils incubated with TLR8 ligands, including R848, produce measurable quantities of extracellular IL-23, as a result of a time-dependent induction of IL-12B and IL23A transcripts.²⁴ Notably, we also found that supernatants from neutrophils, but not CD14⁺-monocytes, treated with R848, promote the differentiation of naïve cord blood T lymphocytes into Th17 cells in an IL-23-dependent fashion,²⁴ thus indicating that neutrophil-derived IL-23 is biologically active in vitro.

In this study, we have investigated whether TLR8 ligands trigger the expression of other IL-12 family members by neutrophils. First of all, we report that highly pure neutrophils incubated in vitro with R848, CL075, or Motolimod/VTX-233, express, produce, and release *EBI3* (as detected either by ELISA or immunoblot), at levels almost comparable to those produced by autologous CD14⁺-monocytes treated under the same experimental conditions. If one takes into account that the amounts of cytokines released from human neutrophils in vitro are, in most cases, orders of magnitude lower than those produced by monocytes,^{1,29} the quantity of *EBI3* produced by activated neutrophils herein found should be considered as really remarkable. This implies that activated neutrophils represent major sources of *EBI3*, and, as such, they must have a potentially undoubted relevance in vivo.

Because *EBI3* typically (but not necessarily) associates with other subunits of the IL-12 family members, namely, IL-12A to form IL-35, IL-27A to form IL-27 and, presumably, IL-23A to form IL-39, we then sought whether activated neutrophils could generate those heterodimeric cytokines. Subsequent experiments uncovered that R848-, as well as LPS-, treated neutrophils neither express *IL27A* mRNA nor, confirming previous data,²⁴ accumulate *IL12A* transcripts, thereby excluding the formation of either IL-27 or IL-35. Under the same experimental conditions, CD14⁺-monocytes were instead found to express both *IL27A* and *IL12A* transcripts, as well as to release IL-12 and IL-27, as expected.^{18,37} The lack of *IL27A* mRNA expression in R848- and LPS-treated neutrophils, further confirmed by the failure to detect

IL-27 in the related supernatants, are in contrast with the results by Rinchai et al.,²³ who reported that neutrophils incubated for 6 and 24 h with *B. pseudomallei*, *E.coli*, and even LPS from *E. coli*, but not with *S. aureus*, release amounts of IL-27A in the range of 2000/3000 pg/ml. Rinchai et al.²³ also found that, in *B. pseudomallei*-treated neutrophils, the use of a high affinity WSX-1 soluble receptor (sIL-27RA) to block the biologic activity of IL-27, partially suppressed neutrophil oxidative burst and cytokine production, in turn reducing their antibacterial activity, and indicating a neutrophil-activating effect by endogenous IL-27.²³ Although *B. pseudomallei* was not used as a stimulus in our study, and even if we utilized the same commercial ELISA, we have no concrete hints explaining why our findings, at least in the case of LPS stimulation, are so much in conflict with those by Rinchai et al.²³ One determining factor could be the purity of the neutrophil populations, which, in the study by Rinchai et al.²³ is declared as “generally as more than 95%,” and thus in our opinion not sufficient enough to guarantee the data veracity.⁷

We also made a few attempts to determine whether TLR8-activated neutrophils could produce and/or release IL-39 in vitro, in light of their known capacity to express *IL23A* mRNA and to produce IL-23.²⁴ Accordingly, we tried to co-immunoprecipitate *EBI3* with IL-23A, and vice versa, from supernatants harvested from R848-treated neutrophils, as well as to develop a specific ELISA measuring human IL-39. Although we only collected a series of negative results on all fronts, we cannot actually draw any definitive conclusion, due to the unavailability of specific reagents/positive controls to certify the quality of our experiments. On the other hand, simply based on the amounts of *EBI3*, IL-23A, and IL-23 measured in supernatants harvested from R848-treated neutrophils, we calculated that—even with the limit of having used ELISAs from three different companies—roughly, the amount of IL-23A produced by activated neutrophils is fully employed to form IL-23. In any case, the issue of IL-39 production by any kind of cell will be likely solved only when reliable reagents specific for the human

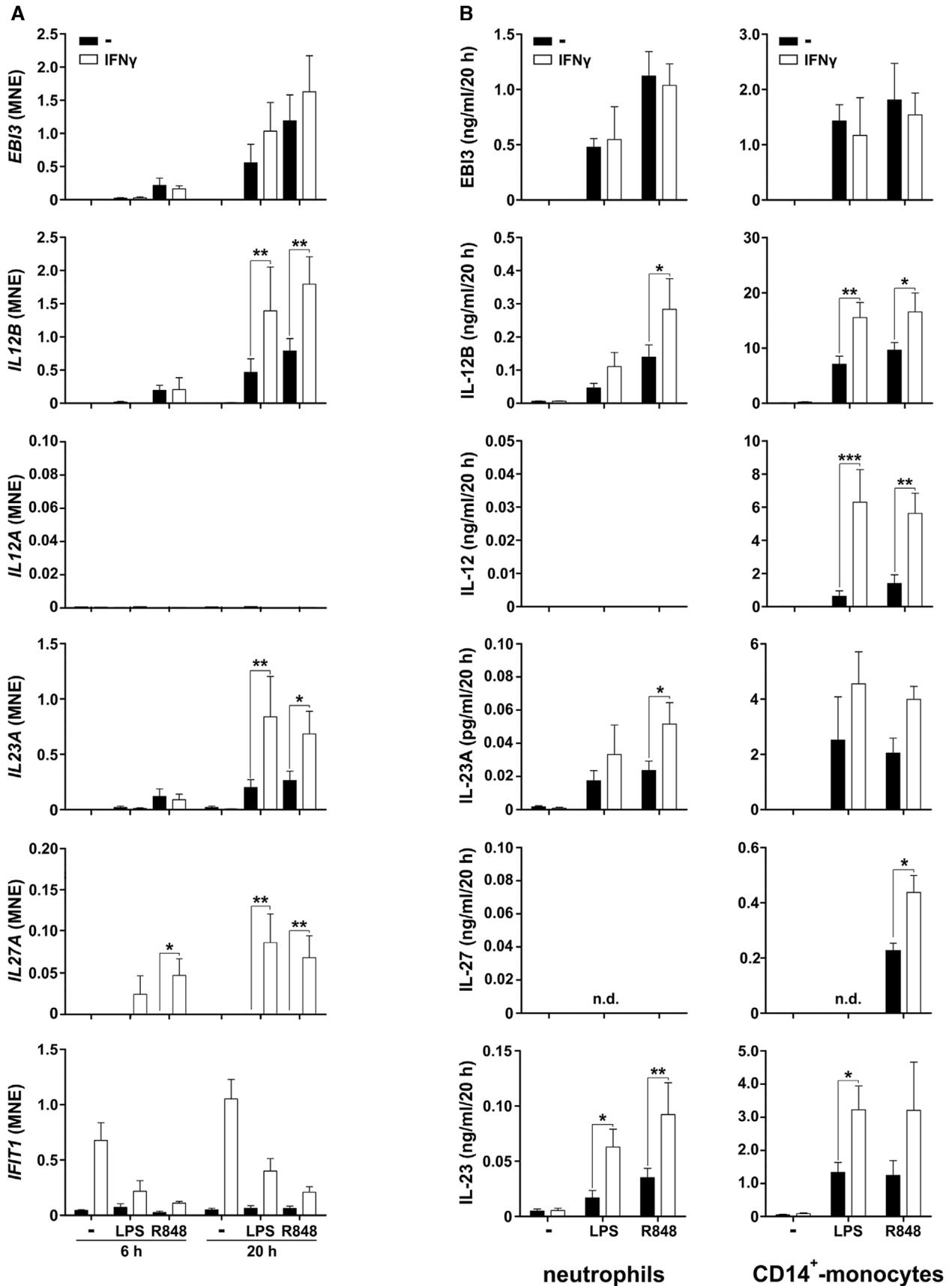


FIGURE 5 Effect of IFN γ on the mRNA expression and production of EB13 and other IL-12 family members by R848- or LPS-treated neutrophils and CD14⁺-monocytes. A total of 5×10^6 /ml neutrophils and 2.5×10^6 /ml CD14⁺-monocytes were cultured with or without 5 μ M R848 and 1 μ g/ml LPS, in combination or not with 200 U/ml IFN γ , for 6 and 20 h, to evaluate: (A) EB13, IL12B, IL23A, IL27A, and IFIT1 mRNA expression in (continued on the next page)

cytokine will be developed, of course assuming that IL-39 really exists in humans. In fact, a recent report focused on the detection of the potential pairings of IL-12 family chains in a system of HEK293T cells co-expressing a set of each possible α -/ β -subunit combination,³⁸ actually failed to detect the EB13/IL-23A combination (alias IL-39), but not other IL-12 family pairings.

In additional experiments, we cultured neutrophils with R848 and IFN γ , with the expectation to find an expression/production of IL-35, based on the presumed capacity of IFN γ to trigger the expression of *IL12A* (and *EBI3*) mRNA by neutrophils.²⁰ However, contrary to our expectations, we found that IFN γ does not actually trigger any expression of *IL12A* mRNA in highly pure neutrophils, as measured by RT-qPCR (which is certainly more sensitive than the RPA used in 1995).²⁰ On the other hand, under the same experimental conditions, IFN γ was found to directly induce *IFIT1* mRNA transcripts, as well as to modulate the expression of *IL12B* and *IL23A* mRNA expression, thus proving its effectiveness. The most likely reason explaining why, currently, we do not observe any *IL12A* mRNA expression in IFN γ -treated cells, consists in the use of highly pure neutrophils that, in 1995, could not be obtained, given the unavailability of technologies such as cell depletion by antibody-coated magnetic beads.³⁹ Accordingly, we have repeatedly evidenced not only how crucial it is to work with highly pure populations of neutrophils for gene expression studies, but also the necessity to unequivocally exclude the possibility that a very low percentage of contaminating cells could determine false positive results ultimately attributed to neutrophils.⁷ In such regard, we have recently attempted to clarify controversies concerning the ability of human neutrophils to produce IFN α ,⁶ IL-10,⁴⁰ and IL-17.⁴¹ Even in our indicted paper²⁰ we made our best in trying to purify neutrophils at the highest possible levels of purity, compatibly with the technological means in possession at those times. Whatever the case is, the unexpected results herein described imply that neutrophils incubated with IFN γ plus either LPS or R848 can produce neither IL-12A nor IL-12, unlike IFN γ plus R848- or LPS-treated monocytes. It follows that our original data on the ability of IFN γ plus LPS-treated neutrophils to produce extracellular IL-12 in vitro must be denied. Even though our current negative data in vitro do not relate to the many studies reporting neutrophil-derived IL-12 in vivo,^{42,43} we apologize for having unintentionally confused the scientific community.

In the experiments in which neutrophils were incubated with IFN γ plus either R848 or LPS, we found an induction of the *IL27A* transcripts. Because the same phenomenon did not occur if the three stimuli were used alone, it is evident that IFN γ plus either R848 or LPS synergize to promote the transcription of the *IL27A* gene. Although the molecular bases of such synergism were not addressed, data prompted us to evaluate whether IL-27 could be released by neutrophils treated for 20 h with IFN γ plus R848, but our ELISA measurement of the related supernatants gave negative data. By contrast, supernatants

from IFN γ plus R848-treated monocytes were found to contain quantities of IL-27 higher than those detected in supernatants from monocytes treated with R848 only. It is therefore possible that the *IL27A* mRNA induced in IFN γ plus R848-treated neutrophils is translated, but not released, because it is stored in intracellular compartments, similar to what was observed for instance in the case of CXCL8,⁴⁴ BAFF,⁴⁵ or TRAIL.⁴⁶ An alternative possibility is that *IL-27* mRNA induced by IFN γ plus R848-mRNA is not translated at all, maybe because it is subjected to microRNA-dependent regulatory controls.⁴⁷ The latter possibility might be in line with other findings observed in neutrophils incubated with R848 in combination with TNF α . Accordingly, R848 plus TNF α were found to additively induce the levels of *EBI3* mRNA, but not those of *EBI3* production, suggesting that regulatory mechanisms controlling also *EBI3* translation or intracellular accumulation/release might take place in neutrophils.

In sum, the present study demonstrates that human neutrophils incubated with TLR8 ligands and TNF α are able to produce remarkable amounts of *EBI3* in vitro. Importantly, our experiments also suggest that R848, LPS and, to a lesser extent, TNF α , all induce *EBI3* mRNA also in murine neutrophils isolated from peritoneum or BM. This indicates that the capacity of neutrophils to respond to proinflammatory stimuli in terms of *EBI3* mRNA induction is conserved among species and represents an important phenomenon. Moreover, immunostaining studies support our in vitro findings, as they evidence an in vivo expression of *EBI3* by neutrophils infiltrating tissues from discrete pathologies, such as Gorham disease, diverticulitis, cholecystitis, and *Bartonella Henselae* infection, but not others, including primary carcinomas, psoriasis, Chron's disease, and COVID-19 pneumonia. Because *EBI3* mRNA expression were found absent in peripheral neutrophils freshly isolated from cohorts of active systemic lupus erythematosus (SLE) or CMV-infected patients (our unpublished observations), it is clear that, as in vitro, also in vivo, *EBI3* expression becomes detectable in neutrophils only in certain pathologies. Why the latter occurs remains to be investigated and explained. Given that as a self-standing molecule *EBI3* has been, for example, involved in the pathogenesis of rheumatoid arthritis,⁴⁸ inflammatory bowel disease,⁴⁹ systemic sclerosis,¹² and cardiac inflammation,⁵⁰ as well in growth-promoting activity of lung⁵¹ breast,⁵² and colorectal cancer,⁵³ other than representing a novel diagnosis marker in Burkitt and diffuse large B-cell lymphoma,⁵⁴ further studies are necessary to clarify what is the effective function and biologic meaning of neutrophil-derived *EBI3* under these latter and other circumstances.

AUTHORSHIP

E.G. and F.A.-S. contributed equally. M.A.C., N.T., W.V., F.L., and O.D. contributed to experimental design; N.T., E.G., F.A.-S., F.B.-A., S.G., M.B., and F.L. contributed to experimental work; M.A.C., N.T., E.G., F.A.-S., F.B.-A.,

neutrophils by RT-qPCR; (B) *EBI3*, IL-12B, IL-12, IL-27, IL-23, and IL-23A extracellular release by ELISA. Gene expression data (mean \pm SEM, $n = 4-8$) are depicted as mean normalized expression (MNE) units after *GAPDH* mRNA normalization, whereas ELISA values stand for the mean \pm SEM ($n = 4-8$). Asterisks indicate a significant increase exerted by IFN γ : *** $P < 0.001$, by 2-way ANOVA followed by Bonferroni's posttest. Data are normalized per equal number of cells

M.B., W.V., F.L., and O.D. contributed to data analysis; and M.A.C., N.T., E.G., and O.D. contributed to manuscript preparation.

ACKNOWLEDGMENTS

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro (AIRC, IG-20339), Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR-PRIN 2015YYKPNP and 20177J4E75_004), and Fondazione CARIPO (2015-0584). The authors thank Centro Piattaforme Tecnologiche (University of Verona, Verona, Italy) for technical support in RNA-seq experiments, and Prof. P. Scapini (University of Verona) for helping with mouse experiments.

DISCLOSURES

The authors declare no conflicts of interest.

ORCID

Marco A. Cassatella  <https://orcid.org/0000-0001-7824-1806>

REFERENCES

- Tamassia N, Bianchetto-Aguilera F, Arruda-Silva F, et al. Cytokine production by human neutrophils: revisiting the "dark side of the moon". *Eur J Clin Invest*;2018:e12952.
- Ley K, Hoffman HM, Kubes P, et al. Neutrophils: new insights and open questions. *Sci Immunol*. 2018;3.
- Fortin CF, Ear T, McDonald PP. Autocrine role of endogenous interleukin-18 on inflammatory cytokine generation by human neutrophils. *FASEB J*. 2009;23:194-203.
- Zimmermann M, Aguilera FB, Castellucci M, et al. Chromatin remodelling and autocrine TNFalpha are required for optimal interleukin-6 expression in activated human neutrophils. *Nat Commun*. 2015;6:6061.
- Arruda-Silva F, Bianchetto-Aguilera F, Gasperini S, et al. Human neutrophils produce CCL23 in response to various TLR-agonists and TNFalpha. *Front Cell Infect Microbiol*. 2017;7:176.
- Zimmermann M, Arruda-Silva F, Bianchetto-Aguilera F, et al. IFNalpha enhances the production of IL-6 by human neutrophils activated via TLR8. *Sci Rep*. 2016;6:19674.
- Calzetti F, Tamassia N, Arruda-Silva F, Gasperini S, Cassatella MA. The importance of being "pure" neutrophils. *J Allergy Clin Immunol*. 2017;139:352-355 e6.
- Hunter CA. New IL-12-family members: iL-23 and IL-27, cytokines with divergent functions. *Nat Rev Immunol*. 2005;5:521-531.
- Hasegawa H, Mizoguchi I, Chiba Y, et al. Expanding diversity in molecular structures and functions of the IL-6/IL-12 heterodimeric cytokine family. *Front Immunol*. 2016;7:479.
- Teng MW, Bowman EP, McElwee JJ, et al. IL-12 and IL-23 cytokines: from discovery to targeted therapies for immune-mediated inflammatory diseases. *Nat Med*. 2015;21:719-729.
- Wang X, Wei Y, Xiao H, et al. A novel IL-23p19/Ebi3 (IL-39) cytokine mediates inflammation in Lupus-like mice. *Eur J Immunol*. 2016;46:1343-1350.
- Kudo H, Wang Z, Jinnin M, et al. EBI3 downregulation contributes to type I collagen overexpression in scleroderma skin. *J Immunol*. 2015;195:3565-3573.
- Espigol-Frigole G, Planas-Rigol E, Ohnuki H, et al. Identification of IL-23p19 as an endothelial proinflammatory peptide that promotes gp130-STAT3 signaling. *Sci Signal*. 2016;9:ra28.
- Cooper AM, Khader SA. IL-12p40: an inherently agonistic cytokine. *Trends Immunol*. 2007;28:33-38.
- Yan J, Mitra A, Hu J, et al. Interleukin-30 (IL27p28) alleviates experimental sepsis by modulating cytokine profile in NKT cells. *J Hepatol*. 2016;64:1128-1136.
- Chan SH, Perussia B, Gupta JW, et al. Induction of interferon gamma production by natural killer cell stimulatory factor: characterization of the responder cells and synergy with other inducers. *J Exp Med*. 1991;173:869-879.
- Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity*. 2000;13:715-725.
- Pflanz S, Timans JC, Cheung J, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells. *Immunity*. 2002;16:779-790.
- Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature*. 2007;450:566-569.
- Cassatella MA, Meda L, Gasperini S, et al. Interleukin-12 production by human polymorphonuclear leukocytes. *Eur J Immunol*. 1995;25:1-5.
- Cassone A, Chiani P, Quinti I, Torosantucci A. Possible participation of polymorphonuclear cells stimulated by microbial immunomodulators in the dysregulated cytokine patterns of AIDS patients. *J Leukoc Biol*. 1997;62:60-66.
- Amedei A, Cappon A, Codolo G, et al. The neutrophil-activating protein of *Helicobacter pylori* promotes Th1 immune responses. *J Clin Invest*. 2006;116:1092-1101.
- Rinchai D, Khaenam P, Kewcharoenwong C, et al. Production of interleukin-27 by human neutrophils regulates their function during bacterial infection. *Eur J Immunol*. 2012;42:3280-3290.
- Tamassia N, Arruda-Silva F, Wright HL, et al. Human neutrophils activated via TLR8 promote Th17 polarization through IL-23. *J Leukoc Biol*. 2019;105:1155-1165.
- Siemsen DW, Kirpotina LN, Malachowa N, et al. Isolation of neutrophils from nonhuman species. *Methods Mol Biol*. 2020;2087:43-59.
- Tamassia N, Cassatella MA, Bazzoni F. Fast and accurate quantitative analysis of cytokine gene expression in human neutrophils by reverse transcription real-time PCR. *Methods Mol Biol (Clifton, NJ)*. 2020;2087:243-260.
- Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol*. 2011;29:24-26.
- Devergne O, Coulomb-L'Hermine A, Capel F, Moussa M, Capron F. Expression of Epstein-Barr virus-induced gene 3, an interleukin-12 p40-related molecule, throughout human pregnancy: involvement of syncytiotrophoblasts and extravillous trophoblasts. *Am J Pathol*. 2001;159:1763-1776.
- Cassatella MA. Neutrophil-derived proteins: selling cytokines by the pound. *Adv Immunol*. 1999;73:369-509.
- Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP. Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther*. 2008;117:244-279.
- Gorden KB, Gorski KS, Gibson SJ, et al. Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8. *J Immunol*. 2005;174:1259-1268.
- Lu H, Dietsch GN, Matthews MA, et al. VTX-2337 is a novel TLR8 agonist that activates NK cells and augments ADCC. *Clin Cancer Res*. 2012;18:499-509.
- Hayes MP, Wang J, Norcross MA. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. *Blood*. 1995;86:646-650.

34. Bridgewood C, Alase A, Watad A, et al. The IL-23p19/EBI3 heterodimeric cytokine termed IL-39 remains a theoretical cytokine in man. *Inflam Res*. 2019;68:423-426.
35. Xue Z-w, Shang X-m, Lv S-h, et al. Effects of Shenshao decoction () on the inflammatory response in the aorta of a rat atherosclerotic model. *Chin J Integr Med*. 2013;19:347-352.
36. Cho K-A, Lee J-K, Kim Y-H, et al. Mesenchymal stem cells ameliorate B-cell-mediated immune responses and increase IL-10-expressing regulatory B cells in an EBI3-dependent manner. *Cell Mol Immunol*. 2017;14:895-908.
37. Ehrnstrom B, Beckwith KS, Yurchenko M, et al. Toll-like receptor 8 is a major sensor of group B streptococcus but not *Escherichia coli* in human primary monocytes and macrophages. *Front Immunol*. 2017;8:1243.
38. Detry S, Składanowska K, Vuylsteke M, Savvides SN, Bloch Y. Revisiting the combinatorial potential of cytokine subunits in the IL-12 family. *Biochem Pharmacol*. 2019;165:240-248.
39. Pelletier M, Maggi L, Micheletti A, et al. Evidence for a cross-talk between human neutrophils and Th17 cells. *Blood*. 2010;115:335-343.
40. Tamassia N, Zimmermann M, Castellucci M, et al. Cutting edge: an inactive chromatin configuration at the IL-10 locus in human neutrophils. *J Immunol*. 2013;190:1921-1925.
41. Tamassia N, Arruda-Silva F, Calzetti F, 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;9:795.
42. Romani L, Bistoni F, Puccetti P. Initiation of T-helper cell immunity to *Candida albicans* by IL-12: the role of neutrophils. *Chem Immunol*. 1997;68:110-135.
43. Bliss SK, Butcher BA, Denkers EY. Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. *J Immunol*. 2000;165:4515-4521.
44. Meda L, Gasperini S, Ceska M, Cassatella MA. Modulation of proinflammatory cytokine release from human polymorphonuclear leukocytes by gamma interferon. *Cell Immunol*. 1994;157:448-461.
45. Scapini P, Carletto A, Nardelli B, et al. Proinflammatory mediators elicit secretion of the intracellular B-lymphocyte stimulator pool (BLYS) that is stored in activated neutrophils: implications for inflammatory diseases. *Blood*. 2005;105:830-837.
46. Cassatella MA, Huber V, Calzetti F, 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*. 2006;79:123-132.
47. Shu C, Yan D, Chen C, et al. Metformin exhibits its therapeutic effect in the treatment of pre-eclampsia via modulating the Met/H19/miR-148a-5p/P28 and Met/H19/miR-216-3p/EBI3 signaling pathways. *Int Immunopharmacol*. 2019;74:105693.
48. Iranshahi N, Assar S, Amiri SM, et al. decreased gene expression of Epstein-Barr virus-induced gene 3 (EBI-3) may contribute to the pathogenesis of rheumatoid arthritis. *Immunol Invest*. 2019;48:367-377.
49. Wetzel A, Scholtka B, Gerecke C, Kleuser B. Epigenetic histone modulation contributes to improvements in inflammatory bowel disease via EBI3. *Cell Mol Life Sci*. 2020.
50. Medina TS, Oliveira GG, Silva MC, et al. Ebi3 prevents *Trypanosoma cruzi*-induced myocarditis by dampening IFN-gamma-driven inflammation. *Front Immunol*. 2017;8:1213.
51. Nishino R, Takano A, Oshita H, et al. Identification of Epstein-Barr virus-induced gene 3 as a novel serum and tissue biomarker and a therapeutic target for lung cancer. *Clin Cancer Res*. 2011;17:6272-6286.
52. Jiang J, Liu X. Upregulated EBI3 correlates with poor outcome and tumor progression in breast cancer. *Oncol Res Treat*. 2018;41:111-115.
53. Liang Y, Chen Q, Du W, et al. Epstein-Barr virus-induced gene 3 (EBI3) blocking leads to induce antitumor cytotoxic T lymphocyte response and suppress tumor growth in colorectal cancer by bidirectional reciprocal-regulation STAT3 signaling pathway. *Mediators Inflamm*. 2016;2016:3214105.
54. Gonin J, Larousserie F, Bastard C, et al. Epstein-Barr virus-induced gene 3 (EBI3): a novel diagnosis marker in Burkitt lymphoma and diffuse large B-cell lymphoma. *PLoS One*. 2011;6:e24617.

How to cite this article: Cassatella MA, Gardiman E, Arruda-Silva F, et al. Human neutrophils activated by TLR8 agonists, with or without IFN γ , synthesize and release EBI3, but not IL-12, IL-27, IL-35, or IL-39. *J Leukoc Biol*. 2020;1-12. <https://doi.org/10.1002/JLB.3MA0520-054R>