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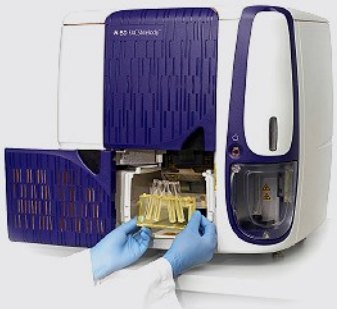

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ARTICLE

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

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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 IFNγ 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, TNFα 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 IFNγ in combination with either TLR8 or TLR4 ligands express/produce neither IL-12, nor IL-35, due to the inability of IFNγ, contrary to previous findings, to activate *IL12A* transcription. Even IL-27 was undetectable in supernatants harvested from IFNγ 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, TNFα

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.

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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 TNFα, 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-27A[23] 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 IFNγ in combination with either TLR8 or TLR4 ligands express/produce neither IL-12 nor IL-35, due to the inability of IFNγ 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 μM R848 (InvivoGen, San Diego, CA, USA); 1 μg/ml ultrapure LPS (*E. coli* 0111:B4 strain, InvivoGen); 10 ng/ml human TNFα (R&D Systems, Minneapolis, MN, USA); 200 U/ml IFNγ (R&D Systems); 10 ng/ml GM-CSF (Miltenyi Biotec); 1000 U/ml G-CSF (Myelostim, Italfarmaco Spa, Milano, Italy); 0.2–10 μM CL075 (InvivoGen); 0.2–10 μM VTX-2337 (Selleck Chem, Boston, MA, USA); or 10 μg/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% CO_2), with or without 5 μM R848 or 1 μg/ml LPS or 10 ng/ml TNFα. 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

	Sequences	
RT-qPCR primers	**Forward primers**	**Reverse primers**
Human primer sets		
IL12A	CTGGACCACCTCAGTTTGG	TTTGTCGGCCTTCTGGAG
IL12B	GGACATCATCAAACCTGACC	AGGGAGAAGTAGGAATGTGG
IL23A	GGACACATGGATCTAAGAGAAGAG	CTATCAGGGAGCAGAGAAGG
RPL32	AGGGTTCGTAGAAGATTCAAGG	GGAAACATTGTGAGCGATCTC
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
IL27A	AGGGAGTTCACAGTCAGCCT	GGTGGAGATGAAGCAGAGACG
EBI3	ACAGAGCACATCATCAAGCC	CCACTTGGACGTAGTACCTG
IL1ra	TTCCTGTTCCATTCAGAGACGAT	AATTGACATTTGGTCCTTGCAA
Mouse primer sets		
EBI3	TACACTGAAACAGCTCTCGTG	CATTTAGCATGTAGGGCACC
IL12B	TCAGGGACATCATCAAACCAG	CGAACAAAGAACTTGAGGGAG
GAPDH	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 2.02. 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 IFNγ 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 H_2SO_4. 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 procedures[4] using anti-human EBI3 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 (Bio-Genex, Fremont, CA, USA) and permanent red as chromogen, or using Novolink Polymer (Leica Microsystems, Wetzlar, Germany) followed by DAB (3,3'-diaminobenzidine). EBI3 was detected by using the mouse monoclonal anti-EBI3 Ab (clone 2G4H6)[28] at 2–4 μg/ml. Sections were counterstained with Mayer hematoxylin.

2.4 | Statistical analysis

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

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 EBI3 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 μM R848, for 6 and 20 h, revealed a remarkable induction of *EBI3* mRNA under R848-activating conditions (Fig. 1A). Given that EBI3 may form IL-27, IL-35, and IL-39, we performed RT-qPCR kinetic experiments of *EBI3*, *IL27A*, and *IL12A* mRNA expression in neutrophils and autologous CD14$^+$-monocytes incubated not only with or without R848, but also with 1 μg/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 *EBI3* 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. *EBI3* 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 EBI3 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 EBI3 (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 EBI3 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 EBI3-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 EBI3-negative.

In another series of experiments, only TNFα (10 ng/ml), but not G-CSF (1000 U/ml) or GM-CSF (10 ng/ml), was found able to trigger the expression of *EBI3* 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 TNFα-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 TNFα,[4] we then examined whether endogenous TNFα could autocrinally regulate the expression of *EBI3* mRNA, and found it to be the case. Indeed, incubation of neutrophils with R848 for 20 h, in the presence of 10 μg/ml adalimumab (a TNFα neutralizing antibody)[30] revealed a 44.7 ± 11.7% inhibition of *EBI3* mRNA expression as compared to cells incubated in its absence ($n = 4$). Interestingly, we also found that, whereas the amounts of extracellular EBI3 detected in supernatants from neutrophils incubated with R848 and/or TNFα for 20 h were substantially similar (Fig. 3B), at the same time point the *EBI3* 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

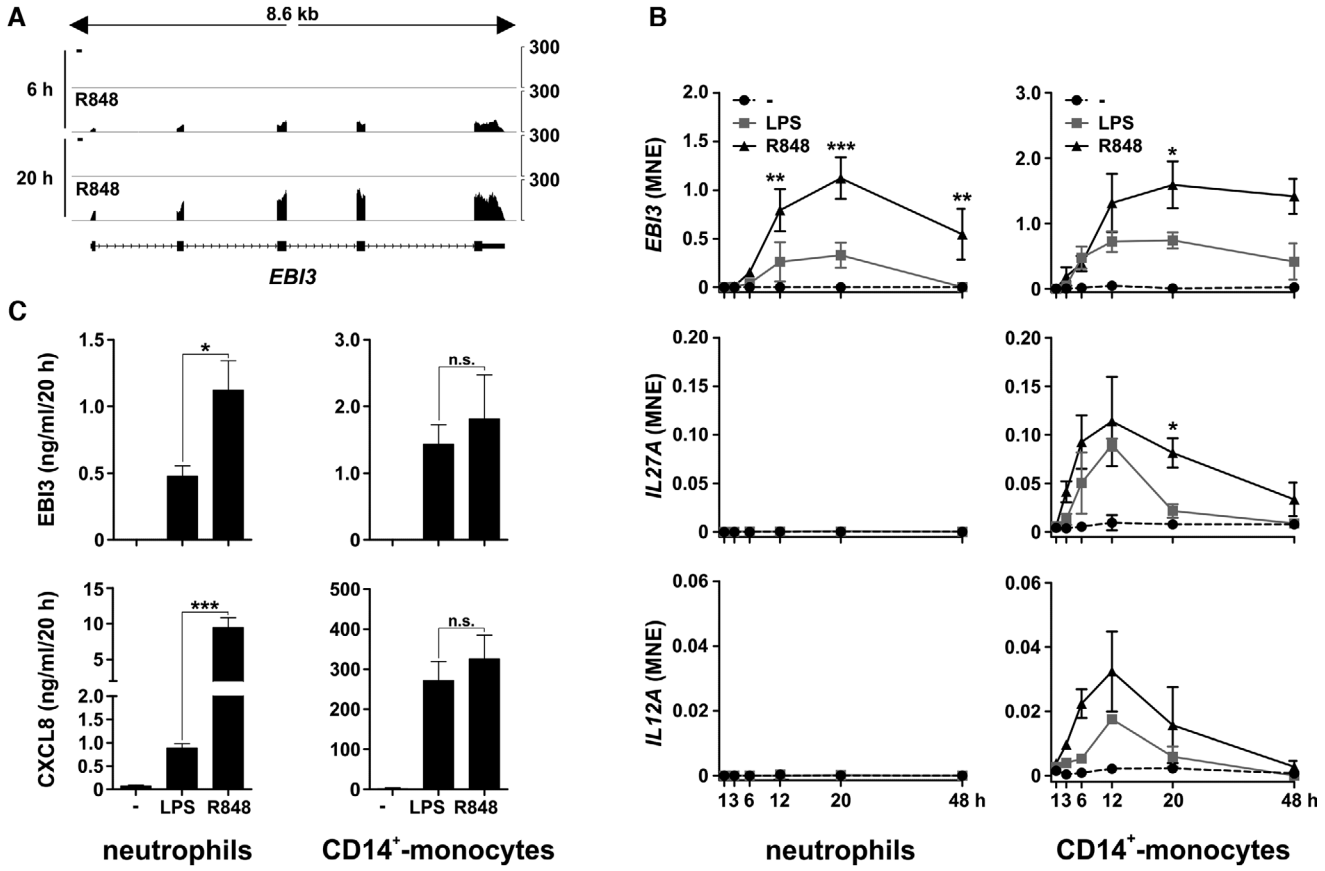

FIGURE 1 ***EBI3*** **mRNA and protein expression in neutrophils and CD14$^+$-monocytes incubated with R848 or LPS.** In (**A**), highly pure neutrophils (5×10^6/ml) were incubated for 6 or 20 h in the absence (–) or the presence of 5 μM R848. Expression levels of *EBI3* transcripts were measured by 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 μM R848 or 1 μg/ml LPS. Plots show kinetics of *EBI3*, *IL27A*, and *IL12A* mRNA expression, as measured by RT-qPCR and depicted as mean normalized expression (MNE) units after normalization to *GAPDH* mRNA (mean ± SEM, $n = 4$–8). Asterisks indicate significant differences between LPS and R848 in terms of *EBI3*, *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 EBI3 and CXCL8 extracellular proteins measured by ELISA ($n = 4$–6). Asterisks indicate significant differences between R848 and LPS in terms of induced EBI3 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 *EBI3*, *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 TNFα genuinely express and produce EBI3. 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 EBI3 transcripts in murine neutrophils

To evaluate whether *EBI3* 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 μM R848, 1 μg/ml LPS and 10 ng/ml TNFα. 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 *EBI3* transcripts, 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 IFNγ on the expression of EBI3 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 IFNγ produce IL-12, given the ability of LPS to induce the expression of *IL12B* (but not *IL12A*, mRNA) and of IFNγ to induce that of *IL12A* (but not *IL12B*) mRNA.[20] We therefore incubated highly pure neutrophils with IFNγ in the presence of either R848 or LPS, with the expectation of detecting,

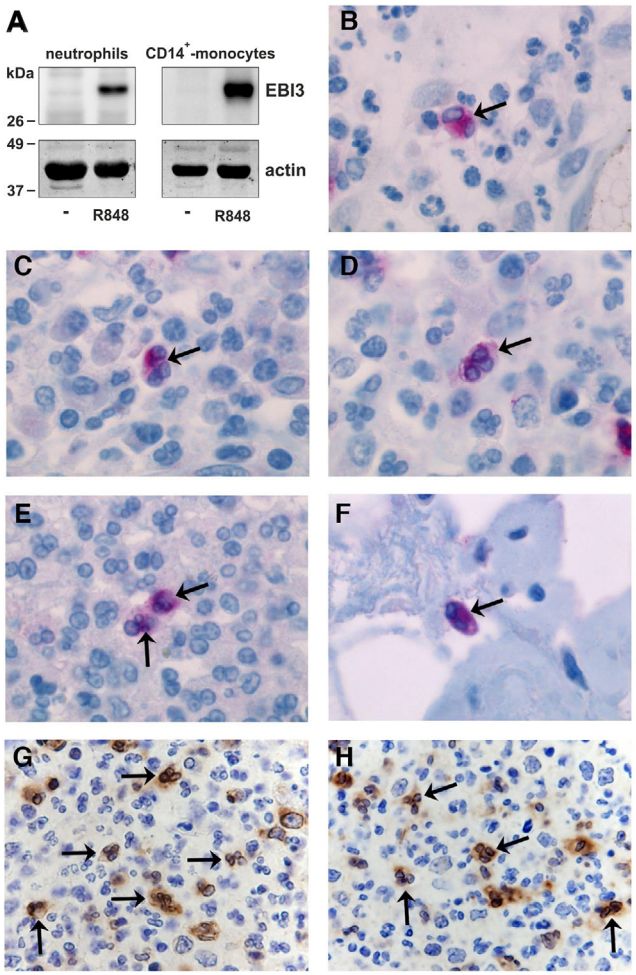

FIGURE 2 Antigenic EBI3 expression in human neutrophils. (**A**), Immunoblot displaying antigenic EBI3 and actin expression in lysates of neutrophils and autologous CD14$^+$-monocytes cultured for 20 h with or without 5 μM R848 (representative experiment, $n = 2$). (**B-H**), Sections from tissues of 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× (**B–F**) and 60× (**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 IFNγ, 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 IFNγ were found unable to induce *IL27A* mRNA expression in neutrophils, IFNγ plus either R848 or LPS triggered detectable levels of *IL27A* transcripts (Fig. 5A). In any case, the efficacy of IFNγ was confirmed by its direct, time-dependent, induction of IFN-induced protein with tetratricopeptide 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 IFNγ 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, IFNγ 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 IFNγ of the scarce IL-12 release by CD14$^+$-monocytes treated with either R848 or LPS[7] (Fig. 5B). Even under "priming" conditions for CD14$^+$monocytes,[33] namely, after a 6 h preincubation with IFNγ 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 IFNγ (Fig. 5B).

Altogether, data show that IFNγ 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 IFNγ plus either R848 or LPS. Finally, data also negate previous publications on the in vitro production of IL-12 by human neutrophils incubated with IFNγ plus LPS,[20] extending such inability to the treatment with IFNγ 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-23A[24] 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 IFNγ 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 immunoblotting[35]], followed by either rabbit polyclonal or mouse monoclonal anti-EBI3 antibodies[36] [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 μg) of rhIL-23A and rhEBI3 as control, as it was previously found to form a stable complex.[11] Then,

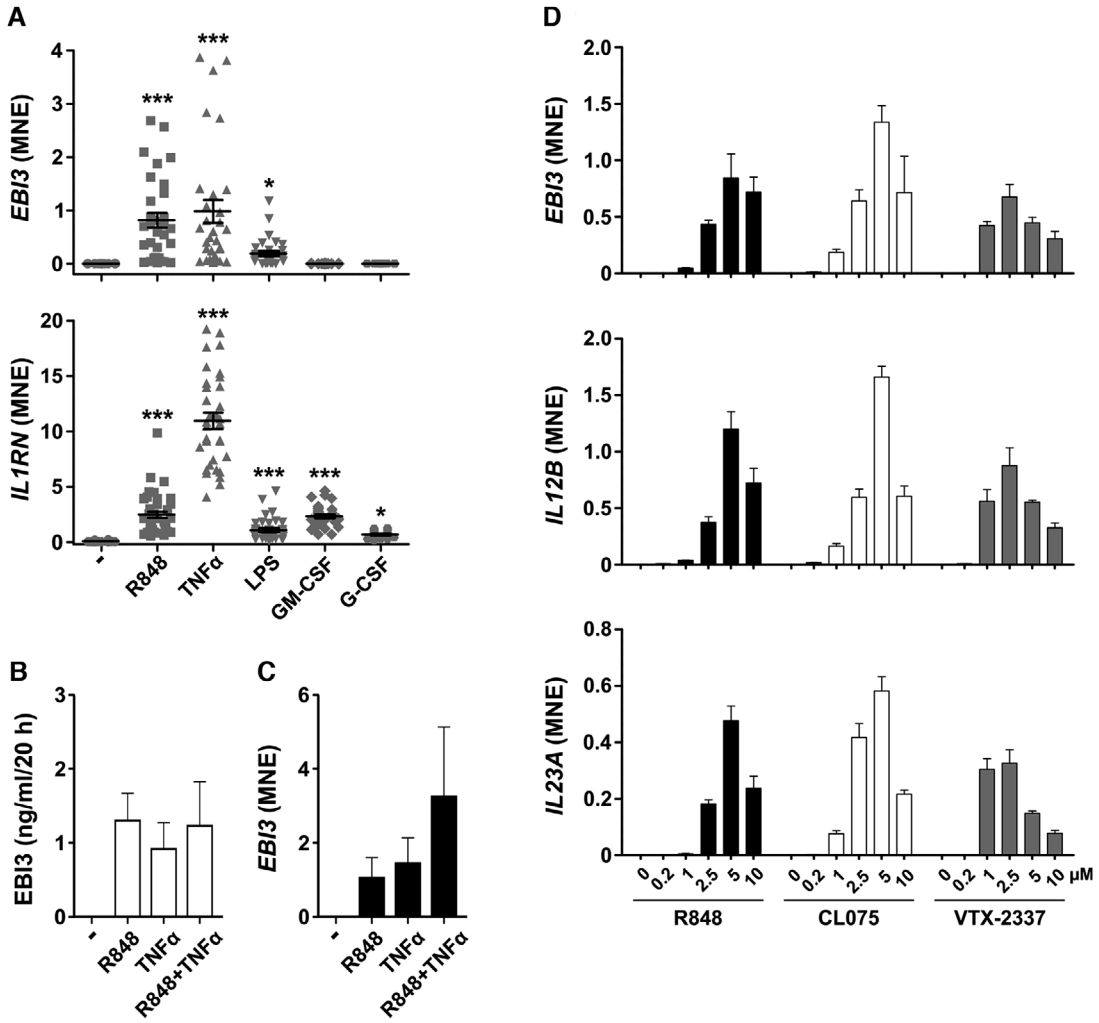

FIGURE 3 **Effect of TLR8 ligands and other agonists on *EBI3* mRNA expression by neutrophils.** (**A**) 5×10^6/ml neutrophils were cultured with or without 5 μM R848, 10 ng/ml TNFα, 1 μg/ml LPS, 1000 U/ml G-CSF and 10 ng/ml GM-CSF to evaluate *EBI3* and *IL1ra* mRNA expression by RT-qPCR ($n = 32$). 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 μM R848 and/or 10 ng/ml TNFα for 20 h, to evaluate their EBI3 release by ELISA (**B**) and *EBI3* mRNA expression by RT-qPCR (**C**). Values represent the means ± SEM ($n = 2–4$). Asterisks indicate a significant increase exerted by R848 plus TNFα as compared with either R848 or TNFα singly used: $^*P < 0.05$, $^{***}P < 0.001$, by Student's *t*-test. (**D**) Neutrophils were cultured with or without R848 (0.2–10 μM), CL075 (0.2–10 μM), or VTX-2337 (0.2–10 μM), to evaluate *EBI3*, *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 IFNγ plus R848-treated neutrophils, as well as from IFNγ plus LPS-treated monocyte-derived DCs, which, in our hands, accumulate the highest levels of *EBI3* and *IL23A* mRNA (data not shown).

In sum, our experiments on a presumed association of extracellular EBI3 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 IFNγ 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 EBI3 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 IFNγ 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 IFNγ induces *IL12A*, but not *IL12B*, transcripts.[20] Our paper was then followed by other in vitro studies, in which the requirement of IFNγ in combination with another agonist to trigger heterodimeric IL-12 production was substantially confirmed.[21] More recently, confirming previous notions,[22]

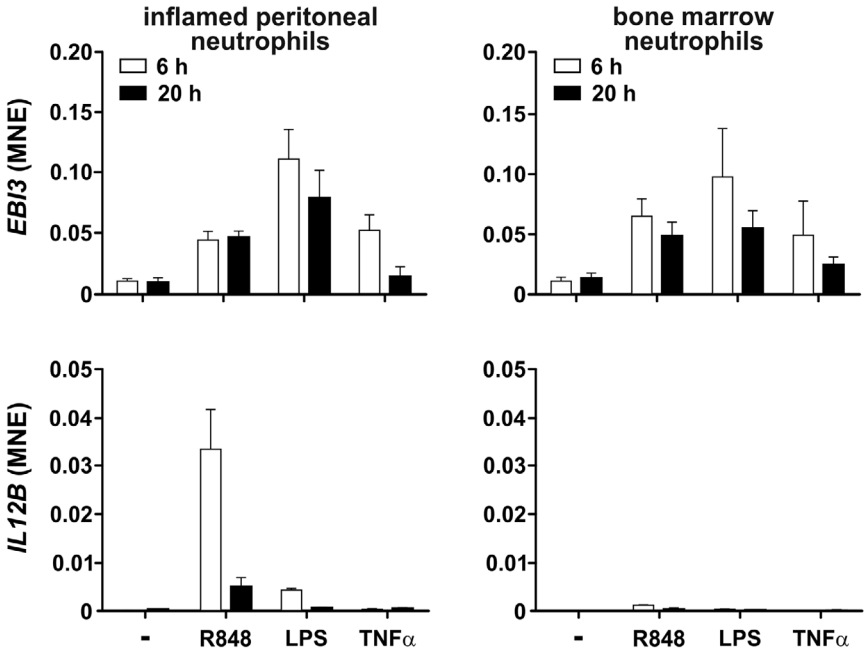

FIGURE 4 ***EBI3*** **and** ***IL12B*** **mRNA expression in murine neutrophils incubated with R848, LPS, and TNFα.** Neutrophils were isolated from peritoneal exudates and bone marrow of C57BL/6 mice, suspended at 5×10^6/ml, and cultured with or without 5 μM R848, 1 μg/ml LPS, and 10 ng/ml TNFα for 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

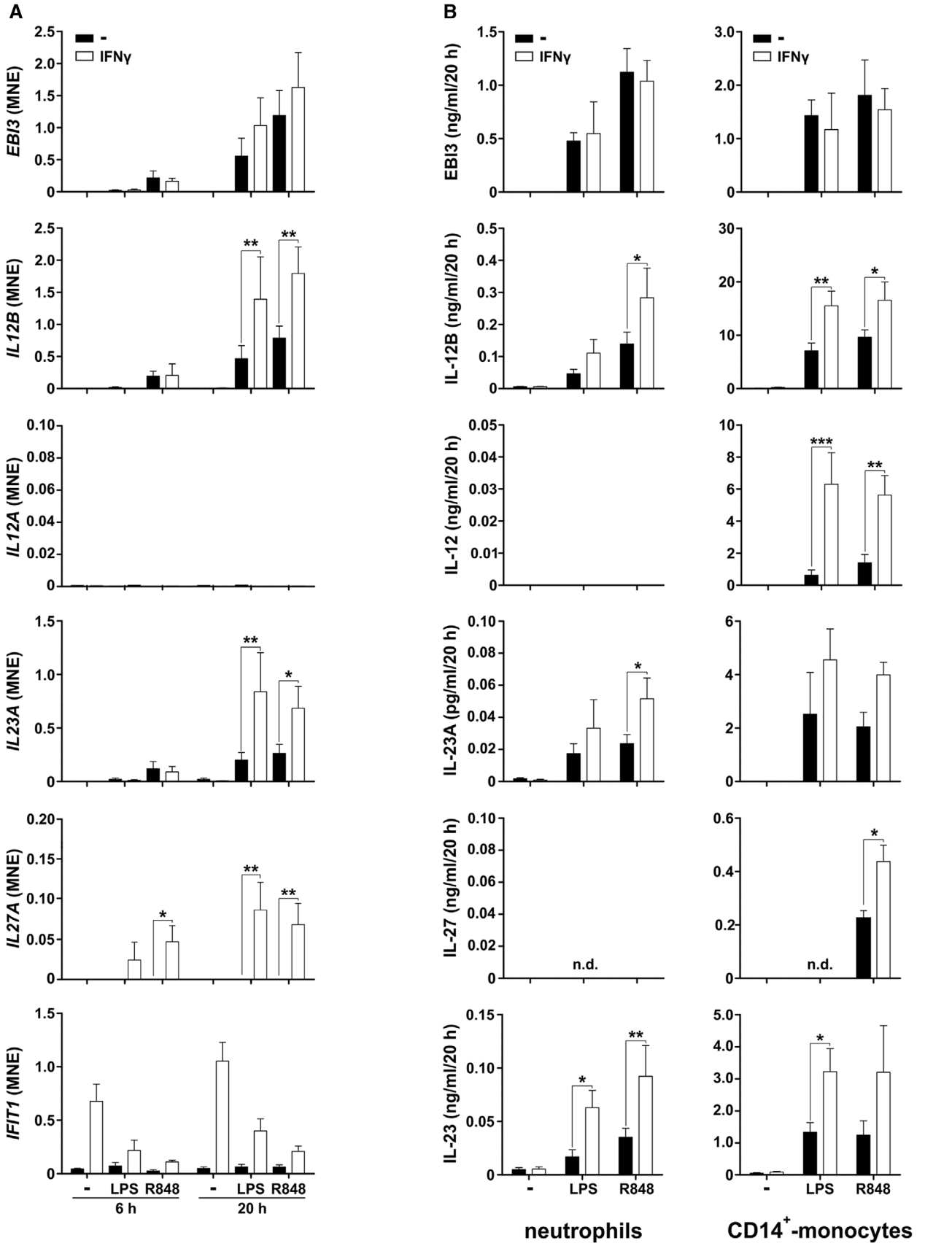

FIGURE 5 **Effect of IFNγ on the mRNA expression and production of EBI3 and other IL-12 family members by R848- or LPS-treated neutrophils and CD14$^+$-monocytes.** A total of 5×10^6/ml neutrophils and 2.5×10^6/ml CD14$^+$-monocytes were cultured with or without 5 μM R848 and 1 μg/ml LPS, in combination or not with 200 U/ml IFNγ, for 6 and 20 h, to evaluate: (**A**) *EBI3*, *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 EBI3/IL-23A combination (alias IL-39), but not other IL-12 family pairings.

In additional experiments, we cultured neutrophils with R848 and IFNγ, with the expectation to find an expression/production of IL-35, based on the presumed capacity of IFNγ to trigger the expression of *IL12A* (and *EBI3*) mRNA by neutrophils.[20] However, contrary to our expectations, we found that IFNγ 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, IFNγ 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 IFNγ-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 IFNα,[6] IL-10,[40] and IL-17.[41] Even in our indicted paper[20] 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 IFNγ plus either LPS or R848 can produce neither IL-12A nor IL-12, unlike IFNγ plus R848- or LPS-treated monocytes. It follows that our original data on the ability of IFNγ 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 IFNγ 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 IFNγ 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 IFNγ plus R848, but our ELISA measurement of the related supernatants gave negative data. By contrast, supernatants from IFNγ 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 IFNγ 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 *IFNγ* 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 TNFα. Accordingly, R848 plus TNFα 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 TNFα are able to produce remarkable amounts of EBI3 in vitro. Importantly, our experiments also suggest that R848, LPS and, to a lesser extent, TNFα, 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 lung[51] 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 ± SEM, $n = 4$–8) are depicted as mean normalized expression (MNE) units after *GAPDH* mRNA normalization, whereas ELISA values stand for the mean ± SEM ($n = 4$–8). Asterisks indicate a significant increase exerted by IFNγ: ***$P < 0.001$, 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.

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DISCLOSURES

The authors declare no conflicts of interest.

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