Gain-of-function *CEBPE* mutation causes noncanonical autoinflammatory inflammasomopathy

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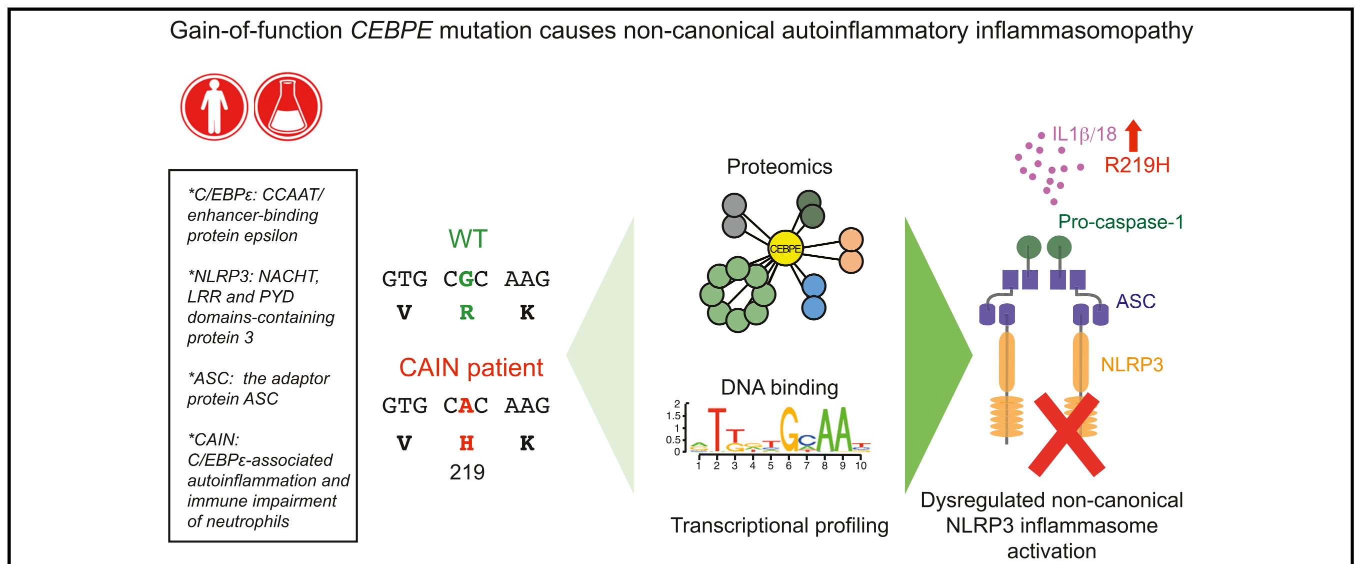
GRAPHICAL ABSTRACT



Background: CCAAT enhancer–binding protein epsilon (C/EBPε) is a transcription factor involved in late myeloid lineage differentiation and cellular function. The only previously known disorder linked to C/EBPε is autosomal recessive neutrophil-specific granule deficiency leading to severely impaired neutrophil function and early mortality. Objective: The aim of this study was to molecularly characterize the effects of C/EBPε transcription factor

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Arg219His mutation identified in a Finnish family with previously genetically uncharacterized autoinflammatory and immunodeficiency syndrome.

Methods: Genetic analysis, proteomics, genome-wide transcriptional profiling by means of RNA-sequencing, chromatin immunoprecipitation (ChIP) sequencing, and assessment of the inflammasome function of primary macrophages were performed.

Results: Studies revealed a novel mechanism of genome-wide gain-of-function that dysregulated transcription of 464 genes. Mechanisms involved dysregulated noncanonical inflammasome activation caused by decreased association with transcriptional repressors, leading to increased chromatin occupancy and considerable changes in transcriptional activity, including increased expression of NLR family, pyrin domain-containing 3 protein *(NLRP3)* and constitutively expressed caspase-5 in macrophages.

Conclusion: We describe a novel autoinflammatory disease with defective neutrophil function caused by a homozygous Arg219His mutation in the transcription factor C/EBPε. Mutated C/EBPε acts as a regulator of both the inflammasome and interferome, and the Arg219His mutation causes the first human monogenic neomorphic and noncanonical inflammasomopathy/immunodeficiency. The mechanism, including widely dysregulated transcription, is likely not unique for C/EBPε. Similar multiomics approaches should also be used in studying other transcription factor–associated diseases. (J Allergy Clin Immunol 2019;144:1364-76.)

Key words: *Immunologic deficiency syndromes, autoinflammatory diseases, hereditary, chemotaxis, interferons, inflammasomes, NLR family, pyrin domain-containing 3 protein, gain-of-function mutation, neomorphic mutation*

Abbreviations used

AID:	Autoinflammatory disease
CAIN:	C/EBPε-associated autoinflammation and immune impairment of neutrophils
C/EBP:	CCAAT enhancer–binding protein
ChIP:	Chromatin immunoprecipitation
ChIP-seq:	ChIP, Chromatin immunoprecipitation sequencing
FC:	Fold change
FDR:	False discovery rate
GOF:	Gain of function
JAK:	Janus kinase
LOF:	Loss of function
NF-κB:	Nuclear factor κB
NLRP3:	NLR family, pyrin domain-containing 3 protein
PID:	Primary immunodeficiency
PPI:	Protein-protein interactions
RNA-seq:	RNA sequencing
SGD:	Neutrophil-specific granule deficiency
SMRC2:	SWI/SNF complex subunit SMARCC2
STAT:	Signal transducer and activator of transcription
WT:	Wild-type

Primary immunodeficiencies (PIDs) are caused by inherent defects in the immune system and offer a unique glimpse into regulation of the human immune system. Findings in patients with PIDs can help in development of treatments for immune dysregulation, which is known to lead to various common diseases, including atherosclerosis, asthma, and diabetes.[1] Of the more than 350 currently known PIDs, approximately 20 are caused by germline autosomal dominant gain-of-function (GOF) mutations.[2-4] In general, GOF mutations can be caused by hyperactivating or neomorphic mechanisms. To date, all known GOF PIDs have been hypermorphic (ie, resulting in increased protein activity).[4] No neomorphic mutations resulting in completely novel molecular functions have been described in patients with PIDs.

Many autoinflammatory diseases (AIDs), such as cryopyrinopathies, are driven by activation of the canonical NLR family, pyrin domain-containing 3 protein (NLRP3) inflammasome through production of the highly proinflammatory IL-1β and IL-18. The noncanonical caspase-4/5 (murine ortholog, caspase-11) inflammasome is a recently discovered inflammasome activated by intracellular bacterial LPS and involved in first-line defense against gram-negative bacteria. Similar to canonical activation, noncanonical inflammasome activation results in increased IL-1β and IL-18 production.[5] In mice induction of caspase-11 expression by type I interferon signaling is required for activation of the noncanonical inflammasome.[6-9] In human subjects local activation of the noncanonical inflammasome has been shown to contribute to the pathogenesis of age-related macular degeneration.[10] However, the potential role of the noncanonical inflammasome in systemic human diseases remains to be explored.

The CCAAT enhancer–binding protein epsilon (C/EBPε), encoded by *CEBPE*, is a transcription factor expressed in myeloid and lymphoid lineage cells and is known to be involved in cellular differentiation and function of late myeloid lineages.[11] The only

This study was supported by the Orion Research Foundation (to H.G.); the Paulo Foundation ja Maire Lisko Foundation (to K.R.); Helsinki University Hospital Research funds (to A.R. and M.R.J.S.), an Academy of Finland Post-doctoral Fellowship (274555, to B.S.); the Päivikki and Sakari Sohlberg Foundation and the Yrjö Jahnsson Foundation (to K.N.); the Division of Intramural Research, National Institute of Health, National Institutes of Health, Bethesda, Maryland (to L.D.N.); the Folkhälsan Research Foundation and the Novo Nordisk (to M.L.); the Academy of Finland (288475 and 294173, to M.V.); the Sigrid Jusélius Foundation (to M.V.); the Finnish Foundation for Pediatric Research (to M.R.J.S.); a University of Helsinki Three-year Research Grant, Biocentrum Helsinki, Biocentrum Finland, and HiLIFE (to M.V.); the Instrumentarium Research Foundation (to M.V. and K.K.E.); and Finska Läkaresällskapet (to K.K.E. and D.N.).

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

Received for publication November 2, 2018; revised May 6, 2019; accepted for publication June 4, 2019.

Available online June 13, 2019.

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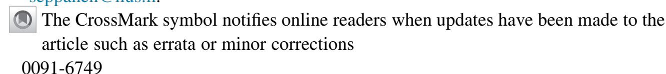
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https://doi.org/10.1016/j.jaci.2019.06.003

previously known *CEBPE*-associated disorder, autosomal recessive neutrophil-specific granule deficiency (SGD), is caused by frameshift mutations resulting in abrogation of at least 2 of the 4 known C/EBPε isoforms. This leads to a complete loss of specific neutrophilic granules and consequently to severely impaired neutrophil function with pronounced susceptibility to bacterial infections and early mortality.[12,13] A milder autosomal dominant *CEBPE* (Val218Ala) variant leads to total SGD with recurrent deep-seated abscesses.[14] Early studies in *Cebpe* knockout mice showed *Cebpe* to be integral for maintenance of constitutive levels of several cytokines, including IFN-γ.[15]

We report an autosomal recessive GOF PID, C/EBPε-associated autoinflammation and immune impairment of neutrophils (CAIN), in a family with a genetically uncharacterized autoinflammatory syndrome.[16-18] According to our results, C/EBPε acts as a regulator of both the inflammasome and interferome. Homozygous missense mutations in *CEBPE* (14:23586886 C->T;p.Arg219His) resulted in markedly decreased C/EBPε association with transcriptional repressors and increased occupancy on chromatin, leading to dysregulated C/EBPε-mediated transcription of interleukin and interferon response genes in neutrophils. Patients' macrophages displayed aberrant caspase-5–mediated activation of the noncanonical inflammasome pathway. Patients with CAIN display a loss-of-function (LOF) mechanism in protein-protein interactions (PPIs) and a novel GOF mechanism in DNA binding and transcriptional regulation. Furthermore, CAIN is the first PID and systemic AID involving a noncanonical inflammasome.

METHODS

Written informed consent was obtained from study participants, and the study was approved by the institutional ethical review board (138/13/03/00/2013).

DNA from 3 affected family members (Fig 1, *A*) were analyzed by using whole-exome sequencing, and Sanger sequencing was further used to confirm the mutation status of the other family members. Before whole-exome sequencing was available, patients were tested against *LBR*, *MVK*, and *NLRP3* mutations; no rare cosegregated variants in known AID genes were noted. Further details of the genetic analysis are available in the Methods section in this article's Online Repository at www.jacionline.org.

Functional studies of *CEBPE* mutation

Flp-In T-REx 293 cell lines stably expressing mutant or wild-type (WT) C/EBPε were generated and used to investigate PPIs by using proximity-dependent biotin identification coupled to mass spectrometry, as described by Liu et al[19] and in the Methods section in this article's Online Repository.

Functional studies of patients' primary cells

Granulocytes were isolated from 2 homozygous patients, 2 heterozygous carriers, and 3 sex- and age-matched control subjects, and chromatin immunoprecipitation sequencing (ChIP-seq) was used to assess C/EBPε binding to chromatin in freshly isolated and LPS-stimulated cells.

RNA sequencing (RNA-seq) was used for genome-wide transcriptional profiling of freshly isolated untreated granulocytes from patients and control subjects. Given the clinical phenotype of increased bacterial infections, we also used 5′-end RNA-seq to identify genes differentially expressed after 3 different stimulations: (1) DNA extracted from *Pseudomonas aeruginosa*, (2) LPS extracted from *P aeruginosa*, or (3) LPS extracted from *Escherichia coli*. Moreover, granulocytes were stimulated with IFN-α2 and IFN-γ, followed by transcriptional profile analysis using 5′-end RNA-seq.

Nanostring[20] analysis was used for direct digital detection of the mRNA levels of selected genes from PBMCs.

Caspase-5 levels were analyzed by using quantitative PCR and Western blotting to access inflammasome activation and inflammasome-mediated cytokine secretion more deeply. Caspase-1 activity was measured by using flow cytometry from blood immune cells, and IL-1β/IL-18 secretion was measured by using ELISA from cultured macrophages.

We also assessed granule exocytosis, granulocyte responsiveness, and neutrophilic and monocytic nuclear factor κB (NF-κB) phosphorylation using flow cytometry. Subcellular morphology of granulocytes and granule abundance of platelets were assessed by using transmission electron microscopy, as well as Wright staining of granulocytes. Further details of the methods are available in the Method section in this article's Online Repository.

RESULTS

Case reports

In the 1970s, affected members of the index family were thought to have atypical Pelger-Huët anomaly because they presented with neutrophil hyposegmentation, aberrant neutrophil responsiveness, and impaired chemotaxis.[16-18] Patients experienced recurrent attacks of abdominal pain, aseptic fever, and systemic inflammation lasting 4 to 5 days. These were accompanied by an acute-phase response and occasionally by nailbed, tongue, submandibular and gluteal abscesses; intra-abdominal granulomas; pyoderma gangrenosum; and buccal ulcerations. Furthermore, they experienced frequent episodes of purulent paronychia complicated by lymphangitis, superficial skin, and mucosal and purulent upper respiratory tract infections. Their autoinflammatory symptoms manifested more clearly during puberty and subsided after menopause (Table I). All affected members further had mild bleeding diathesis with frequent nosebleeds and a tendency toward hematomas after needle sticks and procedures. Extended case reports are available in this article's Online Repository at www.jacionline.org.

Genetic analysis found a novel homozygous *CEBPE* mutation

To identify the causative mutation, we analyzed DNA from 3 surviving members (II.2, II.7, and II.13) of the index family by performing whole-exome sequencing. In exome data we identified between 20,638 and 21,313 single nucleotide variants and small insertions/deletions in each patient. A total of 149 variants were very rare (ie, those not seen in the 6500 National Heart Lung, and Blood Institute Exomes 1000 Genomes database [April 2012 data release] and 2500 exomes analyzed internally by using the same bioinformatics pipeline). Of these, 22 variants were shared between all 3 patients. Only one of these variants was homozygous in all 3 patients (ENSG00000092067:ENST00000206513:exon2:c.G656A;p.Arg219His) in the *CEPBE* gene. The novel homozygous *CEBPE* 14:23586886 C>T mutation cosegregated perfectly with the disease phenotype (Fig 1, *A*, and see Fig E1 in this article's Online Repository at www.jacionline.org).[16-18] Arg219His was predicted to be detrimental and not listed in major public or in-house databases. It resided in the highly conserved basic zipper region's carboxyl terminal DNA-binding domain shared by all 4 C/EBPε isoforms (Fig 1, *B* and *C*).[11,21,22] Identified homozygous or heterozygous novel germline mutations were validated by using Sanger sequencing (Fig 1, *A*).

Widely altered C/EBPε PPIs

To understand the functional effects of the p.Arg219His mutation, we assayed PPIs of WT and mutant C/EBPε using

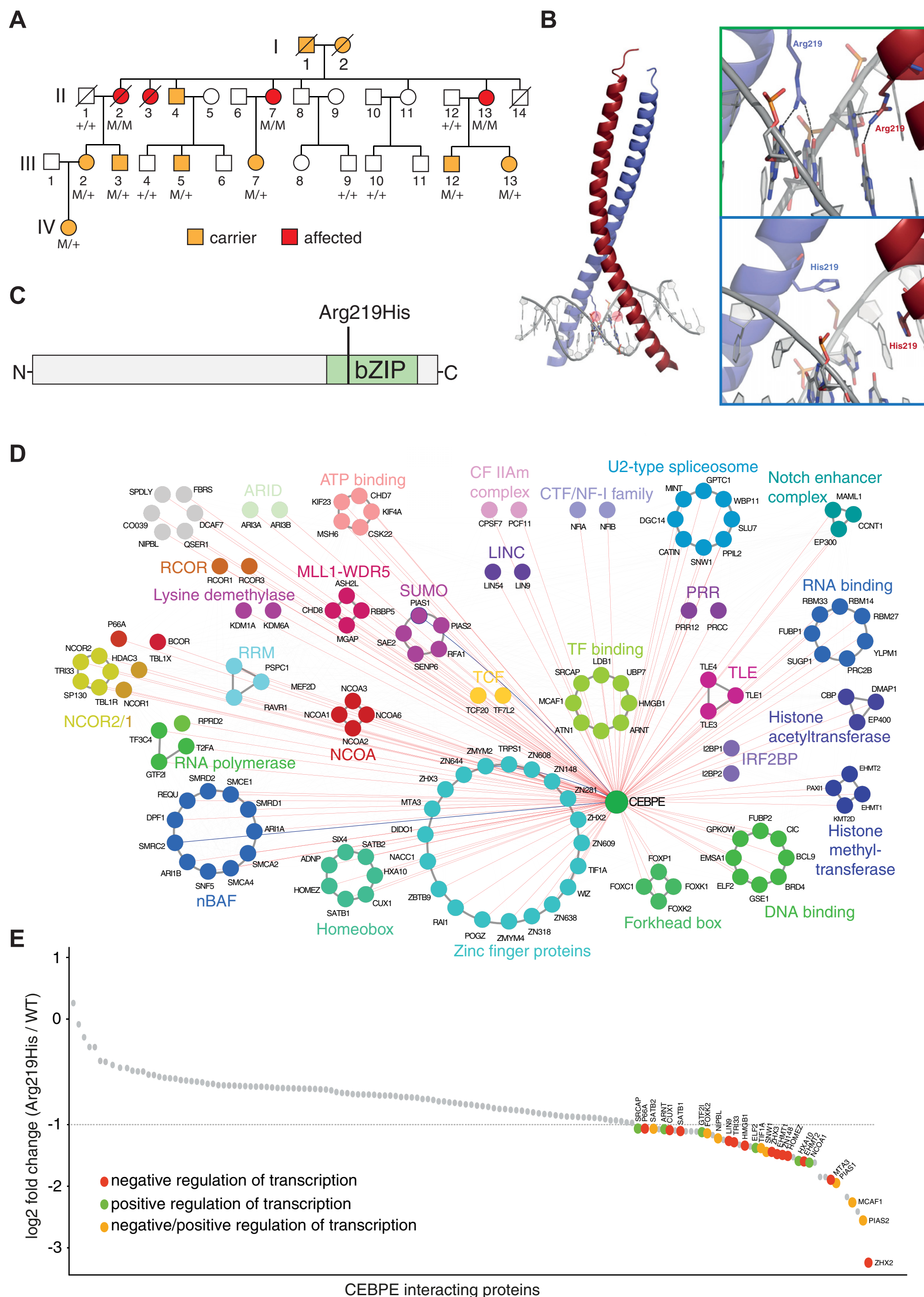

FIG 1. Pedigree of the index family and changes in PPIs. **A,** Pedigree of the index family with the C/EBPε Arg219His mutation. Homozygous subjects are shown in red, heterozygous carriers are shown in orange, and deceased subjects are indicated by *diagonal bars*. **B,** Three-dimensional structure of a C/EBPε dimer (*blue* and *red helixes*) bound to a DNA fragment. The *top right panel* shows the WT Arg219-DNA interaction, and the *right bottom panel* shows the mutated 219His interaction. **C,** Schematic illustration showing the Arg219His mutation within the basic zipper *(bZIP)* region of the DNA-binding domain of C/EBPε. **D,** C/EBPε PPIs detected by using proximity-dependent biotin identification coupled to mass spectrometry. Interacting proteins were classified by using the CORUM and UniProt databases, and the complex and/or functional group membership are depicted by different colors. Novel interactions are shown with red edges, and the 2 previously known C/EBPε interactions are shown in blue. **E,** The Arg219His mutation in C/EBPε causes decreased associations with transcriptional repressors. Log_2 FCs of PPIs (mean, n = 4) between Arg219His and WT C/EBPε are shown. Color coding highlights the transcription regulation action (UniProt) of proteins with more than 2-fold decrease in interaction (log_2 FC < −1).

TABLE I. Clinical characteristic of patients

Patient	II.2	II.7	II.13
Age at onset of symptoms	17 y	17 y	Youth
Current age	Deceased at age 78 y	74 y	69 y
Main symptoms	Abdominal pain, high fever Crater-like ulcers of buccal mucosa occasionally during periodic fever	Abdominal pain, high fever	Abdominal pain, high fever Crater-like ulcers of buccal mucosa occasionally during periodic fever
Duration of attacks (average)	4-5 d	4-5 d	4-5 d
Frequency of attacks (average)	Every 2-4 wk, later more seldom	Every 2-4 wk, later more seldom	Every 2-4 wk, later more seldom
Other symptoms during attacks	Lymphangitis Vomiting Myalgia Pleurisy Arthralgia Ileitis diagnosed by laparotomy	Lymphangitis Pleurisy Episcleritis Scleritis Aphthous colitis	Lymphangitis Vomiting Myalgia
ESR (mm/h) at attacks	70-80	>50	70-80
Other major clinical events	Myocardial infarction at age 46 y	Operated on for ileal leiomyosarcoma at age 35 y Pyoderma gangrenosum at age 43 y	Mesenterial lymph node biopsy at laparotomy at age 55 y showed granulomatous inflammation Recurrent respiratory tract infections since age 42 y Laparotomy at age 55 y showed ileitis and mesenterial lymph node biopsy granulomatous inflammation
Infections	Severe recurrent tongue abscesses when very young Easily acquired purulent wounds with delayed healing Paronychia Gluteal and submandibular abscesses		
Bleeding diathesis	Moderately severe nose bleeds, as well as need for prolonged compression after needle sticks Postoperative hematomas		

In addition, patient II.3 was likely a carrier with similar systemic symptoms, periodic fever, and skin and mucosal sequelae. At age 18 years, she acquired rheumatic fever, causing mitral and aortic valve insufficiency and cardiac arrhythmias. At age 23 years, she had subacute endocarditis caused by *Streptococcus viridans* and died at age 34 years of ventricular fibrillation. Postmortem autopsy showed "substantial" numbers of calcified mesenteric lymph nodes with "nonspecific inflammation."
ESR, Erythrocyte sedimentation rate.

proximity-dependent biotin identification coupled to mass spectrometry (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). This identified 144 C/EBPε interaction partners, 141 of which were previously not reported (Fig 1, *D*, and see Table E1 in this article's Online Repository at www.jacionline.org). Based on quantitative interaction analysis, 108 PPIs were significantly ($P < .05$) altered; 106 showed decreased and 2 showed increased interaction with mutant compared with WT C/EBPε (Fig 1, *E*, and see Table E1). Importantly, many of the diminished interactors were transcriptional repressors, suggesting widely dysregulated C/EBPε-driven transcription (Fig 1, *E*, and see Table E1). Twenty-four of the novel interactions were also seen in Jurkat T cells (see Table E1). We observed similar loss of transcriptional repressors when analyzing the mouse Arg219His C/EBPε mutant, suggesting a high degree of conservation of C/EBPε functions and effects of the mutation (Fig E2, *B*).[15]

One of the known interactions was C/EBPε interaction to SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin remodeling complex subunit SMARCC2 (SMRC2), which is an important regulator of myeloid differentiation.[23] This affinity was reduced in mutant compared with WT SMRC2 ($\log_2$ Mut/WT = -0.41). In addition to SMRC2, we found C/EBPε to interact with 4 other SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily members (SNF5, SMRD1, SMRD2, and SMCE1), all of which were downregulated in patients compared with control subjects (see Table E1).

To see how specific the changes in PPIs were for the Arg219His-mutated C/EBPε, we generated a cell line expressing Val218Ala C/EBPε, which is known to cause SGD,[14,24] and compared the interaction changes between these 2 mutants (see Fig E2, *C*, and Table E1). Most of the studied interactions did not differ between Val218Ala and WT C/EBPε.

Increased chromatin occupancy of Arg219His C/EBPε

Mapping of the p.Arg219His mutation to the DNA-binding domain of C/EBPε prompted us to profile the chromatin occupancy of C/EBPε. We performed ChIP-seq from granulocytes without and with LPS stimulation (Fig 2 and see Table E2 in this article's Online Repository at www.jacionline.org). Peak calling and overlap analysis from biological replicates revealed 3391 C/EBPε-binding sites in control subjects, 4686 in Arg219His heterozygote carriers, and 10322 in homozygous patients (Fig 2, *A*). Similar results were observed on LPS stimulation (Fig 2, *B*). Increased occupancy was also evident in

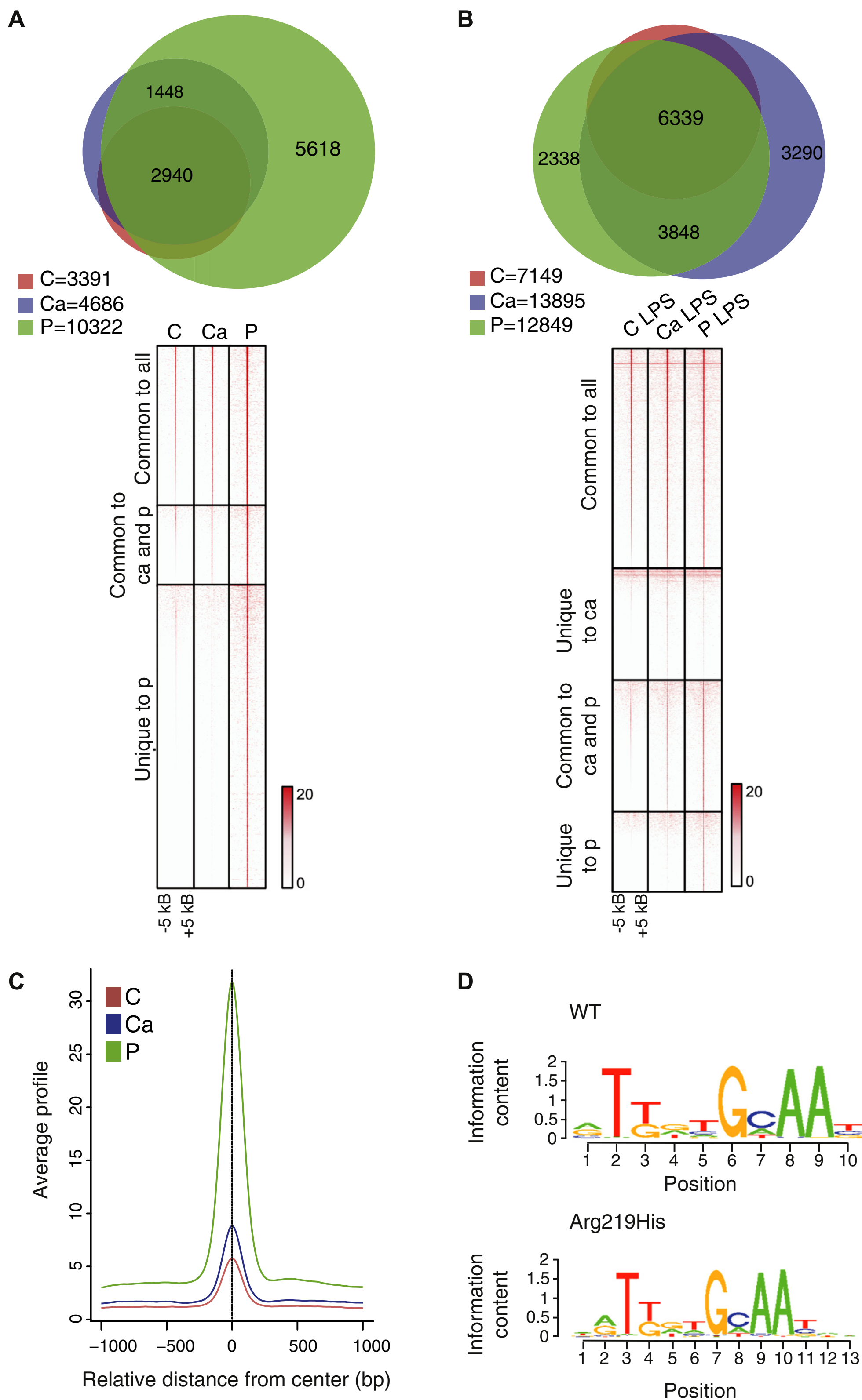

FIG 2. ChIP-seq analysis of C/EBPε DNA binding. **A** and **B,** Area-proportional Venn diagrams of C/EBPε ChIP-seq binding sites and tag density maps of C/EBPε-binding events flanking ± 5 kb in the absence (Fig 2, *A*) and presence (Fig 2, *B*) of LPS treatment. **C,** Average C/EBPε ChIP-seq signal profiles. **D,** Binding motif, as determined by using ChIP-seq. No significant changes were seen in the binding site of the Arg219His mutant. All ChIP-seq experiments were carried out in freshly isolated human granulocytes. *C*, Control subjects; *Ca*, heterozygous carriers; *P*, homozygous patients.

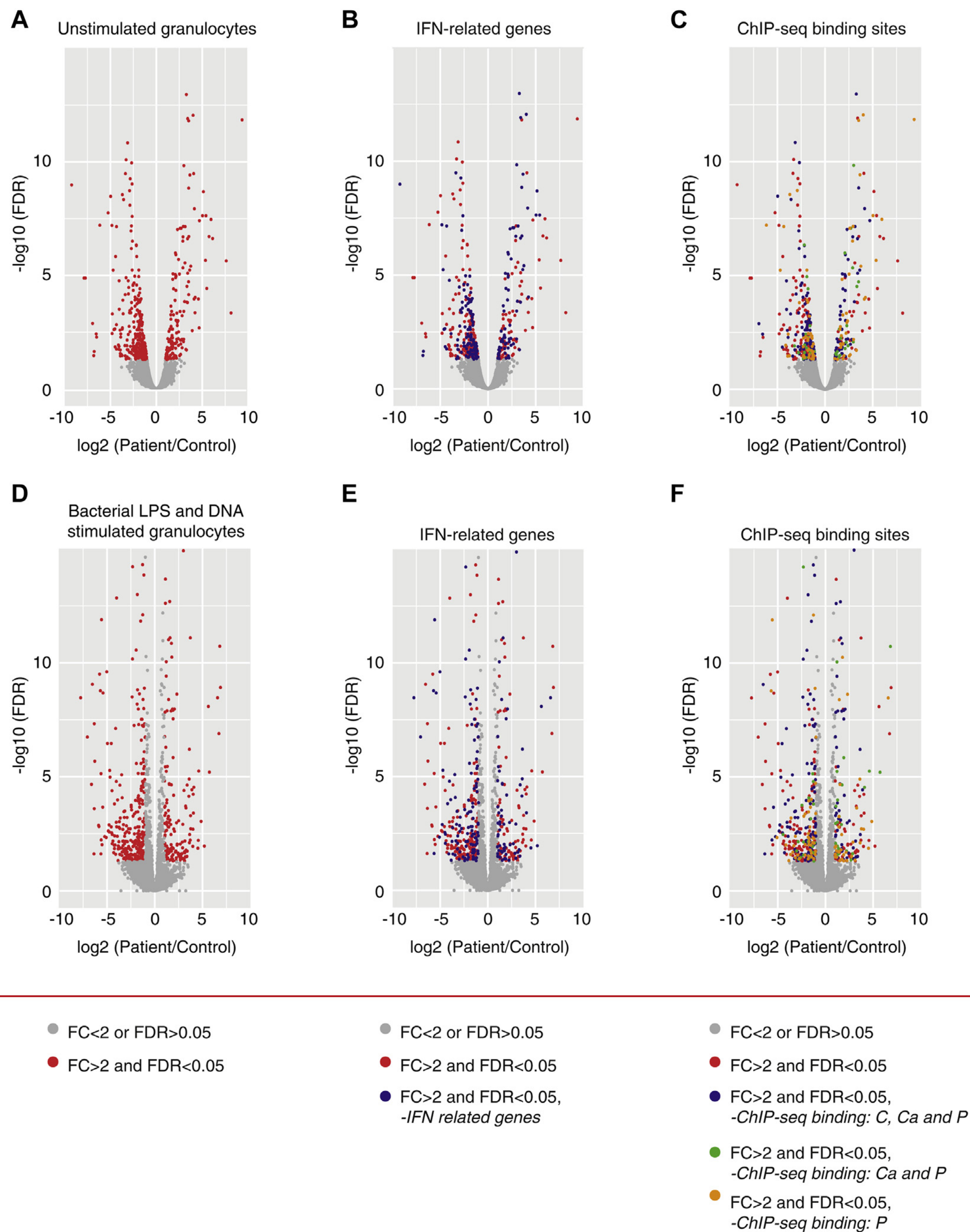

FIG 3. Transcriptomic analysis of unstimulated **(A-C)** and bacterial LPS- and DNA-stimulated **(D-F)** granulocytes using RNA-seq. Fig 3, *A*, RNA-seq revealed 464 differentially transcribed genes (FC > 2 and FDR < 0.05) between the patients and control subjects in unstimulated granulocytes. Of these, 198 (Fig 3, *C*) were identified to be interferon related by using Interferome (version 2.01, www.interferome.org), and 271 (Fig 3, *D*) had C/EBPε-binding sites (mapping to nearby genes within ±50 kb). Importantly, 80 of these 271 genes were associated with patient-specific C/EBPε binding. Fig 3, *D*, Similarly, 470 genes were differentially transcribed in bacterial LPS- and DNA- stimulated granulocytes. Fig 3, *E* and *F*, Of these, 183 (Fig 3, *E*) were identified to be interferon related, and 289 (Fig 3, *F*) had C/EBPε binding-sites. Eighty-one genes had patient-specific C/EBPε binding. Volcano plots represent $\log_2$ FCs and $-\log_{10}$ FDRs of transcripts between the patients and control subjects. Different groups are color coded. *C*, Control subjects; *Ca*, heterozygous carriers; *P*, homozygous patients.

average ChIP-seq signal intensities (Fig 2, *C*). Interestingly, *de novo* motif analyses identified highly similar consensus DNA-binding sequences in WT and mutant C/EBPε (Fig 2, *D*). This suggests that the increased C/EBPε chromatin occupancy was caused by mechanisms other than the altered DNA-binding motifs usually seen in neomorphisms.[25]

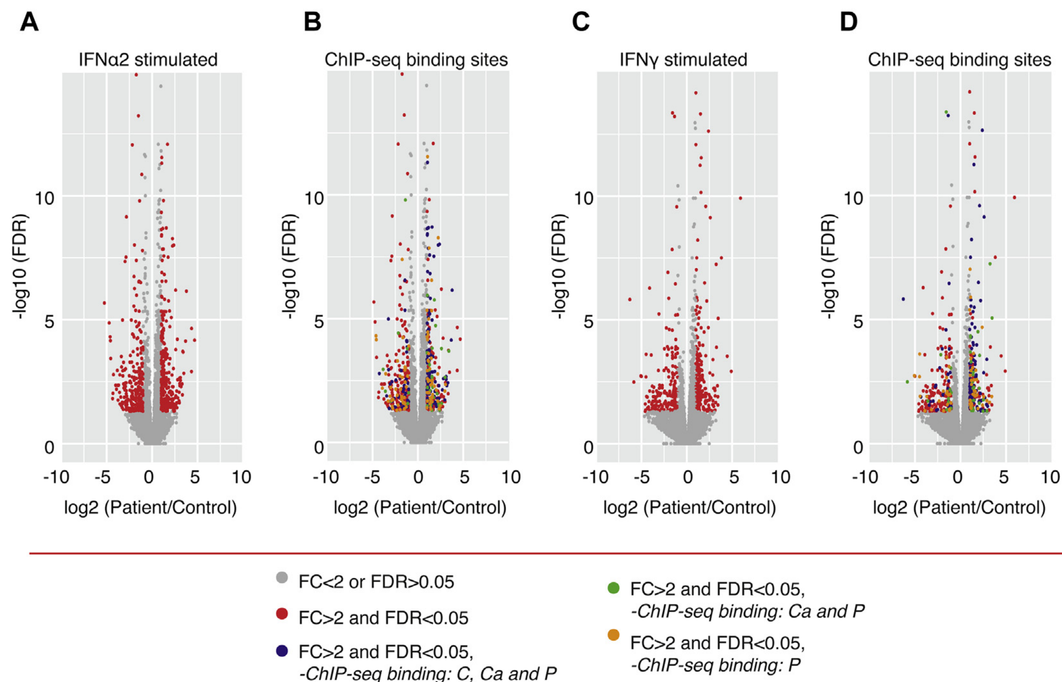

FIG 4. Transcriptomic analysis of IFN-α2–stimulated (**A** and **B**) and IFN-γ–stimulated (**C** and **D**) granulocytes by using RNA-seq. Fig 4, *A*, RNA-seq revealed 534 differentially transcribed genes (FC > 2 and FDR < 0.05) between patients and control subjects after IFN-α2 stimulation. Fig 4, *B*, Of these, 266 had C/EBPε-binding sites (mapping to nearby genes within ±50 kb), and importantly, 83 of these were associated with patient-specific C/EBPε binding. Fig 4, *C*, Similarly, 427 genes were differentially transcribed in IFN-γ–stimulated granulocytes. Fig 4, *D*, Of these, 208 had C/EBPε-binding sites, with 54 being associated with patient-specific C/EBPε binding. Volcano plots represent $\log_2$ FCs and $-\log_{10}$ FDRs of transcripts between patients and control subjects. Different groups are color coded. *C*, Control subjects; *Ca*, heterozygous carriers; *P*, homozygous patients.

Pronounced transcriptional changes in mutated unstimulated granulocytes

Increased mutant C/EBPε binding to DNA and decreased association with transcription repressors can lead to dysregulated transcriptome. Thus we compared transcriptomes of patients with CAIN and control subjects in unstimulated granulocytes using RNA-seq (Fig 3, *A*). This identified 464 significantly differentially transcribed (fold change [FC] ≥ 2 or ≤ 0.5, false discovery rate [FDR] ≤ 0.05) genes, 198 of which, including *NLRP3* (FC = 8.25), were interferon related (Fig 3, *B*, and see Table E3 in this article's Online Repository at www.jacionline.org).

Furthermore, Gene Ontology analysis revealed upregulation of genes involved in inflammatory responses, transcription, chemotaxis, and LPS response (see Table E4 in this article's Online Repository at www.jacionline.org). One of the upregulated genes was *PRTN3*, which encodes the ubiquitous, especially in neutrophils, serine protease proteinase 3.[26] Activated neutrophils secrete PR3, which, among its other functions, cleaves structural proteins and activates the inflammasome-regulated cytokines IL-1β and IL-18.[26,27] Upregulation of *PRTN3* was verified by using RT-PCR (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). *CEBPE* was not among the differentially expressed genes in granulocytes; even slightly increased expression was detected by using RT-PCR in PBMCs (see Fig E3, *B*).

To investigate whether these differentially transcribed genes were under C/EBPε control, we mapped ChIP-seq peaks to nearby genes within ±50 kb range and performed overlap analysis. Of the 464 differentially expressed genes, 271 had C/EBPε-binding sites, and importantly, 80 genes had patient-specific binding (Fig 3, *C*, and see Table E3). We noted increased occupancy and novel C/EBPε-binding sites in patients for overexpressed inflammasome-interleukin–related genes (eg, *NLRP3* [see Fig E4, *A*, in this article's Online Repository at www.jacionline.org], *NFKBIA*, and *IL1R2*), suggesting aberrant inflammasome activation.

Comparison of results for differentially expressed genes with those of other studies

Recently, Serwas et al[24] performed a proteomics analysis of neutrophils from patients with SGD (C/EBPε mutation p.Val218Ala). They detected decreased expression of several granule proteins and increased expression of proteins linked to the nucleoskeleton and cytoskeleton, such as nesprin, vimentin, and lamin B2. Because our proteomic analysis was performed to identify changes on PPIs and not changes in the proteome, we compared the differentially expressed proteins from Serwas et al with our differentially expressed genes in RNA-seq experiments. In our data, opposite to proteins of patients with SGD, nesprin-2 *(SYNE2)* was detected with decreased expression in neutrophils from patients with CAIN (see Table E3). Vimentin was detected with a 2.2-fold increase in patients with CAIN, but because of an FDR of 0.09 it was filtered out from the differentially expressed genes (FDR cutoff = 0.05). Lamin B2 was not differentially expressed in RNA-seq analysis, but we found

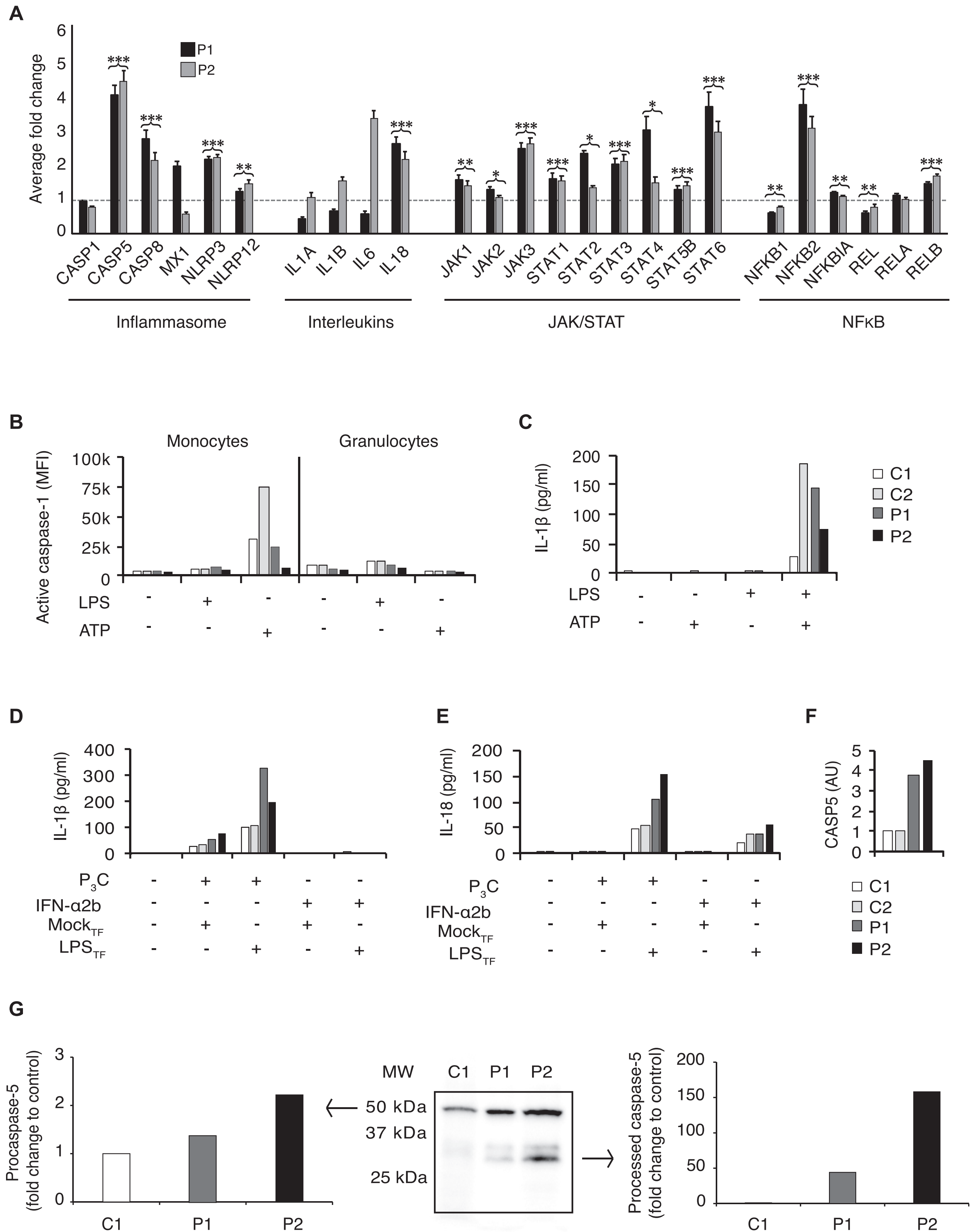

FIG 5. Transcriptomic analysis and characterization of changes in inflammasome activation. **A,** mRNA levels of PBMCs from patients and subjects analyzed by using Nanostring technology. Custom gene panel with selected inflammasome, interleukin, JAK/STAT, and NF-κB1 pathway–related genes were used in analysis, and average FCs of 3 technical replicates are presented. Statistically significant changes (Student *t* test)

Lamin G1 *(LAMC1)* to be upregulated in patients (logFC = 5.0, see Table E3).

Serwas et al[24] also identified lactotransferrin and neutrophil gelatinase-associated lipocalin to be downregulated in patients using both proteomics methods and quantitative PCR.[24] In RNA-seq experiments in patients with CAIN, lactotransferrin levels were also decreased (FC = 0.34), although with a high FDR value of 0.84. Similarly, LCN2 levels were slightly decreased but with a high FDR value.

Khanna-Gupta et al[14] detected increased expression of PU.1 and decreased expression of Gfi-1 in neutrophils from patients with SGD (C/EBPε mutation Val218Ala). We did not find Gfi-1 *(GFI)* and PU.1 *(SPI1)* to interact with C/EBPε in PPI analysis. However, PU.1 expression was detected in RNA-seq, but it did not show changes between patients with CAIN and healthy control subjects (data not shown). Gfi-1 expression was not found in RNA-seq analysis.

The clinical characters of patients with different C/EBPε mutations or SGD are compared in Table E5 in this article's Online Repository at www.jacionline.org.

Transcriptional changes after LPS and bacterial DNA stimulation

Given the clinical phenotype of frequent bacterial infections and neutrophilic skin symptoms (Table I), we stimulated patients' granulocytes with bacterial LPS and DNA. A total of 470 genes were found to be significantly differentially transcribed between patients and control subjects (FC ≥ 2 or ≤ 0.5, FDR ≤ 0.05; Fig 3, *D*, and see Table E6 in this article's Online Repository at www.jacionline.org). Of these, 183 were involved in interferon signaling (Fig 3, *E*, and see Table E6). In particular, gene transcription of the IFIT family (*IFIT1*, *IFIT3*, and *IFIT5)* was decreased, and that of *NLRP3* was increased in patients' cells.

Overlap analysis between ChIP-seq–mapped genes and RNA-seq results identified 289 of 470 differentially transcribed genes having C/EBPε-binding sites, 81 of them showing patient-specific binding (Fig 3, *F*, and see Table E6). Analogous to differentially transcribed genes in unstimulated granulocytes, various inflammasome-related genes, such as *IL18* and *NLRP3*, showed increased C/EBPε occupancy on chromatin in patients. Results again highlight dysregulated interleukin and inflammasome signaling in patients with CAIN.

Transcriptional changes after interferon stimulation

Dysregulated interferon signaling led us to test the direct effects of type I (IFN-α2b) and type II (IFN-γ) interferon stimulation on granulocytes. In response to IFN-α2b stimulation, 534 genes were differentially transcribed (FC ≥ 2 or ≤ 0.5, FDR ≤ 0.05) between patients and control subjects (Fig 4, *A*, and see Table E7, *A*, in this article's Online Repository at www.jacionline.org), with significant alterations in immune response and inflammasome-related gene expression. These included a significant 13.6-fold increase in *NLRP12* expression in patients. NLRP12, a known suppressor of neutrophil migration and chemotaxis,[28] also had increased C/EBPε occupancy on chromatin in patients (see Fig E4, *B*). Overall, 266 of 534 differentially transcribed genes had C/EBPε-binding sites; 83 genes had patient-specific binding (Fig 4, *B*, and see Table E7, *A*).

In response to IFN-γ stimulation, 427 genes were differentially expressed (FC ≥ 2 or ≤ 0.5, FDR ≤ 0.05) between the patients and control subjects (Fig 4, *C*, and see Table E7, *B*), with significant alterations in immune response and inflammasome-related genes. These included a modest 1.7-fold increase in NLRP3 expression under both conditions and a significant 3.8-fold increase in NLRP12 expression after IFN-γ stimulation. Comparison with ChIP-seq–mapped genes showed that 49% (208/427) of differentially expressed genes after IFN-γ treatment had C/EBPε-binding sites. Of these, 54 showed patient-specific binding, respectively (Fig 4, *D*).

Importantly, interferon-related genes, such as *NLRP3*, *TLR4*, *NLRP12*, *IL1RAP*, and *IL1R2*, showed increased transcription and C/EBPε chromatin binding in patients after IFN-α2b and IFN-γ stimulations. A similar effect was observed with *IL18*, *IL13RA1*, *IL17RA*, and *MEFV* after IFN-α2b stimulation.

mRNA levels in PBMCs

Next, we performed direct digital detection of mRNA molecules using Nanostring technology. This does not require conversion of mRNA to cDNA by using reverse transcription or amplification of the resulting cDNA by using PCR. Nanostring results showed increased mRNA levels of both Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway genes and inflammasome components (eg, *CASP5*, *NLRP3*, and *CASP8)*, as well as dysregulation of the NF-κB pathway (Fig 5, *A*). Interestingly, changes in mRNA levels were not concordant with autoinflammation caused by type I interferonopathy (see Table E8 in this article's Online Repository at www.jacionline.org). Importantly, inflammasome and inflammation-related genes, such as *CASP8* (Fig E5, *A*), *NLRP3* (Fig E4, *A*), *NLRP12* (Fig E4, *B*), *IL18*, *NFKB1*, and *STAT6*, were found to be significantly overexpressed with aberrantly greater C/EBPε chromatin binding in patients.

Moreover, Arg219His-mutated C/EBPε functions as an NF-κB2 regulator, with NF-κB2 binding only observed in patients' cells (Fig E5, *B*), resulting in a 3- to 4-fold increase in *NFKB2* transcription (Fig 5, *A*) in patients. Further studies are required to elucidate which role C/EBPε plays in *NFKB2* regulation. Our results suggest neomorphism through enhanced chromatin occupancy of mutant C/EBPε, leading to increased transcription, aberrant inflammasome activation, and dysregulated interleukin, NF-κB2, and interferon signaling.

of combined patients to control subjects are highlighted. *$P < .05$, **$P < .01$, and ***$P < .001$. **B,** Levels of active CASP1 were measured by using flow cytometry after canonical activation of the NLRP3 inflammasome with ATP in whole blood. **C-E,** Peripheral blood monocyte-derived macrophages were primed with the Toll-like receptor ligands LPS/P_3C or IFN-α2b, followed by canonical (Fig 5, *C*) or noncanonical (Fig 5, *D* and *E*) activation of the NLRP3 inflammasome with ATP or transfection of LPS to cytoplasm, respectively. IL-1β or IL-18 cytokine secretion was measured from culture supernatants by means of ELISA. **F,** Relative expression of *CASP5* mRNA in resting macrophages, as determined by means of quantitative PCR. **G,** Expression of pro-caspase-5 (48 kDa) and the processed intermediate form of caspase-5 (approximately 30 kDa) assessed from nonstimulated PBMC cell lysates by using Western blotting. Quantification of bands as FCs compared with control values are showed next to the blot. *AU*, Arbitrary units; *MFI*, median fluorescence intensity; *P_3C*, Pam_3Cys-SKKKK; *TF*, transfected.

NLRP3 inflammasome activation in monocytes and macrophages by a noncanonical inflammasome

Differences in transcription of inflammasome components led us to investigate functional changes in canonical and noncanonical inflammasomes. We detected no differences between patients and control subjects in whole-blood canonical NLRP3 activation or in inflammasome-triggered caspase-1 activity in monocytes (Fig 5, *B*). In cultured macrophages secretion of both IL-1β and constitutively expressed IL-18 was similar after canonical activation (Fig 5, *C*) but was markedly enhanced after noncanonical caspase-4/5–mediated NLRP3 activation in patients compared with control subjects (Fig 5, *D* and *E*). This was caused by aberrantly induced caspase-5 expression in resting macrophages of patients (Fig 5, *F*). In agreement with macrophage results, the increased protein expression of pro-caspase-5 was also detected from nonstimulated PBMCs from patients (Fig 5, *G*). Moreover, considerably more caspase-5 was processed in the patient's PBMCs, as shown by a clear intermediate band of caspase-5 of approximately 30 kDa (Fig 5, *G*). Aberrant activation of patients' macrophages required no priming by interferon response because priming with IFN-α2b instead of a Toll-like receptor agonist abolished any differences in IL-18 expression (Fig 5, *E*) and *CASP5* mRNA levels (see Fig E6, *A*, in this article's Online Repository at www.jacionline.org). Results further suggested increased expression of other interferon-regulated genes (see Fig E6, *B*).

Neutrophils displayed impaired CD66b expression

Patients were known to have impaired granulocyte function and chemotaxis, suggesting a hypomorphic LOF mutation.[16,18] We used flow cytometry to assay the expression of CD66a, CD66b, and CD11b involved in cellular adhesion and migration found within neutrophil granules and with a subsequent increase in surface expression after exocytosis.[29] CD66a and CD11b are found in secondary and tertiary granules, respectively. On flow cytometry, CD66a and CD11b expression was unaffected, which is consistent with a non-SGD disease (see Fig E7 in this article's Online Repository at www.jacionline.org). Additionally, patients' granulocytes displayed side-scattered light within the normal range, unlike in patients with SGD.[12,13]

CD66b is involved in neutrophil localization and activation and has been implicated in interaction of granulocytes with each other. Patients' granulocytes displayed impaired expression of CD66b compared with those from age- and sex-matched control subjects (see Fig E7). The aberrantly low CD66b expression likely contributes, with increased transcription of NLRP12, to the impaired chemotaxis previously reported.[16] Taken together, these data suggest a deficiency in specific granule function.

NF-κB plays a key role in inflammation and immune response.[30-32] Therefore we assessed NF-κB activity in whole blood. No differences were seen in NF-κB phosphorylation at baseline or in response to stimulation with bacterial LPS and DNA, suggesting that their periodic inflammation was not caused by dysregulated NF-κB activity.

Normal neutrophil and platelet morphology

To determine the possible structural changes in neutrophils, we analyzed endoplasmic reticulum size and shape and mitochondrial size by using transmission electron microscopy. No clear differences were seen between patients and control subjects. In addition, electron microscopy showed that patients' neutrophils contained normal-sized azurophilic specific and tertiary granules in normal numbers (data not shown).

Similar results were seen with staining using the Wright method.[33] From Wright-stained smears of both patients and control subjects, 200 consecutive leukocytes were studied for granules and morphology. Patients' neutrophils presented both primary and secondary granules, and no difference was observed in comparison with healthy control subjects (see Fig E8, *A*, in this article's Online Repository at www.jacionline.org). However, as seen earlier,[16] 20% of patients' neutrophils were hyposegmented (see Fig E8, *B*). Eosinophils and basophils also showed normal granules compared with those of healthy control subjects.

C/EBPε is known to control platelet granule formation.[15,21] Patients displayed mild bleeding diathesis, and there we investigated platelet morphology and platelet granule abundance in patients with CAIN. No morphologic aberrancies were found in α granule and overall platelets structure. Dilated open canalicular systems were detected in both patients. Patient II.7 had some multivesicular bodies, which are more common in megakaryocytes. Platelets were slightly activated based on the detected pseudopodia but mainly displayed a normal discoid form (data not shown).

Posttranscriptional compensatory mechanisms

Compared with RNA-seq and ChIP-seq results, patients are bafflingly mildly symptomatic but with mixed neomorphisms and hypomorphisms. Analysis of heterozygous relatives without reported clinical manifestations of disease showed intermediate cellular changes. Thus results suggest a novel autosomal recessive inheritance pattern in which the disease in the heterozygote state lacks expressivity because of unknown posttranscriptional compensatory mechanisms and requires the homozygous state to become clinically manifest. The reduced heritability of disease phenotype, even in the presence of the severely altered DNA binding seen in asymptomatic carriers, likely reflects the complex nature of compensatory mechanisms and underscores the astonishing extent to which the human body can maintain homeostasis, even in the presence of an otherwise pathogenic mutation.

To address this, we analyzed DNA methylation in patients (n = 2) compared with control subjects and found no changes that would explain the patients' symptoms or the relative mildness of them compared with RNA-seq and ChIP-seq analysis (data not shown).

Results from RNA-seq and ChIP-seq raise the possibility that histone methylation could be one compensatory posttranscriptional mechanism. This and open chromatin binding will need to be explored in knock-in animal models, allowing for larger numbers of mutation carriers.

DISCUSSION

We report a novel GOF mechanism caused by a transcription factor mutation. Homozygous Arg219His *CEBPE* mutation leads to CAIN, which was clinically characterized by a combination of autoinflammation, immunodeficiency, and neutrophil dysfunction. To our knowledge, this is the first mutation to cause such widely dysregulated transcription in patients with PIDs. It is also the first AID that involves aberrant expression and activation of noncanonical caspase-4/5 inflammasome. After Li-Fraumeni syndrome, CAIN seems to be only the second germline

neomorphic human disease caused by transcription factor mutations.[25] Our results highlight the potential for germline mutations in transcription factors to cause widespread and complex genome-wide mechanism, with only limited concomitant morbidity. Such changes will not readily be evident by using targeted functional assessment and typical nongenomic types of analyses, causing probable underdiagnosis. For example, transcription factor mutations with pronounced overlap between known clinical GOF and LOF phenotypes (eg, *STAT1*)[34] might need to be studied by using similar data-driven systems-level approaches.

The Arg219His mutation in the DNA-binding domain of C/EBPε was predicted to lead to loss of DNA binding and possible LOF. However, the mutation resulted in a pronounced increase in C/EBPε chromatin binding, likely caused by a decreased association with multiple transcriptional repressors. This decreased transcriptional repressor association could have been driven by C/EBPε Arg219His–induced conformational changes to protein structure because no difference was seen in the consensus DNA recognition motif between control subjects and patients. This mechanism has previously been described for a single (not orthologous) transcriptional repressor in patients with autosomal dominant SGD and in *Drosophila* species.[35]

Our results reveal the role of C/EBPε in complex interlinked signaling cascades. The reported deficiency in neutrophil chemotaxis[18] is likely caused by multiple factors, including differentially transcribed genes (ie, *CD66B*; see Fig E7) affecting cellular maturation and movement and increased expression of NLRP12, a known suppressor of neutrophil migration.[28]

Results also show that C/EBPε regulates interferon pathways and noncanonical inflammasome target genes (eg, *NLRP3*, *CASP5*, and *IL18*), further highlighting the overlap between the inflammasome and interferon signaling. Pathway components associated with noncanonical inflammasome activation were aberrantly transcribed, together with decreased NF-κB1 and increased NF-κB2 and JAK/STAT components (Fig 5, *A*).[36] Although caspase-4 is known to be constitutively expressed, human caspase-5 expression is interferon dependent.[8,9,37] Notably, we found increased baseline expression of caspase-5 in patients' macrophages and increased IL-1β and IL-18 secretion on stimulation of the noncanonical caspase-4/5 inflammasome with intracellular LPS. Because of constitutively expressed caspase-5, interferon priming was not required. Consequently, IFN-α2b priming before intracellular LPS transfection induced caspase-5 expression also in control cells and thus abolished the difference in cytokine secretion between patients and control subjects (Fig 5, *E*, and see Fig E6, *A*). Thus our data imply that the *CEBPE* Arg219His neomorphic mutation was alone sufficient to maintain constitutive caspase-5 expression. This sensitized the patients' macrophages to respond pronouncedly to intracellular LPS - clinically causing hyperinflammation after bacterial stimuli. Investigating both the inflammasome and interferon pathways together in patients with inflammatory conditions might lead to a deeper understanding of their cause and to targeted treatments.[38-40] In patients with CAIN, anti–IL-1β and anti–IL-18 seem likely treatment modalities but remain untested.

The index family has been studied since the 1970s.[16-18] A systems-level approach combining genomics, transcriptomics, and proteomics finally made it possible to unravel the causative pathogenic mechanisms. In conclusion, CAIN reveals GOF mechanisms resulting in autoinflammation and immunodeficiency, which are potentially relevant for various transcription factor–related diseases. Also, C/EBPε seems to be involved in regulation of the noncanonical inflammasome and interferon signaling, suggesting a novel target for drug development. The widely dysregulated transcription seen in asymptomatic heterozygous carriers compared with control subjects suggests extremely effective posttranscriptional regulatory capacity in human subjects that requires further investigation. Given their apparent roles in idiopathic inflammation, roles of both C/EBPε and the noncanonical inflammasome should be further explored in the context of autoimmune diseases and AIDs.

We thank Eira Leinonen, Auli Saarinen (Folkhälsan Institute, Helsinki, Finland), Sini Miettinen (HiLIFE, Institute of Biotechnology, University of Helsinki, Helsinki, Finland), Alli Tallqvist (Skin and Allergy Hospital, Helsinki University Hospital, Helsinki, Finland), Dr Tuomo Honkanen (Päijät-Häme Central Hospital, Lahti, Finland), Riitta Lassila (Coagulation Disorders Unit, Helsinki University Hospital and University of Helsinki, Helsinki, Finland), Leena Saikko (Department of Pathology, University of Helsinki, and HUSLAB, Helsinki University Hospital, Helsinki, Finland), and Fang Zhao (Advanced Microscopy Unit, Medicum, Faculty of Medicine, University of Helsinki, Helsinki, Finland). We also thank Sture Andersson, Petri Auvinen, Olli Ritvos, and Tiina Öhman for critical reading of the manuscript.

Key messages

- **Patients with biallelic *CEBPE* (14:23586886 C>T; p.Arg219His) missense mutations displayed a noncanonical inflammasome–mediated autoinflammatory and immunodeficiency disease, leading to an aberrantly activated noncanonical inflammasome.**
- **C/EBPε acts as a regulator of both the inflammasome and interferome.**
- **We describe a novel GOF mechanism caused by a missense transcription factor mutation, which leads to widely dysregulated transcription. This mechanism is most likely not unique for the transcription factor C/EBPε, and similar multiomics approaches should be applied in patients with other diseases associated with transcription factors.**

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