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RESEARCH ARTICLE

Characterization and biological significance of IL-23-induced neutrophil polarization

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Neutrophils are heterogeneous with distinct subsets, and can switch phenotypes to exert regulatory functions on immunity. We herein demonstrate that IL-23-treated neutrophils selectively produce IL-17A, IL-17F and IL-22, and display a distinct gene expression profile in contrast to resting and lipopolysaccharide-treated neutrophils. IL-17$^+$ neutrophils are present in the colons of mice with dextran sulfate sodium-induced colitis. Adoptive transfer of IL-23-treated neutrophils significantly promotes pathogenesis in this model. IL-23 induces neutrophil polarization through STAT3-dependent RORγt and BATF pathways. Thus, IL-23-induced neutrophil polarization expresses a unique cytokine-producing profile, which may contribute to IL-23-mediated inflammatory diseases.
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INTRODUCTION

Polymorphonuclear neutrophil granulocytes (also called neutrophils) are fully equipped with a variety of granules that are essential for host defense against intruding microorganisms and play a critical role in initiating inflammation and innate immunity.[1–4] However, neutrophils also significantly contribute to various types of acute and chronic pathological damage during infections, autoimmune diseases and graft rejections in either a positive or a negative manner.[5–8] In fact, in addition to their direct cytotoxicity against invading pathogens, neutrophils can switch phenotypes and exert regulatory functions in both innate and adaptive immunity.[9–12] Neutrophils from rheumatoid arthritis patients upregulate MHC-II expression and increase their antigen-presenting ability, leading to T lymphocyte activation.[13] New evidence has also shown that in certain situations neutrophils can be activated to play a role as antigen-presenting cells.[14] Recent studies have indicated that neutrophils exhibit considerable functional plasticity and polarization. Similar to macrophage activation pathways, neutrophils have distinctive subsets, as presented in infections and tumors.[15–17] A subpopulation of neutrophils present in the spleen produces CD40L, BAFF, APRIL and interleukin-21 (IL-21) and has the ability to activate marginal-zone B cells, and subsequently promotes immunoglobulin class-switching, somatic hypermutation and antibody production.[18] Tumor-associated neutrophils have been proposed to be polarized between antitumorigenic and protumorigenic neutrophil phenotypes.[19] However, the extracellular inducing factors, the intracellular molecular regulatory networks, the phenotype characteristics and the biological significance of

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the polarized neutrophils are unclear and require further determination.

IL-23 is crucial in the pathogenesis of psoriasis, experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease (IBD) and collagen-induced arthritis (CIA).[20–22] Genetic polymorphisms in the IL-23 receptor (IL-23R) are important susceptibility factors for Bechet's disease, ankylosing spondylitis and IBDs such as Crohn's disease and ulcerative colitis.[23,24] It is known that IL-23 is essential for the terminal differentiation of IL-17-producing T effector cells.[25,26] However, IL-23R is predominantly found on natural killer cells, innate lymphoid cells (ILCs) and activated memory T cells, and at lower levels on dendritic cells (DCs), monocytes and macrophages in humans, whereas mouse IL-23R is expressed on activated T cells, ILCs, γδ T cells, macrophages and DCs.[27–29] Importantly, studies have demonstrated that IL-23 induces these innate cells to secrete IL-17 and/or IL-22.[30,31] In the present study, we demonstrate that IL-23-treated neutrophils selectively produce IL-17A, IL-17F and IL-22, and display a distinct lineage gene expression profile in sharp contrast to resting and lipopolysaccharide (LPS)-treated neutrophils. IL-17-expressing neutrophils are present in the intestines of mice with dextran sulfate sodium (DSS)-induced colitis. Moreover, adoptive transfer of IL-23-treated neutrophils significantly promotes the severity of the pathogenesis of DSS-induced colitis in WT or IL-17 knockout (KO) recipient animals. IL-23 induces neutrophil polarization through STAT3-dependent BATF and RORγT pathways. Thus, our present studies reveal neutrophil polarization with a unique cytokine-producing gene profile driven by IL-23. This IL-23-induced neutrophil subpopulation may make significant contributions to IL-23-related pathologies.

MATERIALS AND METHODS

Mice

BALB/c and C57BL/6 (B6) mice were purchased from the Beijing University Experimental Animal Center (Beijing, China). IL-17 KO mice were kindly provided by the Key Laboratory of Human Diseases Comparative Medicine, the Ministry of Public Health; Institute of Laboratory Animal Science, CAMS & PUMC (Beijing, China).[32] All mice were bred and maintained in specific pathogen-free conditions. Six-to-twelve-week-old sex-matched littermate mice were mainly used for the experiments unless otherwise noted. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals, Institute of Zoology.

Reagents

Anti-mCD11b-PE-Cy5 was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-mLy6G-PE was purchased from eBioscience (San Diego, CA, USA). Anti-mIL-17A-PE was purchased from Biolegend (San Diego, CA, USA). Anti-mIL-23R-AF488 mAb was purchased from R&D Systems (Minneapolis, MN, USA). Bacterial lipopolysaccharide (*E. coli* 055:B5) was purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant mouse cytokines were purchased from PeproTech (Rocky Hill, NJ, USA). A selective STAT3 inhibitor (S3I-201) was purchased from Sigma-Aldrich. Primary antibodies against p-STAT3 (705), BATF were purchased from Cell Signaling Technology (Danvers, MA, USA). RORγT antibody was purchased from Millipore Biotechnology (Billerica, MA, USA). These antibodies were diluted at 1:1000 in 5% bovine serum albumin (BSA). Anti-β-actin mAb was purchased from Sigma-Aldrich and its working concentration (1:50 000) was determined in previous studies.[33,34]

Recombinant mouse cytokines were used at the following concentrations based on our previous studies:[35] recombinant mouse IL-1β (100 ng/ml, PeproTech), IL-2 (10 ng/ml, PeproTech), IL-4 (10 ng/ml, R&D Systems), IL-6 (50 ng/ml, PeproTech), IL-10 (20 ng/ml, PeproTech), IL-12 (5 ng/ml, PeproTech), IL-13 (50 ng/ml, PeproTech), IL-17A (100 ng/ml, PeproTech), IL-21 (50 ng/ml, R&D Systems), IL-23 (10 ng/ml, R&D Systems), IL-33 (100 ng/ml, R&D Systems), LPS (1 μg/ml, Sigma-Aldrich), IFN-γ (100 ng/ml, PeproTech), TNF-α (100 ng/ml, PeproTech), TGF-β1 (5 ng/ml, R&D Systems), GM-CSF (40 ng/ml, R&D Systems) and G-CSF (100 ng/ml, Biovision, Milpitas, CA, USA).

Isolation and differentiation of neutrophils *in vitro*

Tibiae and femora from BALB/c or B6 mice were removed using sterile techniques, and bone marrow was flushed with PBS. Red blood cells were lysed with ammonium chloride. Primary neutrophils ($CD11b^+Ly6G^+F4/80^-$) were isolated from bone marrow cells with 52% percoll (GE Healthcare Life Sciences, Piscataway, NJ, USA, 17-0891-01) and sorted by MoFlo XDP (Beckman Coulter) in several cases.[36] Primary neutrophils (2×10^6 cells per ml in 48-well plates) were cultured with IL-23 (50 ng/ml) for different time courses as indicated in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, 150 U/ml streptomycin, 200 U/ml penicillin and 5% heat-inactivated FBS at 37 °C and 5% CO_2. The cytokine expressions and phenotypes of neutrophils were characterized by real-time PCR, ELISA assays and flow cytometric analysis.

Microarray hybridization and data acquisition

The neutrophils were stimulated by LPS (1 μg/ml) and IL-23 (50 ng/ml). TRIzol Reagent (Life Technologies, Gaithersburg, MD, USA, 15596-018) was used to extract total RNA following the manufacturer's instructions. Total RNA was amplified and labeled with a Low Input Quick Amp Labeling Kit, One-Color (Agilent Technologies, 5190-2305), and further purified with an RNeasy mini kit (Cat. No. 74106, Qiagen, Valencia, CA, USA). Each slide was hybridized with 1.65 μg Cy3-labeled cRNA using a Gene Expression Hybridization Kit (Cat. No. 5190-2305, Agilent Technologies, Santa Clara, CA, USA, 5188–5242) in a Hybridization Oven (Agilent Technologies, G2545A), according to the manufacturer's instructions. After 17 h of hybridization, slides were washed in staining dishes (Cat. No. 121, Thermo Shandon, Watertown, MA, USA) with a Gene Expression Wash Buffer Kit (Agilent Technologies, 5188–

5327), following the manufacturer's instructions. Slides were scanned by an Agilent Microarray Scanner (Agilent Technologies, G2565CA) with default settings: dye channel—green, scan resolution—5 μm, PMT—100%, 10%, 16 bit. Data were extracted with Feature Extraction software 10.7 (Agilent Technologies). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent Technologies).

Microarray data analysis

Lineage-restricted genes were defined as the genes the expression levels of which were at least two-fold greater in one cell type than in any other cell type. R (version 3.0.2) was used to draw the heatmap of lineage-restricted genes. The fold-changes were calculated as the ratio of the expression level in each cell type versus the second-highest expression level in all cell types. Differentially expressed genes were defined as genes with at least two-fold variance of expression levels in LPS- and IL-23-treated neutrophils compared with untreated neutrophils.

Quantitative PCR analysis

The neutrophils were stimulated by LPS (1 μg/ml) and IL-23 (50 ng/ml) at different time points. Total RNA was isolated with TRIzol (Invitrogen) and reverse transcription was performed with M-MLV superscript reverse transcriptase as described previously. Real-time PCR was performed using multiple kits (SYBR Premix Ex Taq, DRR041A, Takara Bio) on CFX96 (Bio-Rad Laboratories, Hercules, CA, USA). The primers utilized are summarized in Table 1. The mRNA expression level of each gene was normalized to the housekeeping gene hypoxanthinephosphoribosyl transferase (HPRT), as reported previously.[33,37] For the inhibitor assay, cells were pretreated with various inhibitors for 30 min, and then treated with IL-23 for 12 h.

Flow cytometry

For flow cytometric analysis of surface markers, cells were stained with antibodies in PBS containing 0.1% (w/v) BSA and 0.1% NaN_3 as described previously.[38] For the detection of intracellular cytokines, neutrophils were treated with GolgiPlug (BD Biosciences, San Jose, CA, USA) for the last 4–6 h of incubation. Cells were fixed and permeabilized according to the manufacturer's protocol (BD Bioscience) and stained with anti-IL-17 mAb (1:100). Flow cytometry data were acquired on a FACSCalibur (BD Biosciences) or Epics XL (Beckman Coulter, Brea, CA, USA) and analyzed with FCS Express Version 3.0 software (*De Novo* Software) or FlowJo Version 7.6.5 software (TreeStar Inc., Ashland, OR, USA).

Western blotting

Neutrophils were cultured in RPMI 1640 medium with 5% FCS in 12-well plates. Cells were treated with IL-23 (50 ng/ml) or LPS (1 μg/ml) for the indicated times. After stimulation, cells were washed once in cold PBS, lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA pH 7.4) with protease and phosphatase inhibitor cocktails (Sigma) for 10 min on a rocker at 4 °C as described previously.[39] Protein concentration was determined using a BCA assay. Protein samples were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore, Ternicula, CA, USA). Each polyvinylidene fluoride membrane was blocked with TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween20) with 5% non-fat dried milk for 1 h, then incubated with primary antibodies overnight on a shaker at 4 °C. The appropriate HRP-conjugated secondary antibody was then added and was detected through a Tanon 1600R Gel Image System (Tanon Co., Ltd, Shanghai, China). Actin was used as a protein loading control.

ELISA assays

IL-17A (432508), IL-17F (436107) and IL-22 (436307) ELISA assay kits were purchased from Biolegend. The ELISA assay was performed following the manufacturer's instructions. Briefly, pre-coated ELISA plates were blocked with 5% BSA/PBS at room temperature for $\geqslant$1 h. After washing three times with PBS/Tween20, standards and samples were added to pre-coated ELISA plates and incubated at room temperature for 2–4 h. After washing, specific cytokine detection antibodies were added for 1 h at 30 °C followed by Avidin-HRP A for 1 h at 30 °C. Plates were developed using Substrate Solution E. The reaction was stopped with the Stop Solution and optical densities were determined at 450 nm using a plate reader.

Immunofluorescent staining

Cells were cultured on coverslips for the indicated times and fixed in 4% paraformaldehyde for 10 min.[35] Cells were permeabilized with 0.2% Triton X-100 (v/v) in PBS for 10 min at room temperature and blocked for 1 h in 5% BSA/PBS, and incubated overnight in indicated primary mAbs (1:100 dilution in blocking buffer) at 4 °C. Following PBS washes, secondary Ab (FITC- or PE-labeled goat anti-rabbit Ab, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 dilution) was applied for 1 h followed by Hoechst 33342 (2 μg/ml) staining for 10 min before the coverslips were washed in PBS and mounted. Photomicrographs were taken using an LSM510META Laser Scanning Microscope (Zeiss, Germany).

IBD model and histological examination

Recipient B6 mice were i.v.-injected with 5×10^6 sorted neutrophils stimulated with IL-23 for 12 h on days 0, 2, 5 and 8. Colitis was induced by the administration of dextran sulfate sodium (2.5%) in drinking water. The disease activity index (DAI)[40] and body weight of each animal were monitored daily. DAI was determined by scoring the loss of body weight, characteristics of the stool, and occult blood in stool or hematochezia (over the entire period), according to the classic scoring system by Cooper with modification. The scoring process is given as follows: body weight loss (0, none; 1, 1–5%; 2, 5–20%; 3, $>$20%), stool consistency (0, normal; 2, loose stool; 3, diarrhea) and stool blood (0, negative; 2, fecal occult blood test positive; 3, gross bleeding). The animals were

Table 1 Primers used for qRT-PCR analysis

Genes	*Primer sequence (5′–3′)*
HPRT	
Forward primer:	AGTACAGCCCCAAAATGGTTAAG
Reverse primer:	CTTAGGCTTTGTATTTGGCTTTTC
TNF-α	
Forward primer:	GAGTGACAAGCCTGTAGCC
Reverse primer:	CTCCTGGTATGAGATAGCAAA
CXCL4	
Forward primer:	AGAAAGCGATGGAGATCTTAGC
Reverse primer:	ACACAATTGACATTTAGGCAGC
IL-6	
Forward primer:	AACCGCTATGAAGTTCCTCTC
Reverse primer:	AATTAAGCCTCCGACTTGTGAA
CCL3	
Forward primer:	ACACCAGAAGGATACAAGCAG
Reverse primer:	CGATGAATTGGCGTGGAATC
iNOS	
Forward primer:	CACCAAGCTGAACTTGAGCG
Reverse primer:	CGTGGCTTTGGGCTCCTC
IL-17A	
Forward primer:	CTCAGACTACCTCAACCGTTCC
Reverse primer:	ATGTGGTGGTCCAGCTTTCC
IL-17F	
Forward primer:	CATACCCAGGAAGACATACTTAGAAG
Reverse primer:	AGTCCCAACATCAACAGTAGC
IL-22	
Forward primer:	CTGAGAAATGCTTGCGTCTG
Reverse primer:	CGTTAGCTTCTCACTTTCCTTTAG
CCL4	
Forward primer:	AAACCTAACCCCGAGCAAC
Reverse primer:	CTGTCTGCCTCTTTTGGTCA
CCL7	
Forward primer:	TCTCTCACTCTCTTTCTCCACC
Reverse primer:	GGTGATCCTTCTGTAGCTCTTG
CCL9	
Forward primer:	CCTTTTCATACTGCCCTCTCC
Reverse primer:	GTGAGTTATAGGACAGGCAGC
CXCL10	
Forward primer:	CGTCATTTTCTGCCTCATCC
Reverse primer:	GCAATGATCTCAACACGTGG
CXCL11	
Forward primer:	CAGGAAGGTCACAGCCATAG
Reverse primer:	GCACCTTTGTCGTTTATGAGC
CXCL12	
Forward primer:	TGCATCAGTGACGGTAAACCA
Reverse primer:	CACAGTTTGGAGTGTTGAGGAT
CXCL14	
Forward primer:	TGAAGCCAAAGTACCCACAC
Reverse primer:	TTCTTTCCATGATCGTCCACC

Table 1 (Continued)

Genes	*Primer sequence (5′–3′)*
CXCL16	
Forward primer:	AGAATTGGCTGGATGTCGG
Reverse primer:	GCTTTCAGGTATTTTCGGATGTG
CCL21a	
Forward primer:	GTGATGGAGGGGGTCAGGA
Reverse primer:	GGGATGGGACAGCCTAAACT
CCL21c	
Forward primer:	CAGTATTGTCCGAGGCTATAGG
Reverse primer:	CTTTCCAGACTTAGAGGTTCCC
CCL22	
Forward primer:	CCCTCTGCCATCACGTTTAG
Reverse primer:	GACCACAACCAGACCCATG
CCL25	
Forward primer:	TTACCAGCACAGGATCAAATGG
Reverse primer:	CGGAAGTAGAATCTCACAGCAC
CSF2	
Forward primer:	CATGCCTGTCACGTTGAATG
Reverse primer:	TGAAATTGCCCCGTAGACC
BATF	
Forward primer:	GCAGTGACTCCAGCTTCAG
Reverse primer:	TGTCGGCTTTCTGTGTCTG
RORγT	
Forward primer:	ACAGGGAGCCAAGTTCTCAGTCAT
Reverse primer:	CGTGCAGGAGTAGGCCACATTA
IRF7	
Forward primer:	TTGATCCGCATAAGGTGTACG
Reverse primer:	GCATCACTAGAAAGCAGAGGG
IRF4	
Forward primer:	CTTTGAGGAATTGGTCGAGAGG
Reverse primer:	GAGAGCCATAAGGTGCTGTCA
Sox5	
Forward primer:	CCCGTGATCCAGAGCACTTAC
Reverse primer:	CCGCAATGTGGTTTTCGCT
C-Maf	
Forward primer:	AAGGAGAAATACGAGAAGCTGG
Reverse primer:	CGGAGCATTTAACAAGGTGG
CCL17	
Forward primer:	GGAAGTTGGTGAGCTGGTATAAG
Reverse primer:	GTCACAGGCCGTTTTATGTTG
CXCL3	
Forward primer:	GAAGTCATAGCCACTCTCAAGG
Reverse primer:	AAAGACACATCCAGACACCG
CXCL1	
Forward primer:	AGAACATCCAGAGCTTGAAGG
Reverse primer:	TCAGAAGCCAGCGTTCAC
CXCL2	
Forward primer:	GAAGTCATAGCCACTCTCAAGG
Reverse primer:	ATAAGTGAACTCTCAGACAGCG

Table 1 (Continued)

Genes	*Primer sequence (5′–3′)*
Ifi204	
Forward primer:	GGAGCAGTGTTCTATGGAGTG
Reverse primer:	ACTTTGGTTGCCTGTCAATTG
Ifit1	
Forward primer:	AGAGTCAAGGCAGGTTTCTG
Reverse primer:	AAGCAGATTCTCCATGACCTG
Ifit3	
Forward primer:	AGCACAGAAACAGATCACCAT
Reverse primer:	CACCCTGTCTTCCATATGACTG
Ifih1	
Forward primer:	AGAAGTTCGCAGTCCTCAATG
Reverse primer:	TTCTCGTATTTTGGGTTTTCAGC

killed on day 7 of the experiment, and the colonic tissue was removed and cleaned, and then subjected to cell culture, followed by quantitative real-time PCR and histological analyses.

Statistical analysis

All data are presented as the mean ± s.d. Two-way ANOVA analysis was used for comparisons among multiple groups with SPSS 16.0 software. A Mann–Whitney *U*-test was used for comparisons between the two groups. A *P*-value <0.05 was considered statistically significant.

RESULTS

IL-23 selectively induces Th17 cytokine expression in neutrophils

In addition to participating in primary defense against infections, neutrophils produce and release a large number of cytokines and chemokines either constitutively or upon microenvironmental stimulations that play a critical role in infections and pathologies.[41–44] To explore the roles of different cytokines on the expression of IL-17 family members in neutrophils, we first detected the mRNA expression levels of these genes in freshly isolated neutrophils from mouse bone marrow after different cytokine and LPS stimulation for 24 h by real-time PCR. Among the 17 cytokines and LPS stimulations studied, only IL-23 significantly promoted IL-17A and IL-17F expression, while resting neutrophils expressed nearly undetectable levels of IL-17A and IL-17F ($P<0.001$, Figure 1a). In addition, IL-23 also specifically induced significant IL-22 expression compared with other cytokines and LPS ($P<0.001$, Figure 1a). IL-23 induced IL-17A, IL-17F and IL-22 mRNA expression in dose- and time-dependent manners, as determined by real-time PCR (Figures 1b and c). The possible direct effects of IL-23 on neutrophils were supported by the expression of IL-23R on neutrophils as detected by flow cytometry, which showed that approximately 10% of neutrophils are positive for IL-23R (Supplementary Figure 1). To exclude the potential contamination of other immune cells such as T cells, B cells and ILCs during the differentiation process *in vitro*, we sorted the neutrophils of naïve mice to obtain highly purified $CD11b^+Ly6G^+$ cells with more than 99% purity and repeated the same induction experiments. Indeed, the sorted $CD11b^+Ly6G^+$ cells expressed high levels of IL-17A, IL-17F and IL-22 after IL-23 treatment at both the mRNA and the protein levels ($P<0.001$, Figure 1d, Supplementary Figure 2). The protein levels of IL-17A in the culture medium of IL-23-treated sorted neutrophils were significantly increased with increasing IL-23 doses, as detected by ELISA assays, indicating that IL-23-treated neutrophils have the ability to secrete IL-17A ($P<0.01$, Figure 1e). The expression of IL-17A in IL-23-treated neutrophils was further confirmed by flow cytometry and confocal microscopy staining (Figures 1f and g). By contrast, the sorted IL-23R-deficient neutrophils failed to express IL--17A, IL-17F and IL-22 after IL-23 treatment (Supplementary Figure 3). Thus, IL-23, but not other cytokines or LPS, has the ability to promote the expression of the Th17-type cytokines IL-17A, IL-17F and IL-22 in resting mouse neutrophils.

IL-23 induces a distinct gene expression profile in neutrophils

To investigate whether IL-23 induces a unique neutrophil polarization profile, in contrast to the LPS-induced inflammatory neutrophil subpopulation, we determined the gene expression profiles of LPS-polarized neutrophils and IL-23-induced neutrophils by microarray analysis. Indeed, IL-23-induced neutrophils express a unique panel of genes in sharp contrast to resting and LPS-stimulated neutrophils (Figure 2a). Specifically, 1362 genes were upregulated and 1438 genes were downregulated in IL-23-treated neutrophils compared with untreated and LPS-polarized neutrophils (Figures 2a and b). Interestingly, LPS- and IL-23-induced neutrophils express distinctive gene profiles and kinetics of cytokine induction, as indicated by mRNA microarray analysis and confirmed by real-time PCR assays (Figures 2c–e). LPS induces TNF-α and IL-6 expression in neutrophils at the mRNA level (Figure 2d). However, IL-23 fails to induce the expression of TNF-α and IL-6 (Figure 2d). By contrast, IL-23 specifically promotes the expression of IL-17A, IL-17F and IL-22 at both the mRNA and the protein levels in neutrophils ($P<0.01$, Figures 2d and e). Altogether, these data lead us to speculate that IL-23 may drive the polarization of resting neutrophils into a subpopulation with a distinct panel of cytokines compared with LPS-induced inflammatory neutrophils.

IL-23 induces Th17 cytokine expression in neutrophils via STAT3-dependent pathways

It has been reported that RORγT, RORα, IRF-7, IRF4, BATF, Sox5 and C-maf are critical transcription factors for Th17 cell induction.[45–47] The expression of BATF, RORγT, IRF4 and IRF7 is enhanced in IL-23-treated neutrophils at the mRNA level, and the protein level expression of BATF and RORγT is upregulated in IL-23-treated neutrophils (Figures 3a and b). However, the expression of Sox5 and C-maf in IL-23-treated

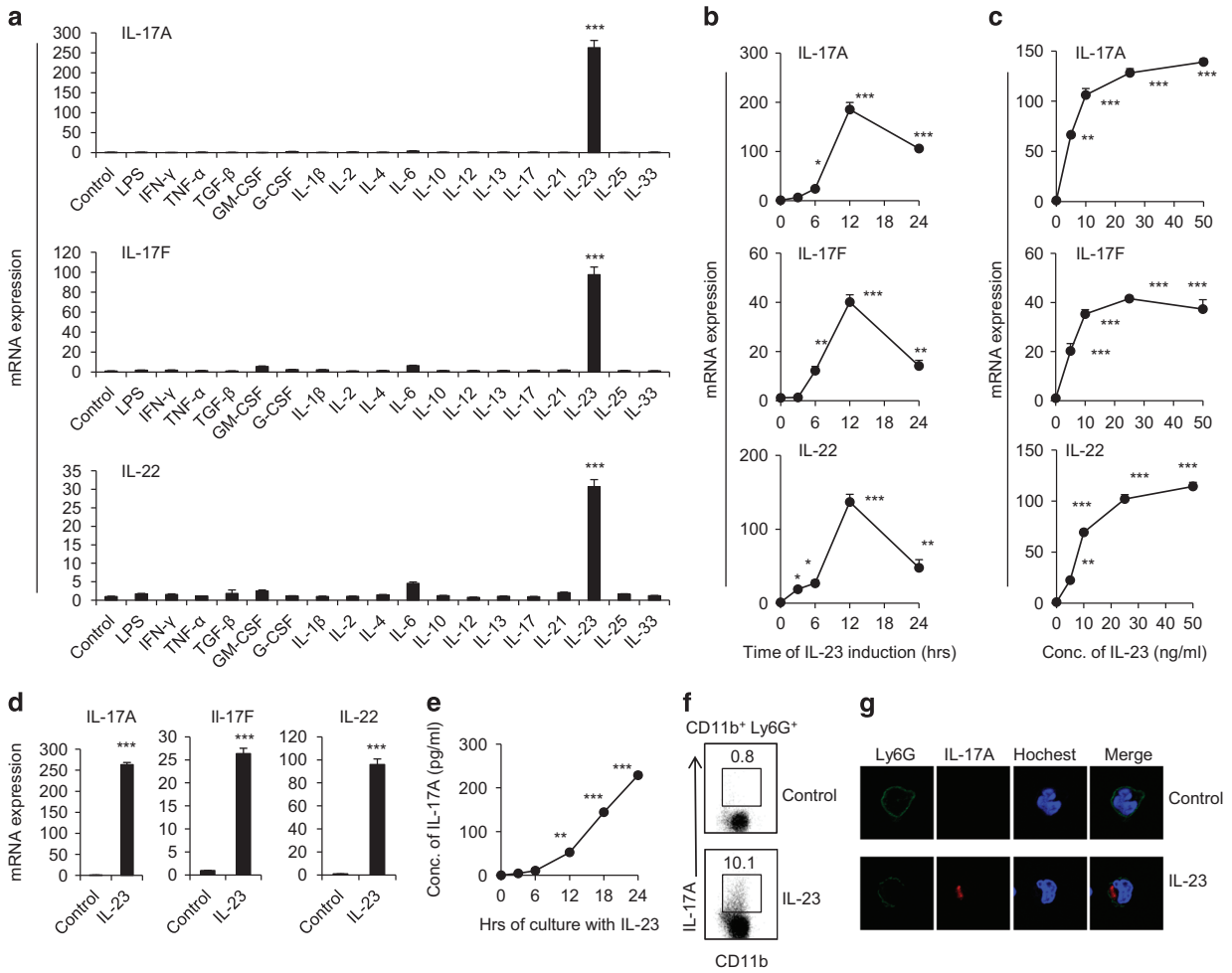

Figure 1 The expression of IL-17A, IL-17F and IL-22 in neutrophils is selectively induced by IL-23. (**a**) Primary neutrophils freshly isolated from the bone marrow of C57BL/6 mice were treated with various cytokines and LPS for 24 h. IL-17A, IL-17F and IL-22 mRNA expression levels were determined by real-time PCR. (**b**) Quantitative PCR analysis of IL-17A, IL-17F and IL-22 mRNA expression in neutrophils treated with IL-23 (50 ng/ml) for different times. (**c**) mRNA expression levels of IL-17A, IL-17F and IL-22 in primary neutrophils treated with different concentrations of IL-23 for 12 h were determined by real-time PCR. (**d**) mRNA expression levels of IL-17A, IL-17F and IL-22 in sorted CD11b$^+$Ly6G$^+$ neutrophils treated with IL-23 (0–50 ng/ml) for 12 h were determined by real-time PCR. (**e**) The protein levels of IL-17A in the culture medium of sorted CD11b$^+$Ly6G$^+$ neutrophils treated with IL-23 (50 ng/ml) for different times were determined by ELISA assays. The protein expression of IL-17A in neutrophils treated with 50 ng/ml IL-23 for 24 h was determined by flow cytometry (**f**) and confocal microscopy (**g**). Data are shown as the mean ± s.d. ($N=3$), representing one of three independent experiments. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with control group. Data were analyzed by Mann–Whitney *U*-test for two group comparisons or one-way ANOVA analysis for multi-group comparisons using SPSS software. LPS, lipopolysaccharide.

cells did not appear to be significantly changed (Figure 3a). Thus, the enhanced expression of BATF and RORγT suggested that they are likely involved in IL-23-stimulated neutrophil polarization. It has been reported that IL-23 promotes STAT3 activation in T cells based on the enhanced levels of p-STAT3 (at amino acids Y705 and S727).[21,48] The level of p-STAT3 in neutrophils is increased after IL-23 treatment (Figure 3c). Inhibition of STAT3 activation by the STAT3-specific inhibitor S31-201 significantly decreases IL-17A, IL-17F and IL-22 protein expression and IL-17A and IL-17F mRNA expression in IL-23-induced neutrophils in a dose-dependent manner ($P<0.001$, Figures 3d and e). The STAT3-specific inhibitor S31-201 also decreased STAT3 activation in IL-23-treated neutrophils, as indicated by the levels of p-STAT3 detected by western blot (Figure 3f). Meanwhile, inhibiting STAT3 activity significantly blocks the IL-23-induced expression of BATF, RORγT, IFR4 and, to a lesser degree, IRF7 in neutrophils (Figures 3g and h), indicating that BATF, RORγT, IFR4 and IRF7 are down-stream molecules in an IL-23-activated STAT3 pathway in neutrophils. Thus, IL-23 induces IL-17A, IL-17F and IL-22 expression in neutrophils through a STAT3-dependent pathway.

The roles of IL-17-expressing neutrophils in colitis in mice

We observed the presence of IL-17-producing neutrophils in a mouse model of DSS-induced colitis, in which Th17 cells

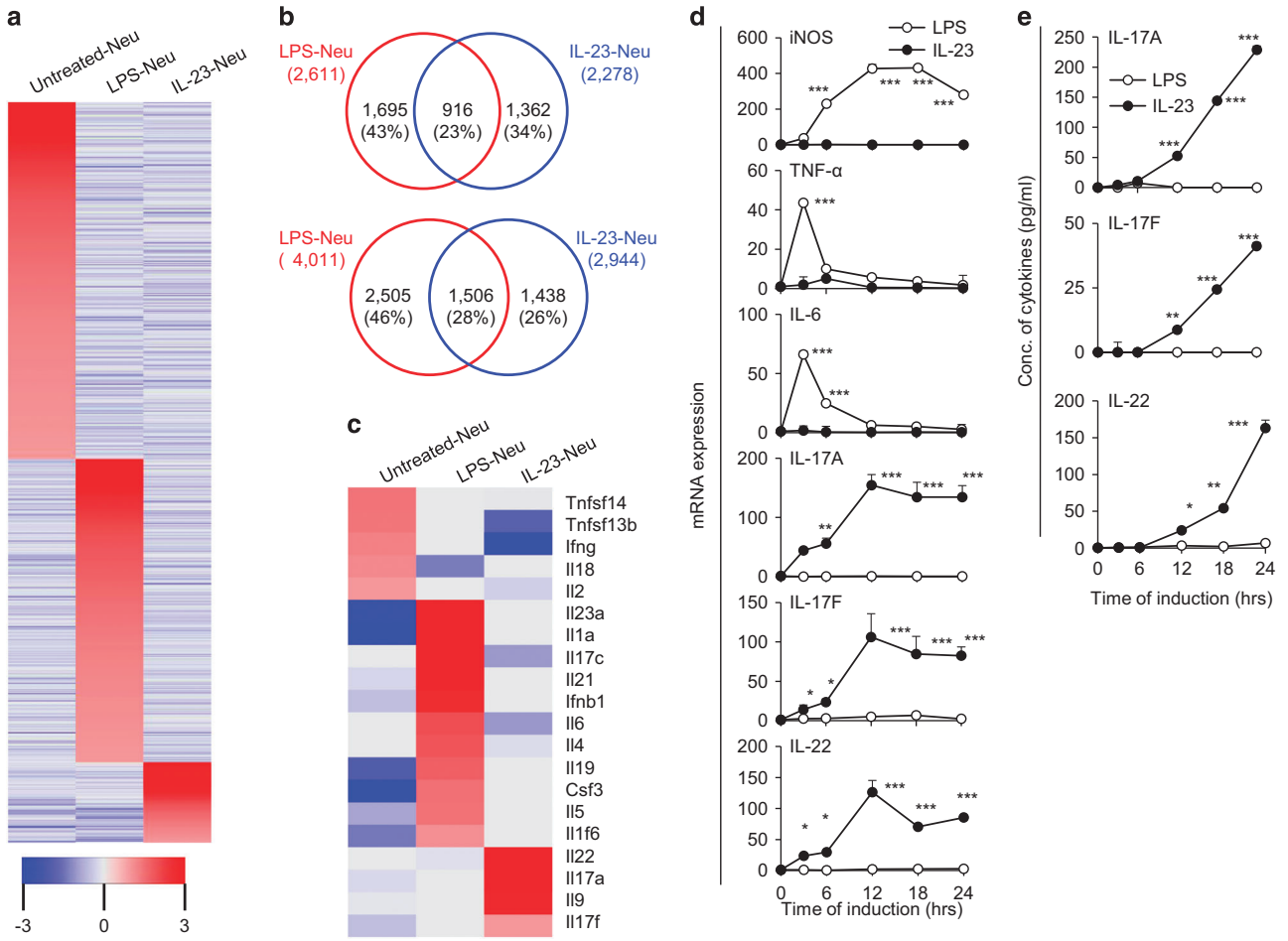

Figure 2 IL-23-treated neutrophils display a distinct gene expression profile. Sorted primary neutrophils were cultured with LPS or IL-23 *in vitro*, and microarray analyses of gene expression profiles were performed. (**a**) A heatmap comparison of gene expression profiles identifies lineage-restricted genes in untreated-Neu, LPS-Neu and IL-23-Neu cells. Genes with predominant expression in one cell type (at least two-fold greater than any other cell type) are organized in the heatmap. (**b**) Venn diagram showing upregulated genes 1695+916 +1362=3973 and downregulated genes 2505+1506+1438=5449 in LPS-Neu and IL-23-Neu compared with untreated neutrophils. Approximately 34% of upregulated genes and 26% of downregulated genes are specifically differentially expressed in IL-23-Neu compared with untreated neutrophils. (**c**) Expression profiling of related cytokines in untreated-Neu, LPS-Neu and IL-23-Neu cells. Genes with predominant expression in one cell type (at least two-fold change compared with other cell types) are organized in the heatmap. Colors represent genes greater than (red) or less than (blue) the second-highest expression in three cell types. (**d**) The expression levels of cytokines including iNOS, TNF-α, IL-6, IL-17A, IL-17F and IL-22 were determined by real-time PCR after the isolated neutrophils were induced with LPS and IL-23 for different times. (**e**) The concentrations of IL-17A, IL-17F and IL-22 in the culture medium of isolated neutrophils induced with LPS and IL-23 for different times were measured by ELISA assays. Data are expressed as the mean ± s.d. ($N=3$–5), with one representative experiment from three independent experiments shown. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ for comparisons between the indicated groups. Data were analyzed by the Mann–Whitney *U*-test or two-way ANOVA analysis using SPSS software.

and neutrophils have been reported to play a critical role.[14] Mice with DSS-induced colitis showed enhanced IL-17A and IL-22 expression in colon tissues (Figures 4a and b). Importantly, IL-17A^{+} neutrophils were present in the colons of DSS-induced colitis mice, as detected by flow cytometry (Figure 4c). Using the neutrophil-depleting antibody RB6-8C5, we found that neutrophil depletion significantly reduced the expression of IL-17A, IL-17F and IL-22 in colon tissues (Supplementary Figures 4a and b), which supports the contribution of neutrophils to these cytokines in colitis. To identify the role of IL-17-producing neutrophils in the pathogenesis of colitis, we adoptively transferred either induced IL-23-treated or resting neutrophils into recipient mice during the administration of DSS. Mice that received IL-23-treated neutrophils displayed more severe symptoms of acute colitis than mice that either received untreated neutrophils or did not receive neutrophils, as shown by significantly shorter survival times (Figure 4d), rapid body weight loss (Figure 4e), increased macroscopic scores (Figure 4f) and shorter colon lengths (Figure 4g) in the mice that received IL-23-treated neutrophils. The median survival times for mice not receiving neutrophils, and for mice receiving untreated, or IL-23-treated neutrophils

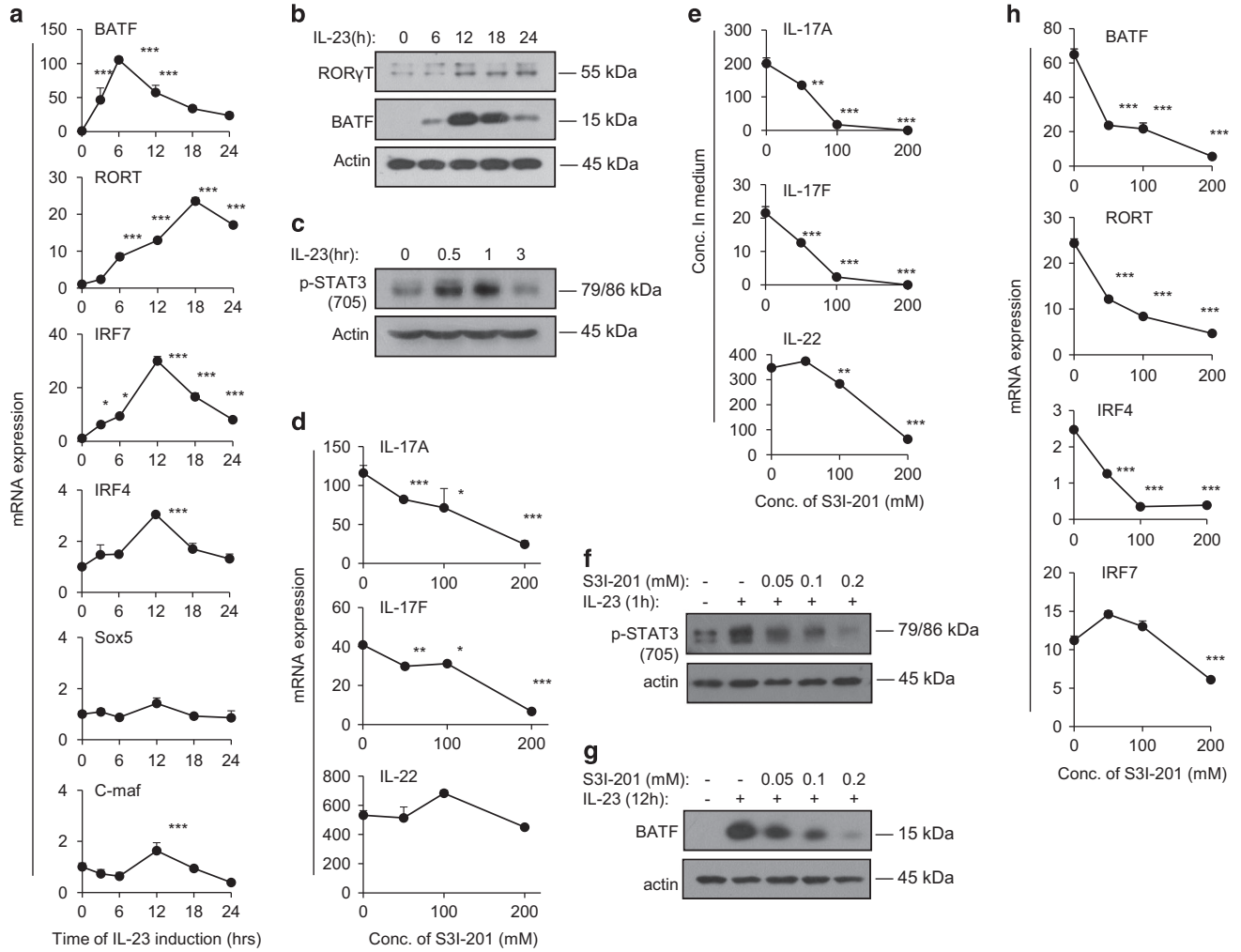

Figure 3 The Th17-type cytokine expression of IL-23-treated neutrophils is mediated by STAT3-dependent signaling pathways. (**a**) mRNA expression levels of BATF, RORγT, IRF7, IRF4, Sox5 and C-maf in neutrophils treated with IL-23 for different times were determined by real-time PCR. (**b**, **c**) The BATF, RORγT and p-STAT3 protein levels in neutrophils after IL-23 treatment for the indicated times were determined by western blot. (**d**) Neutrophils were pretreated with the indicated concentrations of STAT3 inhibitor (S3I-201) for 0.5 h and then stimulated with IL-23 for 12 h. The expression levels of IL-17A, IL-17F and IL-22 were detected by real-time PCR. (**e**) The concentrations of IL-17A, IL-17F and IL-22 in the supernatants of neutrophils pretreated with STAT3 inhibitor (S3I-201) for 0.5 h and then stimulated with IL-23 for 24 h were determined by ELISA assay. (**f**, **g**) p-STAT3 and BATF expression in neutrophils treated with or without IL-23 and/or S3I-201 was determined by western blotting. (**h**) mRNA expression levels of BATF, RORγT, IRF4 and IRF7 in neutrophils pretreated with STAT3 inhibitor (S3I-201) for 0.5 h and then stimulated with IL-23 for 12 h were determined by real-time PCR. Assays were performed more than three times. Data are shown as the mean ± s.d. ($n=3$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ for comparisons between the indicated groups. Data were analyzed by one-way ANOVA analysis using SPSS software.

were 19, 19 and 16 days, respectively. Histological analysis after 7 days of DSS treatment revealed severe colitis in the mice that received IL-23-treated neutrophils, including massive inflammatory infiltrates and thickened walls (Figure 4h). Meanwhile, the colonic tissues of mice that received IL-23-treated neutrophils expressed significantly more IL-17 and IL-22 than those in mice that received resting neutrophils (Figures 4i and j). Interestingly, the adoptive transfer of IL-23-treated neutrophils with an IL-17A deficiency partially but significantly decreased the enhancement of colitis observed with IL-23-treated neutrophils, as supported by mouse survival time (Figure 4k), body weight loss (Figure 4l), macroscopic score (Figure 4m), colon length (Figure 4n) and histological analysis (Figure 4o) in WT and IL-17A-deficient IL-23-neutrophil-transferred mice. To further demonstrate the endogenous role of IL-17-producing neutrophils in colitis progression, IL17A KO mice were treated with DSS and administered either IL-23-treated or untreated neutrophils. IL-17A KO mice receiving IL-23-treated neutrophils displayed more severe symptoms of colitis compared with mice either receiving control neutrophils or not receiving neutrophils, as shown by survival time (Figure 5a), macroscopic score (Figure 5c), colon length (Figure 5d) and histological analysis (Figure 5e). IL-17 and IL-22 expression levels in colonic tissues

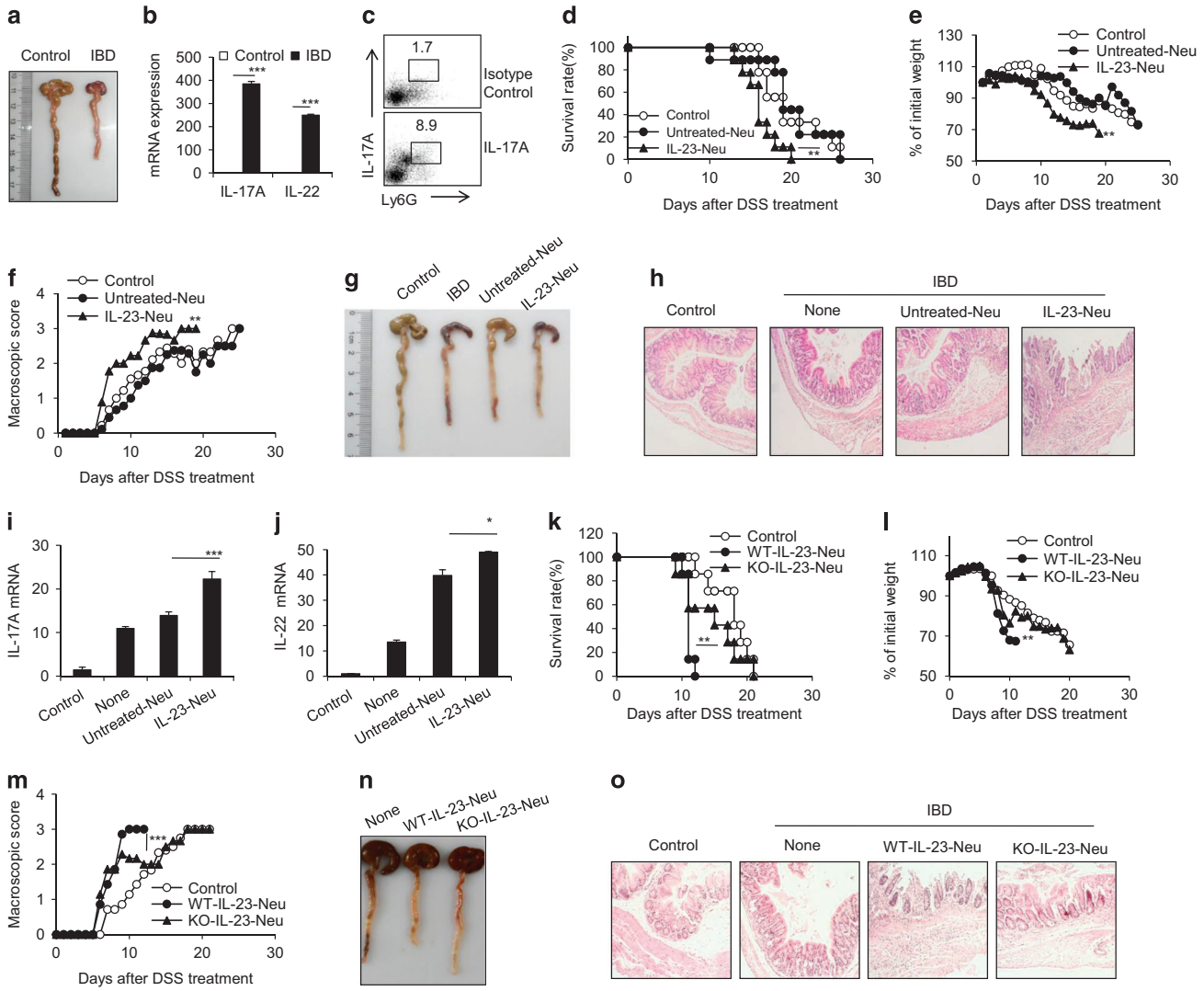

Figure 4 IL-23-treated neutrophils promote dextran sulfate sodium (DSS)-induced colitis. Colitis was induced by adding DSS (2.5%) to drinking water as described in the Materials and Methods. (**a**) Morphological analysis of colons from wild-type and DSS-induced colitis mice. (**b**) mRNA expression levels of IL-17A and IL-22 in the sorted CD11b$^+$Ly6G$^+$ neutrophils from control and DSS-treated colonic tissues were detected by real-time PCR. (**c**) IL-17$^+$ neutrophils in colons of control and DSS-induced colitis mice were detected by flow cytometry. Survival rate (**d**), body weight (**e**), macroscopic score (**f**) and representative pictures (**g**) of colons from control and DSS-induced colitis mice adoptively transferred with PBS, 5×10^6 untreated-Neu or IL-23-treated-Neu cells (sorted CD11b$^+$Ly6G$^+$ neutrophils) per mouse on day 0, 2 and 5 are summarized. (**h**) H&E staining of colonic tissues from control or DSS-induced colitis mice treated as described above. mRNA expression levels of IL-17A (**i**) and IL-22 (**j**) in colonic tissues from mice treated as described above were detected by real-time PCR. Survival rate (**k**), body weight (**l**), macroscopic score (**m**) and representative pictures (**n**) of colons of control and DSS-induced colitis mice adoptively transferred with PBS, 5×10^6 untreated neutrophils, IL-23-treated neutrophils or IL-17A-deficient IL-23-treated neutrophils (sorted CD11b$^+$Ly6G$^+$ neutrophils) per mouse on days 0, 2 and 5. (**o**) H&E staining of colonic tissues from control or DSS-induced colitis mice treated as described above are presented. Data are expressed as the mean ± s.d. ($n=3$–5) and one representative example from three independent experiments with similar results is shown. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ for comparisons between the indicated groups. Data were analyzed by the Mann–Whitney *U*-test or two-way ANOVA using SPSS software. Survival curves were analyzed by the log-rank test.

were higher in IL-17 KO mice receiving IL-23-treated neutrophils than in mice receiving resting neutrophils (Figures 5f and g). Thus, IL-23-induced neutrophils are present in the colonic tissues of colitis mice and have the capacity to promote colitis pathogenesis in mice.

DISCUSSION

In the present study, we have identified a unique neutrophil subpopulation induced by IL-23 with the following properties: (a) to display distinctive gene expression profiles compared with resting neutrophils and LPS-activated neutrophils; (b) to

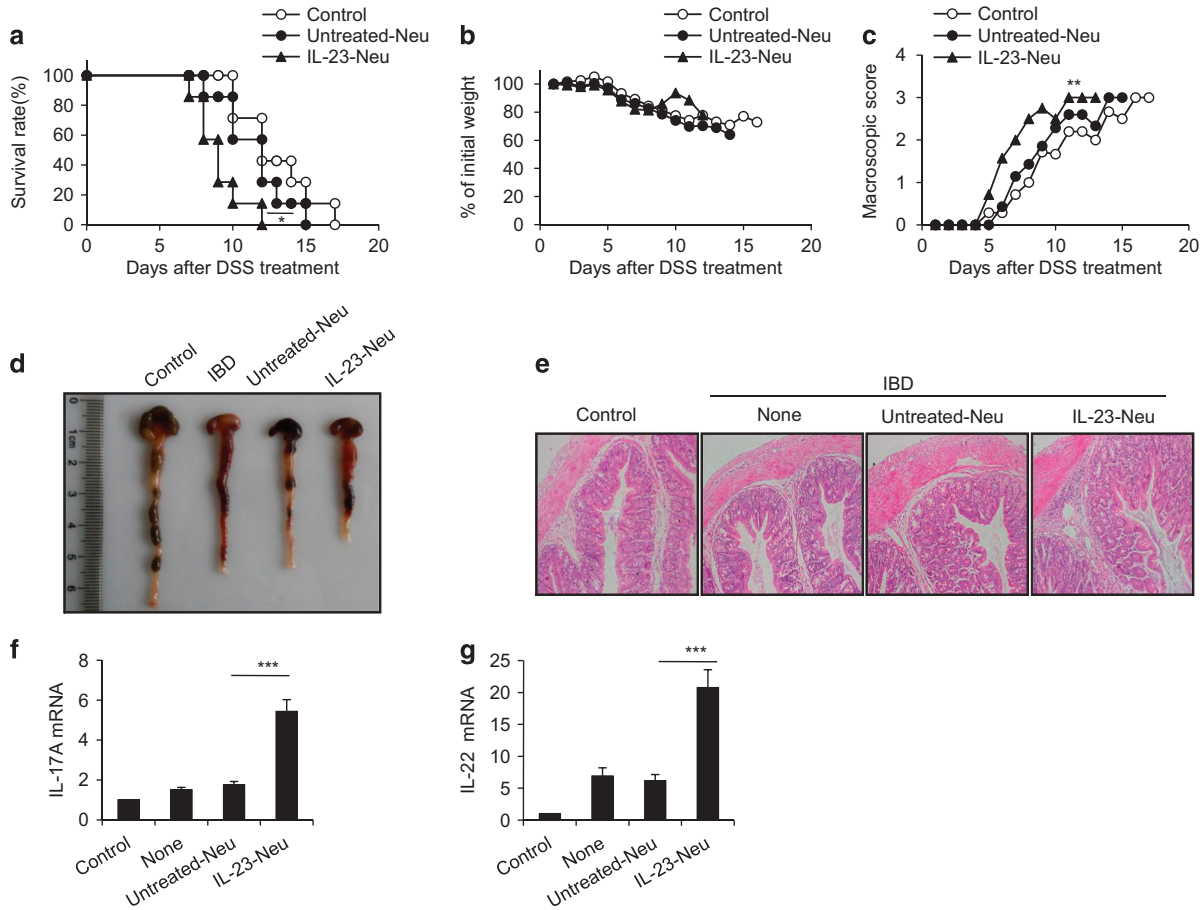

Figure 5 IL-23-treated neutrophils promote dextran sulfate sodium (DSS)-induced colitis in IL-17 knockout (KO) recipients. DSS (2.5%) was administered to IL-17A KO mice for colitis induction. PBS, 5×10^6 untreated or IL-23-treated neutrophils (sorted CD11b$^+$Ly6G$^+$ neutrophils) per mouse were i.v.-injected on days 0, 2 and 5. The survival rate (**a**), body weight (**b**), macroscopic score (**c**) and representative colon pictures (**d**) of control and DSS-induced colitis mice are shown. (**e**) H&E staining of colonic tissues from control or DSS-induced colitis mice treated as described above are presented. The mRNA expression levels of IL-17A (**f**) and IL-22 (**g**) in colonic tissues from mice treated as described above were detected by real-time PCR. Data are expressed as the mean ± s.d. ($n=3$–5) and one representative example from three independent experiments with similar results is shown. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ for comparisons between the indicated groups. Data were analyzed by the Mann–Whitney *U*-test or two-way ANOVA using SPSS software. Survival curves were analyzed by the log-rank test.

produce more Th17-type cytokines including IL-17A, IL-17F and IL-22 and less TNF-α and IL-1β; (c) to promote colitis pathogenesis; and (d) to activate the specific transcription factors RORγT and BATF downstream of STAT3. Thus, we provide additional evidence for differentially polarized neutrophils with respect to cytokine production. We believe that relevant studies may provide new insights into the contribution of neutrophils to the pathogenesis of inflammation and immune disorders and may provide new therapeutic approaches to treat neutrophil-related diseases.

Genetic studies have strongly indicated that IL-23 may significantly contribute to inflammatory disease risk in humans.[49,50] Deficiency of IL-23 in mice causes resistance to experimental immune-mediated diseases such as IBD.[51,52] The promotion of Th17 subsets is highly recognized as a key player for mediating the critical role of IL-23 in these inflammatory diseases.[20,53] Our present study showed that IL-17A$^+$ neutrophils are present in colonic tissues with colitis. IL-23 acts directly on neutrophils to induce IL-17A and IL-17F, and the adaptive transfer of IL-23-treated neutrophils significantly increases the severity of colitis in mice, indicating that IL-17A-producing neutrophils may promote the pathogenesis of colitis in mice. The significance of IL-23-induced neutrophil polarization in Th17 cytokine-related inflammatory diseases needs to be studied further. However, it has recently been reported that neutrophils can release IL-17 in areas with amyloid-β deposits and contribute to disease pathogenesis in Alzheimer's disease-like mouse models.[54] It is interesting and suggestive that the serum concentration of IL-23 has been positively correlated with IL-17-producing neutrophils in patients with fungal keratitis.[55] Neutrophils in the circulation of arthritic mice spontaneously produce IL-17 *in vitro*.[56] IL-17 produced by neutrophils also participates in inflammation-induced airway neutrophilia.[20] Higher levels of IL17$^+$CD177$^+$ neutrophils in the peripheral blood in allergic asthma patients might contribute to disease progression.[57]

IL-17A-producing RORγt$^+$ neutrophils in the peripheral blood may amplify liver tissue injury in a hepatic ischemia-reperfusion injury mouse model.[58] Thus, IL-17A-producing neutrophils contribute to a wide range of inflammatory diseases.

The biological significance and the pro-inflammatory activities of IL-23 in inflammatory and autoimmune diseases include but are not limited to the induction of IL-17 production by Th17 cells and the inhibition of immunosuppressive CD4$^+$CD25$^+$ regulatory T cells.[59,60] We observed that IL-23 can significantly induce IL-17A, IL-17F and IL-22 production in neutrophils. Our data are in line with observations showing that human neutrophils also express RORγt and upregulate the expression of IL-17A, IL-17RC and dectin-2 following IL-6 and IL-23 stimulation.[44] IL-17 production by neutrophils can be induced in various models including mucosal candidiasis, fusarium corneal infections, kidney ischemia-reperfusion injury and psoriasis.[44,61–63] Intra-articular administration of zymosan (a TLR2 ligand) results in IL-17 production by neutrophils in an arthritis mouse model.[56] Activated peritoneal neutrophils *in vitro* produce IL-17A and IL-23 in response to myeloperoxidase-specific anti-neutrophil cytoplasmic autoantibodies via their Fc-regions and the classical complement pathway, which contributes to the occurrence of chronic autoimmune inflammation and ultimately results in the development of local Th17-mediated autoimmunity.[64] These data collectively illustrate that neutrophils are one of the major sources of IL-17 under specific inflammatory conditions. It is known that neutrophils can produce many different cytokines such as IL-1β, TNF-α, IL-12, IL-4 and IL-10 under different stimulations.[41,42] Therefore, based on the specific cytokine production, neutrophils may have different types of functional polarizations, similar to macrophages.

In addition to producing IL-17, IL-23-induced neutrophils also produce IL-22. The large amount of IL-17 produced by this neutrophil subset significantly contributed to the pathogenesis of DSS-induced colitis in mice. However, it has been reported that IL-22 may have opposing effects on the pathogenesis of colitis by not only enhancing inflammation and antimicrobial immunity but also increasing epithelial cell proliferation and tissue repair.[65] Thus, it is clear that the role of IL-22 produced by IL-23-induced neutrophils in colitis needs to be clarified in future studies.

IL-23 signals through IL-23R and IL-12Rβ1 to activate JAK and predominantly leads to phosphorylation and activation of STAT3 in T cells,[66] which promotes the transcription of Il23r and Rorc (encoding RORγ) and stabilizes the expression of genes encoding pro-inflammatory effector molecules including IL17A, IL17F, IL22 and CSF2.[42,43] RORγT, RORα, IRF-7, IRF4, BATF, Sox5 and C-maf are critical transcription factors for Th17 cell induction.[45–47] Our results show that IL-23 uses the classical STAT3–RORγT/BATF pathway to induce a pro-Th17 cytokine gene expression profile in neutrophils.

In summary, our present study demonstrates a Th17 type-like polarization of neutrophils induced by IL-23. These polarized neutrophils promote colitis pathogenesis in a mouse model. These findings shed further light on the existence of neutrophil functional plasticity and polarization, which have previously been neglected. The biological significance of polarized neutrophils in other diseases needs to be investigated and may offer novel therapeutic approaches to treat neutrophil-related immune disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

YL, LZ, ZC and TY designed and carried out the experiments, analyzed the data and wrote the manuscript; H-XS analyzed the microarray data; FY, WW and YH performed the animal model experiments and real-time PCR assays; PW performed the ELISA assays; QZ and YT performed the flow cytometry; LZ, XZ and YZ designed the experiments, analyzed the data, wrote the manuscript and provided overall supervision.

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