IL-17A–responsive neutrophils expressing CD49d and VEGFR1 promote synovium lesions by enhancing the migratory and invasion behavior of synovial fibroblasts at the early stage of rheumatoid arthritis

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Research Article

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Abstract

Background

Emerging evidences indicate there are different subsets of neutrophils whose biological functions vary in different physiological or pathological status. However, the function of the neutrophil subsets in the etiology of rheumatoid arthritis (RA) is rarely reported.

Methods

The presence of $CD49d^{+}VEGFR1^{+}Ly6G^{+}$ neutrophil in blood, joint and bone marrow (BM) were determined in Collagen-induced arthritis (CIA) mice. $CD49d^{-}VEGFR1^{-}$PMN, $CD49d^{+}VEGFR1^{-}$PMN and $CD49d^{+}VEGFR1^{+}$PMN were isolated. The effect of IL-17A on CD49d and VEGFR1 expression was investigated in vitro. WT and IL-$17A^{-/-}$ mice were immunized with bovine type II collagen. Disease activity was evaluated using clinical score and diameter of ankle. The level of MMP-3 and MMP-13 were detected by ELISA. The expression of $CD49d^{+}VEGFR1^{+}$PMN in RA patients and healthy individual (HC) were determined by flow cytometer.

Results

We identified a unique $CD49d^{+}VEGFR1^{+}$neutrophil subset and investigated them in the pathogenesis of RA. The proportion of this neutrophil subset changed dynamically during the arthritis development. *In vivo* and *in vitro* experiments indicated the increased $CD49d^{+}VEGFR1^{+}$neutrophil population was IL-17A-dependent. $CD49d^{+}VEGFR1^{+}$neutrophils, localized in the inflamed synovium of CIA mice, possessed pro-inflammatory features with elevated expressions of TNF-α, VEGF, IL-18, CXCL9, CCL3 and CCL4. $CD49d^{+}VEGFR1^{+}$neutrophils promote the migration and the invasion ability of fibroblast like-synoviocytes (FLS), accompanied with a significant elevation in MMP-3 and MMP-13 release.

Conclusion

We identified a unique neutrophil subset with high expression of CD49d and VEGFR1, which are induced by IL-17A in RA. This unique subset plays a critical role in synovium pathogenesis by activating FLS at the early stage of RA.

Background

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by pronounced synovial hyperplasia, pannus formation, bone destruction[1]. Synovitis is a frequent sign in RA, however some patients display this symptom in the early stage of RA or even prior to clinical arthrtitis. Therefore,

early diagnosis and treatment of synovitis are crucial to improve the joint inflammation and consequent cartilage or bone damage in RA[2]. Numbers of studies demonstrate that there are various infiltrating cells including T, B cells, neutrophils and macrophages in inflamed joint [3]. They can induce synovial hyperplasia, neovascularization and degradative enzymes release, leading to joint inflammation and bone damage in RA[3]. However, it is still unclear which type of infiltrating cells triggers abnormity of synovial fibroblasts' behavior, critical for synovitis and cartilage erosion at the early stage of RA.

Polymorphonuclear neutrophils (PMNs) are one of the most abundant cell types of the innate immune system. In addition to their classical functions as the first line of defense against injury or pathogen infection, neutrophils serve as effectors and modulators of the overarching network of the immune system[4, 5]. There is emerging evidence indicating that neutrophils are the first cells migrating to the inflamed joint where they phagocytose immune complexes and release a wide variety of cytokines, chemokines, immunoregulatory molecules, reactive oxygen species (ROS) and neutrophil extracellular traps (NETs) enhancing damage to the joint in RA[6, 7]. At the onset of clinical disease, neutrophils are reported to highly expressing very late antigen-4 (VLA-4) along with their emigration through endothelium[8]. Recombinant rat VLA-4 (α4, CD49d) antibody can effectively reduce neutrophil migration to inflamed joints and prevent development of arthritis in adjuvant arthritis model[9].

It is reported that neutrophils change their function and phenotype in the development of RA[10–14]. They express high levels of tumor necrosis factor (TNF)[10] and the membrane-bound receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL)[11, 12]. The activation of neutrophils in the synovium are strongly influenced by proinflammatory cytokines like TNF-α and interleukin-8 (IL-8) in the synovial cavity microenvironment[13, 14]. In turn, numerous chemokines are released by activated neutrophil to stimulate osteoclasts and B, T lymphocytes[11, 15]. The altered phenotypic of neutrophils might be associated with the specific function. It is reported that neutrophils in RA patients had a distinct phenotype with high expression of CD147, which are correlated with increased matrix metalloproteinases (MMPs) production of synoviocytes[16]. In allergic asthma model, neutrophils with high expression CXCR4 promote the initiation of allergic asthma by releasing NETs[17]. Moreover, it is reported that $CD49d^{+}VEGFR1^{high}CXCR4^{high}$ neutrophil subset, detected in the circulation of mice and humans, display proangiogenic ability, and stimulate angiogenesis at sites of hypoxia[18].

In this study, we identified a specific neutrophil subset, with high expression of CD49d and VEGFR1, by using a well-established RA model, collagen-induce arthritis (CIA) in mice. In addition, we explored the dynamic changes in proportions of this neutrophil subset in various stages of arthritis as well as its distinctive transcriptional expression. Importantly, we found that this subset could infiltrate into inflamed joint in the early stage of arthritis, and promote FLS migration and erosion ability, which provides a novel mechanism to clarify neutrophils' role in the early pathogenesis of RA.

Materials And Methods

Ethics statement

The study was approved by the Biomedical Research Ethics Committee, West China Hospital of Sichuan University (ChiCTR1900027947) Chengdu, China, and the written consents were obtained from all the participants according to the Declaration of Helsinki. All animal care and experimental procedures strictly adhered to the guidelines of the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the ethics committee of University of Sichuan University, Chengdu, China.

Sample collection.

Blood samples were collected from 13 RA patients (mean age 49.30 years), 9 RA patients complicated with Synovial fluid(SF) (mean age 52.75 years) and 14 Healthy Control (HC) donors (mean age 48.77 years). Clinical traits of donors are shown in Table 1. Synovial tissues from 5 RA patients (mean age 43.50 years) were obtained during knee joint arthroscopy. RA patients fulfilled the 1987 revised criteria of the American College of Rheumatology or the 2010 ACR/EULAR classification criteria.

Table 1
Baseline demographics and clinical traits.

	HC (n = 14)	RA without SF (n = 13)	RA with SF (n = 9)	P^1 Value	P^2 Value
Age (years)	48.77 ± 3.845	49.30 ± 3.587	52.75 ± 5.455	0.9227	0.7637
Gender, Men/women (n)	5/9	4/9	1/8	0.7021	0.3602
Disease duration (month)		6.08 ± 4.668	120.13 ± 4.924		
DAS28-CRP		3.854 ± 0.3203	3.925 ± 0.7376		
CRP(mg/ml)		12.21 ± 3.063	70.79 ± 41.90		

Data are presented as means ± SEM, unless otherwise specified. DAS 28 = disease activity score 28 joints; CRP = C-reactive protein. P^1 (HC VS RA without SF), P^2(HC VS RA with SF).

Mice.

Male DBA/1 and C57BL/6 (6–8 weeks) mice with a mean weight of 20–25 g were purchased from Chengdu dossy experimental animals company (Chengdu, China). IL-17A$^{-/-}$ mice with C57BL/6 background were kindly provided by Professor Jingsong Xu, Guangzhou Regenerative Medicine and Health Guangdong Laboratory, Guangzhou, China.

CIA model.

Mice were intradermally injected at the base of the tail with 100 μL chicken type II collagen (C9301, sigma) emulsified in complete Freund's adjuvant(F5881, sigma) containing 5 mg/ml killed *Mycobacterium tuberculosis* (R19021, REbio) on Day 0. On day 21, mice were boosted with an

intradermal injection of 100 μg of type II collagen in the base of the tail. The clinical severity of arthritis were monitored every two days after the second immunization and joint swelling was scored according to the manufacture's protocol (Hooke Laboratories). CIA control group received sterile phosphate-buffered saline (PBS). DBA/1 control group and CIA group were sacrificed on days 30, 36, and 42, respectively. WT and IL-17A$^{-/-}$ mice were sacrificed on day 42. Blood, bone marrow, ankle and synovial tissues were collected for subsequent measurements.

Cytokine analysis.

The levels of IL-17A, IL-6, IL-10, and TNF-α in serum were assessed using a TH1/TH2/TH17 CBA kit from BD Bioscience (560484, BD Bioscience). The levels of MMP3(EHC078.96.5, Neobioscience) and MMP13 (70-EK1M13-96, MultiSciences) in the culture supernatant and IL-17A level in serum from healthy individuals and RA patients were quantified using enzyme-linked immunosorbent assay (ELISA) kits (EHC170, Neobioscience) according to the manufacturer's protocol.

Cell Culture and Transfection.

The fibroblast-like synoviocytes (FLS) were isolated from synovial tissues as previously described[19] .Briefly, synovial tissues were minced into small pieces and digested with 1.5 mg/mL type II collagenase (#C6885, Sigma) in DMEM (SH30243.01,hyclone) medium for 2 hours at 37°C. The harvested cells were re-suspended in high-glucose DMEM (SH30243.01, hyclone) supplemented with 10% fetal bovine serum (FBS) (10099141, Gibco) containing 1% penicillin/streptomycin and 2% L-glutamine. The culture medium was changed every two days until cells reached 80–90% confluence. Cells were passaged at a ratio of 1:3 and cells within passages 3 to 7 were used in the subsequent experiments.

IL-17A receptor (IL-17R) was silenced in the HL-60 cell line as previously described[20]. In brief, IL-17R shRNA plasmid was bought from Santa cruz (sc-40037, Santa cruz) and the viruses were packaged in HEK293T cells (bio-73410). HL-60 cells (bio-73051) were infected and screened by puromycin and further sorted by flow cytometry. HL-60 and HL-60 $^{IL-17R-/-}$were cultured in RPMI 1640 medium (SH30809.01, Hyclone) containing 10% FBS (10099141, Gibco), 1% penicillin/streptomycin and 2% L-glutamine, at 37℃ and 5% CO_2. The medium was changed every 2–3 days. For cell differentiation, cells were treated with 1.25% dimethyl sulfoxide (DMSO) for 5 days.

Differentiated HL-60 cells (d HL-60) were harvested and washed three times with PBS, and primed with recombinant human IL-17 (96-200-17-25, Peprotech) for 24 hours before co-cultured with FLS. IL-17-primed and unstimulated HL-60 cells were then co-incubated with FLS (5×10^4 cells) in 24-well plates and supernatants were collected after 24 hours.

Histological assessment.

On day 30, DBA/1 mice were sacrificed, and the knees were fixed in 10% formalin solution for 24 hours. The knees were then decalcified in 10% EDTA for 14 days, embedded in paraffin, sectioned (5 μm) and

stained with hematoxylin and eosin.

Synovium from DBA/1 mice was snap-frozen, embedded in optimal cutting temperature compound (OCT) and cut into 6 μm sections. Samples were permeabilized with 0.1% Triton x-100 for 5 minutes, blocked with 3% BSA for 1 hour, and then incubated with rabbit anti–CD49d (AB1924, merckmillipore), rat anti-Ly6G (ab25377, abcam) and goat anti–VEGFR1(AF471, R&D) at 4℃ overnight. After washed three times with PBS-Tween20 (PBST), sections were incubated with Alexa Fluor 488-conjugated affinipure donkey anti-rat IgG (A-21208, Life Technologies), Alexa Fluor 555-conjugated affinipure donkey anti-rabbit IgG (A-31572, Life Technologies) and Alexa Fluor 647-conjugated affinipure donkey anti-goat IgG (A-21447, Life Technologies) in 3% BSA at room temperature for 1 hour. The sections were then washed with PBST, and nuclei were stained with DAPI. Subsequently, the stained sections were analyzed with a confocal laser-scanning fluorescence microscope (Leica).

Isolation of neutrophils.

Murine neutrophils were isolated from the bone marrow of mice euthanized by CO_2 inhalation. Bone marrow cells were obtained by flushing femurs and tibias with PBS containing 0.1% BSA. Cells were filtered through a 70-μm mesh (352350, BD), and neutrophils were isolated by Ficoll density gradient centrifugation according to a previous report[20].

Human neutrophils were isolated from peripheral blood of patients with RA and healthy volunteers by Ficoll density gradient centrifugation as previously described[21]. Neutrophils were collected after eliminating erythrocytes by hypotonic lysis.

Cell sorting, treatments and Flow cytometry analysis.

To quantify Ly6G, CD49d, and VEGFR1 neutrophils in CIA, single-cell suspensions were prepared from bone marrow (femurs), joints and peripheral blood obtained on days 30, 36, and 42, respectively. Ankle cells were isolated by mincing ankles into small pieces and digesting with 1.5 mg/mL collagenase A (10103578001, Roche) in DMEM medium(SH30243.01, hyclone) for 1 hour at 37°C. The solution was filtered through a 70-μm cell strainer (352350, BD). Single cells suspensions were prepared after red blood cells were lysed. The cells were stained with allophycocyanin (APC)-VEGFR1 (FAB4711A, R&D), Alexa Fluor 488-CD49d (11-0492-81, eBioscience), PerCP-Cy5.5-Ly6G (560602, BD) antibodies according to manufacturer's instructions for cell sorting (BD) or flow cytometry analyses (BD). Neutrophils obtained from the bone marrow were treated with murine sera collected from control mice or CIA mice sacrificed on day 30, 36, 42. In order to investigate the effect of IL-17A on expression of CD49d, VEGFR1 on the neutrophils, murine neutrophils were co-cultured with murine serum from CIA mice sacrificed on day 30 solely, together with IL-17A antagonist (3 μg/ml)(MAB421, R&D) ; or stimulated with recombinant mouse IL-17A (10 μg/ml)(210 − 17, peprotech) for 8 hours.

Cells were harvested and washed twice with PBS, then stained with allophycocyanin (APC)-VEGFR1 (FAB4711A, R&D), Alexa Fluor 488-CD49d (11-0492-81, eBioscience), PerCP-Cy5.5-Ly6G (560602, BD)

antibodies for 20 mins in the dark at room temperature. Cells were analyzed by a FACS Calibur flow cytometer (BD). HL-60/ HL-60 $^{IL-17\ R-/-}$ either untreated or treated with recombinant human IL-17 (4 μg/ml)(96-200-17-25, Peprotech) for 8 hours and RA peripheral blood neutrophils were stained with allophycocyanin (APC)-VEGFR1 (R&D)(FAB321A, R&D), PE-CD49d (12-0492-82 ,eBioscience), PE-Cy7-CD15 (560827, BD) antibodies for 20 mins at room temperature. Flow cytometry was applied to analyse the positive cell counts and the mean fluorescence intensity (MFI) of VEGFR1in neutrophils.

Quantitative real-time polymerase chain reaction (RT-PCR).

Total RNA was isolated from sorting-purified $CD49d^{-}VEGFR1^{-}Ly6G^{+}$, $CD49d^{+}VEGFR1^{-}Ly6G^{+}$, $CD49d^{+}VEGFR1^{+}Ly6G^{+}$ cells and human FLS using TRIzol reagent (15596026, thermo). RNA (1μg) was reverse-transcribed by using PrimeScript RT reagent kit (RR047Q, Takara Biomedical). Quantitative real-time PCR was performed employing SYBR Premix Ex Taq (RR420A, Takara Biomedical) according to the manufacturer's protocol. The primers used are displayed in Table 2. The β-actin genewas used as reference housekeeping gene. The thermocycling programs consisted of 40 cycles at 95°C 2 min, 95°C 5 sec, and 60°C 10 sec for all reactions. The $2^{-\Delta\Delta Ct}$ expressing the fold change of the relative mRNA expression in the test samples was recorded.

Table 2
Primer sequences used in the real-time PCR experiment.

Gene	Forward	Reverse
mice		
IL18	GACAGCCTGTGTTCGAGGATATG	TGTTCTTACAGGAGAGGGTAGAC
VEGF	CTGCTGTAACGATGAAGCCCTG	GCTGTAGGAAGCTCATCTCTCC
CCL4	ACCCTCCCACTTCCTGCTGTTT	CTGTCTGCCTCTTTTGGTCAGG
CXCL10	GGATCCCTCTCGCAAGGA	ATCGTGGCAATGATCTCAACA
CXCL9	CGGACTTCACTCCAACACAG	TAGGGTTCCTCGAACTCCAC
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
IL1β	TGGACCTTCCAGGATGAGGACA	GTTCATCTCGGAGCCTGTAGTG
CCL3	ACTGCCTGCTGCTTCTCCTACA	ATGACACCTGGCTGGGAGCAAA
β-actin	CATTGCTGACAGGATGCAGAAGG	TGCTGGAAGGTGGACAGTGAGG
human		
MMP1	CCTGGAGGAAATCTT GCTCATGCT	GTCCAAGAGAATGGCCGAGTTCA
MMP3	CACCAGCATGAACCTTGTTCAGA	TCACCTCCAATCCAAGG AACTTCT
MMP13	CCTTGATGCCATTACCAGTCTCC	AAACAGCTCCGCATCAACCTGC
β-actin	GTCCACCGCAAATGCTTCTA	TGCTGTCACCTTCACCGTTC

RA-FLS invasion and migration assay.

For analysis of invasion, we used the Matrigel invasion chambers (pore size 8 μm) (354480, Corning) following manufacturer's instructions. The Matrigel (354234, Corning) (1:3 dilution in RPMI-1640) was added into the upper chambers and complete medium 1640 was added to the lower chamber. RA-FLS (5×10^4 cells) were seeded in the upper chamber and cultured for 19 hours.

To determine the migration, RA-FLS (3×10^4 cells) were seeded in the upper chambers (pore size 8 μm) (354480, Corning) in the presence of complete medium PRMI1640 in the lower chambers and incubated for 7 hours. The cells remaining in top membrane surface were completely removed with cotton swabs, and those that penetrated to the bottom chamber were fixed and stained with crystal violet reagent. The images were analyzed by direct light microscopy (Olympus).

Statistical analysis.

Descriptive data are presented as the means ± SEM. Differences between two groups were assessed by unpaired Student's t-tests. One-way ANOVA was applied for comparisons between more than two groups.

The Pearson's test was used to analyze correlations. All tests were two-tailed and conducted with 95% confidence. A p value of less than 0.05 was regarded as significant (*P < 0.05; **P < 0.01; ***P < 0.001). Statistical analyses were determined by using GraphPad Prism v.6, SPSS v.22.

Results

Neutrophils expressing CD49d expand in the early stage of CIA.

CIA mice had significantly higher clinical scores of arthritis and ankle thickness compared to control groups as early as day 28 (supplement Fig. 1A). Research evidence suggests an influential role of neutrophils in the pathogenesis of RA. In order to examine the heterogeneity of neutrophils in CIA, single cells solution was prepared from blood, bone marrow and ankle at the early, onset and remission stages of arthritis, respectively (day 30, 36, and 42, respectively; supplement Fig. 1B). Besides an increase in the percentage of neutrophils at the development phase of arthritis (Fig. 1A, B, C, E; supplementary Fig. 2A,B), flow cytometry analyses further indicated that there was a dramatically higher proportion of $CD49d^+$ neutrophil in blood, ankle and bone marrow on day 30 (supplementary Fig. 1C,E, supplementary Fig. 2C). Furthermore, we observed that the percentages of $CD49d^+VEGFR1^-$ neutrophils and $CD49d^+VEGFR1^+$ neutrophils, but not $CD49d^-VEGFR1^-$ neutrophils, were significantly higher than that in control mice at the early stage of arthritis (D30) (Fig. 1D, F,supplementary Fig. 1D,F and supplementary Fig. 2D), suggesting that these two neutrophil subsets may be involved in the early RA pathogenesis.

Gene expression analysis predicts distinct $CD49d^+VEGFR1^+$ neutrophil subset functions.

Key chemokines and cytokines that are differentially expressed by neutrophil subsets could predict distinct functions among neutrophil subsets (Fig. 2A). Therefore, we sorted neutrophil subsets including $CD49d^-VEGFR1^-$, $CD49d^+$ $VEGFR1^-$ and $CD49d^+$ $VEGFR1^+$ from the bone marrow of DBA/1 mice, and determined the transcriptional profile of key chemokines (CCL3, CCL4, CXCL9, CXCL10), cytokines (TNF-α, IL-18, IL-1β), MMP9 and VEGF in these three neutrophil subsets. We found that gene expressions of cytokines, including VEGF, TNF-α, IL-18, were significantly increased in $CD49d^+VEGFR1^+$ population (Fig. 2A), suggesting an elevated pro-inflammatory potential of this subset in inflammatory response of arthritis. Meanwhile, the $CD49d^+VEGFR1^+$ neutrophil subset was enriched in chemokines CCL3, CCL4, CXCL9 gene expressions (Fig. 2B). In contrast, $CD49d^+VEGFR1^-$ and $CD49d^-VEGFR1^-$ populations exhibited significantly lower mRNA levels of CCL3, CCL4, CXCL9, VEGF, TNF-α, IL-18 compared to $CD49d^+VEGFR1^+$ neutrophil subset (Fig. 1A, B). Taken together, these results suggest that $CD49d^+VEGFR1^+$ neutrophils have a distinctive pro-inflammatory transcriptional expression and may be positively involved in RA disease progression.

IL-17A induces expression of CD49d and VEGFR1 expression on neutrophils in vitro and in vivo.

In order to identify the factor responsible for enhancement of the $CD49d^+VEGFR1^+$neutrophil subset in the early stage of arthritis, we isolated neutrophil from mice bone marrow, and treated with culture

medium containing 5% of serum from CIA mice sacrificed on days 30, 36, 42 and naïve mice, respectively. In contrast, sera collected on day 30 significantly increased the frequency of D49d$^+$VEGFR1$^+$neutrophils, whereas no significant change of this subset was observed in other groups (Fig. 3A). To better understand the effect of CIA sera on CD49d and VEGFR1 expression in neutrophils, we determined IL-17A, IL-6, IL-10, and TNF-α concentrations in serum (Fig. 3B). Compared to the control group, cytokines levels in sera from CIA mice were dynamically changed. Importantly, unlike other cytokines, IL-17A level in sera from CIA mice showed a parallelly dynamic change as the frequency of CD49d$^+$ VEGFR1$^+$ neutrophils in blood after stimulation with sera from CIA mice sacrificed on day 30 (Fig. 3C).

IL-17A are known to have a pivotal role in the pathogenesis of RA [22], and secukinumab AIN457, a fully human antibody targeting IL-17A, is rapid and sustained symptom reductions in RA[23]. Meanwhile, IL-17A could modulate granulopoiesis and expression in neutrophils[24]. Therefore, we make a hypothesis that IL-17A maybe the key molecular factor enhancing CD49d$^+$VEGFR1$^+$neutrophils population. To test it we used neutralizing antibodies directed against IL-17A. The blockage of IL-17A significantly reduced the increase in CD49d$^+$VEGFR1$^+$ neutrophils population caused by serum from CIA mice (Day 30), demonstrating that IL-17A is responsible for the CD49d$^+$VEGFR1$^+$ neutrophils expansion in vitro (Fig. 3D). The direct effects of IL-17A on CD49d$^+$VEGFR1$^+$ neutrophils expansion were further confirmed by the increased proportions of CD49d$^+$VEGFR1$^+$ neutrophils in the presence of recombinant mouse IL-17A compared with IL-17A poor environment (Fig. 3E).

To determine whether IL-17A was essential for CD49d$^+$VEGFR1$^+$neutrophils expansion in vivo, we determined this subset change in IL-17A deficient mice with CIA. Compared to WT group, IL-17A-deficient mice were completely protected against the development of CIA (supplementary Fig. 3A). Meanwhile, we observed that the loss of IL-17A signaling led to a significant decrease in the abundance of CD49d$^+$VEGFR1$^+$ neutrophils in blood compared to WT mice (supplementary Fig. 3. B, C), which is consistent with the in vitro results using IL-17A-neutralizing antibodies (Fig. 3D). Taken together, above data demonstrate IL-17A contributes to the increase of CD49d$^+$VEGRF1$^+$ neutrophils population in CIA.

CD49d$^+$VEGFR1$^+$neutrophils localize in the synovium and contribute to synovium pathogenesis.

Synovial hyperplasia are important mediators of end-organ pathology in RA. It is known that synovial hyperplasia is mediated by various infiltrating cell types. The increase in the proportion of CD49d$^+$VEGFR1$^+$ neutrophils at the early stage of CIA suggest that they might be one of the first infiltrating cell types into the synovium, inducing subsequent pathological changes in RA joint. Firstly, we applied H&E staining to profile the abundance of neutrophils in knee excised from D30 CIA mice. Compared to the control group, neutrophils in knees from CIA mice were significantly increased as we expected (Fig. 4A).

Next, we determined whether CD49d$^+$VEGFR1$^+$ neutrophils are localized in the synovium and contribute to synovial hyperplasia. Consistent with the percentage change of CD49d$^+$VEGFR1$^+$ neutrophils in the blood,

this subset was solely observed in synovium of CIA mice sacrificed on day 30 (Fig. 4B). Then, we set up neutrophil and RA FLS co-culture system in vitro to detecte the impact of $CD49d^{+}VEGFR1^{+}$ neutrophils on FLS biological function. The human leukemia cell line HL-60 can be differentiated into neutrophil-like and has been used to study neutrophil functions[25]. Differentiated HL-60 (dHL-60) cells, expressing high level of CD49d, were used in our system (Fig. 4C). Though, VEGFR1 expression on dHL-60 was low, it's expression was significantly enhanced when dHL-60 was primed with IL-17A($P < 0.05$) (Fig. 4C). In contrast, no significant change in MFI of VEGFR1 was observed on dHL-60 $^{IL-17R-/-}$ treated with IL-17 (Fig. 4C), demonstrating that IL-17 signaling is indispensable for VEGFR1 expression on $CD49d^{+}$ neutrophils.

Next, to confirm the effect of cell–cell interactions in vitro, RA FLS were isolated and co-cultured with $CD49d^{+}VEGFR1^{+}$ neutrophils. The ability of $CD49d^{+}VEGFR1^{+}$ neutrophils to enhance the migration and invasion potential of FLS was tested using cell migration and invasion assays. Co-culturing with IL-17-primed dHL-60 was able to enhance RA FLS migration and invasion ability, evidence by much higher numbers of FLS migrating through the transwell chambers, compared to RA FLS co-cultured with unstimulated dHL-60 ($P < 0.05$, Fig. 4D).

In addition, MMP3 and13 genes, which are critical for invasion of RA FLS, were also upregulated when RA FLS ared co-cultured with IL-17-primed dHL-60 ($P < 0.05$)(Fig. 4E). ELISA further confirmed the higher levels of MMP3 and 13 in the supernatant of RA FLS co-cultured with IL-17 primed dHL-60 compared with other groups(Fig. 4F). Taken together, these results indicate that this neutrophil subset expressing CD49d and VEGFR1 is able to elevate FLS' MMPs production, leading to the increases in their invasive and migration potential.

Correlation between serum IL-17A and the frequency of $CD49d^{+}VEGFR1^{+}$ neutrophils in patients with RA.

To test whether $CD49d^{+}VEGFR1^{+}$neutrophils can also be found in RA patients, we applied flow cytometry to measure the frequency of $CD49d^{+}VEGFR1^{+}$ neutrophils in the peripheral blood from RA patients. Consistent with the findings in CIA mice, $CD49d^{+}$ neutrophils also showed a higher mean fluorescence intensity (MFI) of VEGFR1 in blood from RA patients compared to healthy donors (Fig. 5A). In addition, the level of serum IL-17A in RA patients was significantly higher than that of healthy donors (Fig. 5B). The Pearson correlation analysis was performed to examine whether IL-17A and disease activity were associated with $CD49d^{+}$ $VEGFR1^{+}$ neutrophils. The MFI of VEGFR1 on $CD49d^{+}$ neutrophils were positively correlated with IL-17A levels (Fig. 5C), but had no significant correlation with Disease Activity Score 28 (DAS 28) and C-Reactive Protein (CRP) (supplementary Fig. 4A, B). We further detected the $CD49d^{+}VEGFR1^{+}$ neutrophils in the synovial fluid from RA patients (n = 9) with knee joint effusion using flow cytometry. Concordant with the upregulation of $CD49d^{+}VEGFR1^{+}$ neutrophils in RA blood, $CD49d^{+}VEGFR1^{+}$ neutrophils were also detected in synovial fluid from RA patients (Fig. 5D). These results further indicate that Il-17 is highly associated with the increase of circulating $CD49d^{+}VEGFR1^{+}$ neutrophil in RA patients.

Discussion

In the early stage of RA, large numbers of neutrophils accumulate within articular cavity where they contribute to the pathogenesis of RA[7]. Meanwhile, neutrophils are well known for their diverse phenotypes and functions in different contexts. However, it is largely unknown about the effects of unique neutrophil subsets on RA pathogenesis. Here, we explored a distinct $CD49d^{+}VEGFR1^{+}$ neutrophil subset whose proportion was dynamically changed along with the disease development in blood and joints of CIA mice, and particularly reached the peak at the early stage of disease. In addition, this neutrophil subset expansion was to a large extent depend on serum IL-17A level, and displayed distinct gene expressions of pro-inflammatory cytokines and chemokines. More importantly, these neutrophil subsets localize in the synovium of CIA mice in the early phase of arthritis, and are able to promote the migration and invasion ability of FLS via up-regulating MMP3 and 13 production (Fig. 6). This study provides new evidence for the implication of specific neutrophil subset involved into synovium pathogenesis in CIA mice.

Studies have demonstrated that cytokines and chemokines secretion from cells recruited into the joint contribute to a more proinflammatory environment and enhance tissues damage and development of RA[26]. There are high concentrations of CCL3, CCL4, CXCL9, VEGF, IL-18 and TNF-α in the synovial tissue and fluid from RA patients[27–30]. Among these factors, CCL3, CCL4, and CXCL9 play vital roles in recruiting and activating monocytes, lymphocytes and neutrophils[31–34]. Additionally, TNF-α and IL-18 are known proinflammatory cytokines which play critical roles in the progression of RA by inducing immune cells activation, synovitis pannus formation, and bone erosion[35–37]. Although the percentage of $CD49d^{+}$ $VEGFR1^{-}$ neutrophils and $CD49d^{+}$ $VEGFR1^{+}$ neutrophils were shown to dynamically changed than that in control mice at the early stage of arthritis, $CD49d^{+}$ $VEGFR1^{+}$ neutrophils have more specific transcriptional profile, with much higher mRNA expressions of CCL3, CCL4, CXCL9, TNF-α and IL-18. This indicates $CD49d^{+}VEGFR1^{+}$ neutrophils, instead of $CD49d^{+}$ $VEGFR1^{-}$ and $CD49d^{+}$ $VEGFR1^{+}$ neutrophils, might exert more important functions to trigger inflammatory cells trafficking and aggravate the inflammatory status in RA. However, more studies are warranted to address this point.

IL-17A, which was first described in 1993[38], is produced by innate or adaptive immune cells and characterised by inducing the production of chemokines and other cytokines which initiate neutrophil mobilization and recruitment[39] at the site of T cell activation[40], increase the granulopoiesis and product the antimicrobial peptides[41] [42]. More importantly, IL-17A is involved in the activation of neutrophils[43].This study demonstrates that the increased level of serum IL-17 is directly associated with the increased expression of $CD49d^{+}VEGFR1^{+}$ neutrophils in experimental arthritis model and RA. Ex vivo co-culture experiments with bone marrow neutrophils and arthritic sera showed that the raise in the $CD49d^{+}VEGFR1^{+}$neutrophil induced was likely driven by IL-17A, the most biologically active cytokine detected in serum in the early phase of CIA, and IL-17A inhibition also specifically targeted this neutrophil subset. Moreover, in addition of IL-17A-stimulated $CD49d^{+}VEGFR1^{+}$neutrophil appearance, we also observed the reduced frequencies of these neutrophils in blood from IL-17A-deficient mice which were

protected from the development of overt arthritis. In addition, there was a significant positive correlation between serum IL-17A and high frequencies of $CD49d^{+}VEGFR1^{+}$neutrophils in RA patients, further demonstrating the importance of IL-17A signaling to the appearance of the $CD49d^{+}$ $VEGFR1^{+}$neutrophils.

Kinetic analyses of CD49d, VEGFR1 expression on BM, blood and ankle neutrophils indicated that $CD49d^{+}VEGFR1^{+}$ neutrophils might be recruited to ankles at the early stage of RA. This point was indeed proved by immunofluorescence staining of joints from CIA mice, showing the infiltration of $CD49d^{+}VEGFR1^{+}$ neutrophils in the synovium. In RA patients, we also found $CD49d^{+}VEGFR1^{+}$ neutrophils increased exprssion both in the peripheral blood and synovial fluid. Fibroblast-like synovial cells (FLS), the primary cells in synovium tissue, produce cytokines, chemokines, and matrix metalloproteinases (MMPs) that invade adjacent articular cartilage and subchondral bone, causing RA damage[44]. Additionally, FLS can enhance inflammatory chemokines expression to recruit inflammatory cells within the inflamed joint[45]. Current studies report accessing leukocyte subpopulations infiltrated in RA synovium are interacted with FLS, leading to the biological behavior changes of FLS and the consequential articular structures destruction[44]. Synovial joint neutrophils can form neutrophil extracellular traps (NETs), and promote FLS to synthesize various proinflammatory cytokines and chemokines and upregulate the expression of MHC Class II (MHCII)[46, 47]. In this study, we indicate that $CD49d^{+}VEGFR1^{+}$ neutrophils enhance the migratory and invasive behavior of FLS from patients with RA. Importantly, when co-cultured with $CD49d^{+}VEGFR1^{+}$ neutrophils, RA-FLS could release the higher levels of MMP3 and 13. MMPs, a family of Zn^{2+}-containing enzymes, are involved in cartilage invasion and bone destruction in RA. MMP3 and 13 present in RA synovium contribute considerably to the articular cartilage destruction[48, 49]. Collectively, these data suggest that $CD49d^{+}VEGFR1^{+}$ neutrophils may accelerate the synovium pathological progress in RA by activating FLS and the subsequent MMP3 and 13 secretion.

Taken together, our findings suggest that $CD49d^{+}VEGFR1^{+}$neutrophils expansion is in a IL-17A-dependent manner, and this subset displays distinct mRNA expressions of pro-inflamamtory cytokines and chemokines. Importantly, they are involved in the pathogenesis of RA by enhancing FLS migration and invasion activity in the early stage of arthritis. Based on our study, the inhibition of the neutrophil subset $CD49d^{+}VEGFR1^{+}$ may be a potential target for blocking synovium pathogenesis and bone destruction in the early phase of RA.

Abbreviations

RA:rheumatoid arthritis; CIA:Collagen-induced arthritis; BM: bone marrow; HC:healthy individual; FLS: fibroblast like-synoviocytes; PMNs: Polymorphonuclear neutrophils; VLA-4:very late antigen-4; MMPs: matrix metalloproteinases; dHL-60: Differentiated HL-60; MFI: mean fluorescence intensity; CRP: C-Reactive Protein ; DAS 28: Disease Activity Score 28; RT-qPCR: Reverse transcription quantitative polymerase chain reaction; HE: Hematoxylin and eosin; ELISA: enzyme linked immunosorbent assay.

Declarations

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Author contributions

YHL and YBL contributed to the conception and design of the study, and wrote the paper. YHL, YBL, YZ, LYC, HY and QPZ carried out the principal experiments. YHL and YB completed the acquisition or preparation of clinical samples. YHL, TC and YBL contributed to the analysis and interpretation of the data. YZ, YL and Luis E. Muñoz contributed to the critical revision for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during the current study are available.

Ethics approval and consent to participate

The study was approved by the Biomedical Research Ethics Committee, West China Hospital of Sichuan University (ChiCTR1900027947) Chengdu, China, and the written consents were obtained from all the participants according to the Declaration of Helsinki. All animal care and experimental procedures strictly adhered to the guidelines of the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by the ethics committee of University of Sichuan University, Chengdu, China.

Competing Interests

The authors have declared that no conflict of interest exists.

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Figures

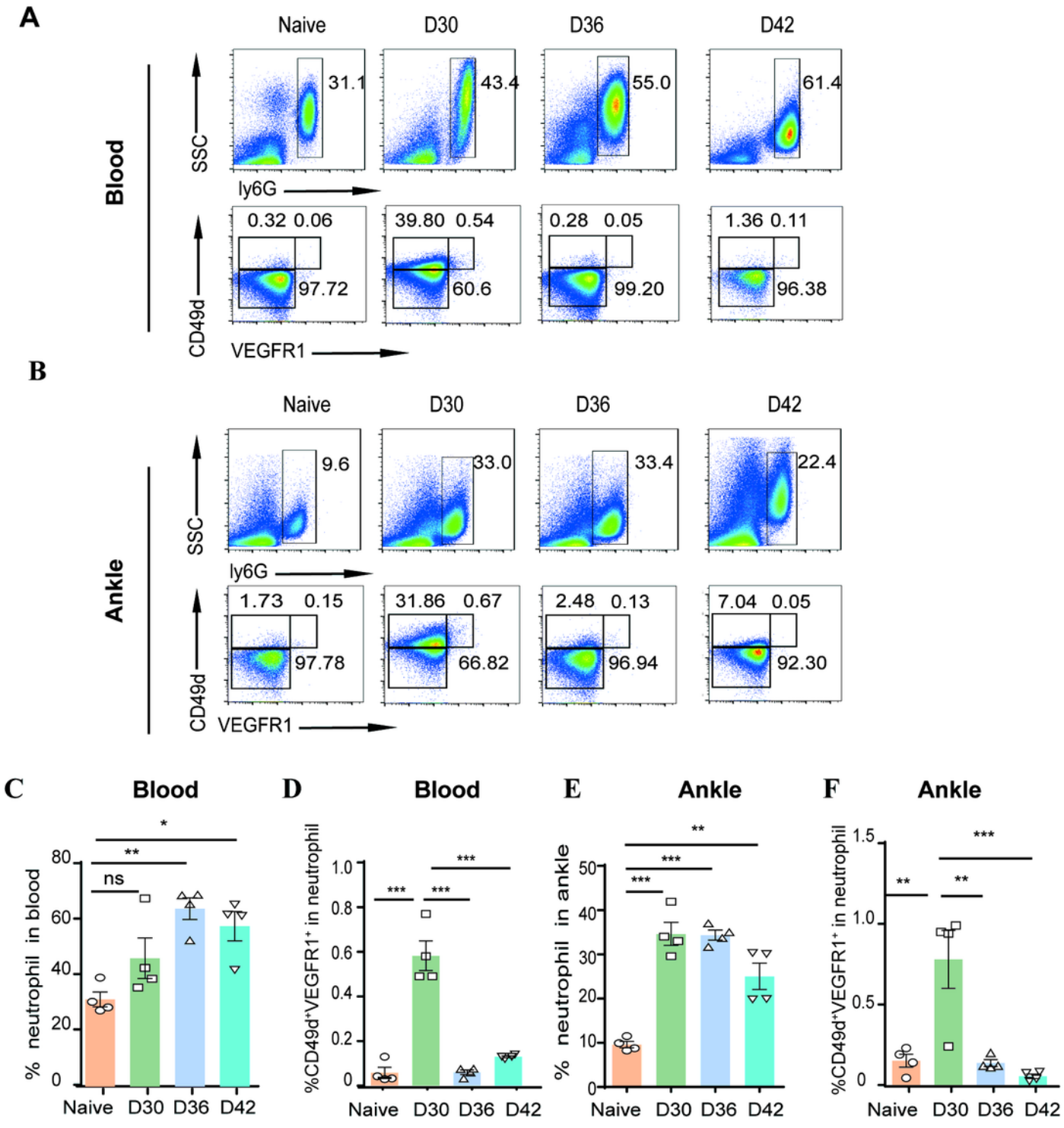

Figure 1

Dynamic changes in the percentages of neutrophil subsets in CIA model.

A and B. Representative flow cytometry plots of CD49d^{+}VEGFR1^{+}neutrophil, CD49d^{+}VEGFR1^{-} neutrophil, CD49d^{-}VEGFR1^{-} neutrophil subpopulation in peripheral blood (A) and ankle (B). C and E. Proportions of neutrophil in peripheral blood (C) and ankle (E), D and F.CD49d^{+}VEGFR1^{+} neutrophil subpopulation in peripheral blood (D) and ankle (F) from naïve and CIA mice sacrificed on D30, 36, 42. (n=4 for each time point). All values are expressed as mean ± SEM. *P < 0.05; **P < 0.01, ***P < 0.001, and n.s., no significant.

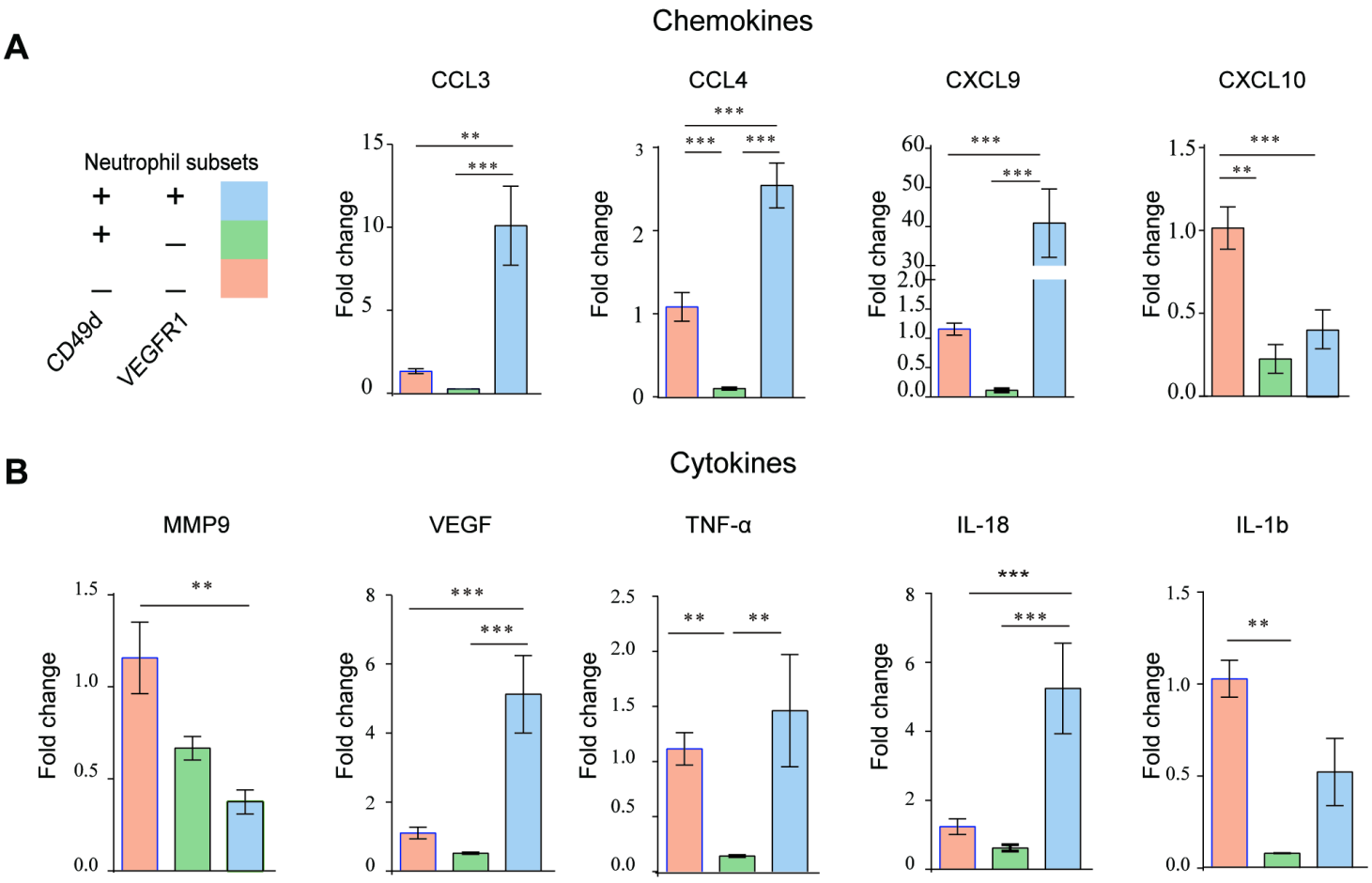

Figure 2

Differences in genes expression between neutrophil subsets.

Genes expression of chemokines (A) and cytokines (B) in CD49d^{-}VEGFR1^{-}, CD49d^{+}VEGFR1^{-} and CD49d^{+}VEGFR1^{+} neutrophil subsets determined by RT-PCR. Data are expressed as mean ± SEM. *P < 0.05; **P < 0.01, ***P < 0.001, and n.s., no significant.

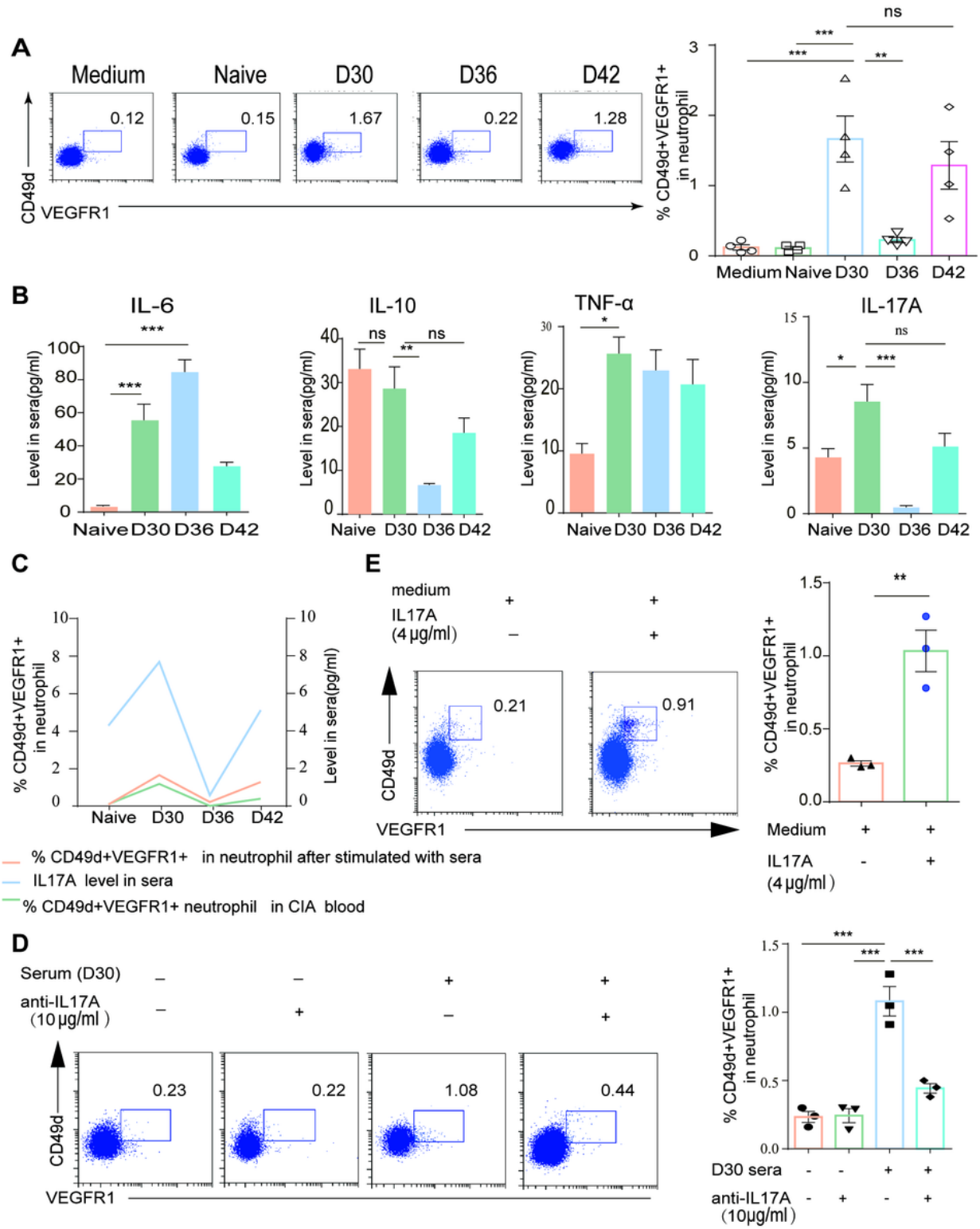

Figure 3

Elevated IL-17A is associated with higher expression of CD49d and VEGFR1 on neutrophils.

A. Neutrophils purified from DBA/1 mice bone marrow were stimulated with 5%

serum collected from naïve and CIA mice sacrificed on D30, 36, 42 for 8 hours. Representative plots and quantification of $CD49d^{+}VEGFR1^{+}$ neutrophils. B. Concentrations of serum IL-17A, IL-6, IL-10, TNF-α assessed by CBA. C. A parallel change between serum IL-17A concentration and the frequency of $CD49d^{+}VEGFR1^{+}$neutrophil subpopulations in peripheral blood of CIA mice. D. Representative plots and quantification of the frequency of $CD49d^{+}VEGFR1^{+}$ subset in neutrophils isolated from bone marrow in response to 5% D30 serum, medium or serum neutralized by anti-IL-17A antibodies (10 μg/ml). E. Representative plots and quantification of the frequency of $CD49d^{+}VEGFR1^{+}$ subset in bone marrow neutrophils after stimulation with medium or recombinant mouse IL-17A (4 μg/ml) for 8 hours. All values are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and n.s., no significant.

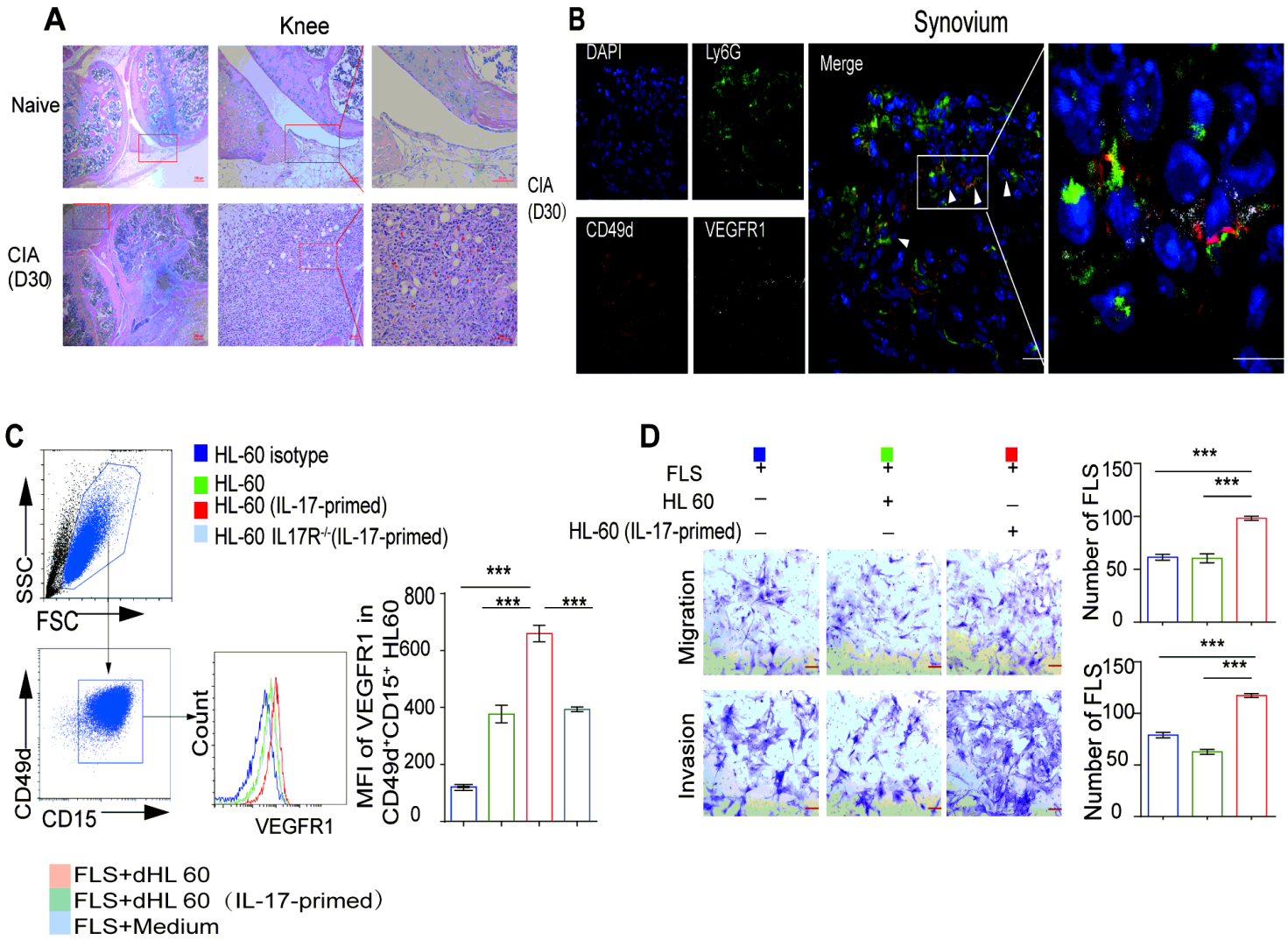

Figure 4

The migration and invasive potential of FLS increased after co-cultured with CD49d$^+$VEGFR1$^+$neutrophils.

A. Microphotograph of knee excised from naïve and CIA mice sacrificed on Day 30

stained with H&E (4 × magnification). Neutrophils infiltrated in synovial tissues (red arrows). B. Representative microphotographs of CD49d, VEGFR1, and Ly6G immunofluorescence staining of synovial tissue from D30 CIA mouse's knee (Scale bars, 50 μm). CD49d$^+$VEGFR1$^+$neutrophils infiltrate

synovial tissues (white arrows). DAPI: blue, VEGFR1: white, CD49d: red, ly6G: green. C. Gating strategy and representative flow cytometry histograms of VEGFR1 expression on dHL-60 and dHL-60$^{17R-/-}$ (IL-17 receptor deficient) cells stimulated either with recombinant human IL-17 (4 μg/ml) or with medium for 8 hours. D. The migration and invasion potential of RA-FLS in co-culture with dHL-60 which were pretreated with or without IL-17 (RA-FLS, RA-FLS+dHL-60, RA-FLS+IL-17 primed dHL-60). E. Gene expression of MMP 1, MMP 3 and MMP 13 in RA FLS after co-cultured with medium, dHL-60, or IL-17 primed dHL-60. F. Quantification of released MMP 3 and MMP13 levels in the suspension in three groups (RA FLS co-culture with medium, dHL-60, or IL-17 primed dHL-60) by ELISA. Data are expressed as mean ± SEM. n = 3 in each group. n=3 independent experiments. n.s., no significant; *P < 0.05 and **P < 0.01.

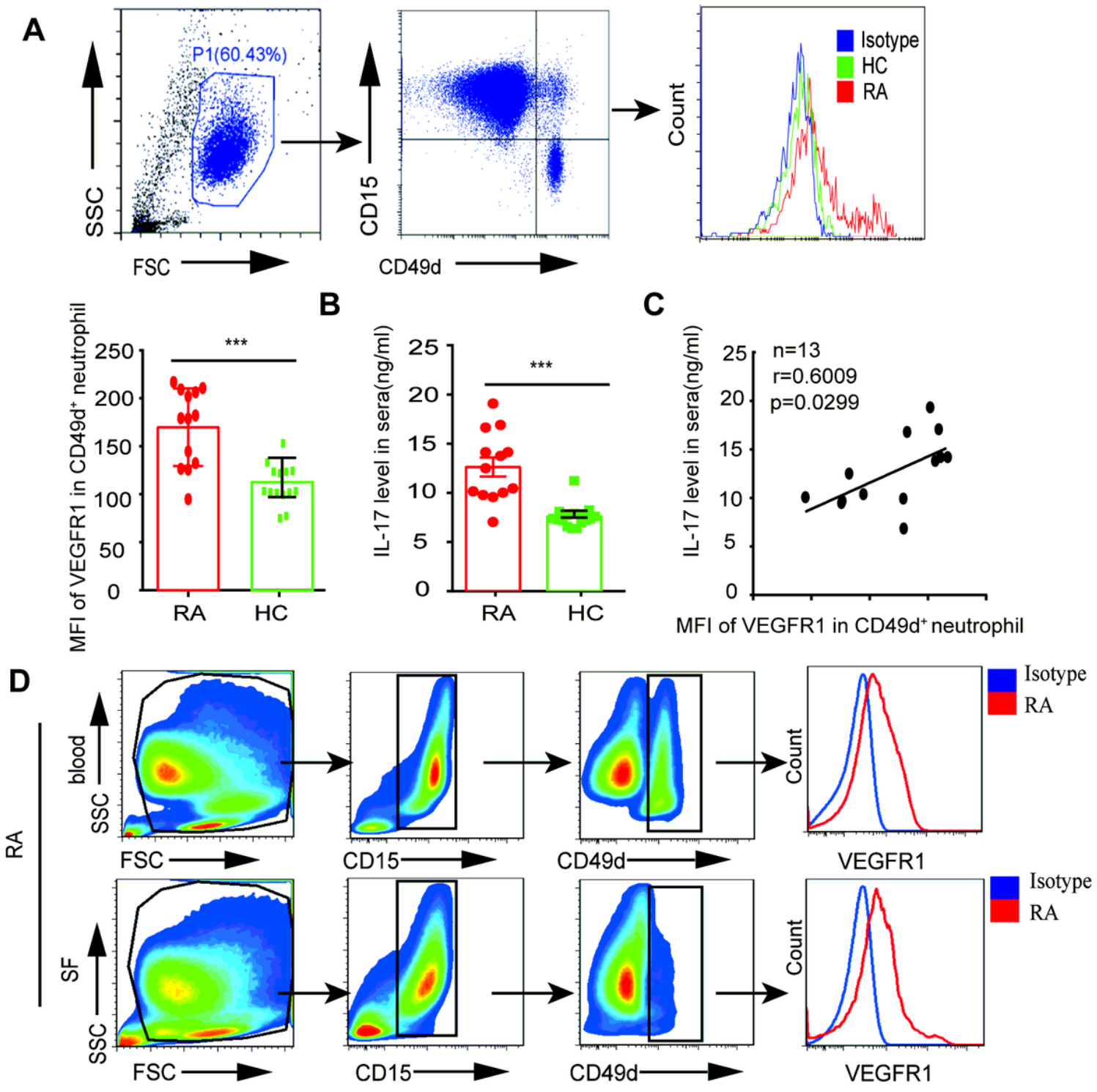

Figure 5

The expression of CD49d and VEGFR1 in neutrophils from RA patients correlates with serum IL17 level.

A. Representative flow cytometry plots and gating strategy of human neutrophils

isolated from blood from RA patients (n =13) and healthy controls (HC, n =14). Representative histograms and quantification of the mean fluorescence intensities (MFIs) of the VEGFR1 expression on

CD49d$^+$ neutrophils. B. Concentrations of IL-17 in sera. C. Correlation between the concentration of IL-17 and the MFI of VEGFR1 expression on CD49d$^+$ neutrophils of RA patients. D. Representative flow cytometry plots and gating strategy of human neutrophils isolated from blood and synovial fluid from RA patient. Each data point represents an individual case. All values are expressed as mean ± SEM. *P < 0.05; **P < 0.01, ***P < 0.001, and n.s., no significant.

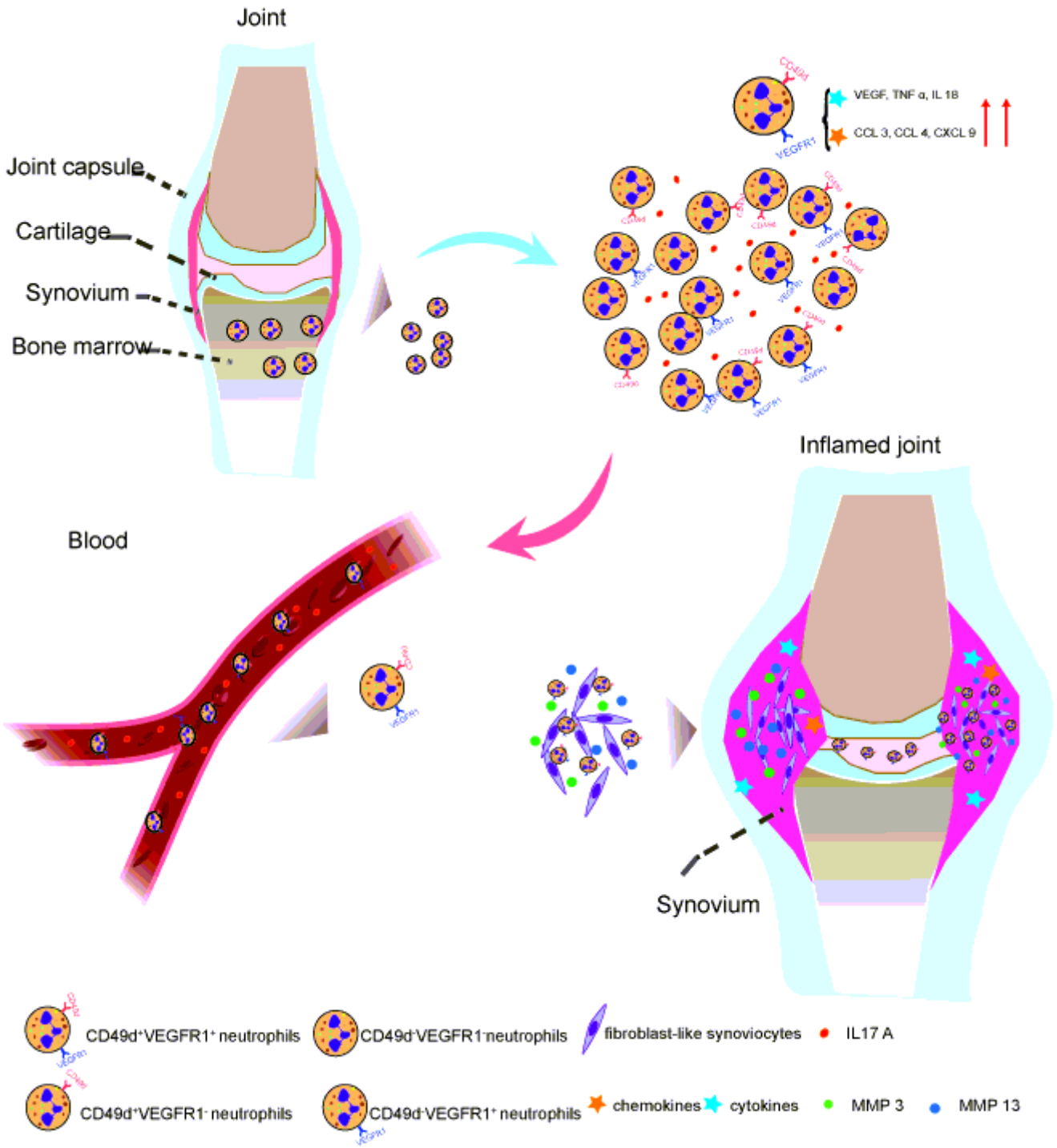

Figure 6

Schematic illustration of the role of $CD49d^{+}VEGFR1^{+}$ neutrophils in the pathogenesis of RA.

$CD49d^{+}VEGFR1^{+}$neutrophils proportion was increased in a IL-17A-dependent manner in bone marrow during the early stage of RA, and they produce the distinct genes of cytokines (VEGF, IL-18 and TNF-α) and chemokines (CCL3, CCL4 and CXCL9). $CD49d^{+}VEGFR1^{+}$ neutrophils migrate into the inflamed synovium, and enhance FLS's migration and infiltration activity and MMP3 and 13 productions, which contributing to the synovium pathogenesis of RA.

Supplementary Files

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