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ORIGINAL RESEARCH

Qing-Luo-Yin Eases T Cells-Mediated Angiogenesis in Adjuvant-Induced Arthritis Rats by Activating PPARγ

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Introduction: Qing-Luo-Yin (QLY) is an anti-rheumatic herbal formula potentially activating PPARγ. The study investigated if and how this property contributes to its anti-angiogenesis effects.

Methods: Adjuvant-induced arthritis (AIA) rats were orally treated by QLY or rosiglitazone (a PPARγ agonist), and their monocytes and lymphocytes were co-cultured reciprocally in vitro with different sera. Healthy littermates received blood transfusion from QLY-treated or AIA model rats. Two days ahead of sacrifice, a matrigel plug was implanted in the recipients. AIA serum-incubated THP-1 monocytes and Jurkat T cells were treated by a mixture comprised sinomenine, berberine and palmatine. Jurkat T cells-related media and T0070907 were used to stimulate human umbilical vein endothelial cells (HUVECs).

Results: QLY and rosiglitazone similarly alleviated joint injuries, synovial angiogenesis and metabolic disorders in AIA rats. Although QLY impaired inflammatory phenotype of AIA rat monocytes in vivo, it cannot be achieved or sustained in vitro. Lymphocytes of QLY-treated AIA rats had a weak inflammatory phenotype and failed to induce inflammatory polarization of monocytes. AIA blood-induced angiogenesis in the matrigel plug, a phenomenon invisible in QLY group. QLY therapy inhibited pathogenic functions of AIA rats' lymphocytes, shown by changes of cytokines network in the recipients' joints, where these cells accumulated. The related compounds affected secretion of Jurkat T cells cultured in AIA serum, which lost the potential in activating HUVECs. This effect disappeared in presence of T0070907, a PPARγ inhibitor.

Conclusion: Angiogenesis amelioration during QLY therapy is an indirect result from PPARγ activation-caused functional changes of T cells.

Keywords: rheumatoid arthritis, Traditional Chinese Medicine, immune, monocyte, vascular endothelial cell, angiogenesis

Introduction

The successful treatment of rheumatoid arthritis (RA) is very challenging due to its elusive etiological mechanism. Many pathogenic factors contribute to its progress and lead to arthritic manifestations. Synovitis is the driving force underlying RA-related structural degradation in joints.[1] In addition to synovial invasion, angiogenesis fuels this symptom directly.[2] Therefore, synoviocytes and vascular endothelial cells are ideal therapeutic targets. Despite many studies have validated this idea, no such drugs are introduced into clinical practice.[3,4] A fundamental reason is that RA pathogenesis is driven by immune hyper-activation rather than specific local changes.[1] Various outcomes from effective anti-RA therapies can be explained by improved immune status, and immune-regulating therapies bring more benefits than the above strategy.

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Thanks to accumulating knowledge about the immune characteristics of RA, biological agents flourish.[5] Compared to conventional drugs, they exhibit obvious clinical advantages by targeting immune cells and signals controlling their differentiation. But the biological agents are usually unaffordable in developing countries. Herbal medicines are used as alternatives to these drugs worldwide. They might be even more effective, benefited from complex chemical composition and multiple therapeutic targets, despite these advantages are largely unexplored. As a representative, Traditional Chinese Medicine (TCM) formula Qing-Luo-Yin (QLY) has been successfully used for over 4 decades. Its therapeutic efficiency was tested by clinical and pharmacological researches.[6–10] It showed eye-catching effects on synovial angiogenesis.[11] A great endeavor is devoted to clarify its anti-rheumatic mechanism, and we have known that SIRT1/PPARγ is one of its molecular targets.[9,10]

Interestingly, SIRT1 and PPARγ function as a pair of rivals, from both the immune and metabolism perspectives. Furthermore, they negatively regulate each other's expression.[12,13] QLY shows potentials in inhibiting SIRT1 and activating PPARγ.[10] Since SIRT1 is generally recognized as a negative regulator of inflammation, its effects on PPARγ would bring more benefits. Actually, PPARγ expression/function deficiency is common in rheumatic subjects.[13] It does not only affect metabolism but also reshapes status of the immune system, as PPARγ down-regulation aggravates imbalanced differentiation of immune cells.[13,14] The factors make PPARγ an anti-rheumatic target. Many compounds with PPARγ-activating potentials have been confirmed with beneficial effects on RA. Rosiglitazone (RSG) is the most well-known representative.[15] Thanks to pleiotropic functions of PPARγ, the related treatments cause series of improvements, including angiogenesis inhibition.[15,16] Given the crucial impacts of immune cells on RA pathology, these anti-angiogenesis effects would be largely immune-relevant.

According to these clues, it is highly possible that QLY eases immune disruption-related angiogenesis by activating PPARγ. This hypothesis was tested here. We further ascertained involvement of PPARγ activation during QLY treatment on adjuvant-induced arthritis (AIA) rats by comparing its effects to RSG. Furthermore, we identified T cells as its preferential target, and revealed that PPARγ agonists in QLY improved the overall immune environment and consequently inhibited synovial angiogenesis via this approach.

Materials and Methods

Chemicals and Reagents

Incomplete Freund's Adjuvant (IFA) and Bacillus Calmette-Guérin (BCG) were purchased from Chondex (Redmond, WA) and Rebio Scientific (Shanghai, China), respectively. ELISA kits of rheumatoid factor (RF), hypoxia inducible factor-1 (HIF-1) and arginase-1 (ARG-1) were supplied by Jianglai Biotechnology (Shanghai, China). ELISA kits of interleukin 1 beta (IL-1β), IL-6, IL-10, IL-17A, transforming growth factor beta1 (TGF-β1), interferon gamma (IFNγ), vasoactive endothelial growth factor (VEGF), intercellular cell adhesion molecule 1 (ICAM-1), C-X-C motif ligand 1 (CXCL1), monocyte chemotactic protein 1 (MCP-1) and insulin-like growth factor 1 (IGF-1) were supplied by Multi-Science (Hangzhou, Zhejiang, China). Quantification kits of nonestesterified fatty acid (NEFA), glucose, lactic acid, pyruvic acid, triglyceride (TG), reduced glutathione (GSH), malonaldehyde (MDA), inducible nitric oxide synthase (iNOS), catalase (CAT), NADH oxidase (NOX), alkaline phosphatase (AKP) and aspartate aminotransferase (AST) were bought from Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). ReverAid first-strand cDNA synthesis kit and universal qPCR master mix were purchased from Thermo Fisher Scientific (Rockford, IL) and New England Biolabs (Ipswich, MA), respectively. Primary β-ACTIN, GAPDH, TLR4, STAT3, P-STAT3, JNK, p-JNK, CPT1A, CD36, PPARγ and ATGL antibodies were obtained from ABclonal Technology (Wuhan, Hubei, China) or Affinity Biosciences (Changzhou, Jiangsu, China). HRP-conjugated secondary antibodies were purchased from Boster Biological Technology (Wuhan, Hubei, China). Red donkey anti-rabbit and orange donkey anti-mouse secondary antibodies were bought from Abbkine (Wuhan, Hubei, China). PE-tagged CD172a, APC-tagged CD43, PE-tagged CD16, FITC-tagged CD3, PE-tagged CD4 antibodies were procured from BioLegend (SanDiego, CA) or Invitrogen (Carlsbad, CA). Culture medium, polyformaldehyde and crystal violet staining solution were procured from Biosharp (Beijing, China). Fetal bovine serum (FBS) was the product of Wisent (Montreal, Quebec, Canada). Pure compounds sinomenine, berberine, and palmatine were purchased from Bencao Yikang (Nanjing, Jiangsu, China). PPARγ selective

inhibitor T0070907 was purchased from Aladdin (Shanghai, China). Rat peripheral blood lymphocyte isolation kit and rat/human peripheral blood monocyte isolation kit were procured from Solarbio Biotech (Beijing, China). Fluorescent dye CFDA-SE and matrigel were bought from MedChemExpress (Monmouth Junction, NJ) and Thermo Fisher Scientific (Rockford, IL), respectively.

QLY Extract Preparation

QLY is composed of 4 ingredients (Kushen, Qingfengteng, Huangbo and Bixie, 1.5:1.2:1:1). Its composition and herbal resources are detailed in Table 1. The crude drugs were purchased from Bozhou Herbal Medicine Market (Anhui, China) and authenticated by Prof. Jian Zuo (Wannan Medical College). Their voucher specimens were stored in the Herbarium Center of Wannan Medical College (ID: 2022–013). These herbal ingredients were underwent reflux extraction with 95% ethanol twice with a weight ratio of 1:5. Afterwards, the residues were extracted by boiling water for one time. The combined filtrates were dried. Finally, a sticky extract with a relative density about 1.2 kg/m^3 was obtained.

General Design of in vivo Experiments

In the first experiment, AIA rats were orally treated by QLY extract to observe its therapeutic effects and clarify the relevance to PPARγ activation. In the second experiment, whole blood of QLY-treated or model AIA rats was transfused into healthy recipients. Based on the latters' responses, we aimed to consolidate the conclusion that QLY impairs pathogenic properties of rheumatic subject's immune cells. These experiments were based on Wistar rats, which were supplied by Tianqin Biotech (Changsha, Hunan, China). To avoid gender-related interference, AIA models were developed selectively using male rats. In the blood transfusion experiment, the male donators and female recipients were littermates to avoid graft rejection and ensure survival of transplanted immune cells. All the rats were kept in a qualified SPF lab and fed by commercial rodent chow and boiled tap water. They were acclimated at least for 1 week and reach 8-week-old before any experiments. Invasive operations were conducted under an unconscious status, and these animals were anesthetized by an intraperitoneal injection of pentobarbital sodium (30 mg/kg). The greatest efforts were devoted to minimize experimental animals' suffering.

AIA Induction and Treatments

A stable emulsion of complete Freund's adjuvant (CFA) was prepared by finely grounding IFA and heat-inactivated aqueous BCG suspension in a glass mortar. The concentration of BCG was 10 mg/mL. Those rats in model and treatment groups were intracutaneously injected with 0.1 mL of CFA at planter surface of the right hind paw. The date was recorded as day 0. Since then, the rats were treated by various agents based on the following grouping: healthy controls (normal, 0.5% CMC-Na), AIA controls (AIA, 0.5% CMC-Na), QLY-treated AIA rats (QLY, QLY extract in 0.5% CMC-Na, equivalent to 10 g/kg of crude drugs), and RSG-treated AIA rats (RSG, RSG in 0.5% CMC-Na, 2 mg/kg). All treatments were administrated by gavage once a day (about 7–8 pm). During this treatment period of 30 days, arthritis scores and body weights were periodically recorded.[6–10]

Therapeutic Outcomes Evaluation

One hour after the last administration, about 4 mL of blood was collected through abdominal aorta of the rats before hemorrhage-caused euthanasia, and plasma and serum were prepared by centrifugation. Levels of cytokines, metabolites and diagnostic indicators in blood were detected by commercial quantification kits. Monocytes and lymphocytes were

Table 1 The Formula Composition of QLY

Herbal Drug	Chinese Name	Plant Source
Radix *Sophorae flavescentis*	Kushen	*Sophora flavescens* Aiton
Caulis *Sinomenium acutum*	Qingfengteng	*Sinomenium acutum* (Thunb.) Rehd. Et Wils.
Cortex *Phellodendri chinensis*	Huangbo	*Phellodendron chinense* C. K. Schneid.
Rhizoma *Dioscoreae hypoglaucae*	Bixie	*Dioscorea hypoglauca* Palibin

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prepared using anti-coagulation blood using isolation kits. Blood serum and immune cells were used in vitro experiments. The liver, spleen and hind paws were dissected and weighted. The joints were fixed in 10% formalin and decalcified by EDTA for 3 weeks. After being dehydrated with gradient ethanol, they were embedded in paraffin and cut at a thickness of 5 μm. The slices were mounted on glass slides, dewaxed, rehydrated and dyed with Hematoxylin–Eosin (H&E) or Safranine O-Fast Green (SOFG). Cartilage erosion and synovial hyperplasia were observed using an Olympus BH-2 microscope. The livers were homogenized in ice-cold normal saline by a handheld homogenizer. Metabolites and cytokines in the homogenates were detected by the corresponding kits. Expression of immune and metabolism-related proteins in the liver and spleen was studied by WB method using routine procedures.

Assessment of QLY Therapy-Caused Impacts on Monocytes and T Cells in vivo

Monocytes/macrophages and T cells are accountable for the majority of pathological changes in RA.[1] QLY therapy potently affects their differentiation and function, leading to improvement of the immune milieu.[6–10] We validated this conclusion here. Distribution of monocyte subsets was characterized by a flow cytometry (FCM) method. Whole blood was spiked into red cells lysis buffer, incubated, and centrifuged. The precipitates were re-suspended and incubated with fluorescein-tagged antibodies (APC-CD43 and PE-CD172a) in the dark. These stained cells were washed by PBS, filtered, and fed to a BD FACSVerse instrument. Some isolated monocytes from the experimental rats were cultured in a complete RPMI-1640 medium for 24 h. Levels of certain cytokines in medium were determined by ELISA kits. The media from AIA and QLY groups were used to incubate healthy rats' lymphocytes, whose immune status was assessed by PCR with routine procedures. The primers were synthesized by Universal Biology (Chuzhou, Anhui, China), with the sequences displayed in Supplementary Table S1. Afterwards, we treated AIA monocytes with different sera. To prepare QLY-containing serum, 5 healthy rats were treated by QLY for 3 days at the dose mentioned above. Five untreated rats from the same batch were killed at the same day to harvest blood serum. After AIA monocytes were cultured in these sera for 24 h, levels of IL-1β and IL-6 in medium were determined. The rinsed monocytes were treated by Triton X-100, bovine serum albumin, primary anti-rat antibodies (IL-1β and PPARγ) and fluorescein-tagged secondary antibodies in turns. Finally, cell nucleus was stained by DAPI. In a replicate experiment, RA patients' monocytes were used instead. In addition to ELISA assays, $CD16^{+}$ monocyte subset was analyzed by the FCM method. Subsequently, we investigated impacts of QLY therapy on T cells and their interaction with monocytes. Lymphocytes of QLY-treated or AIA model rats were cultured for 24 h. Representative cytokines in medium were detected. Supernatant of the media was used to culture healthy rats' monocytes. Their secretion capacity was evaluated by ELISA tests. To further confirm the effects of QLY therapy on PPARγ expression in T cells, we performed WB assays using the spleen, where lymphocytes accumulate.

Blood Transfusion Experiment

If the pathogenic phenotype of immune cells is impaired by QLY therapy, their transplant would be unable to provoke pathological changes in recipients, including angiogenesis. Guided by this theory, we conducted the whole blood transfusion experiment. Six pairs of sex-matured male and female rats were kept separately for breeding. Ten male offspring were induced with AIA, and half of them received QLY treatment. Since inflammation became obvious on day 14, 1 mL of peripheral blood was sampled from each donator through fossa orbitalis vein every 3 days, and injected into the caudal vein of its female littermate. On the day of the 8^{th} blood injection, 0.4 mL of matrigel supplemented with VEGF (10 ng/mL) was injected on abdomen subcutaneously. The matrigel was thawed under 4°C overnight beforehand. Three days later, the recipients were sacrificed. The formed plugs were retrieved, photographed, and subjected to H&E histological examination. Blood was collected through abdominal aorta for FCM analysis. Hind paws were homogenized. Levels of cytokines and biochemical indicators in the homogenates and blood were determined. To clarify the distribution of implanted immune cells, whole blood of an AIA rat was used to prepare peripheral blood mononuclear cells based on a gradient centrifugation method, which were then stained with CFDA-SE. After injection with the cells, a healthy recipient was continually observed by an Optima Max-XP instrument at pre-determined intervals. The excitation and emission wavelengths were set at 500 and 520 nm, respectively.

Characterization of Chemical Components in QLY-Containing Serum

To precipitate protein, 100 μL of the rat serum was spiked into 400 μL of methanol. After a centrifugation at 12000 rpm for 10 min, supernatant was collected and directly injected into a 1260 Agilent Infinity II LC system for quantitative analysis. The chromatographic separation was achieved on a Waters C18 column (4.6 × 250 mm, 8 μm). A gradient elution program was used. Sodium dihydrogen phosphate aqueous solution (10 mm, pH = 8.0) and acetonitrile served as mobile phases A and B, respectively: 0–5 min, 20% A; 5–10 min, 50% A; 10–15 min, 20% A. The flow rate, injection volume and detection wavelength were set at 1 mL/min, 20 μL and 270 nm, respectively. The quantification curves for sinomenine, berberine and palmatine were developed beforehand by analyzing a serial of diluted solutions, which contained all the reference compounds.

Evaluation of Immune-Mediated Anti-Angiogenesis Effects of QLY-Related Compounds

Monocytes and T cells are the potential targets of QLY therapy, which possibly mediated the favorable outcomes including angiogenesis inhibition. Next, we simulated these cells directly with the related compounds to observe their reactions. THP-1 and Jurkat cells were purchased from Pricella Biotech (Wuhan, Hubei, China) and OriCell Biotech (Guangzhou, Guangdong, China), respectively. All cells were cultured and passaged under ordinary conditions. THP-1 monocytes and Jurkat T cells in 6-well plates were allowed to grow for 12 h. Supernatant in the wells was replaced by fresh RPMI-1640 medium supplemented with 10% healthy or AIA rats' serum then. Some AIA serum-stimulated cells were treated by a mixture of sinomenine, berberine and palmatine at various concentrations. Twenty-four hours later, certain cytokines in medium were detected.

To know impacts of QLY therapy on T cells-mediated angiogenesis, we cultured human umbilical vein endothelial cells (HUVECs, bought from the Cell Bank of Chinese Academy of Sciences, Shanghai, China) with the media from below. Jurkat T cells were cultured with a similar arrangement as above. Differently, only one optimal treatment of the compounds was adopted; half compounds-treated cells were stimulated by T0070907 (18 μM). At beginning of this culture, monolayer of HUVECs was scratched by a pipette tip. Twelve hours later, the wound was observed once again. VEGF levels in medium were detected. Next, we seeded HUVECs in a matrigel-coated 6-well plate. After attachment, they were treated by the media for 24 h. Capillary tubules were observed and photographed. The HUVECs were then seeded in matrigel-coated transwells (8 μm). The lower chambers were filled with the media. After 24 h, the cells infiltrated through pores were stained with crystal violet and photographed.

Statistical Analysis

All the data were presented as mean ± standard deviation. Results of WB experiments were semi-quantified by Image J (1.52a, NIH, Bethesda, MD). Shapiro–Wilk test and Bartlett's test were used to check normality and homogeneity of variance beforehand. Statistical difference among/between groups was evaluated by one-way ANOVA followed by Tukey's post hoc test or *T*-test by the help of Graph Pad Prism 8.0 (Cary, NC).

Results

QLY and RSG Alleviated AIA-Related Angiogenesis in Rats

AIA-related articular manifestations reach peak about day 14. Since then, therapeutic effects of the two treatments became obvious, causing profound decrease in arthritis scores. But they did not prevent AIA-caused decrease in body weight gain (Figure 1A). By the end of this in vivo experiment, paw swelling was still evident in AIA rats, while this situation was greatly eased in all the treated rats (Figure 1B). Hepatomegaly and splenomegaly were significant in AIA rats, which were reduced by QLY and RSG (Figure 1C). This change serves as a sign of the improved immune status, which was confirmed by the following ELISA tests.

Compared to normal controls, all the RA-related indicators increased a lot in AIA rats' blood. RF levels were increased from 139.9 ± 56.2 to 757.2 ± 255.9 IU/L. Levels of CXCL1, IL-1β, IL-6 and MCP-1 were increased by 42.1%, 79.9%, 240.7% and 48.8%, respectively. Interestingly, levels of two anti-inflammatory cytokines IL-10 and TGF-β1 were also elevated. A significant increase of VEGF (from 25.0 ± 0.9 to 34.9 ± 4.3 pg/mL) in AIA rats exhibits an environment

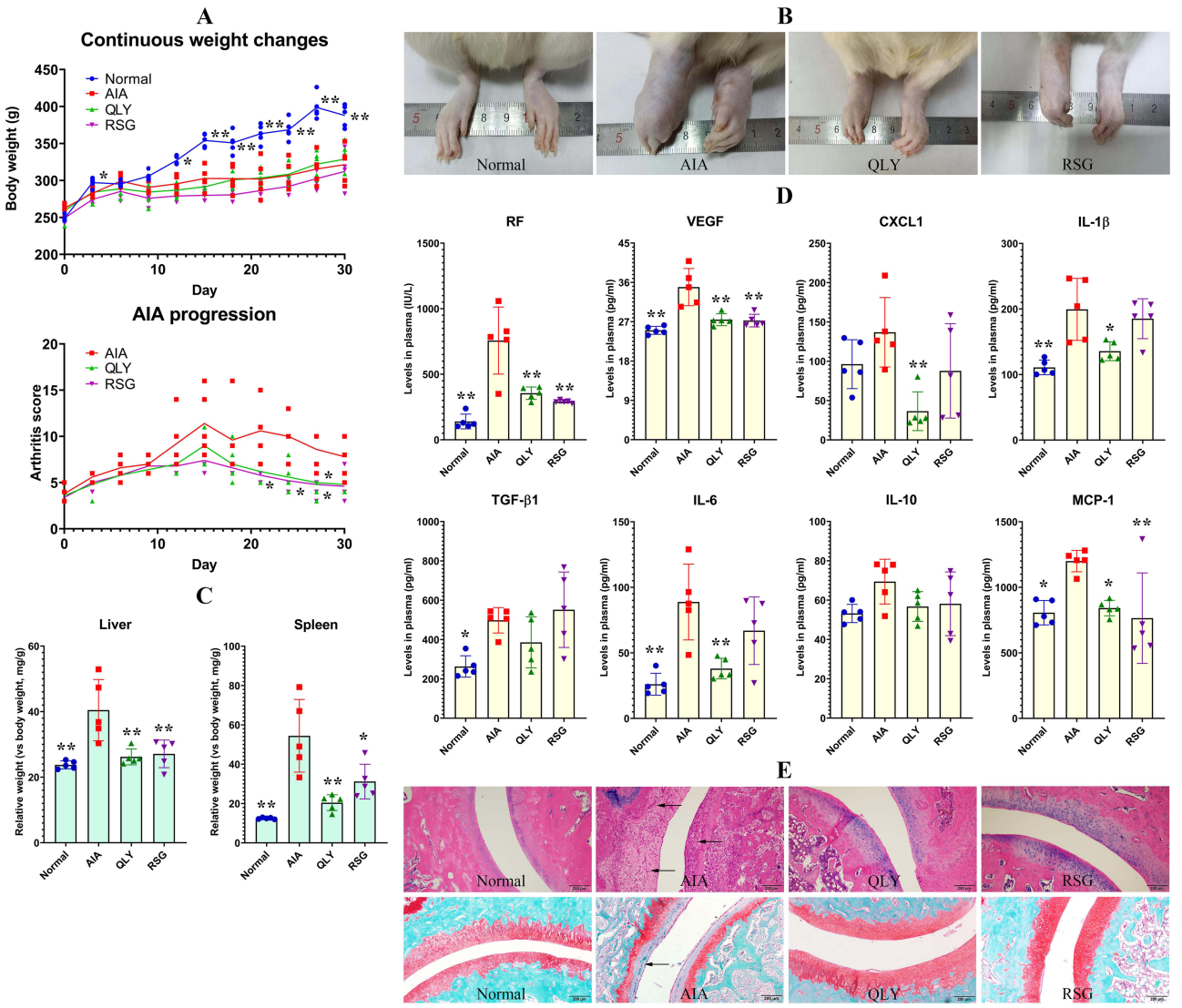

Figure 1 QLY reduced AIA severity in rats. (**A**) periodic changes of arthritis scores and body weights; (**B**) morphological changes of hind paws; (**C**) relative weights of the liver and spleen; (**D**) levels of RA diagnostic indicators in blood; (**E**) injuries of knee joints (20 × magnification; arrows in the above H&E-stained and below SOFG-stained sections indicate angiogenesis and cartilage erosion, respectively). Statistical significance: *p < 0.05 and **p < 0.01 compared with AIA model rats.

favorable for angiogenesis. All the abnormalities were restored by the treatments. Although QLY performed much better than RSG in decreasing CXCL1, IL-1β and IL-6, they were similarly effective in reducing VEGF. Its blood levels in the two groups were 27.5 ± 1.4 and 27.3 ± 1.4 pg/mL, respectively (Figure 1D). Benefited from that, the treatments eased angiogenesis (H&E staining) and cartilage degradation (SOFG staining) in joints (Figure 1E). These facts demonstrate that the therapeutic actions of QLY were similar to RSG. They both improved the immune environment and arthritic manifestations.

QLY and RSG Relieved AIA-Related Metabolic Abnormalities by Activating PPARγ

If QLY and RSG similarly activated PPARγ, they must affect metabolic status of AIA rats. We noticed an increase in lactic acid and pyruvic acid as well as a decrease in glucose in AIA rats. It indicates glycolysis acceleration, a sign of inflammation.[17] TG levels increased a bit. QLY and RSG narrowed the AIA-caused metabolic changes (Figure 2A). The altered metabolism condition would impact oxidative balance in vivo. The increased ratio of MDA/CAT exhibits oxidative stress escalation, which was restored by QLY and RSG more or less. GSH levels were increased in AIA rats

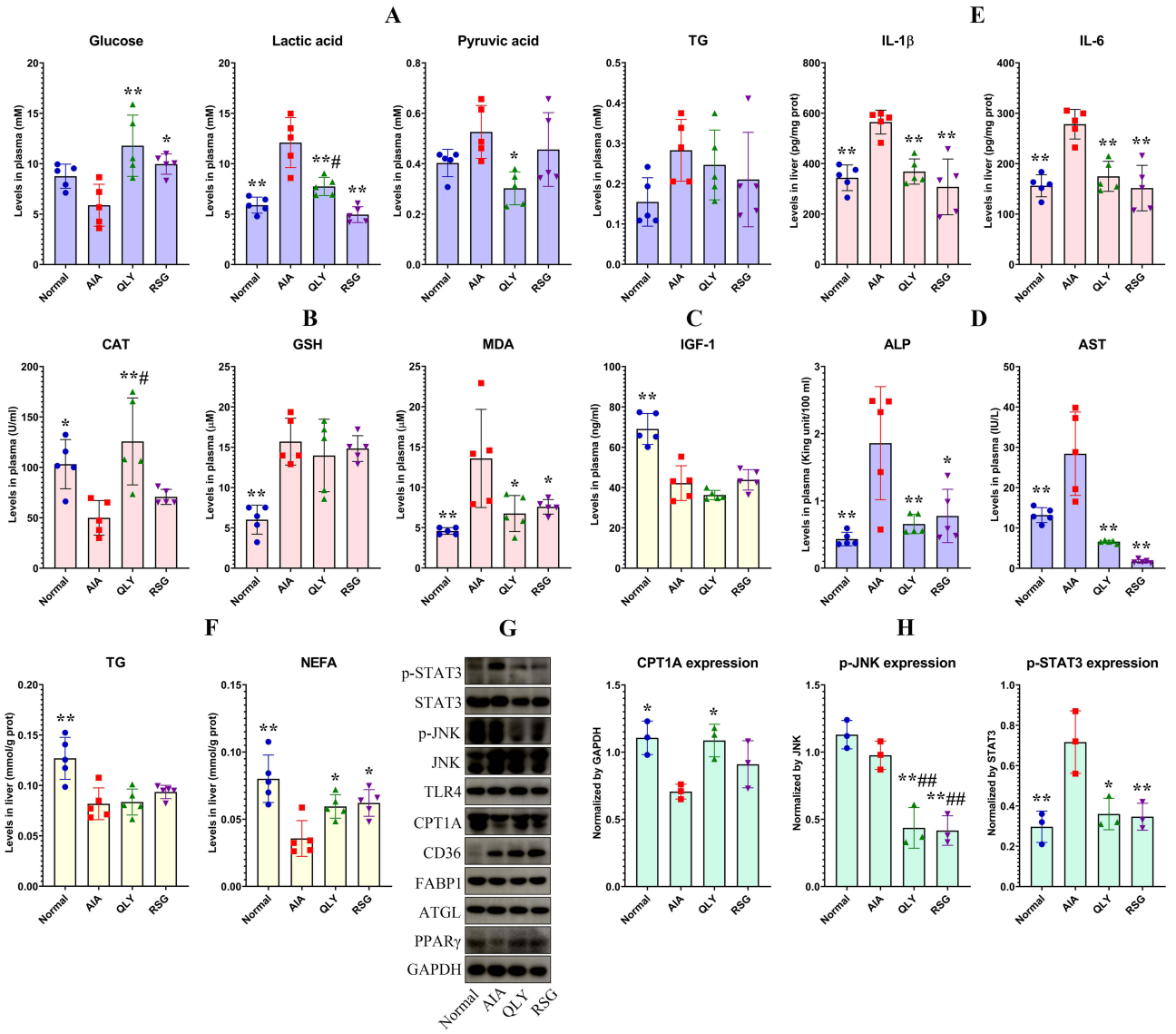

Figure 2 QLY improved metabolic disorders in AIA rats by activating PPARγ. (**A**) levels of representative metabolites in blood; (**B**), levels of oxidative indicators in blood; (**C**) levels of IGF-1 in blood; (**D**) levels of liver injuries indicators in blood; (**E**) levels of inflammatory cytokines in the liver; (**F**) levels of representative lipid metabolites in the liver; (**G**) WB assays performed on liver samples; (**H**) the quantification results of assay G. Statistical significance: *p < 0.05 and **p < 0.01 compared with AIA model rats; #p < 0.05 and ##p < 0.01 compared with normal healthy rats.

too but barely affected by the two treatments (Figure 2B). IGF-1 is a novel RA-related indicator, controlling metabolism of glucose and mineral.[18] Its levels decreased in AIA rats. Neither of QLY nor RSG improved this situation (Figure 2C).

Because metabolism is largely controlled by the liver, we next focused on changes there. Compared with normal controls, levels of ALP and AST in AIA rats were increased by 329.8% and 115.3%, respectively. Their huge increase indicates substantial hepatic injuries (Figure 2D). It was attributed to AIA-related inflammation. AIA rats were detected with higher levels of IL-1β (564.8 ± 47.4 vs 343.9 ± 51.0 pg/mL) and IL-6 (278.4 ± 29.4 vs 156.2 ± 21.8 pg/mL) in their liver than the healthy counterparts (Figure 2E). After QLY and RSG treatments, liver injuries and inflammation were thoroughly cured (Figure 2D and E).

Presumed PPARγ activation induced by the treatments did not only ease inflammation but would also profoundly reshape metabolism. Levels of TG and NEFA in the liver of AIA rats were much lower than normal controls. Although the treatments did not affect TG, they effectively restored NEFA levels (Figure 2F). To find the reason for the metabolic changes, we performed WB assays, and the uncropped images are included in Supplementary Figure S1. In AIA rats'

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liver, expression of ATGL, CD36 and CPT1A was unchanged, up-regulated, and down-regulated respectively. It can be inferred that the liver took in more NEFA but utilized less in AIA rats, when fat mobilization remained unchanged.[19] Hence, the decline of TG and NEFA was caused by impaired synthesis. It reflects functional deficiency of PPARγ. Indeed, compared to normal controls, PPARγ expression in AIA rats' liver was down-regulated. QLY and RSG promoted expression of PPARγ and one of its downstreams CPT1A (Figure 2G). Expression of TLR4 and JNK was hardly affected by AIA, while STAT3 phosphorylation was promoted (Figure 2H). Hence, Th17 cells but not monocytes/macrophages plausibly initiated inflammation there.[20] RSG and QLY treatments greatly reduced p-STAT3 expression but showed weak effects on TLR4/JNK. It was assumed that PPARγ regulation preferentially affected T cells rather than monocytes/macrophages during these therapies.

T Cells Were a Preferential Target of QLY Treatment in AIA Rats

We performed FCM analysis to investigate impacts of the treatments on monocytes (Figure 3A). It was revealed that non-classic $CD43^{+}CD172a^{+}$ subset was increased by 3-fold in AIA rats, but classic $CD43^{+}CD172a^{-}$ subset was reduced. The treatments restored these abnormal changes (Figure 3B). Also, they showed potentials in down-regulating secretion of NOX and iNOS, two indicators of inflammatory monocytes/macrophages (Figure 3C).[21] To confirm if the immune phenotype of these cells was fundamentally changed, we isolated and cultured them in vitro. Phenotype of AIA monocytes was greatly changed. They released more IL-1β (182.2 ± 57.2 vs 73.1 ± 10.9 pg/mL), IL-6 (53.4 ± 13.4 vs 40.1 ± 8.7 pg/mL), TGF-β1 (1133.4 ± 112.1 vs 480.9 ± 152.2 pg/mL) and IL-10 (160.1 ± 24.8 vs 81.8 ± 5.2 pg/mL) than normal controls. However, no significant improvement concerning these changes was observed in the monocytes isolated from QLY-treated AIA rats (Figure 3D). This fact questioned if PPARγ activation can substantially affect monocytes/macrophages during QLY and RSG therapies.

The following experiments answered this question. Expression of immune genes (*IL-6, IFNγ, IL-10, IL-17A, FOXP3* and *ROR-γT*) in normal lymphocytes cultured in the media from monocytes of AIA and QLY groups were similar (Figure 4A). Furthermore, QLY-containing serum showed no different impacts to the normal control on IL-1β and IL-10 secretion of AIA monocytes in vitro (Figure 4B). Immunofluorescence assay validated this result. This serum neither reduced IL-1β secretion nor promoted PPARγ expression in the cells (Figure 4C). We noticed that expression of IL-1β

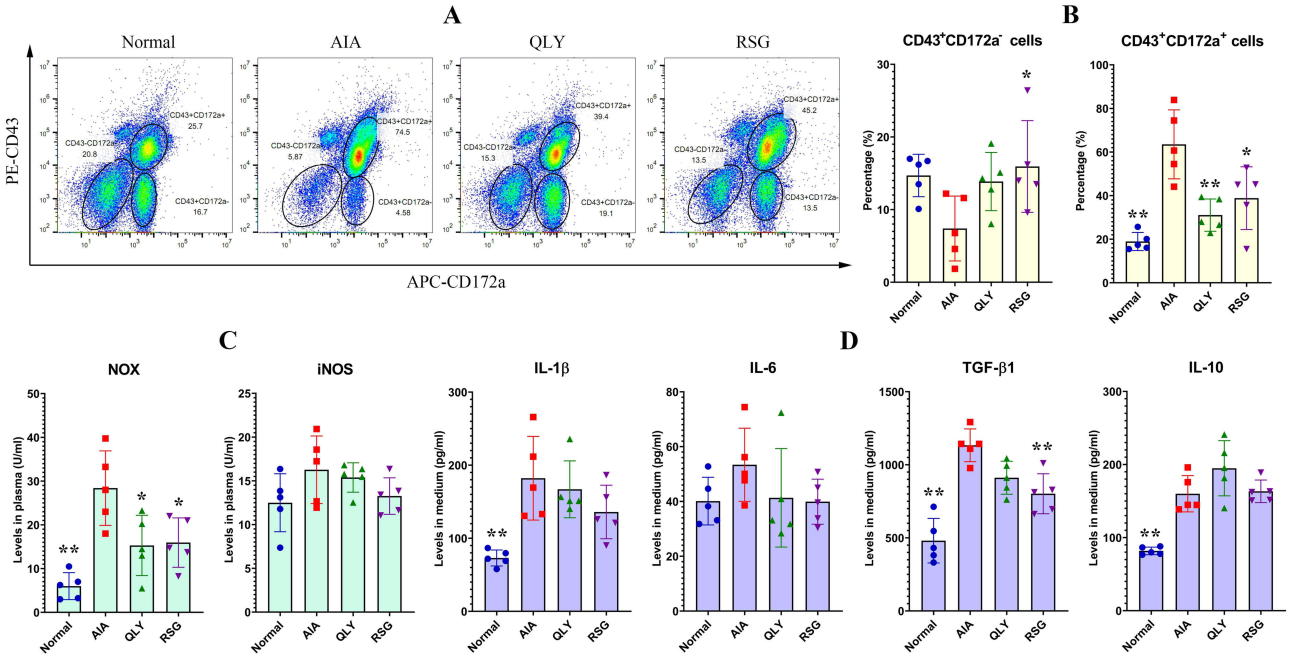

Figure 3 QLY-induced monocyte phenotype changes were unsustainable in vitro. (**A**) FCM analysis of different monocyte subsets in the rats' blood; (**B**) the quantification results of assay (A); (**C**) levels of inflammatory monocyte indicators in blood; (**D**), levels of cytokines released by the in vitro cultured rat monocytes. Statistical significance: *p < 0.05 and **p < 0.01 compared with AIA model rats.

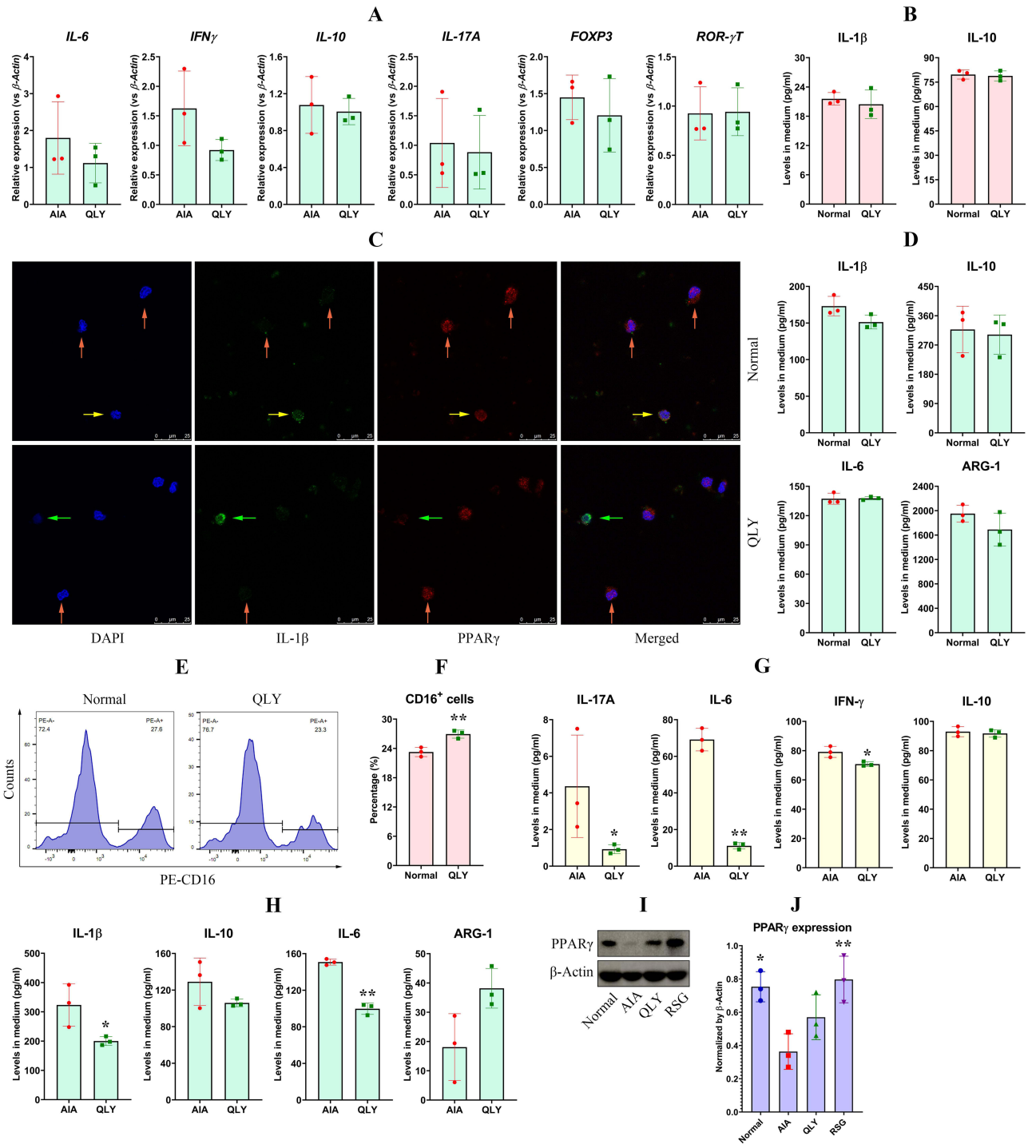

Figure 4 QLY preferentially targeted T cells. (**A**) expression of various T cell subsets-related genes in normal lymphocytes grew in the media from the above monocyte culture systems; (**B**) levels of cytokines released by AIA monocytes cultured by normal or QLY-containing serum; (**C**) expression and localization of IL-1β and PPARγ in these monocytes (100 × magnification; Orange, yellow and green arrows indicate IL-1β^{-}PPARγ^{+}, IL-1β^{+}PPARγ^{+} and IL-1β^{+}PPARγ^{-} cells, respectively); (**D**) levels of cytokines released by RA monocytes cultured by normal or QLY-containing serum; (**E**) FCM analysis of these cells; (**F**) the quantification results of assay E; (**G**) levels of cytokines released by the in vitro cultured lymphocytes from different rats; (**H**) levels of representative cytokines released by normal monocytes grew in the media from these lymphocyte culture systems; (**I**) WB assays performed on spleen samples; (**J**), the quantification results of assay I. Statistical significance: *p < 0.05 and **p < 0.01 compared with AIA group.

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and PPARγ was not always relevant. Monocytes may highly express IL-1β and PPARγ at the same time. Then, we repeated the culture experiment using RA patients' monocytes. QLY-containing rat serum showed no effects on immune indicators IL-1β, IL-10, IL-6 and ARG-1 (Figure 4D). It even increased distribution of $CD16^+$ cells, the presumed inflammatory monocytes (Figure 4E and F).[22] Regardless of this mystery, we conclude that QLY exerts no direct anti-inflammatory effects on monocytes.

Therefore, the observed changes in monocytes above would be mediated by T cells, the dominate role in RA pathology.[1] Indeed, the lymphocytes of QLY-treated AIA rats released less IL-17A (0.9 ± 0.2 vs 4.4 ± 2.8 pg/mL), IL-6 (11.1 ± 1.8 vs 69.2 ± 6.2 pg/mL) and IFN-γ (70.8 ± 1.5 vs 79.1 ± 3.8 pg/mL) than AIA controls. It shows impaired Th1/17 differentiation. Treg differentiation was unaffected, as IL-10 levels were similar (Figure 4G). When cultured in media from these lymphocytes, normal monocytes showed diversified reactions. Compared to AIA controls, the monocytes in QLY group produced less IL-1β (200.3 ± 15.1 vs 323.7 ± 72.4 pg/mL), IL-10 (106.0 ± 4.2 vs 129.1 ± 26.0 pg/mL) and IL-6 (99.7 ± 6.2 vs 150.8 ± 3.3 pg/mL). The opposite outcome occurred on ARG-1 levels (Figure 4H). These clues hint that QLY-caused PPARγ up-regulation indirectly affected polarization of monocyte by inhibiting Th1/17 differentiation, which was supported by WB assays (Figure 4I). PPARγ expression in AIA rats' spleen was reduced and then restored by QLY and RSG treatments (Figure 4J).

T Cells Mediated Anti-Angiogenesis Effects of QLY in AIA Rats

Although monocytes and T cells in blood are the two major players in RA, outcomes from transfusion of the experiment rats' blood should be largely attributed to the latter, considering the population advantage. Impacts from short-lived granulocytes are negligible. Immune cells of AIA rats were widely distributed in the body through circulation channels after transplant. Viscera, spine and knee joints were the enrichment sites. Those cells in chest were gradually eliminated. The remaining cells accumulated in the knee and spine (Figure 5A). It suggests that T cells were the main long-lived immune cells from AIA rats in the recipients, as they exhibit chemotaxis to joints due to autoimmune reaction to cartilage.[1] QLY-treated AIA rats' blood transfusion caused a decline in $CD43^+CD172a^+$ cell counts, albeit it was not significant (Figure 5B and C). $CD3^+CD4^+$ T cell counts were similar (Supplementary Figure S2). We analyzed a panel of cytokines to demonstrate the immune consequences. Disappointingly, transfusion of QLY-treated AIA rats' blood did not cause very notable changes. Among all the investigated indicators, only IL-1β (154.6 ± 24.6 vs 198.1 ± 18.6 pg/mL) and ARG-1 (11.7 ± 1.4 vs 7.9 ± 3.0 ng/mL) showed significantly different levels between QLY and AIA groups (Figure 5D). Their changes basically indicate phenotype shift of monocytes. But iNOS reduction in QLY group was not obvious (Figure 5E). There was no level difference of MDA, GSH and CAT, confirming the similar immune and metabolism status of these recipients (Figure 5F).

Given the selective distribution of donators' immune cells, changes in joints would be more obvious. Hence, we repeated the above analyses using knee homogenates. It verified the hypothesis. Level differences of IL-1β and ARG-1 there between the groups were similar to those found in blood. TGF-β1 decrease did not only indicate altered immune condition, but also promised eased angiogenesis in the recipients receiving QLY-treated AIA rats' blood. In line with this, levels of some other angiogenesis-related cytokines including VEGF (82.0 ± 23.0 vs 137.8 ± 34.3 pg/mg prot), HIF-1α (5.9 ± 1.7 vs 12.5 ± 3.1 pg/mg prot) and ICAM-1 (331.8 ± 106.9 vs 793.9 ± 207.3 pg/mg prot) in QLY group were also decreased, compared to AIA group. The significant decrease of IL-17A (0.5 ± 0.2 vs 1.6 ± 1.6 pg/mg prot) and IFNγ (95.6 ± 16.4 vs 125.0 ± 9.9 pg/mg prot) in QLY group confirm that Th1/17 differentiation in their donors was hampered by QLY (Figure 6A). The microenvironment differences affected functions of monocytes/macrophages. As a result, QLY group showed lower levels of iNOS (0.3 ± 0.1 vs 1.0 ± 0.5 U/mg prot) than AIA group (Figure 6B). Despite these immune changes, oxidative stress was unaffected (Figure 6C). These data confirm that angiogenesis is a T cells-mediated event in AIA rats. AIA immune cells caused angiogenesis in the implanted matrigel plug, indicated by dark red color. Their potentials were weakened by QLY therapy (Figure 6D). Dense vessels formed there in the recipients of AIA group. A large amount of immune cells were infiltrated through these channels. These situations were improved in QLY group (Figure 6E). The collective evidence shows that QLY reshaped functions of T cells and then exerted anti-angiogenesis effects in AIA rats.

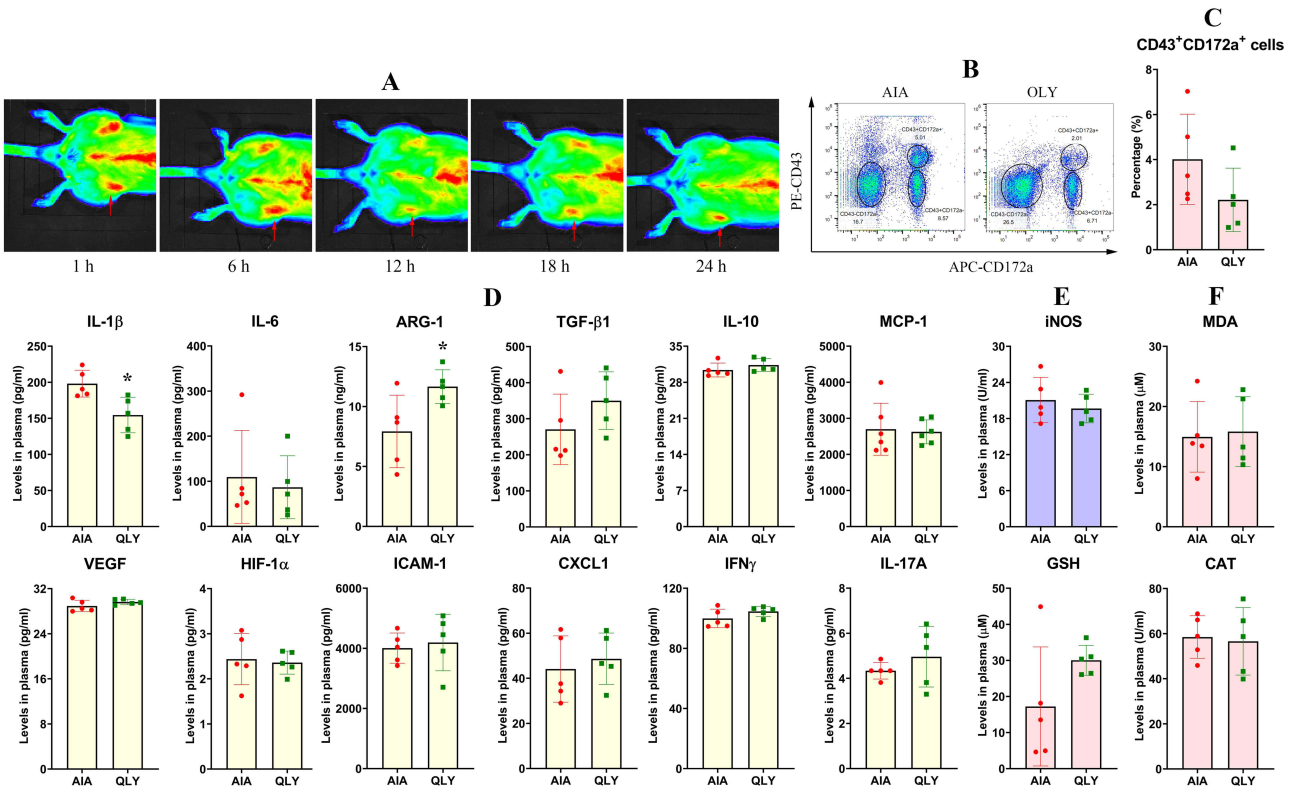

Figure 5 No obvious difference about internal environment of recipients was observed after transfusion with different AIA rats' blood. (**A**) dynamic distribution of AIA immune cells in the recipient after blood transfusion; (**B**) FCM analysis of monocyte subsets in the recipients' blood; (**C**) the quantification results of assay B; (**D**) a comprehensive evaluation of cytokine network alteration; (**E**), iNOS levels in the recipients' blood; (**F**) levels of oxidative indicators in the recipients' blood. Statistical significance: *p < 0.05 compared with AIA group.

QLY-Derived PPARγ Agonists Inhibited Angiogenesis by Targeting T Cells

Main bioactive components in QLY are alkaloid derivatives, which contribute significantly to its anti-rheumatic effects.[6–10] In QLY-containing serum, we detected 3 alkaloids, sinomenine, berberine and palmatine (Figure 7A). With the quantification curves (Figure 7B), their blood concentrations were calculated. The mean levels of sinomenine, berberine and palmatine were 2.08, 0.51 and 1.12 μg/mL, respectively (Figure 7C). The values were adopted for the medium dose of the compounds treatment in the following experiments. The low and high doses were divided and multiplied by 2, respectively. This mixture failed to reduce the secretion of IL-1β and TGF-β1 in AIA rat serum-cultured THP-1 cells (Figure 7D). Differently, AIA serum-induced production of IFNγ and IL-17A in Jurkat T cells was reduced, generally in a dose-dependent manner (Figure 7E). Then, we treated Jurkat cells with the compounds at the high dose only. The medium of AIA serum-incubated T cells greatly augmented VEGF secretion in HUVECs (from 462.3 ± 70.4 to 826.4 ± 60.1 pg/mL). The compound mixture impaired this capacity, but T0070907 abrogated this effect (Figure 7F). VEGF levels affected angiogenesis status eventually. Wound healing occurred in AIA group, but the compounds treatment on Jurkat T cells impaired capacity of the medium in activating HUVECs (Figure 7G). Subsequently, we confirmed that potentials of HUVECs infiltrating through transwells and forming tubes were reinforced when cultured by the medium of AIA serum-cultured T cells, which were inhibited by the compounds (Figure 7H and I). T0070907 showed antagonistic effects to the compounds mixture in all the above experiments. Hence, T cells-mediated anti-angiogenesis effects of QLY rely on PPARγ activation.

Discussion

In RA field, humankind has entered a targeted therapy era. The knowledge about pathogenic functions of immune cells does not only lay a foundation for developing biological agents but also explains the therapeutic mechanisms of

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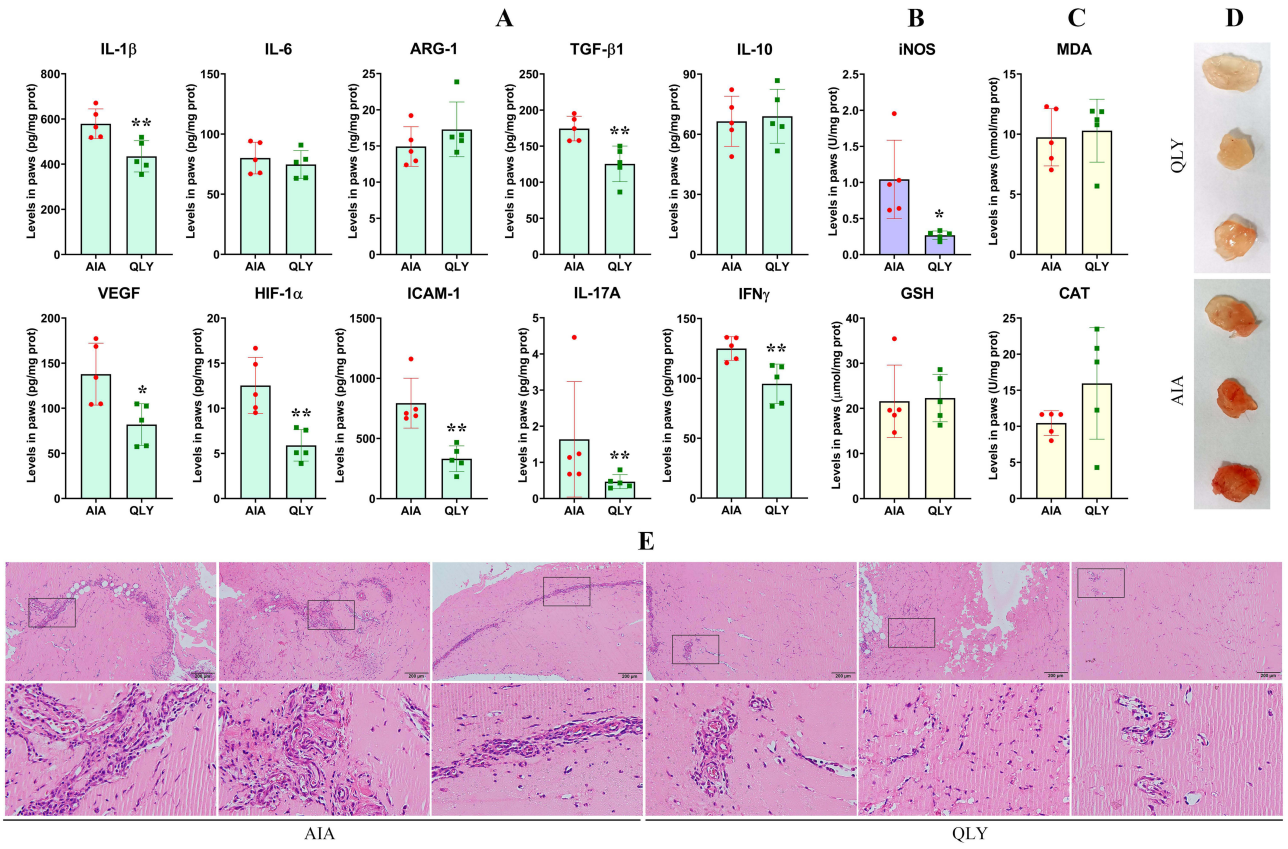

Figure 6 QLY impaired the ability of AIA rats' lymphocytes inducing angiogenesis in vivo. (**A**) levels of immune/angiogenesis-related cytokines in the recipients' joints; (**B**) iNOS levels in joints; (**C**) levels of oxidative indicators in joints; (**D**) matrigel plugs from the recipients; (**E**) histological examination of H&E-stained plugs (20 × magnification). Statistical significance: *p < 0.05 and **p < 0.01 compared with AIA group.

conventional drugs. Unfortunately, despite many available immune-regulating drugs, RA still cannot be thoroughly cured. This fact hints that some other factors may also be important in RA, in addition to immune alteration. The answer came from clinical observations, which discovered metabolic disorders as the most common complication of RA.[23] The evolving theory of immunometabolism provides an explanation for how metabolic changes affect RA progress.[24] Glucose metabolism disturbance was apparent in AIA rats (Figure 2A). Meanwhile, IGF-1, a regulator promoting glucose oxidation, was down-regulated (Figure 2C). This signal is anti-inflammatory, and its compromise amplifies inflammation in RA.[25] Comparatively, dyslipidemia is even more common.[23] Active RA is always accompanied with lipids decrease.[26,27] We observed a similar phenomenon in AIA rats here (Figure 2A). The signals controlling adipogenesis therefore become interesting research subjects. PPARγ is very important in this aspect. It decides differentiation of adipocytes and promotes fat synthesis.[13] Its functional deficiency in the liver is the main cause of RA-related blood lipid depletion.[19]

Inspired by this, we examined changes in the liver to clarify effects of QLY on PPARγ. QLY did not only restore altered lipid levels there but also promoted expression of PPARγ and its downstream CPT1A (Figure 2G). Contrarily, IGF-1 levels in blood were not restored. That is, QLY selectively modulated PPARγ-mediated lipids metabolism. This effect would be attributed to certain alkaloids.[9] Sinomenine, berberine and palmatine were the representatives with high blood concentrations after oral QLY therapy (Figure 7C). In fact, all of them were confirmed with PPARγ activating properties.[28–30] As for mechanism underlying the beneficial outcomes, it was related to PPARγ activation-caused inhibition of inflammatory transcription factors. Among them, AP-1 and NF-κB are the best known.[13,14] In fact, STAT3 is also one of The PPARγ targets, a regulator of Th17 differentiation.[31] According to these clues, QLY would inhibit inflammatory activation of both monocytes/macrophages and T cells. But interestingly, its effects seem to be selective. It effectively repressed expression of p-STAT3 in AIA rats' liver, where distribution of inflammatory

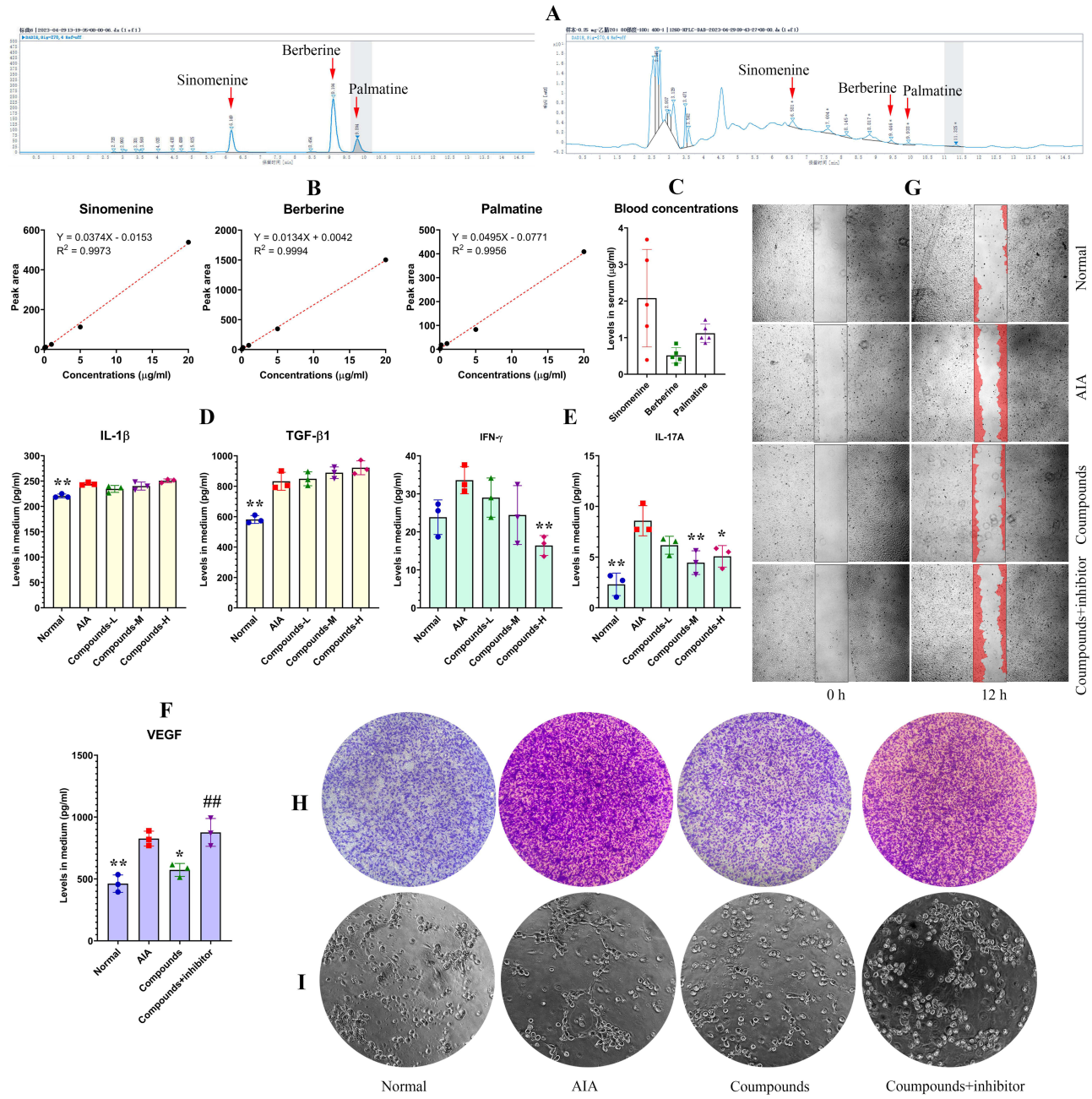

Figure 7 QLY-related compounds inhibited T cells-mediated angiogenesis in vitro. (**A**) HPLC detection of the compounds in QLY-containing serum; (**B**) the quantification curves; (**C**) serum concentrations of the compounds; (**D**) levels of cytokines released by THP-1 monocytes stimulated by normal/AIA serum and the compound mixture; (**E**) levels of cytokines released by Jurkat cells received the same stimuli; (**F**) levels of VEGF released by HUVECs grew in the media from Jurkat cells treated by normal/AIA serum and the compounds or in combination with a PPARγ inhibitor T0070907; (**G**) impacts of these media on wound healing of HUVECs (20 × magnification); (**H**) impacts of these media on transwell infiltration of HUVECs (50 × magnification); (**I**) impacts of the media on tube-forming of capacities (100 × magnification). Statistical significance: *$p < 0.05$ and **$p < 0.01$ compared with AIA group; $^{\#\#}p < 0.01$ compared with compounds treatment group.

macrophages was barely affected, as indicated by unchanged TLR4 expression (Figure 2G). It implies that therapeutic effects of QLY on AIA rats were mainly mediated by T cells.

Our previous works found that QLY improved the whole immune microenvironment of RA models.[7–9] But T cells were more sensitive to this therapy than monocytes/macrophages. The related compounds cannot affect the latters' inflammatory polarization in vitro at normal treatment concentrations. Whereas, they significantly inhibited the development of Th1/17 cells.[8] Moreover, QLY could even exacerbate macrophages-related inflammation because of SIRT1 inhibition.[6] The clues hint that T cells rather than monocytes/macrophages are the preferential target of QLY therapy.

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This study consolidates the conclusion. QLY reduced secretion of all inflammatory cytokines in AIA rats (Figure 1D). However, QLY-induced monocyte changes cannot be sustained in vitro (Figure 3D). Neither QLY-containing serum nor QLY-related compounds inhibited inflammatory secretion of monocytes in AIA condition (Figures 4B and 7D). Comparatively, T cells responded to these treatments well (Figures 4G and 7E). More importantly, QLY effectively suppressed the release of Th1/17 cells-derived cytokines in vivo, as evidenced in the blood transfusion experiment (Figure 6A). Via intercellular communication, these changes reshaped functions of co-existing monocytes (Figure 4H). The immune milieu was then improved as a whole. Benefited from this, angiogenesis was attenuated (Figure 6E). In fact as early as 2018, we had noticed that QLY would preferentially target inflammatory T cells, suggested by down-regulation of pentose phosphate pathway.[7] Because T cells initiate RA-related autoimmune reactions and most of pathological changes, this selective effect on T cells is essential for overall anti-rheumatic effects of QLY.

Conclusion

Similar to RSG, QLY effectively reduced AIA severity in rats by activating PPARγ. T cells were more sensitive to this therapy than monocytes both in vivo and in vitro. Due to inhibition on Th1/17 differentiation, the immune microenvironment was rebalanced. As a result, the activation of vascular endothelial cells was undermined. The collective data show that QLY inhibits immune-mediated abnormal angiogenesis in AIA rats by activating PPARγ in T cells.

Abbreviation

WAT, white adipose tissues; AIA, adjuvant-induced arthritis; QLY, Qing-Luo-Yin; RA, rheumatoid arthritis; TCM, Traditional Chinese Medicine; IFA, Incomplete Freund's adjuvant; BCG, Bacillus Calmette-Guérin; RSG, rosiglitazone; RF, rheumatoid factor; HIF-1α, hypoxia inducible factor 1 alpha; ARG-1, arginase 1; IL-1β, interleukin 1 beta; TGF-β1, transforming growth factor beta 1; IFNγ, interferon gamma; VEGF, vasoactive endothelial growth factor; ICAM-1, intercellular cell adhesion molecule 1; CXCL1, C-X-C motif ligand 1; MCP-1, monocyte chemotactic protein 1; IGF-1, insulin-like growth factor 1; TG, triglyceride; NEFA, nonestesterified fatty acid; CAT, catalase; NOX, NADH oxidase; GSH, reduced glutathione; MDA, malonaldehyde; iNOS, inducible nitric oxide synthase; AKP, alkaline phosphatase; AST, aspartate aminotransferase; FBS, fetal bovine serum; FCM, flow cytometry.

Data Sharing Statement

All data generated or analyzed during this study are included in this article and its Supplementary Information. Other relevant data are available from the corresponding author upon reasonable request.

Ethics Approval

The animal study was approved by the Ethical Committee of Wannan Medical College (LLSC-2022-223). The use of animals was adhered to the National Institutes of Health Guide for the Care and Use of Laboratory animals (2011), and all the experiment methods were in accordance with ARRIVE guidelines.

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Disclosure

The authors declare no conflict of interest in this work.

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