Gut Microbiota Dysbiosis in High-Risk Individuals For Rheumatoid Arthritis Triggers Mucosal Immunity Perturbation and Promotes Arthritis in Mice

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Abstract

Background: Intestinal microbial dysbiosis in patients with rheumatoid arthritis (RA) is associated with enhanced inflammation in the joint. Recent studies indicate intestinal microbiota changes prior to clinical signs of arthritis in the joints. However, it remains undefined whether gut microbiota dysbiosis is triggering the initiation and progression of RA during the preclinical stages. Here, we describe the gut microbiome characteristics in preclinical high-risk individuals for RA, and investigate their consequences on the mucosal immunes system and involvement in the pathogenesis of arthritis.

Results: 16S rRNA sequencing was performed on faeces from human participants, comprising preclinical high-risk for RA individuals (Pre-RA, n=53), who were anti-citrullinated protein antibodies positive, established RA patients (RA, n=30) and healthy individuals (HCs, n=38). Pre-RA individuals showed lower fecal microbial diversity comparing with HCs. The bacterial community structure dynamically shifted from HCs to Pre-RA individuals and to RA patients, with the relative abundance changes of genera including *Bacteroides*, *Ruminiclostridium_5*, and *Streptococcus*. To further decipher the effect of these gut microbiota alterations in mucosal immune response, we transplanted pooled human fecal samples from each group into antibiotics pre-treated mice. The inoculation of faeces from the Pre-RA group increased fluorescent-labelled dextran uptake, decreased ZO-1 gene expression in the intestine, and changed typical microvilli structure of the small intestine of the recipient mice. Moreover, Th17 cells in the mesenteric lymph nodes and Peyer's patches were also increased in mice following fecal microbiota transplantation (FMT) from Pre-RA and RA compared to HC group. We also applied the collagen induced arthritis (CIA) mice model to test the effect of FTM from Pre-RA individuals on arthritis severity. Importantly, we revealed that the FMT from Pre-RA and RA group similarly enhanced clinical CIA symptom in recipient mice as compared with HC-FMT mice.

Conclusions: Gut microbial dysbiosis is already present in high-risk individuals for RA with autoimmune signature. FMT from preclinical individuals triggers the intestinal barrier dysfunction and changed mucosal immunity further contributing to arthritis development.

Background

Rheumatoid arthritis (RA) is characterized by chronic inflammation and progressive destruction in synovial joints, accompanied with systemic manifestations and autoantibodies production {i.e., anti-citrullinated protein antibodies (ACPAs) and rheumatoid factor (RF)}[1]. Prior to the onset of clinically or histologically identified arthritis, genetically predisposed individuals may go through prolonged preclinical stages during which environmental exposures work in concert with genetic factor (i.e., 'shared epitope 'alleles) to drive the initiation and progression of disease[2]. For those with seropositive RA, a hallmark in their preclinical stage is the appearance of ACPA[3, 4]. The presence of ACPA in serum is predicting a high risk for developing seropositive RA[5, 6].

The fact that circulating autoantibodies appear in the absence of clinical symptoms of arthritis indicate the initiation of the disease or 'loss of tolerance' in other sites but the joints. The microbial dysbiosis among RA patients and arthritis animal models have been extensively reviewed [7, 8]. Accumulating evidences point that initiating events may occur at mucosal surfaces colonized by the local microbiome. Studies utilizing inflammatory arthritis models have demonstrated that germ-free mice or experimentally reduction of the gut microbiome by antibiotics treatment protects animals from inflammatory arthritis and that mucosal microbial imbalance, rather than pathogens invasion, contributes to disease onset[9–14]. There is a direct promoting role of gut microbiota from established RA patients in arthritis development in SKG mice. Maeda Y *et al.* have found that dysbiotic microbiota from RA patients accelerated arthritis in SKG mice by activating autoreactive T cells in the intestine [14]. Another study comparing RA patients with first-degree relatives suggests altered fecal microbes are associated with disease course and autoantibody production. The expansion of a rare genus of *Collinsella* positively correlates with IL-17A level, increased gut permeability and disease severity in CIA mice following FMT [15]. Although the 'mucus origins hypothesis' has been raised based on findings of mucosal inflammation in early RA patients, it is still unclear how the microbial changes and mucosal inflammation are contributory to RA pathogenesis during the preclinical stage. In fact, we have already noticed that oral microbial dysbiosis occurs in early RA patients[16]. D Alpizar-Rodriguez *et al.* reported the difference in intestinal microbiome in European populations with high risk for RA[17]. It is an urgent yet unmet need to identify the biomarkers, especially the intestinal microbiota profile for preclinical high-risk RA since early treatments or precautions are associated with better outcomes and less socioeconomic burden. Exploring the specific mucosal processes undergoing at early RA would also help us to understand how microbiota may contribute to arthritis development.

Therefore, we undertook this study to better understand the impact of described microbial characteristics in high-risk RA individuals. Using FMT transplantation experiments in the CIA murine model, we show mechanism triggered specifically by pre-RA microbiota that contributes to the onset of clinical RA symptoms.

Methods

Study cohort, patient characteristics and sample collection

The study procedure was approved by the Biomedical Research Ethics Committee, West China Hospital of Sichuan University (ChiCTR1900022605), and informed written consents were obtained from all participants according to the Declaration of Helsinki. Individuals at high-risk for RA were recruited from West China Hospital, Sichuan University, China. These subjects had a positive serum ACPA, with or without arthralgia at the time of enrollment. Absence of arthritis was confirmed by physical examination of 44 joints. RA patients were diagnosed according to the American College of Rheumatology (ACR) 2010 classification for RA. Most RA patients were receiving oral disease-modifying anti-rheumatic drugs (DMARDs) and/or corticosteroids at the time of enrollment (Table 1). All patients were biological agents naive. Age, gender and ethnicity-matched healthy controls (HCs) with no personal history of inflammatory

arthritis were recruited. ACPA-negative profiles for HCs were obtained from the health management center. Subjects from all three groups were ≥ 18 years old. Exclusion criteria were as follows: having a history of antibiotics treatment or surgery in the last three months, current extreme diet, major organ dysfunction, cancer, other rheumatic or autoimmune diseases including osteoarthritis, systemic lupus erythematosus,Sjögren syndrome or diabetes. 121 participants were enrolled, comprising 53 high-risk individuals, 30 RA patients and 38 HCs. Detailed demographic and clinical parameters are given in Table 1.

Table 1
Demographic and clinical features of human participants

Characteristic	RA (n = 30)	Pre-RA (n = 53)	Healthy controls (n = 38)
Age, mean (median) years	50.6 (53)	48.5 (49)	48.2 (49)
Female, %	67	55	63
BMI, mean (median)	24.5 (24.0)	23.0 (23.6)	23.9 (24.0)
Disease duration, mean (median) months	17.7 (13.0)	-	-
Disease activity parameter			
ESR, mean (median) mm/h	43.13 (40.00)	-	-
CRP, mean (median) mg/L	17.07(3.66)	-	-
DAS28, mean (median)	4.91 (4.61)	-	-
Autoantibody status			
ACPA positive, n (%)	29 (97)	53 (100)	0
IgM-RF positive, n (%)	26 (87)	13 (25)	0
ACPA titer, mean (median) U/ml	330.3 (371.8)	175.3(83.2)	-
IgM-RF titer, mean (median) IU/ml	413.2 (170.0)	74.8 (24.8)	-
Medication use, %			
DMARDs (MTX, LEF, HCQ)	25(83)	0	0
Prednisone	20(67)	0	0
Biologic agent	0	0	0
NSAIDs	23(77)	0	0
Smoking, n (%)	11(37)	62	15(39)

RA: rheumatoid arthritis; Pre-RA: preclinical RA; BMI: body mass index; ESR: Erythrocyte Sedimentation Rate; CRP: C-reactive protein; DAS28: Disease Activity Score in 28 joints; ACPA: anti-citrullinated protein antibodies; IgM-RF: IgM rheumatoid factor; DMARDs: Disease-modifying anti-rheumatic drugs; MTX: Methotrexate, LEF: Leflunomide, HCQ: Hydroxychloroquine.

Stool samples were freshly collected from each participant within one hour after excretion, and were immediately frozen and stored at − 80 °C for further analyses.

Bacterial DNA Extraction and PCR amplification

Microbial DNA was isolated from stool using the FastDNA® SPIN Kit and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to manufacturer's protocols. Quality of DNA were determined by NanoDrop 2000 (Thermo Scientific, USA), and quality checked by 1% agarose gel electrophoresis. The V3-V4 hypervariable regions of the bacteria 16S rRNA gene were amplified with primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') by thermocycler PCR system (GeneAmp 9700, ABI, USA). The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol.

Illumina MiSeq sequencing and processing of sequencing data

Purified amplicons were pooled in equimolar and paired-end sequenced on an Illumina MiSeq platform (Illumina, San Diego, USA). Raw fastq files were quality-filtered by Trimmomatic and merged by FLASH with the following criteria: (i) The reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window. (ii) Sequences whose overlap being longer than 10 bp were merged according to their overlap with mismatch no more than 2 bp. (iii) Sequences of each sample were separated according to barcodes (exactly matching) and Primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE(version 7.1)with a novel 'greedy' algorithm that performs chimera filtering and OTU clustering simultaneously. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU132) 16S rRNA database using confidence threshold of 70%. Sequence count table was rarefied to the smallest number of reads per sample to reduce the effects of variable sequencing depths on downstream analyses.

Experimental animals

C57BL/6J mice were bred and raised in a specific-pathogen-free (SPF) animal facility at the Department of Laboratory Animal Center of West China Hospital, Sichuan University and housed under a 12 hours light-dark cycle. All animal experiments were approved by the Sichuan University Animal Care and Use Committee and followed the recommendations from the Guide for the Care and Use of Laboratory Animals of Chinese Association for Laboratory Animal Science.

Fecal microbiota transplantation (FMT)

For fecal microbiota transplantation, adult male mice (7 weeks of age) were pre-treated with antibiotics by oral gavage of 200 μl sterile phosphate-buffered saline (PBS) containing 1 mg/ml ampicillin, neomycin, metronidazole, and 0.5 mg/ml vancomycin daily for 3 days. Same dosages of above antibiotics were also added into drinking water during antibiotics treatment. Twenty-four hours after cessation of the antibiotics, the mice were divided into 4 groups each of 5 to 8 mice, and were fed every other day for two weeks with fecal from patients with RA, high-risk subjects (Pre-RA), healthy controls (HC), or PBS. The mice were sacrificed 1 day after the last gavage.

To prepare the fecal microbiota for transplantation, 5 samples from each population group (patients with RA, Pre-RA, HCs) were selected at random and mixed at equal weight. One gram of the mixed stool was then suspended in 5 ml PBS and centrifuged to obtain the supernatant. An aliquot of 200 ml suspension was orally administrated to each mouse.

Induction of collagen-induced arthritis (CIA)

Before CIA induction, six-week old male DBA/1 mice were first treated with antibiotics as described, and inoculated with faeces from 3 groups or PBS every other day for one week. The mice were then intradermalyl immunized at the base of the tail with 100 μl of immunization-grade chicken type II collagen (C9301, Sigma, USA) emulsified in complete Freund's adjuvant containing 5 mg/ml killed *Mycobacterium tuberculosis* (Biological product institute, Beijing, China) on day 0[18]. Twenty-one days after the first immunization, the mice received a booster in the base of the tail, and were monitored for clinical signs of arthritis.

Assessment of arthritis and histological analysis

The severity of arthritis was scored in a blinded manner as described previously[19]. The clinical scores for each paw were evaluated every other day and scored individually on a scale of 0–4, which results in a maximum score of 16 in one mouse. Each paw is scored as follows: 0, no evidence of erythema and swelling; 1, erythema and mild swelling confined to the tarsals or ankle joint; 2, erythema and mild swelling extending from the ankle to the tarsals; 3, erythema and moderate swelling extending from the ankle to metatarsal joints; 4, erythema and severe swelling encompass the ankle, foot, and digits, or ankylosis of the limb. For histology, whole paw joints were isolated and fixed in 4% paraformaldehyde, decalcified in EDTA, and then embedded in paraffin. Tissue sections (4 μm) were stained using hematoxylin and eosin (H&E). Synovial inflammation and erosion of hyaline cartilage were scored in a blinded manner on a scale of 0–3[20].

Flow cytometry analysis

Flow cytometry was performed to study the immune cells alterations in various immune organs including the small intestinal lamina propria (LP), Peyer's patches (PP), mesenteric lymph nodes (mLN), spleen, bone marrow (BM), and blood in the mice receiving stool transplantation. For the small intestine, mesenteric fat was removed and Peyer's patches were collected. The rest tissues were dissected and incubated in Hank's balanced salt solution with 5 mM EDTA to remove epithelial cells, and subsequently digested with 2 mg/ml collagenase type IV and 2.5 U/ml Nuclease. Lamina propria lymphocytes were harvested at the interphase between 40% and 80% Percoll gradient. Cells were then resuspended in staining solution (PBS with 2% bovine serum albumin, BSA) for flow cytometry. Bone marrow (BM) cells were collected by flushing the long bones with sterile PBS. For the blood, BM and spleen tissues, red blood cells were removed with lysis buffer. The single cell suspension was filtered through a 70 μm cell strainer, washed with PBS, and resuspended in staining solution for flow cytometry analysis.

Cells were stimulated with 50 ng/ml PMA (Sigma) and 1 μg/ml ionomycin for 4 hours with brefeldin A addition before T helper cells staining. Cells were treated with Fc receptor blocker rat anti-mouse CD16/CD32 (Biolegend) for 30 minutes before staining. Fluorochrome-conjugated monoclonal antibodies utilized include: Alexa Fluor 700 anti-mouse CD45, APC-Cy7 anti-mouse CD3e, Percp-Cy5.5 anti-mouse CD4, FITC anti-mouse IFN-γ, APC anti-mouse IL-4, PE anti-mouse IL-17A, BB515 anti-mouse CD25, APC anti-mouse Foxp3, BV510 anti-mouse CD19, FITC anti-mouse CD11b, Percp-Cy5.5 anti-mouse Ly6G. All antibodies and relative isotype controls were purchased from BD Biosciences. For intracellular IFN-γ, IL-4 and IL-17A staining, we used the Intracellular Fixation & Permeabilization buffer set (BD), and Foxp3 / Transcription Factor Staining Buffer Set (eBioscience) was used for Foxp3 staining. Samples were analyzed by BD FACS Canto and subsequent analysis was performed with FlowJo.

Bacterial IgA coating assay

50 mg of frozen fecal pellets were incubated with 1 ml of PBS for 30 min and then homogenized 30 seconds using a bead beater. 10 μl of supernatant was harvested and added to 1 ml PBS with 2% BSA. Bacteria were then pelleted at 8000 g for 5 min at 4 °C and supernatant was discarded. After blocking with 2% BSA, samples were stained with PE anti-mouse IgA (eBioscience), washed twice, and then stained with nuclear stain SYTO BC (Invitrogen). Samples were analyzed by BD FACS Celesta.

Quantitative RT-PCR

Total RNA was isolated from the small intestine using the TRIzol (Invitrogen). RNA was digested with DNaseI and reverse transcribed into cDNA using an oligo d(T) primer. Quantitative real-time polymerase chain reaction (RT-PCR) was performed using SYBR Green master mix (Vazyme). Samples were analyzed in triplicate, and the relative RNA expression was normalized to that of beta-actin. Primer sequences were shown in Supplementary Table 1.

RT^2 Profiler PCR Array Gene Expression

Total RNA was isolated from the small intestine using the TRIzol Reagent (Invitrogen) and purified with RNeasy Mini Kit and RNase-Free DNase Set (Qiagen). RNA quality was determined using a spectrophotometer and was reversely transcribed using RT^2 First Strand Kit. The complementary DNA was used on the real-time RT2 Profiler PCR Array Mouse Inflammatory Response and Autoimmunity (catalog no. PAMM- 077Z; QIAGEN). Cycle threshold values were exported to a table for analysis, which were normalized based on a full panel of reference genes. The fold changes were calculated using the delta-delta cycle threshold method.

Gut permeability test

Intestinal permeability was evaluated by measuring the fluorescence intensity of FITC-conjugated dextran uptake. Mice were deprived of food and water for 4 hours, and then administered with non-digestible FITC-conjugated dextran, 4 kDa (Sigma) at a dose of 0.6 mg/g body weight. Eighty microliters of serum were collected from the tail vein and diluted with PBS (1:1). The fluorescence intensity of FITC in serum

was measured using the synergy plate reader (Biotek). The gene expression of tight junction protein (ZO-1) and mucin (MUC2) in the small intestine was determined by RT-PCR as described above.

Immunofluorescent staining

Paraffin-embedded small intestine tissues were used for analyzing the expressions of CD4 and IL-17. After deparaffinization, the slides were heated in an autoclave with sodium citrate for antigen repairing, followed by 5% goat serum for blocking. Slides were then incubated with primary antibody {Rat Anti-mouse CD4 antibody at 1:50 (Invitrogen), Rabbit Anti-mouse IL-17 antibody at 1:400 (Abcam)} at 4 °C overnight. Anti-rat Alexa Fluor 555 (1:1000) and Anti-rabbit Alexa Fluor 647 (1:500) were used as respective second antibody. Signals were observed using confocal microscopy (A1R MP+, Nikon).

Statistical analysis

To determine statistically different bacterial taxa among the three groups, we applied the Kruskal-Wallis test, with multiple test corrected by Benjamini-Hochberg false discovery rate (FDR) test. The linear discriminant analysis (LDA) effect size (LefSe) analysis was applied to dissect the most discriminatory taxa among groups. Different features with an LDA score cut-off of 3.0 were identified. Analysis of similarities (ANOSIM) test was used to assess the difference in microbial compositions due to group factors. For cross-sectional analyses of differences in baseline characteristics, diversity indexes and other numerical variables among three or more groups, one-way ANOVA test was applied followed by Bonferroni correction. Chi-square test or Fisher's exact test was used to evaluate the proportional difference in categorical variables among three or more groups. Data were presented as means ± SEM. For animal studies, statistical significance was determined routinely using one-way ANOVA followed by Bonferroni post hoc test for multiple comparisons. Two-tailed P values < 0.05 were considered significant. Statistical tests were performed with GraphPad Prism 7.0 software (GraphPad, La Jolla, CA).

Results

Cohort demographics and clinical features

A total of 121 human participants provided fecal samples including 38 healthy controls (HC), 53 ACPA positive preclinical high-risk subjects (Pre-RA), and 30 diagnosed RA patients (RA). Basic demographics (age, gender,BMI and smoking status) were similar across all groups (Table 1). Eighteen of the 53 Pre-RA subjects reported history of arthralgia, although no arthritis based on physical examination of 44 joints was detected when first enrolled. Among Pre-RA subjects, ACPA and IgM-RF positivity were 100% and 25%, respectively, compared to 97% and 87% in RA patients (ACPA, $P = 0.36$; IgM-RF, $P < 0.0001$). The sera antibodies titers in RA group, were 1.88-fold higher for ACPA (median level 330.3 U/ml vs. 175.3 U/ml) and 5.52-fold higher for IgM-RF (median level 413.2 IU/ml vs. 74.8 IU/ml), compared to Pre-RA group. The mean disease duration in RA patients was 17.7 months (median 13.0 months) and the mean DAS28 was 4.91, reflecting the presence of moderate disease in these early RA patients. Most of the patients with RA were on treatment but none with biological agents.

Profiles of gut microbiota shift between Pre-RA and RA in human

By 16S rRNA sequencing of fecal microbiota, comparable community richness (Sobs index) and slightly lower bacterial diversity (Shannon index) were found in Pre-RA subjects compared with HC (Fig. 1A). The majority of OTUs (640) were shared by all groups, with 65 OTUs detected only in Pre-RA and RA groups. The four dominate phyla across all groups were *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (Fig. 1B, C). For beta diversity, principal coordinate analysis (PCoA) based on Bray-Curtis dissimilarity metrics revealed significantly different bacterial community structures and compositions of the fecal microbiomes of the three groups (ANOSIM test, $P < 0.001$, R = 0.12). Interestingly, the overall intestinal microbiota communities dynamically shifted from HCs to Pre-RA subjects and to RA patients (Fig. 1D and **Supplementary Fig. 1)**. Discriminatory genera including *Bacteroides* and *Ruminiclostridium_5*, were dynamically decreased in relative abundance (Fig. 1E). On the contrary, *Streptococcus*, *Lactobacillus*, *Lactococcus*, *Ruminococcaceae_UCG-004* and *Weissella* were significantly enriched in RA patients when compared to HC (Fig. 1E).

To determine taxonomic biomarkers that characterize the differences in the progression of RA, linear discriminant analysis (LDA) analysis was performed (Fig. 2A). We identified potential disease stage specific bacteria, including 1) *Clostridia* and *Bacteroidetes* in HCs, 2) *Blautia* and norank genus of the family *Lachnospiraceae* in Pre-RA individuals, and 3) *Bacilli*, *Lactobacillales* and *Streptococcaceae* in RA patients. Utilizing random forest analysis, a panel of six species were selected as bacterial biomarker to build ROC curve, which yield an AUC of 0.78 (95%CI = 0.66–0.9) in discriminating RA patients from HCs (Fig. 2B, C). Taken together, these data indicated that RA patients had significantly altered intestinal microbiota which may serve as diagnostic biomarkers, and these changes had already begun since preclinical stage.

Fecal microbiota from high-risk individuals impairs gut barrier function in mice

To explore whether the altered fecal microbiota in preclinical high-risk individuals has an effect on gut barrier function, we transferred the stool samples from HCs, Pre-RA and RA patients (or PBS alone as control) to antibiotics-treated mice. The intestinal microbial profiles of mice were determined by 16S rRNA sequencing after inoculation. The recipient mice exhibited significantly different microbial community structures by non-metric multidimensional scaling analysis (NMDS) ($P = 0.001$) (Fig. 3A, B). *Fusobacterium* and *Escherichia-Shigella* genera were enriched in Pre-RA and RA group of mice, separately. *Akkermansia* and *Staphylococcus* genera were enriched in both Pre-RA and RA groups compared with HC (Fig. 3C). In addition, there were increases in the intestinal permeability of the mice receiving stool transplantation from Pre-RA individuals compared with HC, evidenced by the significantly increased dextran uptake (Fig. 3D), although a higher dextran uptake were found in all three groups of stool-transferred mice than the PBS group. Reduced mRNA expressions of tight junction protein (*Zo1*)

and mucin (*Muc*2) were also detected, accompanied with injured microvilli of the small intestine in Pre-RA fecal transplanted mice (Fig. 3D-F). Whereas the mRNA level of *Claudin1*, *Occludin*, *Tff3* and *Cdx2* were comparable among all stool-transferred groups (**Supplementary Fig. 2**). These data collectively reflected increased permeability and impaired mucosal barrier of the gut in mice receiving Pre-RA stool inoculation.

Fecal microbiota from high-risk individuals triggers intestinal mucosal immune perturbation

We then sought to detect whether fecal microbiota dependent induction of gut permeability would affect local mucosal or systemic immunity status. To do so, we measured T helper cells subsets (Th1, Th2, Th17), regulatory T cells (Tregs), B cells and neutrophils in various immune organs including mesenteric lymph nodes (mLN), Peyer's patches (PP) and lamina propria (LP) of the small intestine, spleen and bone marrow (BM) by flow cytometry. Increased frequencies of Th17 cells, but not Th1 or Th2 cells, were found in mesenteric lymph nodes and Peyer's patches in Pre-RA and RA group of mice (Fig. 4A-C, **Supplementary Fig. 3**). Although no significant differences were found in the frequencies of Th17, Th1 and Th2 cells in the spleen and LP of the small intestine across all groups (**Supplementary Fig. 4–5**). Immunofluorescent staining of CD4 and IL-17 in the small intestine indicated an enhanced CD4 and IL-17 positive signals (Fig. 4D). The proportions of Tregs in Peyer's patches, mesenteric lymph nodes and LP of the small intestine, B cells in Peyer's patches and spleen, neutrophils in peripheral blood and bone marrow were comparable across all groups (**Supplementary Fig. 6–8**). Taking together, these data suggest that fecal microbiota from Pre-RA individuals are able to cause the imbalance of intestinal mucosal immunity, accompanied with dramatic Th17 cell subset skewing.

To gain insights into the molecular basis underlying the pro-inflammatory effect of the stool from patients with RA and Pre-RA subjects, we used the Mouse Inflammatory Response and Autoimmunity PCR Array to profile in small intestine the expression of 84 key genes involved in inflammatory response. The data showed up-regulation of 15 genes for more than 1.5-fold in Pre-RA and/or RA groups, many of which encode for cytokines and their receptors. The list included the genes interleukin 17a (*Il17a*), *Il22*, *Il23a*, C-C motif chemokine ligand 20 (*Ccl20*), *Il1b*, and *Il6*, corresponding to the immune system involving chemotaxis, proinflammatory response, and in particular Th17 activation pathway (Fig. 4E, F). Quantitative RT-PCRs were further performed to verify these genes differences among different groups. Significant increases in mRNA expressions of *Il17a*, *Il22* and *Tnfa* were confirmed in the small intestine of mice receiving Pre-RA and RA stool inoculation (Fig. 4G). RA fecal transplanted mice also had notably increased *Il6* mRNA expression, compared with HC and Pre-RA fecal microbiota inoculated mice. The mRNA expression of *Il23a* did not reach statistical significance among three groups (Fig. 4G).

Since secretory IgA (SIgA) serves as a critical first-line barrier that regulates host-commensal homeostasis, limiting the access of pathogens and toxins, we next applied a flow-cytometry based approach to enumerate the frequency of IgA-coated bacteria in stool transferred mice. The proportions of SIgA coated bacteria in fecal were also elevated in Pre-RA and RA groups compared with HC, which has similar level with PBS group (Fig. 4H, I).

FMT from high-risk individuals increased CIA severity

Based on the observations that human fecal microbiota changed prior to the onset of clinical RA and that it could cause intestinal immunity perturbation in recipient mice, we hypothesized that the microbiota dysbiosis in the gut mucosa may be a driving factor in the pathogenesis of RA. We therefore inoculated the mice with human fecal microbiota (or PBS) and subsequently induced arthritis using the collagen-induced arthritis (CIA) model (Fig. 5A). Severer arthritis was observed in Pre-RA and RA groups of mice after the second boost immunization compared with the HC group (Fig. 5B). On the whole, Pre-RA and RA groups of mice had the higher arthritis score, more inflammatory cells infiltration and severer cartilage erosion by histological assessment, although cartilage erosion score between HC and Pre-RA mice was not statistically significant (Fig. 5C-E). Taken together, these studies suggest that fecal microbiota from high-risk for RA individuals are able to promote CIA in mice.

To test our hypothesis that intestinal barrier impairment and mucosa immune perturbation play a critical role in disease initiation and pathogenesis of arthritis, we first studied the intestine mucosa structure under disease state by H&E staining. As observed in FMT study, injured and unorganized structure of microvilli of small intestine was found in Pre-RA and RA groups (Fig. 6A). Based on the results from FMT study, and to further pinpoint the way in which microbiota driven Th17 cell immune abnormality promotes arthritis in mice, we examined the frequency of Th17 cells in mesenteric lymph nodes and Peyer's patches 20 days after the first immunization (preclinical stages). Consistent with previous results, increased frequencies of Th17 cells were detected in mLN and Peyer's patches in Pre-RA and RA groups (Fig. 6B, C). Enhanced CD4 and IL-17 positive signals were also identified by immunofluorescent staining of the small intestine (Fig. 6D), showing increased intestinal infiltration of Th17 cells prior to arthritis symptoms.

Discussion

The need for the microbiota to drive disease in various arthritis animal models underscores its importance in RA pathogenesis[9–14], yet comprehensive investigations on the intestinal microbiome of preclinical seropositive RA and their mechanism of action on RA development are still lacking. Our present study focusing on preclinical high-risk for RA individuals identified imbalanced intestinal microbiota, which could increase gut permeability, lower the intestinal barrier function and promote mucosal immunity perturbation after transfer into mice. More importantly, the altered intestinal microbiota from high-risk for RA individuals is able to drive inflammatory arthritis development in the initial stage, leading to exacerbated CIA in mice. These findings provide direct evidence for the 'mucus origins hypothesis' that intestinal microbial dysbiosis may drive the pathogenesis of RA in the initial stages.

We previously reported a lower bacterial diversity in saliva microbiome in high-risk individuals for RA[16]. Consistent with the change in saliva, there was a significant decrease in the intestinal bacterial diversity in Pre-RA individuals compared with HC. The bacterial community richness was comparable in all groups.

Interestingly, no difference in the alpha diversity of fecal microbiota was found between established RA patients and HCs, which are also reported by other studies investigating oral or fecal samples[16, 21, 22]. These findings likely reflect a distinct microbial profile in preclinical individuals. Importantly, we identified that the bacterial community structures dynamically shifted during disease progression as represented by the three groups of individuals. Comparative analysis of the relative abundance identified gradual reductions of some genera including *Bacteroides and Ruminiclostridium_5*, as disease progression. *Bacteroides* spp., especially *Bacteroides fragilis* has been implicated in directing Tregs differentiation in the gut to maintain mucosal tolerance[23]. The reduction of reportedly beneficial *Bacteroides* in new-onset RA patients was correlated to the expansion of *Prevotella copri*[24], a species that closely associated with RA[17, 25] and metabolic disorders[26]. During disease progression, a genus of the class *Clostridia — Ruminiclostridium_5* was also reduced. *Clostridia* is most famous for the clusters IV, XIVa and XVIII of *Clostridia* in promoting differentiation and function of Tregs[27]. Although potentially beneficial taxa such as *Bacteroides* and *Ruminiclostridium_5* was reduced, the Tregs remain unchanged in the intestine, suggesting a relatively redundant role of these taxa in Tregs regulation. The present study did not find enrichment of *Prevotella* in neither high-risk individuals nor RA patients as indicated by other studies[17, 24]. In fact, despite suggesting pathogenically expanded in RA patients from western countries, our study and several previous studies based on Chinese populations failed to reveal such a change of *Prevotella* in RA[22, 28]. It is probably due to the distinct ethnical, dietary or clinical features of the subjects involved. However, we did find *Streptococcus* enriched in RA patients. Recently, Boer *et al* found in the Rotterdam Study that the abundance of intestinal *Streptococcus* species is associated with increased knee pain and inflammation[29]. While many streptococcal species are symbiotic, some are pathogens responsible for various disorders, including pneumonia, sepsis, and endocarditis[30]. Another piece of evidence linking *Streptococcus* infection (among other pathogens) with joint disease is reactive arthritis, which is commonly taken as sterile inflammatory arthritis as a sequel to extra-articular bacterial infection, often in the gastrointestinal or urogenital tract[31]. In vitro study found that *Streptococcus pyogenes* may promote osteoclastogenesis and bone destruction by stimulating receptor activator of NF-κB ligand (RANKL) expression by osteoblasts[32]. Collectively, these findings demonstrated an altered fecal microbiota in preclinical high-risk individuals, with beneficial taxa reduced and potential pathogenic taxa elevated, although the exact functions of each specific genus or species in such a scenario can be quite complicated.

We also examined the intestinal microbiota of mice by 16S rRNA sequencing after stool transplantation. The recipient mice exhibited significantly different microbial community structures. *Fusobacterium* were enriched in Pre-RA group of mice compared with HC, while *Akkermansia* and *Staphylococcus* were enriched in both Pre-RA and RA groups. Interestingly, *Fusobacterium* has been implicated in metabolic disorder[33, 34], osteoarthritis[35] and Behcet's disease[36]. One of the most well-known pathogenic species within the *Fusobacterium* genus, *Fusobacterium nucleatum*, are associated with the occurrence of multiple conditions including colorectal carcinoma[37], IBD[38], and RA[39]. The last decade has witnessed a growing interest in *Akkermansia*, of which *Akkermansia muciniphila* is the only species isolated until now and was originally identified by its capacity of degrading mucin in human intestine[40].

The enrichment of this mucin-degrading bacterium in both Pre-RA and RA groups corresponded to the decreased mucin level, which may promote the breakdown of gut barrier and antigen exposure. Although taken as a beneficial bacterium in IBD[41] and anti-PD-1 immunotherapy against tumor[42], *A. muciniphila* was significantly enriched in patients with MS[43], spondyloarthritis (SpA)[44] and RA[28]. A study from Taiwan revealed increased abundance of *A. muciniphila* in RA patients, especially ACPA positive patients[28]. Additionally, *A. muciniphila* stimulates the proinflammatory immune phenotype of PBMC[43], aggravates intestinal inflammation in infected mice[45], promote arthritis in K/BxN mice[46], and its enrichment is associated with the onset of arthritis in transgenic rat model of SpA[47]. These findings imply a mucin-degrading and proinflammatory role of *A. muciniphila* in inflammatory disorders, including RA. We are aware of the mismatch of differentially abundant bacteria in human groups (aforementioned) and stool transferred mice, as has been seen in many well-conducted studies[36, 48, 49].There are several possible explanations for these discrepancies: 1) Although human and mice intestinal microbiota share considerable similarity at the genus level (around 80 genera in common), they have large quantitative differences[50]. Not all human gut microbiome can colonize germ-free or antibiotic treated mice likely due to inter-organism variations in diet, environment and the small-intestine: colon length ratio[51, 52]; 2) The microbe interaction network can be altered in the absence or presence of some genera, which would change the differentially abundant genera cross organisms. Therefore, it's more precise to conclude that the colonized microbiota in mice receiving stool from Pre-RA and RA patients drive the pathogenesis of arthritis.

Gut microbiota alteration commonly influences the intestinal barrier function and local mucosa immunity status of an individual[15, 53]. In fact, inoculation of stool from high-risk individuals indeed increased gut permeability for FITC/dextran 4 kDa and impaired the intestinal mucosal barrier as revealed by increased dextran uptake, decreased mRNA expression of the tight junction protein (ZO-1) and mucin (MUC2), and histologically identified injured microvilli of the small intestine in mice. The disturbance of intestinal first-line physical barrier likely boosts the exposure of bacterial antigens at the interface and subsequent activation of the intestinal immune response. Consistently, our data suggest that the Pre-RA and RA microbiota may educate the immune system inducing secretory IgA response (increased IgA coated bacteria), chemotaxis (up-regulation of *Ccl20*), proinflammatory response (*Il1b*, *Tnfa* and *Il6*), and in particular Th17 activation pathway (*Il17a*, *Il22*). Th17 effector cytokines, IL-17 and IL-22, promote intestinal barrier function and induce both antimicrobial peptide and IgA secretion[54]. Increased infiltration of Th17 cells in the small intestine were found in Pre-RA and RA groups compared with HC, especially in Peyer's patches and the draining mesenteric lymph nodes. In fact, several studies have shown that the mucosal Th17 populations are highly dependent on the microbial composition. Colonization of mice with *segmented filamentous bacterium* (SFB) induces Th17 cells accumulation in small intestine lamina propria (SI LP)[55]. Periodontal pathogens such as *Porphyromonas gingivalis* and *Prevotella nigrescens* promote CIA by inducing Th17 response driven by TLR2 and IL-1[56]. The severity of arthritis was significantly mitigated in Th17-deficient CIA mice, in which the disease-promoting effect of Th17 highly rely on microbiome constitution[57]. Furthermore, translocation of *Enterococcus*

gallinarum into systemic organs to drive autoimmune pathogenesis are associated with gut barrier breakdown and skewed T cells towards Th17 cells in both SI LP and mLN in autoimmune-prone host[58].

Accumulating evidences indicate the important role of Th17 cells in mucosal immunity[59]. Genetic mutations interrupting Th17 development or function are associated with increased mucosal bacterial and fungal infections[60, 61]. However, Th17 cells have potent inflammatory potential and contribute to multiple inflammatory disorders, including inflammatory bowel disease (IBD), multiple sclerosis (MS) and arthritis[13, 62]. Moreover, studies have indicated that microbiota are indispensable for arthritis development, with Th17 cells being a vital bridge in this process[13, 57]. Indeed, autoimmune inflammatory arthritis in RA and Pre-RA stool-transferred mice were more serious, and associated with injured intestine microvilli structure and intestinal Th17 cells accumulation.

There are limitations in our study. Firstly, our study investigated ACPA positive established RA patients (except one case) and high-risk individuals, which did not take the ACPA negative RA into consideration. In established RA patients, the proportion of ACPA-IgG positivity can be as high as 60 ~ 80%[28, 63–65]. Although other autoantibody positive and even seronegative RA exists, they constitute a minor proportion, and preclinical cases can hardly be identified in seronegative RA without neither symptoms nor serum biomarkers. ACPA positivity has been identified as a powerful marker for future risk for RA and most of the RA preclinical studies have focused on the ACPA positive group. Second, most of our enrolled RA patients were on treatment, which may affect the gut microbiota. Early treatment-naïve RA patients would help provide more precise information of RA gut microbiota. Fortunately, our main studying focus of preclinical cases were treatment naïve. Finally, although we proposed here the ‘intestinal microbiota-Th17-arthritis’ model, the exact mechanism of how intestinal immune perturbation elicits systemic inflammation was not fully clarified in this study. More studies are needed to further explore the potential mechanisms.

Conclusions

In summary, we demonstrated that (1) the fecal microbiota dysbiosis occurred in the high-risk individuals for seropositive RA, with gradual alteration of community structures and the abundance of several genera during disease progression; (2) transplantation of the dysbiotic fecal microbiota impaired the gut barrier function and triggered Th17-featured intestinal immune perturbation; (3) fecal microbiota from high-risk RA exacerbated arthritis in CIA model, with preclinical enrichment of Th17 cells in the gut. These results suggested that, through breakdown of gut barrier, intestinal microbiota dysbiosis triggered mucosal immune perturbation may represent the early event driving the development of RA during preclinical stage. This study provides the possibilities to predict or prevent RA development by monitoring the intestinal bacterial community and/or interfering with the altered gut microbiota in the preclinical high-risk individuals.

Declarations

Ethics approval and consent to participate

The study procedure was approved by the Biomedical Research Ethics Committee, West China Hospital of Sichuan University (ChiCTR1900022605), and informed written consents were obtained from all human participants according to the Declaration of Helsinki. All animal experiments were approved by the Sichuan University Animal Care and Use Committee and followed the recommendations from the Guide for the Care and Use of Laboratory Animals of Chinese Association for Laboratory Animal Science.

Consent for publication

Not applicable

Availability of data and material

The fastq files of 16S rRNA sequencing were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA573829).

Competing interests

Not applicable

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Authors' contributions

YL.T., HT.N., HR.T., YH.L., LC.S. and YX.W. performed the experiments. All authors provided editorial input. All authors made substantial contributions to the acquisition, analysis, and interpretation of data described in this article. All authors critically reviewed the report and approved the final version.

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Not applicable

References

1. McInnes, I.B. and G. Schett, *The pathogenesis of rheumatoid arthritis.* N Engl J Med, 2011. **365**(23): p. 2205-19.

2. Smolen, J.S., D. Aletaha, and I.B. McInnes, *Rheumatoid arthritis.* Lancet, 2016. **388**(10055): p. 2023-2038.
3. Nielen, M.M.J., et al., *Specific autoantibodies precede the symptoms of rheumatoid arthritis: A study of serial measurements in blood donors.* Arthritis & Rheumatism, 2004. **50**(2): p. 380-386.
4. Jorgensen, K.T., et al., *Cytokines, autoantibodies and viral antibodies in premorbid and postdiagnostic sera from patients with rheumatoid arthritis: case-control study nested in a cohort of Norwegian blood donors.* Ann Rheum Dis, 2008. **67**(6): p. 860-6.
5. Rantapaa-Dahlqvist, S., et al., *Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis.* Arthritis Rheum, 2003. **48**(10): p. 2741-9.
6. van de Stadt, L.A., et al., *The extent of the anti-citrullinated protein antibody repertoire is associated with arthritis development in patients with seropositive arthralgia.* Ann Rheum Dis, 2011. **70**(1): p. 128-33.
7. Luo, Y. and W.C. Blackledge, *Microbiome-based mechanisms hypothesized to initiate obesity-associated rheumatoid arthritis.* Obes Rev, 2018. **19**(6): p. 786-797.
8. Tong, Y., et al., *Microbiota and metabolites in rheumatic diseases.* Autoimmun Rev, 2020: p. 102530.
9. Jubair, W.K., et al., *Modulation of Inflammatory Arthritis in Mice by Gut Microbiota Through Mucosal Inflammation and Autoantibody Generation.* Arthritis Rheumatol, 2018. **70**(8): p. 1220-1233.
10. Rehaume, L.M., et al., *ZAP-70 genotype disrupts the relationship between microbiota and host, leading to spondyloarthritis and ileitis in SKG mice.* Arthritis Rheumatol, 2014. **66**(10): p. 2780-92.
11. Taurog, J.D., et al., *The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats.* J Exp Med, 1994. **180**(6): p. 2359-64.
12. Abdollahi-Roodsaz, S., et al., *Stimulation of TLR2 and TLR4 differentially skews the balance of T cells in a mouse model of arthritis.* J Clin Invest, 2008. **118**(1): p. 205-16.
13. Wu, H.J., et al., *Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells.* Immunity, 2010. **32**(6): p. 815-27.
14. Maeda, Y., et al., *Dysbiosis Contributes to Arthritis Development via Activation of Autoreactive T Cells in the Intestine.* Arthritis Rheumatol, 2016. **68**(11): p. 2646-2661.
15. Chen, J., et al., *An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis.* Genome Med, 2016. **8**(1): p. 43.
16. Tong, Y., et al., *Oral Microbiota Perturbations Are Linked to High Risk for Rheumatoid Arthritis.* Frontiers in Cellular and Infection Microbiology, 2020. **9**(475).
17. Alpizar-Rodriguez, D., et al., *Prevotella copri in individuals at risk for rheumatoid arthritis.* Ann Rheum Dis, 2019. **78**(5): p. 590-593.
18. Meng, X., et al., *Hypoxia-inducible factor-1α is a critical transcription factor for IL-10-producing B cells in autoimmune disease.* Nat Commun, 2018. **9**(1): p. 251.
19. Meng, X., et al., *Hypoxia-inducible factor-1alpha is a critical transcription factor for IL-10-producing B cells in autoimmune disease.* Nat Commun, 2018. **9**(1): p. 251.

20. Yamanishi, Y., et al., *Regulation of joint destruction and inflammation by p53 in collagen-induced arthritis.* Am J Pathol, 2002. **160**(1): p. 123-30.
21. Scher, J.U., et al., *Periodontal disease and the oral microbiota in new-onset rheumatoid arthritis.* Arthritis Rheum, 2012. **64**(10): p. 3083-94.
22. Zhang, X., et al., *The oral and gut microbiomes are perturbed in rheumatoid arthritis and partly normalized after treatment.* Nat Med, 2015. **21**(8): p. 895-905.
23. Round, J.L. and S.K. Mazmanian, *Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota.* Proc Natl Acad Sci U S A, 2010. **107**(27): p. 12204-9.
24. Scher, J.U., et al., *Expansion of intestinal Prevotella copri correlates with enhanced susceptibility to arthritis.* Elife, 2013. **2**: p. e01202.
25. Pianta, A., et al., *Evidence of the Immune Relevance of Prevotella copri, a Gut Microbe, in Patients With Rheumatoid Arthritis.* Arthritis Rheumatol, 2017. **69**(5): p. 964-975.
26. Pedersen, H.K., et al., *Human gut microbes impact host serum metabolome and insulin sensitivity.* Nature, 2016. **535**(7612): p. 376-81.
27. Atarashi, K., et al., *Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota.* Nature, 2013. **500**(7461): p. 232-6.
28. Chiang, H.I., et al., *An Association of Gut Microbiota with Different Phenotypes in Chinese Patients with Rheumatoid Arthritis.* J Clin Med, 2019. **8**(11).
29. Boer, C.G., et al., *Intestinal microbiome composition and its relation to joint pain and inflammation.* Nat Commun, 2019. **10**(1): p. 4881.
30. Patterson, M.J., *Streptococcus*, in *Medical Microbiology. 4th edition*. 1996, University of Texas Medical Branch at Galveston.
31. Hannu, T., *Reactive arthritis.* Best Pract Res Clin Rheumatol, 2011. **25**(3): p. 347-57.
32. Okahashi, N., et al., *Infection by Streptococcus pyogenes induces the receptor activator of NF-kappaB ligand expression in mouse osteoblastic cells.* Infect Immun, 2003. **71**(2): p. 948-55.
33. Liu, R., et al., *Gut microbiome and serum metabolome alterations in obesity and after weight-loss intervention.* Nat Med, 2017. **23**(7): p. 859-868.
34. Haro, C., et al., *The gut microbial community in metabolic syndrome patients is modified by diet.* J Nutr Biochem, 2016. **27**: p. 27-31.
35. Huang, Z., et al., *Faecal microbiota transplantation from metabolically compromised human donors accelerates osteoarthritis in mice.* Ann Rheum Dis, 2020. **79**(5): p. 646-656.
36. Ye, Z., et al., *A metagenomic study of the gut microbiome in Behcet's disease.* Microbiome, 2018. **6**(1): p. 135.
37. Kostic, Aleksandar D., et al., *Fusobacterium nucleatum Potentiates Intestinal Tumorigenesis and Modulates the Tumor-Immune Microenvironment.* Cell Host & Microbe, 2013. **14**(2): p. 207-215.
38. Strauss, J., et al., *Invasive potential of gut mucosa-derived fusobacterium nucleatum positively correlates with IBD status of the host.* Inflammatory Bowel Diseases, 2011. **17**(9): p. 1971-1978.

39. Moen, K., et al., *Synovial inflammation in active rheumatoid arthritis and psoriatic arthritis facilitates trapping of a variety of oral bacterial DNAs.* Clin Exp Rheumatol, 2006. **24**(6): p. 656-663.
40. Derrien, M., et al., *Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium.* Int J Syst Evol Microbiol, 2004. **54**(Pt 5): p. 1469-1476.
41. Png, C.W., et al., *Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria.* Am J Gastroenterol, 2010. **105**(11): p. 2420-8.
42. Routy, B., et al., *Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors.* Science, 2018. **359**(6371): p. 91-97.
43. Cekanaviciute, E., et al., *Gut bacteria from multiple sclerosis patients modulate human T cells and exacerbate symptoms in mouse models.* Proc Natl Acad Sci U S A, 2017. **114**(40): p. 10713-10718.
44. Stoll, M.L., et al., *Altered microbiota associated with abnormal humoral immune responses to commensal organisms in enthesitis-related arthritis.* Arthritis Res Ther, 2014. **16**(6): p. 486.
45. Ganesh, B.P., et al., *Commensal Akkermansia muciniphila exacerbates gut inflammation in Salmonella Typhimurium-infected gnotobiotic mice.* PLoS One, 2013. **8**(9): p. e74963.
46. Stoll, M.L., et al., *Akkermansia muciniphila is permissive to arthritis in the K/BxN mouse model of arthritis.* Genes Immun, 2019. **20**(2): p. 158-166.
47. Asquith, M.J., et al., *Perturbed Mucosal Immunity and Dysbiosis Accompany Clinical Disease in a Rat Model of Spondyloarthritis.* Arthritis Rheumatol, 2016. **68**(9): p. 2151-62.
48. Baxter, N.T., et al., *Structure of the gut microbiome following colonization with human feces determines colonic tumor burden.* Microbiome, 2014. **2**(1): p. 20.
49. Wong, S.H., et al., *Gavage of Fecal Samples From Patients With Colorectal Cancer Promotes Intestinal Carcinogenesis in Germ-Free and Conventional Mice.* Gastroenterology, 2017. **153**(6): p. 1621-1633.e6.
50. Hugenholtz, F. and W.M. de Vos, *Mouse models for human intestinal microbiota research: a critical evaluation.* Cell Mol Life Sci, 2018. **75**(1): p. 149-160.
51. Turnbaugh, P.J., et al., *The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice.* Science translational medicine, 2009. **1**(6): p. 6ra14-6ra14.
52. Nguyen, T.L.A., et al., *How informative is the mouse for human gut microbiota research?* Disease models & mechanisms, 2015. **8**(1): p. 1-16.
53. Sorini, C., et al., *Loss of gut barrier integrity triggers activation of islet-reactive T cells and autoimmune diabetes.* Proceedings of the National Academy of Sciences of the United States of America, 2019. **116**(30): p. 15140-15149.
54. Schnupf, P., et al., *Segmented filamentous bacteria, Th17 inducers and helpers in a hostile world.* Current Opinion in Microbiology, 2017. **35**: p. 100-109.
55. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.

56. de Aquino, S.G., et al., *Periodontal pathogens directly promote autoimmune experimental arthritis by inducing a TLR2- and IL-1-driven Th17 response.* J Immunol, 2014. **192**(9): p. 4103-11.
57. Evans-Marin, H., et al., *Microbiota-Dependent Involvement of Th17 Cells in Murine Models of Inflammatory Arthritis.* Arthritis Rheumatol, 2018. **70**(12): p. 1971-1983.
58. Manfredo Vieira, S., et al., *Translocation of a gut pathobiont drives autoimmunity in mice and humans.* Science, 2018. **359**(6380): p. 1156-1161.
59. Ost, K.S. and J.L. Round, *Communication Between the Microbiota and Mammalian Immunity.* Annu Rev Microbiol, 2018. **72**: p. 399-422.
60. Okada, S., et al., *IMMUNODEFICIENCIES. Impairment of immunity to Candida and Mycobacterium in humans with bi-allelic RORC mutations.* Science, 2015. **349**(6248): p. 606-613.
61. Puel, A., et al., *Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity.* Science, 2011. **332**(6025): p. 65-8.
62. Brown, E.M., D.J. Kenny, and R.J. Xavier, *Gut Microbiota Regulation of T Cells During Inflammation and Autoimmunity.* Annu Rev Immunol, 2019. **37**: p. 599-624.
63. Yin, J., et al., *Influence of Cigarette Smoking on Rheumatoid Arthritis Risk in the Han Chinese Population.* Front Med (Lausanne), 2017. **4**: p. 76.
64. Johansson, K., et al., *Mediterranean diet and risk of rheumatoid arthritis: a population-based case-control study.* Arthritis Res Ther, 2018. **20**(1): p. 175.
65. Rönnelid, J., et al., *Longitudinal analysis of citrullinated protein/peptide antibodies (anti-CP) during 5 year follow up in early rheumatoid arthritis: anti-CP status predicts worse disease activity and greater radiological progression.* Ann Rheum Dis, 2005. **64**(12): p. 1744-9.

Figures

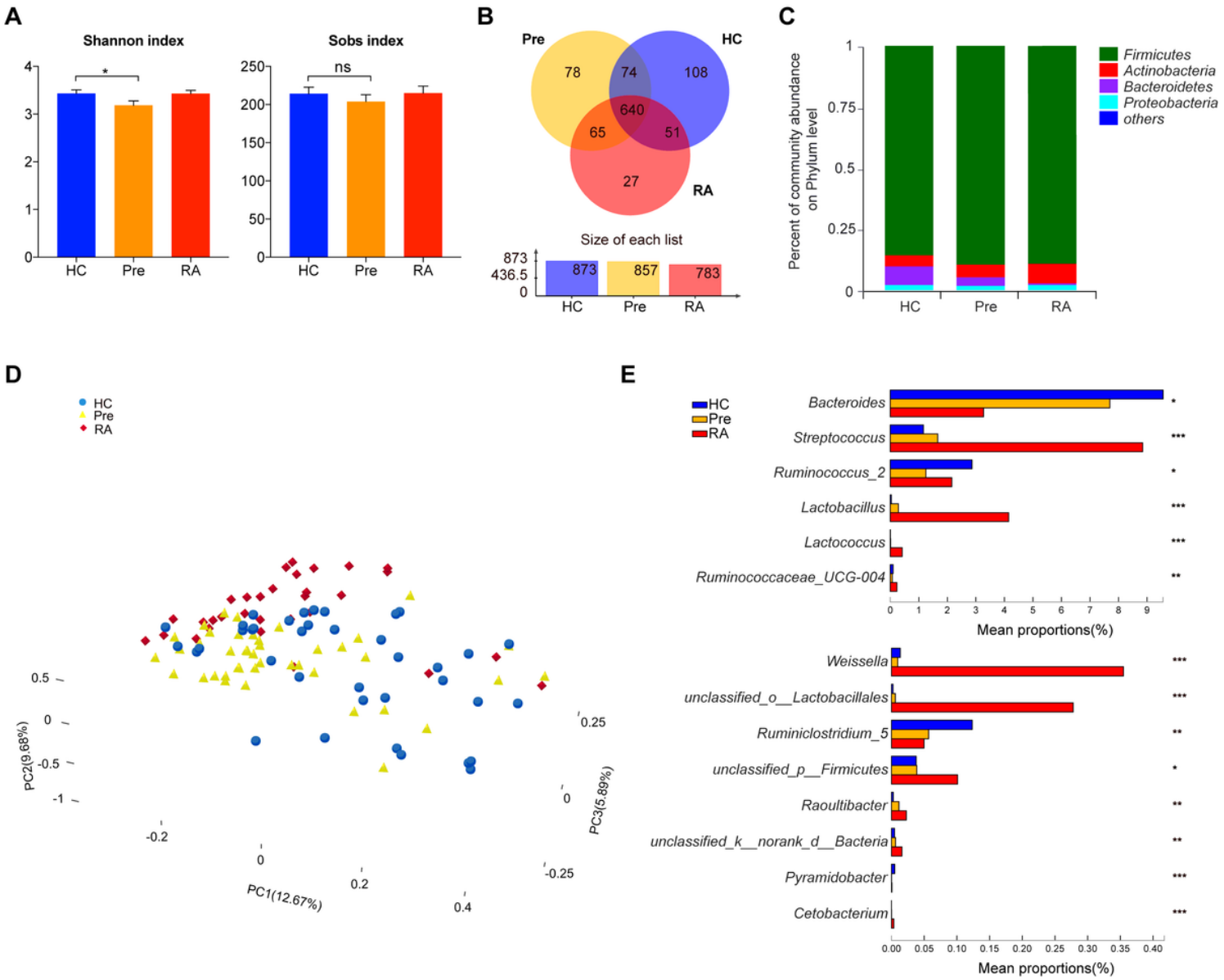
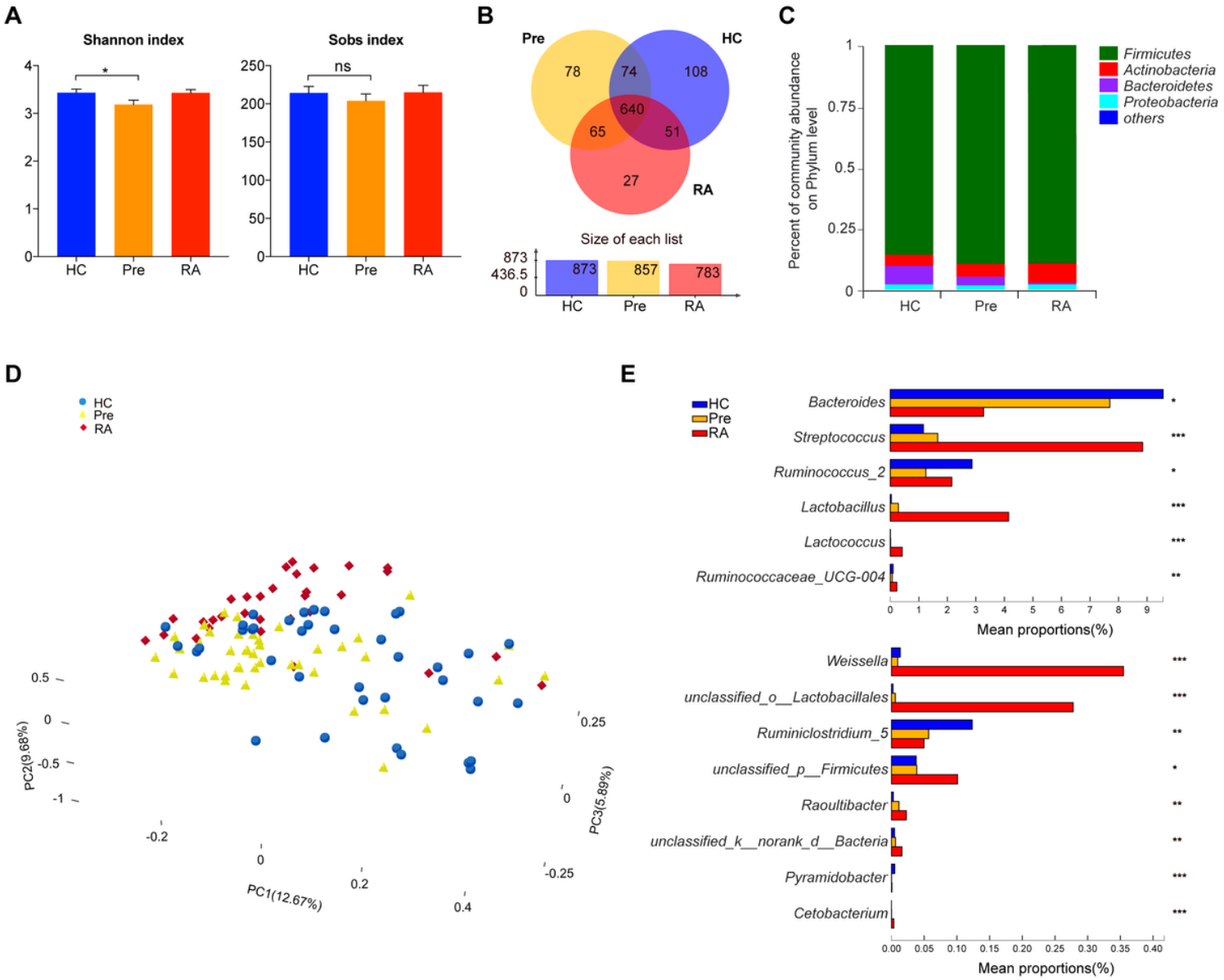


Figure 1

Fecal microbiota alterations in the high-risk for RA individuals. (A) Comparison of microbial diversity (Shannon index) and community richness (Sobs index) in healthy controls (HC), preclinical high-risk subjects (Pre-RA) and RA patients. (B) A Venn diagram showing the shared or unique OTUs in the three groups. (C) Community bar-plot analysis showed relative abundance of fecal microbiota in each group at the phylum level. (D) Three dimensional PCoA analysis of bacterial community distance based on Bray-Curtis dissimilarity metrics of relative abundance profiles. (ANOSIM test, P<0.001) (E) Differentially abundant genera between subjects from the HC, Pre-RA and RA groups. Data were compared by the Kruskal-Wallis test (p values were adjusted by the FDR correction). PCoA, principal coordinate analysis; RA, rheumatoid arthritis. *p < 0.05; **p < 0.01; ***p < 0.001.



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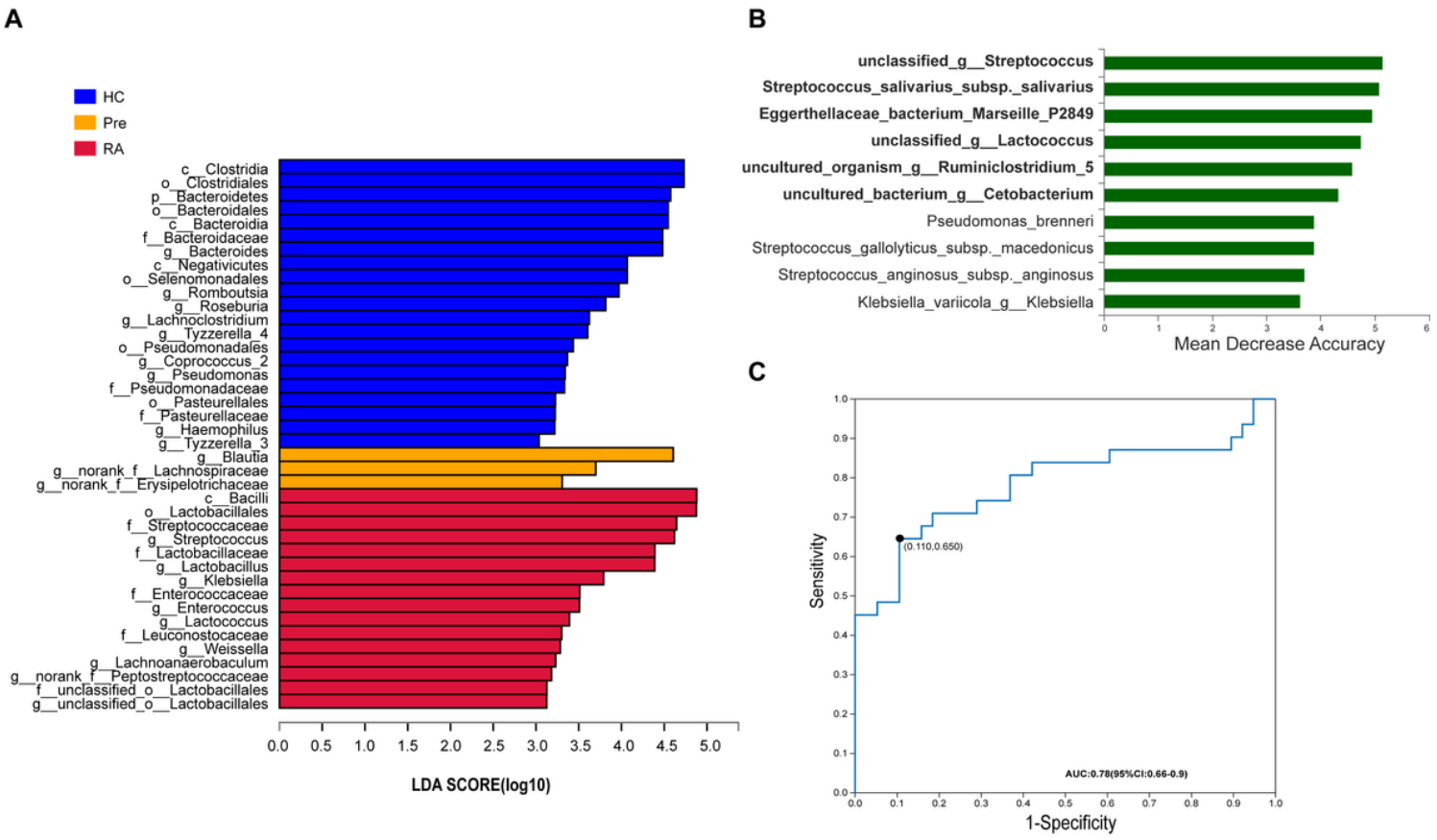
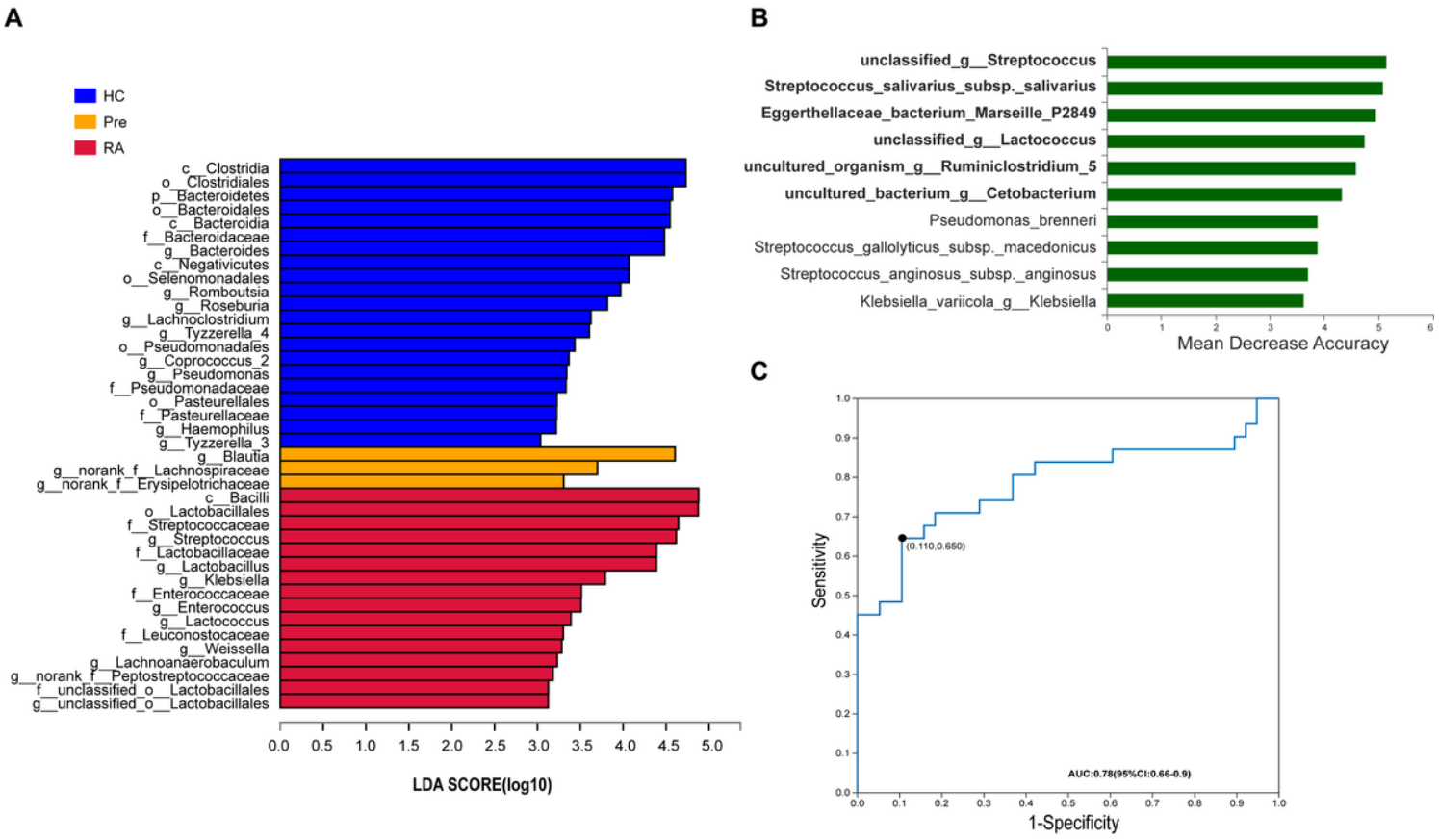


Figure 2

Taxonomic biomarkers characterizing the differences in the progression of RA. (A) LefSe analysis of differentially abundant taxa in healthy control (HC), preclinical high-risk subjects (Pre-RA) and RA patients. LDA cutoff scores was three. (B-C) Random forest model was applied to identify bacterial biomarkers for RA patients. Ranked lists of species in order of random forests reporting feature importance scores were obtained and an AUC-validation method was used to determine the optimal species set of six (B). The built ROC curve based on the selected panel of six species yield an AUC of 0.78 (C). LDA, Linear discriminant analysis; LefSe, the LDA effect size; AUC, Area under the curve; ROC, Receiver operating characteristic curve.



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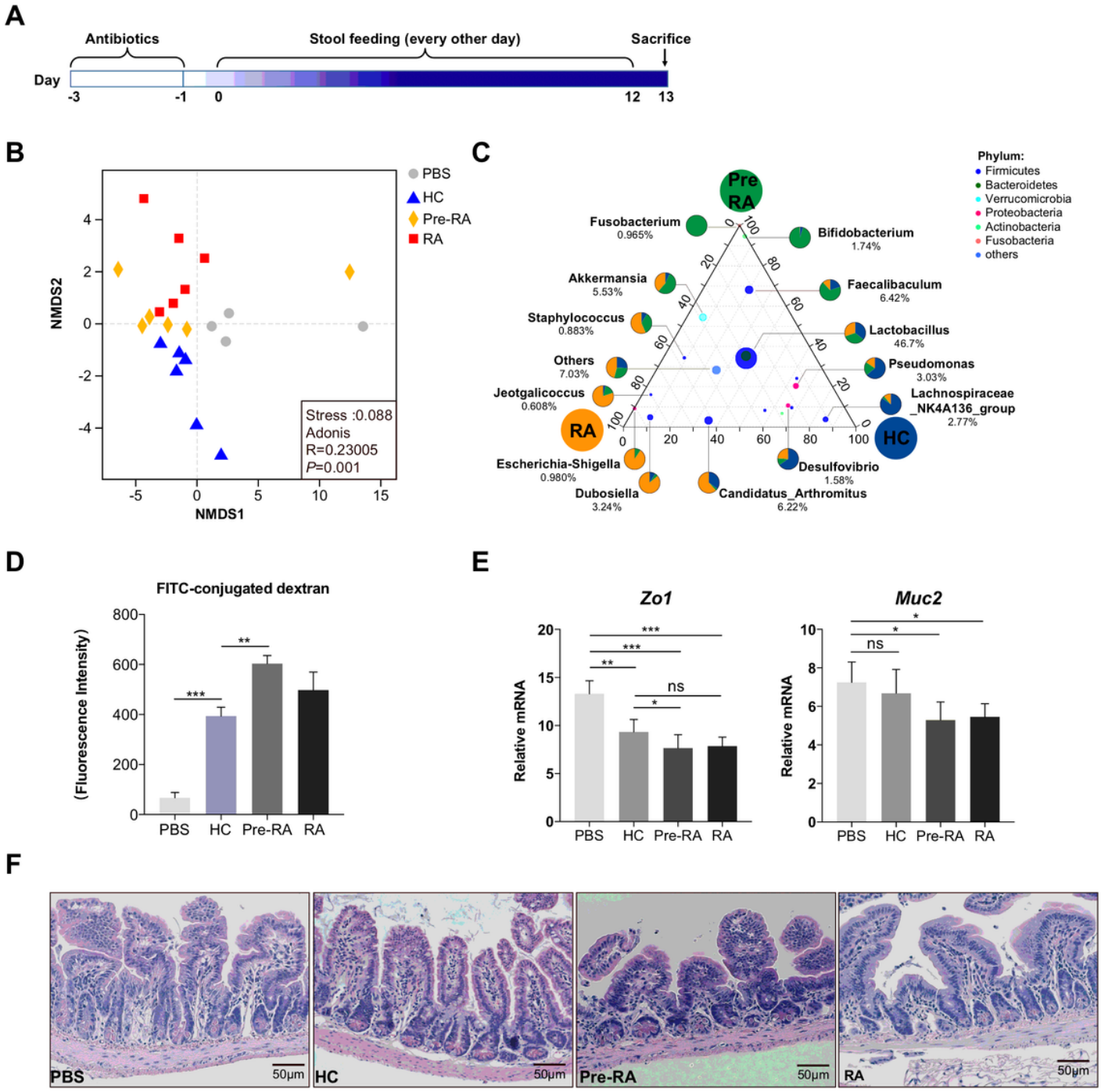
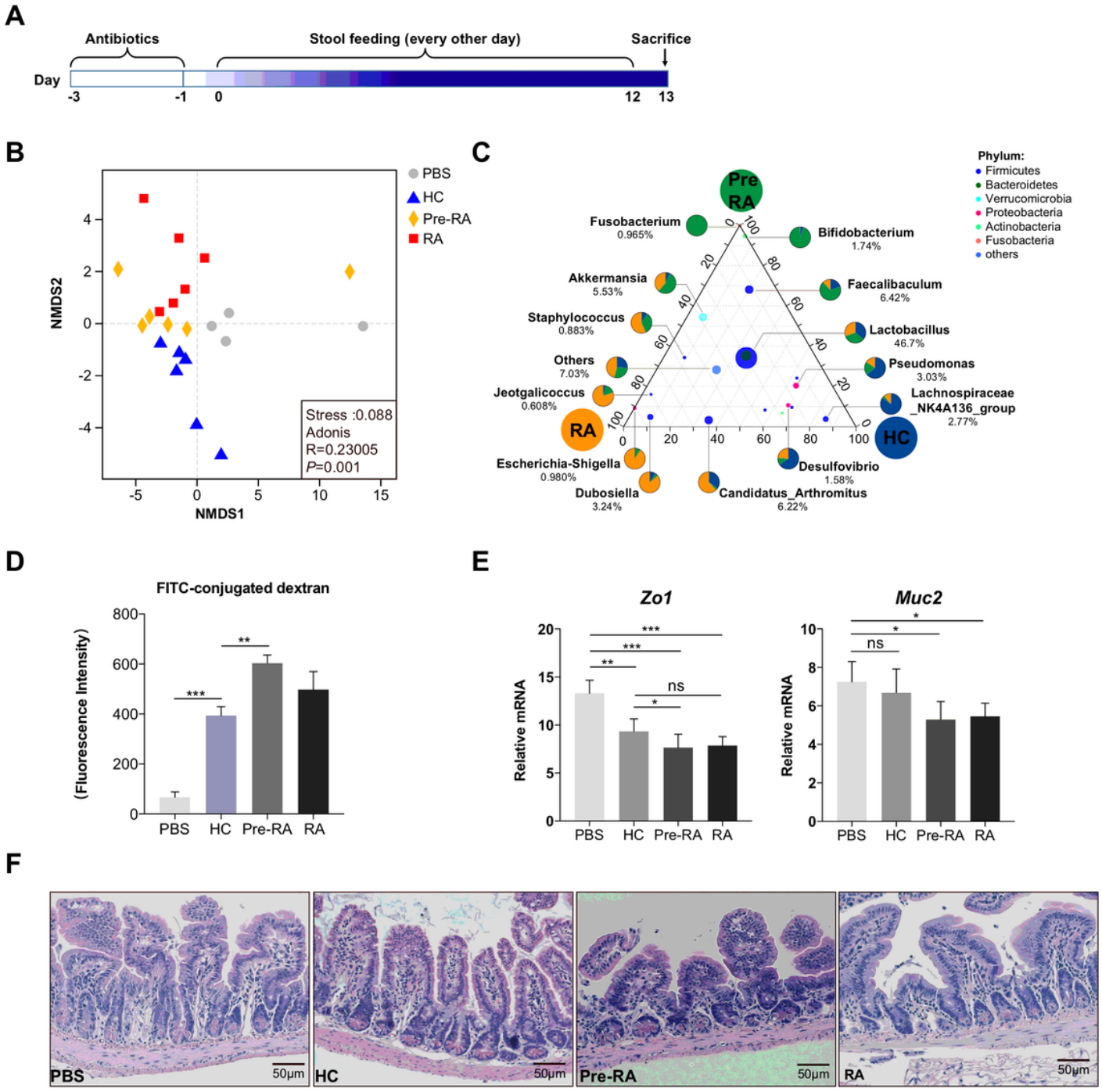


Figure 3

Gut barrier function impairs after fecal microbiota transplantation. (A) Schematic representation of fecal microbiota transplantation. SPF mice were first orally inoculated with antibiotics and afterwards with fecal contents from three different groups of people (HC, Pre-RA and RA) or sterile PBS. After 13 days, the mice were sacrificed for further analysis. (B) NMDS analysis showed significant differences between PBS, HC, Pre-RA and RA groups. (C) A ternary diagram showing the distribution and proportion of different genera in each groups of mice. (D) Fluorescence intensity of FITC-conjugated dextran in serum.

(E) (F)Representative histological slides showing normal and injured microvilli in small intestine of each group of mice. *p < 0.05; **p < 0.01; ***p < 0.001.



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PBS, HC, Pre-RA and RA groups. (C) A ternary diagram showing the distribution and proportion of different genera in each groups of mice. (D) Fluorescence intensity of FITC-conjugated dextran in serum. (E) (F)Representative histological slides showing normal and injured microvilli in small intestine of each group of mice. *p < 0.05; **p < 0.01; ***p < 0.001.

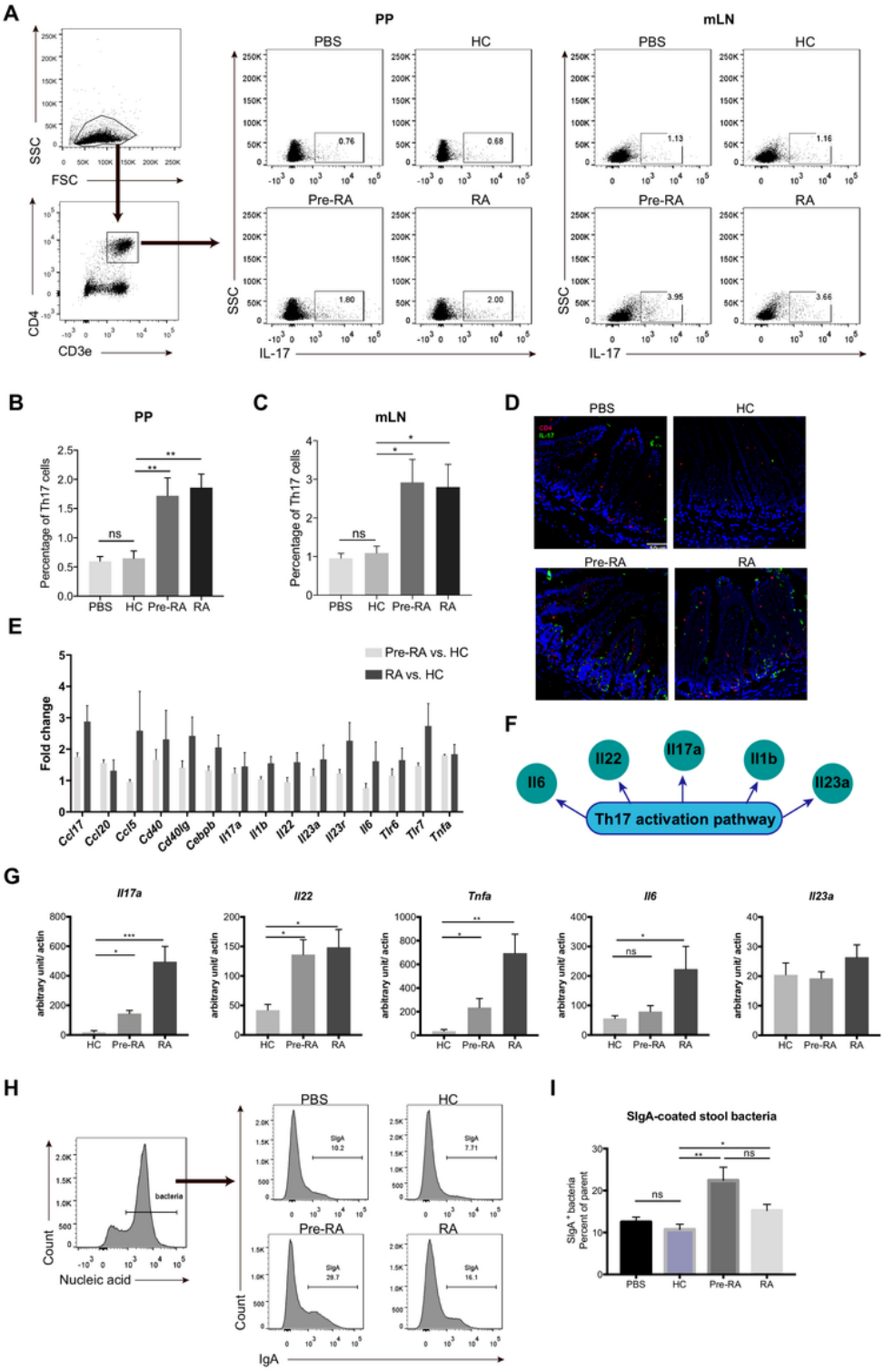
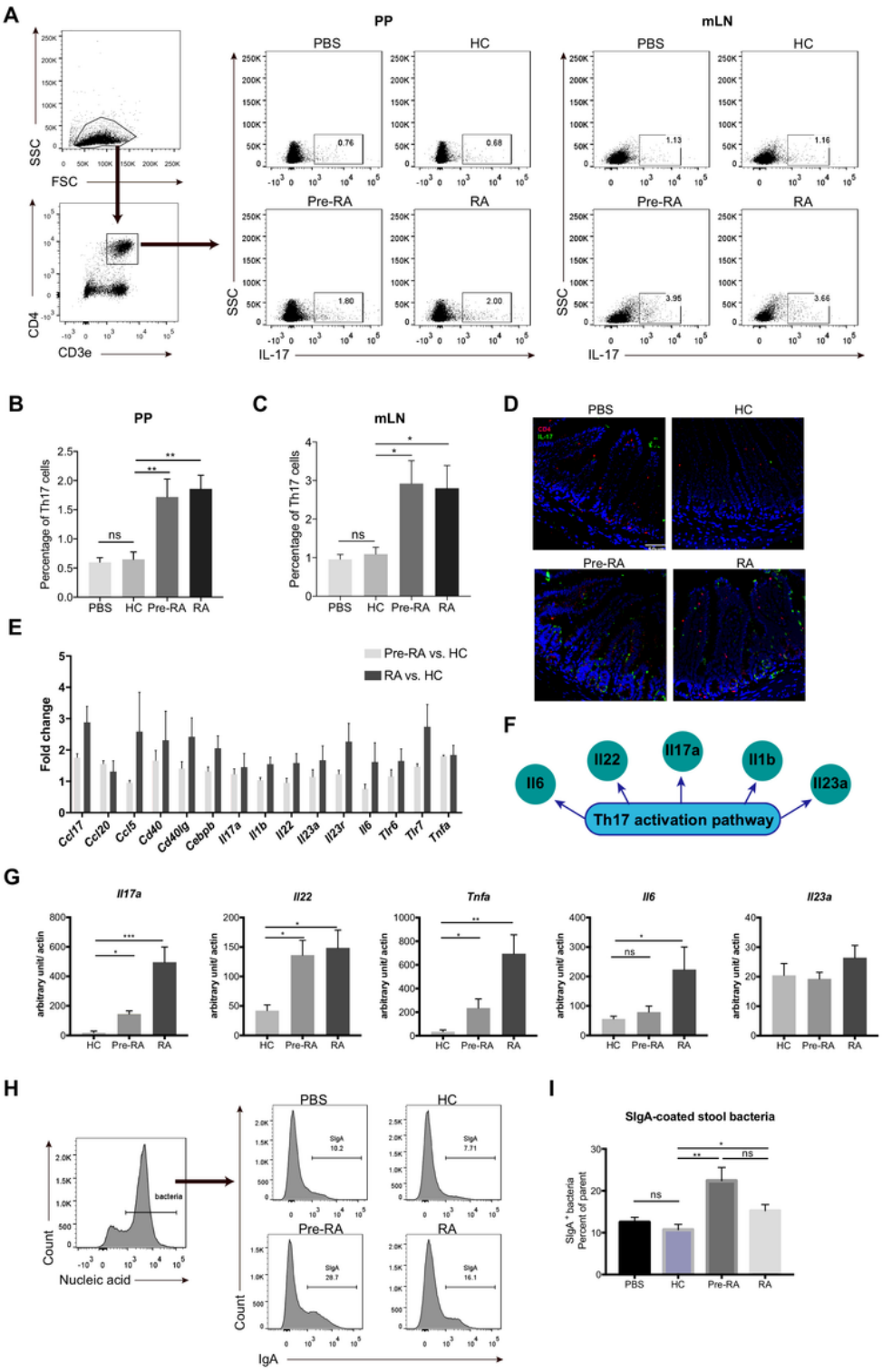


Figure 4

Fecal microbiota from high-risk individuals promotes intestinal immune perturbation. (A) Flow cytometry analysis and (B-C) bar charts showing increased Th17 cell infiltration in the small intestine Peyer's patches (PP) and mesenteric lymph nodes (mLN) in mice receiving Pre-RA and RA stool gavage. Percentages of Th17 cells were compared using the one-way ANOVA test followed by Bonferroni correction. (D) Immunofluorescent staining of CD4 (Red) and IL-17 (Green) in paraffin-embedded small intestine tissues. Nucleus were counterstained with DAPI. (E) Upregulation in the expression of 15 transcripts in the small intestine by the Mouse Inflammatory Response and Autoimmunity PCR Array, after gavage of Pre-RA and RA stool. (F) A diagram showing Th17 activation pathway implicated by upregulated genes identified by the PCR array. (G) Quantitative RT-PCR validation was performed to confirm changes in expression of genes including Il17a, Il22, Tnfa, Il6 and Il23a. Expression levels were compared using the one-way ANOVA test followed by Bonferroni correction. (H) Flow cytometry analysis and (I) bar charts showing increased frequency of IgA-coated bacteria in mice receiving Pre-RA and RA stool gavage. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.



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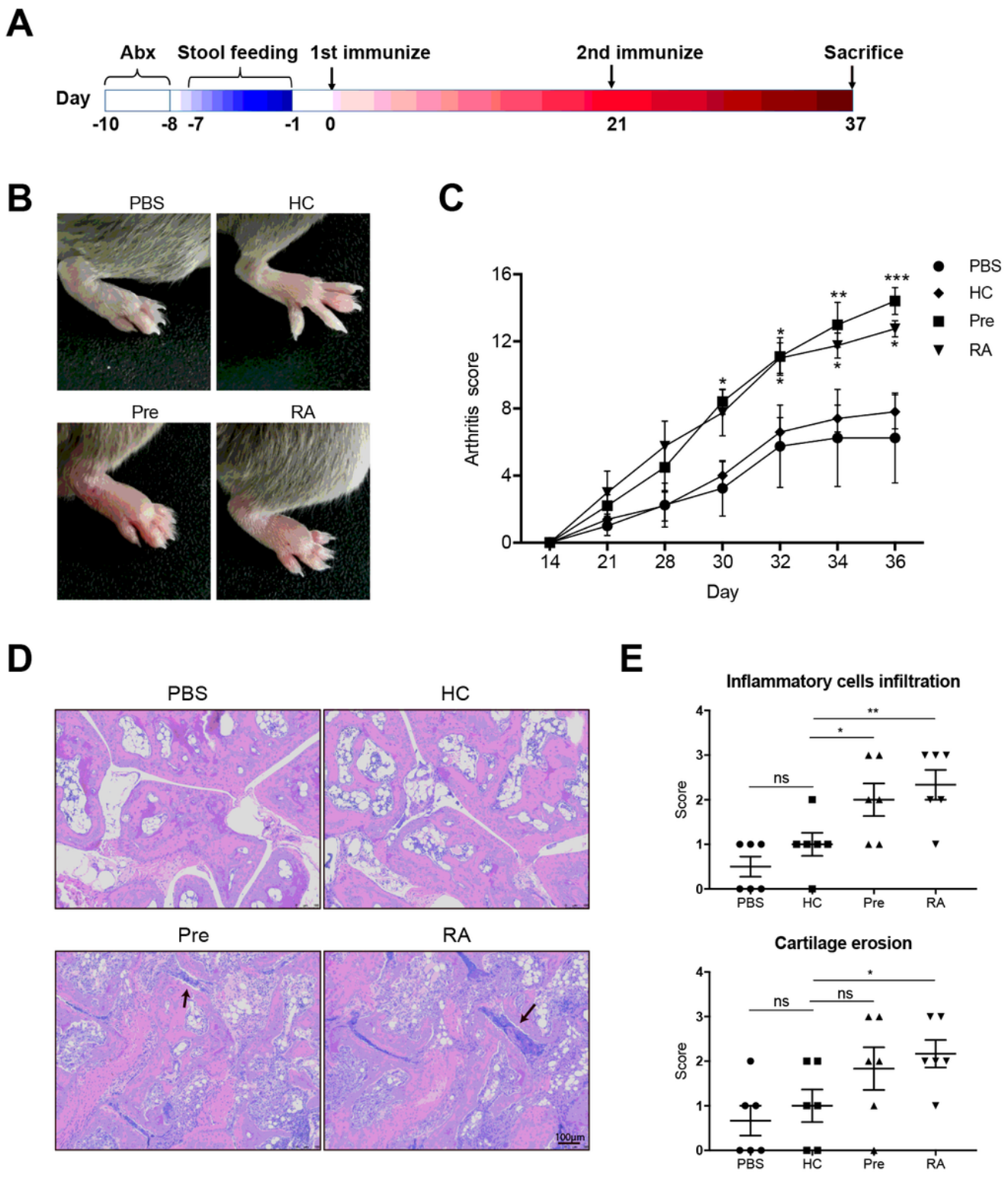
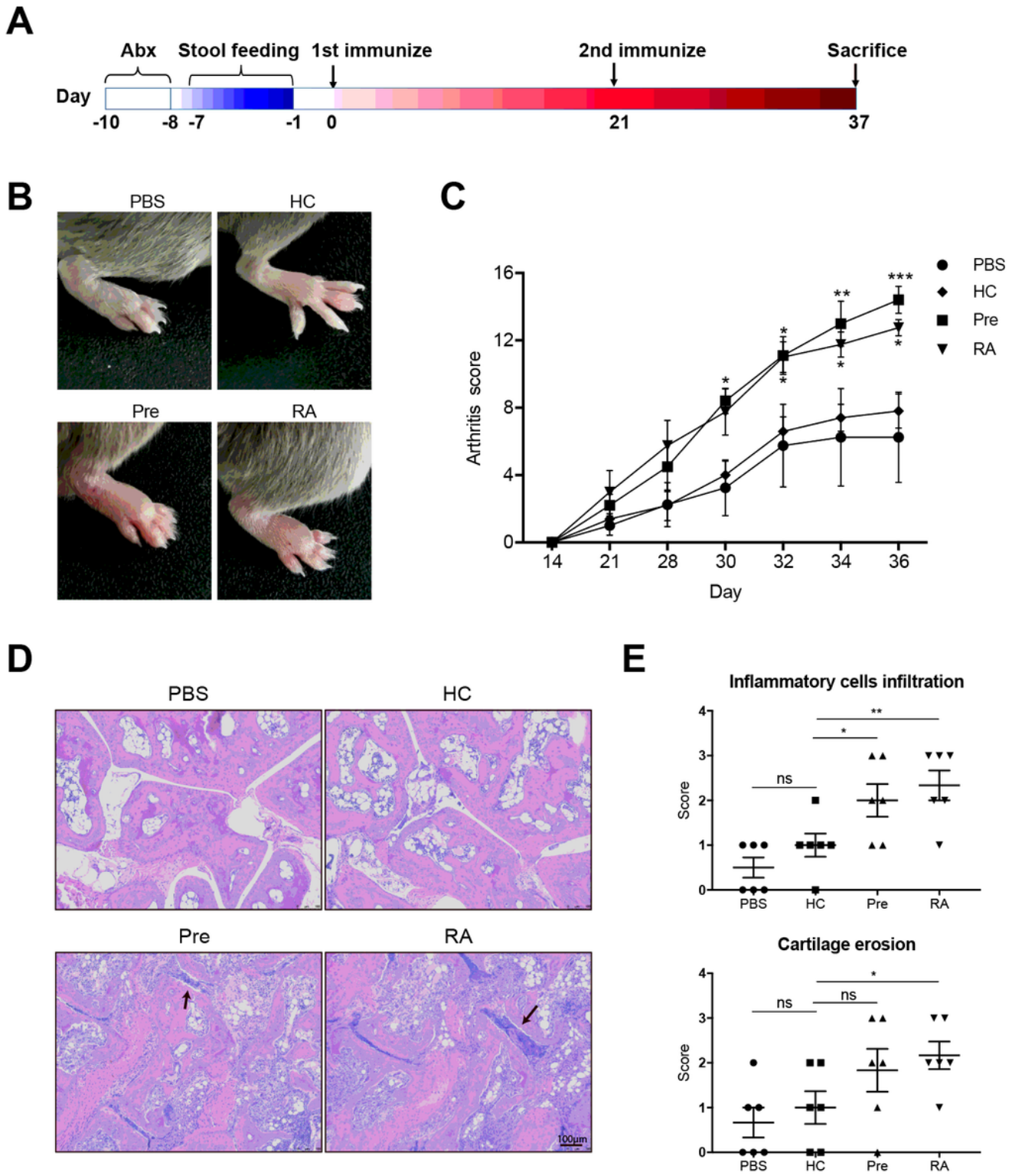


Figure 5

Exacerbated arthritis in CIA mice inoculated with fecal from high-risk individuals. (A) Schematic representation of fecal microbiota transplantation and induction of arthritis. SPF mice were first orally inoculated with antibiotics, then with fecal contents from three different groups of people (HC, Pre-RA and RA) or sterile PBS, and immunized twice (day 0 and day 21) with collagen type II to induce arthritis. The mice were sacrificed for further analysis on day 37. (B) Representative image of the mice hind paw after

the second immunization (day 34). (C) Arthritis score indicating disease severity in the four groups of mice. (D) Representative histological images and (E) quantification of inflammatory cells infiltration and cartilage erosion of the paw in each group of mice. Statistics were calculated using the one-way ANOVA test followed by Bonferroni correction. *p < 0.05; **p < 0.01; ***p < 0.001.



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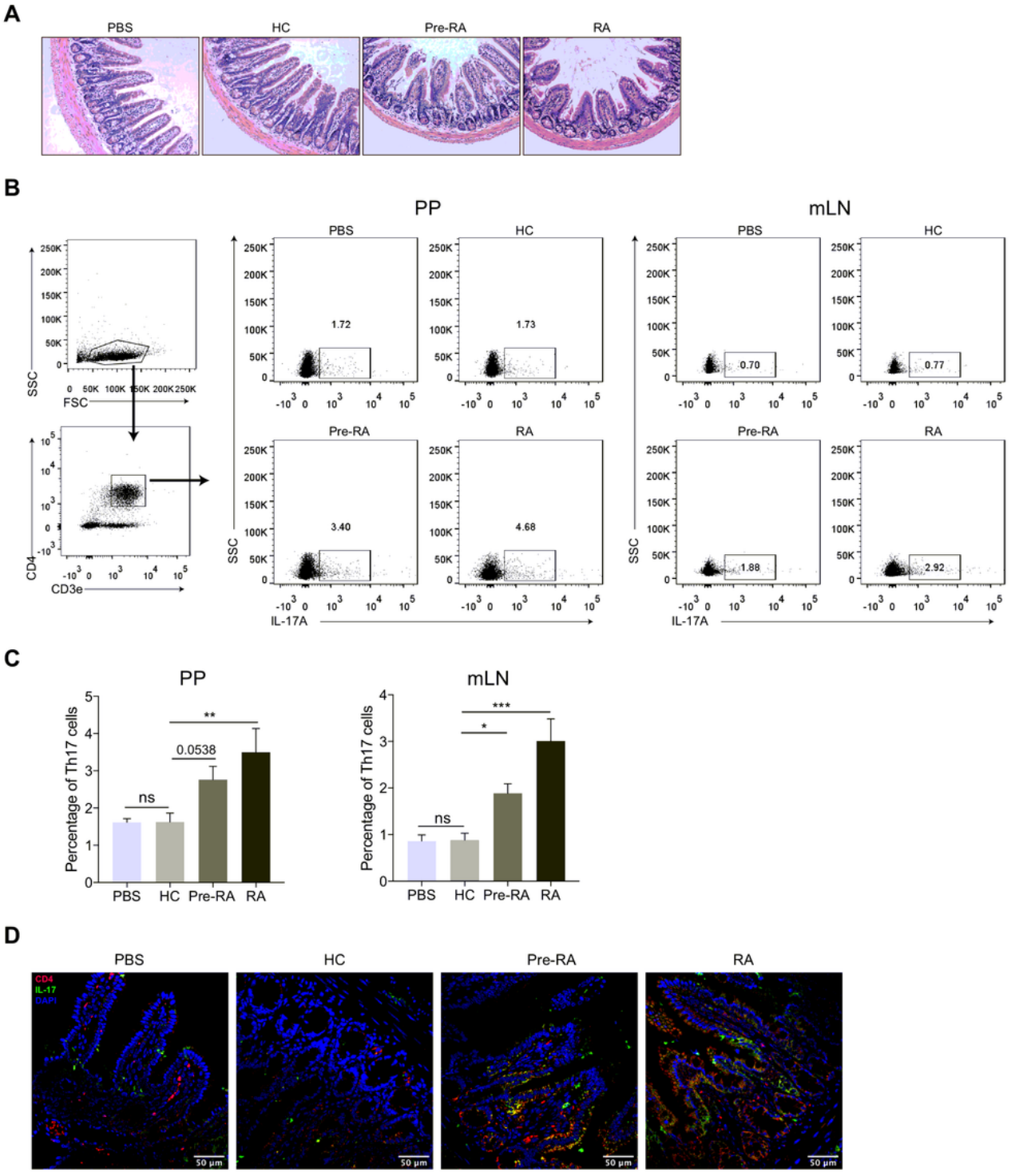

Figure 6

Fecal microbiota from high-risk individuals promotes intestinal Th17 cells infiltration in CIA mice. (A) Histological assessment of the small intestine structure in CIA mice after stool transfer. (B) Flow cytometry analysis and (C) bar charts showing increased Th17 cell infiltration in the small intestine Peyer's patches (PP) and mesenteric lymph nodes (mLN) in CIA mice receiving Pre-RA and RA stool gavage. Percentages of Th17 cells were compared using the one-way ANOVA test followed by Bonferroni

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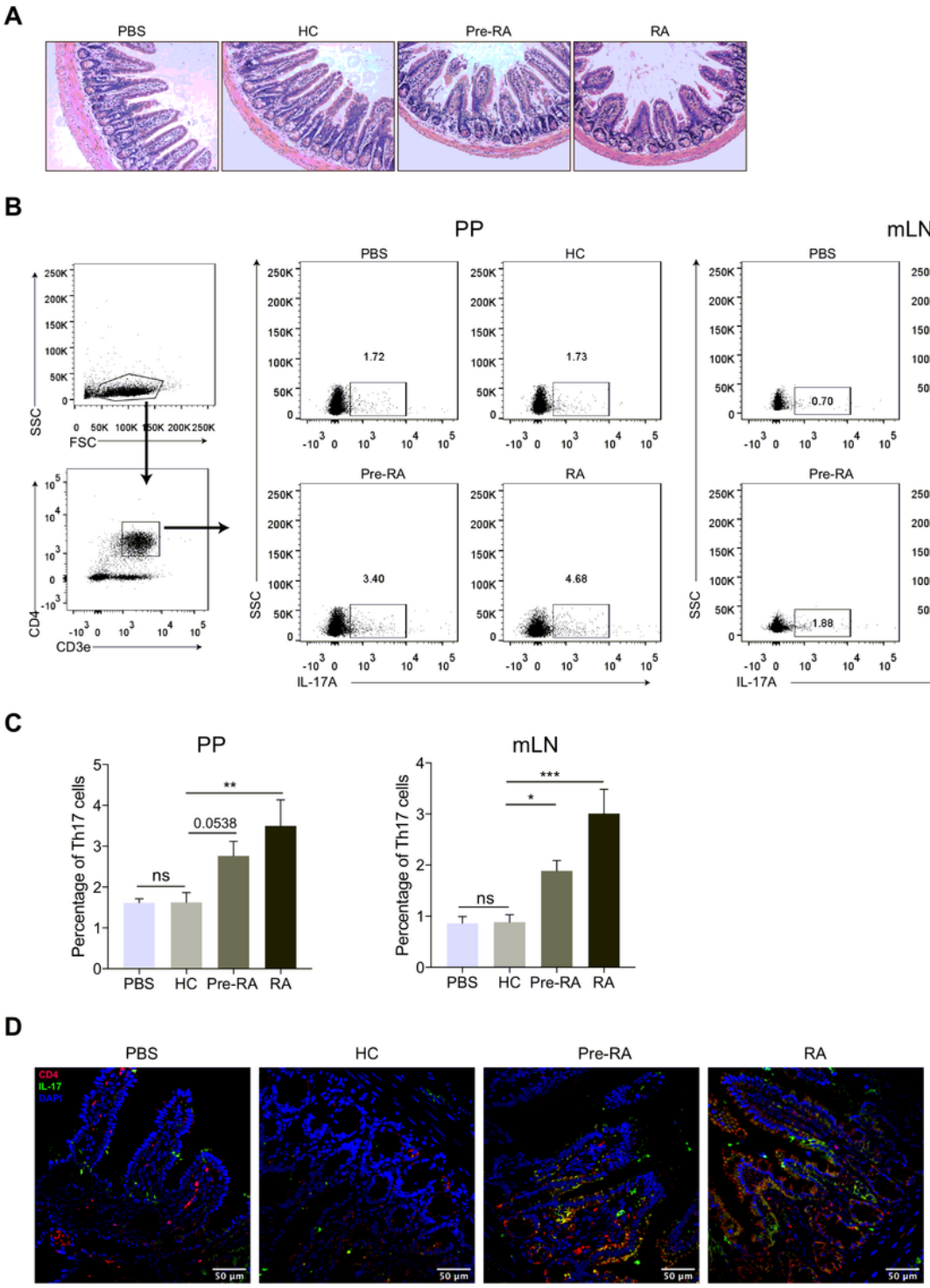

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Fecal microbiota from high-risk individuals promotes intestinal Th17 cells infiltration in CIA mice. (A) Histological assessment of the small intestine structure in CIA mice after stool transfer. (B) Flow cytometry analysis and (C) bar charts showing increased Th17 cell infiltration in the small intestine

Peyer’s patches (PP) and mesenteric lymph nodes (mLN) in CIA mice receiving Pre-RA and RA stool gavage. Percentages of Th17 cells were compared using the one-way ANOVA test followed by Bonferroni correction. (D) Immunofluorescent staining of CD4 (Red) and IL-17 (Green) in paraffin-embedded small intestine tissues. Nucleus were counterstained with DAPI. *p < 0.05; **p < 0.01; ***p < 0.001.

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