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DOI:
10.1016/j.phrs.2019.104351

Document Version
Peer reviewed version

Citation for published version (Harvard):
Raucci, F, Iqbal, AJ, Saviano, A, Minosi, P, Piccolo, M, Irace, C, Caso, F, Scarpa, R, Pieretti, S, Mascolo, N & Maione, F 2019, 'IL-17A neutralizing antibody regulates monosodium urate crystal-induced gouty inflammation', *Pharmacological Research*, vol. 147, 104351. https://doi.org/10.1016/j.phrs.2019.104351

Accepted Manuscript

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PII: S1043-6618(19)30973-9
DOI: https://doi.org/10.1016/j.phrs.2019.104351
Article Number: 104351

Reference: YPHRS 104351

To appear in: *Pharmacological Research*

Received date: 30 May 2019
Revised date: 10 July 2019
Accepted date: 10 July 2019

Please cite this article as: Raucci F, Iqbal AJ, Saviano A, Minosi P, Piccolo M, Irace C, Caso F, Scarpa R, Pieretti S, Mascolo N, Maione F, IL-17A neutralizing antibody regulates monosodium urate crystal-induced gouty inflammation, *Pharmacological Research* (2019), https://doi.org/10.1016/j.phrs.2019.104351

IL-17A neutralizing antibody regulates monosodium urate crystal-induced gouty inflammation

Running title: IL-17A and gouty inflammation.

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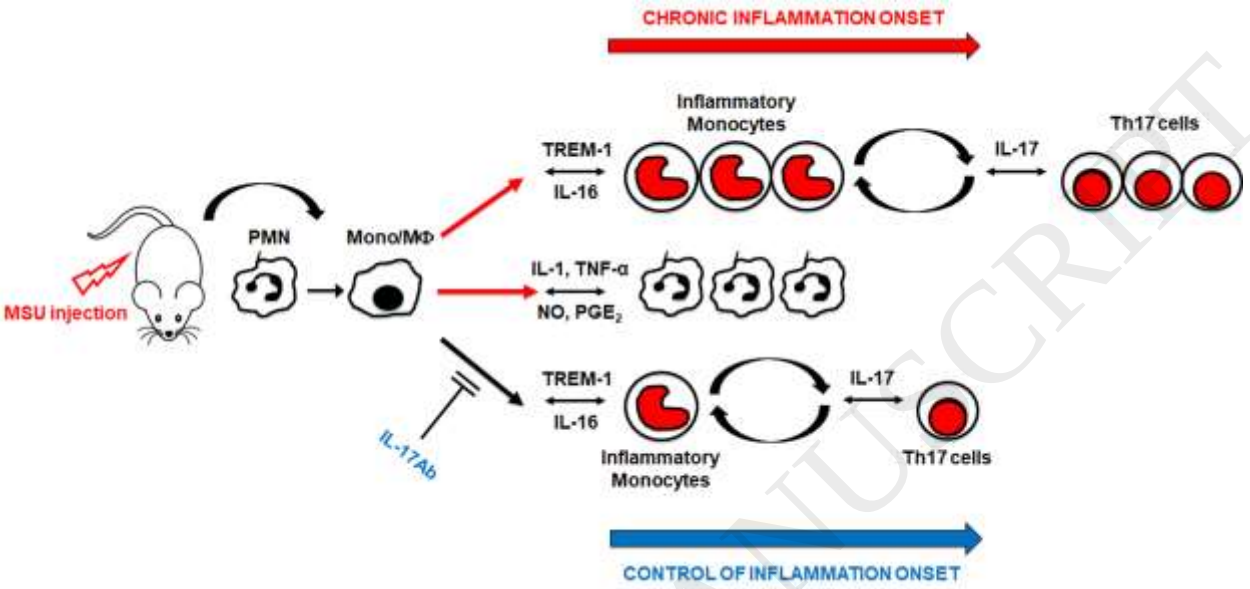
Graphical abstract



Abstract

Gout is a paradigm of acute, self-limiting inflammation caused by the deposition of monosodium urate (MSU) crystals within intra-and/or peri-articular areas, which leads to excruciating pain, joint swelling and stiffness. The infiltration of leukocytes drives the inflammatory response and remains an attractive target for therapeutic intervention. In this context, emerging evidence support the view that systemic differentiation of Th17 cells and their *in-situ* infiltration as one of the potential mechanisms by which these cells, and their main product IL-17, causes damage to the target tissues. To test if IL-17 was having a detrimental role in gouty onset and progression we targeted this cytokine, using a neutralizing antibody strategy, in an experimental model of gout.

Joint inflammation was induced in CD-1 mice by the intra-articular (i.a.) administration of MSU crystals (200 µg/20 µl). Animals from IL-17Ab-treated groups received 1, 3 and 10 µg (i.a.) in 20 µl of neutralizing antibody 30 minutes after MSU crystals administration. Thereafter, joints were scored macroscopically, and knee joint oedema determined with a caliper. Histological analyses, myeloperoxidase assay and western blots analysis for COX-2/mPGEs-1/IL-17R pathway were conducted at the 18 h time-point (peak of inflammation) to evaluate leukocyte infiltration and activation, followed by the analysis, *in situ*, of ~40 pro/anti-inflammatory cytokines and chemokines. Flow cytometry was also used to evaluate the modulation of infiltrated inflammatory monocytes and systemic Th17 and Treg profile. Treatment with IL-17Ab revealed a dose-dependent reduction of joint inflammation scores with maximal inhibition at 10 µg/20 µl. The neutralizing antibody was also able to significantly reduce leukocyte infiltration and MPO activity as well the expression of JE, IL-1α, IL-1β, IL-16, IL-17, C5a, BLC and, with a less extent IP-10, Rantes, KC, TIMP-1, SDF-1 and metalloproteinases in inflamed tissues. Biochemical analysis also revealed that IL-17Ab treatment modulated COX-2/mPGEs-1 pathway (and related PGE_2 production) without interfering with IL-17R expression.

Furthermore, flow cytometry analysis highlighted a selective modulation of infiltrating inflammatory monocytes ($B220^{-}/GR1^{hi}$-$F480^{hi}/CD115^{+}$) and circulating Th17, but not Treg, cells after IL-17Ab treatment.

Collectively the results of this study report for the first time, that i.a. injection of MSU crystals stimulates *in vivo* production of Th17 cells and Th17-related inflammatory cyto-chemokines. In addition, we have demonstrated that the administration of a neutralizing antibody against IL-17 attenuates joint symptoms, swelling and leukocytes infiltration to the inflamed tissue, possibly providing a new strategy for the treatment of gouty inflammation and/or arthritis.

Abbreviations: Ab, Antibody; BLC, B lymphocyte chemoattractant; BSA, bovine serum albumin; Cyto-chemokines, cytokines and chemokines; COX, cyclogeneses; C5a, complement component 5a; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FACS, fluorescence activated cell sorting; FBS, fetal bovine aGA, gouty arthritis; H&E, haematoxylin and eosin; i.a, intra-articular; IL-, interleukin-; IP-10, inducible protein 10; KC, keratinocyte chemoattractant; MCP-1, monocyte chemoattractant protein-1; mPGEs, microsomal prostaglandin synthetase; MHC, major histocompatibility complex; MIP-, macrophage inflammatory proteins-; MPO, myeloperoxidase; MSU, monosodium urate; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; PMSF, phenylmethansulfonyl fluoride; RA, rheumatoid arthritis; RANTES, regulated on activation normal T expressed and secreted; RT, room temperature; SDF-1, stromal cell-derived factor 1; TIMP-1, metallopeptidase inhibitor-1; TNF-α, tumor necrosis factor alfa; TREM-1, triggering receptor expressed on myeloid cells-1.

Keywords: Gout; IL-17A; Inflammation; Monosodium urate crystal; Neutralizing antibody.

Chemical compounds studied in this article: Ethanol (PubChem CID:702); PGE_2 (PubChem CID:5280360); Phosphate-buffered saline (PubChem CID: 24978514); Sodium chloride (PubChem CID:5234); Sodium urate (PubChem CID: 23697816).

1. Introduction

Gout is a paradigm of acute, self-limiting inflammation caused by increased blood uric acid levels and damaging effects of monosodium urate (MSU) crystals accumulation within intra-and/or peri-articular areas [1]. Under long-standing hyperuricemia, MSU crystals deposits, usually associated with tophi, further induce chronic inflammatory response that may lead to joint structure damage, often referred to as gouty arthritis (GA) or chronic gout [2].

During the progression of the inflammatory response and gouty attack, MSU crystals induce a significant leukocyte infiltration into the joint cavity [3]. This early pathologic hallmark is mainly characterized by neutrophil influx into the joint fluid followed by monocytes/macrophages that, in turn, phagocytose MSU crystals resulting in membranolytic and inflammasome activation [4,5], generation of oxygen derived free radicals, release of lysosomal enzymes, pro-inflammatory interleukins (mainly IL-1, IL-6 and TNF-α) and PGE_2 [6,7].

PGs and leukotrienes are powerful bioactive lipid mediators involved in numerous homeostatic functions but also in the onset of inflammation [8]. Their biosynthesis is initialized by COXs and

then finalised PGE_2 synthases (by the inducible isoform mPGEs-1) that convert PGH_2 [9] to PGE_2 [10,11]. Recently, several biochemical and pharmacological studies demonstrated a molecular interaction between COX and PGEs isoenzymes [12,13] resulting in preferential functional coupling activity of COX-2/mPGEs-1/PGE_2, which triggers the inflammatory response associated with conditions such as gout [14,15,16,17]. However, it is still unclear how this process is being regulated and which molecular mechanisms are detrimental for gouty inflammation onset [18,19].

In this context, emerging evidences support the view that systemic differentiation and maturation of Th17 cells and local recruitment as one of the potential mechanisms by which these cells cause damage to target tissues. Liu et al. [20] reported significantly elevated levels of IL-17 in the serum of GA patients early in the onset of symptoms of gout and decrease gradually as symptoms diminish. Moreover, recent work highlighted that, although the genetic variants in IL-17 do not appear to be involved in the development of gout [21], systemic Th17/Treg imbalance changes over time during the development of acute gouty arthritis and a decrease in ratio, favouring Th17 cells is consistent with inflammation development in the joints [22].

We and others have also highlighted the importance of IL-17 (and IL-17 related cytokines), [23,24,25] in sustaining the release of multiple mediators such as IL-1, IL-6, IL-8, PGE_2 and MCP-1 (also known as JE) by a wide variety of cells involved both directly and indirectly, on GA physio-pathology, including fibroblasts, endothelial cells, neutrophils and inflammatory monocytes. [26,27,28].

Collectively these findings suggest a detrimental role for IL-17 in GA onset and progression and helped us to formulate the hypothesis that targeting IL-17, by a neutralizing antibody strategy, could provide a novel treatment strategy to target gouty inflammation and/or arthritis.

2. Materials and methods

2.1 Reagents

Flow cytometry fixation and permeabilization buffer Kit I, PGE_2 Elisa kit, proteome profiler mouse cytokine array Kit, recombinant mouse IL-17 (also known as IL-17A) neutralizing antibody (IL-17Ab, monoclonal rat IgG_{2A}, clone 50104) and its related isotype control (IgG_{2A}, clone 54447) were purchased from R&D System (Milan, Italy). Collagenase (Type VIII), fetal bovine serum (FBS), hyaluronidase, monosodium urate crystals, E-Toxate™ reagent from Limulus Polyphemus and RPMI-1640 cell medium were purchased from Sigma- Aldrich Co. (Milan, Italy) whereas FACS buffer and conjugated antibodies from BioLegend (London, UK). Ficoll-Paque Plus (endotoxin tested, $\leq$ 0.12 EU/ml) was obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). For western blot analysis, the primary antibodies were obtained from Elabioscience (Milan, Italy) whereas the HRP-conjugated IgG secondary antibodies from Dako (Copenhagen, Denmark). Unless otherwise stated, all the other reagents were from BioCell (Milan, Italy).

2.2 Animals

All animal care and experimental procedures were carried out in compliance with the international and national law and policies and approved by Italian Ministry of Health. Animal studies were reported in compliance with the ARRIVE guidelines and with the recommendations made by EU Directive 2010/63/EU for animal experiments and the Basel declaration including the 3Rs concept [29,30]. CD- 1 male mice (10–14 weeks of age, 25–30 g of weight) were purchased from Charles River (Milan, Italy) and kept in an animal care facility under controlled temperature, humidity, and on a 12 hr:12 hr light:dark cycle, with *ad libitum* access to water and standard laboratory chow diet. All procedures were carried out to minimize the number of animals used (n=6 per group) and their suffering.

2.3 Preparation of MSU crystals

The MSU crystals were prepared as previously described [31]. Briefly, 800 mg of MSU were dissolved in 155 ml of boiling milli-Q water containing 5 ml of NaOH, and after the pH was adjusted to 7.2, the solution was cooled gradually by stirring at room temperature and crystals collected after centrifugation at 3.000 g for 5 min at 4ºC. The obtained crystals were washed twice with 100% ethanol, dried, autoclaved (180ºC for 2 hours), and weighed under sterile conditions. Crystals were resuspended in PBS by sonication and stored in a sterile environment until use. MSU crystals were confirmed as endotoxin-free by a commercial test kit of limulus polyphemus lysate assay (<0.01 EU/10 mg).

2.4 Induction of MSU-induced knee joint inflammation

Joint inflammation was induced by the intra-articular (i.a.) administration of MSU crystals (200 µg/20 µl) into the right knee joint of mice under isoflurane anaesthesia. Control animals received an

i.a. injection of PBS (20 µl) or 20 µl of IL-17Ab isotype control. Animals from IL-17Ab-treated groups received 1, 3 and 10 µg (i.a.) in 20 µl of neutralizing antibody 30 minutes after MSU crystals administration.

2.5 Joint scoring and evaluation

In a preliminary experiment finalized to evaluate the dose-responsive effect of IL-17Ab, the joints from different experimental conditions were scored macroscopically on a scale from 0 to 3, where 0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, and 3 = severe inflammation, in increments of 0.25. A score of 0.25 was given when the first signs of swelling and redness were present. Joint swelling scoring was performed by two authors without knowledge of the experimental groups [32]. After macroscopic scoring, knee joint oedema was determined with a caliper (Mitutoyo) before (zero time), and after the i.a. injection with MSU crystals or MSU crystals plus IL-17Ab at the times indicated. Knee joint oedema was determined for each mouse by subtracting the initial paw value to the paw value measured at each time point and presented as Δ mm/joint [31].

2.6 Histology

Histological analysis was conducted at the 18 h time-point. Joints were trimmed, placed in decalcifying solution (EDTA 0.1 mM in PBS) for 14 days and then embedded in paraffin. Sections (5 µm) were deparaffinized with xylene and stained with haematoxylin and eosin (H&E) as previously described [23] for conventional morphological evaluation. A dimension used for the analysis of the slices was 569 x 633 pixels and magnification ×20. In all cases, a minimum ≥3

sections per animal were evaluated. Phase-contrast digital images were taken using the Image Pro image analysis software package.

2.7 Myeloperoxidase assay

Leukocyte myeloperoxidase (MPO) activity was assessed by measuring the H_2O_2- dependet oxidation of 3,3′,5,5′- tetramethylbenzidine as previously reported [23]. Tissues (knee joint) were homogenized for 35 s in a solution composed of hexadecyltrimethylammonium bromide (0.5% w/v) in 50 mM sodium phosphate buffer pH 5.4. After homogenization, samples were centrifuged (4000 g) for 20 min and the supernatant used for the assay. Aliquots of 20 µl were incubated with 160 µl of 3,3′,5,5′- tetramethylbenzidineand 20 µl of H_2O_2 (in 80 mM phosphate buffer, pH 5.4) in 96- well plates. Plates were incubated for 5 min at room temperature, and OD was read at 620 nm using a plate-reader (Biorad, Italy). Assay were performed in duplicates and normalized for protein content.

2.8 Western blot analysis

Whole knee joints homogenates (35 µg of protein) were subjected to SDS-PAGE (12% gel) using standard protocols as previously described [33]. The proteins were transferred to PVDF membranes in the transfer buffer [25 mM Tris–HCl (pH 7.4) containing 192 mM glycine and 20% v/v methanol] at 400 mA for 2 h. The membranes were saturated by incubation for 2 h with non-fat dry milk (5% wt/v) in PBS supplemented with 0.1% (v/v) Tween 20 (PBS-T) for 2 h at RT and then incubated with 1:2000 dilution of primary antibodies over-night at 4°C as such as mouse monoclonal anti-COX-2 (E-AB-27666), rabbit polyclonal anti-mPGEs-1 (E-AB-32563), rabbit polyclonal anti-IL-17R (E-AB-63080) and mouse monoclonal anti-actin (E-AB-20094) and then

washed 3 times with PBS-T. Blots were then incubated with a 1:3000 dilution of related horseradish peroxidase-conjugated secondary antibody for 2 h at RT and finally washed 3 times with PBS-T. Protein bands were detected by using the enhanced chemiluminescence (ECL) detection kit and Image Quant 400 GE Healthcare software (GE Healthcare, Italy). Protein bands were quantified using GS 800 imaging densitometer software (Biorad, Italy) and normalized with respective actin.

2.9 Isolation and characterization of joint-infiltrating cells

According to the protocol described by Akitsu and colleagues [34] ankle joints were cut out and digested with hyaluronidase (2.4 mg ml^{-1}) and collagenase (1 mg ml^{-1}) in RPMI 1640 plus 10% FBS for 1 h at 37 °C. The cells were filtered through a cell strainer with a 70-µm nylon mesh (Becton Dickinson, Franklin Lakes, NJ, USA) and washed with RPMI 1640 plus 10% FBS. Thereafter, collected cells were washed in PBS for total cell count before flow cytometry analysis.

2.10 Cytokines and Chemokines Protein Array

All mice were killed at indicated time-point and knee joint immediately removed and collected into a 2 ml tube for an immediate preservation in liquid nitrogen and a successive storage at −80°C. The isolated tissues were homogenized in ice- chilled Tris–HCl buffer (20 mM, pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM sodium orthovanadate, and one protease inhibitor tablet per 50 ml of buffer. Protein concentration was determined by the BioRad protein assay kit (BioRad, Italy). Equal volumes (1.5 ml) of the pulled homogenates were then incubated with the precoated proteome profiler array membranes according to the manufacturer's instructions. Dot plots were detected by using the enhanced chemiluminescence detection kit and Image Quant 400

GE Healthcare software (GE Healthcare, Italy) and successively quantified using GS 800 imaging densitometer software (Biorad, Italy).

2.11 ELISA assay

Enzyme-linked immunosorbent assay for PGE_2 was carried out on whole knee joints homogenates. Briefly, 100 µl of tissue supernatants, diluted standards, quality controls and dilution buffer (blank) were added to a pre-coated plate with monoclonal anti-PGE_2 for 2 h. After washing, 100 µl of biotin labeled antibody was added for 1 h. The plate was washed and 100 µl of streptavidin-HRP conjugate was added and the plate was incubated for a further 30 min period in the dark. The addition of 100 µl of the substrate and stop solution represented the last steps before the reading of absorbance (measured at 450 nm) on a microplate reader. Antigen levels in the samples were determined using a standard curve of PGE_2 and expressed as pg ml^{-1}.

2.12 Flow cytometry

Lymphocytes were isolated from whole blood (collected by intracardiac puncture) by Ficoll-Paque Plus gradient method were washed in FACS buffer (PBS containing 1% BSA and 0.02% NaN_2) and directly stained with the following conjugated antibodies (all from BioLegend, London, UK): CD4 (1:200; clone GK1.5), CD8 (1:200; clone 5H10-1), CD25 (1:200; clone 3C7) for 60 minutes at 4ºC. After washing, cells were fixated, permeabilized, and stained intracellularly with IL-17A (1:200; clone TC11-18H10.1) and Foxp3 antibody (1:200: clone MF-14). Th17 and Treg population were defined as $CD4^+IL\text{-}17^+$ and $CD4^+CD25^+Foxp3^+$ cells respectively. For the characterization of joint-infiltrating cells we adopted a similar procedure followed by the incubation with GR1 (1:300; clone RB6-8C5), F480 (1:300; clone BM8), B220 (1:200; clone RA3-6B2), CD115 (1:200; clone AFS98)

for 60 minutes at 4ºC. T cell, neutrophils, macrophage and resident/inflammatory monocytes were defined according to the flow cytometry procedure previously described [25,35]. At least $1x10^4$ cells were analysed per sample, and determination of positive and negative populations was performed based on the staining attained with irrelevant IgG isotypes. Flow cytometry was performed on BriCyte E6 flow cytometer (Mindray Bio-Medical Electronics, Nanshan, China) using MRFlow and FlowJo software operation.

2.13 Data and statistical analysis

The data and statistical analysis in this study comply with the international recommendations on experimental design and analysis in pharmacology [36] and data sharing and presentation in preclinical pharmacology [37,38]. The results obtained were expressed as the mean ± SD. Statistical analysis were performed by using one- way ANOVA followed by Bonferroni or Dunnett's post- test, when comparing more than two groups. GraphPad Prism 7.0 software (San Diego, CA, USA) was used for analysis. Data were considered statistically significant when a value of $P \leq 0.05$ was achieved.

3. Results

3.1 Neutralization of IL-17 reduces the severity of MSU-induced gouty inflammation

I.a. injection of MSU crystals into mouse knee joints was used to mimic the etiologic cause of human gouty inflammation [39]. MSU crystals injection produced an intense and reproducible joint inflammatory score (that peaked between 18 and 24h) that was dose-dependently attenuated by the administration of IL-17Ab (1-10 µg/20µl), with a maximum inhibition rate observed at dose of 10µg. A significant, but lesser, effect was observed when IL-17Ab was administrated at dose of 3µg, then abrogated at dose of 1µg (Fig. 1A). Administration of IL-17Ab isotype control (IgG_{2A}) had no significant effects on clinical scores (data not shown).

Based on these results, we evaluated joint swelling in a time course experiment with the most active dose of IL-17Ab (10µg). As shown in Fig. 1B, neutralization of IL-17 remarkably decreased joint swelling between 18 and 24h. All inflammatory parameters subsided by 24–48h after MSU crystals injection.

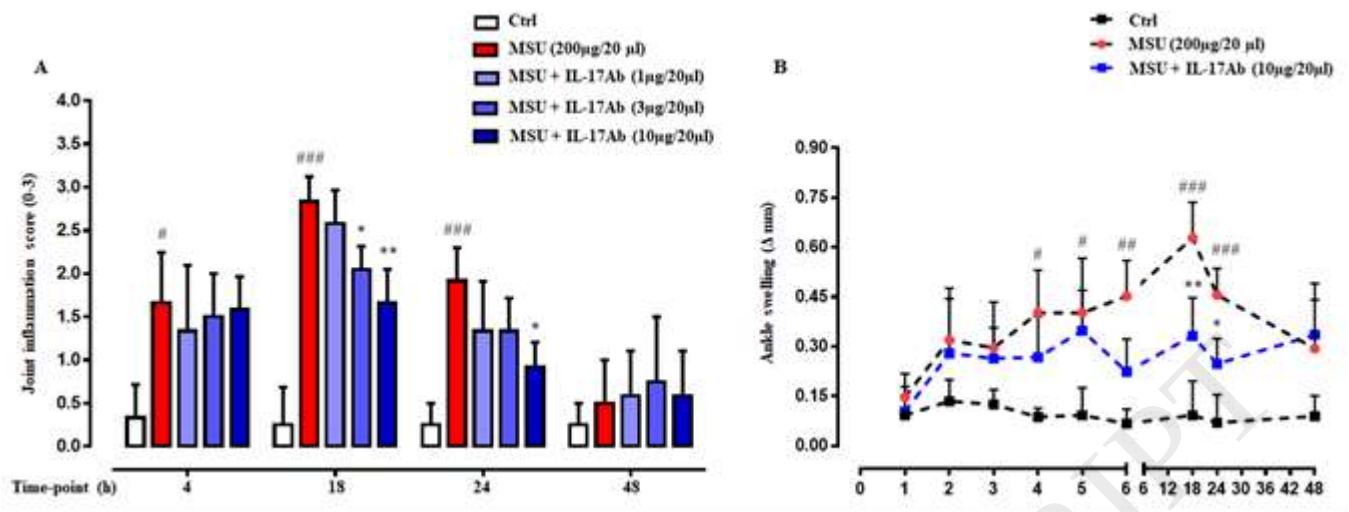


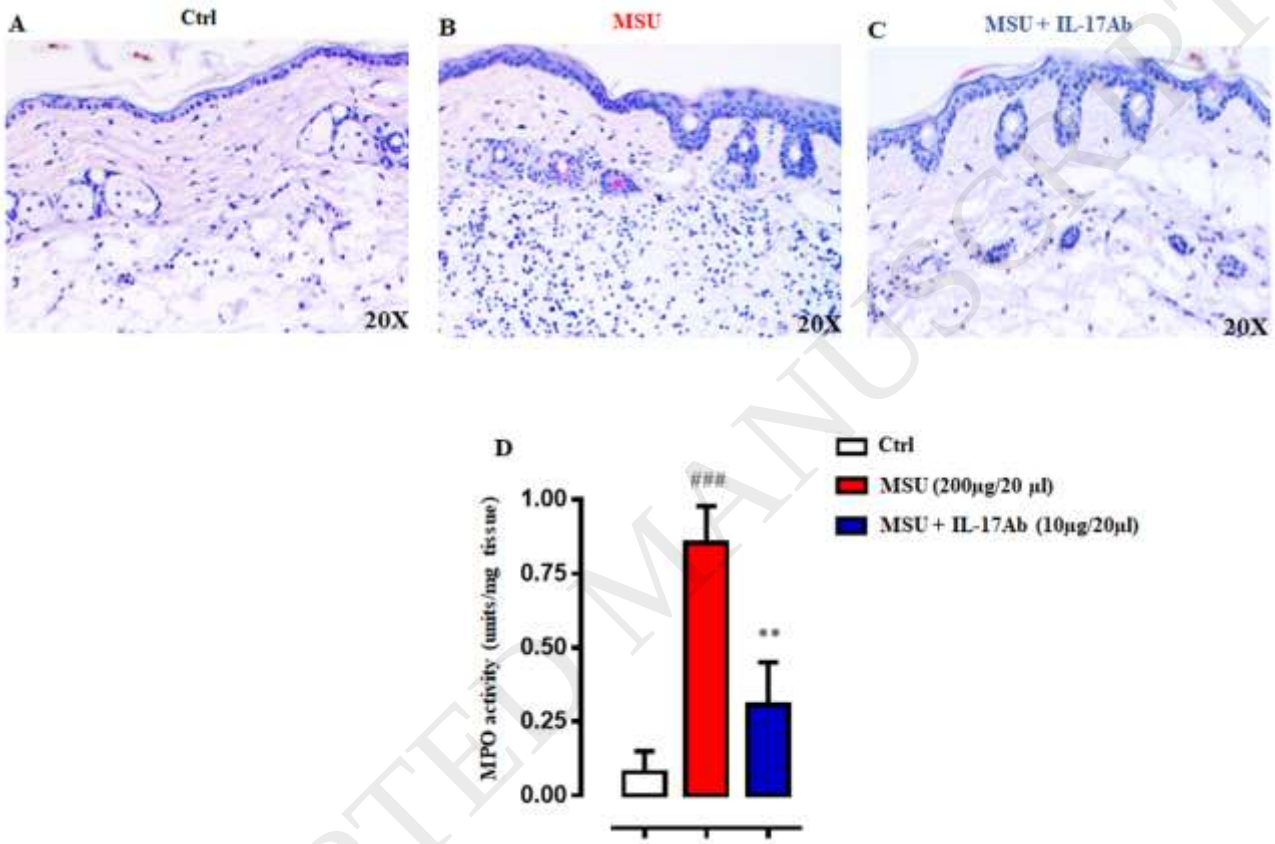
Fig. 1. IL-17Ab modulates MSU crystals-induced gouty inflammation and oedema. Mice were treated with IL-17Ab (1.0, 3.0 and 10µg/20µl) or IL-17Ab vehicle (20µl PBS) 30 min after to intra-articular stimulation with MSU crystals (200µg/20µl) in the right knee joint. (**A**) Joint inflammation score (0-3 in increments of 0.25) was evaluated at 4, 18, 24 and 48 h and (**B**) joint inflammation oedema was evaluated at 1,2,3,4,5,6,18, 24 and 48 h after the stimulus with MSU. Data (expressed as joint inflammation score and Δ increase of knee joint mm) are presented as means ± SD of n=6 mice per group. Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's multiple comparisons. $^{\#}P\leq0.05$, $^{\#\#}P\leq0.01$, $^{\#\#\#}P\leq0.005$ *vs* Ctrl group; *P≤0.05, **p≤0.01 *vs* MSU group.

3.2 IL-17Ab ameliorates MSU-induced leukocytes recruitment and activation to the knee joints

Leukocyte recruitment into the knee joint is a hallmark of gout inflammation and arthritis pathology [40,41]. Thus, based on previous results, we selected the most active doses (10µg) of IL-17Ab to test the modulation of leukocyte recruitment and activation. The histopathological (H&E) findings

(Fig. 2A, 2C) show that IL-17Ab reduced inflammatory cell recruitment to the knee joint (Fig. 2C) compared to MSU crystals-treated mice (Fig. 2B), suggesting a reduction in synovitis. We also observed a significant reduction in MPO activity (a surrogate marker for granulocyte infiltration) in the knee joints of IL-17Ab-treated mice compared to MSU crystals group alone (Fig. 2D).

In line with these observations, it has been reported that in several tissues and different cell types including leukocytes, fibroblasts, osteoblasts and chondrocytes, mPGEs-1 expression is enhanced by a variety of inflammatory factors including LPS, IL- 1β, TNF- α, and IL-17, the latter of which was targeted in this study [42,43,44]. Western blot analysis performed on whole knee joint homogenates showed that COX-2 (Fig. 3A) and mPGEs-1 (Fig. 3B) were up-regulated during MSU



crystals condition and were both significantly decreased after IL-17Ab treatment (Fig. 3E, 3F respectively). Moreover, we observed that IL-17 receptor (IL-17R) expression (increased in MSU-treated animals compared to Ctrl group) was not influenced after IL-17Ab administration (Fig. 3C, 3G). Notably, we observed a strong reduction of soluble PGE_2 levels in IL-17Ab-treated group compare to MSU-treated animals (Fig. 3H).

Fig. 2. IL-17Ab treatment moderates leukocyte infiltration in the mouse knee joint. Mice were treated with IL-17Ab (10µg/20µl) or IL-17Ab vehicle (20µl PBS) 30 min after to intra-articular stimulation with MSU crystals (200µg/20µl) in the right knee joint. Thereafter, at 18h time-point knee joint tissues were stained for haematoxylin-eosin (**A-C**) and tested for myeloperoxidase activity (**D**). H&E pictures are representative of three independent experiments with similar results. Data of MPO activity (expressed as Units for mg of tissue) are presented as means ± SD of n=6 mice per group. Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's multiple comparisons. $^{###}P\leq0.005$ *vs* Ctrl group; **p≤0.01 *vs* MSU group.

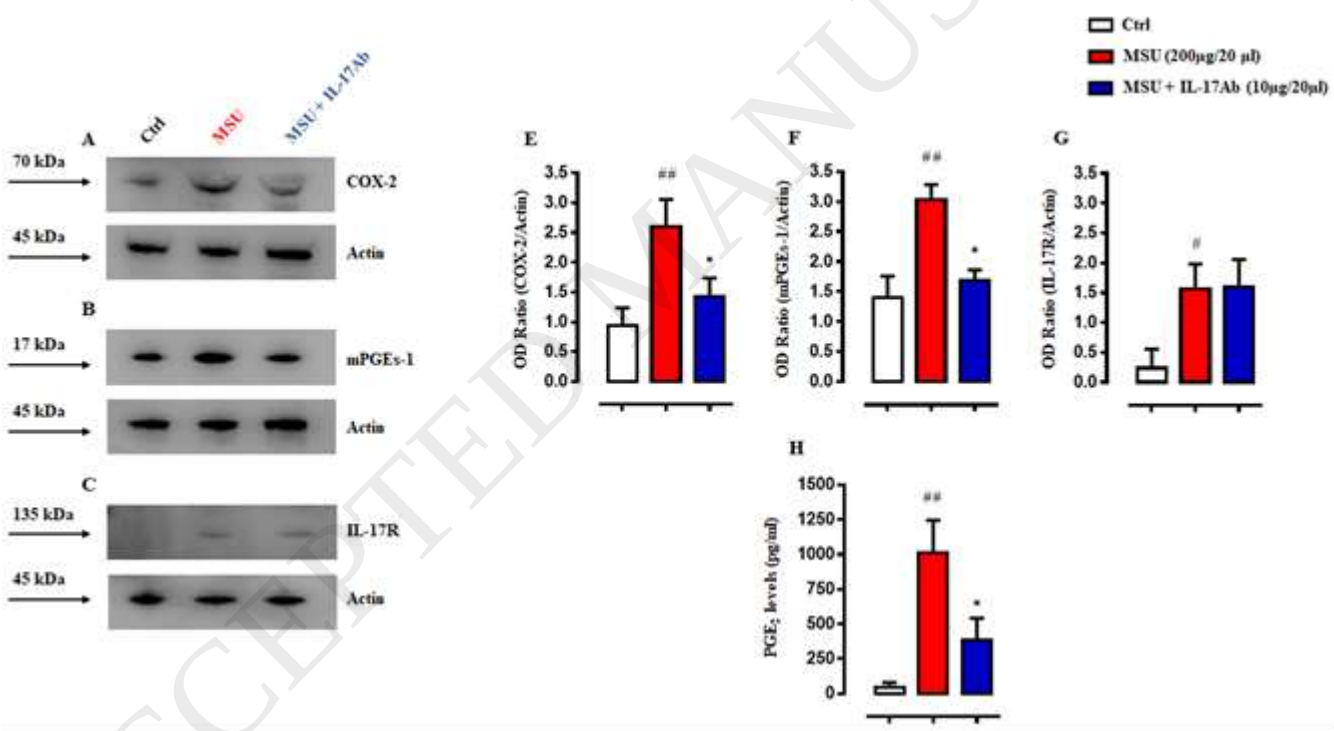


Fig. 3. IL-17Ab modulates COX-2 and mPGEs-1 expression in MSU crystals-induced gouty inflammation. Whole knee joints homogenates from Ctrl, MSU and MSU + IL-17Ab group were analysed by western blot for COX-2 (**A**), mPGEs-1 (**B**), IL-17R (**C**) and their actin expression with related cumulative densitometric values (**E-G**). WB pictures are representative of three separate experiments with similar results. Thereafter, whole homogenates from different experimental

conditions were assayed by ELISA for PGE_2 levels (**E**). Data of ELISA assay (expressed as pg/ml) are presented as means ± SD of n=6 mice per group. Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's multiple comparisons. $^{\#\#}P \leq 0.01$ *vs* Ctrl group; $^{*}p \leq 0.05$ *vs* MSU group.

3.3 Injection of IL-17Ab into the knee joints reduces the recruitment of inflammatory monocytes

To investigate and compare the phenotype of inflammatory leukocytes recruited by MSU injection we stained cells with an anti-B220, anti-CD115, anti-F480, anti-GR1 antibodies and analysed by flow cytometry. Specifically, to identify potential differences in monocyte subpopulations, we first gated on the $B220^-$ population (Gate R1, Figure 4A, 4D and 4G) and then determined GR1 and F480 expression (Fig. 4B, 4E, 4H). Double positive population for these markers (Gate R2) was then further interrogated for CD115 (Fig. 4C, 4F, 4I) as their expression level is commonly correlated with the degree of maturation of inflammatory monocytes [45,46]. Our results show that in MSU-injected mice, the majority of cells recovered were $B220^-/GR1^{hi}$-$F480^{hi}/CD115^+$ (88.30±2.48 compared to 71.70±2.57 of Ctrl) with a significant lower expression in IL-17Ab-treatd group (75.30±2.29) (Fig. 4J). These values were strengthened by an irrelevant percentage of positive cells found in the staining for the isotype control antibodies (data not shown).

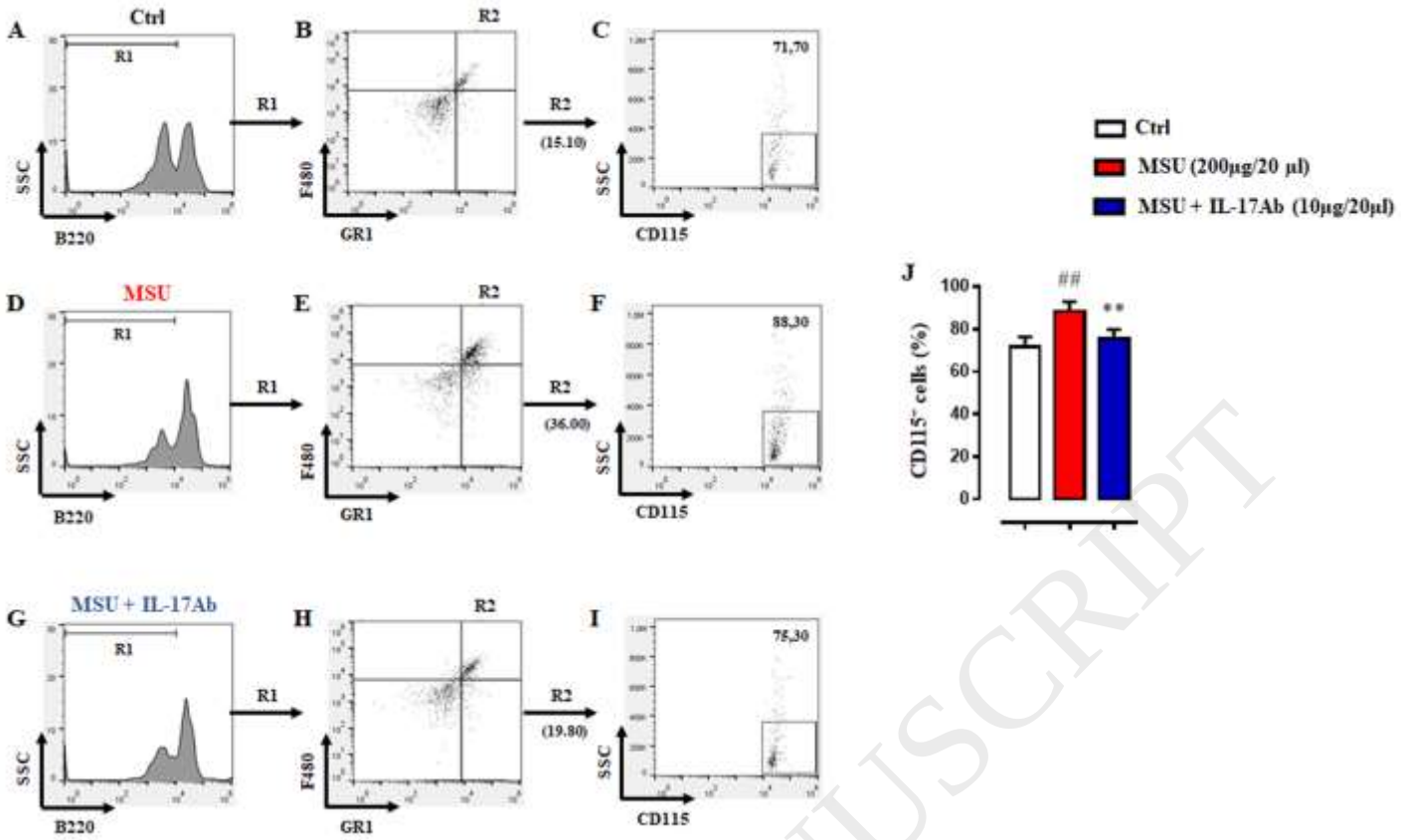
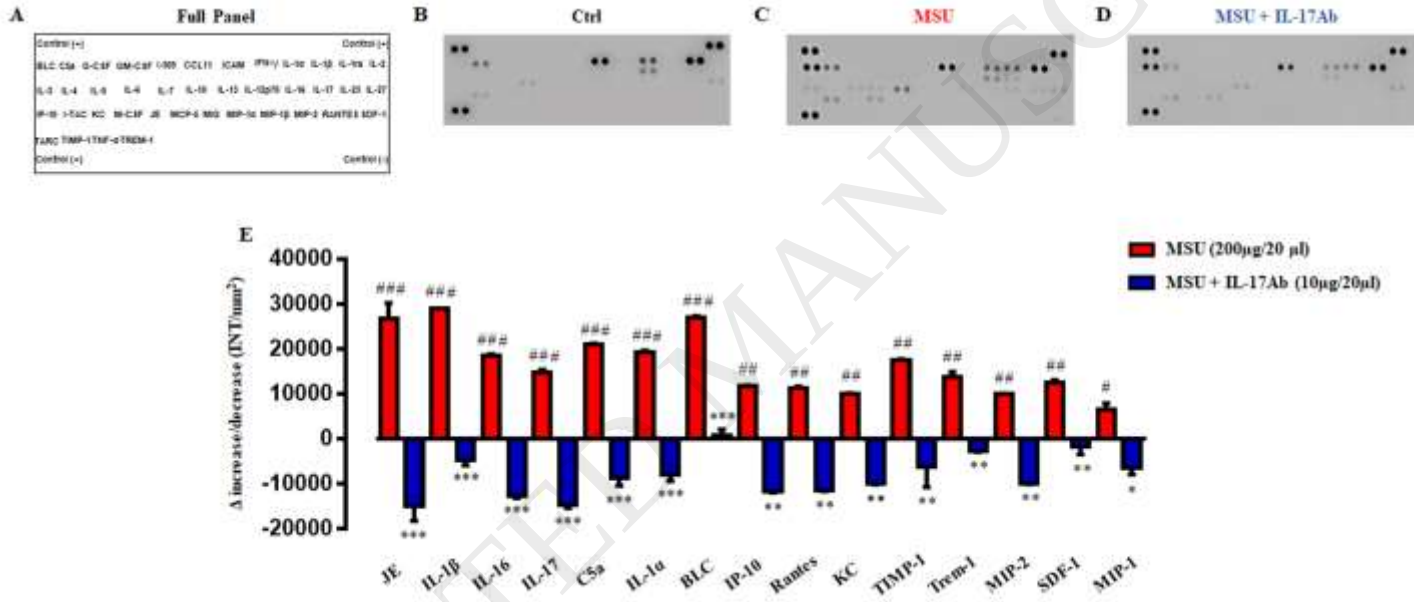


Fig. 4. Flow cytometry strategy applied to identify the modulation of inflammatory monocytes in MSU and MSU + IL-17Ab-tretaed groups. Ankle joints were digested, and single cell suspensions were obtained. Cells were washed and stained with the following panel of antibodies; anti-B220, anti-CD115, anti-F480, anti-GR1. Cells negative for B220 (R1, **A**, **D**, **G**) were plotted for GR1-F480 (**B**, **E**, **H**) in order to get a double positive population (R2) followed by further characterization based on CD115 (**C**, **F**, **I**). The numbers in the dot plots indicated the percentage of positively stained cells after gating strategy whereas histograms values (**J**) indicated the percentage of CD115 positive stained cells. FACS plots are representative of three independent experiments

with similar results. Data are presented as means ± SD of n=6 mice per group. Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's multiple comparisons. ##P≤0.01 *vs* Ctrl group; **p≤0.01 *vs* MSU group.

We next sought to determine the effect of IL-17Ab on MSU crystals-induced pro-inflammatory cyto-chemokines production (Fig. 5A) given the importance of pro-inflammatory mediators to gout pathology onset [47]. MSU crystals administration induced a robust increase of JE, IL-1α, IL-1β,



IL-16, IL-17, C5a, BLC and, with a less (but still significant) extent IP-10, Rantes, KC, TIMP-1, SDF-1 and metalloproteinases (Fig. 5C) compared to Ctrl group (Fig. 5B). Interestingly, treatment with IL-17Ab (Fig. 5D) reverted this cyto-chemokines over-production next to MSU crystals-treated mice (Fig. 5E). IL-17Ab isotype control did not alter the levels of any of the mediators measured (data not shown)

Fig. 5. Survey of inflammatory mediators collected from mice receiving MSU and IL-17Ab injection in the knee joint. Inflammatory fluids obtained from homogenates knees joints were

assayed using a Proteome Profiler cytokine array (**A**) for Ctrl (**B**), MSU (**C**) and MSU + IL-17Ab (**D**) group. The bar graph (**E**) shows the densitometric analysis of the arrays in **B–D**. Bars show mean changes ± SD. of positive spots of three independent experiments with n=6 mice obtained as a Δ of increase/decrease in the INT/mm^2 between MSU and Ctrl (red bar) and MSU + IL-17Ab and MSU (blue bar). Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's multiple comparisons. $^{\#}P\leq0.05$, $^{\#\#}P\leq0.01$, $^{\#\#\#}P\leq0.005$ *vs* Ctrl group; *P≤0.05, **p≤0.01, ***p≤0.005 *vs* MSU group.

3.4 *In situ* administration of MSU and IL-17Ab influences systemic Th17 and Treg balance

In order to clarify whether the differing inflammatory profiles observed in the knee joints were correlated to different systemic Th17 and Treg balance, we stained isolated lymphocytes cells with an anti-CD4, an accessory protein for MHC class-II antigen/T-cell receptor interaction, an anti-CD8, a marker that identifies cytotoxic/suppressor T-cells that interact with MHC class I and then with an anti-CD4/IL-17 and anti-CD4/CD25/Foxp3 in order to identify Th17 and Treg population respectively according to previous protocols [48,49].

The results obtained did not reflect any significant difference in the levels of $CD4^+$ and $CD8^+$ cells in all experimental conditions (Fig. 6A, 6D). However, as shown in Fig. 6E and 6G, MSU crystals-treated mice displayed a strong increase in the percentage of $Th17^+$ cells (12.70±3.85) compared to Ctrl group (0.01±0.002) which was significantly reduced after IL-17Ab treatment (2.55±1.10). This systemic lymphocyte profile change (Fig. 6G) did not affect the Treg repertoire, given that we did not observe any significant difference in terms of $CD4^+CD25^+Foxp3^+$ cells (Fig. 6F and 6H). These values were strengthened by irrelevant percentage of positive cells found in all the staining for the isotype control antibodies (data not shown).

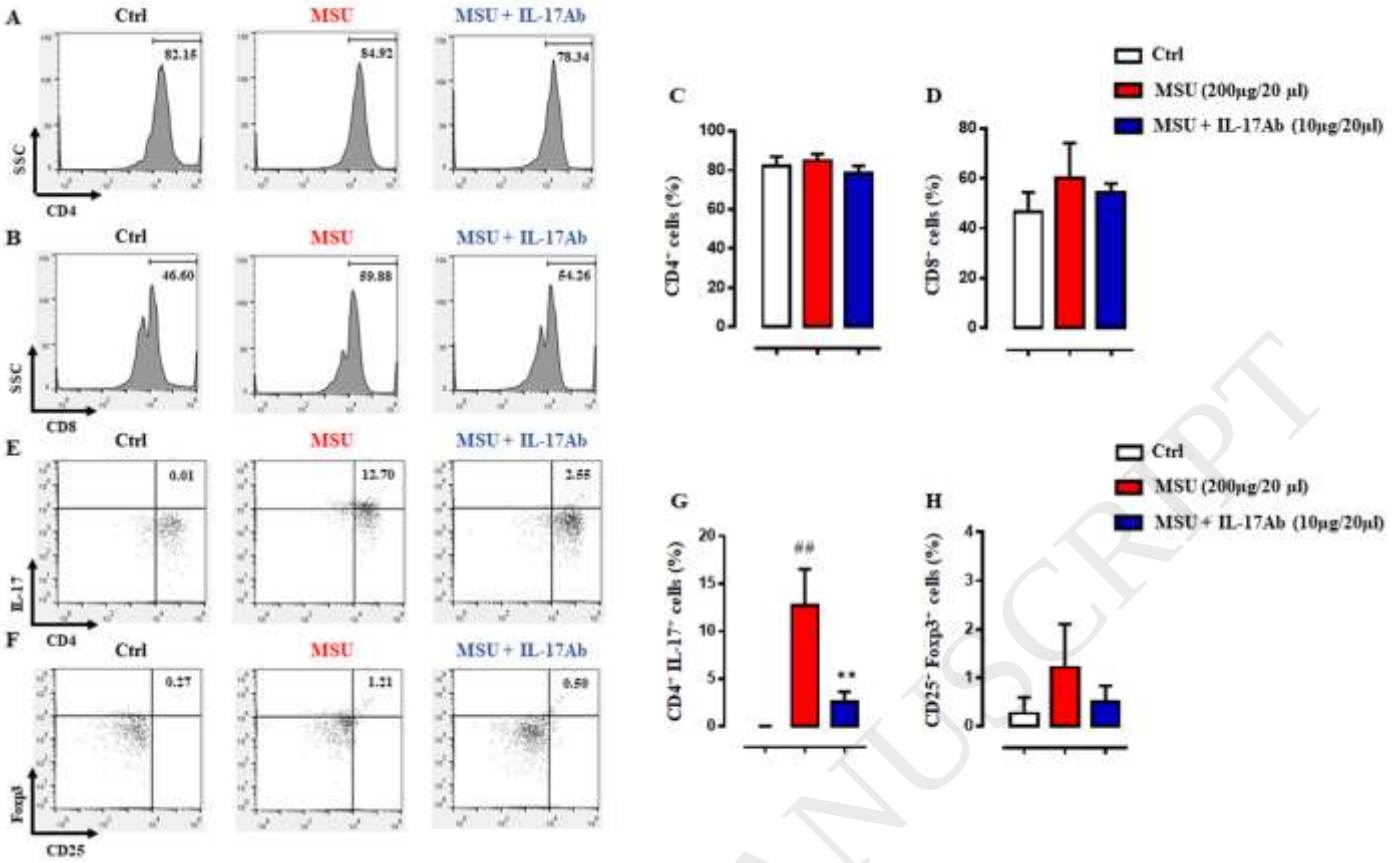


Fig. 6. IL-17Ab influences Th17 and Treg ratio on peripheral blood. Lymphocytes isolated from whole blood by Ficoll-Paque Plus gradient method were washed, stained with CD4 (**A**), CD8 (**B**) and CD4/IL-17 (**E**). Finally, $CD4^+$ cells were plotted for CD25/Foxp3 (**F**) and analysed by FACS. Histograms values (**C**, **D**, **G** and **H**) indicated the percentage of positively stained cells in the different experimental conditions. FACS pictures are representative of three independent experiments with similar results. Data are presented as means ± SD of n=6 mice per group. Statistical analysis was conducted by one-way ANOVA followed by Bonferroni's multiple comparisons. $^{\#\#}P \leq 0.01$ *vs* Ctrl group; $^{**}p \leq 0.01$ *vs* MSU group.

4. Discussion

The results of this study show, for the first time, that i.a. injection of MSU crystals stimulates *in vivo* production of Th17 cells and Th17-related inflammatory cyto-chemokines. In addition, we have provided evidence that the administration of a neutralizing antibody against IL-17 attenuates joint symptoms, swelling and leukocytes infiltration/activation to the inflamed tissues, possibly providing a new strategy for the treatment of gouty inflammation and/or arthritis.

Gout is characterized by the deposition of MSU crystals in joints, which is associated with the rise of serum urate content, leading to excruciating pain and inflammatory events [50, 51]. MSU crystals deposits or tophi further induce chronic inflammatory responses that may lead to joint damage, often referred to as gouty arthritis or chronic gout [52]. Moreover, MSU crystals can also cause acute, self-limited, inflammatory flares, which are likely triggered by crystal shedding from the cartilage surface into the joint space, where they can interact with resident cells.

All these processes are mainly related to activated neutrophils and macrophages which are responsible for the release of PGE_2, IL-1β, TNF-α, and, activate nociceptor neuron and thereby producing pain [53,54,55]. Indeed, targeting IL-1β, TNF-α or PGE_2 in rheumatic diseases reduce neutrophil recruitment and associated pain [40].

Recent evidences have highlighted that serum IL-17 levels are significantly elevated in GA patients [20] and that systemic Th17/Treg imbalance is consistent with inflammation development in the joints. Accordingly, the changes of Th17/Treg ratio decrease at an earlier stage followed by an increase at a later stage, suggesting a photogenic role of this specific $CD4^+$ subset in gouty onset and development [22]. In line with these observations, here we have demonstrated that MSU

crystals injection in mice induce the up-regulation of different mediators strictly correlated with biological activity of IL-17 and Th17 cells profile [56]. We found that MSU crystals administration induced the modulation of i) typical pro-inflammatory cytokines such as IL-1α, IL-1β, IL-16, IL-17 and Trem-1, ii) different chemokines such as BLC, IP-10, JE, SDF-1 and Rantes involved in leukocytes recruitment and activation and iii) other activators of inflammation such as C5a, the chemotactic cytokines MIP-1/2 and the tissue inhibitor of metalloproteinase TIMP-1.

In line with these observations, it has been reported that in several tissues and different cell types including leukocytes, fibroblasts, osteoblasts and chondrocytes mPGEs-1 expression is enhanced by a variety of inflammatory factors including LPS, IL- 1β, TNF- α, and IL-17, the latter of which was targeted in this study [42,43,44]. COX-2/mPGEs-1 is a very complex enzymatic process that initiate the formation of PGE_2 and lengthening of the pro-inflammatory and pro-nociceptive stimuli [11,57]. The concept of a changeable PGs production pathway (including the modulation of both COX-2 and mPGEs-1) could have important implications for understanding the inflammatory events typical of certain illness including gouty inflammation and/or arthritis. Interestingly, we have demonstrated that COX-2 and mPGEs-1 were up-regulated during MSU administration and both decreased after IL-17Ab treatment. This finding was also confirmed by a strong reduction of PGE_2 levels observed in IL-17Ab-treated group. The selective modulation of COX-2/mPGEs-1 pathway was also correlated with the observation that IL-17 receptor expression (increased in MSU-treated animals compared to Ctrl group) was not influenced by IL-17Ab administration. All these biochemical changes were consistent with those observed macroscopically. Indeed, administration of a neutralizing monoclonal antibody against IL-17 prevented swelling of the inflamed joints between 18 and 24 h, but it failed to have a consistent effect at other time points of observation.

Successively, to investigate and compare the phenotype of the inflammatory leukocytes recruited by MSU injection into knee joints, we first gaited on cells isolated from knee joints for $B220^-$

population, followed by GR1-F480 expression to finally identify the level of $CD115^{+}$, commonly correlated with the degree of maturation of inflammatory monocytes [45,46]. Our results show that in MSU-injected mice, most of the cells recovered were inflammatory monocytes with a significant lower expression of mentioned markers in IL-17Ab-treatd group.

The inflammatory effects of MSU crystals have been recently suggested to involve the activation of the NLRP3 inflammasome and IL-1β [58]. The role of this cytokine in the pathogenesis of inflammation in gout has been strengthened by studies showing that the biologic Anakira (recombinant IL-1Ra) has beneficial therapeutic effects in gout [59,60]. However, in the literature the role of other specific inflammatory components necessary for gouty onset as such as IL-16, TREM-1, JE, Rantes and SDF-1 that we found up-regulated and modulated in MSU crystals-and IL-17Ab-treated mice respectively, is less clear. In this context the presence of IL-17 could justify the overproduction of these soluble mediators released from activated monocytes/macrophage in the later stage of gouty inflammation. In fact, an aspect that should not be omitted is that the presence of IL-17, at the later stages of an inflammatory response, has been proposed to be the main contributor to chronic inflammation due its ability to sustain the recruitment of neutrophils and inflammatory monocytes [61,62], mainly by the selective production of JE, IL-16 and TREM-1 [23,24,25,63]. Given that this cytokine is thought to contribute to the pathogenesis of this disease, targeting the IL-17 axis could represent an attractive option for inflammatory-related diseases.

IL-16 and TREM-1 are classically considered key players in rheumatoid arthritis and inflammatory-related diseases due their ability to modulate neutrophil migration across the epithelium [64] and macrophage infiltration at pathological sites [65,66]. Recent studies also demonstrated that fibroblast-like synoviocytes from RA patients express higher levels of IL-16 compared to those from osteoarthritis patients [67] and that TREM-1 is involved in both neutrophil migration and

macrophage infiltration in chronic inflammatory pathologies [64,68] thus providing a possible explanation for the amplified inflammatory response we observed in our study.

Considering this scenario, it makes sense that patients with RA display high level of IL-17 and are more sensitive to an anti-IL-17 inhibitor. Accordingly, in most of these patients, anti-IL-17 treatment leads to the reduction of symptoms, inflammation and bone destruction [69,70]. Moreover, it should be noted that IL-17 has been shown to play a key role in other forms of arthritis, such as Psoriatic Arthritis (PsA), in which the induction of IL-17 cytokines axis (IL-17A, IL-17F and IL-22) promotes intra-and/or peri-articular inflammation [71,72,73]. To better understand this previous observation and viewing the coin from other side a flow cytometry bioassay was carried out on peripheral blood to evaluate the balance Treg/Th17 after MSU crystals induction and IL-17Ab treatment. Interestingly, our results show that IL-17Ab selectively reverted Th17 systemic positive cells "normally" up-regulated after MSU crystals injection, without interfering with Treg population.

5. Conclusion

Overall, our findings and previously reported evidences suggest that IL-17 may play a crucial role in the joint pathology at the late phase of crystal-induced acute inflammation/arthritis. What we think as most interesting and novel in this study is the possibility of the existence of two mechanisms of inflammatory amplification during gouty inflammation: one local and the second systemic that in turn amplify and sustain the inflammatory onset but, intriguingly, both related to Th17 and IL-17 biology. Future elucidation of the pathophysiological roles of IL-17 in gouty inflammation will be crucial in the understanding the precise mechanism of crystal-induced inflammation and will possibly provide a new strategy for the treatment of this pathology.

Conflict of interest

The authors declare no competing conflict of interests.

Author contributions

FR, AJI, AS, PM, MP and FM performed the experiments. FM designed the study, drafted and wrote the manuscript. CI, FC, RS, SP and NM edited and revised the manuscript. All Authors gave final approval to the publication.

Funding and Acknowledgments

This work was supported by MIUR (PRIN 2017; 2017A95NCJ/2017A95NCJ_002, "Stolen molecules - Stealing natural products from the depot and reselling them as new drug candidates"). We would like to thank Dr. Antonio Baiano and Mr. Giovanni Esposito and Mr. Angelo Russo for animal care and technical assistance.

Declaration of interest: The authors declare no competing conflict of interests.

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