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RESEARCH ARTICLE

# Intra-articular injection of triamcinolone acetonide sustains macrophage levels and aggravates osteophytosis during degenerative joint disease in mice

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**Background and purpose:** Corticosteroids such as triamcinolone acetonide (TAA) are potent drugs administered intra-articularly as an anti-inflammatory therapy to relieve pain associated with osteoarthritis (OA). However, the ability of early TAA intervention to mitigate OA progression and modulate immune cell subsets remains unclear. Here, we sought to understand the effect of early intra-articular injection of TAA on OA progression, local macrophages, and peripheral blood monocytes.

**Experimental approach:** Degenerative joint disease was induced by intra-articular injection of collagenase into the knee joint of male C57BL/6 mice. After 1 week, TAA or saline was injected intra-articularly. Blood was taken throughout the study to analyse monocyte subsets. Mice were killed at days 14 and 56 post-induction of collagenase-induced OA (CiOA) to examine synovial macrophages and structural OA features.

**Key results:** The percentage of macrophages relative to total live cells present within knee joints was increased in collagenase- compared with saline-injected knees at day 14 and was not altered by TAA treatment. However, at day 56, post-induction of CiOA, TAA-treated knees had increased levels of macrophages compared with the knees of untreated CiOA-mice. The distribution of monocyte subsets present in peripheral blood was not altered by TAA treatment during the development of CiOA. Osteophyte maturation was increased in TAA-injected knees at day 56.

**Conclusion and implications:** Intra-articular injection of TAA increases long-term synovial macrophage numbers and osteophytosis. Our findings suggest that TAA accentuates the progression of osteoarthritis-associated features when applied to an acutely inflamed knee.

KEYWORDS
inflammation, macrophage, monocyte, osteoarthritis, osteophytes corticosteroid

**Abbreviations:** ACL, anterior cruciate ligament; CiOA, collagenase-induced osteoarthritis; FC, flow cytometry; OA, osteoarthritis; OARSI, osteoarthritis research society international; SBP, subchondral bone plate; TAA, triamcinolone acetonide.

## 1 | INTRODUCTION

Joint instability as caused by ligament injury is a risk factor for the development of **osteoarthritis** (OA) (Akhtar et al., 2016; Driban et al., 2014; Wilder et al., 2002). OA is the most common form of arthritis affecting the entire joint, including the articular cartilage, subchondral bone, ligaments, capsule, synovium, and peri-articular muscles (Bijlsma, 2001). Intra-articular injection of glucocorticoids, such as **triamcinolone acetonide** (TAA), is frequently applied clinically as an anti-inflammatory therapy to alleviate knee pain associated with OA (Bellamy et al., 2006; McAlindon et al., 2014; Miller et al., 1958; van Middelkoop et al., 2016) and is strongly recommended by the American College of Rheumatology/Arthritis Foundation (Kolasinski et al., 2020a). However, evidence on the long-term benefit of TAA treatment is conflicting, with reports highlighting a potential association of intra-articular glucocorticoid treatment with accelerated structural progression of OA (Kompel et al., 2019; McAlindon et al., 2017; Zeng et al., 2019). Interestingly, early intervention with intra-articular TAA treatment following anterior cruciate ligament (ACL) injury has been reported to reduce the levels of cartilage degeneration biomarkers in the synovial fluid of patients (Lattermann et al., 2017). However, the long-term outcome of early TAA treatment following injury and its ability to alleviate OA progression in the knee requires further elucidation.

Low-grade inflammation in OA is associated with progression of structural changes characteristic of knee OA (Robinson et al., 2016). Macrophages residing within the synovial membrane are considered primary mediators of the inflammatory response and have been recognised as crucial regulators of OA progression (Zhang et al., 2020). Macrophages are highly plastic cells that exist as diverse phenotypes which dynamically respond to changing environmental stimuli and can be classified as pro-inflammatory (M1), anti-inflammatory, or tissue repair-associated (M2) subsets (Murray et al., 2014). TAA is postulated to mediate its therapeutic effect in OA due to its capacity to modulate inflammatory gene transcription and potential to alter macrophage activation (Nixon et al., 2013; Siebelt et al., 2015). However, local intra-articular injection of TAA also leads to systemic absorption, with detectable serum plasma concentrations observed in the blood of patients up to 6 weeks post-injection (Kraus et al., 2018). Interestingly, glucocorticoid treatment has been previously reported to alter the functional profile of peripheral blood monocytes in vitro and in vivo, inducing an anti-inflammatory, migratory phenotype (Ehrchen et al., 2007; Varga et al., 2008; Varga et al., 2014). Blood monocytes which originate in the bone marrow may migrate from the circulation into tissues under inflammatory conditions and differentiate into macrophages (Geissmann et al., 2003; Sunderkötter et al., 2004). Like macrophages, monocytes can also be categorised according to their cell surface receptor expression and function, comprising classical and non-classical/intermediate monocyte subsets (Ziegler-Heitbrock, 2014). Interestingly, circulating non-classical monocytes were shown to be recruited to the joint upon injury, driving the development and progression of inflammation in a murine model of rheumatoid arthritis (Misharin et al., 2014). Intra-articular glucocorticoid injection has been shown to alter blood monocyte phenotype as well as leucocyte trafficking in patients with rheumatoid arthritis (Steer et al., 1998). However, the responsiveness of systemic as well as local immune cell subsets to local administration of TAA during OA progression remains unclear.

Knowledge on the impact of an early intervention with short-term TAA treatment on local and systemic inflammatory processes, as well as the progression of structural damage following joint injury, could provide important insights for OA therapy. Intra-articular injection of collagenase is a well-known model to study instability-induced OA, characterised by a high synovial inflammatory component (van Osch, van der Kraan, Blankevoort, et al., 1996; van Osch, van der Kraan, van Valburg, & van den Berg, 1996). Therefore, the aims of this study were to examine the effect of early intra-articular injection of TAA on local macrophages and peripheral blood monocytes, as well as cartilage degeneration, subchondral bone changes, and osteophyte formation during OA progression in the collagenase-induced OA (CiOA) mouse model.

### What is already known

- Intra-articular injection of TAA is applied as an anti-inflammatory therapy for the management of osteoarthritis.
- Evidence on the long-term benefit of TAA treatment on disease progression is conflicting.

### What this study adds

- Early intervention with short-term TAA treatment leads to long-term synovial macrophage numbers and aggravates osteophytosis.

### What is the clinical significance

- Intra-articular TAA treatment may not be used in an acutely inflamed joint following injury

## 2 | METHODS

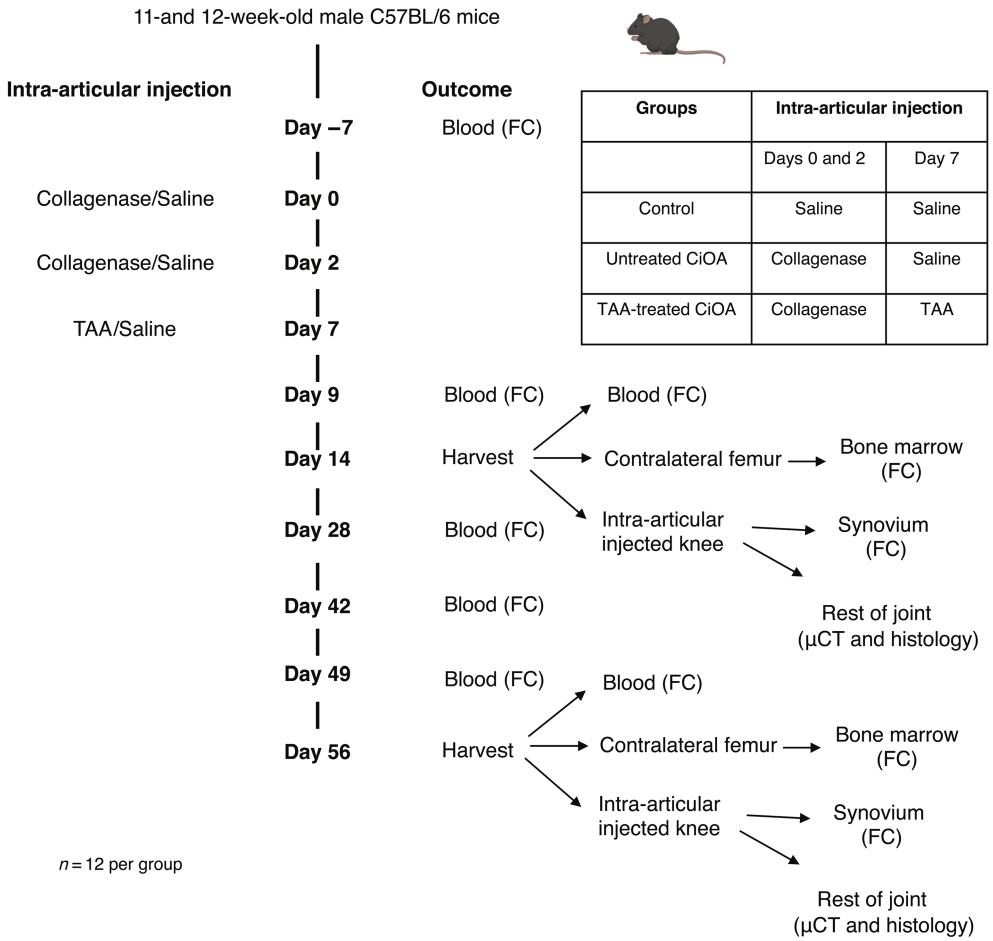
### 2.1 | Animal model

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All animal experimentation procedures were conducted with approval by the

Animal Ethical Committee of Erasmus University Medical Center (Licence number AVD101002015114, protocol number 16-691-03). The 11- and 12-week-old male C57BL/6 mice (C57BL/6J0laHsd, 27.66 g ± 1.91 g; Envigo, Cambridgeshire, UK) were housed in groups of 4 in individually ventilated cages and maintained on a 12-h light/dark cycle with ad libitum access to standard diet and water at the Experimental Animal Facility of the Erasmus MC. Mice were randomly divided into three experimental groups ($N = 12$ per time point): control, untreated CiOA, and TAA-treated CiOA. For all procedures, mice were anaesthetised using 3% isoflurane/0.8 L $O_2$/min (Pharmachemie BV, Haarlem, the Netherlands), and a 3- to 4-mm dermal incision was made to the right knee at the height of the patellar tendon. OA was induced unilaterally by two intra-articular injections of 3 U of highly purified collagenase type VII from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, USA) in 6 μl of saline (0.9% NaCl; Sigma-Aldrich) at days 0 and 2. Control mice received intra-articular injections of 6 μl of saline only. All intra-articular injections were administered using a 50-μl syringe (Hamilton, Bonaduz, Switzerland) and 30G needle (BD Medical, New Jersey, USA). TAA (Kenacort; Bristol-Myers Squibb, Woerden, The Netherlands) was diluted to a concentration of 4.16 mg·ml$^{-1}$ with saline. TAA-treated OA mice received an intra-articular injection of 6 μl (containing 25 μg of TAA) to the right knee at day 7 post-induction of CiOA. This dose was based on previous rodent studies and the dose applied in humans considering the volume of the synovial fluid (Kroin et al., 2016; Siebelt et al., 2015). Untreated CiOA and control mice received an intra-articular injection of 6 μl of saline as a vehicle control. The order of intra-articular injection administration was performed randomly at each time-point. Mice were killed in agreement with the Directive 2010/63/EU by cervical dislocation under isoflurane anaesthesia 14 or 56 days following induction of CiOA. A schematic representation of the study is summarised in Figure 1.

## 2.2 | Flow cytometric analysis of peripheral blood monocytes, synovial tissue and bone marrow

Peripheral blood was harvested from the facial vein of mice 7 days prior to and 9, 14, 28, 42, 49, and 56 days post-induction of OA. Blood sampling order was performed randomly at each time-point. Fifty microlitres of whole blood were pre-incubated with purified rat anti-mouse CD16/CD32 (BD Biosciences Cat# 553140, RRID: AB_394655, New Jersey, USA) for 5 min on ice. Blood was stained for the expression of CD11b (BioLegend Cat# 101228, RRID: AB_893232), CD115 (BioLegend Cat# 135505, RRID:AB_1937254), Ly-6C (BioLegend Cat# 128005, RRID:AB_1186134), and CD62L (BioLegend Cat# 104412, RRID:AB_313099) to identify myeloid cells and specific monocyte subsets, as well as CD3 (BioLegend Cat#




| Groups | Intra-articular injection | |
|---|---|---|
| | Days 0 and 2 | Day 7 |
| Control | Saline | Saline |
| Untreated CiOA | Collagenase | Saline |
| TAA-treated CiOA | Collagenase | TAA |

**FIGURE 1** Experimental set-up of the in vivo experiment. The table on the right outlines the intra-articular injection scheme applied to the right knee of mice for each experimental group ($n = 12$). On the left, a timeline showing the outcome measurements. At day 14, a group of 36 mice were killed ($n = 12$ per group) to assess the early effects of TAA. Another group of 36 mice was used to assess the long-term effects of TAA at the end point, day 56. CiOA, collagenase-induced osteoarthritis; FC, flow cytometry; TAA, triamcinolone acetonide. Created with BioRender.com

100220, RRID:AB_1732057), NK1.1 (BioLegend Cat# 108713, RRID:AB_389363), CD19 (BioLegend Cat# 115520, RRID:AB_313655), and Ly-6G (BioLegend Cat# 127618, RRID:AB_1877261) to eliminate T-cells, natural killer cells, B cells, and neutrophils (all antibodies from BioLegend, San Diego, USA; Table S1). Cells were stained for 30 min at 4°C in the dark, followed by incubation with 2 ml of 1× FACS lysing solution (BD Biosciences) for 10 min to lyse red blood cells. Following centrifugation at 400 × *g* for 10 min, supernatant was removed, and cells washed and resuspended in FACSFlow buffer (BD Biosciences).

To evaluate macrophage subsets within knee joints at days 14 and 56 post-induction of OA, the patella with surrounding synovial tissue was dissected from the right knee and enzymatically digested. Tissue was incubated with 2 mg·ml$^{-1}$ collagenase type IV (Life Technologies, California, USA), 2.4 mg·ml$^{-1}$ dispase II (Roche, Penzberg, Germany), and 0.2 mg·ml$^{-1}$ DNase I (Sigma-Aldrich) in Hanks' buffered salt solution (Thermo Fisher Scientific, Massachusetts, USA) at 37°C for 60 min. The resulting cell suspension was filtered through a 100-μm cell strainer, and cells were washed and resuspended in FACSFlow buffer. Cells were stained for the expression of CD11b (BioLegend Cat# 101228, RRID:AB_893232), F4/80 (BioLegend Cat# 123108, RRID:AB_893502), CD86 (BioLegend Cat# 105013, RRID:AB_439782), CD206 (BioLegend Cat# 141707, RRID:AB_10896057) (all BioLegend; Table S1), and CD163 (Thermo Fisher Scientific Cat# 12-1631-82, RRID:AB_2716924) to identify macrophage subsets and a LIVE/DEAD™ fixable dead cell stain (1:1000 dilution; Life Technologies) to exclude dead cells. Cells were stained for 30 min at 4°C in the dark, washed, and resuspended in FACSFlow.

Bone marrow was isolated from the contralateral femur at days 14 and 56 post-induction of OA. The femur was cut at both ends, and bone marrow was flushed out over a 100-μm cell strainer using a 25G needle attached to a 5-ml syringe (All BD Medical) filled with Roswell Park Memorial Institute 1640 (RPMI) media (Thermo Fisher Scientific). The resulting cell suspension was spun at 500 × *g* for 5 min, and cells were resuspended in FACSFlow and stained for the expression of Ly-6C (BioLegend Cat# 128005, RRID:AB_1186134) and CD31 (BioLegend Cat# 102410, RRID:AB_312905) (all BioLegend; Table S1) to assess bone marrow composition as previously described (de Bruijn et al., 1998) and with a LIVE/DEAD™ fixable dead cell stain (1:1000 dilution; Life Technologies). Cells were incubated with antibodies for 30 min at 4°C in the dark, washed, and resuspended in FACSFlow.

All samples were analysed using a FACSJazz cytometer (BD Biosciences) and FlowJo software version 10.0.7 (FlowJo LLC, Oregon, USA). The gating strategies applied for blood monocytes, macrophages, and bone marrow composition analysis are presented in Figures S1 and S2.

## 2.3 | Histological analysis

Knees were fixed in 4% formalin (v/v) for 1 week and scanned for μCT analysis. Afterwards, knees were decalcified in 10% EDTA for 2 weeks and embedded in paraffin. Coronal sections of 6 μm were cut for analysis and sections were stained with Safranin O and Fast Green. Images were acquired using the NanoZoomer Digital Pathology program (Hamamatsu Photonics, Ammersee, Germany).

Cartilage damage was evaluated by two observers blinded to the treatment groups using the Osteoarthritis Research Society International (OARSI) scoring system described by Glasson et al. (2010). Briefly, this score ranges from 0, for normal cartilage, to 6, for cartilage with clefts and erosion to the calcified cartilage in >75% of the articular surface. For each knee, cartilage quality in the lateral and medial compartment—both femur and tibia—of the knee was scored and averaged with three sections at standardised locations in the knee with 180 μm between sections. For each knee, the average score assigned by two blinded observers (MFB and NK) was averaged.

Osteophyte maturation was scored in the lateral and medial compartment of femur and tibiae, from 0 where no osteophyte was observed, 1 for a cartilaginous osteophyte, and 2 for an ossified osteophyte and averaged with three sections at standardised locations in the knee with 180 μm between the sections. For each knee, the average score assigned by two blinded observers (MFB and NK) was averaged. Osteophyte size was assessed using the NanoZoomer Digital Pathology program (Hamamatsu Photonics) by measuring the area of the osteophyte in three sections and calculating the average per joint, similarly to the osteophyte maturation score. We selected osteophytes at the medial side of the tibiae plateau, the location where the incidence of osteophytes was highest in CiOA knees.

## 2.4 | Micro CT scans and analysis

Knees were imaged using a Quantum GX micro-computed tomography (μCT) scanner (PerkinElmer, Waltham, MA) with the following settings: time = 5 min, voxel size = 7 μm, tube voltage of 90 kV, and tube current = 180 μA. Three-dimensional reconstructed images were obtained using software AccuCT 1.0 (PerkinElmer), and the proximal tibia was selected for further analysis. Subchondral bone plate thickness was measured in frontal plane cross sections of the weight-bearing region of the medial tibia plateau.

## 2.5 | Data and statistical analysis

The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* (Curtis et al., 2018) and are in accordance with the ARRIVE guidelines (Kilkenny et al., 2010). The study was designed to generate groups of equal size, using randomisation and blinded analysis. Statistical analysis was undertaken only for studies where each group size was at least $n = 5$. No values were excluded for the data analysis and presentation, except for the micro CT analysis of the knees at day 56 where a low resolution during acquisition led to inability of image reconstruction. In this case, we still had $n = 8$–10 left. The declared group size is the number of independent values, and statistical analysis was done using these independent values.

Sample size was determined considering a change in peripheral blood monocyte subsets of 30% resulting from treatment to be relevant in our study. As per power calculation (using a standard deviation of 25%) with a statistical power level of 0.8 and significance level (α) of 0.05, our sample size per group for a two-tailed hypothesis test was 11 mice. One additional mouse per group was included to allow for unforeseen loss, yielding $n = 12$ mice per treatment group for each time-point, resulting in 72 mice in total. Statistical evaluation was performed using GraphPad Prism 9.0 and IBM SPSS 24 (IBM). Normality testing of data was performed using the Shapiro–Wilk test. For parametric data, a one-way ANOVA with Bonferroni post hoc test was conducted. For nonparametric data, a Kruskal–Wallis test with Bonferroni or Dunn's post-test correction for multiple comparisons was used, depending on the nature of the data. Statistically significant differences of all post hoc tests were found at $P < 0.05$. All post hoc tests were performed only if the $F$ value for the ANOVA achieved statistical significance and there was no significant variance inhomogeneity.

### 2.6 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

## 3 | RESULTS

### 3.1 | Macrophage-mediated inflammation is sustained in knee joints following TAA treatment

To identify whether TAA affects macrophages after joint injury in vivo, the patella with surrounding synovial tissue was isolated from the knee joints of mice and macrophage composition was examined. At day 14, synovitis was present in this model as observed by a significant increase in macrophages within CiOA-induced knees (Figure 2a). Intra-articular injection of TAA 1 week prior to harvesting the knees, however, did not significantly affect the proportion of macrophages present at this time point. At day 56 post-induction of CiOA, macrophage levels in untreated-CiOA knees did not significantly differ compared with control knees. However, TAA-treated CiOA knees contained significantly higher levels of macrophages compared with both control and untreated CiOA mice at this later time point (Figure 2b), suggesting that inflammatory processes in CiOA knees were sustained following TAA treatment. To further investigate the disruption of inflammatory responses by TAA, the expression of activation markers by macrophages in the knee joint was examined at each time-point (Figure 2c). At day 14, flow cytometric analysis identified a significant increase in the expression of the activation marker CD86, associated with a pro-inflammatory phenotype, by macrophages in the knees of CiOA mice compared with control animals, irrespective of TAA treatment. Expression of the marker CD206, indicative of a tissue-repair phenotype, also did not differ between groups at this timepoint. However, expression of the marker CD163, which is associated with an anti-inflammatory phenotype, was significantly decreased by macrophages of untreated-CiOA knees compared with control knees. Interestingly, expression of CD163 was significantly higher in TAA-treated compared with untreated CiOA mice and did not significantly differ from control animals at this time point. At day 56, macrophages isolated from TAA-treated CiOA joints were associated with higher expression of CD86 compared with untreated-CiOA knees, though it was not different compared with control. The expression of CD206 and CD163 did not differ between experimental groups at this time point. These data suggest that a single intra-articular injection of TAA early in the pathogenic process has long-term effects in the joint, sustaining increased levels of macrophages with slightly elevated activation marker profile during OA development.

### 3.2 | TAA aggravates osteophyte formation during OA progression

We next interrogated whether TAA injection modulates osteophytosis and cartilage damage in the joint. Cartilage damage was significantly increased at both days 14 and 56 in CiOA knees compared with control knees and was not altered by TAA injection (Figure 3c,h). After 2 weeks of CiOA, osteophytes were present particularly at the margins of the medial tibia plateau but no differences were observed between untreated and TAA-treated CiOA knees (Figure 3d,e). Interestingly, osteophyte maturation was significantly increased in TAA-injected knees at day 56, compared with control and untreated-CiOA joints, though no changes were observed with respect to size (Figure 3i–k). Together these results show that injection of TAA increased osteophyte maturation in CiOA joints.

### 3.3 | Subchondral bone plate thickness is not modulated by TAA

To assess subchondral bone plate (SBP) changes, we performed μCT scans of the knees ex vivo at days 14 and 56 and analysed bone morphometry in the medial tibial plateau (Figure 4a). At day 14, the thickness of the subchondral bone plate was decreased in untreated CiOA knees compared with the knees of control animals, confirming previous studies in this model (Botter et al., 2011). Also in TAA-treated CiOA knees, the mean subchondral bone plate thickness was diminished compared with the knees of control mice, but this did not reach statistical difference (Figure 4b). At day 56, subchondral bone plate thickness was similar between all experimental groups (Figure 4c). These results suggest that intra-articular TAA injection does not interfere with OA subchondral bone plate changes in vivo.

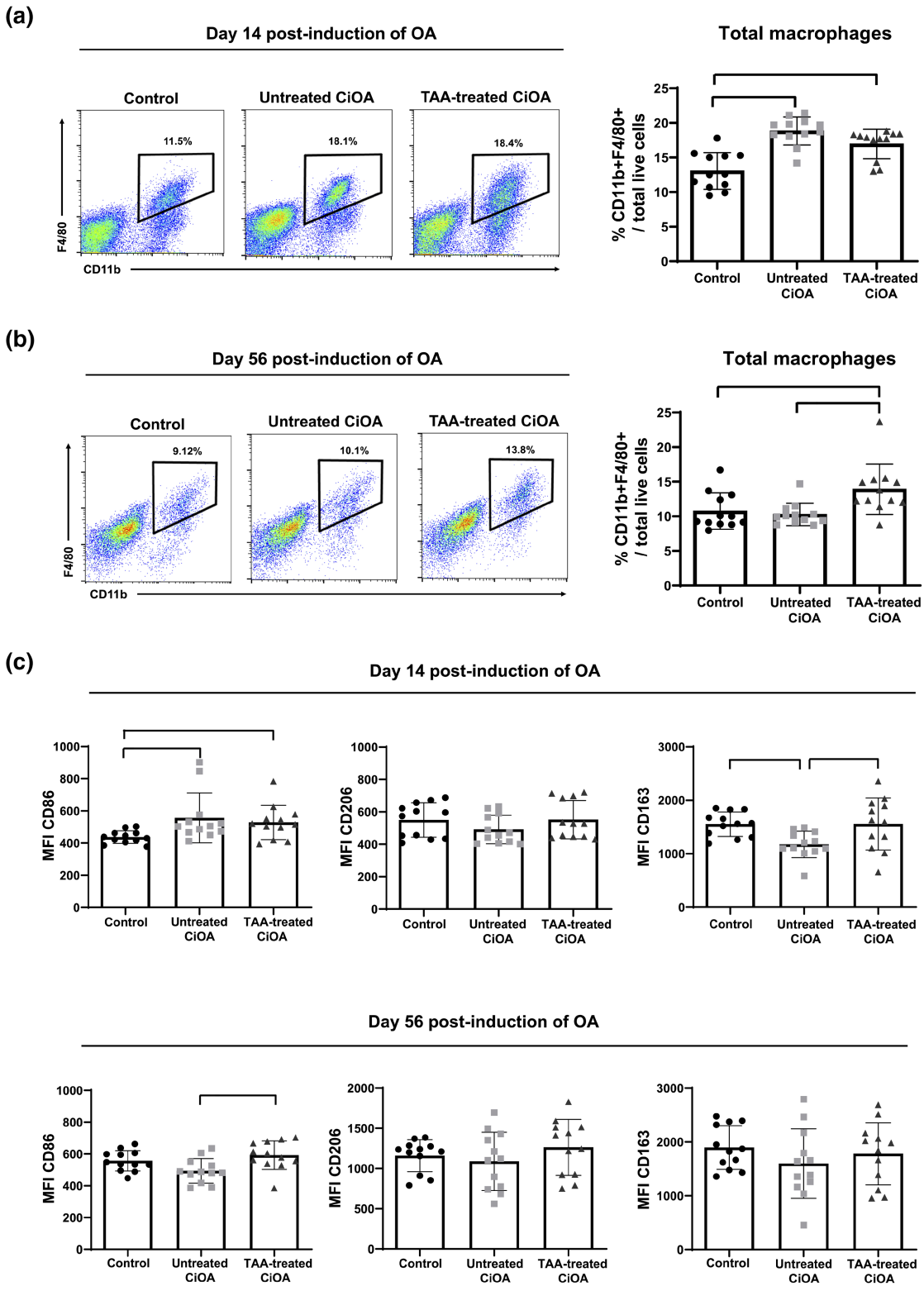



**FIGURE 2** TAA sustained macrophage-mediated inflammation in the knee joint and altered the expression of macrophage activation markers following induction of collagenase-induced osteoarthritis (CiOA). Analysis of flow cytometry data showing the percentage of CD11b+F4/80+ macrophages present within digested patellar/synovial tissue isolated from the right knee joint of mice at day 14 (a) and day 56 (b) post-induction of CiOA. Data represent the mean ± SD. (c) Expression of the macrophage activation markers CD86, CD206, and CD163 by macrophages present in the knee joint at days 14 and 56 post-induction of CiOA, as determined by flow cytometry. Values represent the mean ± SD for median fluorescence intensity (MFI) for each marker. Each dot represents data of an individual mouse ($n = 12$ per timepoint). Non-parametric data were evaluated using a Kruskal–Wallis test with Bonferroni post hoc test. For parametric data, a one-way ANOVA with Bonferroni post hoc test was conducted. * $P<0.05$

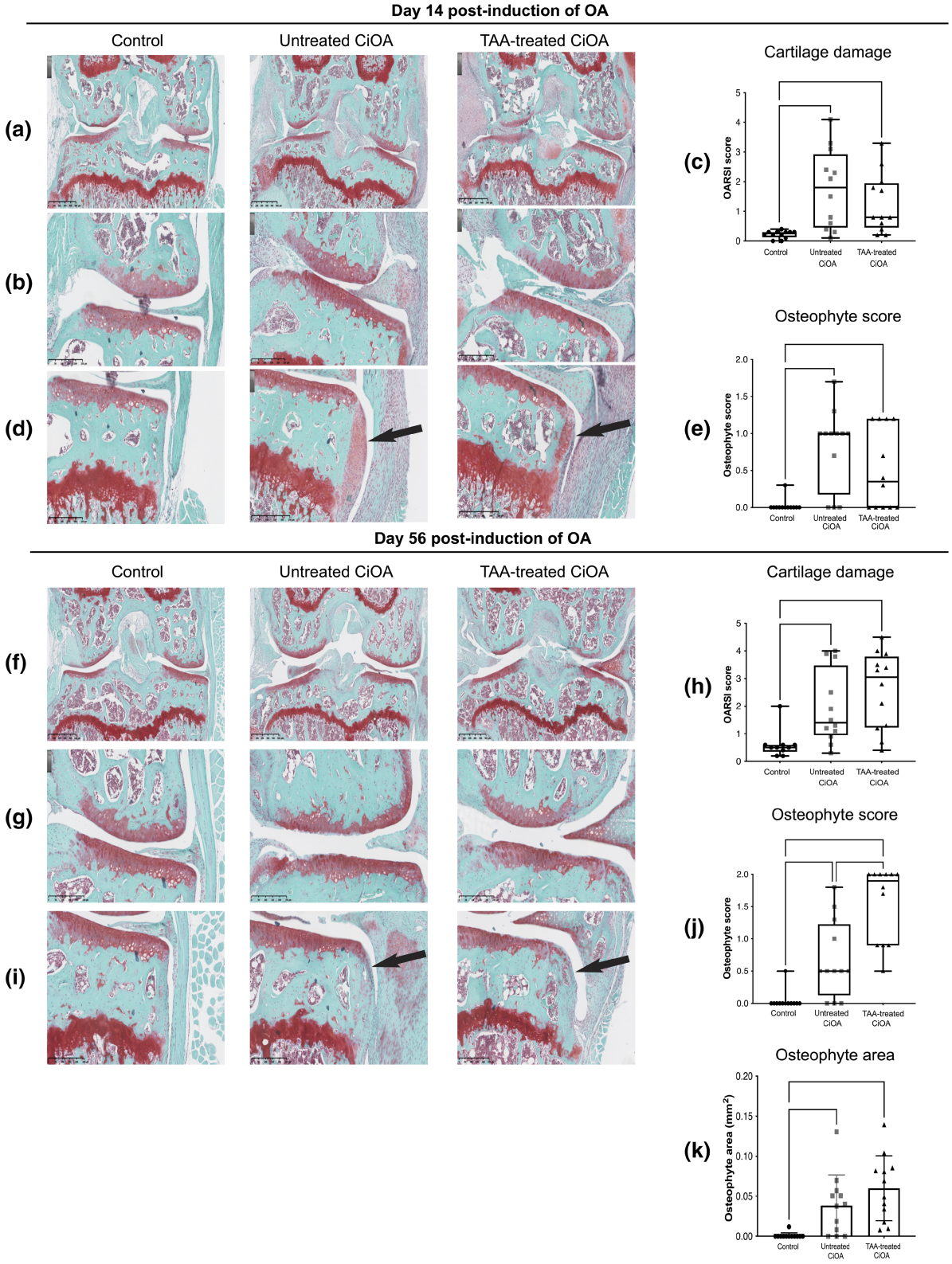



**FIGURE 3** TAA exacerbated osteophyte formation in CiOA knees. Histological analyses of cartilage damage and osteophyte formation at day 14 and day 56 after induction of CiOA. (a, f) Safranin O/Fast Green staining of control, untreated CiOA, and TAA-treated CiOA knees with magnification of the medial side (b, g), showing osteophyte formation adjacent to the medial tibiae plateau (d, i). Arrows indicate osteophytes. Graphs for cartilage damage (c, h) and osteophyte scores (e, j) are box-and-whiskers plots, with line indicating the median and error bars spanning maximum to minimum values. (k) Osteophyte area adjacent to the medial tibiae plateau is represented by the mean ± SD. Each dot represents data of an individual mouse ($n = 12$ per timepoint). Kruskal–Wallis test with Dunn's post-test correction for multiple comparisons was used for OARSI and osteophyte score. For osteophyte area, a one-way ANOVA with Bonferroni post hoc test was conducted. * $P<0.05$

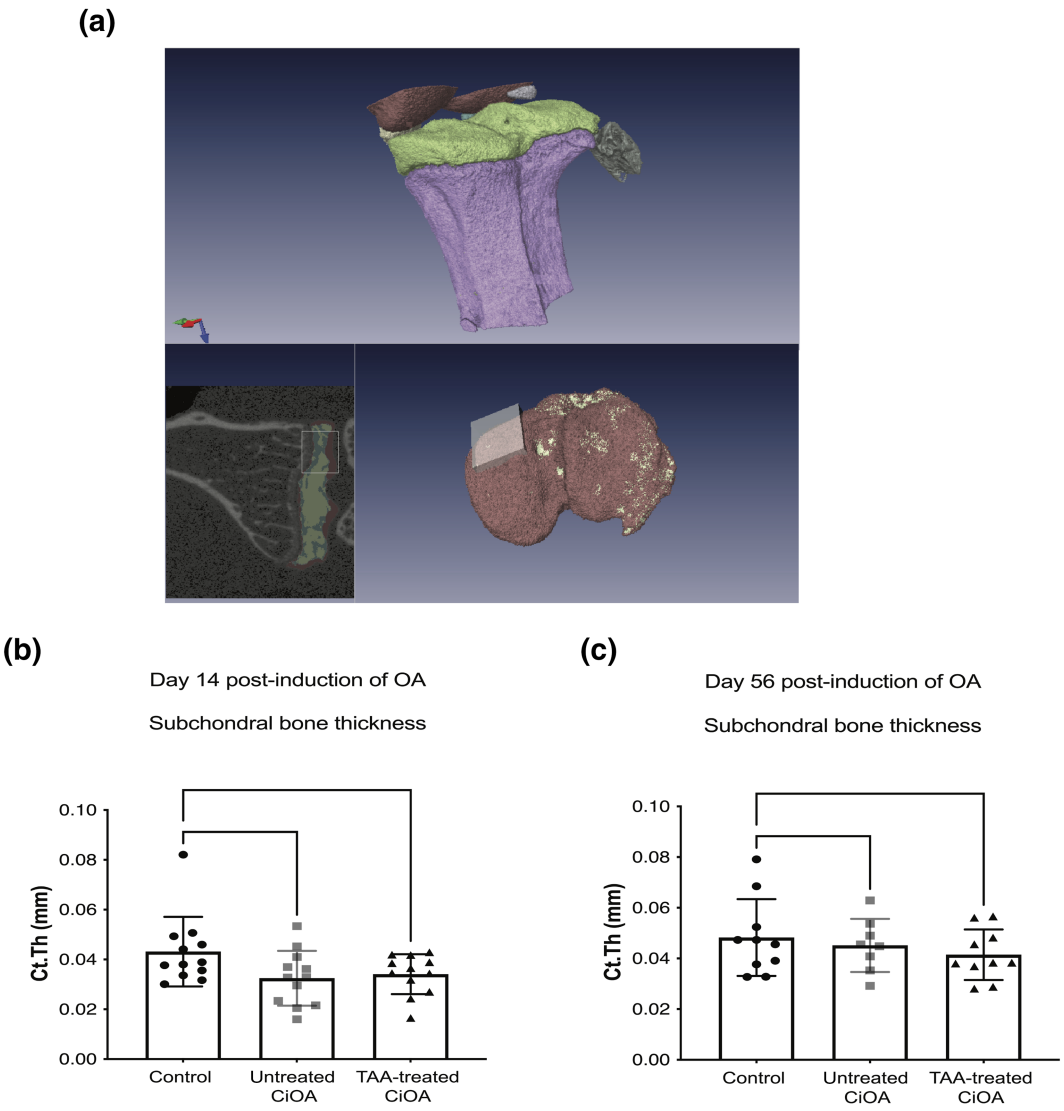



**FIGURE 4** TAA treatment did not alter subchondral bone plate thickness during CiOA development. (a) Bone morphometric analysis in medial tibiae plateau of the right knee of mice, showing the region of interest (ROI). Cortical thickness (Ct. Th.) in subchondral bone plate at day 14 (b) and day 56 (c). Each dot represents data of an individual mouse, with $n = 12$ mice per group at day 14 and $n = 8–10$ per group at day 56. Some samples were excluded at day 56 due to low resolution during acquisition that led to inability of image reconstruction. A one-way ANOVA with Bonferroni post hoc test was conducted. * $P<0.05$. ns, non-significant

### 3.4 | Intra-articular injection of TAA does not exert systemic effects on monocytes in peripheral blood and bone marrow

Having observed an effect of intra-articular injection of TAA on local macrophage-mediated inflammation in the joint, we also sought to examine potential systemic effects of TAA treatment. Therefore, we longitudinally assessed the responsiveness of peripheral blood monocyte subsets to intra-articular injection of TAA during the development of CiOA. The distribution of monocyte subsets was not altered in the blood of mice following induction of CiOA compared with control mice, with the exception of day 28 where a significant relative increase in classical monocytes, consequently resulting in a decrease of non-classical/intermediate monocytes, was observed (Figure S3). Intra-articular injection of TAA did not significantly change monocyte subsets in the peripheral blood at an early time point of 2 days post-injection, or at days 14 and 56 (Figure 5a,b). Finally, the percentage of monocytes and myeloid progenitors, granulocytes and lymphocytes in the bone marrow of the contralateral femur of TAA-treated mice, did not significantly differ compared with untreated-CiOA mice or control animals at days 14 and 56 (Figure S4), further indicating that short-term local delivery of TAA does not exert systemic effects at the level of monocyte and bone marrow precursor composition.

## 4 | DISCUSSION

Despite the long-standing use of intra-articular glucocorticoid injections, there is ongoing debate about their benefits on structural OA features (Juni et al., 2015). The dampening activity of TAA on

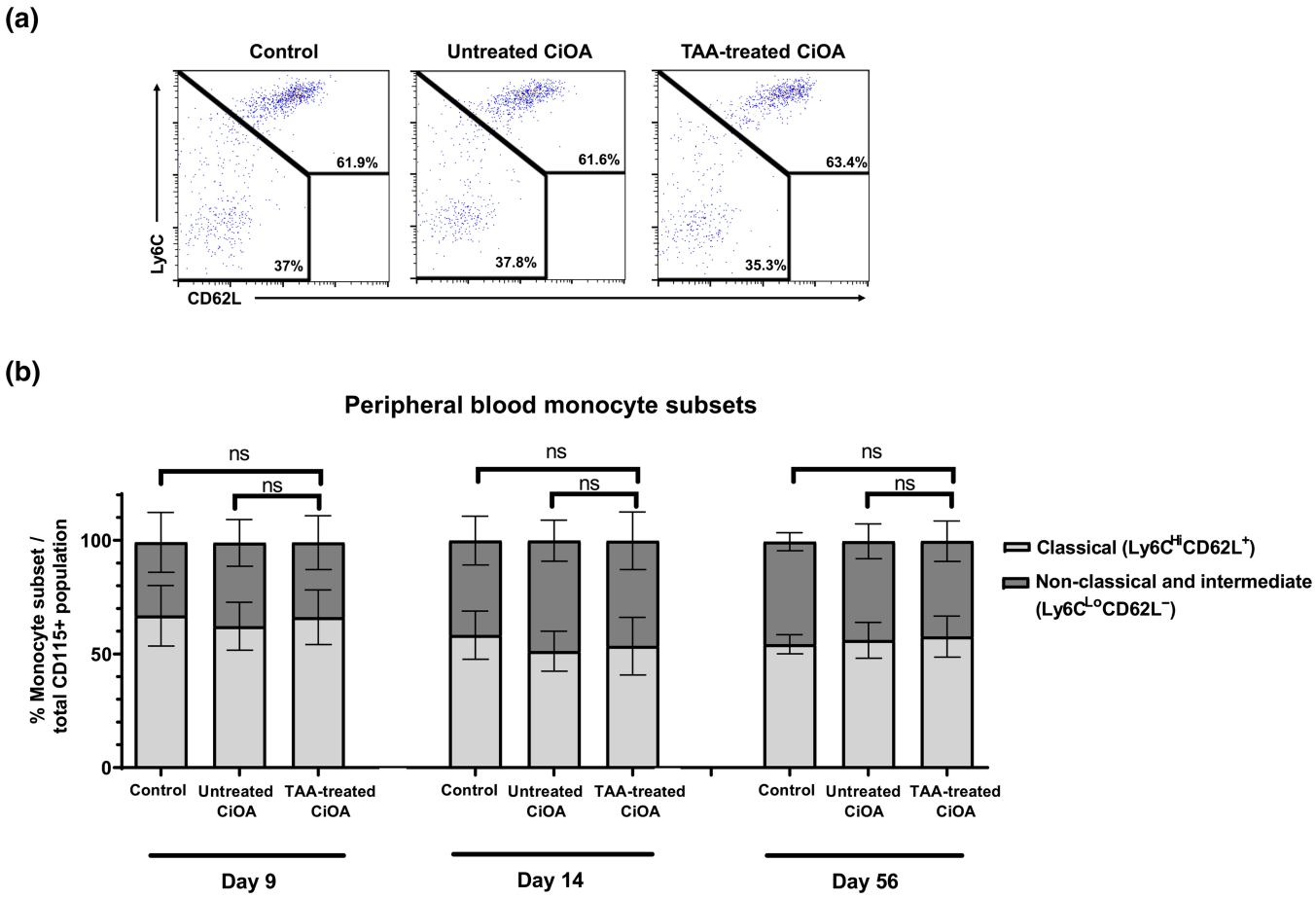



**FIGURE 5** Intra-articular injection of TAA did not exert systemic effects on peripheral blood monocyte subsets. (a) Representative dotplots of flow cytometric analysis of peripheral blood monocyte subsets 48 h following intra-articular injection of TAA (day 9 post-induction of CiOA). (b) Percentage of classical ($Ly6C^{hi}CD62L^{+}$) and non-classical and intermediate ($Ly6C^{lo}CD62L^{-}$) monocyte subsets present in the peripheral blood of mice at days 9, 14, and 56 post-induction of CiOA. Data represent the mean ± SD, with $n = 24$ mice per group at days 9 and 14, and $n = 12$ mice per group at day 56. ns, non-significant. Differences in the percentage of monocyte subsets between experimental groups were evaluated per timepoint using a one-way ANOVA with Bonferroni post hoc test

inflamed synovium following joint injury and progression of OA has been postulated to mediate its therapeutic effect (Nixon et al., 2013; Paik et al., 2019). However, the biological mechanisms underlying the potential impact of TAA on OA progression are currently not well understood. Additionally, the potential of early TAA treatment following injury to alter OA progression is not clear. The findings of this study identify the presence of sustained macrophage-mediated inflammation as well as increased osteophytosis in the knee joint following short-term local TAA treatment (Figure 6). These data suggest a negative effect of TAA administration in acutely inflamed joints following trauma, and provide further insight into cellular mechanisms which may govern the balance between therapeutic benefit and potential harm of TAA treatment during OA progression.

Previous studies have highlighted the potential of TAA to alter macrophage activation in the knee joint (Siebelt et al., 2015). Our findings that TAA increased synovial macrophage numbers are in accordance with the data of Siebelt and colleagues, which showed that TAA increased macrophage activation in a rat papain-induced OA model (Siebelt et al., 2015). Rudnik-Jansen et al. observed that TAA did not modulate synovitis in an anterior cruciate ligament and medial transection rat model, when administered at 4 weeks post-OA induction (Rudnik-Jansen et al., 2019). Additionally, intra-articular injection of TAA to the knees of patients with rheumatoid arthritis does not reduce the level of macrophages present within synovial tissue (van der Goes et al., 2012). Therefore, the modulation of synovial inflammation by TAA might be dependent on the pathological environment in the joint and time of administration.

In the present study, we also investigated whether TAA modulated the phenotype of macrophages present within the joint following induction of CiOA. In this regard, macrophage expression of activation markers CD86, CD206, and CD163, which are known markers associated with pro-inflammatory, tissue repair, and anti-inflammatory macrophage phenotypes (Fischer-Riepe et al., 2020; Johnston et al., 2012; Lech & Anders, 2013), respectively, were analysed by flow cytometry. In line with previous reports, macrophage CD163 expression was decreased following induction of CiOA and

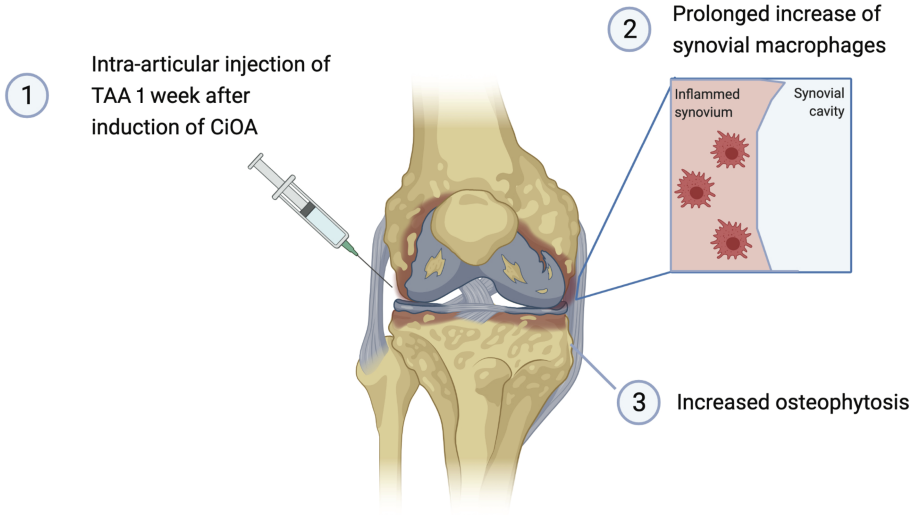



**FIGURE 6** Graphical representation of main conclusions. Created with BioRender.com

significantly increased in response to TAA treatment (Khatab et al., 2018; Siebelt et al., 2015). Furthermore, macrophages present within the joints of TAA-treated CiOA mice had increased expression of CD86 at the later time-point compared with untreated-CiOA knees, indicating a potential shift towards a pro-inflammatory phenotype. Glucocorticoids are known to induce expression of CD163 and CD206 by human and rodent macrophages in vitro (Buechler et al., 2000; Shepherd et al., 1985; Siebelt et al., 2015). However, discrepancies in the expression of markers associated with polarised macrophage phenotypes between in vitro-generated and in vivo populations has been previously described (Orecchioni et al., 2019). Moreover, expression of M2-associated markers does not indicate anti-inflammatory activity by definition. A human study showed adipose tissue macrophages expressing CD163 and CD206 are capable of high level proinflammatory cytokine production when triggered (Zeyda et al., 2007). Future studies may be required evaluating the functionality of the macrophage populations present in vivo, including their capacity to produce mediators influencing inflammation and osteogenesis, to fully determine their response to TAA treatment and subsequent impact on OA progression.

Current clinical reports on the benefit of TAA treatment are conflicting, with previous studies describing accelerated disease progression following repeated intra-articular TAA injections in patients with mild to moderate OA (McAlindon et al., 2017; Zeng et al., 2019). In line with our findings, Rudnik-Jansen et al. have observed that a single intra-articular bolus injection of TAA did not reduce cartilage damage in an anterior cruciate ligament and medial transection rat model, when administered at 4 weeks post-OA induction (Rudnik-Jansen et al., 2019). Furthermore, here, we have observed increased osteophytosis in TAA-treated CiOA joints. In contrast to our observations, Siebelt et al. (2015) found reduced osteophyte formation in rats in response to TAA-treatment. This divergence in outcomes may be due to differences between the models, considering that the use of papain results in cartilage matrix degradation, whereas collagenase initiates joint instability by degrading the ligaments. Furthermore, Siebelt et al. (2015) applied a weekly TAA intra-articular injection regime which began at the time of OA induction and continued throughout the duration of the experiment, in contrast to TAA administration at a single early time point post-joint injury in the present study. The formation of osteophytes is known to be linked to growth factors which are secreted by macrophages. The increase of osteophyte formation in TAA-treated mice in our study might be associated with the increase of macrophages in the joint, since these cells have been shown to promote osteophytosis in CiOA knees (Blom et al., 2004; van Lent et al., 2004). Additionally, Rudnik-Jansen and colleagues have previously reported increased dystrophic calcification in unstable OA rat knees that were treated with TAA utilising an extended release delivery system (Rudnik-Jansen et al., 2019). Osteophytes and the abnormal bone formation in the ligaments and tendons are caused by endochondral ossification of skeletal stem cells (Roelofs et al., 2020; van der Kraan & van den Berg, 2007). Therefore, TAA might accentuate this process on progenitor cells leading to osteophytosis either via a direct effect or via synovial macrophages (Ferrao Blanco et al., 2021). In this regard, further studies are needed to investigate the relationship between the increase of osteophytosis and macrophages due to TAA.

Glucocorticoids are known to modulate bone loss due to increased osteoclastogenesis or induced apoptosis of osteoblasts and osteocytes (Ahmad et al., 2019; Chotiyarnwong & McCloskey, 2020). However, we found that locally delivered TAA did not alter subchondral bone thickness during CiOA development. Interestingly, non-classical peripheral blood monocytes have been identified as crucial cells for osteoclast differentiation in an experimental model of rheumatoid arthritis (Puchner et al., 2018). In addition to modulating macrophage behaviour, glucocorticoid treatment has also been reported to alter the functionality of peripheral blood monocytes. Glucocorticoid treatment of human monocytes in vitro has been shown to induce an intermediate subset phenotype (Liu et al., 2015), and ex vivo stimulation of monocytes with glucocorticoids reported to induce a distinct monocyte phenotype associated with anti-inflammatory properties (Ehrchen et al., 2007; Varga et al., 2008). High-dose treatment with glucocorticoids induces selective apoptosis

of intermediate and non-classical monocytes (Dayyani et al., 2003). Furthermore, intra-articular glucocorticoid injection to patients with rheumatoid arthritis was shown to alter monocyte trafficking (Steer et al., 1998). Our findings on the distribution of monocyte subsets in peripheral blood, as well as the abundance of monocytes in the bone marrow, indicate that local short-term intra-articular delivery of TAA at the examined concentration does not induce systemic immune responses in vivo during the development of CiOA.

Intra-articular injection of glucocorticoids such as TAA is recommended and routinely applied clinically for the management of OA pain of the knee (Kolasinski et al., 2020b; McAlindon et al., 2014). A limitation of our study is that we did not perform pain measurements, and therefore, further investigation is required to determine the effect of early intervention with TAA on pain relief during OA progression. Additionally, we did not evaluate the impact of intra-articular TAA administration on a healthy joint. Given that our primary focus was on the modulatory effect of TAA on an injured joint and TAA will not likely be injected in a healthy joint, this was not a primary aim of our study. Rudnik-Jansen et al., however, previously reported that intra-articular injection of a microsphere-based extended TAA release system to healthy rat knees did not have any deleterious effects within the joint (Rudnik-Jansen et al., 2019). Interestingly, intramuscular TAA injection into the gluteus muscle has been previously reported to reduce pain compared with placebo injection in patients with hip OA (Dorleijn et al., 2018). Furthermore, a clinical trial evaluating the potential of intramuscular TAA injection as an alternative to intra-articular administration for the treatment of knee OA is underway (Mol et al., 2020). In light of the study of Dorleijn et al. indicating a systemic effect of TAA on joint pain (Dorleijn et al., 2018), intramuscular injection of TAA may potentially serve as a beneficial therapeutic strategy to avoid the negative local effects of TAA within the injured knee joint observed in our study.

In conclusion, the findings of our study suggest that short-term intra-articular TAA treatment may sustain OA-associated local inflammatory processes in the knee mediated by macrophages, which may further contribute to a loss of homeostasis within the joint and exacerbate disease progression. In the context of clinical translation of these findings, caution should be taken when locally applying TAA treatment to an acutely inflamed joint following injury. Further investigation of the impact of TAA on the behaviour of local immune cell populations during active inflammation in the knee may enhance therapeutic outcomes for the treatment of OA.

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## AUTHOR CONTRIBUTIONS

MNFB designed the study, performed experiments, analysed and interpreted the data, and wrote the manuscript. NK and AC performed experiments, analysed the data, and edited the manuscript. RN, PJML, and SMB interpreted the data and edited the manuscript. YMBJ and GJVMvO designed the study, interpreted the data, and edited the manuscript. NF designed the study, performed the experiments, analysed and interpreted the data, and wrote the manuscript. All authors approved the final version of the manuscript.

## CONFLICT OF INTEREST

S.M. Botter has a consultancy agreement with Octapharma AG, Lachen, Switzerland. Other co-authors have nothing to disclose.

## DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

## DATA AVAILABILITY STATEMENT

Raw data are available upon request from the corresponding author.

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