Targeted IL-4 therapy synergizes with dexamethasone to induce a state of tolerance by promoting Treg cells and macrophages in mice with arthritis

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Abbreviations: CTRL, control; DXM, dexamethasone; RA, rheumatoid arthritis

ABSTRACT

F8-IL-4 is a recently developed immunocytokine which delivers IL-4 to sites of inflammation by targeting the neovasculature. We previously reported that F8-IL-4, in combination with dexamethasone, provides a durable therapy in mice with collagen-induced arthritis (CIA). Therefore, the objective of this study was to identify the mechanism by which IL-4 and dexamethasone combination therapy provides long-lasting disease remission.

F8-IL-4 alone attenuated inflammation in CIA and this was associated with increased T_H2 and decreased T_H17 cell numbers in the joints. Similarly, dexamethasone alone had an anti-inflammatory effect associated with lower T_H17 cell numbers. In both cases, these therapeutic benefits were reversed once treatment was stopped. On the other hand, combination therapy with F8-IL-4 plus dexamethasone led to a synergistic increase in the percentage of regulatory T (Treg) cells and anti-inflammatory macrophages in the arthritic joint and spleen as well as IL-10 levels in serum and spleen. The net result of this was a more pronounced attenuation of inflammation and, more importantly, protection from arthritis relapse post-therapy retraction.

In conclusion, F8-IL-4 plus dexamethasone, is a durable treatment for arthritis that acts by promoting Treg cells in a synergistic manner, and by producing a sustained increase in anti-inflammatory macrophages.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting joints, in which the delicate balance between pro- and anti-inflammatory cells and their cytokines is perturbed. This is illustrated by the fact that targeting pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in RA patients and in mouse models of RA, as well as boosting levels of anti-inflammatory cytokines (IL-10 and IL-4) leads to a reduction in disease severity [1]–[3]. IL-4 is an anti-inflammatory cytokine which has been under investigation for the treatment of autoimmune arthritis since the late 1990's. IL-4 downregulates the production of pro-inflammatory cytokines such as IL-1, TNF-α and IL-6 which have been implicated in the pathogenesis of RA [4],[5]. The *ex vivo* delivery of IL-4 has been shown to be effective in attenuating inflammation in mouse models of arthritis [2],[6],[7]. Furthermore, IL-4 has been identified in mice with arthritis during disease remission [8] which suggests that this cytokine also plays a role in the resolution of disease. IL-4 has an effect on a wide range of immune cells as it's receptor is present on both T and B cells, mast cells, macrophages, monocytes and fibroblasts [9]. IL-4 inhibits $T_H17$cells [10],[11] which are associated with joint inflammation and cartilage damage [12]. IL-4 plays an integral part in the induction of CD4 T helper 2 (T_H2) cells, by activating STAT6 and the transcription factor GATA3. IL-4 is also the signature cytokine secreted by T_H2 cells which are known for their role in antibody-mediated immunity, responses against extracellular parasites and in the development of allergic reactions such as asthma. It is well established that IL-4 in conjunction with IL-13 induces a state of alternate activation in macrophages, which are generally considered to be immunosuppressive [9],[13]–[16]. IL-4 has been directly linked to the up-regulation of genes associated with alternatively activated macrophages such as arginase, mannose receptor (CD206), FIZZ1 and Ym1/2 [9]. These findings suggest that the exogenous delivery of IL-4 is a viable therapeutic option in RA as well as in other inflammatory diseases. However, although recombinant IL-4

therapy has shown efficacy in psoriasis [17], there are no documented successful trials in RA patients, possibly due to the fact that continuous delivery using miniosmotic pumps [2] or by repeated daily injections [17] is needed to reach a therapeutic concentration level at the site of inflammation. More importantly, nearly 50% of patients with psoriasis reported side-effects, such as headaches, with high doses of recombinant IL-4 [17]. The use of immunocytokines bypasses this problem as it enables the delivery and accumulation of cytokines at the site of disease [18]–[20]. F8-IL-4 is an immunocytokine (antibody + cytokine) which delivers recombinant IL-4 specifically to sites of inflammation by targeting the neovasculature. The F8 antibody recognizes the alternatively spliced extra domain A of fibronectin, which is expressed at sites of inflammation while being undetectable in healthy organs with the exception of sites of physiological angiogenesis [21].

Sustained treatment-free remission in patients with autoimmune diseases has long been the unattainable holy grail of autoimmune disease treatment. Tolerising strategies with dexamethasone (DXM) and immunogenic peptides have shown great promise in mouse models of diabetes [22]. We showed previously that F8-IL-4 attenuates collagen-induced arthritis (CIA) in DBA/1 mice, a preclinical model for RA [23]. More importantly, F8-IL-4 in combination with DXM, led to prolonged disease remission; however the mechanism behind this phenomenon is not known. The preparation for phase I clinical trials in RA patients with F8-IL-4 are presently underway. The aim of the present study was to understand the mechanisms underlying the sustained therapeutic effect of F8-IL-4 and DXM by comparing the effect of monotherapy versus combination therapy on cells of the immune system, including myeloid and lymphoid cells. Our findings confirm those of others showing a significant therapeutic benefit with IL-4 alone [2],[7],[23]. In addition, we are the first to demonstrate that combination therapy leads to prolonged disease remission by the expansion of Treg cells and macrophages of an anti-inflammatory and toleragenic phenotype.

RESULTS

IL-4 delivery to arthritic joints attenuates inflammation in a mouse model of rheumatoid arthritis

First, we confirmed the ability of F8-IL-4 to attenuate joint inflammation in a well-established pre-clinical mouse model of RA. During the treatment regime, no adverse effects of F8-IL-4 or DXM were recorded. After 10 days of treatment, mice with collagen-induced arthritis (CIA) were culled and the influence of F8-IL-4 on cytokine levels in serum as well as on cells at the site of disease was determined (Figure 1A). As reported previously [23], F8-IL-4 treatment reduced the arthritic scores in mice with arthritis. The therapeutic effect of F8-IL-4 was significant from day 4 of treatment onwards. During the 10 day treatment period, there was no significant difference in the arthritic scores of mice receiving DXM monotherapy and F8-IL-4 + DXM combination therapy. Both DXM monotherapy and combination (F8-IL-4 + DXM) treated mice showed a significant attenuation of disease in comparison to control animals from day 2 of treatment onwards (Figure 1B).

As expected, increased levels of IL-4 were detected in the joints and serum of F8-IL-4-treated animals but not in the control (PBS treated) or DXM monotherapy group. Of note was an induction of the anti-inflammatory cytokine IL-10 in combination treated mice. The levels of serum IL-10 in these mice correlated with IL-4 levels, suggesting that mice that respond to IL-4 treatment with the induction of IL-4 also induce IL-10 (Figure 1C). Supernatants from arthritic joint cultures of F8-IL-4 + DXM treated mice had a significant decrease in the production of pro-inflammatory cytokines: IL-1β, IL-6, GM-CSF and IL-12 (Figure 1D). In addition, serum levels of IL-6, TNFα and IL-12 were also significantly lowered by F8-IL-4 + DXM treatment (Supporting Information Fig. 1A). Surprisingly, the levels of TNFα were higher in the paw cultures from mice that received F8-IL-4 monotherapy (Figure 1D). These findings suggest that although F8-IL-4 monotherapy can attenuate inflammation by inducing

the expression of anti-inflammatory cytokines such as IL-4 and IL-10, possibly some of its therapeutic effects are hindered by the induction of TNF-α in the inflamed joint.

F8-IL-4 synergizes with DXM to decrease T_H17 / Treg cell ratios in arthritic joints

As F8-IL-4 had a significant influence on cytokines secreted by cells of the myeloid as well as lymphoid lineages, in our next set of experiments we examined the phenotype of cells found at the site of inflammation in more detail. The gating strategy used to identify lymphocyte populations using flow cytometry is shown in Supporting Information Fig. 2. We found that the total number of cells isolated from joints on day 10 was comparable between treatment groups (Supporting Information Fig. 3A). As predicted, F8-IL-4-treated mice had a significant increase both in the total number and the percentage of IL-4-secreting T_H2 cells in the joint confirming the biological effect of therapy (Figure 2A and Supporting Information Fig. 3B). F8-IL-4 treatment had no effect on IFN-γ levels in serum, in paw culture supernatants or on T_H1 cell numbers in the paw (Supporting Information Fig. 3C and data not shown). Conversely, the numbers of $CD4^+$ and TCRγδ T cells secreting the pro-inflammatory cytokine, IL-17A, as well as the level of IL-17A detected in the paws was decreased by treatment with F8-IL-4 alone, DXM alone or F8-IL-4 + DXM (Figure 2B-D).

It has been reported that IL-4 has a negative effect on the percentage of Treg cells among $CD4^+$ T cells [24],[25]. We found that there was no significant effect of IL-4 on the Treg population (Figure 2E). Although the total number of T_H17 was significantly reduced by F8-IL-4 alone, the ratio between pro-inflammatory T_H17 and suppressor Treg cells was not significantly changed (Figure 2F). Dexamethasone is a well-known immunosuppressant which induces Treg cells [26]. Therefore, we hypothesized that DXM would support Treg numbers in F8-IL-4 treated mice and thereby improve clinical outcomes. DXM monotherapy led to a trend towards an increased proportion of Treg cells among $CD4^+$ T cells in the paws of CIA

mice, but the increase was not significant. However, F8-IL-4 + DXM-induced a significantly higher percentage of Treg cells in comparison to DXM monotherapy. This contributed to a significant decrease in the T_H17:Treg cell ratio in the joints of arthritic mice.

F8-IL-4 plus DXM induces an anti-inflammatory phenotype in macrophages in arthritic mice

As our previous results suggested that F8-IL-4 in combination with DXM has an effect on cytokines secreted by cells of the myeloid lineage, such as IL-1β, IL-6 and IL-12 we investigated the effects on myeloid cell number, phenotype and activation. First, we examined the effect of F8-IL-4 treatment on macrophage, neutrophil and dendritic cell numbers in mice with CIA. The gating strategy used to identify myeloid populations using flow cytometry was described in [27] and is shown in Supporting Information Fig. 4. All treatment groups had an increase in the percentage of F4/80 expressing myeloid cells (data not shown). A reduction in the total number of paw macrophages was observed in DXM-treated animals (Figure 3A). F8-IL-4 + DXM as well as DXM monotherapy did not affect the total numbers of neutrophils or dendritic cells in the paw (Supporting Information Fig. 5A and B). In our next set of experiments we addressed the hypothesis that the combination of F8-IL-4 plus DXM supports macrophages of a tolerogenic phenotype while inhibiting pro-inflammatory phenotypes. Indeed, combination treated mice showed a significant increase in the M2-associated marker, CD206 (Figure 3B). There was a significant decline in the percentage of macrophages expressing MHC class II in combination treated mice, suggesting a lower level of cell recruitment from other myeloid cell compartments such as the bone marrow. Combination treated mice also showed a significant decrease in the percentage of MHC class II high macrophages which suggests that combination therapy leads to a reduction in macrophage activation (Figure 3C). In addition, F8-IL-4 + DXM mice showed a higher expression of other M2-

associated markers such as *Arg1*, *Il10* and *Ym1* as well as a lower expression of M1-associated markers (*Il12b*). On the other hand, DXM induced the expression of *Nos2* (Figure 3D). Arginase mRNA (*Arg1*) expression levels in combination treated mice correlated with their arthritic score (Figure 3E), but not their serum IL-4 levels (Supporting Information Fig. 5C). This suggests that even though animals with high levels of IL-4 have also a higher expression of IL-10, *Arg1* is best correlated with disease status of the animal.

F8-IL-4 plus DXM provides protection from arthritis relapse and inhibits IL-17A expression in joints

As combination therapy led to an increase in the proportion of anti-inflammatory cell types, we examined whether these effects translated into clinical benefit and whether they were sustained in the long-term. As in our previous experiments, arthritis was induced in DBA/1 mice. Once the mice showed signs of arthritis they were recruited to one of two treatment groups (F8-IL-4 + DXM or DXM alone) and treated for a limited period of 10 days (Figure 4A). Once treatment was stopped there was a dramatic increase in the arthritic scores of mice receiving DXM alone (Figure 4B). However, mice receiving combination therapy maintained a significantly lower mean arthritic score in comparison to DXM monotherapy mice. Furthermore, withdrawal of DXM monotherapy led to a spread of disease to previously unaffected paws, which was not observed in the F8-IL-4 + DXM combination treated mice. The mice were monitored daily until day 21 at which time point the mice were culled. Consistent with the clinical findings, combination treated mice had lower numbers of cells in their paws at day 21 (Figure 4C). Combination treated mice also continued to have a low number of pro-inflammatory T_H17 and IL-17A$^+$ TCR$\gamma\delta$ T cells, but interestingly these mice no longer showed a significant increase in the proportion of T_H2 cells (Figure 4D-E). Serum and paw

culture supernatant levels of IL-4 were below detection in combination treated mice (data not shown). The number of T_H1 cells and Treg cells in the joints of combination treated mice was identical to those of DXM mice; however combination mice had a significant decrease in the ratio between T_H17 and Treg cells (Figure 4F and Supporting Information Fig. 6A, B). As on day 10 with the exception of TNFα, supernatants from paw cultures of combination treated mice, showed a lower level of pro-inflammatory cytokines (Figure 4G). The anti-inflammatory cytokine, IL-10 was still significantly higher in the serum (Figure 4H).

F8-IL-4 plus DXM-induced anti-inflammatory macrophages persist after therapy retraction

In contrast to DXM monotherapy mice, F8-IL-4 + DXM treated mice continued to have a smaller population of neutrophils in their paws. Post-therapy retraction, there was no difference between the numbers of macrophages and dendritic cells in the paws between treatment groups (Figure 5A-C). However, F8-IL-4 + DXM treated mice continued to have a higher proportion of CD206-expressing macrophages, which suggests a long-term effect of F8-IL-4 + DXM on macrophage phenotype (Figure 5D). Combination-treated mice continued to have a lower proportion of MHC class II positive macrophages (Figure 5E). Similarly as on day 10, on day 21 there was no significant difference in the expression of *Nos2* between treatment groups. DXM-treated mice had a higher expression of *IL-12b* which encodes the p40 subunit of IL-12 and IL-23 and is associated with M1 macrophages. In contrast, genes associated with tolerogenic M2 macrophages (*Arg1, Il10*) were expressed at higher levels in the spleens of combination treated mice in comparison to DXM monotherapy treated mice (Figure 5F). Arginase mRNA (*Arg*) expression levels correlated with the arthritic scores of combination treated mice on day 21 (Figure 5G).

DISCUSSION

In this study, we sought to elucidate the mechanism of action of IL-4 and DXM combination therapy in arthritis. Recombinant IL-4, as well as F8-IL-4, have shown efficacy in mouse models of RA [2],[23],[28]. We confirmed and extended these findings by demonstrating decreased T_H17 and increased T_H2-type responses at the site of disease activity following treatment with F8-IL-4. The anti-inflammatory properties of IL-4 were first described in the early 1990's in which it was linked to the down-regulation of TNF-α, IL-1 and IL-6 [4],[5],[29]. Indeed in our hands, F8-IL-4 treatment alone significantly reduced the levels of IL-1β and IL-6 in arthritic joints. We were intrigued to find that F8-IL-4 increases local TNF-α level in paws, but not in the serum of arthritic mice. There is data suggesting that IL-4 can increase TNF-α production by murine peritoneal macrophages in some circumstances [30], a phenomenon that it not true for LPS-stimulated TNF-α production by these cells [31]. Therefore, it is possible that IL-4 may have contrasting effects on macrophages based on their localization or state of activation and possibly even phenotype. Even species differences have been noted, as human macrophages do not behave in this way as the presence of IL-4 is known to inhibit TNF production [4]. The mechanism behind the increase in TNF-α is unknown, but it could possibly explain the relatively low level of efficacy of F8-IL-4 monotherapy and suggests that F8-IL-4 would act synergistically with TNF-α inhibitors.

Studies have shown that IL-4 can influence T_H17 cells indirectly by the downregulation of IL-23 which is needed for the stability of T_H17 cells [32] or directly by opposing IL-17A expression [10],[11]. All three treatment regimens used in this study decreased T_H17 numbers in inflamed joints. Consistent with this were lower levels of IL-17A detected in paw culture supernatants. Various studies point towards the importance of IL-17A in the pathogenesis of RA (reviewed in [33],[34]). Blocking IL-17A in animal models of arthritis protects from joint and cartilage damage and leads to significant clinical improvements [35]. Surprisingly in con-

trast to what has been observed in animals models, in clinical trials with RA patients targeting IL-17A alone has been relatively disappointing [36], suggesting that other cytokines produced by T_H17 cells (GM-CSF, TNF-α) also may play a role in RA.

IL-4 therapy may have some negative effects on immunosuppressive cells as it has been reported to indirectly influence tolerance-inducing regulatory T (Treg cells) cell numbers [24],[25]. In this study, although F8-IL-4 did not significantly reduce Treg total numbers in arthritic paws, we observed a tendency for IL-4 to reduce the percentage of $FoxP3^+$ Treg cells among the $CD4^+$ T cell population. This result suggests that although IL-4 induces a significant decline in T_H17 cells, the T_H17 to Treg ratio is not significantly changed. We hypothesised that boosting Treg numbers in F8-IL-4 treated mice would further enhance the previously described therapeutic effects of IL-4.

DXM is a synthetic glucocorticosteroid that is well-known for its immunosuppressive effects. We chose to use DXM in this study as it is already used in RA patients [37] and it targets T_H17 cells [38] and supports Treg cells [26],[39] as well as toleragenic macrophages [22],[26],[40]. DXM is often used in RA patients either alone or more often in combination with so-called disease-modifying antirheumatic drugs (DMARDs) or biologics such as anti-TNF. However, severe adverse effects, such as osteoporosis, mood changes including depression and anxiety as well as aggravation of diabetes limit DXM use in patients. More importantly, the risk of these side effects increases with higher doses (>10 mg/day) and the duration of therapy. Therefore, the European League against Rheumatism (EULAR) recommends to only use low-dose glucocorticosteroids for up to six months and to only consider this treatment as part of an initial treatment strategy in combination with one or more DMARDs [41]. Although it is difficult to compare treatment regimens between humans and mice due to inter-species physiological differences, in our study to limit the toxic effect of DXM, we used a relatively low dose of DXM and a treatment period of only 10 days. We

found that combination therapy (F8-IL-4 + DXM) led to a more pronounced attenuation of inflammation than F8-IL-4 monotherapy. Significantly, in comparison to DXM monotherapy, a further expansion in the proportion of Treg cells as well as an increase in the proportion of anti-inflammatory macrophages in the arthritic joint and spleen were observed in combination treated mice. An important question that arises from this study is to whether the anti-inflammatory macrophages that are induced in combination treated mice are dependent on Treg cells. Indeed, studies have shown that Treg cells can induce M2 macrophages [42] in mice and alternatively-activated macrophages in humans [43]. At least, in mice, Treg induction by M2 macrophages seems to be dependent on multiple factors including, arginase, IL-10, and TGF-β [42]. Conversely, the opposite is also true, as anti-inflammatory macrophages can induce Treg cells in mice and in man in processes dependent on IL-10, TGF-β and retinoic acid [44],[45]. Alternatively, a third possibility could be envisioned in our study in which both subsets have developed independently of each other as DXM has been shown to induce both subsets [26],[40].

IL-10 is a potent anti-inflammatory cytokine which protects the host from immune-mediated damage. The exogenous delivery of IL-10 has been shown to attenuate CIA [18],[19],[46],[47] possibly by targeting pro-inflammatory T_H17 cells [48],[49]. Although we do not know which cell type(s) in our study produced the IL-10, it has been shown to be expressed by tolerogenic subpopulations of macrophages as well by T_H2 cells and Treg cells, but under certain circumstances is also produced by T_H1 and T_H17 cells making them less pathologic [50]. It has recently been reported that IL-4 induces IL-12 which in turn is needed for the induction of T_H1 responses [51]. In our study, although F8-IL-4 monotherapy did increase circulating IL-12 levels in the serum, combination therapy drastically decreased these levels. Furthermore, neither F8-IL-4 nor F8-IL-4 + DXM treated mice showed an increase in IFN-γ or T_H1 responses. One possible explanation for these contradictory findings may be

that targeting of IL-4 to sites of inflammation, in contrast to systemic administration, leads to IL-10 induction and thereby counteracts the effects of IL-12 on T_H1 cells.

Inflammatory macrophages have been described in the synovium of RA patients where they secrete pro-inflammatory cytokines such as TNF-α and IL-1β which further support the inflammatory process [52] thus making them valid targets in RA. Macrophage polarisation is orchestrated by the cytokine milieu. Inflammatory macrophages are induced by LPS or bacteria, and/or IFN-γ or GM-CSF. In contrast, macrophages can acquire an alternative state of activation in the presence of IL-4 and/or IL-13, IL-10 and/or M-CSF. Noteworthy is the fact that in our study F8-IL-4 + DXM combination treatment lead to the induction of a gene signature characteristic of alternatively-activated macrophages. Alternatively-activated macrophage markers such as arginase, mannose receptor (CD206), FIZZ1 and Ym1/2 are known to be induced by IL-4 [9]. This would suggest that IL-4 and DXM combination treatment, along with the induction of IL-10, leads to the up-regulation of markers associated with alternatively-activated macrophages, which support tissue healing. In accordance with this observation, mice that had high levels of IL-4 in their serum on day 10 had a high level of IL-10 in their serum.

Unexpectedly, IL-4 levels in serum did not correlate with the total arthritic scores of mice, but to levels of IL-10. On the other hand, arginase did correlate with the total arthritic score of F8-IL-4 + DXM-treated mice. This would suggest that IL-4 has an indirect effect on disease severity, possibly by inducing other anti-inflammatory factors such as arginase in macrophages. This is further supported by the observation that animals with a higher level of arginase expression on day 21 also had a lower arthritic score. In addition, the level of IL-4, in contrary to arginase, decreased once treatment was stopped and it was no longer detected in combination treated mice on day 21.

As combination therapy in comparison to DXM monotherapy led to an increase in the proportion of anti-inflammatory cell types, we hypothesized that these effects could be sustained in the long-term after therapy retraction. As shown previously [23], F8-IL-4 + DXM, but not DXM treated mice, were protected from arthritis relapse post-therapy retraction [23]. The experiment was terminated at day 21 post-disease onset due to the high arthritic scores of DXM monotherapy mice. An examination of the phenotype of cells infiltrating the arthritic joints revealed that combination treated mice still had a significant decrease in the number of T_H17 cells and neutrophils. In addition on day 21, combination treated mice had a higher level of IL-10 and also an anti-inflammatory macrophage gene signature. Although both groups had a similar number of Treg cells at the site of disease, only combination mice were able to maintain a significantly lower ratio between T_H17 and Treg cells.

In conclusion, F8-IL-4 and DXM combination therapy is a durable treatment for arthritis that acts by supporting the generation of Treg cells, and by providing an environment for anti-inflammatory macrophages.

MATERIALS AND METHODS

Induction and assessment of arthritis

Arthritis was induced in DBA/1 mice as described previously [53]. Briefly, bovine type II collagen was purified from articular cartilage and dissolved in 0.1 M acetic acid. Ten to 12-week-old male DBA/1 mice (Harlan UK) received one subcutaneous 100 µL injection of 200 µg bovine type II collagen in complete Freund's adjuvant (BD Biosciences), at the base of the tail and on the flank.

Post-immunization, the mice were monitored daily for signs of arthritis. Once an animal showed signs of arthritis (redness and/or swelling of front or hind paw joints) it was randomly assigned to a treatment group and monitored daily. Arthritis severity was scored as follows: 0 = normal paw, 1 = slight swelling and/or erythema, 2 = pronounced swelling, 3 = ankylosis. All four limbs were scored blindly, giving a maximum possible score of 12 per animal. Hind paw swelling was measured daily with calipers (Krœplin).

Mice were maintained under specific pathogen-free conditions with food and water available *ad libitum*. All procedures were approved by the Clinical Medicine Animal Welfare Ethical Review Board and the UK Home Office in accordance with the Animals (Scientific procedures) Act 1986.

Drugs

The immunocytokine, F8-IL-4 (owned by Philogen Spa) is a fusion protein between murine IL-4 and the single variable chain of the F8 monoclonal antibody, and has been described in more detail elsewhere [23]. Dexamethasone (Sigma-Aldrich) was dissolved in PBS to a concentration of 1 mg/mL and stored at 4°C. Where specified, animals received daily intraperitoneally 100 µL of dexamethasone (100 µg per mouse) or PBS (control). F8-IL-4 treated an-

imals received three doses of 100 µL (100 µg per mouse) of F8-IL-4 on day 1, 4 and 7 post-disease onset. Analgesia was provided in the form of Novalgin (Analgin) (Sanofi-Aventis) added to the drinking water (1.3 g/L). The water bottles were changed twice per week. Mice with long-standing arthritis (longer than 10 days), received additional daily subcutaneous injections of Novalgin (200 µg/kg).

Analysis of immune responses in arthritic mice

The experimental design is depicted in Figure 1A and 4A. All mice with clinical arthritis were treated from day 1 to 10 of arthritis. On day 10 some animals were culled whereas others were kept until day 21 before being culled. In both cases peripheral blood, spleen and paws were taken. A single-cell suspension of joint cells was prepared from paws as follows. After skin removal, each arthritic paw was digested in 1 mL of 0.1 mg/mL DNase I and 0.313 mg/mL Liberase (both from Roche Diagnostics) for 90 minutes. Cells were cultured in RPMI-1640 with L-glutamine, 10% fetal calf serum, and penicillin/streptomycin (all from Life Technologies). To detect cytokine secretion, joint cells were stimulated with an anti-CD3ε antibody (clone 145-2C11, eBioscience). After 48 hours culture supernatants were taken for cytokine quantification.

Flow cytometric analysis

Joint cells were stimulated with 0.02 µg/mL PMA, 0.4 µM ionomycin and 1.25 µg/mL brefeldin A (all from Sigma-Aldrich) for 4 hours. The antibodies used for surface and intracellular stainings of mouse cells, are listed in Supporting Information Table 1. The cells were fixed and permeabilised with the buffers provided with the FoxP3 Staining Buffer Set (eBioscience). Live cells were identified using the Zombie Fixable Viability Kit (BioLegend). Data

was acquired on a CANTO II flow cytometer using FACSDIVA software (all BD Biosciences) and analysed using FlowJo software (Tree Star, Inc.).

Cytokine detection

Cytokines were detected in serum and 48-hour anti-CD3ε stimulated joint cell culture supernatants using the Meso Scale Discovery platform (MSD) according to manufacturer's specifications.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen). cDNA was transcribed using the Reverse Transcription System (Promega). For quantitative PCR, reactions were performed using TaqMan primers and probes (Applied Biosystems). Probes used are listed in Supporting Information Table 2. All procedures were done according to the manufacturer's instructions. The comparative threshold cycle (C_T) method ($\Delta\Delta C_T$) was used for relative quantification of gene expression.

Statistical analysis

Data are presented as the arithmetic mean ± standard error of mean (SEM). Comparisons between two groups were carried out using an unpaired student t-test (two-way). For more than two groups, analysis of variance (ANOVA) was used followed by Tukey's (two-way) or Dunnett's (one-way) *post-hoc* test. Correlations were tested using the Spearman correlation coefficient for non-parametric data and the Pearson correlation coefficient for parametric da-

ta. Probability values (*P*) of less than 0.05 were considered significant. Statistical analysis was performed using Graphpad PRISM (Graphpad Software Inc.).

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ROLE OF THE STUDY SPONSOR

Philochem had no role in the study design or in the collection, analysis, or interpretation of the data, the writing of the manuscript, or the decision to submit the manuscript for publication, except via the authors who were employees of Philochem.

AUTHOR CONTRIBUTION

JK – participated in study design, carried out the experiments, data analysis and interpretation prepared the figures and drafted the manuscript.

ROW – participated in study design, interpretation and edited the manuscript.

TH, FP, MM and DR – participated in data interpretation and edited the manuscript.

All authors read and approved the manuscript.

CONFLICT OF INTEREST

JK and ROW – received a research grant from Philochem Zurich

TH, FP and MM – are Philochem employees

DN – is the co-founder of Philochem

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FIGURE LEGENDS

FIGURE 1. **The delivery of IL-4 to joints of mice with collagen-induced arthritis ameliorates disease severity and reduces pro-inflammatory cytokine levels**

(**A**-**D**) CIA was induced in DBA/1 mice. Once arthritic, the mice received on days 1, 4 and 7 injections of PBS (CTRL) or F8-IL-4. DXM was given daily for 10 days. On day 10 animals were sacrificed and their paw and serum samples taken for further analysis. (**A**) Schema of the experimental approach is shown. (**B**) During the treatment, all four paws of mice with CIA were assessed daily for signs of arthritis and their arthritic scores calculated. Representative photographs of paws on day 10 are shown. *P* values were calculated via two-way ANOVA with Tukey's *post-hoc* test. (**C**) Concentrations of IL-4 and IL-10 in serum samples from CIA mice on day 10 were determined by Meso Scale Discovery (MSD). Correlations between IL-4 and IL-10 levels were tested using the Spearman correlation coefficient for non-parametric data. (**D**) Concentrations of cytokines in 48-hour paw cell culture supernatants from CIA mice on day 10 were determined by MSD. (**C**-**D**) Data are shown as mean ± SEM (n = 16-17 mice/group) and are pooled from two independent experiments. *P* values were calculated via one-way ANOVA with Dunnet's *post-hoc* test *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

FIGURE 2. **F8-IL-4 synergizes with dexamethasone to attenuate inflammation by decreasing T_H17 / Treg cell ratios in arthritic paws**

(**A-F**) On day 10, cells were isolated from arthritic joints of mice with CIA and characterised by flow cytometry. (**A-B**) Absolute numbers of T_H2 ($CD4^+$ $IL\text{-}4^+$) and T_H17 ($CD4^+$ $IL17A^+$) in arthritic paws with representative flow cytometry dot plots are shown. The gating strategy for T cells is shown in Supporting Information Fig. 2. (**C**) Concentrations of IL-17A in 48-hour anti-CD3-stimulated paw culture supernatants from CIA mice on day 10 were determined by MSD. (**D**) Absolute numbers of $IL\text{-}17A^+$ TCRγδ T cells in arthritic paws on day 10. (**E**) The percentage of Treg cells ($CD25^+$ $FoxP3^+$) among $CD4^+$ T cells found in the paws of CIA mice with representative flow cytometry dot plots is shown. (**F**) The ratio between total numbers of T_H17 and Treg cells in paws on day 10 is shown. (**A-F**) Data are shown as mean ± SEM (n = 16-17 mice/group) and are pooled from two independent experiments. *P* values were calculated via one-way ANOVA with Dunnet's *post-hoc* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant.

FIGURE 3. **F8-IL-4 plus DXM treatment induces an anti-inflammatory phenotype in macrophages**

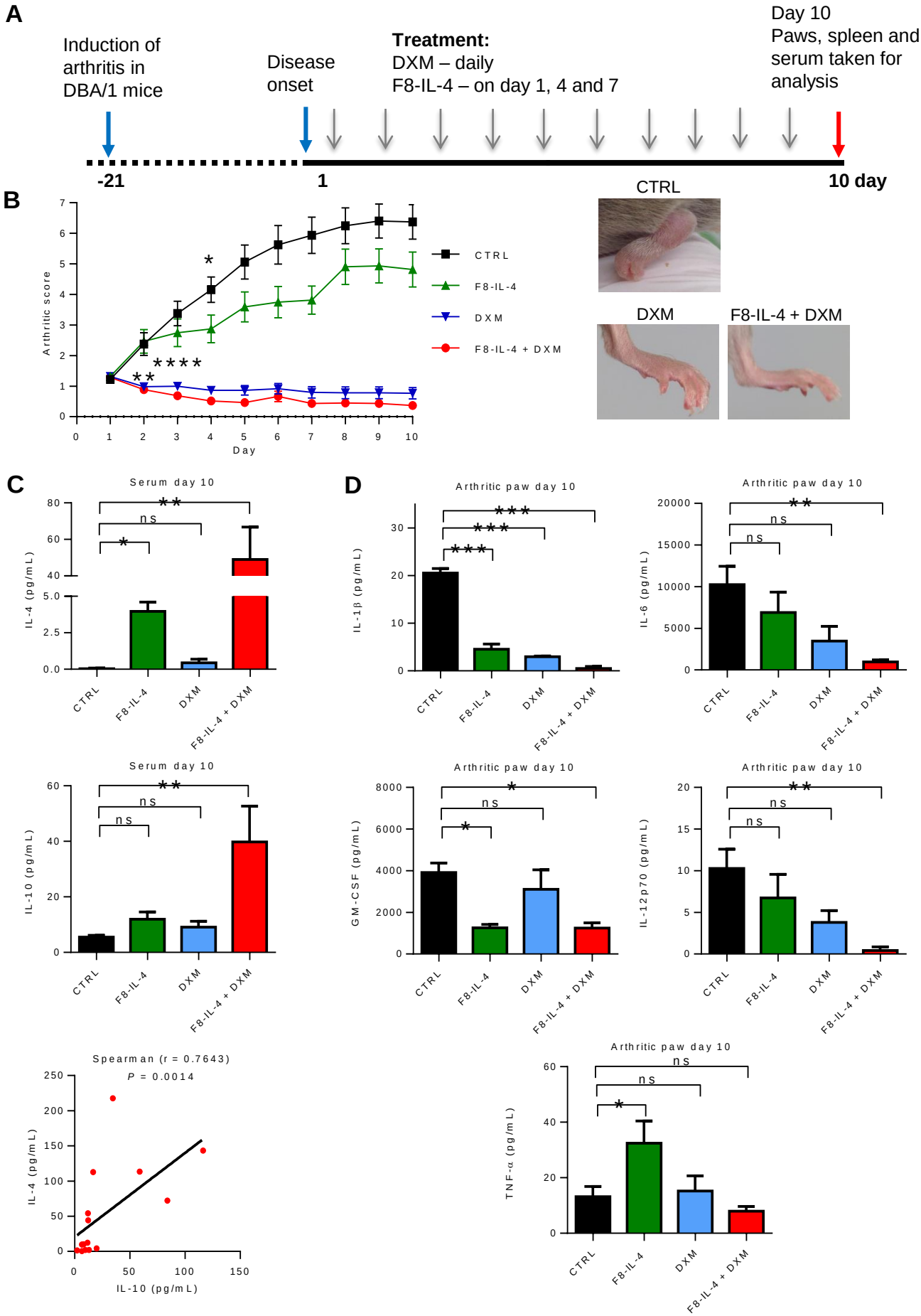
(**A-E**) On day 10 arthritic paws of mice with CIA were counted and characterised by flow cytometry. Spleens were taken for quantitative RT-PCR. (**A**) Shown are total numbers of macrophages found in paws of CIA mice at day 10 (n = 16-17 mice/group). Representative flow cytometry plot is also shown. The whole gating strategy for macrophages is shown in Supporting Information Fig. 4. (**B**) The percentage of $CD206^{+}$ macrophages in arthritic paws was determined by flow cytometry (n = 5 mice/group). (**C**) The percentage of total MHC class II and MHC class II high expressing macrophages in arthritic paws was determined by flow cytometry (n = 5 mice/group). (**D**) CIA day 10 spleen cells were analysed for M1 and M2 macrophage markers using quantitative RT-PCR. Levels of marker expression were determined via quantitative RT-PCR are expressed as the mRNA level normalized to *Gapdh* expression and compared with control mice. (**B-D**) Error bars represent ± SEM (n = 5 mice/group). *P* values were calculated via one-way ANOVA with Dunnet's *post-hoc* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant. (**E**) Correlations between *Arg1* expression and the arthritic score (calculated as the total area under the curve from 10 days) in combination treated mice, were tested using the Pearson correlation coefficient for parametric data.

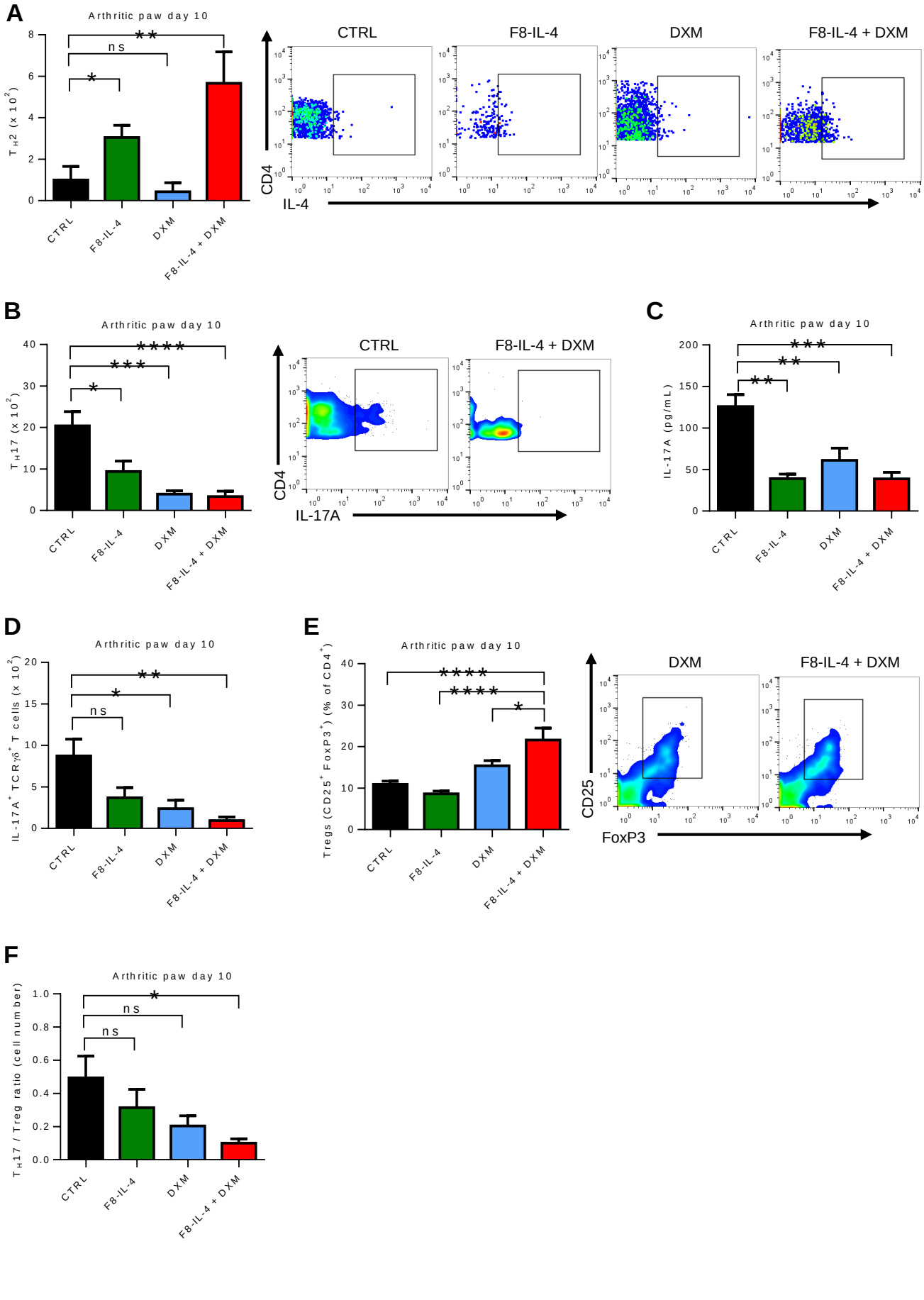
FIGURE 4. **F8-IL-4 plus DXM but not DXM alone provide protection from arthritis re-occurrence by inhibiting IL-17A induction in joints**

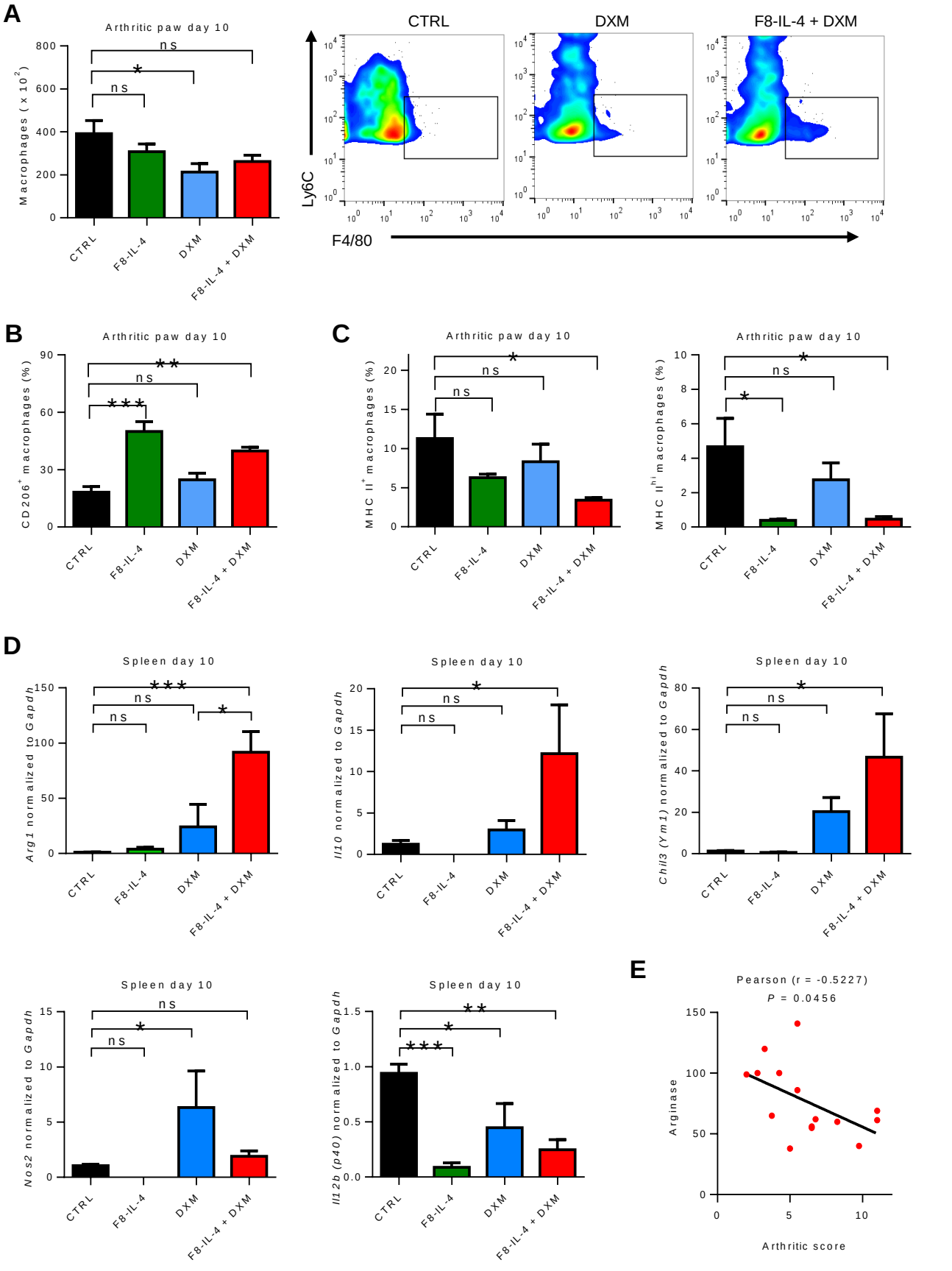
(**A-H**) Arthritic CIA mice were treated with F8-IL-4 + DXM or DXM alone until day 10. On day 10 all therapy was stopped. On day 21 animals were sacrificed and serum and paw samples taken for further analysis. (**A**) A schematic view of the experimental workflow showing the time points of treatment, therapy retraction and animal sacrifice. (**B**) All four paws were assessed daily for signs of arthritis and the arthritic score for each animal was calculated. Representative photographs of paws on day 21 are shown. (**C**) The total number of cells found in arthritic paws on day 21 is shown. (**D**) Absolute numbers of IL-17A$^+$ T_H17 (CD4$^+$ IL17A$^+$) and IL-17A$^+$ TCRγδ T cells in arthritic paws were determined by flow cytometry. Concentrations of IL-17A in 48-hour anti-CD3-stimulated paw cell culture supernatants from CIA mice were determined by MSD. (**E**) Absolute numbers of T_H2 (CD4$^+$ IL-4$^+$) cells in arthritic paws are shown. (**F**) The ratio between total numbers of T_H17 and Treg cells in paws is shown. (**G**) Concentrations of pro-inflammatory cytokines in 48-hour anti-CD3-stimulated paw cell culture supernatants from CIA mice were determined by MSD. (**H**) CIA day 21 serum samples were analysed for IL-10 by MSD. (**B-H**) Error bars represent ± SEM (n = 10 mice/group). *P* values were calculated via unpaired student t-tests.

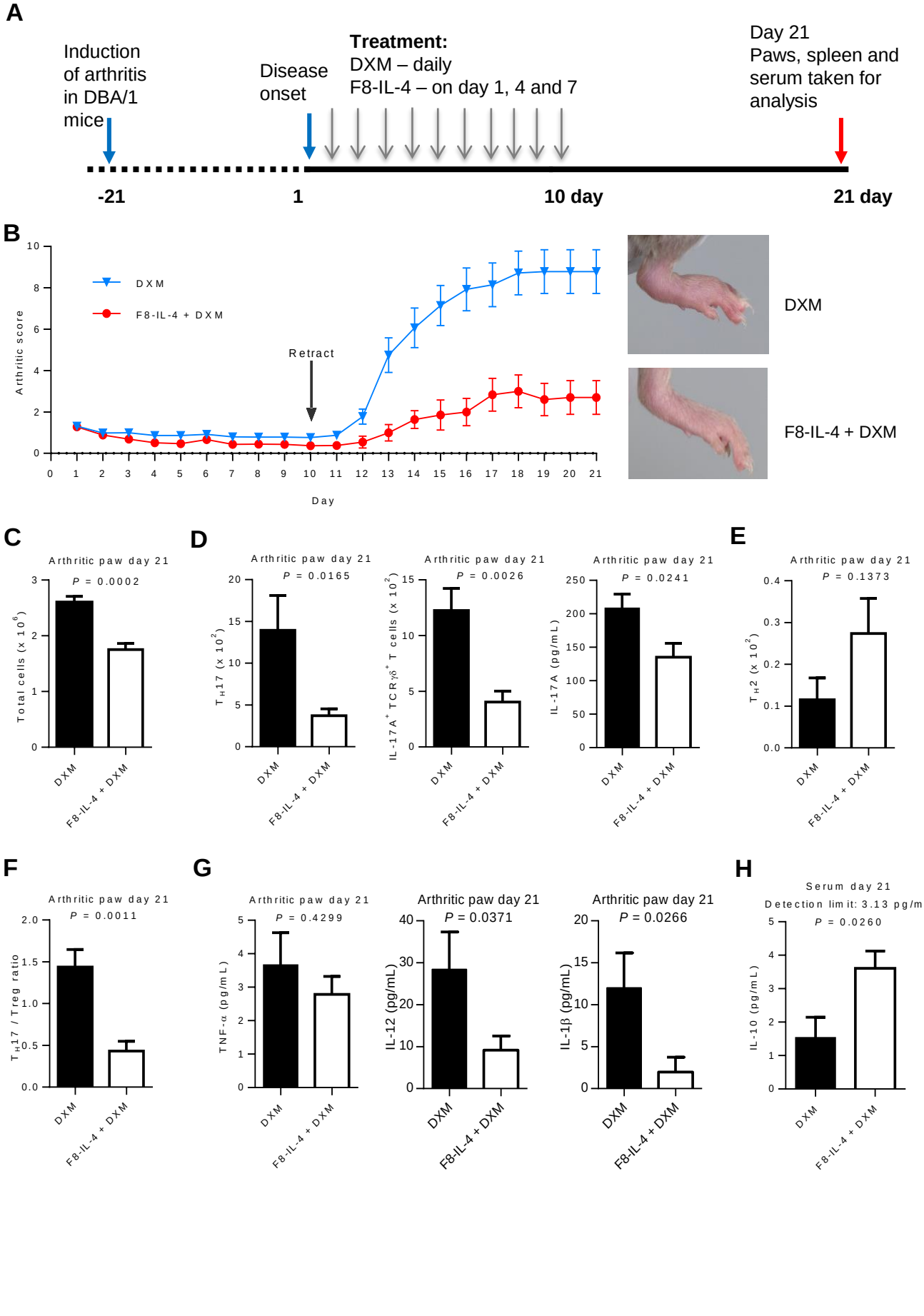
FIGURE 5. **Anti-inflammatory macrophages induced by F8-IL-4 plus DXM are found in CIA mice after therapy retraction**

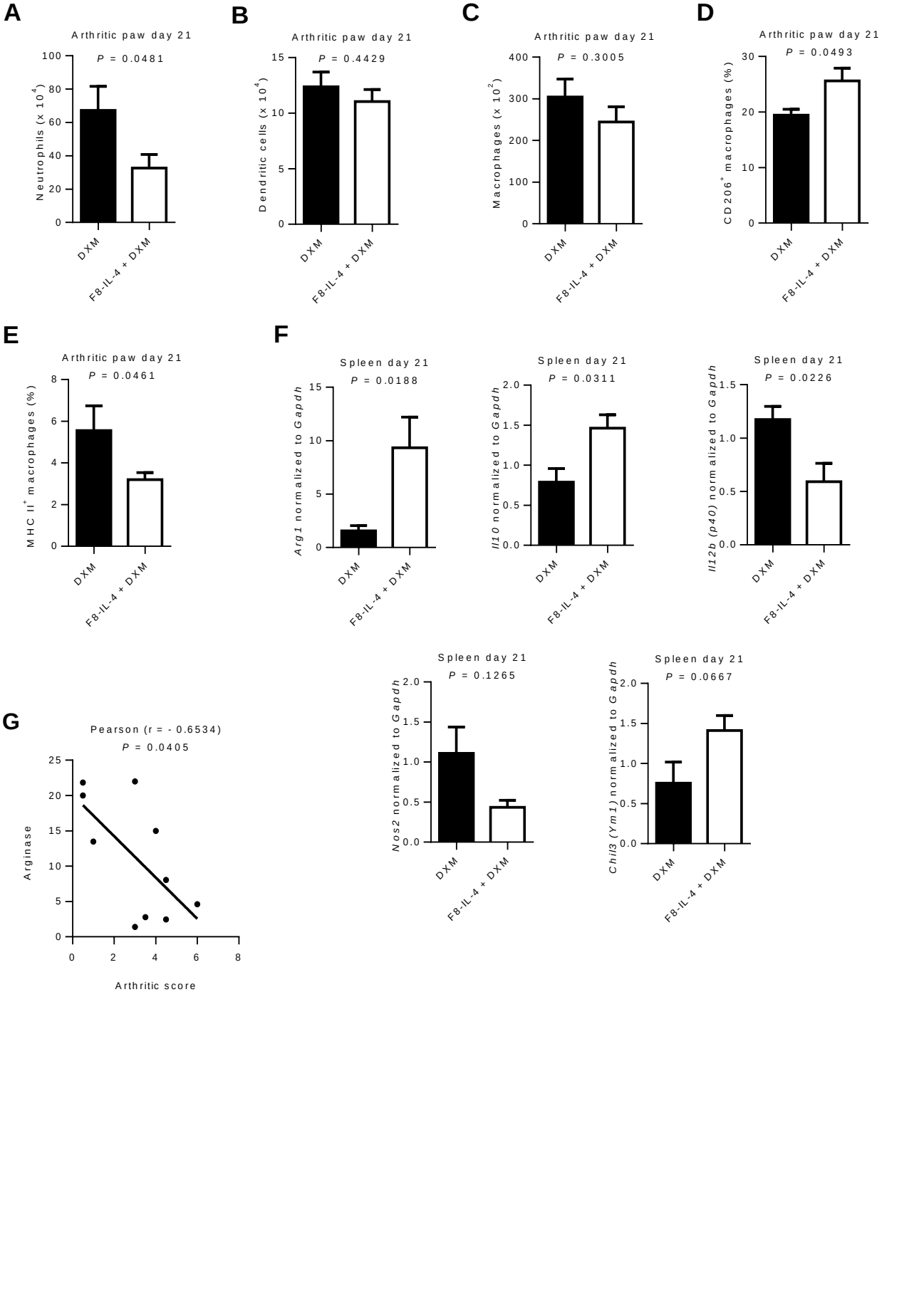
(**A-G**) Arthritic CIA mice were treated with F8-IL-4 + DXM or DXM alone until day 10. On day 10 all therapy was retracted. Spleen and paw samples taken for further analysis on day 21. (**A-B**) Total numbers of dendritic cells and neutrophils found in paws of CIA mice at day 21. **(C**) Shown are total numbers of macrophages found in paws of CIA mice at day 21. (**D**) The percentage of $CD206^+$ macrophages in arthritic paws was determined by flow cytometry. (**E**) The percentage of MHC class II^+ macrophages in arthritic paws was determined by flow cytometry. (**F**) CIA day 21 arthritic spleen cells were analysed for M1 and M2 macrophage markers with quantitative RT-PCR. Levels of mRNA determined via quantitative RT-PCR are expressed as the mRNA level normalized to *Gapdh* expression and compared with DXM treated mice. (**A-F**) Error bars represent ± SEM (n = 10 mice/group). *P* values were calculated via unpaired student t-tests. (**G**) Correlations between *Arg1* expression and the arthritic score on day 21 in combination treated mice were tested using the Pearson correlation coefficient for parametric data.


A
Induction of arthritis in DBA/1 mice
Disease onset
Treatment:
DXM – daily
F8-IL-4 – on day 1, 4 and 7
Day 10
Paws, spleen and serum taken for analysis
-21
1
10 day
B
Arthritic score
Day
CTRL
F8-IL-4
DXM
F8-IL-4 + DXM
C
Serum day 10
IL-4 (pg/mL)
IL-10 (pg/mL)
Spearman (r = 0.7643)
P = 0.0014
D
Arthritic paw day 10
IL-1β (pg/mL)
IL-6 (pg/mL)
GM-CSF (pg/mL)
IL-12p70 (pg/mL)
TNF-α (pg/mL)
ns


A
Arthritic paw day 10
T_H2 (x 10^2)
ns
*
**
CTRL
F8-IL-4
DXM
F8-IL-4 + DXM
CD4
IL-4
B
Arthritic paw day 10
T_H17 (x 10^2)
*
***
****
IL-17A
C
Arthritic paw day 10
IL-17A (pg/mL)
**
***
D
Arthritic paw day 10
IL-17A$^+$ TCR$\gamma\delta^+$ T cells (x 10^2)
E
Arthritic paw day 10
Tregs (CD25$^+$ FoxP3$^+$) (% of CD4$^+$)
CD25
FoxP3
F
Arthritic paw day 10
T_H17 / Treg ratio (cell number)

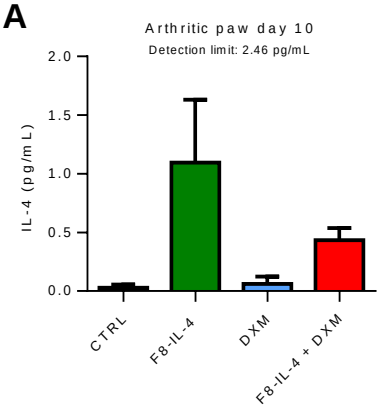

A
Arthritic paw day 10
Macrophages (x 10²)
800
600
400
200
0
ns
*
ns
CTRL
F8-IL-4
DXM
F8-IL-4 + DXM
CTRL
DXM
F8-IL-4 + DXM
Ly6C
F4/80
B
Arthritic paw day 10
CD206⁺ macrophages (%)
90
60
30
0
**
ns
***
C
Arthritic paw day 10
MHC II⁺ macrophages (%)
20
15
10
5
0
*
ns
ns
Arthritic paw day 10
MHC II^hi macrophages (%)
10
8
6
4
2
0
*
ns
*
D
Spleen day 10
Arg1 normalized to Gapdh
150
100
50
0
***
ns
ns
*
Spleen day 10
Il10 normalized to Gapdh
20
15
10
5
0
*
ns
ns
Spleen day 10
Chil3 (Ym1) normalized to Gapdh
80
60
40
20
0
*
ns
ns
Spleen day 10
Nos2 normalized to Gapdh
15
10
5
0
ns
*
ns
Spleen day 10
Il12b (p40) normalized to Gapdh
1.5
1.0
0.5
0.0
**
*
***
CTRL
F8-IL-4
DXM
F8-IL-4 + DXM
E
Pearson (r = -0.5227)
P = 0.0456
Arginase
150
100
50
0
0
5
10
Arthritic score

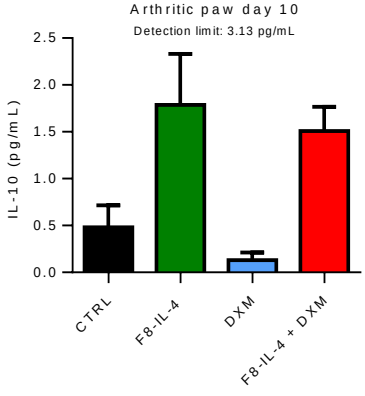

A
Induction of arthritis in DBA/1 mice
Disease onset
Treatment:
DXM – daily
F8-IL-4 – on day 1, 4 and 7
Day 21
Paws, spleen and serum taken for analysis
-21
1
10 day
21 day
B
DXM
F8-IL-4 + DXM
Retract
Arthritic score
Day
DXM
F8-IL-4 + DXM
C
Arthritic paw day 21
P = 0.0002
Total cells (x 10^6)
D
Arthritic paw day 21
P = 0.0165
TH17 (x 10^2)
Arthritic paw day 21
P = 0.0026
IL-17A+ TCRγδ+ T cells (x 10^2)
Arthritic paw day 21
P = 0.0241
IL-17A (pg/mL)
E
Arthritic paw day 21
P = 0.1373
TH2 (x 10^2)
F
Arthritic paw day 21
P = 0.0011
TH17 / Treg ratio
G
Arthritic paw day 21
P = 0.4299
TNF-α (pg/mL)
Arthritic paw day 21
P = 0.0371
IL-12 (pg/mL)
Arthritic paw day 21
P = 0.0266
IL-1β (pg/mL)
H
Serum day 21
Detection limit: 3.13 pg/mL
P = 0.0260
IL-10 (pg/mL)
DXM
F8-IL-4 + DXM


A
Arthritic paw day 21
P = 0.0481
Neutrophils (x 10^4)
100
80
60
40
20
0
DXM
F8-IL-4 + DXM
B
Arthritic paw day 21
P = 0.4429
Dendritic cells (x 10^4)
15
10
5
0
DXM
F8-IL-4 + DXM
C
Arthritic paw day 21
P = 0.3005
Macrophages (x 10^2)
400
300
200
100
0
DXM
F8-IL-4 + DXM
D
Arthritic paw day 21
P = 0.0493
CD206+ macrophages (%)
30
20
10
0
DXM
F8-IL-4 + DXM
E
Arthritic paw day 21
P = 0.0461
MHC II+ macrophages (%)
8
6
4
2
0
DXM
F8-IL-4 + DXM
F
Spleen day 21
P = 0.0188
Arg1 normalized to Gapdh
15
10
5
0
DXM
F8-IL-4 + DXM
Spleen day 21
P = 0.0311
Il10 normalized to Gapdh
2.0
1.5
1.0
0.5
0.0
DXM
F8-IL-4 + DXM
Spleen day 21
P = 0.0226
Il12b (p40) normalized to Gapdh
1.5
1.0
0.5
0.0
DXM
F8-IL-4 + DXM
Spleen day 21
P = 0.1265
Nos2 normalized to Gapdh
2.0
1.5
1.0
0.5
0.0
DXM
F8-IL-4 + DXM
Spleen day 21
P = 0.0667
Chil3 (Ym1) normalized to Gapdh
2.0
1.5
1.0
0.5
0.0
DXM
F8-IL-4 + DXM
G
Pearson (r = - 0.6534)
P = 0.0405
Arginase
25
20
15
10
5
0
0
2
4
6
8
Arthritic score

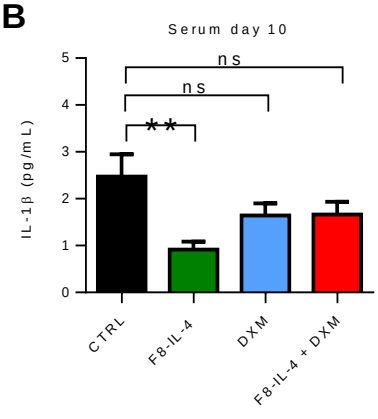
SUPPLEMENTARY FIGURE 1

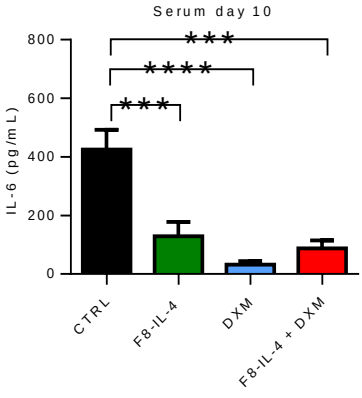
A

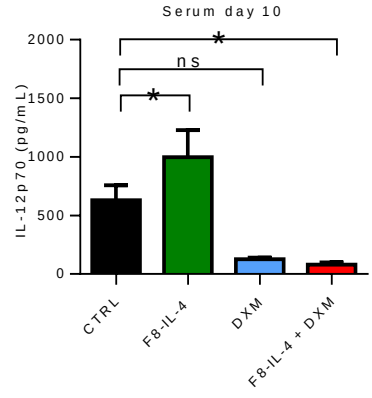


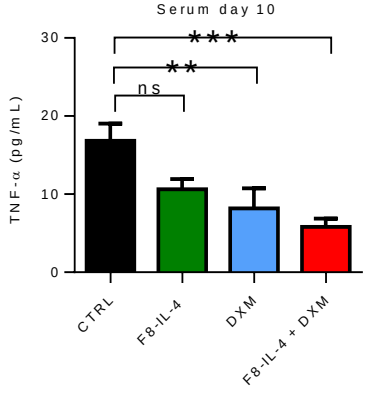


B

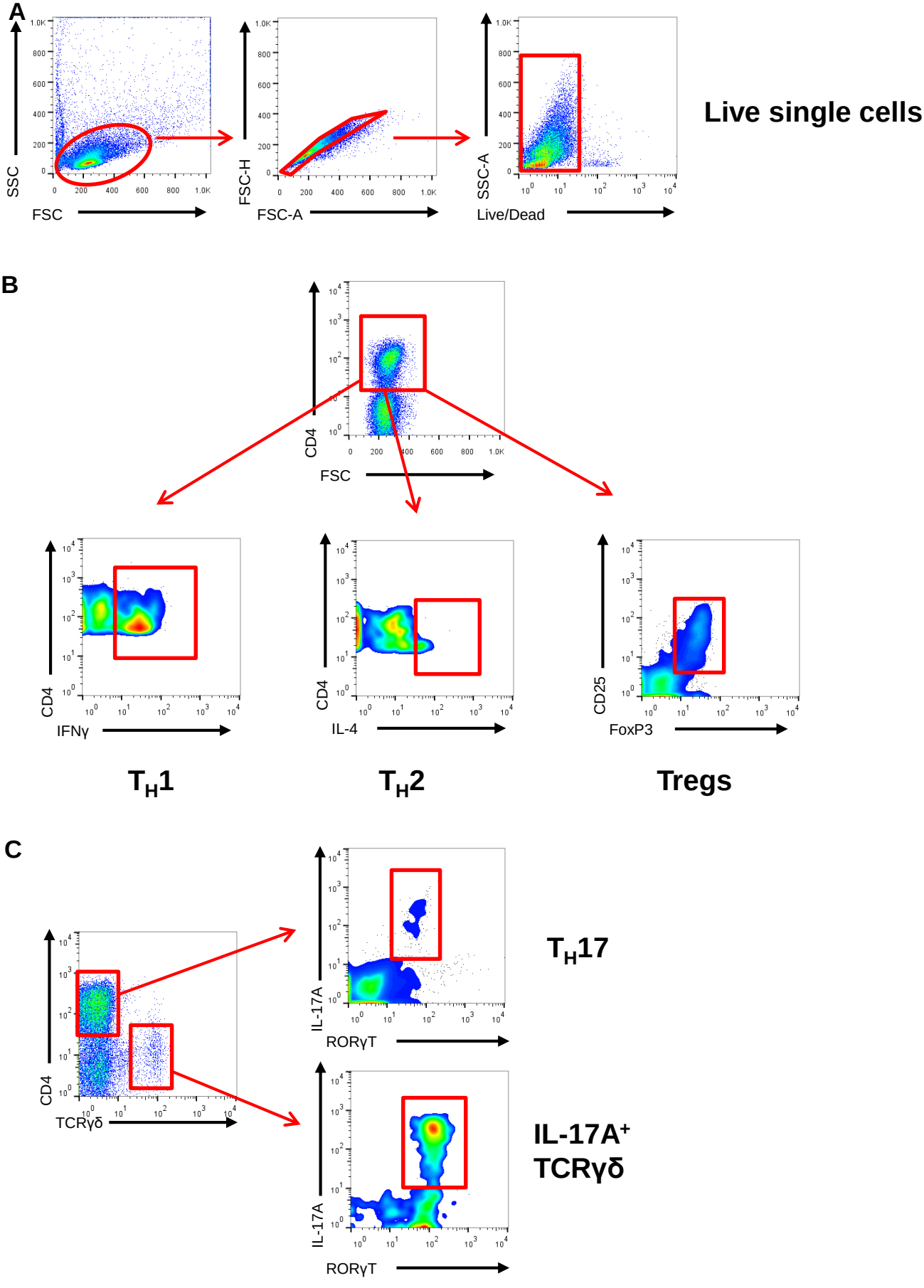






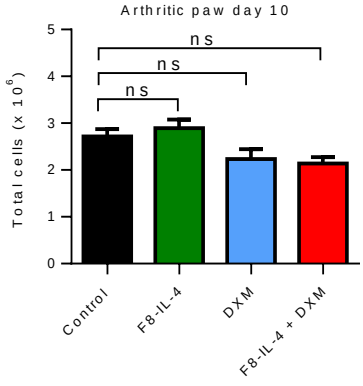


SUPPLEMENTARY FIGURE 2



SUPPLEMENTARY FIGURE 3

A



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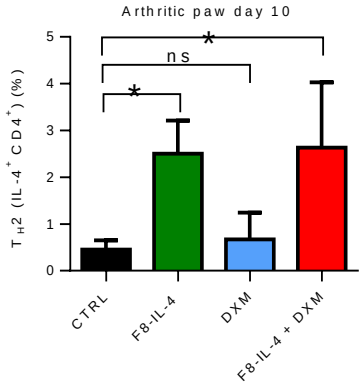

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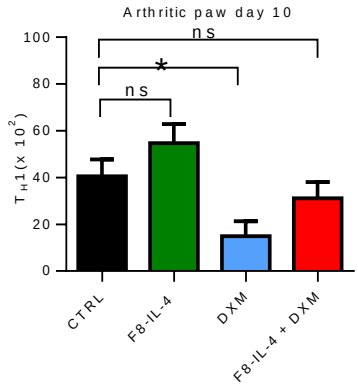
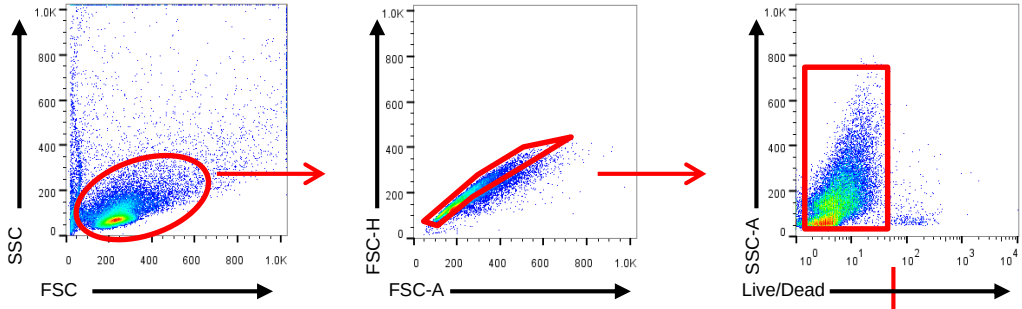
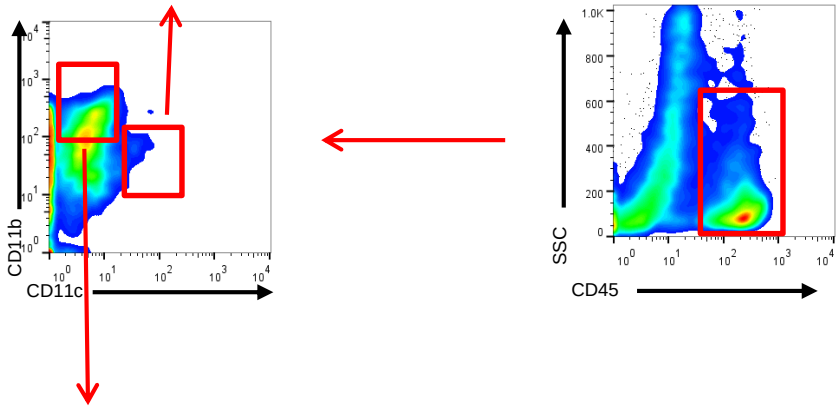
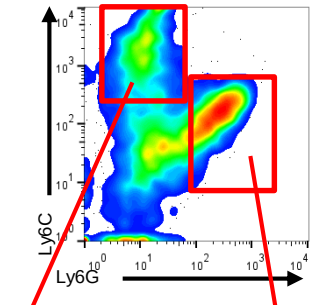


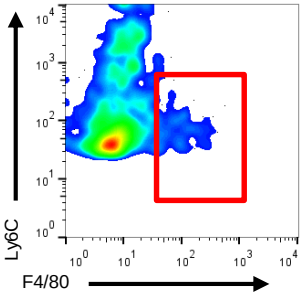
SUPPLEMENTARY FIGURE 4



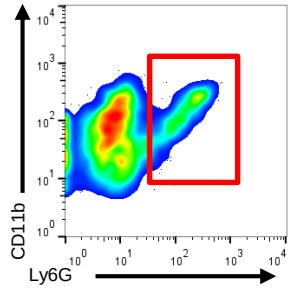
Dendritic Cells







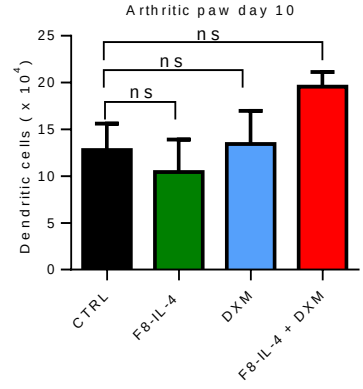
Macrophages



Neutrophils

SUPPLEMENTARY FIGURE 5

A



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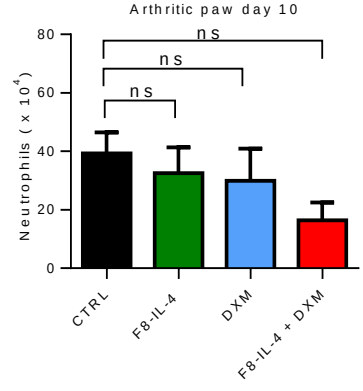

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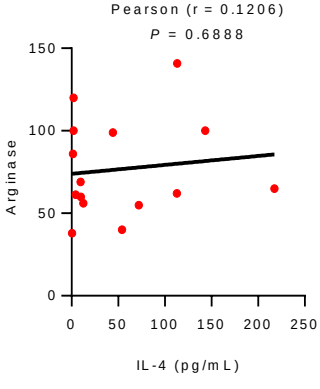
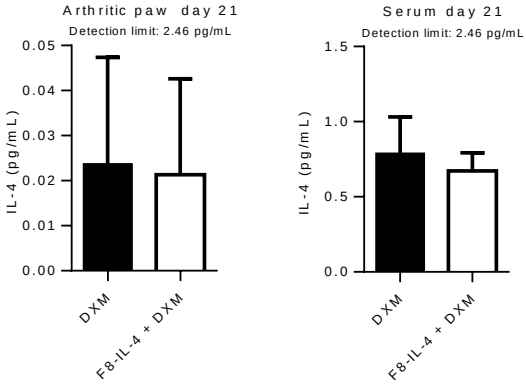


SUPPLEMENTARY FIGURE 6

A



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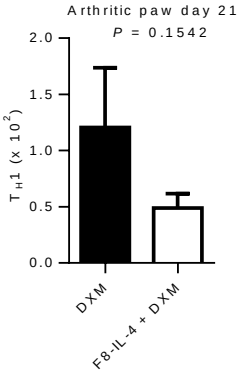

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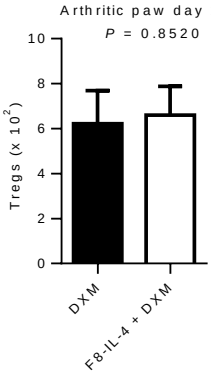

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