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An *in vitro* model for osteoarthritis using long-cultured inflammatory human macrophages repeatedly stimulated with TLR agonists

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Osteoarthritis (OA) is characterized by an abundance of inflammatory M1-like macrophages damaging local tissues. The search for new potential drugs for OA suffers from the lack of appropriate methods of long-lasting inflammation. Here we developed and characterized an *in vitro* protocol of long-lasting culture of primary human monocyte-derived macrophages differentiated with a combination of M-CSF+GM-CSF that optimally supported long-cultured macrophages (LC-Mϕs) for up to 15 days, unlike their single use. Macrophages repeatedly stimulated for 15 days with the TLR2 ligand Pam3CSK4 (LCS-Mϕs), showed sustained levels over time of IL-6, CCL2, and CXCL8, inflammatory mediators that were also detected in the synovial fluids of OA patients. Furthermore, macrophages isolated from the synovia of two OA patients showed an expression profile of inflammation-related genes similar to that of LCS-Mϕs, validating our protocol as a model of chronically activated inflammatory macrophages. Next, to confirm that these LCS-Mϕs could be modulated by anti-inflammatory compounds, we employed dexamethasone and/or celecoxib, two drugs widely used in OA treatment, that significantly inhibited the production of inflammatory mediators. This easy-to-use *in vitro* protocol of long-lasting inflammation with primary human macrophages could be useful for the screening of new compounds to improve the therapy of inflammatory disorders.

Keywords: *In vitro* model · Inflammation · Macrophages · Osteoarthritis · TLR ligands

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

Among numerous chronic inflammatory diseases, osteoarthritis (OA) is nowadays the most widely diffused age-related medical issue for which definitive therapies are not yet available [1–5]. The pathophysiology of OA involves a complex network of altered cell types, changes in molecular pathways, and damaged mechanical structures (i.e. bone or extracellular matrix). Among the cellular components present in the microenvironment of the diseased joint, macrophages play a key role in orchestrating the inflammatory response. It has been demonstrated that macrophages are extremely plastic immune cells [6], with the ability to acquire different immunomodulatory behaviors depending on the surrounding microenvironment and to strongly influence other cell types in the joint [7]. In healthy conditions, tissue-resident synovial macrophages protect the joint from inflammation. However, during OA, synovial macrophages acquire an M1-like proinflammatory phenotype and are detrimental to the joint's structure [8]. This knowledge is in line with additional reports showing how the continuous recruitment of monocytes (precursors of macrophages) toward the joint exacerbates inflammation and disease severity [9]. OA-associated M1-like macrophages trigger inflammation and tissue damage by producing a wide range of proinflammatory cytokines such as IL-1β, TNF-α, IL-6, as well as chemokines able to recruit other immune cells, such as CCL2 (monocytes) or CXCL8 (neutrophils) [9–11]. Extensive studies demonstrated the activation of specific molecular pathways in M1 macrophages, such as those downstream to the toll-like receptor 4 (TLR4) receptor (through MyD88 to activate NF-κB), as well as other TLRs [12] or interferon regulatory factors and transcription factors of the STAT family (while STAT1 and STAT5 have been involved in the M1 polarization, the family members STAT4 and STAT6 were observed in the M2-like phenotypes) [13]. This state of chronic inflammation affects the synovia, bone osteoclasts, and chondrocytes, eventually resulting in bone reabsorption and remodeling [14], thus worsening OA conditions.

A main limitation to the study of macrophages in OA is related to the availability of appropriate *in vitro* and *in vivo* models, reliably mimicking the inflamed joints observed in the clinic. While *in vivo* models in mice, rabbits, or sheep, have been used to study the disease or for the pre-clinical testing of therapeutic strategies [7, 15], *in vitro* assays are also needed for the analysis of molecular signaling pathways and drug screening [16]. The literature reports numerous *in vitro* systems for OA [17], some including macrophages [18–28] (Supporting information Table), but with some limitations. First, some of them rely on the use of myeloid leukemia cell lines, such as THP-1 [18, 20], J774 [19], RAW 264.7 [22, 23] or U937 [24], which present mutations in molecular signaling pathways and a high rate of continuous proliferation; these features may affect their immunotoxicity behavior, thus becoming a less reliable model. Second, when murine [21] or human [25–28] primary cells were employed, the experimental time points were rather short (up to 2–7 days). Ideally, an *in vitro* culture system should reproduce the chronic stimulation of macrophages, thus modeling the pathological environment of the OA joint. Here we have set up and optimized a new *in vitro* model of long-lasting inflammation using primary human monocyte-derived macrophages cultured with a combination of M-CSF and GM-CSF that maximized macrophage survival up to 15 days (M-/GM-Mϕ or long-cultured macrophages, LC-Mϕ), and repeatedly stimulated with either LPS+IFN-γ or Pam3CysSerLys4 (Pam3CSK4). These long-cultured stimulated macrophages (LCS-Mϕs) produced sustained levels of IL-6, CXCL8, and CCL2 over time and could be successfully down-modulated by anti-inflammatory drugs such as dexamethasone (DEX) and celecoxib (CEL), two compounds commonly used in the clinic for the treatment of OA [29]. On the whole, our *in vitro* model with LCS-Mϕs could be useful for the screening of new anti-inflammatory approaches.

Results

Inflammatory mediators in the synovial fluid from OA patients and peripheral blood of healthy donors

We first detected the presence of several inflammatory mediators in synovial fluid (SF) samples obtained from 11 patients with OA, to get an insight into the inflammatory markers to be tested in our *in vitro* macrophage culture model. To measure low cytokine concentrations commonly found in biological fluids, we used a novel multiplex ELISA method (Ella Automated Immunoassay System®) with a high sensitivity [30]. Quantification of panel of 8 inflammatory mediators by Ella® showed detectable levels for all the cytokines and chemokines tested (Fig. 1). Comparison with the peripheral blood of a cohort of 20 healthy donors indicated that IL-6 and the chemokines CCL2 and CXCL10 were more represented in the SFs of patients with OA (Fig. 1A–C), while an opposite trend was observed for TNF-α and IFN-γ (Fig. 1D, E). No significant changes were observed for IL-1β, CXCL8, or CCL5 (Fig. 1F, H). Overall, these results guided us in the selection of the inflammatory markers to test in the *in vitro* cultured macrophages.

Monocyte treatment with M-CSF or GM-CSF and TLR agonists to obtain proinflammatory macrophages

In preliminary experiments, we sought to determine the best combination of growth/differentiation factors and proinflammatory stimuli to obtain M1-like inflammatory macrophages *in vitro*. Human Monocyte-Derived Macrophages (HMDMs) were differentiated for 5 days, either with hrM-CSF (M-Mϕs), to obtain M0 non-polarized macrophages, or with hrGM-CSF (GM-Mϕs) to precondition macrophages toward a proinflammatory phenotype [30–32]. Fully differentiated macrophages were stimulated for 24 h with LPS, engaging TLR4, in combination with IFN-γ, to obtain classical M1 macrophages, or with Pam3CSK4, binding to TLR2. TLR2 is considered the main receptor involved in the recognition of damage-associated molecular patterns, such as fragments

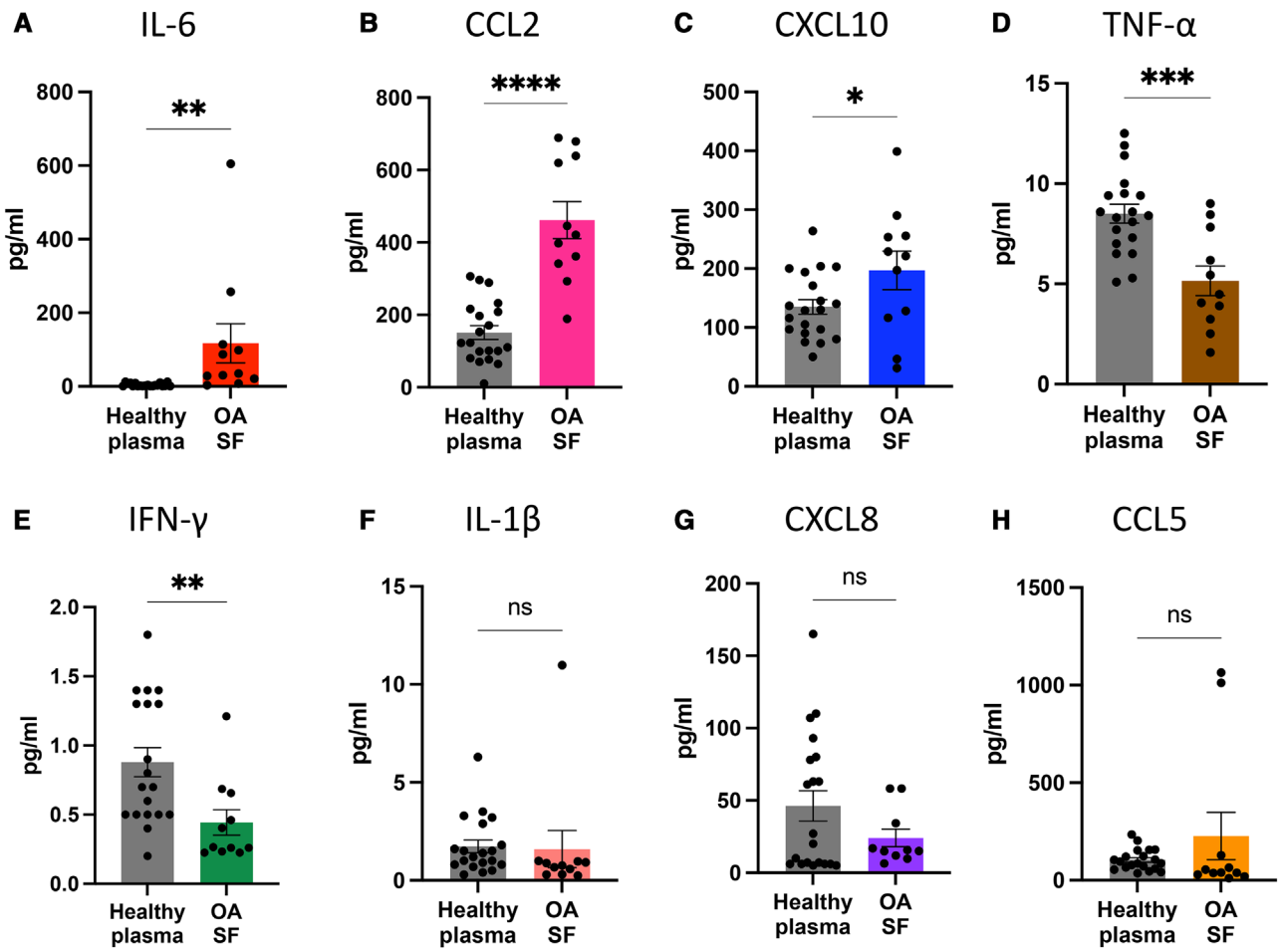

Figure 1. Comparison of inflammatory mediators measured in the synovial fluids collected from knee joints of patients with osteoarthritis. The synovial fluids (SF) from 11 OA patients were tested with the Ella Automated Immunoassay System® to measure the concentrations of (A) IL-6, (B) CCL2, (C) CXCL10, (D) TNF-α, (E) IFN-γ, (F) IL-1β, (G) CXCL8, (H) CCL5. Plasma from healthy donors (n = 20) was also tested. Dots represent the value measured in each single donor. Bars represent mean ± s.e.m. Statistical comparisons were performed using unpaired t-test. Statistically significant differences are represented as follows: $^{*}p$ <0.05, $^{**}p$ < 0.01, $^{***}p$ < 0.001, and $^{****}p$ < 0.001.

of matrix molecules degraded upon tissue damage, a condition found in joints affected by OA [33].

Exposure of macrophages to different CSFs plus different proinflammatory stimuli resulted in their polarization toward a heterogeneous phenotype, reflected by their ability to produce different amounts of cytokines (Fig. 2A–H). In particular, though IL-6 production appears in response to all tested conditions, the stimulation by GM-Mϕs with LPS+IFN-γ led to a 5-fold increase in the production of IL-6 compared with the other conditions (Fig. 2B). Macrophages differentiated with GM-CSF also secreted more IL-1β compared to M-CSF-differentiated cells, and less IL-10, confirming the notion that GM-CSF promotes proinflammatory macrophages (Fig. 2C, D). In macrophages stimulated with the TLR2 agonist Pam3CSK4, CXCL8 levels were higher compared with macrophages stimulated with LPS+IFN-γ (Fig. 2E). Similarly, CCL2 was much higher after Pam3CSK4 stimulation but only in M-Mϕs (Fig. 2F). Conversely, the concentration of CXCL10, the production of which is known to be induced by IFN-γ, was coherently increased with LPS+IFN-γ but not with Pam3CSK4 in M-Mϕs, although detectable levels were produced in GM-Mϕs (Fig. 2G). Finally, the primary proinflammatory cytokine TNF-α presented higher levels after LPS+IFN-γ stimulation (Fig. 2H).

On the whole, GM-CSF induced a differentiation in macrophages that were more prone to inflammation, and TLR2 engagement by Pam3CSK4 stimulated higher secretion of inflammatory mediators, especially CXCL8 and CCL2. In parallel, we exposed macrophages to a pool of synovial fluids from 7 patients (used as an endogenous source of proinflammatory stimuli) or a cocktail of primary inflammatory cytokines (TNF-α + IL-1β + IFN-γ). As shown in Supporting information Fig. S1A and B, the secreted levels of IL-6 and IL-10 were minimal, indicating that macrophages were not sufficiently stimulated. These results clearly demonstrated that to optimize an *in vitro* assay of activated proinflammatory macrophages the use of TLR-agonists is necessary.

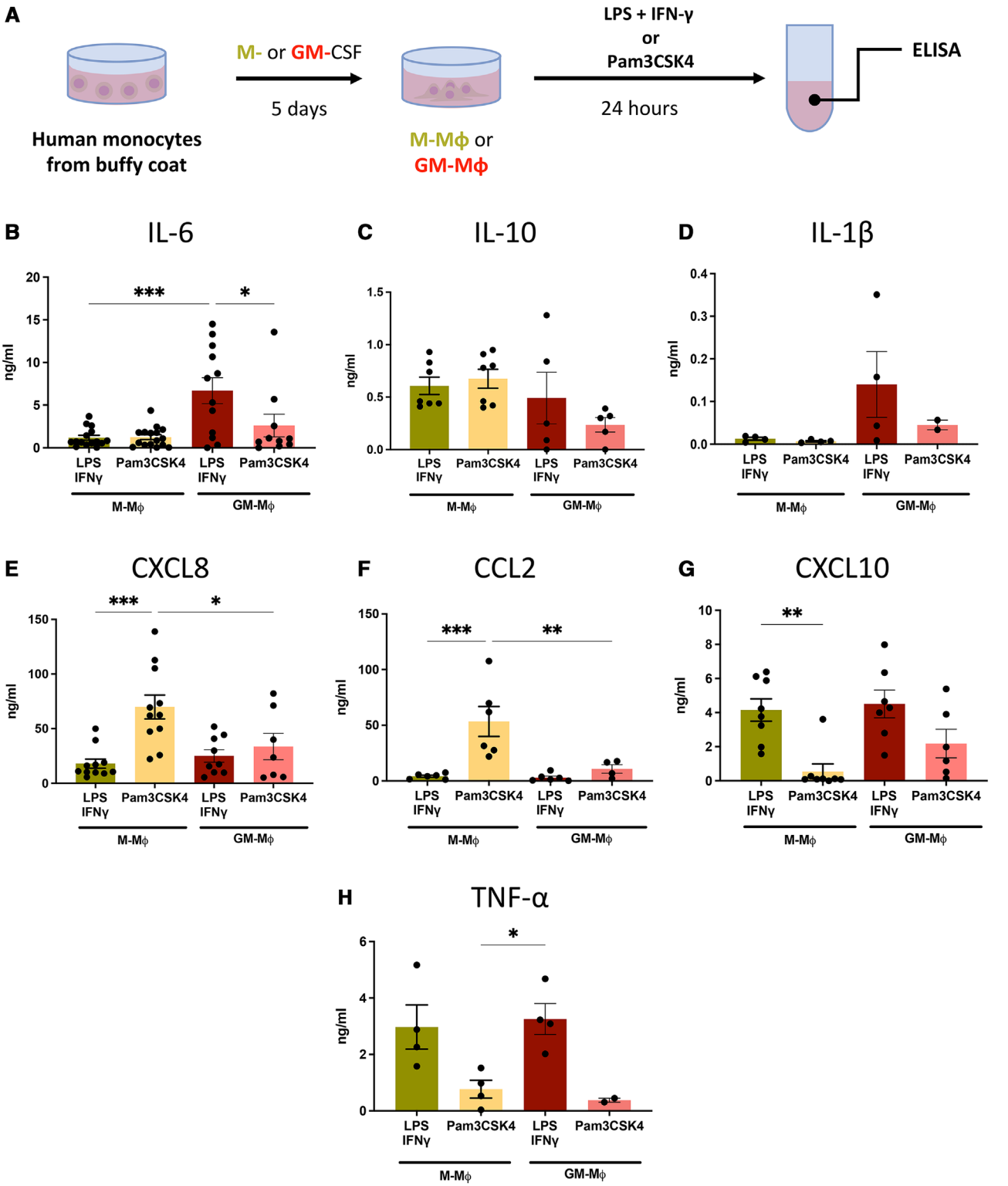

Figure 2. Proinflammatory activity of macrophages activated with different TLR ligands in the short-term culture assay. (A) Experimental protocol. Purified human monocytes were cultured for 5 days with either M-CSF or GM-CSF (both used at 25 ng/mL) and stimulated with LPS 100 ng/mL + IFN-γ 50 ng/mL or Pam3CSK4 100 ng/mL. Quantification of cytokines secreted after 24 h: (B) IL-6, (C) IL-10, (D) IL-1β, (E) CXCL8, (F) CCL2, (G) CXCL10, and (H) TNF-α. Bars represent mean ± s.e.m; N = up to 15 per group (mean of seven donors). Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison tests. Statistically significant differences are represented as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.001$. Unstimulated macrophages were always assayed for each donor showing nondetectable levels of the cytokines.

Concurrent use of M-CSF and GM-CSF increases survival of macrophages *in vitro* in a long-term assay

The above experiments were performed with monocytes differentiated for a short period of 5 days and exposed to stimuli only once. To better mimic *in vitro* the conditions found in arthritic joints, we set up a longer assay (15 days) with repeated exposure to stimuli. We therefore set up a model using hrM-CSF in combination with repeated exposure to hrGM-CSF. Monocytes were first primed with M-CSF (from day 0 to day 5), next with GM-CSF (from day 2 to day 5), and then stimulated with GM-CSF every 2–3 days (Fig. 3A, M-/GM-Mϕ or long-cultured macrophages, LC-Mϕ); we compared this protocol with the stimulation with single CSFs (Fig. 3A, M-Mϕ or GM-Mϕ). We first checked macrophage viability, as M-CSF or GM-CSF-macrophages do not usually survive *in vitro* for more than 8–9 days. Using the Alamar Blue viability assay (Fig. 3B) and bright-field microscopy examination (Fig. 3C) we observed that most M-Mϕs or GM-Mϕs do not survive up to 15 days. Conversely, LC-Mϕ presented the highest viability at day 15, compared with cells differentiated with single CSFs, independently from the set of proinflammatory stimuli used (LPS+IFN-γ or Pam3CSK4). Thus, the culture of LC-Mϕs for up to 15 days was used for the subsequent long-lasting experiments.

LC-Mϕs retain the long-lasting ability to produce proinflammatory cytokines

Next, we explored whether these LC-Mϕ macrophages maintained the ability to produce inflammatory cytokines after repeated exposure either to the cocktail of recombinant cytokines (IL-1β, IFN-γ, TNF-α) or to LPS+IFN-γ or to Pam3CSK4, collecting supernatants at different timepoints (days 6, 8, 10, 13, 15). As for the short assay, we found that repeated stimulation with IL-1β, IFN-γ, and TNF-α did not sufficiently activate macrophages to secrete inflammatory mediators (Supporting information Fig. S2), while the use of TLR ligands led to a prolonged inflammatory activity up to day 15. In particular, LC-Mϕs repeatedly exposed to Pam3CSK4 showed a sustained production overtime of IL-6 and CXCL8, with no significant decrease at any time point except on day 8, while the stimulation with LPS+IFNγ led to a sustained production of CXCL8, but not IL-6 (Fig. 3D, E). On the contrary, the chemokine CCL2 was the only one to remain stable over time in LC-Mϕs stimulated both with LPS+IFN-γ or Pam3CSK4 (Fig. 3F). We also compared the production of IL-10 and TNF-α in LC-Mϕs stimulated either with LPS+IFN-γ or with Pam3CSK4. The anti-inflammatory cytokine IL-10 gradually decreased in LC-Mϕs stimulated with LPS+IFN-γ, while in those stimulated with Pam3CSK4, the production was partially restored after a significant drop on days 8 and 10 (Supporting information Fig. S3A). Surprisingly, with both stimuli, TNF-α was highly secreted on day 6, then not detectable anymore on day 8, but a recovery was registered on days 10 and 13 before a final drop on day 15 (Supporting information Fig. S3B). The decrease of some inflammatory mediators on day 8 is likely to be explained by the long-recognized phenomenon of "endotoxin tolerance". It is known that pre-activated monocytes/macrophages show hyposensitivity to secondary stimulation due to the inactivity of NF-kB (caused by high levels of inhibitory p50 homodimers) and lower production of proinflammatory cytokines [34, 35]. In the subsequent stimulations, however, macrophages recovered their ability to produce inflammatory mediators, and Pam3CSK4 performed significantly better than LPS+IFN-γ at most time points tested, leading us to choose this condition (denominated as LCS-Mϕs) for the next experiments.

LCS-Mϕs present a similar phenotype to macrophages isolated from the knee joint of OA patients

To understand the reliability of our long-lasting *in vitro* model, mimicking the pathological condition of chronic inflammation, we isolated macrophages from the synovial tissue of two OA patients (OA-Mϕs) using CD14+ beads and characterized their inflammatory profile in comparison to our model (LCS-Mϕs) both at the level of secreted cytokines and RNA expression (Fig. 4A). As observed for our LCS-Mϕs, IL-6 was secreted by freshly isolated synovial macrophages (OA-Mϕs) and higher amounts were detected following their ex vivo stimulation Pam3CSK4 for 24 h (OAS-Mϕs; Fig. 4B). Synovial macrophages also produced considerable levels of CXCL8 and CCL2, though to a lower extent than our LCS-Mϕs (Fig. 4B–D). IL-10 and TNF-α were very low or barely detected in the supernatant of macrophages from OA patients, as in LCS-Mϕs (Supporting information Fig. S3C and D). In addition, to further increase the characterization of our *in vitro* model, we screened publicly available datasets of gene expression comparing the synovial tissue of healthy patients versus OA patients and we selected a panel of genes to test in our samples, as they top-ranked in most of the dataset explored (Table 1). In particular, we assayed *ALOX5* (involved in the biology of leukotrienes), *C5AR1* (receptor for the anaphylatoxin C5a), *COX2* (enzyme for PGE2 production), *PFKFB3* (a glycolytic enzyme upregulated in inflammation), *TYROBP* (activating proinflammatory signaling of different immune cells), *FCGR2B* and (receptor for the Fc fragment of IgG involved in phagocytosis), the inflammatory genes *IL6*, *CXCL8* and *CCL2*. All these genes expressed in synovial macrophages from OA patients were also similarly expressed in LCS-Mϕs (Fig. 4E–M), with few exceptions: higher *CXCL8* and *CCL2* RNA levels in LCS-Mϕs and lower for the *TYROBP* gene. Taken together, these results demonstrate that our long-term *in vitro* model with LCS-Mϕs resembles quite well the actual condition of OA-Mϕs.

Modulation of inflammation in LCS-Mϕs

For the validation of our long-lasting *in vitro* protocol using macrophages as an effective screening platform to test new therapeutic strategies, we investigated whether the chronic inflammation expressed by LCS-Mϕs could be downmodulated

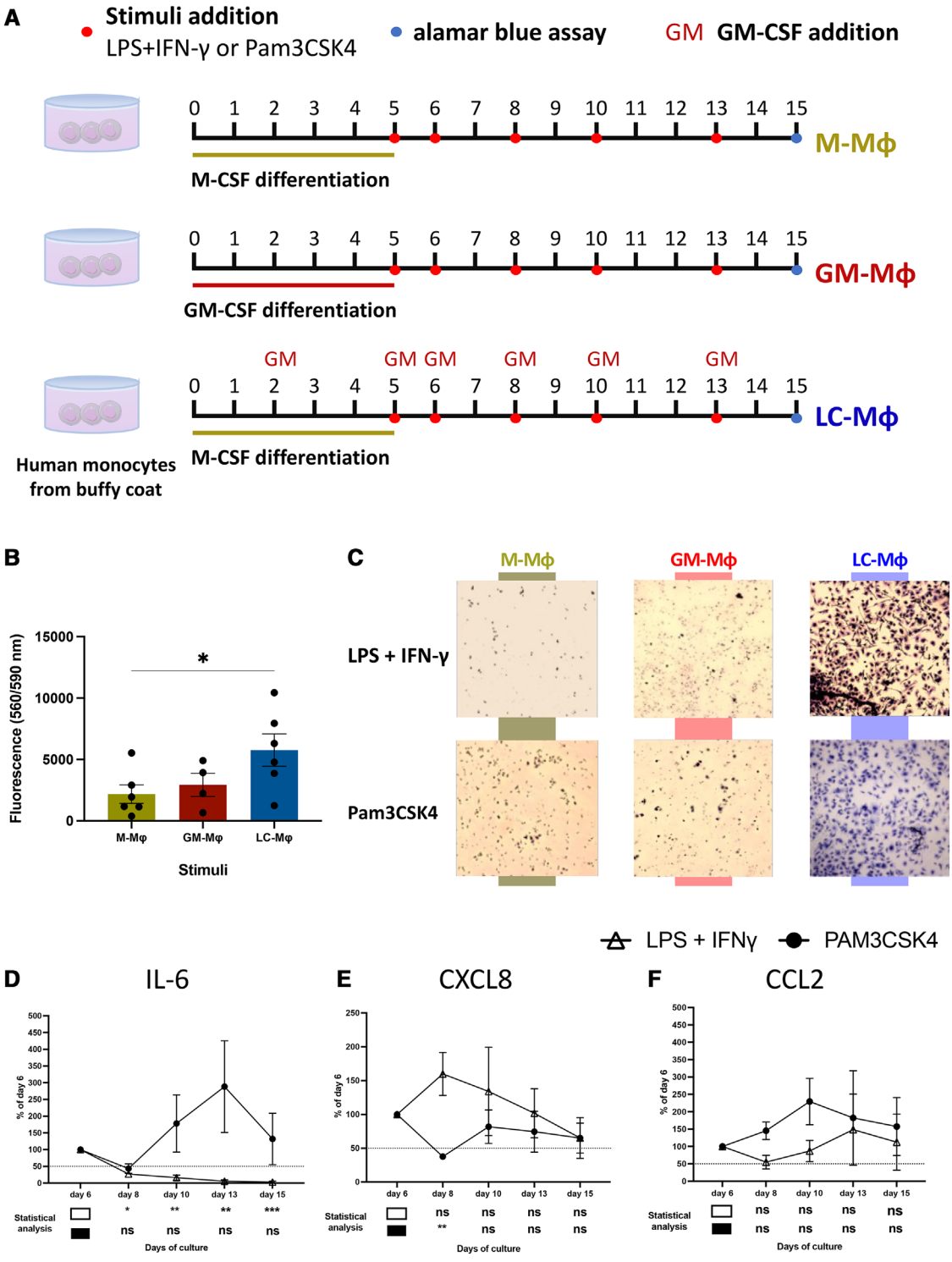

Figure 3. Viability and cytokine production of human primary macrophages chronically stimulated in long-term *in vitro* culture (15 days). (A) Three experimental protocols to differentiate and polarize human primary macrophages chronically activated to an inflammatory phenotype, mimicking OA-conditions *in vitro*: macrophages differentiated with M-CSF (25 ng/mL) (M-Mϕ), macrophages differentiated with GM-CSF (25 ng/mL) (GM-Mϕ), macrophages differentiated with M-CSF + GM-CSF and continuously re-stimulated with GM-CSF (LC-Mϕ); all cells were treated with LPS (100 ng/mL) + IFN-γ (50 ng/mL) or Pam3CSK4 100 ng/mL at indicated days. (B) Cell viability was evaluated by Alamar Blue Assay of M-Mϕ, GM-Mϕ, and LC-Mϕ on day 15. Data are represented as fluorescence measured at 560/590 nm; (C) Optical microscopy (Diff Quick staining), at the same magnification, obtained with each protocol at day 15 for a representative donor plotted in 3B. Concentrations at different time points (day 6, 8, 10, 13, 15) of (D) IL-6, (E) CXCL8, and (F) CCL2 in LC-Mϕ stimulated either with LPS+IFN-γ or with Pam3CSK4. Bars represent mean ± s.e.m; N = up to 6 per group. The statistical comparison was performed using one-way ANOVA followed by Dunnett's multiple comparison tests. Statistically significant differences are represented as follows: $^{*}p < 0.05$.

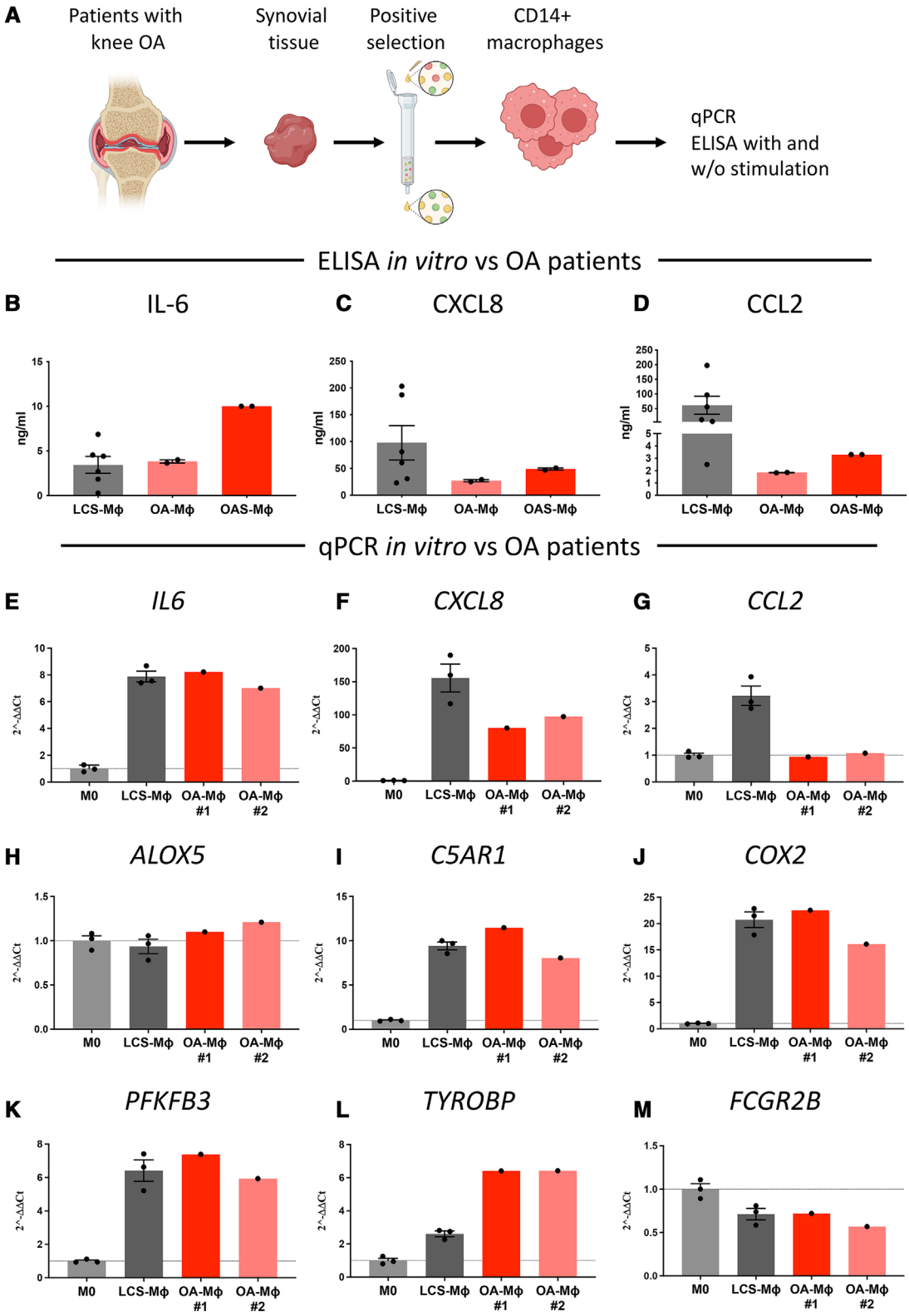

Figure 4. Immune characterization of macrophages from OA patients and comparison with the *in vitro* long-cultured macrophages stimulated with Pam3CSK4 (LCS-Mϕ). (A) Experimental procedure to obtain macrophages from the synovial tissue of patients with OA. Concentrations of (B) IL-6, (C) CXCL8, and (D) CCL2 in macrophages from OA patients compared to LCS-Mϕ. Macrophages from OA patients were plated for 24 h and assayed both unstimulated (OA-Mϕ) or upon stimulation with M-CSF, GM-CSF, and Pam3CSK4 (OAS-Mϕ). Quantitative PCR data for (E) *IL6*, (F) *CXCL8*, (G) *CCL2*, (H) *ALOX5*, (I) *C5AR1*, (J) *COX2*, (K) *PFKFB3*, (L) *TYROBP*, (M) *FCGR2B* normalized for the average values of M-Mϕ at day 5 (here indicated as M0), representative of resting conventional macrophages.

Table 1. Top differentially expressed genes in inflamed synovium and in macrophages from OA patients.

				References	GEO accession
DEGs inflamed synovium	**Inflammation**	Cytokines / Chemokines	***IL6, IL10, TNF,*** *IFN,* ***CCL2, CXCL8, CCL5, CXCL10,*** *CXCL13, CXCL2, CXCL5*	[50-52]	GSE89408
		Enzymes	*NOS2,* ***COX2***	[50]	GSE1919
	Metabolism		***ALOX5****, COL3A1, CTSS, HAS1, MMP9, MMP3,* ***PFKFB3***	[50,51,53,54]	GSE82107 GSE55457
DEGs OA macrophages			***IL1B, C5AR1, FCGR2B, IL10, IL6, TYROBP***	[55]	GSE123492

Note: Tested genes are in bold.
Abbreviations: DEGs, differentially expressed genes; OA, osteoarthritis.

by dexamethasone (DEX) and celecoxib (CEL), two well-characterized anti-inflammatory drugs widely used in OA therapy. First, we examined the viability of monocytes and macrophages exposed for 24 h to these drugs (Supporting information Figs. S4 and S5), showing no significant toxicity for DEX up to 100 μM or for CEL up to 50 μM. Both drugs, especially DEX, demonstrated good anti-inflammatory efficacy on treated monocytes or macrophages, inhibiting the secretion of IL-6, IL-1β, CXCL8, and IL-10 (Supporting information Figs. S4 and S5). Similarly, following the long-lasting protocol, we studied the viability of LCS-Mϕs exposed to DEX or CEL at different concentrations (five doses), added 1 h before the stimulation with Pam3CSK4 (every 2–3 days, as described in Fig. 5A). Extensive toxicity was observed in LCS-Mϕs exposed to DEX concentrations between 10 μM and 25 nM at days 10 and 15 (Fig. 5B), leading us to select the lower dose of 10 nM for the next experiments. Instead, no toxicity was observed for any concentration of CEL from 10 nM to 25 μM (Fig. 5C), nor for the combined exposure to DEX 10 nM + CEL 25 μM (Fig. 5D).

Thus, LCS-Mϕs were treated in the long-lasting assay with DEX 10 nM and/or CEL 25 μM, at each timepoint, 1 h before stimulation with Pam3CSK4; levels of proinflammatory mediators were tested at days 6, 8, 10, 13, and 15. As shown in Fig. 5E, a significant reduction in IL-6 levels was observed for DEX with or without CEL at each time point, while CEL alone only showed a trend to IL-6 inhibition (not significant versus control). For CXCL8, the best anti-inflammatory activity was observed with DEX either alone or in combination with CEL at days 8, 10, and 13, while no significant efficacy was found for CEL alone, except at day 6 (Fig. 5F). In a similar manner, CCL2 was inhibited just in the conditions with DEX (alone or together with CEL), but not with CEL monotherapy (Fig. 5G).

Finally, to better define the anti-inflammatory activity of CEL, we also investigated the secretion of PGE_2, as this is the final product of the COX-2 enzyme inhibited by CEL treatment (Supporting information Fig. S6). Differently from the previous mediators, PGE_2 was detectable in the controls just on days 6, 8, and 15; however, at these timepoints, a clear inhibition was observed in macrophages treated with CEL alone and/or in combination with DEX, showing a greater efficacy mediated by CEL. As a whole, these data demonstrate that the inflammatory mediators produced by LCS-Mϕs can be modulated and inhibited by DEX and CEL, indicating that our *in vitro* protocol could be useful for the testing of new anti-inflammatory pharmacological strategies.

Discussion

OA represents a hot topic in biomedical research, as a growing number of people is affected worldwide, and its therapeutic management remains challenging and high-cost demanding for developed countries. The introduction of appropriate *in vitro* models of chronic inflammation could aid in dissecting the pathophysiology of this disabling condition, and at the same time allow the testing of new treatments to ameliorate the therapeutic management of the disease, alleviate the symptoms, and protect the joints from prolonged damage. To this scope, we have established a long-lasting *in vitro* protocol of chronic inflammation using repeatedly stimulated human primary macrophages, which can mimic OA conditions and response to treatment. Our novel approach using LCS-Mϕs differs from previously described *in vitro* models. Firstly, most of the investigations have so far relied on short time points (maximum 7 days) as detailed in Supporting information Table [18–28], while our long-term model of macrophage cultures shows satisfactory cell viability and continuous secretion of inflammatory mediators up to 15 days; this is a crucial aspect, as acute and chronic inflammation activate distinct molecular signaling pathways and result in the production of different proinflammatory mediators. Secondly, most *in vitro* strategies have used established myeloid leukemia cell lines which are not fully representative of human chronically inflamed macrophages [18–20, 22–24, 36, 37].

It is well known that M-CSF and GM-CSF induce the differentiation of monocytes into mature macrophages, but their effects showed some distinct features [38]. M-CSF is physiologically

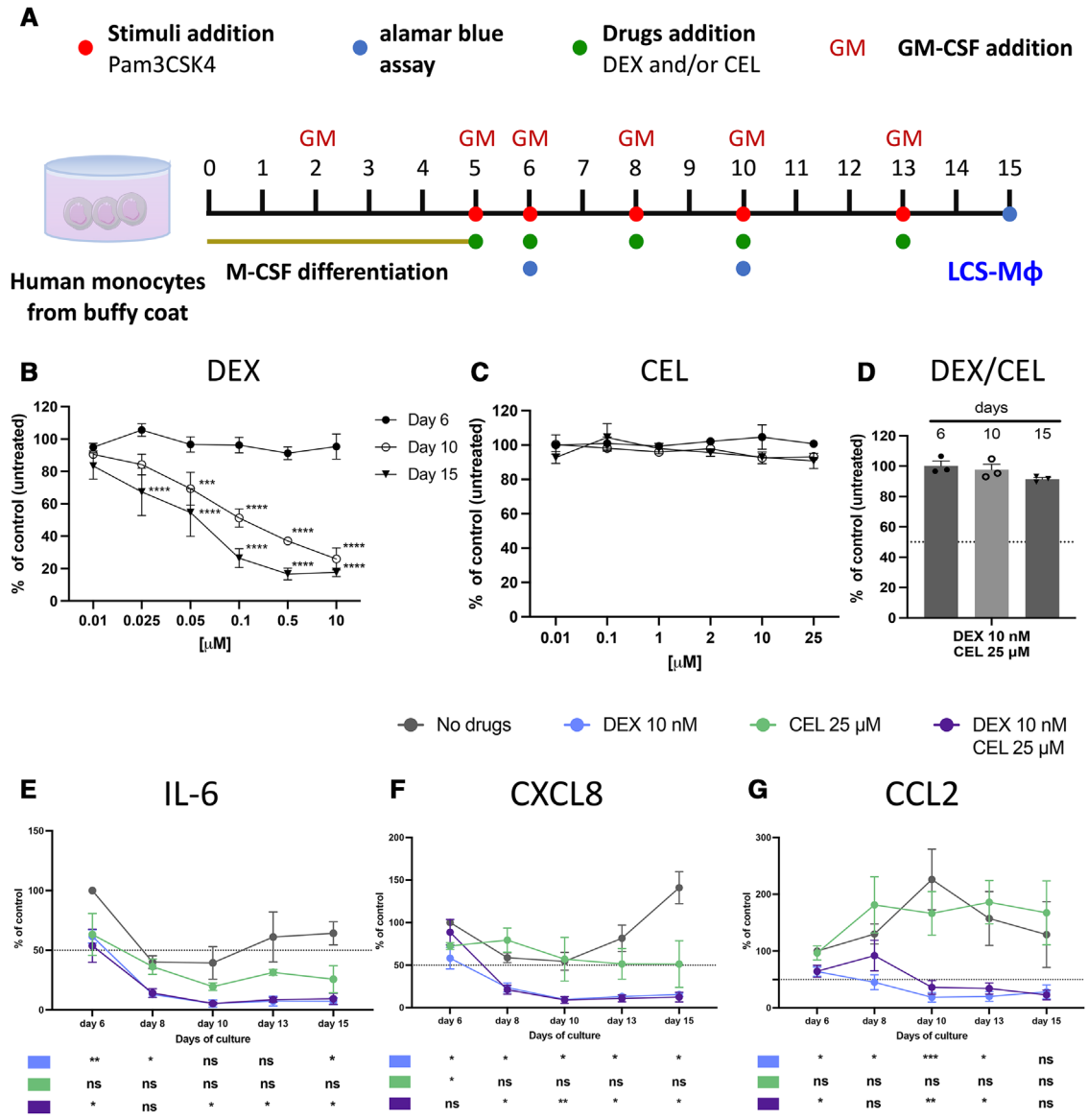

Figure 5. Toxicity and anti-inflammatory activity of dexamethasone and celecoxib on *in vitro* LCS-Mϕ. (A) Experimental protocol used. Human primary macrophages cultured with M-CSF + GM-CSF and stimulated with Pam3CSK4 (LCS-Mϕ) were exposed to the anti-inflammatory drugs: dexamethasone (DEX) or celecoxib (CEL) 1 h before adding the proinflammatory stimuli at each time point except on day 15, in which supernatant was just collected and Alamar Blue assay performed. Toxicity of indicated doses of (B) DEX or (C) CEL at different time points (day 6, 10, and 15). Data for each timepoint are normalized for the untreated. (D) Toxicity of selected nontoxic doses of DEX and CEL combined at different time points (day 6, 10, and 15). Concentrations of (E) IL-6, (F) CXCL8, (G) CCL2 measured by ELISA at each timepoint (days 6, 8, 10, 13, and 15) compared with untreated cells at day 6 (24 h after the first proinflammatory stimulation with Pam3CSK4). Bars represent mean ± s.e.m; N = 4–7 per group. Statistical comparisons for 5B, C, D wer performed using two-way ANOVA followed by Dunnett's multiple comparison tests, while for 5E, F, G the comparison was carried out by comparing each DEX and/or CEL group individually with the control at the respective day. For conditions on day 6, one sample t-test was used, while for conditions on day 8 Mann–Whitney test was used. Statistically significant differences are represented as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.001$.

produced in steady-state conditions [39] and is mostly used in experimental protocols to obtain fully differentiated nonpolarized M0-macrophages [40], while GM-CSF is released in pathologic conditions, and supports the differentiation of macrophages prone to an inflammatory phenotype [30–32, 41]. Our results are in line with the existing literature: GM-Mϕs produced higher concentrations of IL-6 and IL-1β while reducing the levels of the anti-inflammatory cytokine IL-10. Plus, in the long-lasting model, the continuous addition of GM-CSF promoted the survival of LC-Mϕs with sustained proinflammatory activity, a condition that we could not achieve by replacing GM-CSF with M-CSF (data not shown).

Cultured macrophages were stimulated with two TLR agonists: LPS in combination with IFN-γ, to obtain classically activated M1-macrophages, and Pam3CSK4 engaging the TLR2, best mimicking the damage-associated molecular patterns present in

the OA-inflammatory milieu [42]. We also used synovial fluids from OA patients as a source of endogenous inflammatory mediators and a cocktail of cytokines (IL-1β + TNF-α + IFN-γ), but the two latter approaches did not sufficiently activate macrophages in an "inflammatory mode", even in the long-term assay with repeated stimulation. Both LPS+IFN-γ and Pam3CSK4 lead to an M1-like polarization of macrophages but with different patterns of secreted cytokines. In brief, while the use of LPS+IFN-γ induced the typical acute-like inflammatory response, with strong production of TNF-α, IL-6, and CXCL10, the agonist Pam3CSK4 promoted higher release of chemokines such as CXCL8 and CCL2, involved in the continuous recruitment of leukocytes. This latter finding is in accordance with a study that used repeated stimulations of alveolar macrophages with either Pam3CSK4 or LPS [43]. Of note, a study by Xue et al. showed that macrophages stimulated with Pam3CSK4 together with TNF-α and PGE_2 (referred to as M^{TPP}) acquired a phenotype that differed from the classical M1-like macrophages obtained with LPS, and resembling that of cells in chronic inflammatory conditions (such as granulomatous diseases) [44]. Another investigation carried out with murine bone marrow-derived macrophages demonstrated that Pam3CSK4 activates primarily the MAPK pathway, while LPS is particularly involved in type I interferon responses [45]. In the same study, repeated stimulation of TLR2 did not result in reduced expression of *IL6* or *IL10* genes, contrary to what was observed for LPS. In addition, it has been shown that LPS and Pam3CSK4 induce apoptosis of macrophages through different mechanisms, such as generating an autocrine loop of TNF-α [46] or through pyroptosis of macrophages, with the release of the proinflammatory mediators IL-1β and IL-18 [47]. These findings may explain the severe toxicity we observed on macrophages primed either with M-CSF or GM-CSF and then challenged with LPS + IFN-γ or Pam3CSK4. Indeed, the combined priming with M-CSF and GM-CSF and the continuous addition of GM-CSF resulted in minimal toxicity even when LC-Mϕs were repeatedly stimulated. In an effort to compare the chronic inflammation of our LCS-Mϕs with that expressed in the biological samples of OA patients, we first collected synovial fluids from patients and measured several inflammatory mediators that were produced also by our LCS-Mϕs in the long culture, including IL-6, CXCL8, and CCL2. The same mediators, especially IL-6, were also expressed and produced by macrophages purified from surgical synovial samples of OA patients; furthermore, we also checked the expression of some myeloid-related genes in OA macrophages and in our LCS-Mϕ (e.g. *C5AR1, FCGR2B*), as well as inflammation-related genes (e.g. *ALOX5*, *COX2*, *PFKFB3*, and *TYROBP)*, overall finding quite a good similar profile in the two macrophage populations, thus giving good credit to our *in vitro* protocol.

To validate the flexibility of our long-term *in vitro* model of chronic inflammation with repeated stimulation by Pam3CSK4 (LCS-Mϕs), we tested the anti-inflammatory activity of DEX and CEL, two drugs commonly used in patients with OA. DEX, which is known to deeply alter the transcription profile of macrophages [48], showed a profound anti-inflammatory effect, but also severe toxicity at higher doses in LCS-Mϕs. In contrast, CEL, selectively inhibiting the COX-2 enzyme [49], presented a milder anti-inflammatory activity, but no toxicity at higher concentrations. It is interesting to note that a single treatment of monocytes or macrophages showed different effects by the two drugs, both in terms of toxicity and antitumor efficacy. For example, CEL inhibited, at least partially, IL-6, CCL2, and IL-1β production in short-term cultured macrophages and monocytes, while being almost inefficient in long-term cultured macrophages. This apparently negative result has anyway relevance in the pathological context because new myeloid cells are continuously recruited in the inflamed joints from the bloodstream; from these data, it is inferred that newly arrived monocytes should be more sensitive to the anti-inflammatory effect of CEL. Furthermore, it should be considered that CEL in the long-term assay maintained its ability to efficiently inhibit PGE_2, which is also an important mediator of inflammation. Regarding DEX, while monocytes and macrophages tolerate a single dose of 100 μM, repeated exposure to concentrations higher than 10 nM, (i.e. 1000 times less) is toxic for these cells. Regarding the anti-inflammatory effect, even in the long-term culture with LCS-Mϕs, DEX showed good efficacy and significantly inhibited IL-6, CXCL8, and CCL2.

In conclusion, this long-lasting culture model of chronically inflamed macrophages is simple and reproducible and may constitute a useful platform for the screening of new therapeutic strategies for OA and other inflammatory diseases. Taken together, our experimental results suggest that a chronologically distinct use of DEX and CEL could be implemented in a clinical situation. DEX could be used in the short-term at higher doses, inducing a deep reprogramming of macrophages toward an anti-inflammatory phenotype,while a combination of low doses of DEX with higher doses of CEL could result in a sustained amelioration of the inflammation, with no significant toxicities.

Materials and methods

Isolation of primary human monocytes from healthy blood donors, differentiation in macrophages, and short-term protocol

Human monocytes were isolated from buffy coats of blood donations of healthy donors using two sequential separation media, Ficoll and Percoll, as already described previously [40]. "Complete RPMI" consists of RPMI (Euroclone, Cat#ECB2000), supplemented with 10% fetal bovine serum (Euroclone, Cat#ECS0186L), 0.5% penicillin/streptomycin 10,000 U/mL (Gibco, Cat#15140122) and 0.5 % UltraGlutamine Supplement 200 mM (Lonza, Cat#BE17-605E/U1). For experiments employing monocytes, cells were directly challenged with proinflammatory stimuli and/or anti-inflammatory compounds (as detailed below). To obtain HMDMs, monocytes were resuspended in warm (37°C) RPMI without serum and plated ($1.1*10^6$ cells/well in 24-well plates, 1.6×10^6 cells/well in 12-well plates) to allow adhesion for 30 minutes at 37°C. Media was replaced

with complete RPMI, containing the selected CSF. To obtain M-Mϕs media containing hrM-CSF 25 ng/mL (Peptrotech, Cat#300-25) was added, while GM-Mϕs were obtained using hrGM-CSF (Peptrotech, Cat#300-03) at the same concentration. Monocytes, M-Mϕs and GM-Mϕs were then stimulated in the short-term (24 h) with three different sets of stimuli: (1) LPS 100 ng/mL (Sigma, Cat#L4005) in combination with hrIFN-γ 50 ng/mL (Peprotech, Cat#300-02); (2) Pam3CSK4 100 ng/mL (InvivoGen, Cat#tlrl-pms); (3) a cocktail of proinflammatory recombinant cytokines, hrIL-1β (Peprotech, Cat#200–01B), hrTNF-α (Peprotech, Cat#300–01A), and hrIFN-γ, all at the same concentration of 50 ng/mL.

The anti-inflammatory drugs used were DEX (Acofarma, Cat#M0013570) and CEL (Merck, Cat#PHR1683). The cells were exposed to these drugs at indicated concentrations for 1 hour before the addition of the proinflammatory stimuli and kept in the cell culture media until the end of the experiment.

Protocol for *in vitro* chronic stimulation of macrophages in the long-term

The long-lasting protocol was performed by replacing the culture media from day 5 to 15 every 2–3 days (specifically on days 5, 6, 8, 10, 13). Plus, for this set of experiments, long-cultured macrophages (LC-Mϕs) were prepared by adding hrGM-CSF 25 ng/mL on day 2, on monocytes already plated with hrM-CSF 25 ng/mL on day 0, and by the supplement of hrGM-CSF every time the culture media was replaced. For the proinflammatory stimulation, the same three different sets of stimuli introduced for the short-term protocol were used and the stimuli were added at each time point (described in each figure). Similarly, DEX and/or CEL were added 1 h before the proinflammatory stimuli and kept in the media until their replacement at the following time point.

Collection and processing of peripheral blood from healthy donors and synovial samples from the knee joint of OA patients

SF samples were obtained from the knee of 11 patients (4 males, 7 females, 66.2 ± 11.2, mean ± SD) undergoing TKA in the Knee Surgery unit at Humanitas Research Hospital. Synovial tissue (ST) samples were obtained from two patients and collected similarly. Informed consent was acquired from all the patients following the Declaration of Helsinki and the approval from the Ethical committee of our institute (study number: 2515; ID experimentation: 1341). All synovial fluid samples were collected under sterile conditions and centrifuged (430 rcf for 10 min at room temperature). The supernatant was then collected, and two sets of samples were prepared: a small aliquot (0.4 mL) was stored at −80°C and used directly for the ELISA assays (see next section) while the remaining supernatants of all the samples were mixed to create a pool for direct stimulation of macrophages.

ST samples were immediately processed and disaggregated following an enzymatic/mechanic procedure. Briefly, after removing the adipose tissue and cutting synovium into small pieces, tissue was digested with 0.5 mg/mL Collagenase IV from *Clostridium histolyticum* (Sigma, Cat#C5138) in RPMI without serum for 40 min at 37°C in an orbital shaker incubator. The solution was further disaggregated by up–down pipetting with 5 mL syringes and the cell suspension was passed through a 70-μm cell strainer to remove any undigested tissue. Filtered cell suspensions were then centrifuged at 4°C, 300 rcf for 5 min, the pellet was resuspended in complete RPMI, and cells were counted with Trypan Blue staining 0.04%. Macrophages from the synovial cell suspension (OA-Mϕs) were isolated with the CD14 MicroBeads Human kit (Miltenyi Biotec, Cat#130-050-201), following the manufacturer's instructions. Isolated synovial macrophages were processed either directly or following *in vitro* plating (1.1×10^6 cells/well in 24-well plates) and stimulation, for RNA isolation or ELISA assays, as described in the next paragraphs.

Samples of peripheral blood of healthy donors were obtained from peripheral veins, centrifuged (450 rcf for 10 min) and plasma was stocked at −20°C before being processed by ELISA.

Toxicity and ELISA assays

All toxicity assays were performed using Alamar blue reagent (ThermoFisher, Cat#DAL1100) following the manufacturer's protocol. ELISA assays were performed using commercial kits of the DuoSet series provided by Bio-Techne SRL: IL-6 (Cat#DY206), CXCL8 (Cat#DY208), IL-1β (Cat#DY201), TNF-α (Cat#DY210), IL-10 (Cat#DY217B), CXCL10 (Cat#DY266), CCL2 (Cat#DY279). Synovial fluid aliquots were analyzed using the multiplex ELISA assay platform Ella (Bio-Techne SRL), using two different sets of cytokines: CCL2, CCL5, IL-6, IFN-γ on one plate, CXCL10, CXCL8, TNF-α, IL-1β on the other one. These combinations were selected to match the standard curves for the different cytokines. The assays for PGE_2 were provided by Cayman (Cat#004CA514010-96).

Quantitative polymerase chain reaction

Total RNA from part of synovial macrophages was isolated with 200 μL/ million cells of PureZol RNA Isolation Agent (Bio-Rad, Cat#7326890), purified with Direct-zol RNA Miniprep kit (Zymo Research, Cat#R2052) following the manufacturer's protocol and quantified with a spectrophotometer (Nanodrop 8000, ThermoFisher). RNA was retrotranscribed to cDNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cat#4368813), following manufacturers' indications. RT-quantitative PCR was conducted using the Fast SYBR Green Master Mix (Applied Biosystems, Cat#4385612), as indicated by the manufacturer, in a QuantStudio 7 Real-Time PCR System (ThermoFisher). The following genes were analyzed: *IL6* (F- CTGGATTCAATGAG-

GAGACTTGCC, R- GGTTGGGTCAGGGGTGGTTA), *CXCL8* (F-CACTGTGTGTAAACATGACTTCC, R- GGTGGAAAGGTTTG-GAGTATGT), *CCL2* (F- GTCTTGAAGATCACAGCTTCTTTGG, R- AGCCAGATGCAATCAATGCC), *ALOX5* (F- AAGCGATGGA-GAACCTGTTCA, R- GTCTTCCTGCCAGTGATTCATG), *COX2* (F-TTCAAATGAGATTGTGGGAAAATTGCT, R- AGATCATCTCT-GCCTGAGTATCTT), *PFKFB3* (F-ACAAATGCGACAGGGACTTG, R- GGATGTTCATCAGGTAGTACACG), *C5AR1* (F- TGCTGACCAT-ACCCTCCTTC, R- CCCTAACCACGGACTCTTCA), *FCGR2B* (F-GTTGGGGCTGAGAACACAAT, R- ACAGGGAGCTTCAGGACTCA) and *TYROBP* (F- GACATCCGACCTCTGACCCT, R- CTGGTGCT-GACAGTGCTCAT). *GAPDH* (F- TCGGAGTCAACGGATTTGGT, R-TGAAGGGGTCATTGATGGCA) was regarded as the reference gene for quantitative analysis and results were expressed as fold change to the control using the ΔΔCt method.

Bright-field microscopy and staining

The images of macrophages stimulated with the different conditions were obtained by bright-field microscopy on samples stained with the Diff Quik® Staining Set (Medion Diagnostics AG, Cat#130832) according to the manufacturer's protocol. The fixing agent and the two dyes provided with the kit were sequentially added for 1 minute on the cells, removing each one at the end of the incubation time. In the end, the plates were washed using tap water, dried overnight, and acquired in brightfield microscopy (Inverted Microscope IX53, Olympus).

Statistical analyses

All the statistical tests were performed with GraphPad Prism 9.4.1. Further details are provided in each corresponding figure legend.

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Author contributions: Paola Allavena, Fernando Torres Andón, and Elizaveta Kon decided on the scientific concept behind the designed experiments. Aldo Ummarino and Alba Pensado-López performed the *in vitro* experiments, under the supervision of Paola Allavena, Fernando Torres Andón, and Clément Anfray. Lourdes Alcaide-Ruggiero, Alba Pensado-López, Roberta Migliore, Michele Caputo, Nicholas Calà, and Francesco M. Gambaro helped with the isolation of HMDMs and ELISA. Aldo Ummarino wrote the manuscript with contributions from Paola Allavena, Fernando Torres Andón, Elizaveta Kon, and Flavio L. Ronzoni. All authors agreed on the final version of the manuscript.

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Abbreviations: **CEL**: celecoxib · **DEX**: dexamethasone · **LC-Mϕs**: long-cultured macrophages · **LCS-Mϕs**: long-cultured stimulated macrophages · **LPS**: lipopolysaccharide · **OA**: osteoarthritis · **Pam3CSK4**: Pam3CysSerLys4 · **SF**: synovial fluid · **TKA**: total knee arthroplasty · **TLR**: toll-like receptor

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