Pharmacologic Activation of LXR Alters the Expression Profile of Tumor-Associated Macrophages and the Abundance of Regulatory T Cells in the Tumor Microenvironment

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Abstract

Liver X receptors (LXR) are transcription factors from the nuclear receptor family that are activated by oxysterols and synthetic high-affinity agonists. In this study, we assessed the antitumor effects of synthetic LXR agonist T0901317 in a murine model of syngeneic Lewis lung carcinoma. Treatment with T0901317 inhibited tumor growth in wild-type, but not in LXR-deficient mice, indicating that the antitumor effects of the agonist depends on functional LXR activity in host cells. Pharmacologic activation of the LXR pathway reduced the intratumoral abundance of regulatory T cells (Treg) and the expression of the Treg-attracting chemokine Ccl17 by MHCIId+high tumor-associated macrophages (TAM). Moreover, gene expression profiling indicated a broad negative impact of the LXR agonist on other mechanisms used by TAM for the maintenance of an immunosuppressive environment. In studies exploring the macrophage response to GM-CSF or IL4, activated LXR repressed IRF4 expression, resulting in subsequent downregulation of IRF4-dependent genes including Ccl17. Taken together, this work reveals the combined actions of the LXR pathway in the control of TAM responses that contribute to the antitumoral effects of pharmacologic LXR activation. Moreover, these data provide new insights for the development of novel therapeutic options for the treatment of cancer.

Significance: This study reveals unrecognized roles of LXR in the transcriptional control of the tumor microenvironment and suggests use of a synthetic LXR agonist as a novel therapeutic strategy to stimulate antitumor activity.

Introduction

Nuclear receptors are a family of transcription factors with key functions in health and disease. Many members within this family are activated in a ligand-dependent manner. In particular, liver X receptors (LXR) are activated by cholesterol derivatives, including specific oxysterols, and by synthetic high-affinity agonists. Two LXR subtypes have been identified, LXRα (NR1H3) and LXRβ (NR1H2), which are expressed in tissues in an overlapping but not identical manner. Both LXRs bind to DNA as heterodimers with another subgroup of the nuclear receptor family, the retinoid X receptors (RXR), to regulate transcription of a variety of target genes involved in lipid metabolism (revised in ref. 1) and in immune cell function (2–6). Moreover, upon ligand binding, LXRs repress inflammatory gene expression (revised in ref. 7).

Synthetic LXR agonists activate different mechanisms that translate into antiproliferative effects in a wide variety of cancer cell types (revised in ref. 8). In vivo studies, however, have produced contradictory results on the role of the LXR pathway in controlling tumor growth. In several mouse models of cancer, LXR agonists efficiently reduced primary tumor growth (9–11). Interestingly, while inhibiting the metastasis of melanoma cells in an apolipoprotein E (APOE)-dependent manner (9), the agonist GW3965 exacerbated the dissemination of breast cancer cells to the lung (10). Another study showed...
that endogenous LXR ligands can be secreted by tumor cells as a strategy for immune evasion (12). In that setting, the activation of LXRα repressed the expression of CC chemokine receptor (CCR)7 on dendritic cells, thus impairing their migration to lymphoid organs and compromising the establishment of an antitumor adaptive immune response. Conversely, recent work has shown that LXR agonism, and subsequent APOE production, reduces the levels of myeloid-derived suppressor cells (MDSC), thus enhancing CTL activity (11). These contrasting observations support the need to dissect the roles of the LXR pathway in the tumor microenvironment in different contexts of cancer.

Solid tumors are infiltrated by heterogeneous populations of leukocytes. Among the immune cells within the tumor site, tumor-associated macrophages (TAM) are particularly abundant and present at all stages of tumor progression. Interestingly, TAMs have been associated with poor prognosis in a variety of cancers (13, 14). In most solid tumors, TAMs exhibit protumoral functions by promoting cancer cell survival and proliferation, extracellular matrix remodeling, and angiogenesis that benefit tumor cell migration and dissemination to secondary locations (15). TAMs also adopt immune-suppressive roles within the tumor microenvironment. Through the surface expression of a number of regulatory molecules, TAMs are able to directly suppress immune responses against tumor cells. For instance, TAMs express human leukocyte antigen (HLA)-C, HLA-G, and HLA-E, which inhibit the activation of NK cells, and ligands for programmed cell death protein 1 (PD-1) or CTL antigen 4 (CTLA4; PD-L1 and B7–1, respectively), which inhibit T-cell proliferation and activation as well as the cytolytic activity of CD8 T cells. TAMs can also influence the antitumoral immune response indirectly through the induction of l-arginine–consuming enzymes, namely nitric oxide synthase (NOS)2 and arginase 1 (ARG1), and the secretion of a array of cytokines and chemokines (revised in ref. 16). For example, TAMs secrete CC chemokine ligand (CCL)17 and CCL22 upon stimulation by granulocyte/monocyte-colony stimulating factor (GM-CSF) produced by tumor cells (17, 18). Through their binding to surface, CCR4, CCL17, and CCL22 promote the migration of regulatory T cells (Treg) to the tumor microenvironment (19, 20), thus facilitating the establishment of an immunosuppressive environment.

In mice, different TAM populations have been identified within solid tumors, displaying markers that partially fit with the classical versus alternative macrophage activation paradigm (21). TAMs exhibiting a more proinflammatory gene signature are enriched in normoxic areas of the tumor and express high levels of MHCII, whereas TAMs displaying a more alternative phenotype are located mostly within hypoxic tumor areas, have a superior proangiogenic activity and express low MHCII levels (22). Nevertheless, both TAM subsets are poor antigen-presenting cells, express Ccl17 and Ccl22 (although MHCII+ TAMs produce higher levels of these chemokines), and are able to suppress T-cell activation.

In this work, we demonstrate unrecognized roles of LXRα in the control of TAM gene expression. The synthetic LXR agonist TO901317 (T1317) inhibited the growth of syngeneic Lewis Lung carcinoma in wild-type (WT) but not in LXR-deficient (LXRα−/−) mice, despite the fact that injected cancer cells express LXRα isofoms in both settings and that these cells are sensitive to growth inhibition by high doses of LXR agonists in vitro. This indicates that LXR activity in host cells is essential for the antitumor effects of the synthetic LXR agonist. In this context, several mechanisms used by TAMs for the maintenance of an immunosuppressive environment were downregulated upon pharmacologic LXR activation, including the expression of the chemokine CCL17, which correlated with a decrease in the abundance of intratumoral Tregs in vivo. In addition, LXR activity repressed other genes that are part of the protumoral program of TAMs and reduced partially the capability of these cells to suppress T-cell proliferation in vitro. Moreover, repression of IRF4 expression emerged as a mechanism linking LXR activation with the downregulation of selective genes, such as Ccl17, in different macrophage populations. Taken together, this work provides novel insights about the biological actions of LXR agonists and supports their pharmacologic use as antitumoral drugs.

### Materials and Methods

#### Reagents

The synthetic high-affinity LXR agonists T1317 and GW3965 were purchased from Cayman Europe and Tocris, respectively. Recombinant murine GM-CSF and IL4 and human IL4 and macrophage-colony stimulating factor (M-CSF) were purchased from PeproTech.

#### Animals

C57BL/6 mice were purchased from Harlan and raised as a colony in our animal facility. LXR-deficient mice were initially donated by Dr. David Mangelsdorf (UT Southwestern Medical Center, Dallas, TX) and backcrossed into the C57BL/6 background for more than ten generations. Foxp3EGFP mice were generated by crossing the NOD. Foxp3EGFP strain (23) with C57BL/6 mice for five generations. PyMT mice (24) with an FVB/N background were obtained from The Mouse Models of Human Cancers Consortium Repository (National Cancer Institute, Frederick, MD) and backcrossed into the C57BL/6 background for nine generations. Three-week-old JunB−/−; MxCre mice (25) and Cre-negative control littermates (C57BL/6 background) were administered three intraperitoneal injections of poly I:C at weekly intervals to induce JunB deletion systemically (including the hematopoietic lineage/bone marrow). JUND-deficient mice (26) and WT control littermates were generated by heterozygote intercrosses (C57BL/6 background). IRF4-deficient mice and control C57BL/6 mice were bred at the animal facility of the Biomedical Research Center at the University of Marburg (Marburg, Germany). Unless otherwise stated, the mice were fed a regular chow diet. All the protocols requiring animal manipulation have been approved by the Institutional Animal Care and Use Committees from Parc Científic de Barcelona (#9672), Universitat de Barcelona (#7088), Institut de Recerca de l’Hospital de la Santa Creu i Sant Pau (#7281), and the University of Marburg (R.P. Giessen, Germany).

#### Cells

Bone marrow–derived macrophages were obtained from six to ten-week-old mice as described previously (27). Bone marrow precursors were differentiated to macrophages in DMEM supplemented with 20% heat-inactivated FBS (Sigma-Aldrich) and 30% L929 conditioned media as a source of M-CSF.

Human macrophages were differentiated in vitro from peripheral blood mononuclear cells (PBMC) from healthy donors. See more details in Supplementary Methods. The protocol has been approved by the Bioethics Commission of the University of Barcelona and the blood samples were obtained from the Blood and Tissue Bank from Generalitat de Catalunya.

The 3LL-R cell line (used in our lab since 2016; ref. 28) was maintained in RPMI media with l-glutamine (l-Gln, 0.3 g/L; BioWest) supplemented with 10% FBS. Raw264.7 macrophages (ATCC, RRID:
CVCL_0493, obtained in 2011) were cultured in DMEM-10% FBS. All cell lines were used within 15 passages after thawing.

Tumor progression studies
3LL-R cells (3 × 10⁶) were subcutaneously injected in eight- to ten-week-old WT or LXRαβ-deficient male mice. The tumors were allowed to grow for two weeks. From day 7 to day 15, length (D) and width (d) measures were taken with a digital caliper and tumor volume was calculated using the formula V = π × (d × D)/6 (29).

In some experiments, at day 7, once the tumor was established and lasted for the next 8 days, the animals received a daily dose of T1317 (15 mg/kg) through an intraperitoneal injection. Control animals received an equivalent dose of vehicle (DMSO) diluted in PBS. At day 15, the mice were euthanized and the tumors excised and processed.

Alternatively, tumor development was evaluated in PyMT transgenic mice. After weaning, PyMT female mice were administered either a regular chow diet (A01; Scientiﬁc Animal Food & Engineering) or the same diet supplemented with 30 mg/kg of T1317. The mice were monitored every three days for palpable tumors starting at 6 weeks of age. Tumor latency was defined as the time to the development of the first palpable tumor in each mouse. The mice were euthanized at 22 weeks of age. Total tumor burden was determined after all the mammary glands were excised and weighed, and the mass of the tumor-bearing mammary glands was measured. Each mammary gland was numerically labeled as in ref. 30.

Identification of immune cell populations
3LL-R cells (3 × 10⁶) were injected subcutaneously in recipient mice as described above. At day 15 postinjection, the tumors were dissected and processed as indicated in Supplementary Methods. The final tumor suspension was diluted to a concentration of 10⁶ cells/mL in PBS and incubated first with Fc block (rat anti-mouse CD16/CD32, BD Biosciences; 1:50 dilution, 30 minutes, 4°C). For myeloid cell determination, the cells were incubated with specific antibodies against CD11b, Ly6G, Ly6C, and IA/IE (MHCII; see more details in Supplementary Methods; ref. 21). Cell populations were analyzed using flow cytometry using a FACSaria Fusion cell sorter (BD Biosciences; 1:50 dilution, 30 minutes, 4°C). For lymphoid cell determination, the cells were incubated with specific antibodies against CD4 and CD8 (see more details in Supplementary Methods; Supplementary Fig. S1). The cells were then permeabilized and fixed using the Fossp3/Transcription factor staining buffer set (Invitrogen) following the manufacturer’s recommendations. The cells were analyzed by flow cytometry.

Alternatively, for lymphocyte cell determination, 3LL-R cells were injected subcutaneously in Foxp3EGFP reporter mice. The tumors were collected at day 10 post cancer cell injection. Cell suspension was blocked with Fc block and incubated with specific antibodies against CD4 and CD8 (Supplementary Methods). The cells were analyzed by flow cytometry. Tregs were identified via EGFP expression analysis (Supplementary Fig. S1).

In some experiments, the spleens were also harvested. See details in Supplementary Methods. Lymphocyte populations were analyzed as described above.

Treg depletion
To downregulate the frequency of Tregs within tumors, male Foxp3EGFP reporter mice were administered antibodies anti-CD25 (InVivoMab anti-mouse CD25 (IL2Ra), clone PC-61.5.3, BioXCell (#BE0012); 200 μg per animal diluted in PBS; i.p. injection) at days 2, 5, and 8 post tumor cell injection. Control mice were administered the isotype control (InVivoMab rat IgG isotype control anti-horseradish peroxidase, clone HRPN; BioXCell (#BE0088)). At day 5, and until day 9, the mice received a daily dose of T1317 (15 mg/kg) or vehicle (DMSO) through intraperitoneal injection. From day 5 to day 10, tumor progression was evaluated. At day 10, the mice were euthanized, and the spleens and tumors were recovered and processed as described above.

Isolation of TAMs
3LL-R cells were injected subcutaneously in recipient C57BL/6 mice and, at day 15 postinjection, the tumors were excised and processed as described above. Cells suspensions were generated from pooled tumors (five tumors per sample) and incubated with the antibodies described for myeloid cell determination. MHCII⁺ TAM and MHCII⁺⁺ TAM populations were isolated using a FACSaria Fusion Cell Sorter (BD Biosciences). For ex vivo experiments, TAMs were cultured in RPMI-10% FBS, supplemented with 1-glutamine, HEPES, 10 mmol/L sodium pyruvate, nonessential amino acids (BioWest), and 3.7 mmol/L 2-mercaptoethanol (Sigma-Aldrich).

Isolation of Tregs
3LL-R cells were injected subcutaneously in Foxp3EGFP transgenic mice. At day 10 postinjection, cell suspensions were generated from pooled tumors and Tregs were sorted as EGFP⁺ cells using a FACSaria Fusion cell sorter. The cells were maintained in RPMI-10% FBS for subsequent analysis. Alternatively, Tregs were isolated from the spleens of Foxp3EGFP mice.

Proliferation assays
3LL-R cells were plated in 24-well plates (10⁵ cells/well) and starved in RPMI without FBS during 24 hours in the presence of LXR ligands or vehicle (DMSO). After starvation, the cells were incubated with 10% FBS and 0.3 g/L l-glutamine for 24 hours. Finally, the cells were pulsed with [3H]-thymidine (1 μCi/mL; ICN Pharmaceuticals) for 6 hours. The cells were fixed in 70% methanol, washed in 10% TCA, and lysed in 1% SDS/0.3 mol/L NaOH. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard counter (GMI). Each experimental condition was performed in triplicates.

Puriﬁed Tregs (10⁵ cells/well) were stained with CellTrace CFSE Cell Proliferation Kit (Invitrogen) following the manufacturer’s recommendations and then stimulated with T1317 (1 μmol/L) or DMSO for 18 hours at 37°C. Unstained cells were grown separately as a negative control for cytometry. Treg proliferation was induced during 48 hours using the mouse and T Cell Activation/Expansion Kit (Miltenyi Biotec), which consists of anti-biotin MACSisBead particles and biotinylated antibodies against mouse CD3e and CD28, in the presence of IL2 (100 U/mL). Cell proliferation was analyzed by flow cytometry as the percentage of cells with CFSE dispersion compared with nonactivated Tregs. Murine IL2 was expressed in and puriﬁed from E. coli as described previously (31).

Suppression of T-cell proliferation
TAMs or Tregs were seeded in 96-well plates (200,000 TAMs/well or 100,000 Tregs/well in RPMI-10% FBS) and stimulated with T1317 (1 μmol/L) or DMSO for 18 hours at 37°C. In experiments using TAMs, the medium was then replaced by fresh medium (without LXR agonist). Total splenocytes were obtained from the spleens of C57BL/6 mice as a cell suspension and stained with the CellTrace CFSE Cell Proliferation Kit. Unstained splenocytes were grown separately as a negative control for cytometry. CFSE-stained splenocytes were either
grown alone in RPMI-10% FBS or incubated with TAMs or Treg cells at a 1:1 ratio. T-cell proliferation was induced using the Mouse T Cell Activation/Expansion Kit during 48 hours at 37°C and analyzed by flow cytometry. **Phagocytosis assay**

MHCI<sup>low</sup> TAMs and MHCI<sup>high</sup> TAMs were seeded in 24-well plates (500,000 cells/well) and stimulated with T1317 1 μmol/L or DMSO for 18 hours at 37°C. The cells were incubated with 3-μm
Pharmacologic stimulation of the LXR pathway reduces the abundance of Tregs in the tumor. WT C57BL/6 mice (A–C, E, and G), Foxp3EGFP transgenic mice (D) or LXR-deficient mice (B) were subjected to subcutaneous injection of 3LL-R cells. The mice were treated daily with DMSO or T1317 (15 mg/kg) from day 7 (A–C, E, and G) or day 5 (D) of tumor establishment. The tumors were collected at day 15 (A–C, E, and D, and G) or day 10 (D) after tumor inoculation. The spleens were collected at day 15 (G). The abundance of different immune cell populations was measured by flow cytometry. In A, D, and G, the graphics represent the frequencies of immune cell populations (percentage of gated live singlets) in the tumors (A, C, D, and G) or spleens (G). Horizontal bars indicate mean values from each experimental group. Pooled data from two (C and G) or three (A and D) independent experiments; n = 18–19 (A), n = 11–12 (C and D), n = 10–11 (G) mice/group (Mann–Whitney test). In B, the absolute numbers of intratumoral immune cells normalized to the tumor weight was determined in WT and LXR-deficient mice. n = 5 mice/group. One-way ANOVA test followed by Newman–Keuls post hoc. In E, the absolute amount of Tregs was determined in each tumor and normalized to the tumor volume. Pooled data from two independent experiments; n = 10–11 mice/group (t test). In F, PyMT female mice were administered a chow diet with or without supplementation with T1317 (50 mg/kg). Relative expression levels of Foxp3 mRNA (normalized to L14) in mammary glands at 22 weeks of age (qPCR). n = 8–10 mice/group. t test; *, P < 0.05; **, P < 0.001.
fluorescent microspheres (Fluoresbrite YG microspheres, Polysciences) at a ratio of 20 beads/cell for 30 minutes at 37 °C. After this time, the cells were placed on ice, washed three times with ice-cold PBS, and fixed in PBS-2% PFA. The phagocytosis of microspheres was analyzed by flow cytometry.

**RNA extraction, cDNA synthesis, and quantitative real-time PCR analysis**

Total RNA was extracted from cells or tissues using TRIzol (Invitrogen) as recommended by the manufacturer. For cDNA synthesis, 1 μg of RNA was subjected to reverse transcription using M-MLV Reverse transcriptase RNase H Minus, Point Mutant, oligo (dT)15 primer and PCR nucleotide mix (Promega). Quantitative real-time PCR (qPCR) was performed using the Power SYBR Green Reagent Kit (Applied Biosystems) following the manufacturer’s recommendations. See more details in Supplementary Methods. The data were expressed as mRNA levels relative to ribosomal L14 or to Gapdh expression in murine and human samples, respectively.

**Gene expression profiling**

Total RNA was purified using the RNAeasy Kit (Qiagen) following the manufacturer’s instructions. The samples were processed as described in Supplementary Methods and hybridized to GeneChip Mouse Clarion S Array (Affymetrix). For each sample, expression estimates were calculated from probe intensities and represented as log_{2} values. Heatmaps were produced with Heatmapper (Wishart Research Group, University of Alberta).

**Figure 3.**

The antitumoral action of T1317 is not effective in mice undergoing Treg depletion. **A,** T1317 does not impair the capability of Tregs to suppress T-cell proliferation. Tregs were isolated from the spleens (left) or tumors (right) of Foxp3EGFP mice. Total splenocytes were isolated from the spleens of WT mice, stained with CFSE, and incubated with Tregs at a 1:1 (Treg:splenocyte) ratio. Control cells were grown in the absence of Tregs. T-cell proliferation was induced *in vitro* for 48 hours using antibodies against CD3e and CD28 and analyzed by flow cytometry. The graphics represent the percentage of cells with CFSE dispersion compared with nonactivated splenocytes. Mean ± SD. Left, n = 3 biological replicates. Right, n = 5 biological replicates (pooled data from two independent experiments; t test; **P < 0.05; ***P < 0.01). B and C, Tregs were isolated from the spleen of Foxp3EGFP mice. In B, purified Tregs were incubated with DMSO or T1317 in *vitro* and the expression of Il10 and Tgfb was analyzed by qPCR; mean ± SD (n = 3). In C, Tregs were stained with CFSE, incubated with DMSO or T1317, and induced to proliferate in the presence of a combination of anti-CD3/CD28 antibodies and murine recombinant IL2 (100 U/mL). Mean ± SD, n = 3 (one-way ANOVA). D and E, Male Foxp3EGFP reporter mice were subjected to the subcutaneous injection of 3 × 10^6 3LL-R cells. At days 2, 5, and 8 post tumor cell injection, the mice were administered either antibodies anti-CD25 or control isotype antibodies (200 μg per animal in PBS by intraperitoneal injection). At day 5, and until day 9, the mice received a daily dose of T1317 (15 mg/kg) or vehicle (DMSO). n = 5–6 animals/group. D, At day 10, the mice were euthanized. The frequency of FOXP3-GFP^+ Treg in spleens (top) and tumors (bottom) was evaluated by flow cytometry. Horizontal bars represent mean values. Kruskal–Wallis test followed by Dunn multiple comparison test; **P < 0.05; ***P < 0.01. Selected experimental conditions were also compared using a Mann–Whitney test (#, P < 0.05; ##, P < 0.01). E, Tumor volumes were measured from day 5 to day 10 and represented as fold change. Two-way ANOVA with repeated measures; **P < 0.05; ***P < 0.01; ****P < 0.001.
Identification and cloning of potential enhancer regions with IRF4-binding sites

Potential enhancer regions containing IRF4-binding sites upstream and downstream of the Ccl17 gene were isolated from mouse tail genomic DNA using REDExtract N-Amp PCR Ready Mix (Sigma-Aldrich). See more details in Supplementary Methods. Amplified regions were subsequently cloned between the KpnI and Xhol restriction sites of a pGL3-promoter vector (Promega).

Protein extraction and Western blot analysis

The cells were washed twice in cold PBS and lysed on ice with lysis solution (1% Triton X-100, 10% glycerol, 50 mmol/L HEPES, pH 7.5, 250 mmol/L NaCl, protease inhibitors, 1 mmol/L sodium orthovanadate). Insoluble material was removed by centrifugation at 13,000 x g for 4 minutes at 4°C. Cell lysates were processed for Western blot analysis as described in Supplementary Methods.

Results

Functional LXR expression in host cells is required for the antitumoral actions of the LXR agonist T1317

We and others had previously reported antiproliferative actions of LXR agonists in primary immune cells (36, 37) and in tumor cell lines in vitro (revised in ref. 8). These observations were confirmed in this study using the Lewis lung carcinoma cell line 3LL-R, which expresses both LXRα and β (Fig. 1A and B). Interestingly, however, tumor progression studies based on the subcutaneous injection of 3LL-R cells into syngeneic mice (C57BL/6 background) revealed that the administration of the LXR agonist T1317 was able to inhibit the growth of established tumors in WT mice, but not in mice lacking functional LXRαs (Fig. 1C and D) despite the fact that the injected cancer cells express LXRs. Altogether, these observations suggest that LXR expression in host cells is essential for the inhibitory actions of the synthetic LXR agonist on tumor growth.
The LXR agonist was also effective in preventing tumor growth in PyMT female mice, a model for spontaneous breast adenocarcinoma development and progression (Fig. 1E–G). The oral administration of the agonist through the diet did not affect tumor latency (Fig. 1E), but resulted in a significant decrease in mammary gland weight at 22 weeks of age (Fig. 1F and G). This finding argues for the importance of LXR activity in the microenvironment once tumors are established, rather than affecting cancer cell proliferation and early-stage carcinogenesis.

**Pharmacologic LXR activation reduces the abundance of Tregs within the tumor**

To dissect the actions of the LXR agonist in the tumor microenvironment, we next used flow cytometry to assess, in 3LL-R tumors, the abundance of different intratumoral immune cell populations that have prognostic values. At the level of myeloid cells, several populations were distinguished in 3LL-R tumors: CD11b+/Ly6Chigh/Ly6G− cells (compatible with Ly6Chigh monocytes and monocyctic MDSCs), CD11b+/Ly6Cmed/Ly6G− cells (compatible with neutrophils and polymorphonuclear MDSCs), tumor-associated dendritic cells (TADCs; CD11b+/Ly6C−/Ly6G−/MHCIIhigh), and two TAM subsets (CD11b+/Ly6C+/Ly6G−) expressing different levels of MHCII and termed MHCIIlow TAMs and MHCIIhigh TAMs (gating strategy shown in Supplementary Fig. S1). Pharmacologic LXR activation specifically decreased the frequency of MHCIIhigh TAMs without significantly affecting the frequency of other myeloid cell populations analyzed here (Fig. 2A). However, the total numbers of MHCIIhigh TAMs differed considerably between tumors and only a tendency toward a decreased amount of these cells (when normalized to the weight or volume of the tumor) was observed in the tumors from T1317-treated WT mice (Fig. 2B). These observations suggest that the decrease in the relative frequency of MHCIIhigh TAMs in response to LXR activation does not consistently reflect reduced absolute numbers of these cells in the tumor.

At the level of T lymphocytes, the administration of T1317 significantly decreased the frequency and the total numbers of CD4+FOXP3+ Tregs within the tumor (Fig. 2C–E). In addition, decreased levels of Foxp3 expression were observed in the mammary glands from T1317-treated PyMT mice in comparison with mice fed a regular diet (Fig. 2F), which suggests that T1317 may also downregulate Treg infiltration in spontaneous breast adenocarcinoma. In LXR-deficient mice, T1317 did not inhibit the total numbers of the immune cell populations tested, including Tregs (Fig. 2B), which is in line with the LXR-specific effects of the agonist in the control of tumor growth (Fig. 1C and D).

Because a major goal of this study was to characterize the mechanisms underlying the anti-tumoral actions of synthetic LXR agonists, we further explored the effects of LXR activation in Tregs. First, the treatment of tumor-bearing mice with T1317 did not impact the frequency of Tregs in the spleen (Fig. 2G), suggesting that the decrease in Treg abundance is specific to the tumor. Of note, the frequency of splenic CD4+ FOXP3+ cells was downregulated upon pharmacologic LXR activation (Fig. 2G), in line with previous work showing a role for LXR in the negative control of central T-cell proliferation (36). Next, we evaluated whether the immunosuppressive capacity of Tregs is affected by T1317. Tregs were isolated from either the spleens or tumors of Foxp3EGFP reporter mice and treated with the LXR agonist or vehicle. Their capability to inhibit T-cell proliferation induced by anti-CD3/CD28 antibodies was tested in vitro. Interestingly, the LXR agonist did not impair the suppressive capacity of Tregs on T-cell proliferation (Fig. 3A). Moreover, activation of LXRs did not reduce the expression of the anti-inflammatory cytokines IL10 and Tgfβ in Tregs from the spleen (Fig. 3B). Taken together, these results suggest that pharmacologic LXR activation does not inhibit the immunosuppressive capacity of the Treg itself, but rather results in a decrease in the amount of Tregs within the tumor. In addition, the LXR agonist did not inhibit the polyclonal proliferation of Tregs (Fig. 3C).

Importantly, higher infiltration of Tregs often correlates with less favorable outcomes in different types of tumors and accumulated evidence indicates that the removal of Tregs is able to enhance antitumor immune responses (reviewed in ref. 38). To assess the relevance of the Treg population in the antitumoral actions of T1317, the levels of Tregs were reduced by the intraperitoneal injection of mAbs against CD25 in Foxp3EGFP reporter mice (Fig. 3D). In comparison with control mice (injected with isotype control antibodies), Treg depletion resulted in reduced tumor progression (Fig. 3E). In line with the data in Fig. 2C and D, treatment with T1317 reduced the frequency of intratumoral FOXP3-GFP+ Tregs (Fig. 3D) and inhibited tumor progression in control mice (Fig. 3E). Interestingly, the LXR agonist was not effective in reducing tumor volumes in mice undergoing anti-CD25–mediated Treg depletion (Fig. 3E), which suggests that the actions of T1317 in tumor progression are Treg-dependent.

**Pharmacologic LXR activation downregulates Ccl17 expression in TAMs**

Treg abundance in the tumor has been shown to correlate with the local production of the chemokines CCL17 and CCL22 (19, 20, 39). Interestingly, TAMs, predominantly MHCIIhigh TAMs in mice, highly
contribute to the secretion of these chemokines in the tumor microenvironment (21). Given our observations on Treg abundance, we next sought to investigate the impact of LXR activation on chemokine expression by TAMs from 3LL-R tumor-bearing mice. Interestingly, the treatment of WT mice with T1317 resulted in the reduced expression of Ccl17 in MHCII<sup>high</sup> TAMs, but not in TAMs from LXR-deficient mice (Fig. 4A and B). Culturing these cells in vitro led to a drastic drop in Ccl17 mRNA levels (compare Fig. 4A and C), which suggests that the expression of this chemokine in MHCII<sup>high</sup> TAMs is highly dependent on signals present in the tumor microenvironment. Ex vivo administration of the LXR agonist had little impact on Ccl17 expression under these conditions (Fig. 4C). A tendency for the downregulation of Ccl17 was also observed in MHCII<sup>low</sup> TAMs from T1317-treated WT mice (Fig. 4A). When WT MHCII<sup>low</sup> TAMs were treated ex vivo with the LXR agonist, clear inhibitory effects were observed on Ccl17 and Ccl22 expression (Fig. 4C), suggesting that the production of such chemokines by this TAM population may also be susceptible to downregulation by LXRs provided that the agonist can reach the (hypoxic) areas within the tumor where these cells reside.

We further explored whether the pharmacologic activation of LXRs affects other pathways that may be involved in the maintenance of an immunosuppressed tumor microenvironment. Both MHCII<sup>high</sup> and MHCII<sup>low</sup> TAMs were isolated from established tumors and treated with the LXR agonist ex vivo. When incubated with splenocytes, both TAM subsets were able to suppress T-cell proliferation (Fig. 4D). Interestingly, the pretreatment with the LXR agonist counteracted partially the suppressive capacity of TAMs. In contrast, their capability to phagocytose latex microspheres was not downregulated by the agonist (Fig. 4E).

Microarray studies were performed to evaluate global effects on gene expression and some of the actions were validated in independent experiments through qPCR analysis (Fig. 4F and G; Supplementary Figs. S2–S4). The expression of the isofoms LXRβ and LXRβ of their heterodimeric partners RXRα and RXRβ was confirmed in both TAM subpopulations and stimulation with the LXR agonist did not change their expression levels (Supplementary Fig. S2A), as is the case in many other cellular systems. Low levels of RXRγ were detected in these cells. As expected, several genes previously recognized as direct targets of the LXR-RXR heterodimer were induced in both TAM subsets (Supplementary Fig. S2B). In contrast to observations in other cell types, however, no induction of Apeo expression was observed in TAMs treated with T1317 (Supplementary Figs. S2B and S2C). Similar results were obtained from whole tumors exposed to the LXR agonist in vivo (Supplementary Fig. S2D).

We used the gene expression profiling data to further characterize the effects of the LXR agonist on selected markers of macrophage activation, including surface markers, enzymes, cytokines, and chemokines other than Ccl17/Ccl22 (Supplementary Fig. S3). As demonstrated in previous work (21), MHCII<sup>high</sup> and MHCII<sup>low</sup> TAMs differ in the levels of expression of a number of activation markers. The expression of most of the genes selected for this analysis was unaffected by the LXR agonist. However, significant downregulation was observed in the expression of several markers of activation (Cd68, Nos2, Il1b, Il10, Il4ra, Mmp9, and Ptges) in T1317-treated MHCII<sup>high</sup> TAMs.

In parallel, whole profiling analysis revealed that different subsets of genes were repressed (>25% repression) by T1317 in MHCII<sup>high</sup> and MHCII<sup>low</sup> TAMs (Supplementary Fig. S4A). An unbiased GO analysis of genes repressed by T1317 in MHCII<sup>high</sup> TAMs showed the enrichment of several biological processes, including the positive regulation of nitric oxide and reactive oxygen species (ROS) biosynthesis (Supplementary Fig. S4B), which are important biological mechanisms contributing to the maintenance of an immunosuppressive state by tumor-infiltrated myeloid cells. No significant enrichment of specific biological processes was observed among the genes repressed by T1317 in MHCII<sup>low</sup> TAMs. In addition, we selected a list of genes with reported involvement in immunosuppression in tumors and/or in the acquisition of a macrophage alternative activation phenotype. T1317 repressed the expression of several genes within this category in MHCII<sup>high</sup> TAMs and MHCII<sup>low</sup> TAMs (Fig. 4F). The fact that the agonist was able to repress a larger set of genes in MHCII<sup>high</sup> TAMs (Fig. 4F and G) suggests differences between both cell subpopulations in the repertoire of genes susceptible to LXR-mediated repression.

**Synthetic LXR agonists inhibit the induction of Ccl17 and Ccl22 by IL4 or GM-CSF**

Ccl17 and Ccl22 expression in cells from the monocyte-macrophage lineage is regulated by GM-CSF and IL4 (40–42). Moreover, GM-CSF has been shown to induce Ccl17 expression in MHCII<sup>high</sup> TAMs in vivo (18). Considering the repressive effect of LXR agonism on Ccl17 expression in TAMs, we further explored whether synthetic LXR agonists modulate the actions of GM-CSF or IL4 in macrophages. In these experiments, bone marrow-derived macrophages, obtained from either WT or LXR<sup>−/−</sup> mice, were preincubated with the LXR agonists GW3965 or T1317 and then stimulated with IL4 or GM-CSF for different periods of time. Pharmacologic activation of the LXR pathway inhibited the expression of Ccl17 and Ccl22 induced by IL4 (Fig. 5A and B), whereas other key marker genes of the macrophage response to IL4 were not affected (Fig. 5A). The effects of the agonists on chemokine expression were drastically reduced or abolished in LXR-deficient macrophages (Fig. 5C and D), indicating that these effects largely depend on functional LXR activity. In line with the changes in gene expression, pharmacologic activation of LXRs inhibited the secretion of CCL17 and CCL22 in WT macrophages (Fig. 5E). Interestingly, the induction of these chemokines at different time points after IL4 treatment was significantly higher in LXR-deficient macrophages than in WT cells (Fig. 5E and F), suggesting that LXRs can perform basal repression of these chemokines in the absence of synthetic high-affinity agonists. Of note, the expression of LXR<sup>−/−</sup> was not reciprocally inhibited by IL4 (Fig. 5G) and the effects of the LXR agonist could not be attributed to changes in the pattern of STAT-6 phosphorylation in response to IL4 (Fig. 5H).

Stimulation with T1317 or GW3965 also resulted in lower production of Ccl17 and Ccl22 in response to GM-CSF (Fig. 6A and B). Notably, and contrary to the effects on the IL4 response, LXR agonists negatively impacted other GM-CSF target genes such as Arg1, Retnla, and Mgl2 (Fig. 6A) and GM-CSF itself downregulated Lxra expression (Fig. 6C), suggesting a more general reciprocal negative interaction between GM-CSF signaling and the LXR pathway. Nevertheless, the effects of the agonists were abolished or severely reduced in LXR-deficient cells (Fig. 6B and D).

**Pharmacologic LXR activation inhibits IRF4 expression**

Despite the use of different signaling modules, macrophage responses to IL4 and GM-CSF share the induction of the transcription factor IRF4 (41, 43). The use of IRF4-deficient (IRF4<sup>−/−</sup>) macrophages showed that the functional expression of IRF4 is required for the induction of a subset of the genes evaluated, namely Ccl17, Ccl22, Retnla, and Il10, but not for Arg1, Mrc1, and Mgl2 (Fig. 7A and B). Of note, Ccl17 and Ccl22, which are inhibited by LXR agonists during the
Figure 6.
LXR agonists inhibit the induction of Ccl17 and Ccl22 in response to GM-CSF. Bone marrow–derived macrophages from WT (A–C) or LXRα/β−/− (B and D) mice were treated with vehicle, T1317 or GW3965 (1 µmol/L) for 16 hours and then stimulated with GM-CSF (5 ng/mL) for 24 hours. In C, WT macrophages were treated with GM-CSF for the indicated periods of time. In A, C, and D, gene expression levels were determined by qPCR. Mean ± SEM. n = 3–8 (A), n = 3–4 (C), n = 4 (D) independent experiments using 1–2 biological replicates/experiment (ANOVA–Bonferroni). B, Determination of the secreted levels of CCL17 and CCL22 by ELISA. Mean ± SD; n = 3 biological replicates. Two-way ANOVA–Bonferroni. A–D, **, P < 0.01; ***, P < 0.001. Selected experimental conditions were also compared using a t test (#, P < 0.05; ##, P < 0.01).
Pharmacologic LXR Activation Modulates TAM Gene Expression

macrophage response to both IL4 and GM-CSF, were the most drastically impaired genes in IRF4<sup>−/−</sup> macrophages.

IRF4 has been shown to cooperatively bind activator protein-1 (AP1) complexes in T cells to regulate gene transcription from AP1-IRF composite elements (44). We therefore evaluated whether the induction of IRF4-dependent genes required cooperation with JUNB or JUND. Although we cannot discard redundant roles from these proteins, the upregulation of Ccl17, Ccl2, Retnla, and Il1b in response to IL4 or GM-CSF was not impaired in macrophages deficient for either JUNB or JUND (Supplementary Figs. SSA–SSD).

Interestingly, the expression of IRF4 itself during the macrophage response to IL4 or GM-CSF was inhibited by LXR agonists both at the mRNA and protein level (Fig. 7C–G). It has been recently reported that both cytokines induce the upregulation of the expression of the demethylation jumonji domain-containing protein 3 (JMJD3) upstream of the transcriptional activation of IRF4 (45, 46). However, LXR activity did not repress the expression of this enzyme in response to IL4 or GM-CSF (Supplementary Fig. S6A and S6B), which suggests that the inhibitory effects on IRF4 expression reported here are not related to upstream alterations in JMJD3.

Previous work had analyzed the binding of IRF4 across the murine genome in T cells through ChIP-seq analysis (33). By reanalyzing these data, we identified three sites with enriched binding of IRF4 in the proximity of the Ccl17 gene both in naive CD4<sup>+</sup> T cells and during Th17 cell differentiation (Supplementary Fig. S7A–S7C). To translate this finding to macrophages, we transfected Raw264.7 cells with reporter plasmids containing various IRF4-binding regions. Peak 2, located approximately 6.5 Kb upstream of the Ccl17 transcription start site, displayed strong enhancer activity in response to IL4, which was downregulated in response to IL4 or GM-CSF (Supplementary Fig. S6A and S6B), which suggests that the inhibitory effects on IRF4 expression reported here are not related to upstream alterations in JMJD3.

To determine whether functional IRF4 expression is important for the antitumoral actions of the LXR agonist in vivo, tumor progression studies were carried out in WT and IRF4<sup>−/−</sup> mice treated with either DMSO or T1317. Tumors acquired larger volumes in IRF4<sup>−/−</sup> mice as compared with WT mice (Fig. 7M). Notably, there was lower infiltration of lymphocytes in general (not only Tregs) in the tumors in IRF4<sup>−/−</sup> mice (Fig. 7N), which may explain the increased tumor growth in these mice. Nevertheless, the tumors in the IRF4<sup>−/−</sup> background were not as responsive to T1317 as the tumors grown in WT mice, which supports the importance of IRF4 as a mediator of the antitumoral actions of pharmacologic LXR activation.

**Discussion**

In this study, we have identified novel roles of activated LXRs in the control of tumor growth. Despite the fact that the synthetic LXR agonist T1317 is able to directly inhibit cancer cell proliferation in vitro, its antitumoral potential is compromised in LXR-deficient mice carrying WT tumors, thus highlighting the importance of LXR functional activity in the tumor microenvironment over direct anti-proliferative effects in a cancer cell-autonomous manner. Indeed, most studies, including ours, have only demonstrated significant effects of LXR agonists at directly inhibiting the proliferation of transformed cells when used at relatively high doses (5–10 μmol/L; refs. 47–49), in

![Figure 7](https://example.com/figure7.png)
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contrast to lower doses required for the activation of metabolic pathways or for the repression of inflammatory gene expression in primary cells.

Previous work by Tavazoie and colleagues also placed the focus on the role of LXRs in the tumor microenvironment by showing the downregulation of the frequency of MDSCs and an increased CTL activity within tumors in response to high doses of LXR agonists (11). Mechanistic studies using melanoma B16F10 cells indicated that LXR activation, in an ApeE-dependent manner, resulted in increased apoptosis of MDSCs. In the work presented here, the characterization of infiltrated myeloid cells from lung carcinoma tumors indicated that LXR agonism slightly reduced the relative frequency of MHCII\textsuperscript{high} TAMs without affecting other, more abundant populations in the tumor, including those populations theoretically enriched for MDSCs. It is possible that differences in the type of tumor, in the type of agonist, or in the stage of tumor progression at the time of agonist administration help explain why MDSC frequencies were not decreased in our model. In addition, the dose of agonist used during development of LLC tumors in our study was considerably lower (15 mg/kg/day) than the doses at which LXR agonists inhibited intratumoral MDSC frequencies (80–100 mg/kg/day) in the work mentioned above. In this sense, ApeE mRNA levels were not upregulated in whole tumors from mice treated with the dose of T1317 used in our study nor in TAMs exposed to the agonist in vitro, in contrast to the increase in other well-established LXR targets genes.

TAMs may locally expand in response to M-CSF (50). Although it is not clear which signals specifically modulate the proliferation or survival of MHCII\textsuperscript{high} TAMs, we have previously shown that LXR agonists inhibit macrophage proliferation induced by several growth factors, including M-CSF and GM-CSF (37). For these reasons, we cannot discard some contribution of the LXR pathway to the control of local TAM proliferation. However, the decreased proportion of MHCII\textsuperscript{high} TAMs was not consistently accompanied by reduced absolute numbers of these cells within the tumor, which suggests that the changes in their frequency are mostly influenced by the relative distribution of other intratumoral cells.

The results from this work strongly suggest that combined actions that result from the modulation of TAM responses contribute to the antitumoral effects of pharmacologic LXR activation. The LXR agonist is able to repress several genes that play key roles in the protumoral program of TAMs. In fact, the capability of TAMs to suppress T-cell proliferation in vitro was reduced by the LXR agonist. Notably, different subsets of genes were repressed by T1317 in MHCII\textsuperscript{high} TAMs and MHCII\textsuperscript{low} TAMs. For example, Nos2 expression was selectively repressed in MHCII\textsuperscript{high} TAMs, whereas Trem1 expression was inhibited in MHCII\textsuperscript{low} TAMs. We do not have a clear explanation for these differences, but we cannot discard that hypoxic conditions may result in intracellular changes increasing the resistance of some genes to LXR-mediated repression. In addition, previous work has reported differences in the mechanisms used by these two subpopulations to suppress T-cell activation, with MHCII\textsuperscript{high} TAMs being more dependent on NOS2 activity (21). Therefore, further understanding of the relative contribution of some of these genes in the suppressive activity of TAM subpopulations is still warranted.

In addition to direct suppressive mechanisms, MHCII\textsuperscript{high} TAMs are major contributors to CCL17 and CCL22 production (21), which are important signals for Treg recruitment to the tumor. The analysis of the actions of the LXR agonist on TAM subpopulations in vivo showed the significant repression of Cdc17 in MHCII\textsuperscript{high} TAMs and a tendency for such repression in MHCII\textsuperscript{low} TAMs. The effects of the LXR agonist on the latter population were more evident when the cells were exposed to the agonist ex vivo, suggesting that the availability of the LXR ligand might be lower in hypoxic areas of the tumor. Treatment with the LXR agonist did not impact the frequency of Tregs in the spleen or their capacity to proliferate in vitro, which supports the notion that the repression of CCL17 production in TAMs has an important role in the reduction of the abundance of intratumoral Tregs upon LXR activation. Despite the fact that Tregs exist in low numbers in LLC tumors, depletion of these cells in vivo diminished tumor progression in mice. Importantly, the pharmacologic activation of LXRs did not efficiently reduce tumor growth under these conditions, suggesting that the inhibitory effects on Treg abundance are an important mechanism mediating the antitumoral actions of the LXR agonist. Because LXR activation did not inhibit functional aspects on isolated Tregs, such as the expression of anti-inflammatory cytokines or their capacity to suppress T-cell proliferation ex vivo, we conclude that the importance of this mechanism lies in the ability of the LXR agonist to downregulate the abundance of Tregs within the tumor, in correlation with reduced chemokine expression by TAMs, rather than in compromising the immunosuppressive potential of the Treg itself.

Our in vitro studies indicate that, in the absence of a synthetic LXR agonist, saturating doses of recombinant IL4 or GM-CSF are able to induce higher production of CCL17/22 in LXR-deficient macrophages than in the WT counterparts. However, within a more complex entity, the tumor microenvironment, WT and LXR-deficient MHCII\textsuperscript{high} TAMs express similar levels of these chemokines in the absence of pharmacologic LXR activation, in line with the fact that tumors grown in LXR-deficient mice do not display a drastic increase in Treg abundance. Therefore, whether or not LXR-deficient cells produce higher levels of the chemokine might depend on additional signals that are present in each setting. Similar observations in other cellular/physiologic scenarios (5, 11, 51, 52) support the notions that LXR biology is complex and that genetic ablation and pharmacologic activation of these nuclear receptors do not necessarily result in opposite biological effects.

At the molecular level, the results from this work propose IRF4 targeting as a relevant mechanism that links pharmacologic LXR activation with the downregulation of Ccl17. On one hand, the induction of this chemokine by IL4 or GM-CSF is fully dependent on IRF4 functionality, and LXR activity is able to downregulate IRF4 expression in both settings. On the other hand, IRF4 overexpression blocked the capability of LXR agonists to negatively modulate the transcriptional enhancing activity of a region containing IRF4-binding sites upstream of the Ccl17 promoter (and downstream of Ccl22). It is plausible that the downregulation of IRF4 expression may also contribute to the repressive actions of LXR agonists on other genes induced by GM-CSF.

Within tumors, in correlation with the inhibitory effects on Ccl17 expression, the levels of If4 were downregulated in MHCII\textsuperscript{high} TAMs in response to the administration of the LXR agonist in vivo. However, interpretation of the data from the systemic deficiency in IRF4 should be done with caution. IRF4 is involved in the development and function of different subsets of CD4\textsuperscript{+} T cells, not only Tregs, and in the generation of Th1 responses (reviewed in ref. 53). The reduced infiltration of CD8\textsuperscript{+} and CD4\textsuperscript{+} lymphocytes in LLC tumors most probably compromises the development of antitumoral immune responses, which would explain the increased tumor growth in the IRF4-deficient model despite Treg numbers being downregulated. In line with this notion, systemic IRF4
deficiency promoted an immunosuppressed tumor microenvironment in other models of cancer (54). Importantly, these mice did not respond to pharmacologic LXR activation as efficiently as WT mice, which suggests that IRF4 plays a relevant role in the antitumoral actions of the LXR agonist. However, we are aware that a deficiency of IRF4 specifically in macrophages would help to better answer this question. Future studies will be addressed in this direction.

In this work we have validated that pharmacologic activation of LXRs also exerts inhibitory effects on the induction of the Ifn4/Ccl17 axis in human macrophages. However, whether or not the expression levels of Ifn4 specifically in TAMs have prognostic value in human tumors remains elusive. Importantly, the accumulation of CCL17-expressing macrophages has been described in lung adenocarcinoma, favoring the recruitment of effector Tregs (55). In addition, high expression levels of intratumoral CCL17 have been associated with poorer overall survival rates in hepatocellular carcinoma (56). Moreover, Kaplan–Meier analysis using the R2. Genomics Analysis and Visualization Platform (http://r2.amc.nl) to assess the probability of overall survival in patient cohorts of kidney renal clear cell carcinoma from The Cancer Genome Atlas project, as well as in non–small cell lung cancer (57), indicates that high expression of CCL17 also associates with poorer overall survival probability in these types of cancer (Supplementary Fig. S8A and S8B). On the other side, in a mouse model of subcutaneous tumor development, the downregulation of CCL17 upon transduction of short hairpin RNA in CT26 cancer cells resulted in decreased Treg infiltration within tumors and suppressed tumor growth (58), suggesting that targeting the intratumoral levels of CCL17 may represent a promising strategy against cancer.

Taken together, this work reveals unappreciated roles for pharmacologic LXR activation in the control of several macrophage-mediated mechanisms contributing to the maintenance of an immunosuppressive microenvironment (Supplementary Fig. S9), thus providing novel insights about the mechanisms of action of LXR agonists as therapeutic drugs against cancer.

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**Authors’ Contributions**

J. Carbó: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing. T.E. Leon: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing. J. Font-Díaz: Conceptualization, formal analysis, validation, investigation, visualization, methodology, writing-original draft, writing-review and editing. J.C. Escol-Gil: Formal analysis, funding acquisition, investigation, visualization, methodology, L. Cédó: Formal analysis, investigation, visualization, methodology. J.C. Escol-Gil: Formal analysis, funding acquisition, investigation, visualization, methodology. J. Font-Díaz: Conceptualization, methodology, writing-review and editing. A.F. Valledor: Conceptualization, resources, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing-original draft, project administration, writing-review and editing.

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