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The STAT3 inhibitor galiellalactone inhibits the generation of MDSC-like monocytes by prostate cancer cells and decreases immunosuppressive and tumorigenic factors

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Abstract

Background: The transcription factor signal transducer and activator of transcription 3 (STAT3) is implicated in cancer drug resistance, metastasis, and immunosuppression and has been identified as a promising therapeutic target for new anticancer drugs. Myeloid-derived suppressor cells (MDSCs) play a major role in the suppression of antitumor immunity and STAT3 is involved in the accumulation, generation, and function of MDSCs. Thus, targeting STAT3 holds the potential of reversing immunosuppression in cancer. This study aims to investigate the effect of the small molecule STAT3 inhibitor galiellalactone on prostate cancer cell– induced generation of MDSCs from monocytes and the effect on immunosuppressive factors and inflammatory cytokines.

Methods: Primary human monocytes were cocultured with prostate cancer cells (DU145, PC3, and LNCaP-IL6) or with conditioned medium (CM) from prostate cancer cells in the presence or absence of the STAT3 inhibitor galiellalactone. Monocytes were analyzed by flow cytometry for an MDSC-like phenotype ($CD14^+$ $HLA\text{-}DR^{-/lo}$). The secretion and gene expression of immunosuppressive factors and inflammatory cytokines from prostate cancer cells and monocytes were investigated.

Results: Galiellalactone blocked the prostate cancer cell–induced generation of MDSC-like monocytes with an immunosuppressive phenotype ex vivo. Monocytes cultured with CM from prostate cancer cells showed increased expression of phosphorylated STAT3. Prostate cancer cells increased the expression of interleukin1β (IL1β), IL10, and IL6 in monocytes which was inhibited by galiellalactone. In addition, galiellalactone decreased indoleamine 2,3-dioxygenase gene expression in monocytes. Galiellalactone reduced the levels of IL8 and granulocyte macrophage-colony stimulating factor in prostate cancer cells per se.

Conclusion: The STAT3 inhibitor galiellalactone may prevent the prostate cancer cell–induced generation of MDSCs and reverse the immunosuppressive mechanisms caused by the interplay between prostate cancer cells and MDSCs. This is a potential new immunotherapeutic approach for the treatment of prostate cancer.

KEYWORDS
cancer therapy, castration-resistant prostate cancer, myeloid-derived suppressor cells, signal transducer and activator of transcription 3, STAT3 inhibitor

1 | INTRODUCTION

Prostate cancer is the second most common cancer in men according to the World Health Organization, with 1.1 million newly diagnosed men and 307 000 reported deaths worldwide in 2012.[1] Novel therapeutic agents for treatment of advanced and metastatic castration-resistant prostate cancer (mCRPC) include cabazitaxel, abiraterone acetate, enzalutamide, and radium-223, which all have shown survival benefits. However, most patients become treatment-resistant within a few years.[2,3] Furthermore, immunotherapies have shown little efficacy in prostate cancer until today.[4] There is an urgent need to develop novel therapies for treatment-resistant prostate cancer.

Cancer cells may avoid the immune defense by inhibiting immune responses or by activating immunosuppressive mechanisms, creating a favorable tumor microenvironment that leads to cancer cell survival, growth, and metastatic spread. The transcription factor Signal Transducer and Activator of Transcription 3 (STAT3) is implicated in acquired drug resistance, metastasis, and immune suppression and has been identified as a relevant and promising target for the development of new drugs against cancer.[5-7] The STAT3 signaling pathway is highly involved in the interaction between cancer cells and the tumor microenvironment, particularly in the inhibition of immune responses and induction of immunosuppressive cells, thus promoting immune evasion.[8,9]

Myeloid-derived suppressor cells (MDSCs) are known to play a major role in the suppression of antitumor immunity and STAT3 signaling is involved in the accumulation, generation, and ultimately, functioning of MDSCs.[5,8,10] Elevated levels of MDSCs have been found in the peripheral blood and in tumor tissue of prostate cancer patients, and the levels correlate with disease progression.[11-15] MDSCs are immature myeloid cells with potent immunosuppressive functions in the tumor microenvironment. MDSCs act by inhibiting T cells through various mechanisms including an increased arginase-1 activity causing L-arginine depletion, expression of inducible nitric oxide synthase leading to increased levels of reactive oxygen species and nitric oxide, and by elevated levels of indoleamine 2,3-dioxigenase (IDO) causing a reduction in local tryptophan levels. Furthermore, MDSCs secrete the immunosuppressive factors IL10 and transforming growth factor β. STAT3 has been shown to regulate the expression of both the immunosuppressive proteins IDO and arginase-1 and the secretion of tumor promoting and immunosuppressive factors eg, IL6, IL10, and vascular endothelial growth factor (VEGF)[9,16,17] thus making STAT3 a relevant target for alleviating immunosuppression.

MDSCs have been identified as targets for cancer therapy and to improve immunotherapy and in this context targeting STAT3 in MDSCs is suggested to be a promising therapeutic approach.[5,18-20] Inhibiting STAT3 in MDSCs isolated from patients with prostate cancer using STAT3 small interfering RNA resulted in MDSCs with decreased immunosuppressive activity[12] and the tyrosine kinase inhibitor sunitinib was shown to inhibit STAT3 in MDSCs and to reduce MDSC levels in renal cell carcinoma.[21] Galiellalactone is a STAT3 inhibitor exerting its effect through inhibition of the expression of STAT3 regulated genes by directly binding to STAT3, thus blocking the protein-DNA interaction.[22,23] Galiellalactone inhibits the proliferation of prostate cancer cells expressing active phosphorylated STAT3 (pSTAT3) and induces apoptosis by downregulation of STAT3-activated genes.[23] In previous studies, we have demonstrated that galiellalactone inhibits tumor growth and metastatic spread in an orthotopic prostate cancer mouse model.[24]

There are limited treatment options for metastatic treatment-resistant prostate cancer patients and existing immunotherapies have shown little efficacy in prostate cancer.[4] However, targeting the immunosuppressive environment in prostate cancer may improve treatment outcomes. Thus, inhibition of STAT3 in this disease setting emerges as a very interesting therapeutic approach.

In this study, we aimed to investigate whether the STAT3 inhibitor galiellalactone could block the generation of cells with an MDSC-like phenotype derived from primary human monocytes, in the presence of prostate cancer cells, ex vivo. We also investigated the effect of galiellalactone on immunosuppressive factors in the generated MDSC-like monocytes and the secretion of inflammatory cytokines and tumorigenic factors from both prostate cancer cells and monocytes.

2 | MATERIALS AND METHODS

2.1 | Isolation of peripheral blood monocytes from metastatic prostate cancer patients and MDSC analysis

Peripheral blood was collected in EDTA tubes from patients with mCRPC (n = 9) and analyzed within 24 hours. Peripheral blood mononuclear cells (PBMCs) were enriched by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). Permission was obtained from the Regional Ethical Review Board in Lund (Dnr 2013/400). PBMCs were analyzed for monocytic MDSCs ($CD11b^+$ $CD14^+$ $HLA\text{-}DR^{-/lo}$) using flow cytometry analysis as described below. $CD14^+$ $HLDR^{-/lo}$ cells were isolated from PBMCs from patients with mCRPC using BD FACSAria Cell Sorter and subjected to cytospin and analyzed for pSTAT3 expression as described below.

2.2 | Isolation of monocytes from healthy donors

PBMCs were isolated from healthy donors by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences).

Permission was obtained from the Regional Ethical Review Board in Lund (Dnr 2017/949). Monocytes were isolated from PBMCs by magnetic sorting using Monocyte Isolation Kit II (Miltenyl Biotec).

2.3 | Flow cytometry analysis

Flow cytometry analysis of PBMCs from healthy donors and from patients with mCRPC and isolated monocytes from healthy donors was performed on FACSVerse (BD Biosciences). The antibodies used were anti-CD11b (clone ICRF44), anti-HLA-DR (clone G46-6), and anti-CD14 (clone M5E2) from BD Biosciences. Dead cells were identified by 7-AAD staining and excluded from the analysis. FlowJo v10.0 software was used for subsequent analyses.

2.4 | Prostate cancer cell culture and preparation of conditioned medium

The human prostate cancer cell lines DU145, PC3 (both from the American Type Culture Collection, [ATCC]) and long-term interleukin-6 (IL6) stimulated LNCaP cells (LNCaP-IL6 cells; kind gift from Prof Zoran Culig, Innsbruck)[25] were used. The prostate cancer cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. LNCaP-IL6 cells were maintained in the above medium supplemented with 5 ng/mL IL6 (Sigma Aldrich, St. Louis, MO). All cells were incubated at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. For the preparation of conditioned medium (CM), prostate cancer cells were cultured in RPMI-1640 supplemented with 10% human serum (Sigma Aldrich). After 48 hours the medium was collected, centrifuged at 3000 rpm for 30 minutes at 4°C. The supernatant was stored at −80°C until use. The DU145 and PC3 cell lines were authenticated using Short Tandem Repeat analysis performed by ATCC. Cells were used at low passages and cultured for less than 3 months. Cells were routinely tested for and found free of mycoplasma.

2.5 | Monocyte and prostate cancer cell coculture

Monocytes were cultured in RPM1-1640 supplemented with 10% human serum (Sigma Aldrich) and 1% penicillin-streptomycin. For coculture experiments, monocytes were cultured with the prostate cancer cell lines DU145, PC3, or LNCaP-IL6 in transwells (Costar) allowing cancer cells and monocytes to share culture medium. Monocytes were also cultured with CM from prostate cancer cells without the presence of prostate cancer cells. Monocytes were cultured with or without galiellalactone for 72 hours before further analysis. Galiellalactone was provided by Glactone Pharma AB (Helsingborg, Sweden).

2.6 | Cytometric bead array for human inflammatory cytokines

Monocytes and prostate cancer cells were cultured with or without the presence of galiellalactone for the indicated time points. Cytokine secretion was evaluated in supernatants of prostate cancer cell cultures and monocyte cultures using Cytometric Bead Array Human Inflammatory Cytokines (BD Bioscience) according to the manufacturer's instructions. The cytokines IL8, IL1β, IL6, tumor necrosis factor (TNF), IL10, and IL12p70 were analyzed. The levels of cytokines were evaluated using CytoFLEX from Beckman Coulter Inc with subsequent analysis using FlowJo software.

2.7 | Cytospin and immunocytochemistry

Suspensions of monocytes cultured for 72 hours in CM from prostate cancer cells or MDSCs isolated from mCRPC patients were placed in a cytospin funnel clamped to a glass slide and spun at 800 rpm for 2 minutes. The slides were air-dried, fixed in 4% formaldehyde for 10 minutes, and permeabilized with 1% Triton-X Tris buffer, pH 7.6 for 1 hour. The slides were subjected to immunohistochemistry using Dako Autostainer Plus En Vision™+ Kit (Dako). Cells were stained using the antibody anti-pSTAT3 Tyr-705 (ab76315; Abcam).

2.8 | Western blot

Prostate cancer cell lysates were prepared and analyzed by Western blot analysis as in a previous publication.[22] Primary antibodies used were anti-STAT3 (#4904; Cell Signaling Technology), anti-pSTAT3 Tyr-705 (ab76315; Abcam) and anti-beta-actin (#691001; BioMedicals MP) was used as loading control.

2.9 | Quantitative real-time polymerase chain reaction

The gene expression of IL6, IL8, VEGF, granulocyte macrophage-colony stimulating factor (GM-CSF), IL23, arginase-1, and IDO was investigated in monocytes and prostate cancer cells with quantitative real-time polymerase chain reaction. The messenger RNA (mRNA) expression in monocytes was investigated after 72 hours and in prostate cancer cells after 24 hours. The RNA was extracted with the use of a RNAeasy Kit (Qiagen). For internal control the housekeeping genes *SDHA* (sense: TGGGAACAAGAGGGCATCTG; antisense: CCA CCACTGCATCAAATTCATG) and *GAPDH* (sense: TGCACCACC AACTGCTTAGC; antisense: TGGCATGGACTGTGGTCATGAG) were used for monocytes and *YWHAZ* (sense: ACTTTTGGTACATT GTGGCTTCAA; antisense CCGCCAGGACAAACCAGTAT), and *GAPDH* for prostate cancer cells. Gene expression was normalized against the geomean of the housekeeping genes to determine the relative expression levels of the genes of interest. Genes that were

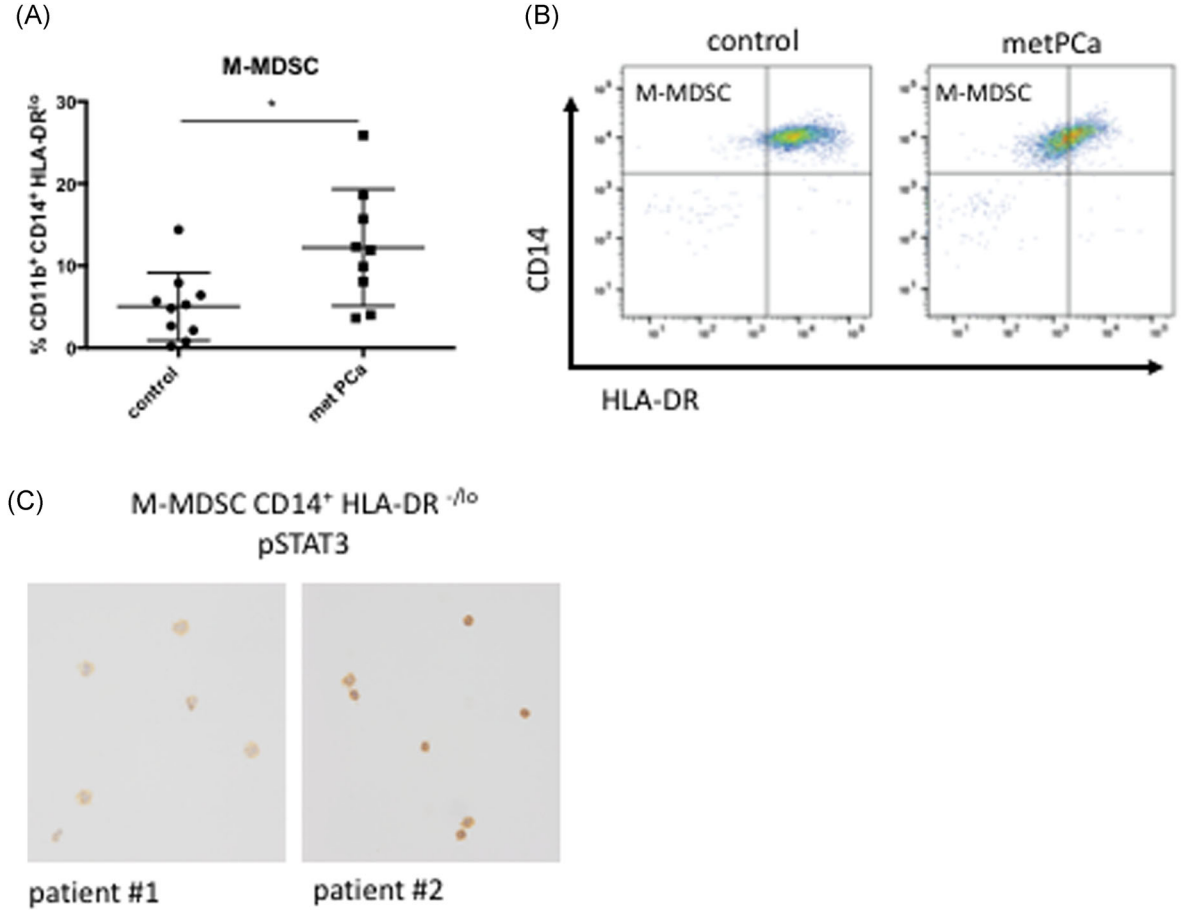

FIGURE 1 Monocytic MDSCs are increased in metastatic prostate cancer patients and express pSTAT3. A, The level of monocytic MDSCs (M-MDSC; $CD11b^+$ $CD14^+$ $HLA\text{-}DR^{-/lo}$) was significantly higher in PBMCs from metastatic prostate cancer patients (met PCa; n = 9) compared to healthy controls (n = 10). B, Representative plot of $CD11b^+$ PMBCs gated for $CD14^+$ $HLA\text{-}DR^{-/lo}$ M-MDSCs from healthy donors (control) and metastatic prostate cancer patients (met PCa). C, Immunohistochemistry for pSTAT3 was performed on isolated M-MDSCs ($CD14^+$ $HLA\text{-}DR^{-/lo}$) from PBMCs of two individual patients with metastatic prostate cancer. MDSCs, myeloid-derived suppressor cells; PBMCs, peripheral blood mononuclear cells; pSTAT3, phosphorylated STAT3 [Color figure can be viewed at wileyonlinelibrary.com]

analyzed were *IL8* (sense: ACTGAGAGTGATTGAGAGTGGAC; antisense: AACCCTCTGCACCCAGTTTTC), *IL6* (sense: GGCACTGGC AGAAAACAACC; antisense: GCAAGTCTCCTCATTGAATCC), *ARG1* (sense: GGCTGGTCTGCTTGAGAAAC; antisense: CTTTTCCCACAG ACCTTGGA), *IDO* (sense: GGCACACGCTATGGAAAACT; antisense: CGGACATCTCCATGACCTTT) and *CSF2* (GM-CSF) (sense: CAGCC ACTACAAGCAGCACT; antisense: CCAGCAGTCAAAGGGGATGA) and *VEGF* (sense: TTTTGCTTGCCATTCCCCAC; antisense: GTCACTC ACTTTGCCCCTGT), and *IL23* (sense: CAAAGCAAGTGGAAGTGGGC; antisense: AGCAACAGCAGCATTACAGC).

2.10 | Statistical analysis

Statistical analysis was performed using GraphPad Prism and analysis of variance followed by Dunnett's multiple comparison test, Kruskal-Wallis uncorrected Dunn's test or Friedman nonparametric paired test. Data are presented as ± standard error of the mean. Statistical significance was considered at $P \leq .05$.

3 | RESULTS

3.1 | Monocytic MDSCs are increased in the peripheral blood of patients with metastatic prostate cancer

The levels of monocytic MDSCs were investigated in PBMCs from nine mCRPC patients and ten healthy controls (Figure 1A). Significantly higher levels of MDSCs were observed in metastatic prostate cancer patients as compared to controls (Figure 1A and 1B). MDSCs isolated from two individual prostate cancer patients were shown to express pSTAT3 by IHC staining (Figure 1C).

3.2 | STAT3 inhibition by galiellalactone prevents prostate cancer cell–induced generation of MDSC-like monocytes

To investigate if prostate cancer cells may generate monocytes with an MDSC surface phenotype ($CD14^+$ $HLA\text{-}DR^{-/lo}$), monocytes isolated

from PBMCs from healthy donors were cultured in vitro in the presence of CM from prostate cancer cells or cocultured together with prostate cancer cells in a transwell system for 72 hours (Figure 2A and 2B). Monocytes cocultured with DU145, LNCaP-IL6, and PC3 cells or in the presence of CM from DU145 and LNCaP-IL6 cells showed an increased population of $CD14^+$ $HLA\text{-}DR^{-/lo}$ cells compared to control monocytes (Figure 2A). The increase in $CD14^+$ $HLA\text{-}DR^{-/lo}$ population was most pronounced in monocytes cultured with DU145 cells or DU145 CM (Figure 2A and 2B).

Next, we investigated whether the small molecule STAT3 inhibitor galiellalactone could affect the generation of MDSC-like monocytes by prostate cancer cells. In the presence of galiellalactone we observed significantly less MDSC-like monocytes generated by prostate cancer cell cocultures or prostate cancer cell CM (Figure 2A). The inhibition of generated MDSCs using galiellalactone in the absence of prostate cancer cells and just CM indicates a direct inhibitory effect by galiellalactone on monocytes in the generation of prostate cancer induced–MDSC-like monocytes. Galiellalactone had the greatest effect on the DU145 CM generated MDSCs. Interestingly, a slight increase in $CD14^+$ $HLA\text{-}DR^{-/lo}$ cells was observed by galiellalactone alone in control monocytes. The amount of dead cells was not increased by galiellalactone treatment as measured by 7-AAD staining (Figure 2C).

Monocytes cultured in the presence of CM from prostate cancer cells showed an increased expression of active pSTAT3 compared to control monocytes (Figure 2D). Immunostaining for pSTAT3 was mainly present in the cytoplasm and occasionally in the nucleus of the monocytes. The strongest expression appeared to occur in DU145 CM cultured monocytes. The prostate cancer cell lines DU145 and LNCaP-IL6 express STAT3 and pSTAT3 constitutively and PC3 cells are negative for STAT3 and pSTAT3 (Figure 2E).

3.3 | Galiellalactone decreases IL8 and GM-CSF in prostate cancer cells

A panel of secreted cytokines (IL8, IL1β, IL6, TNF, IL10, and IL12p70) was investigated in the CM of prostate cancer cells after 48 hours of culture. High levels of IL6 and IL8 were observed in CM from DU145 and PC3 cells and low levels of IL6 and IL8 in LNCaP-IL6 cells (Figure 3A and 3B). TNF, IL12p70, IL1β, or IL10 were not detected in CM of the studied prostate cancer cells. Next, we investigated the effect of STAT3 inhibition by galiellalactone on cytokine secretion and expression in DU145 and LNCaP-IL6 cells after 24 hours of culture. Galiellalactone significantly reduced the levels of secreted IL8 from DU145 and LNCaP-IL6 cells and decreased the IL8 mRNA levels in DU145 cells (Figure 3C). Galiellalactone had no significant effect on IL6 secretion or IL6 mRNA expression in DU145 or LNCaP-IL6 cells. (Figure 3D). The already low levels of IL6 in LNCaP-IL6 cells were not affected by galiellalactone. Galiellalactone did not alter the levels of TNF, IL12p70, IL1β, or IL10 in the supernatant of prostate cancer cells (data not shown). DU145 cells expressed high levels of GM-CSF mRNA compared with LNCaP-IL6 and PC3 cells, and galiellalactone significantly inhibited GM-CSF mRNA expression in DU145 cells (Figure 3E). VEGF mRNA was expressed in DU145 and LNCaP-IL6 cells and at a lower level in PC3 cells (Figure 3F). The VEGF expression was not affected by galiellalactone.

3.4 | Inflammatory cytokine secretion in monocyte cultures and the effect of galiellalactone

Next, we investigated the levels of inflammatory cytokines secreted by monocytes and the effect on cytokine secretion by factors present in prostate cancer cell CM and the presence of galiellalactone. The levels of IL8, IL1β, IL6, TNF, IL10, and IL12p70 proteins were measured by cytometric bead array in supernatants from monocyte cultures (Figure 4). IL6 and IL8 were detected in the supernatant of monocytes as well as low levels of IL1β and IL10. TNF and IL12p70 were not detected. In the presence of prostate cancer cell CM, a significant increase in IL1β and IL10 levels in monocytes was observed (Figure 4A and 4D). Galiellalactone significantly inhibited the prostate cancer CM-induced increase in IL1β secretion and significantly reduced the levels of IL10 in monocytes (Figure 4A and 4D). IL1β and IL10 were not detected in CM from prostate cancer cells indicating that the secretion of these cytokines by monocytes was induced by prostate cancer CM (Figure 3A). IL6 levels in monocyte supernatant were significantly increased by DU145 and PC3 CM which was inhibited in the presence of galiellalactone. There was no effect of galiellalactone on the levels of IL8 in the supernatant of monocytes (Figure 4C).

3.5 | Expression of the immunosuppressive markers arginase-1, IDO, IL6, and IL23 in monocytes

We next investigated the expression of markers in monocytes involved in immunosuppressive functions of MDSCs (Figure 5). The mRNA expression of IDO, arginase-1, IL6, and IL23 was investigated in monocytes cultured with prostate cancer cell CM in the presence and absence of galiellalactone (Figure 5A and 5D). IDO gene expression in monocytes was decreased by galiellalactone, both in control monocytes and monocytes cultured with prostate cancer CM (Figure 5A). Monocytes cultured in the presence of prostate cancer cell CM showed increased IL6 mRNA levels, which was inhibited upon treatment with galiellalactone (Figure 5B), which is in accordance with the observed IL6 protein levels in monocyte supernatants (Figure 4B). Levels of arginase-1 mRNA in monocytes were unaffected by prostate cancer CM or galiellalactone (Figure 5C). IL23 mRNA expression in monocytes was not significantly affected by prostate cancer cell CM or the presence of galiellalactone (Figure 5D).

4 | DISCUSSION

STAT3 activation in monocytes is central for the generation and function of MDSCs. This study shows that the STAT3 inhibitor galiellalactone has the potential to inhibit prostate cancer cell–induced generation of primary human MDSC-like monocytes

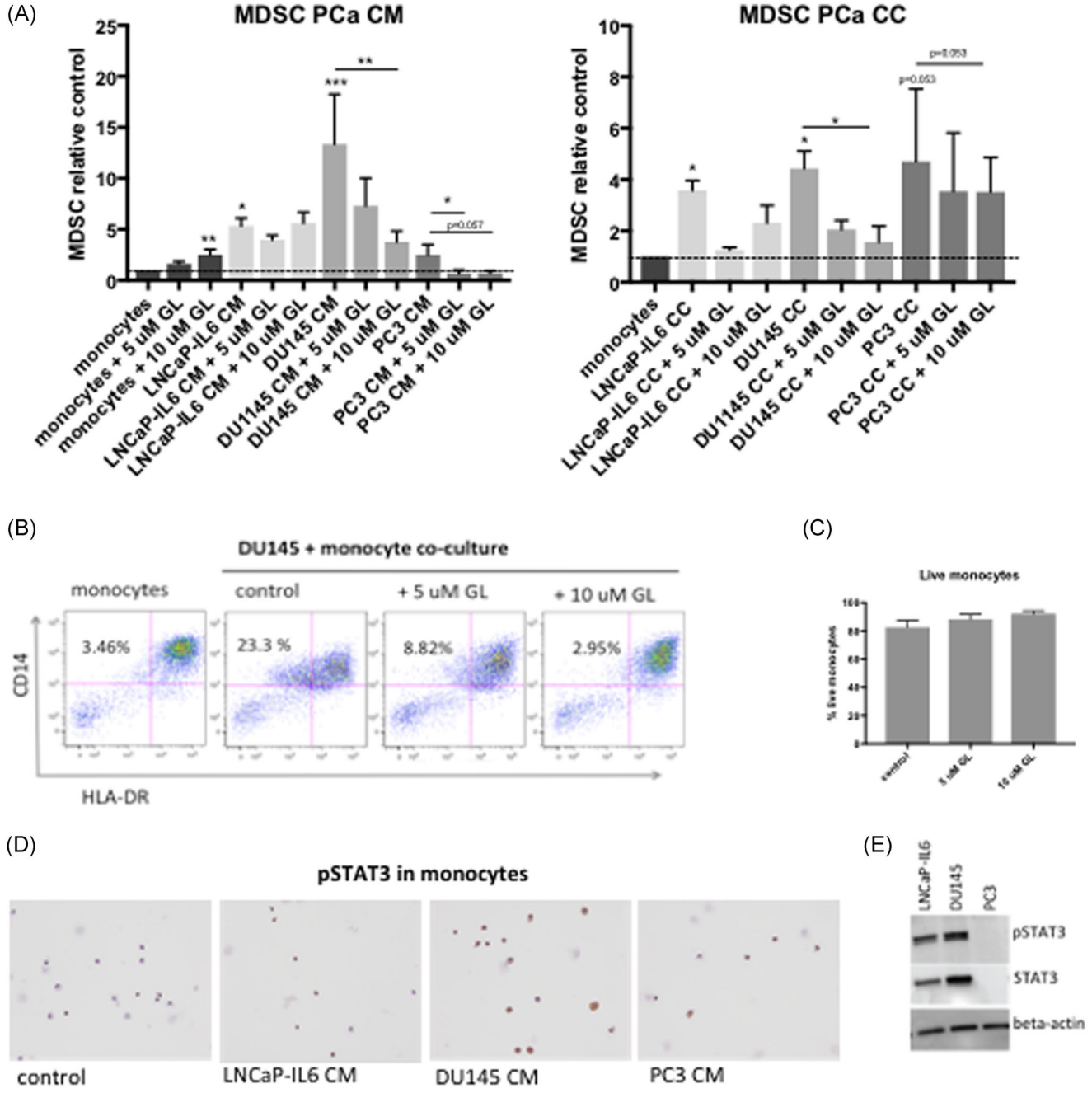

FIGURE 2 STAT3 inhibition by galiellalactone prevents prostate cancer cell–induced generation of MDSC-like monocytes. A, Monocytes cocultured (CC) with the prostate cancer cells DU145, PC3 or LNCaP-IL6 (n = 3-4), or cultured with conditioned medium (CM) from the prostate cancer cells (n = 4-6), with or without the STAT3 inhibitor galiellalactone (GL) for 72 hours and analyzed for MDSC-like monocytes ($CD14^+$ $HLA\text{-}DR^{-/lo}$). MDSC levels are expressed relative monocytes in control medium. B, Gating strategy of monocytes for MDSC-like phenotype ($CD14^+$ $HLA\text{-}DR^{-/lo}$). Representative plot of monocytes cocultured with DU145 cells in the presence and absence of galiellalactone for 72 hours. C, Effect of galiellalactone on viability of isolated monocytes. Isolated monocytes were cultured with and without galiellalactone for 72 hours. Dead cells were identified by 7-AAD staining and FACS analysis. There was no observed decrease in viability by galiellalactone treatment on monocytes (n = 6). D, Immunohistochemistry for pSTAT3 was performed on monocytes subjected to cytospin. Monocytes cultured with CM from prostate cancer cells for 72 hours showed an increased expression of pSTAT3 compared to monocytes cultured in control medium. E, Western blot analysis for the expression of pSTAT3 and STAT3 cell in lysates from the prostate cancer cells PC3, DU145, and LNCaP-IL6. β-Actin is used as a loading control. MDSC, myeloid-derived suppressor cell; pSTAT3, phosphorylated STAT3; STAT3, signal transducer and activator of transcription 3 [Color figure can be viewed at wileyonlinelibrary.com]

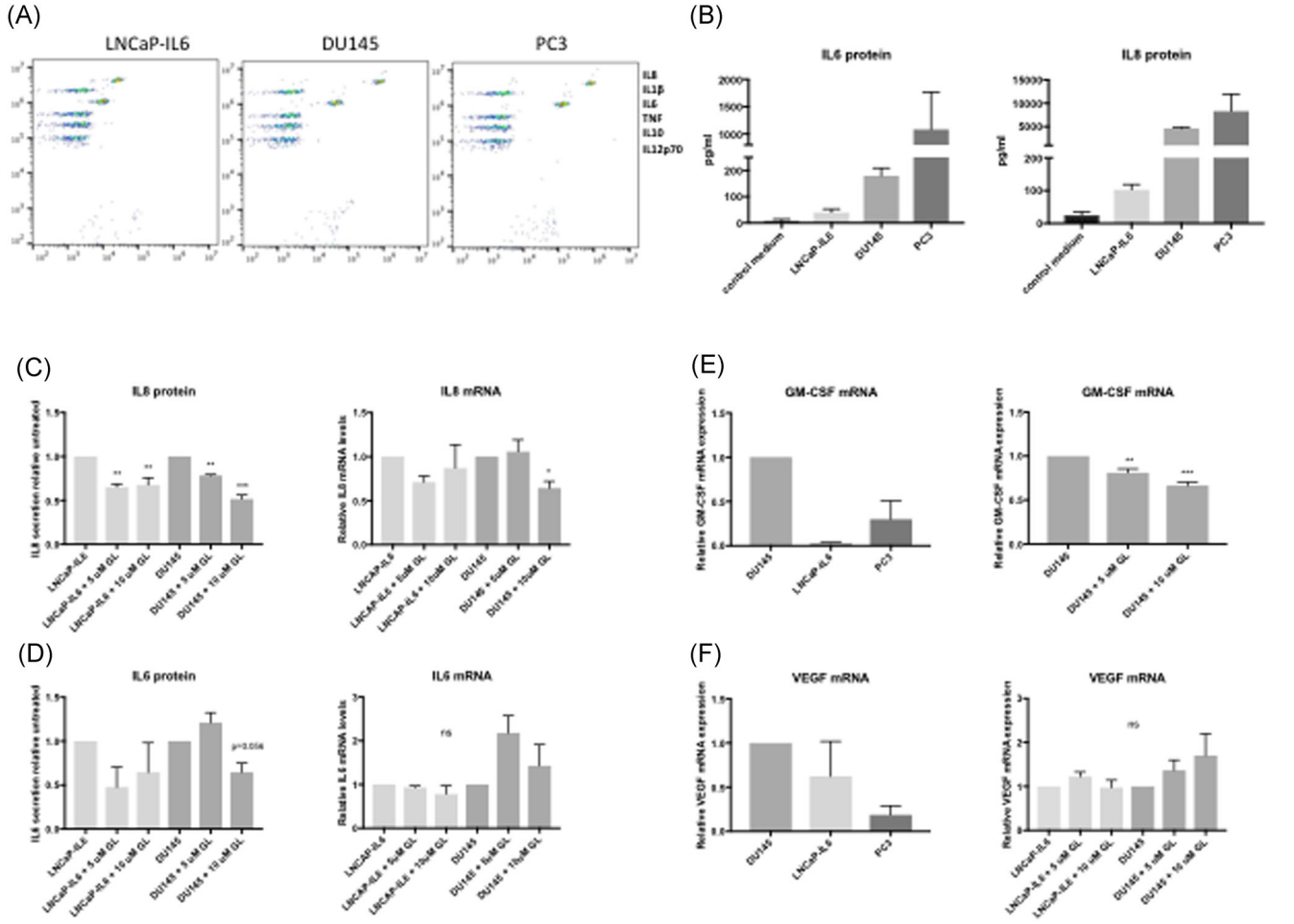

FIGURE 3 Galiellalactone inhibits IL8 and GM-CSF expression in prostate cancer cells. A, Cytokine secretion in conditioned medium (CM) from prostate cancer cells was analyzed using cytometric bead array (CBA). B, IL6 and IL8 protein levels in CM from LNCaP-IL6, DU145, and PC3 cells after 48 hours in culture as measured by CBA (n = 4). C,D, IL6 and IL8 protein levels in cell culture medium relative untreated control cells (n = 3) and IL6 and IL8 mRNA levels (n = 5) in DU145 and LNCaP-IL6 cells after treatment with galiellalactone (GL) at 5 and 10 μM for 24 hours (n = 4). E, GM-CSF mRNA expression levels in prostate cancer cells (n = 2) and the effect of GL on the gene expression of GM-CSF in DU145 cells after 24 hours (n = 4-5). F, VEGF mRNA expression levels in prostate cancer cells (n = 2) and the effect of GL on the gene expression of VEGF in DU145 and LNCaP-IL6 cells after 24 hours (n = 4-5). Data are presented as mean ± SEM. GM-CSF, granulocyte macrophage-colony stimulating factor; IL, interleukin; mRNA, messenger RNA; VEGF, vascular endothelial growth factor [Color figure can be viewed at wileyonlinelibrary.com]

ex vivo and to decrease levels of immunosuppressive and tumorigenic factors in both monocytic MDSCs and cancer cells. We observed an increased level of $CD14^{+}$ $HLA\text{-}DR^{-/lo}$ monocytic MDSCs in the peripheral blood of mCRPC patients compared to healthy controls, which is in line with previous studies in advanced prostate cancer.[13-15] The isolated monocytic MDSCs from mCRPC patients expressed pSTAT3 in accordance with observations made in various cancers.[16,26,27] These results spurred us to investigate the effect of the STAT3 inhibitor galiellalactone on the generation of MDSCs from monocytes by prostate cancer cells ex vivo.

In this study, prostate cancer cells or CM from prostate cancer cells promoted the generation of $CD14^{+}HLA\text{-}DR^{-/lo}$ MDSC-like monocytes from primary human monocytes in culture, and CM from prostate cancer cells increased the expression of pSTAT3 in monocytes. These findings are in line with previous studies, where increased expression of pSTAT3 in monocytes induced by cancer cells ex vivo was associated with MDSC generation.[26,28] In addition, we could show that markers related to MDSC immunosuppressive function such as IL6 and IL10 were increased in monocytes by prostate cancer cell CM, further supporting the generation of an MDSC phenotype in monocytes.

Galiellalactone inhibited the generation of monocytic MDSCs likely by blocking the activation and signaling of STAT3 induced by prostate cancer cell–derived factors. The effect of the STAT3 inhibitor galiellalactone on the generation of MDSC-like monocytes was evident both in cocultures with prostate cancer cells and in cultures with prostate cancer cell CM in the absence of prostate cancer cells, indicating a direct effect of galiellalactone on monocytes in preventing MDSC generation. The decrease in the MDSC-like population in the presence of STAT3 negative PC3 cells following

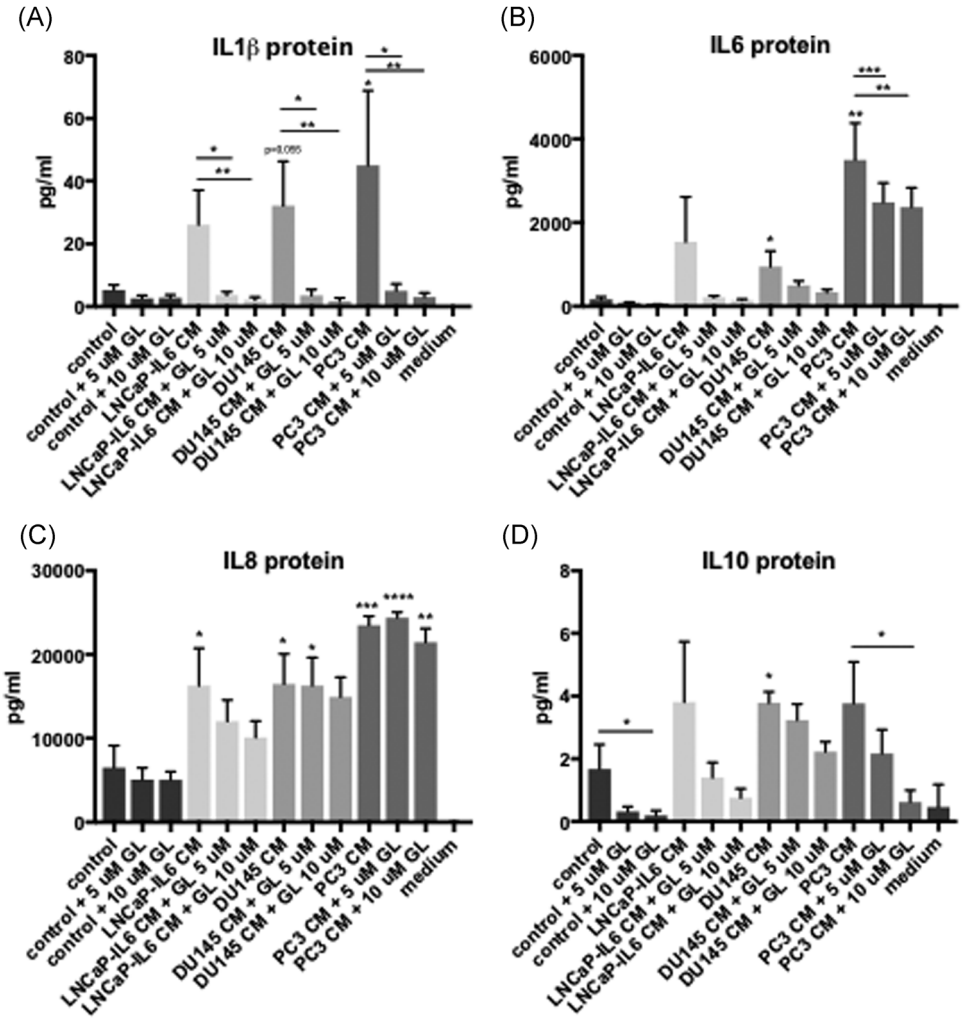

FIGURE 4 Inflammatory cytokine secretion in monocyte cultures and the effect of galiellalactone. A-D, The levels of IL1β, IL6, IL8, and IL10 in supernatants of monocytes were measured with cytometric bead array (CBA). Monocytes were cultured with conditioned medium (CM) from prostate cancer cells with or without galiellalactone (GL) for 72 hours. Data are presented as mean ± SEM; n = 6. IL, interleukin

galiellalactone treatment further suggests that the effects of STAT3 inhibition on MDSC generation are on the monocytes. However, galiellalactone may also directly act on prostate cancer cells to affect prostate cancer cell–derived factors involved in MDSC generation such as GM-CSF, which was significantly decreased by galiellalactone in DU145 cells. A small increase in $CD14^{+}HLA\text{-}DR^{-/lo}$ monocytes was observed by galiellalactone in monocytes but this was however not accompanied by increased MDSC-related functional markers or cytokines indicating that an immunosuppressive phenotype of monocytes was not induced by galiellalactone.

Factors derived from tumor cells promoting MDSC generation include IL6, IL1β, IL10, GM-CSF, granulocyte colony-stimulating factor and VEGF,[10] all known to activate STAT3 that is a central signaling pathway for generation and function of MDSCs.[8,19] Many cytokines induced by STAT3 e. g. IL6, IL10, and VEGF also function as STAT3 activators.[9] The prostate cancer cells in this study were shown to secrete IL6, GM-CSF, and VEGF factors likely involved in the observed generation of MDSC-like monocytes although additional factors not investigated may also be involved. The ability to generate MDSC-like monocytes by the different prostate cancer cell lines varied, likely reflecting the different levels and mediators produced by the cancer cells. GM-CSF is one of the most important factors for generation and survival of MDSCs and tumor derived GM-CSF has even been suggested to be responsible for the increased level of MDSCs detected in patients with cancer.[29] In addition to GM-CSF, IL6 is considered an important inducer of MDSCs.[29] Increased levels of GM-CSF and IL6 are observed in metastatic prostate cancer.[30,31] Galiellalactone had limited effect on prostate cancer cell–derived IL6 and VEGF, factors involved in MDSC generation, although GM-CSF was significantly inhibited. Together this indicates that GM-CSF is the principal effector molecule produced by prostate cancer that is capable of promoting MDSC generation, and that is inhibited by the novel STAT3 inhibitor galiellalactone.

IL10, IL1β, and IL6 expressions were increased in monocytes by CM from prostate cancer cells indicating induction of an immunosuppressive and tumorigenic phenotype. IL10 secreted by MDSCs has a potent immunosuppressive activity promoting various functions such as induction of regulatory T cells and M2 macrophage polarization.[9,10,32]

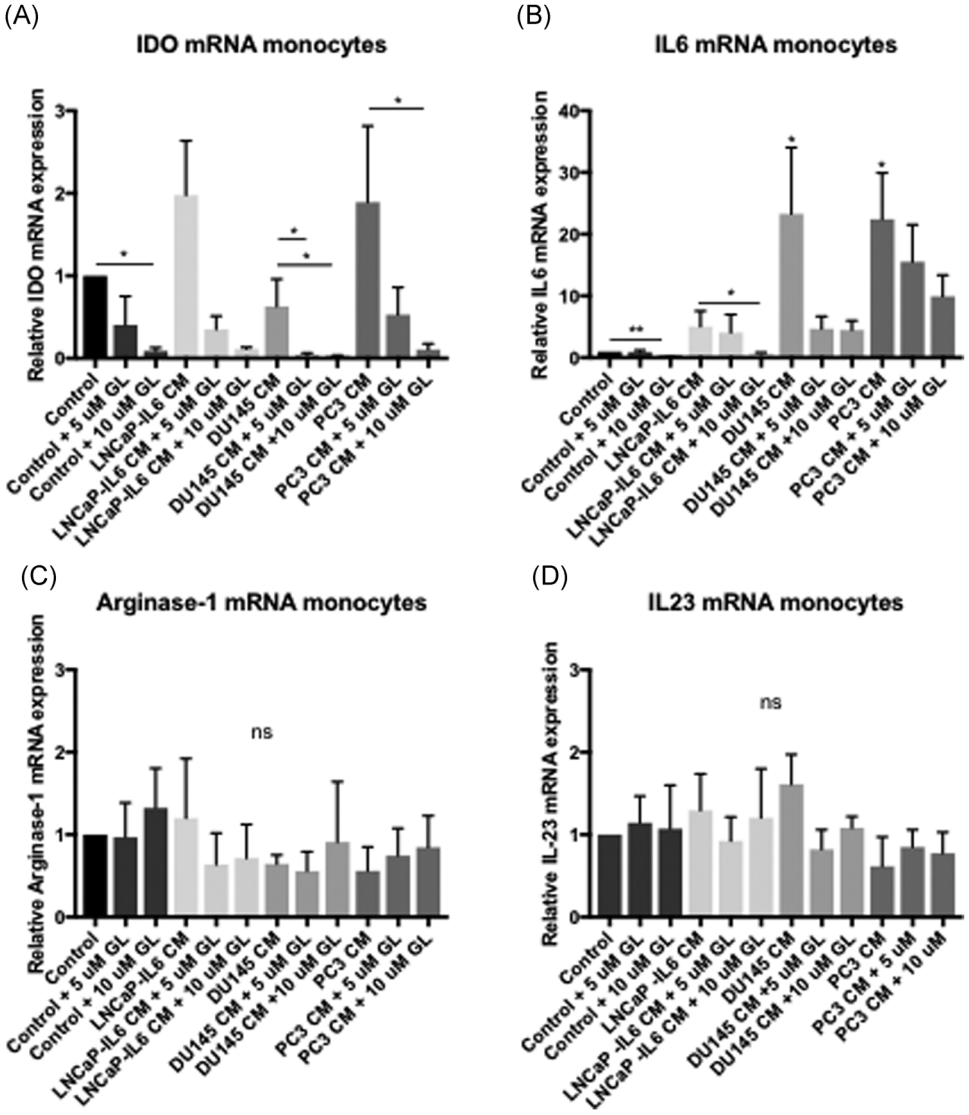

FIGURE 5 Expression of arginase-1, IDO, IL6, and IL23 in monocytes. A-D, The mRNA expression of IDO, arginase-1, IL6, and IL23 in monocytes cultured with prostate cancer cell conditioned medium (CM), with or without galiellalactone (GL) for 72 hours. The gene expression levels are presented as relative control monocytes. n = 3-5. Data are presented as mean ± SEM. IDO, indoleamine 2,3-dioxigenase; IL, interleukin; mRNA, messenger RNA

Galiellalactone significantly decreased the level of secreted IL10 indicating the possibility of inhibiting immunosuppressive actions mediated by MDSCs. IL1β secretion by monocytes was induced by prostate cancer CM. GM-CSF derived from prostate cancer cells may in part be responsible for the induced IL1β expression as GM-CSF is known to induce IL1β expression.[33] The decreased expression of IL1β following galiellalactone treatment is in line with the observation that the STAT3 inhibitor JSI-124 decreased IL1β in MDSCs.[34] IL1β is a potent inducer of several cytokines and chemokines including IL8, IL6, and TNFα. IL1β is involved in MDSC expansion and migration and may promote tumor development and metastasis.[35] Inhibiting IL1β secretion in the tumor microenvironment by galiellalactone may thus inhibit MDSCs and tumor development.

Targeting IL6 is thought to alleviate tumor immunosuppression[36] and IL6 secretion by MDSCs may exert tumorigenic effects on surrounding tumor cells as well as autocrine effects by activating STAT3. Inhibition of IL6 secretion and STAT3 activity in myeloid cells by galiellalactone may thus induce antitumoral responses and reduce MDSC generation and activity. Galiellalactone decreased the CM-induced IL6 mRNA and protein levels in monocytes, in contrast to DU145 cells where galiellalactone lacked effect on IL6 expression indicating that IL6 expression in DU145 cells may be regulated by other transcription factors than STAT3, such as nuclear factor kappa-light-chain-enhancer of activated B cells.[37,38]

Galiellalactone inhibited IDO expression in monocytes, suggesting that galiellalactone may prevent immunosuppressive activities. It has previously been shown that IDO is upregulated by IL6 and GM-CSF in MDSCs via activation of STAT3 and that STAT3 inhibition decreased this immunosuppressive activity of MDSCs.[17,34,39] Arginase-1 expression and activity in monocytes and MDSCs varies considerably between

individuals,[40] which may explain why we did not find a consistent effect on arginase-1 expression in this study. IL23 secreted by granulocytic MDSCs is observed to be involved in CRPC.[41] However, in this study, we focus on monocytic MDSCs and we did not observe a significant increase in IL23 expression in the monocytic MDSCs generated by prostate cancer cell CM. We observed decreased IL8 secretion by galiellalactone in prostate cancer cells. In prostate cancer patients, increased levels of IL8 are observed in the circulation and correlates with aggressiveness and levels of circulating granulocytic MDSCs.[11] IL8 is a potent angiogenic factor and also functions as a chemoattractant of neutrophils and monocytes,[42] thus inhibition of IL8 secretion in prostate cancer cells may inhibit chemoattractant properties and angiogenic effects in the prostate tumor environment.

To date, there are no approved direct STAT3 inhibitors used in the clinical setting. Clinical trials with the small molecule STAT3 inhibitor Napabucasin, also known as BB1608 or BB608, has shown some efficacy in colorectal cancer.[43] Furthermore, attempts to target STAT3 have been made using the STAT3 antisense oligonucleotide AZD9150 in lymphoma with moderate effect[44] and clinical trials are ongoing for additional malignancies (www.clinicaltrials.gov). As for STAT3 inhibition in prostate cancer, attempts have been made to target IL6 signaling by IL6 monoclonal antibody (Siltuximab) and although preclinical studies show positive results, so far limited antitumoral effects have been observed in clinical trials with prostate cancer patients.[45,46]

We have previously shown that galiellalactone inhibits metastatic spread and growth of pSTAT3 expressing prostate cancer cells, both in vivo and in vitro[23,24] indicating that STAT3 inhibition by galiellalactone may target both the tumor suppressive environment and tumor cells directly in prostate cancer. In this study, we have shown that STAT3 inhibition by galiellalactone also may target the myeloid cell compartment of tumors thus interrupting important immunosuppressive and tumor-promoting factors derived from both monocytes and prostate cancer cells and inhibit the generation of MDSC-like monocytes.

5 | CONCLUSION

The STAT3 inhibitor galiellalactone may reverse the immunosuppressive mechanisms caused by the interplay between prostate cancer cells and MDSCs. The results indicate that galiellalactone has powerful effects on immune cells affecting cancer growth and metastasis, further emphasizing that STAT3 inhibitors represent a potential new treatment for advanced prostate cancer.

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CONFLICT OF INTERESTS

Anders Bjartell and Rebecka Hellsten are board members of Glactone Pharma AB. Martin Johansson is the CEO of Glactone Pharma Development AB.

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