Activin A skews macrophage polarization by promoting a pro-inflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers

Running head: Activin A skews macrophage polarization

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ABSTRACT

M-CSF favors the generation of FRβ⁺ IL-10-producing immunosuppressive M2-polarized macrophages [M2 (M-CSF)], whereas GM-CSF promotes a pro-inflammatory M1-polarized phenotype [M1 (GM-CSF)]. Here we describe that activin A is preferentially released by M1 (GM-CSF) macrophages, impairs the acquisition of FRβ and other M2 (M-CSF)-specific markers, downmodulates the LPS-induced release of IL-10, and mediates the tumor cell growth inhibitory activity of M1 (GM-CSF) macrophages, whose Smad2/3 is constitutively phosphorylated. The contribution of activin A to M1 (GM-CSF) macrophage polarization was evidenced by the capacity of a blocking anti-activin A antibody to reduce M1 (GM-CSF) polarization markers expression while enhancing FRβ and other M2 (M-CSF) markers mRNA levels. Moreover, an inhibitor of Activin receptor-Like Kinase (ALK)4/5/7 (SB431542) promoted M2 (M-CSF) marker expression but limited the acquisition of M1 (GM-CSF) polarization markers, implying a role for Smad2/3 activation in macrophage polarization. In agreement with these results, expression of activin A and M2 (M-CSF)-specific markers were oppositely regulated by tumor ascites. Therefore, activin A contributes to the pro-inflammatory macrophage polarization triggered by GM-CSF, and limits the acquisition of the anti-inflammatory phenotype in an Smad2-dependent manner. Our results demonstrate that activin A-initiated Smad signaling skews macrophage polarization towards the acquisition of a pro-inflammatory phenotype.
INTRODUCTION

Tissue resident macrophages are phenotypically and functionally heterogeneous under homeostatic conditions because of their extreme sensitivity to the extracellular cytokine milieu. Although GM-CSF and M-CSF contribute to cell survival and proliferation, they exert distinct actions during macrophage differentiation. The lack of M-CSF alters the development of various macrophage populations, whereas GM-CSF-deficient mice only exhibit altered maturation of alveolar macrophages. Along the same line, both cytokines promote the in vitro differentiation of macrophages with distinct morphology, pathogen susceptibility and inflammatory function. GM-CSF gives rise to monocyte-derived macrophages with high antigen-presenting capacity and which produce pro-inflammatory cytokines in response to TLR stimulation. Conversely, M-CSF leads to the generation of macrophages with high phagocytic activity and IL-10-producing ability in response to pathogens. Based on their respective cytokine profiles, human macrophages generated in the presence of GM-CSF or M-CSF are representative of the classical (M1) or alternative (M2) macrophage polarization states, respectively, and are considered as pro-inflammatory and anti-inflammatory macrophages.

Activins are pluripotent growth and differentiation factors of the TGFβ superfamily, which are structurally composed of two β subunits (activin A, βAβA; activin AB, βAβB; activin B, βBβB) linked by a single covalent disulfide bond, and whose expression is high in inflammatory pathologies, including rheumatoid arthritis and inflammatory bowel disease. Initially characterized as inducers of follicle-stimulating hormone production, activins are now known to regulate the growth of numerous cell types, contribute to maintenance of pluripotency of embryonic stem cells, and exert anti-tumorigenic effects. Activins share their intracellular signaling pathway via Smad2,3 with other members of the TGFβ superfamily. Like TGFβ,
activins also regulate inflammatory responses and exert immunostimulatory and immunosuppressive functions at the T cell level. Within the myeloid lineage, activin A has a central role in innate immunity, and is considered as a crucial modulator of inflammatory responses by virtue of its pro-inflammatory and regulatory activities. In fact, activin A modulates cytokine and chemokine release from myeloid cells, and contributes to Langerhans cell differentiation both in vitro and in vivo. Activin A expression is upregulated upon activation and in response to inflammatory mediators, but suppressed by glucocorticoids. The ability of activin A to induce Arginase-1 expression in murine peritoneal macrophages has led to the proposal of activin A as a Th2 cytokine that promotes macrophage polarization towards the alternative phenotype.

We have previously analyzed the differences in gene expression between GM-CSF-polarized [M1 (GM-CSF)] macrophages and M-CSF-polarized [M2 (M-CSF)] macrophages, and described the preferential expression of Folate Receptor β (FRβ) and Heme-Oxygenase 1 (HO-1) in in vitro derived M2 (M-CSF) macrophages and ex vivo Tumor-Associated Macrophages (TAM). To identify factors mediating the acquisition of their corresponding profiles, we studied whether M1 (GM-CSF)-derived molecules influenced the acquisition of M2 (M-CSF)-specific markers. We found that M1 (GM-CSF) macrophages secrete large amounts of functional activin A, that promotes the expression of M1 (GM-CSF) markers, impairs the acquisition of M2 (M-CSF) markers and downregulates the production of IL-10. These results indicate that activin A contributes to macrophage polarization and shapes the inflammatory behaviour of macrophages. Moreover, given the macrophage ability for re-polarization under appropriate cytokine conditions, activin A might function, in an autocrine or paracrine manner, regulating macrophage switch between polarization states.
MATERIALS AND METHODS

**Macrophage differentiation, cell culture and flow cytometry.** Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from normal donors over a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Monocytes (>95% CD14+ cells) were cultured at 0.5 x 10^6 cells/ml for 7 days in RPMI supplemented with 10% fetal calf serum (FCS) (completed medium), at 37°C in a humidified atmosphere with 5% CO₂, and containing 1000U/ml GM-CSF or M-CSF (10 ng/ml, ImmunoTools GmbH, Friesoythe, Germany) to generate M1 and M2 monocyte-derived macrophages, respectively. Cytokines were added every two days. To generate monocyte-derived dendritic cells (MDDC), monocytes were cultured at 0.7 x 10^6 cell/ml in complete medium containing GM-CSF (1000 U/ml) and IL-4 (1000U/ml, ImmunoTools GmbH, Friesoythe, Germany) for 5-7 days, with cytokine addition every second day. M1 (GM-CSF) macrophages generated in the presence of 1000U/ml GM-CSF were phenotypically identical to those generated in the presence of 50U/ml or 200U/ml GM-CSF, and distinct from MDDC, as they were devoid of cell surface CD1a and CD209, retained CD14 and displayed lower levels of MHC class II (Supplementary Figure 1). Recombinant human activin A (2.5-25 ng/ml, Miltenyi Biotech, Bergisch Gladbach, Germany) was added together with the indicated cytokine. To test their effects on macrophage polarization, a blocking anti-activin antibody (100 ng/ml, R&D Systems) or the inhibitor of ALK4, ALK5 and ALK7, SB431542 (10μM), were added every 24 hours. GM-CSF-free conditioned medium from M1 (GM-CSF) macrophages (at day 5) was generated by extensive washing of the cells 3 hours after GM-CSF addition and a further culture for 48 hours. Murine M1 and M2 Bone Marrow-Derived Macrophages (BMDM) were generated essentially as reported ⁹, ²⁵, using human M-CSF (10 ng/ml, ImmunoTools) or murine GM-CSF (20 ng/ml, Sigma Aldrich),
respectively. The mink lung epithelial cell line Mv1Lu was maintained in DMEM supplemented with 10% FCS. Phenotypic analysis was carried out by flow cytometry as reported, using either rabbit polyclonal antisera anti-human FRβ or rabbit preimmune serum, followed by FITC-labelled Fab goat anti-rabbit IgG. All incubations were done in the presence of 50 µg/ml of human IgG to prevent binding through the Fc portion of the antibodies.

**Western blot.**- Cell lysates were obtained in 10mM Tris-HCl pH 8, 150mM NaCl, 1% NP-40 (NP-40 lysis buffer) containing 2 mM Pefabloc, 2 µg/ml of aprotinin/antipain/leupeptin/pepstatin, 10mM NaF and 1 mM Na3VO4. Ten µg of cell lysates was subjected to SDS-PAGE and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking of the unoccupied sites with 5% bovine serum albumin in a buffer containing 50 mM Tris-HCl pH 7.6, 150 mM NaCl, and 0.1% Tween-20. Protein detection was carried out using polyclonal antisera against phosphorylated Smad2 (pSmad2-Ser465/467, clone A5S, Millipore), Smad2/3 (Millipore), GAPDH (sc-32233, Santa Cruz Biotechnology, Santa Cruz, CA) or β-actin (Sigma-Aldrich, UK ), and accomplished with the Supersignal West Pico Chemiluminescent system (Pierce, Rockford, IL).

**ELISA.**- Supernatants from M1 and M2 macrophages were tested for the presence of cytokines and growth factors using commercially available ELISA for TNF-α, IL-10, IL-6 (all from ImmunoTools), IL-12 p40 (OptEIATM IL-12 p40 set, BD Pharmingen, San Diego, CA), and activin A (R&D Systems, Inc, Minneapolis, USA) following the protocols supplied by the manufacturers. Murine activin A was determined using Quantikine Human/Mouse/Rat activin A Immunoassay (R&D Systems).
**Reporter Gene Assays.** - The FOLR2 gene promoter-based reporter construct pFOLR2-200Luc was generated by PCR amplification of the -210/+2 fragment of the FOLR2 promoter \(^{29}\) using oligonucleotides \(5^{-}\)CCCAGCTTTCCCTCTGGGTGATGAGCATTCTC-3’ \and \(5^{-}\)CCGCTGCCAGAAGCAGCACTGAGTG-3’\), and cloning of the resulting fragment into HindIII/XhoI-digested pXP2. Mv1Lu cells, a well established cellular model to study the signaling of the TGF-β superfamily \(^{26}\), or murine embryo fibroblasts, were transfected with 0.5 μg of the Smad2-dependent p3TP-Lux reporter construct \(^{30}\) or pFOLR2-200Luc, respectively, using Superfect (Qiagen). After transfections, cells were washed, cultured in DMEM plus 0.2% FCS, and treated with undiluted conditioned media from M1 (GM-CSF) or M2 (M-CSF) macrophages, 25 ng/ml recombinant human activin A (Miltenyi Biotec) or 10 ng/ml TGF-β1 (R&D Systems) for 24 hours. When required, cells were preincubated for 30 minutes with 10 μM SB431542 (Sigma) before treatment. To neutralize activin A activity, 0.1 μg/ml of a blocking monoclonal antibody (R&D Systems) was used. In some experiments, cells were cotransfected with 0.4 μg of expression vector encoding a dominant negative mutant of either Smad2 \(^{31}\) or Smad3 \(^{32}\). To normalize transfection efficiency, cells were co-transfected with an SV40 promoter-based β-galactosidase expression plasmid (RSV-βgal). Measurement of relative luciferase units and β-galactosidase activity were performed using the Dual-Glo Luciferase Assay System (Promega) and the Galacto-Ligth kit (Tropix), respectively, in a Varioskan Flash spectral scanning multimode reader (Thermo Scientific).

**Quantitative real-time RT-PCR.** - Oligonucleotides for selected genes were designed according to the Roche software for quantitative real time PCR. Total RNA was extracted using the RNeasy kit (Qiagen), retrotranscribed, and individually amplified cDNA were quantified using the Universal Human Probe Roche library (Roche Diagnostics). Assays were made in triplicates and results normalized according to the expression levels of 18S rRNA and GAPDH RNA. Results were
expressed using the ΔΔCT method for quantitation. All microarray data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE27792.
RESULTS

**M1 (GM-CSF) macrophage--conditioned medium prevents acquisition of Folate Receptor β (FRβ) expression.**—As reported, expression of cell surface FRβ, encoded by the *FOLR2* gene, identifies IL-10-producing *ex vivo* isolated Tumor-Associated Macrophages (TAM) and *in vitro* polarized M2 (M-CSF) (hereafter referred to as M2) macrophages, but not M1 (GM-CSF) (hereafter termed M1) macrophages (Figure 1A). In fact, FRβ mediates Folate-FITC capture by M2 polarized macrophages, but not M1 macrophages (Figure 1B). GM-CSF and M-CSF oppositely modulated *FOLR2* gene expression: GM-CSF greatly downregulated *FOLR2* RNA levels in FRβ-positive M2 macrophages (Figure 1C), whereas M1 macrophage-conditioned medium significantly inhibited *FOLR2* RNA induction (Figure 1D). This latter inhibitory activity was observed when the M1 macrophage-conditioned medium was used either undiluted (*p* = 0.0009), diluted 1/2 (*p* = 0.001) or after a 1/10 dilution (more than 90% reduction, *p* = 0.001) (Figure 1D). Therefore, M1 macrophage-conditioned medium contains factor(s) that prevent the acquisition of the M2-specific marker FRβ.

**M1 macrophages secrete activin A, which downregulates FOLR2 gene expression.**—To identify M1-derived factors that prevent FRβ induction, we searched for soluble factors preferentially produced by M1 macrophages. Gene expression profiling revealed that *INHBA* gene expression, which codes for the inhibin βA subunit, is much higher in M1 than in M2 macrophages (log₂ M1/M2 = 6.1; *p* = 5.3 x 10⁻⁸, Figure 1E), a difference further confirmed by qRT-PCR on independent samples (Figure 1E) and upon polarization of either CD14⁺CD16⁻ or CD14⁺CD16⁺ monocytes (data not shown). Unlike *FOLR2*, *INHBA* RNA expression was induced in M2 macrophages exposed to GM-CSF, and abrogated in M1 macrophages upon replacement of their conditioned medium by M-CSF (*p* < 0.005, Figure 1F). In agreement with RNA data and its inducibility by GM-CSF, *INHBA*-encoded activin A levels were significantly higher in M1
macrophage-conditioned media from six independent donors \((p < 0.05\), Figure 1G), and were continuously increased during the GM-CSF-dependent M1 differentiation/polarization process (Figure 1H). Although LPS increases circulating activin A levels \textit{in vivo} \(18\), the differential production of activin A by M1 and M2 macrophages was maintained after LPS stimulation, regardless of whether cells were maintained in their conditioned medium or not during LPS activation (Figure 1I). Similar results were observed in murine bone marrow-derived macrophages (BMDM), as murine M1 BMDM secrete significantly higher levels of activin A than M2 BMDM (Figure 1J). Altogether, these results indicate that activin A is differentially regulated by GM-CSF and M-CSF, and that activin A expression inversely correlates with \textit{FOLR2} gene expression.

Next, to determine whether activin A affects \textit{FOLR2} gene expression, M2 polarization was accomplished in the presence of recombinant human activin A. The M-CSF-dependent acquisition of \textit{FOLR2} RNA expression was significantly and dose-dependently reduced by activin A, an effect that could be observed both during (3 days) and at the end (7 days) of the polarization process (Figure 2A,B). In fact, activin A inhibited both the basal and the M-CSF-dependent upregulation of \textit{FOLR2} RNA (Figure 2B). Besides, whereas IL-6 had no effect, the activity of the \textit{FOLR2} gene proximal regulatory region was significantly reduced \((p = 0.0008)\) by activin A (Figure 2C), thus indicating a transcriptional effect. Therefore, activin A inhibits the appearance of \textit{FOLR2} RNA in macrophages, and it can be concluded that the differential expression of FR\(\beta\) on M1 and M2 macrophages derives, at least in part, from the opposite actions of GM-CSF and M-CSF on \textit{INHBA} gene expression.

To evaluate the involvement of activin A in the inhibitory effects that GM-CSF and M1 macrophage-conditioned medium have on \textit{FOLR2} mRNA expression, a blocking anti-activin A antibody was used in three different experimental settings. First, the inhibitory action of different
doses of GM-CSF-free M1 macrophage-conditioned medium on FOLR2 mRNA upregulation in cultured monocytes was significantly reversed in the presence of the blocking anti-activin A antibody (Figure 2D). Second, antibody-mediated blockade of activin A also significantly reduced the inhibitory activity of GM-CSF-free M1 macrophage-conditioned medium on the acquisition of FOLR2 mRNA by M2 macrophages (Figure 2E). Finally, and more importantly, the presence of the anti-activin A antibody during the GM-CSF-dependent M1 macrophage polarization also led to significantly higher levels of FOLR2 mRNA (Figure 2F). Altogether, these results indicate that the inhibitory effect of GM-CSF and the GM-CSF-free M1 macrophage-conditioned medium on the expression of the prototypical M2 marker FOLR2 is, at least partly, mediated by activin A, thus suggesting the implication of this member of the TGFβ family in macrophage polarization.

Activin A contributes to M1 (GM-CSF) macrophage polarization. Considering the above results, we assessed the influence of activin A on genes that, like FOLR2, are differentially expressed between M1 (GM-CSF) and M2 (M-CSF) macrophages (Figure 3)\(^{22,23,33}\). Regarding M2-specific markers, and after the 7-day polarization process, the presence of activin A led to a reduction in the M-CSF-dependent induction of MAF, IGF1 and F13A1, blunted that of SERPINB2, but had no inhibitory effect on HS3ST1 expression (Figure 4A). Moreover, the presence of a blocking anti-activin A antibody increased the expression of MAF, IGF1, F13A1 and SERPINB2 mRNA yielded upon polarization in the presence of GM-CSF-free M1 macrophage-conditioned medium (Figure 4B). As a final proof of the activin A involvement in macrophage polarization, the GM-CSF-dependent polarization of M1 macrophages was carried out in the presence of the blocking anti-activin A antibody. As shown in Figure 4C, the expression of M2 (M-CSF)-specific markers MAFB, ETS2, FOLR2, IL10, IGF1 and SERPINB2 was significantly enhanced upon blockade of activin A activity during the polarization process. Furthermore, the presence of the antibody also led to a significant reduction of three M1 (GM-CSF)-specific markers like CCL17, ECSCR and
CCR2 (Figure 4C). These results indicate that activin A plays a relevant role in the GM-CSF-mediated M1 macrophage polarization, as it potentiates the expression of M1 (GM-CSF)-specific markers and inhibits that of M2 (M-CSF) markers. Thus, considering its constitutive expression during GM-CSF-mediated M1 polarization, it is tempting to speculate that activin A shapes the polarization of macrophages in an autocrine/paracrine manner.

Given the inhibitory action of an anti-activin A antibody on the acquisition of M1 markers, and since activin A has been proposed as a Th2-polarizing cytokine \(^{21}\), its effects on the expression of genes preferentially found in both M1 and IL-4-activated macrophages \(^{33}\) were also evaluated. To that end, monocytes were treated with activin A for 72 hours and in the absence of GM-CSF. Activin A treatment did not modify the expression of \(TM4SF1, CCL17\) or \(ILR1N\), the latter being a known activin A target gene \(^{14}\) (Supplementary Figure 2). However, activin A significantly enhanced \(SERPINE1\) RNA levels (Figure 4D), whose expression is significantly higher in M1 (GM-CSF) than in M2 (M-CSF) macrophages (Figure 3). Therefore, while activin A contributes to the GM-CSF-mediated polarization, it is also capable by itself of upregulating the expressing of genes preferentially found in M1 (GM-CSF) macrophages, further confirming its ability to shape the phenotypic polarization of macrophages.

**Activin A mediates the growth inhibitory activity of M1 macrophage-conditioned medium.**

Although originally identified as a gonadal-derived regulators of pituitary follicle-stimulating hormone, activin A exerts multiple actions, including cancer cell growth arrest \(^{10,13,34,35}\). The tumor suppressive properties of activin A prompted us to analyze whether M1 macrophage-conditioned medium affected K562 leukemic cell growth, a function previously adscribed to activin A \(^{36}\). GM-CSF-free M1 macrophage-conditioned medium significantly inhibited the growth of K562 leukemic cells to a greater extent than recombinant activin A (25 ng/ml), and this growth
suppressive activity was significantly reverted in the presence of either a blocking anti-activin A monoclonal antibody or SB431542, an ALK4/5/7 inhibitor known to prevent activin A-induced Smad2/3 phosphorylation \(^{37}\) (Figure 5A). Moreover, in agreement with previous results \(^{36}\), GM-CSF-free M1-conditioned medium increased K562 cell volume, a feature associated with differentiation into hemoglobin-expressing cells, and this differentiation-inducing ability was reduced by a blocking anti-activin A monoclonal antibody (Figure 5B). These results indicate that M1 macrophages release functional activin A, endowing them with tumor-resistance capability, a defining property of M1-polarized macrophages \(^{38}\).

**Activin A modulates the cytokine profile of M2 macrophages.**- Besides their opposite responses towards tumor cells, M1 and M2 macrophages differ in T cell-stimulatory ability and pathogen-stimulated cytokine/chemokine profile \(^{6,7}\). In allogeneic MLR, M1 macrophages induced considerable higher T cell proliferation than M2 macrophages, but exposure of the latter to activin A did not modify their T cell stimulatory ability (Supplementary Figure 3), a result reminiscent of the lack of effect of follistatin on the allostimulatory function of monocyte-derived dendritic cells \(^{20}\). Regarding cytokine release, and in agreement with previous reports \(^{7}\), LPS stimulation of M1 macrophages led to production of pro-inflammatory cytokines (TNF\(\alpha\), IL-12p40, IL-6) (Figure 5C) and the acquisition of Dendritic Cell maturation ability (Supplementary Figure 4), whereas LPS-stimulated M2 macrophages produced high levels of IL-10 (Figure 5C). Activin A also significantly reduced the release of IL-10 from LPS-stimulated M2 macrophages \((p = 0.01)\), but had no effect on the production of TNF\(\alpha\) (Figure 5D). Moreover, macrophages exposed to activin A during M-CSF-driven polarization showed a diminished production of IL-10 in response to LPS \((p = 0.003, \text{Figure 5D})\). Therefore, and in agreement with its effect on \textit{IL10} mRNA levels during macrophage polarization (Figure 4C), activin A negatively regulates IL-10 production from M2
macrophages, and skews macrophage polarization by impairing the acquisition of the anti-inflammatory phenotype and cytokine profile.

**Activin A influences macrophage polarization via Smad2/3 activation.** Since activin A triggers Smad2 phosphorylation $^{13}$, we next determined the extent of Smad2 activation in unstimulated and LPS-stimulated macrophages. Smad2 was found constitutively phosphorylated in M1 macrophages, and remained activated after LPS stimulation (Figure 6A). By contrast, no Smad2 phosphorylation was detected in either untreated or LPS-stimulated M2 macrophages (Figure 6A). The absence of Smad2 activation in M2 macrophages was not due to a defective activin/Smad signaling pathway, since activin A treatment of M2 macrophages led to overt Smad2 phosphorylation (Figure 6B). As a proof of the specificity of the anti-activin A antibody used in preceding experiments, the presence of the antibody reduced the degree of Smad2 phosphorylation triggered by activin A on M2 macrophages (Figure 6C), while the activin A-induced Smad2 phosphorylation was completely prevented in the presence of SB431542, which prevents Smad2/3 phosphorylation $^{37}$ (Figure 6C).

A further support for the role of activin A in shaping M1 macrophage polarization was obtained through evaluation of the transcriptional effects of M1 macrophage-derived activin A. Like recombinant TGFβ1 and activin A, M1-conditioned medium transactivated the p3TP-Lux reporter construct ($p = 0.0008$), whereas supernatants from M2 macrophages had no effect (Figure 6D). Importantly, the transactivation ability of the M1 macrophage-conditioned medium was abolished by 1) SB431542 ($p = 0.015$) (Figure 6D); 2) cotransfection of dominant negative forms of Smad2 ($p = 0.0018$) or Smad3 ($p = 0.0055$) (Figure 6D); and 3) the blocking anti-activin A antibody ($p = 0.0001$) (Figure 6D). Altogether, these results demonstrate that M1-derived activin A activates Smad2/3 in an autocrine/paracrine manner, thus providing a molecular support for its capacity to
shape macrophage polarization. Besides, since the transactivation ability of the M1 macrophage-conditioned medium is completely abolished by an anti-activin A antibody, the basal phosphorylation state of Smad2/3 in M1 macrophages must reflect an autocrine/paracrine effect of activin A.

**Smad signaling modulates macrophage polarization.** The differential status of the basal Smad2/3 phosphorylation between M1 and M2 macrophages prompted us to study the impact of Smad2/3 signaling on macrophage polarization. To that end, M1 macrophages were generated in the presence of the ALK4/5/7 inhibitor SB431542. As expected, blockade of Smad2/3 activation resulted in a robust increase in *FOLR2* expression (>30-fold) (Figure 6E), a finding compatible with the inhibitory effect of activin A on *FOLR2* levels (Figures 2A-B and 4C). The resulting macrophage population also exhibited higher expression of other M2-specific markers, including *IGF1* (>500-fold), *SERPINB2* (>500-fold), *MAFB, MAF* and *ETS2* (10-fold), the latter known to determine the transcriptional profile of tumor-associated macrophages (Figure 6E). By contrast, the presence of SB431542 led to significantly lower levels of the M1-specific markers *CCL17, ECSCR* and *CCR2* (Figure 6E), a result that is in agreement with the sensitivity of these three genes to the presence of the blocking anti-activin A antibody during the GM-CSF-driven M1 polarization (Figure 4C). Noteworthily, a higher level of *IL10* mRNA, whose presence is a defining property of M2-polarized macrophages, was also observed when polarization took place in the presence of SB431542 (Figure 6E). Altogether, these results suggest that the activin A-triggered Smad signaling pathway has an important role in macrophage polarization, as both activin A blockade and inhibition of Smad2/3 activation shift the macrophage gene signature towards the appearance of an anti-inflammatory phenotype, thus posing activin A as a critical determinant for macrophage polarization.
DISCUSSION

Macrophage differentiation and polarization are critically determined by the cellular environment, which also dictates cytokine responsiveness \(^{40}\). The search for the mechanisms underlying GM-CSF- and M-CSF-driven macrophage differentiation and the acquisition of anti-inflammatory M2 (M-CSF) macrophage markers has led to the identification of activin A as a factor that shapes macrophage polarization in response to GM-CSF, and whose presence limits the production of IL-10, contributes to expression of M1 macrophage polarization and prevents the expression of M2-specific markers. The relevance of the activin A expression for macrophage polarization is underscored by the opposite effect that ascitic fluids have on \textit{INHBA} gene expression and the levels of M2-specific markers (data not shown). In addition, and considering the macrophage ability for re-polarization \(^{41}\), activin A might halt the macrophage switch between distinct polarization states. To our knowledge this is the first report describing the relevance of the activin A-Smad2/3 axis in human macrophage polarization, and its involvement in preventing the acquisition of the phenotype and effector functions commonly associated to M2/anti-inflammatory macrophages.

The shift in macrophage polarization is now recognized as a very relevant event in tumorigenesis, wound healing and resolution of inflammation, and its deregulation underlies both tumor progression and chronic inflammatory diseases \(^{41,42}\). Consequently, determination of the molecular mechanisms for macrophage polarization is a very active area of research, and could result in the identification of novel opportunities for manipulating immune and inflammatory responses. The transcriptional basis for the various macrophage polarization states is beginning to be unraveled. Whereas NF\(\kappa\)B and AP1 drive classical M1 macrophage polarization, the alternative M2 state is controlled by STAT6, PPAR\(\gamma\) \(^{42}\), STAT1 and NF\(\kappa\)B p50 \(^{43}\). In the case of murine macrophages,
type I interferon signaling appears essential for phenotypic and functional M2 polarization of BMDM in response to M-CSF and, more recently, the Jmjd3-Irf4 axis has been pointed out as crucial for expression of genes related to the M2 macrophage polarization in BMDM exposed to M-CSF. Given the phenotypic differences between murine and human M2 macrophages in response to IL-4, it cannot be anticipated whether activin A and Smad signaling contribute to murine macrophage polarization. However, the much higher level of activin A produced by GM-CSF-polarized M1 BMDM (Figure 2F) would favor the hypothesis that activin A also modulates murine macrophage polarization.

Tumor-derived factors modulate macrophage polarization, and contribute to the anti-tumor (M1)-to-pro-tumor (M2) shift that takes place in TAM along tumor progression. From this point of view, the preferential expression of activin A by M1 (GM-CSF) macrophages is compatible with its pro-apoptotic, tumor suppressor and anti-angiogenic effects, as well as with its capacity to inhibit proliferation of human tumor cells from various origins. Therefore, tumor cells that inhibit activin A activity or expression in macrophages (thus reducing their M1 phenotype signature) would exhibit a growth advantage, a property commonly adscribed to tumor cells promoting the acquisition of an M2 phenotypic signature. The fact that ascitic fluids from tumors of different origins oppositely modulate INHBA mRNA levels and M2 (M-CSF)-associated markers (data not shown) supports the validity of activin A as a marker for M1 polarized macrophages. On the other hand, the identification of various M2 (M-CSF)-specific markers whose expression is prevented or downmodulated by activin A (Figure 4) implies that activin A negatively affects the acquisition of the macrophage anti-inflammatory gene profile. This effect is particularly relevant in the case of IL-10, whose release constitutes a hallmark of stimulated M2 (M-CSF) macrophages. In fact, IL10 gene transcription in macrophages is dependent on the cMAF transcription factor, whose expression is associated to M2 (M-CSF) polarization, and
whose RNA levels are reduced by activin A (Figure 4) and enhanced by interference with the Smad signaling pathway (data not shown). Consequently, the capacity of activin A to impair MAF RNA expression might underlie its negative regulatory effect on IL10 gene expression.

The ability of activin A to trigger Arginase-1 expression and inhibit IFNγ-induced NO synthase 1 expression has led to the suggestion that it functions as a Th2 cytokine that promotes alternative murine macrophage activation 21. However, in the case of human macrophages, although M1 macrophages release high levels of activin A, they do not display any of the phenotypic markers that characterize alternatively activated human macrophages 33 (Puig-Kröger, Sierra-Filardi, Vega and Corbi, unpublished). Activin A, synthesized by monocytes/macrophages in response to LPS 18, TNFα or IL-1β 49, exhibits both pro- or anti-inflammatory activities 18 as it stimulates the production of IL-1 and TNFα by human monocytes and macrophages and inhibits IL-10 effects on prostatic epithelial cells 50. In fact, activin A blockade by follistatin leads to reduced levels of LPS-induced IL-1 and TNFα 18. The link between pro-inflammatory macrophage polarization and activin A is further illustrated by the fact that activin A expression is inhibited by anti-inflammatory agents like glucorticoids and retinoic acid 19,20. Since factors promoting M1/classical macrophage polarization enhance activin A production, its expression appears to be a common parameter of M1 polarized macrophages, as well as a critical contributor to their phenotype and effector functions. In this regard, the expression of type I (ALK4, ACVR1B) and type II (ACVR2A, ACVR2B) activin receptor mRNA in M1 (GM-CSF) macrophages (data not shown) explains the ability of activin A to influences the gene expression profile of M1 polarized macrophages in a paracrine/autocrine manner. This appears to be true since 1) Smad2 is constitutively phosphorylated in M1 (GM-CSF) macrophages; and 2) polarization in the presence of a blocking anti-activin A antibody or the ALK4/5/7 inhibitor SB431542 not only leads to upregulation/induction of M2-specific markers, but impairs the expression of genes associated with
GM-CSF-driven M1 polarization. Therefore, activin A (like other TGFβ family members) has a prominent role in determining the macrophage polarization state and, consequently, shaping the inflammatory response of macrophages to exogenous stimuli.

In summary, the present manuscript identifies activin A as a relevant contributor to the differential gene expression profiles and effector functions exhibited by pro-inflammatory and anti-inflammatory macrophages. The importance of activin A in macrophage polarization is supported by its ability to reduce IL-10 production by anti-inflammatory macrophages, and to inhibit the acquisition of the IL-10-producing ability during M-CSF-driven polarization. The identification of a set of M2 (M-CSF) macrophage-specific genes whose expression is found in TAM and negatively affected by activin A provides novel potential therapeutic targets for the modulation of the macrophage inflammatory response under pathological conditions.
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E. Sierra-Filardi, A. Puig-Kröger, F. J. Blanco and C. Nieto performed research and analyzed data; R. Bragado and M. I. Palomero contributed vital new reagents; M. A. Vega analyzed data; C. Bernabéu and A. L. Corbí designed the research and analyzed data; A. L. Corbí wrote the paper. The authors declare no conflicts of interest.

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REFERENCES


24. Gratchev A, Kzhyshkowska J, Kothe K, et al. Mphi1 and Mphi2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. Immunobiology. 2006;211(6-8):473-486.


FIGURE LEGENDS

Figure 1. M1 (GM-CSF) macrophages inhibit Folate Receptor β (FRβ) expression on M2 (M-CSF) polarized macrophages and release high levels of activin A. A. Cell surface expression of FRβ on M1 and M2 macrophages, as determined by flow cytometry using a rabbit polyclonal antiserum against human FRβ (empty histogram) or a non-specific rabbit pre-immune antiserum (filled histogram). The percentage of marker-positive cells and the mean fluorescence intensity (in parenthesis) are indicated. B. FRβ function in M1 and M2 macrophages, as demonstrated by confocal microscopy on cells incubated at 37ºC with Folate-FITC (green fluorescence) and Transferrin-Texas red (red fluorescence). C. FOLR2 mRNA expression levels, as determined by qRT-PCR, in M2 (M-CSF) macrophages after replacement of the culture supernatant by either M-CSF- (grey histograms) or GM-CSF-containing complete medium (empty histograms) for 48 hours. Results are expressed as Relative mRNA levels (relative to GAPDH RNA levels and referred to the RNA levels in cells maintained in M-CSF-containing medium). Mean and standard deviation of triplicate determinations are shown (*, p < 0.005). D. FOLR2 mRNA expression levels determined by qRT-PCR on peripheral blood monocytes (Mo.) and monocytes cultured for 72 hours and either in the absence (0) or in the presence of increasing percentages of M1 macrophage-conditioned media (M1 SN %). Results are expressed as Relative mRNA levels (relative to GAPDH RNA levels and referred to levels detected in peripheral blood monocytes). Mean and standard deviation of triplicate determinations are shown (*, p < 0.005). E. Relative INHBA gene expression in M1 and M2 macrophages, as determined by microarray DNA analysis (empty histograms) and qRT-PCR (grey histograms). F. INHBA mRNA expression levels determined by qRT-PCR on the indicated macrophages after replacement of their respective culture supernatant with fresh complete medium containing either M-CSF- (grey histograms) or GM-CSF (empty histograms) for 48 hours. Mean and standard deviation of triplicate determinations are shown. Results are expressed as Relative mRNA levels (relative to GAPDH.
RNA levels) and referred to the INHBA RNA levels in cells treated with M-CSF (*, p < 0.005). G. Activin A levels released by M1 and M2 macrophages from six independent donors, as determined by ELISA. H. Determination of activin A release during the differentiation of M1 and M2 macrophages from two independent donors, as determined by ELISA on culture supernatants removed at the indicated time points. I. Activin A levels released by M1 and M2 macrophages untreated (-) or stimulated with 10 or 100 ng/ml LPS for 24 hours, and either maintained in their conditioned media (no wash) or in fresh culture medium (wash) during the LPS stimulation period. J. Activin A levels released by murine M1 and M2 BMDM from four independent samples, as determined by ELISA. In G-J, each determination was performed in triplicate, and means and standard deviations are shown (*, p < 0.005; **, p < 0.05).

Figure 2.- Activin A inhibits Folate Receptor β (FRβ) expression, and mediates the inhibitory effect of GM-CSF or M1 macrophage-conditioned medium.- A. FOLR2 mRNA expression levels in macrophages differentiated for 3 days (left panel) or 7 days (right panel) in the presence of M-CSF, GM-CSF or M-CSF plus activin A (ActA, 10 ng/ml), as determined by qRT-PCR. B. FOLR2 mRNA expression levels determined by qRT-PCR on monocytes (Mo.) or macrophages cultured for 7 days in the presence of the indicated cytokine combinations. In A and B, results are expressed as mRNA levels relative to GAPDH RNA and referred to the expression level observed in the presence of M-CSF (A) or in monocytes (B). Shown are means and standard deviations of triplicate determinations (*, p < 0.005; **, p < 0.05). C. Transcriptional activity of the pFOLR2-200Luc reporter construct in Mv1Lu cells, incubated in the absence (-) or in the presence of IL-6 or activin A (ActA, 25 μg/ml). For normalization purposes, cells were co-transfected with the RSV-β-gal expression plasmid, and results are presented as RLU (Relative Light Units), which indicate the units of luciferase activity per unit of β-galactosidase activity for each assay condition. Experiments were performed in triplicate, and shown are the means and standard deviations (*, p <
D. FOLR2 mRNA expression levels determined by qRT-PCR on monocytes exposed for 72 hours to increasing concentrations of GM-CSF-free M1 macrophage-conditioned media (GM-CSF-free M1 SN %) and in the presence of 100 ng/ml of a blocking anti-activin A antibody (α-ActA) or an isotype-matched antibody (IgG). Results are expressed as Relative mRNA levels (relative to GAPDH RNA levels). Mean and standard deviation of triplicate determinations are shown (*, p < 0.005). E. FOLR2 mRNA expression levels determined by qRT-PCR on M1 macrophages or M2 macrophages generated in the presence of GM-CSF-free M1 macrophage-conditioned media (50%) and with a daily addition of a blocking anti-activin A antibody (α-ActA) or an isotype-matched antibody (IgG). Results are expressed as Relative mRNA levels (relative to GAPDH RNA levels). Mean and standard deviation of triplicate determinations are shown (**, p < 0.05). F. FOLR2 mRNA expression levels determined by qRT-PCR on M1 macrophages generated in the presence of a blocking anti-activin A antibody (α-ActA) or an isotype-matched antibody (IgG). Results are expressed as Relative mRNA levels (relative to GAPDH RNA levels). Mean and standard deviation of triplicate determinations are shown (**, p < 0.05).

**Figure 3.-** Relative expression of the indicated genes in M1 (GM-CSF) and M2 (M-CSF) macrophages, as determined by microarray DNA analysis (empty histograms) and qRT-PCR (grey histograms). The adjusted p-value for the microarray data of each specific gene is indicated.

**Figure 4.-** Effect of activin A on the acquisition of M1- and M2-specific markers.- A. MAF, IGF1, SERPINB2, F13A1 and HS3ST1 mRNA expression levels as determined by qRT-PCR on macrophages differentiated for 7 days in M-CSF, GM-CSF or M-CSF plus activin A (10 ng/ml). B. MAF, IGF1, SERPINB2, F13A1 and HS3ST1 mRNA expression levels as determined by qRT-PCR on macrophages differentiated for 7 days in GM-CSF or M-CSF plus GM-CSF-free M1-conditioned medium together with either a blocking anti-activin A antibody (M-CSF+50% M1 SN
+ α-ActA) or an isotype-matched antibody (M-CSF+50% M1 SN + IgG). C. Expression of the indicated genes in M1 (GM-CSF) macrophages generated in the presence of a blocking anti-activin A antibody (100 ng/ml), and relative to their expression in M1 (GM-CSF) macrophages generated in the presence of an isotype-matched antibody (IgG), as determined by qRT-PCR using microfluidic cards. The experiment was performed in triplicates (*, p < 0.005; **, p < 0.05), and data is presented on a log scale. The left panel shows the expression of M2 (M-CSF)-specific markers, and these results as expressed relative to the level of expression of each gene in the presence of the isotype-matched antibody (Fold induction α-ActA/IgG). The right panel shows the expression of M1 (GM-CSF)-specific markers, and these results as expressed relative to the level of expression of each gene in the presence of the anti-activin A antibody (Fold repression, IgG/α-ActA). D. SERPINE1 mRNA expression levels as determined by qRT-PCR on macrophages from two independent donors, and treated for 7 days with M-CSF, GM-CSF or activin A. Results are expressed as Relative mRNA levels (relative to GAPDH RNA levels) and referred to the expression level observed in the presence of M-CSF (A), M-CSF+50% M1 SN + IgG (B) or GM-CSF (D). The means and standard deviations of triplicate determinations are shown (*, p < 0.005; **, p < 0.05).

Figure 5.- Effects of activin A on M1 (M-CSF) macrophages effector functions.- (A,B) Growth inhibitory activity. A. Proliferation of K562 cells exposed for 96 hours to activin A (25 ng/ml) or to GM-CSF-free M1 (GM-CSF) macrophage-conditioned media (GM-CSF-free M1 SN), and either in the presence of a blocking anti-activin A antibody (α-ActA, 100 ng/ml), an isotype-matched antibody (IgG, 100 ng/ml), 10 μM SB431542 or a similar amount of vehicle (DMSO) as a control. Results show the mean and standard deviation of the effects of three independent preparations of GM-CSF-free M1 (GM-CSF)-conditioned medium, and are expressed relative to the proliferation measured in untreated cells (Relative cell proliferation). B. Cell volume of K562
cells after exposure for 96 hours to either activin A (25 ng/ml) or to GM-CSF-free M1 (GM-CSF) macrophage-conditioned media (M1 SN), and either in the absence or presence or a blocking anti-activin A monoclonal antibody. Each determination was performed in triplicate, and means and standard deviations are shown (*, p < 0.005; **, p < 0.05). (C,D) LPS-induced cytokine profile.-

C. Determination of IL-12p40, IL-6, IL-10 and TNFα release by ELISA in culture supernatants of M1 and M2 macrophages either untreated or stimulated with LPS (10 ng/ml) for 24 hours. Each determination was performed in triplicate, and means and standard deviations are shown. D. Determination of IL-10 and TNFα release by ELISA in culture supernatant of M2 macrophages differentiated in the absence (M2) or presence of activin A (M2 ActA), and either unstimulated or stimulated with LPS (10 ng/ml) for 24 hours in the presence or absence of activin A. Each determination was performed in triplicate, and means and standard deviations are shown (*, p < 0.005; **, p < 0.05).

Figure 6.- Smad2 is constitutively phosphorylated in M1 (GM-CSF) macrophages, whose activin A release activates Smad-dependent reporter genes.- A. Detection of phosphorylated Smad2 and total Smad2/3 in lysates of untreated or LPS-treated M1 and M2 macrophages, as determined by Western blot. β-actin expression levels were determined in parallel as a loading control. The bands corresponding to Smad2 and Smad3 are indicated by arrowheads (n.s., non-specific band). B. Detection of activated Smad2 in lysates of untreated or activin A-treated M2 macrophages, as determined by Western blot. GAPDH expression levels were determined in parallel as a loading control. C. Detection of activated Smad2 by Western blot on lysates of M2 macrophages subjected to a 30 min treatment with DMSO (-), SB431542 (SB), an anti-activin A antibody (α-ActA) or an isotype-matched antibody (IgG), and then treated with activin A for 1 hour. Total Smad2/3 and GAPDH expression levels were determined in parallel as loading controls. D. Transcriptional activity of the p3TP-Lux reporter construct in Mv1Lu cells either unstimulated or exposed to 10
ng/ml TGFβ1, 25 ng/ml activin A, or conditioned medium from M1 (M1 SN) or M2 (M2 SN) macrophages. When indicated, cells were preincubated for 30 minutes with 10 μM SB431542 before treatment, maintained in culture medium with 0.1 μg/ml of a blocking antibody against activin A (anti-ActA), or cotransfected with expression vectors coding for dominant negative mutants of either Smad2 (Smad2 d.n.), or Smad3 (Smad3 d.n.). For normalization purposes, cells were co-transfected with the RSV-β-gal expression plasmid, and results are presented as RLU (Relative Light Units), which indicate the units of luciferase activity per unit of β-galactosidase activity for each assay condition. Experiments were performed in triplicate, and shown are the mean and standard deviation. Six replicas of each experiment were performed, and means and standard deviations are shown (*, p < 0.005; **, p < 0.05).

E. Expression of the indicated genes in M1 (GM-CSF) macrophages generated in the presence of SB431542 (10 μM), and compared to their expression in M1 (GM-CSF) macrophages generated without the inhibitor (Vehicle, DMSO), as determined by qRT-PCR using microfluidic cards. The experiment was performed in triplicates on macrophages from two independent donors (*, p < 0.005; **, p < 0.05), and data is presented on a log scale. The left panel shows the expression of M2 (M-CSF)-specific markers, and these results as expressed relative to the level of expression of each gene in the presence of DMSO (Fold induction SB431542/Vehicle). The right panel shows the expression of M1 (GM-CSF)-specific markers, and these results as expressed relative to the level of expression of each gene in the presence of the SB431542 inhibitor (Fold repression, Vehicle/SB431542).
Figure 1

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B. 

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E. 

F. 

G. 

H. 

I. 

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**Figure 2**

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**C**

- IL-6 ActA

**D**

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**Legend:**
- M-CSF
- GM-CSF
- ActA 10 ng/ml
- ActA 25 ng/ml
- Mo.
- RLU (Luc/β-Gal)x100
- IgG
- α-ActA
- GM-CSF-free M1 SN (%)

* significant difference
** highly significant difference
Figure 4

A

Relative mRNA levels

MAF

IGF1

SERPINB2

F13A1

HS3ST1

M-CSF  GM-CSF  M-CSF + Activin A

B

Relative mRNA levels

MAF

IGF1

SERPINB2

F13A1

HS3ST1

GM-CSF  M-CSF + 50% M1 SN + IgG  M-CSF + 50% M1 SN + α-ActA

C

Fold induction (α-act/α-Act A)

MAFB  ETS2  FOLR2  IL10  IGF1  SERPINB2

D

Fold repression (IgG/α-act A)

CCL17  ECSCR  CCR2

SERPINE1

GM-CSF  M-CSF  Activin A

Figure 4
Figure 6

Panel A: Western blot analysis of pSmad2, Smad2/3, and β-actin in M1/LPS and M2/LPS samples over 0, 0.5, and 2 hours. The blot shows a significant increase in pSmad2 and Smad2/3 in M2/LPS compared to M1/LPS.

Panel B: Western blot analysis of pSmad2 and GAPDH in M2 samples with and without ActA treatment. The blot shows a greater induction of pSmad2 in M2 samples treated with ActA.

Panel C: Western blot analysis of pSmad2, Smad2/3, and β-actin in cultures treated with activin A and signaling inhibitors. The blot shows a significant reduction in pSmad2 and Smad2/3 in the presence of activin A.

Panel D: Relative light units (RLU) for (Luc/β-Gal)×100 in M1 SN and M2 SN samples treated with SB431542, α-ActA, Smad2 (d.n.), Smad3 (d.n.), and TGF-β1. The graph shows a significant increase in RLU in M2 SN samples treated with TGF-β1.

Panel E: Bar graph showing fold induction and repression of genes (MAFB, ETS2, FOLR2, IL10, IGF1, SERPINB2, CCL17, ECSCR, CCR2) induced by SB431542/Vehicle and repressed by Vehicle/SB431542 in Donor #1 and Donor #2. The legend indicates statistically significant differences (* p < 0.05, ** p < 0.01).
Activin A skews macrophage polarization by promoting a pro-inflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers

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