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New mechanisms regulating *Id3* gene expression and TGFβ signalling in macrophages

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In a healthy organism macrophages perform a number of functions depending on the site of their action and cytokine/hormone microenvironment. Upon damage or infection and in course of inflammatory reaction macrophages are able to change their phenotype and adapt functional activity to provide an organism with efficient protection. It was described previously that in $M\varphi_{IL\text{-}4/dex}$ TGFβ1 induces strong upregulation of *Id3* expression. While the mechanism of BMP-stimulated *Id3* expression regulation is studied in several cell types, its regulation by TGFβ in macrophages is not studied to date. Canonical TGFβ signalling has been shown to influence target gene expression via the Smad2/3 complex binding to Smad binding elements (SBEs). Analysis of *Id3* promoter sequence did not reveal any functional SBE, therefore search for enhancers was done using bioinformatic analysis of *Id3* genomic locus. Two evolutionary conserved regions (ECRs) were found upstream (ECR1) and downstream (ECR2) of human *Id3*. Luciferase assay analysis revealed that both ECRs contain enhancers that amplify the activity of the promoter and work synergistically. The strongest promoter activity amplification by factor 9.5 was observed if both ECRs were used. Analysis of TGFβ dependence, however, revealed that no SBEs are present within ECRs. To test the hypothesis that epigenetic mechanism may mediate TGFβ induced effects in macrophages, the effects of histone deacetylase (HDAC) inhibitors on the expression of TGFβ induced genes were tested. Using qRT-PCR it was found that pan-inhibitor of HDACs trichostatin A and specific inhibitor apicidin cause significant reduction of *Id3* expression, while another specific HDAC inhibitor MS-275 has no effect. These results may indicate that *Id3* expression does not depend on chromatin structure and HDACs-induced effect is rather based on stabilization of inhibitory Smad7. Next, activation of Smad1/5 dependent pathway by TGFβ1 was tested in macrophages. Using Western blot analysis with anti-pSmad1/5 and anti-pSmad2 antibodies it was shown that TGFβ1 stimulation of $M\varphi_{IL\text{-}4/Dex}$ induces not only phosphorylation of Smad2/3, but also phosphorylation of Smad1/5. Interestingly analysis of the effects of BMPs revealed that macrophages are able to respond to BMP4 only and that it induces phosphorylation of Smad2/3, instead of its canonical signalling molecules Smad1/5. To dissect TGFβ signalling in macrophages pharmacologic inhibitors were used. It was found that both Smad1/5 and Smad2/3 mediated signal transduction pathways in macrophages can be inhibited by ALK4/5/7 inhibitor SB431542. Complete inhibition of Smad1/5 phosphorylation was achieved when SB431542 was used at a concentration of 5 µM, while for complete inhibition of Smad2/3 phosphorylation 25 µM was needed. SB431542 as well inhibited phosphorylation of Smad2/3 induced by BMP4. Inhibition of BMP receptors using BML-275 failed to inhibit any effect of any cytokine tested. To test the hypothesis that TGFβ1 activates an additional, Smad2/3 independent pathway that leads to Smad1/5 phosphorylation, inhibitors of MAPK/ERK1, JNK and p38 pathways were used. None of these inhibitors showed an effect on Smad2/3 or Smad1/5 phosphorylation, indicating that these pathways are not involved in the effects observed in macrophages upon TGFβ1 stimulation. Analysis of TGFβ induced genes in macrophages treated with 5µM SB431542 together with bioinformatic analysis of promoter sequences of these genes allowed dissecting Smad1/5 and Smad2/3 mediated effects in macrophages.