

ORIGINAL ARTICLE

Separate roles of IL-6 and oncostatin M in mouse macrophage polarization *in vitro* and *in vivo*

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Abstract

Arginase-1 (Arg-1)-expressing M2-like macrophages are associated with Th2-skewed immune responses, allergic airway pathology, ectopic B16 melanoma cancer growth in murine models, and can be induced by Oncostatin M (OSM) transient overexpression *in vivo*. Here, we compare OSM to the gp130-cytokine IL-6 in mediating macrophage polarization, and find that IL-6 overexpression alone (Ad vector, AdIL-6) did not induce Arg-1 protein in mouse lungs at day 7, nor ectopic melanoma tumor growth at day 14, in contrast to overexpression of OSM (AdOSM). AdOSM elevated levels of IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluid, whereas AdIL-6 did not. Bone marrow-derived macrophages respond with Arg-1 enzymatic activity to M2 stimuli (IL-4/IL-13), which was further elevated in combination with IL-6 stimulation; however, OSM or LIF had no detectable activity *in vitro*. Arg-1 mRNA expression induced by AdOSM was attenuated in IL-6^{-/-} and STAT6^{-/-} mice, suggesting requirements for both IL-6 and IL-4/IL-13 signaling *in vivo*. Ectopic B16 tumor burden was also reduced in IL-6^{-/-} mice. Thus, OSM induces Arg-1⁺ macrophage accumulation indirectly through elevation of Th2 cytokines and IL-6 *in vivo*, whereas IL-6 acts directly on macrophages but requires a Th2 microenvironment, demonstrating distinct roles for OSM and IL-6 in M2 macrophage polarization.

INTRODUCTION

The characteristics of chronic inflammation in different conditions can be influenced by the functions of various sets of adaptive immune cells. These include the effect of immunological skewing influenced by Th1, Th2, Th17, or T-regulatory cells, all of which can lead to different inflammatory consequences.¹ For example, Th17 skewing influences the inflammatory response in the rheumatoid arthritic joint to generate cartilage degradation, whereas Th2 skewing in atopic dermatitis can result in skin barrier dysfunction and promotion of epithelial cell hyperplasia.^{2–4} Thus, further understanding of the mechanistic basis of such complex interaction governing the immune system may enable approaches in treatments

for diverse pathologies. During lung inflammation, immune cell skewing can result in different pathological outcomes. For instance, classical Th1 responses lead to a pro-inflammatory microenvironment and tissue destruction, while Th2 immunity promotes tissue remodeling, airway hyper-responsiveness and eosinophilic infiltration.¹ In addition to T-cell subsets, the role of macrophage diversity is another component that is involved in skewing immune responses and pathology. The current paradigm of macrophage classification describes an association of the “M1” macrophage phenotype with pro-inflammatory secretory products including IL-6, TNF- α , IL-12, IL-1 β and Th1 responses, whereas the “M2” (alternatively activated) macrophage has been linked with Th2 responses and tissue repair.⁵

Subsets of M2 cells have also been proposed in the mouse system.⁶ Generally, M2 cells show enhanced release of IL-10 or TGF- β and cell expression of Arg-1 and mannose receptor (CD206). Although Th1 cytokines (IFN- γ) support M1 skewing and Th2 cytokines (IL-4/IL-13) support M2 development *in vitro*, the participation of other cytokines in M1/M2 skewing is less clear. Thus, here we focus on cytokines within the IL-6-family in this respect.

Gp130 cytokines are elevated during chronic inflammatory lung disease, including IL-6, Leukemia Inhibitory Factor (LIF), Oncostatin M (OSM), IL-11 and IL-31, and these cytokines may participate in immune cell skewing within the lung microenvironment.⁷⁻¹¹ Recently, IL-6 and OSM have been implicated in macrophage polarization *in vitro*. Mauer *et al.*¹² have demonstrated that IL-6 can potentiate IL-4-mediated M2 macrophage differentiation by upregulating IL-4R α expression on bone marrow-derived macrophages. In addition, Komori *et al.* have suggested that OSM can directly enhance an M2 macrophage phenotype in macrophages derived from adipose tissue.¹³ Although these studies independently implicate IL-6 and OSM in augmenting the alternative activation of macrophages *in vitro*, their role in macrophage skewing in lung tissues *in vivo* is less clear. We have demonstrated that pulmonary overexpression of OSM in mice induces a marked increase in the expression of Arg-1 (M2 macrophage marker) and Arg-1⁺ cell accumulation in mouse lungs.¹⁴ We here compare direct and indirect effects of OSM in comparison to IL-6 on M2 cell accumulation *in vivo* and M2 polarization *in vitro*. Since M2-like cells have previously been implicated in promoting tumor growth, invasion and angiogenesis, we also compared the function of OSM and IL-6 on tumor growth of ectopic B16 melanoma in association with M2 accumulation in lungs.¹⁴ The study suggests separate roles of Oncostatin M and IL-6 in elevation of M2 macrophage populations during inflammation of the lung.

RESULTS

Overexpression of OSM, but not IL-6 increased Arginase-1 protein and mRNA expression in mouse lung tissue

Our previous studies showed that pulmonary overexpression of OSM induces Arg-1 expression and Arg-1⁺ cells in C57Bl/6 mouse lungs. Here, we examine mechanisms of OSM function *in vivo*, and first compared the effects of OSM to IL-6 in mediating Arg-1⁺ macrophage accumulation. C57Bl/6 mice were endotracheally intubated with AdDel70 control vector, AdOSM or AdIL-6, to induce transient pulmonary

overexpression of OSM or IL-6, and examined at day 7 as previously.¹⁵ Confirming overexpression of vector-encoded cytokines in the system, we observed increased levels of OSM or IL-6 in the bronchoalveolar lavage fluid (BALF) of mice treated with AdOSM or AdIL-6, respectively, but no increase in AdDel70-treated mice (Figure 1a and b). AdOSM induced IL-6 expression as previously published,¹⁴ but were not as high as levels in AdIL-6 treatments. The IL-6 levels generated by AdOSM in BAL is likely reflecting contributions from multiple cell types, including macrophages and connective tissue cells. We have previously shown that OSM directly induces IL-6 expression *in vitro* in connective tissue cells^{16,17} that, along with leukocytes, will collectively contribute to IL-6 levels detected. OSM levels were not detectable in BALF of AdIL-6 nor AdDel70-treated mice. Arg-1⁺ cells were elevated in the lung tissue of mice intubated with AdOSM as assessed by immunohistochemistry, but not in mice treated with AdIL-6 (Figure 1c). Expression of Arg-1 was primarily localized to macrophages within alveolar spaces. Similarly, western blot analysis of whole lung homogenates demonstrated an induction of Arg-1 protein signal (50-fold) in the lungs of mice treated with AdOSM, compared to AdDel70 or AdIL-6 (Figure 1d and e, please see online Supplementary Material for full western blot images). We observed two bands using the Arg-1 primary antibody, which has been previously reported as two isoform variants of Arg-1 protein.¹⁸ We also observed increased phosphorylation of STAT3 in total lung extracts upon overexpression of OSM or IL-6 (Figure 1f) confirming that sufficient levels of OSM or IL-6 were present to induce biological effects. Arg-1 mRNA levels were significantly increased in total lung homogenates of mice treated with AdOSM, whereas homogenates from AdIL-6-treated mice showed no detectable levels above AdDel70 control (Figure 1g), consistent with Arg-1 protein data. iNOS (Nos2) mRNA levels in lung homogenates among the three treatment groups were similar and detected at significantly lower levels compared to Arg-1. Combined, these data suggest that overexpression of OSM *in vivo* promoted elevation of Arg-1-expressing cells, whereas IL-6 overexpression did not.

OSM stimulates increases in alternatively activated macrophages

We have previously observed overexpression of OSM to promote a T-helper 2 (Th2)-like environment in the lung.¹⁵ As the development of alternatively activated M2-polarized macrophages has been shown to require Th2-type cytokines and we observed here increases in Arginase-1 expression, a marker of M2 macrophages, we

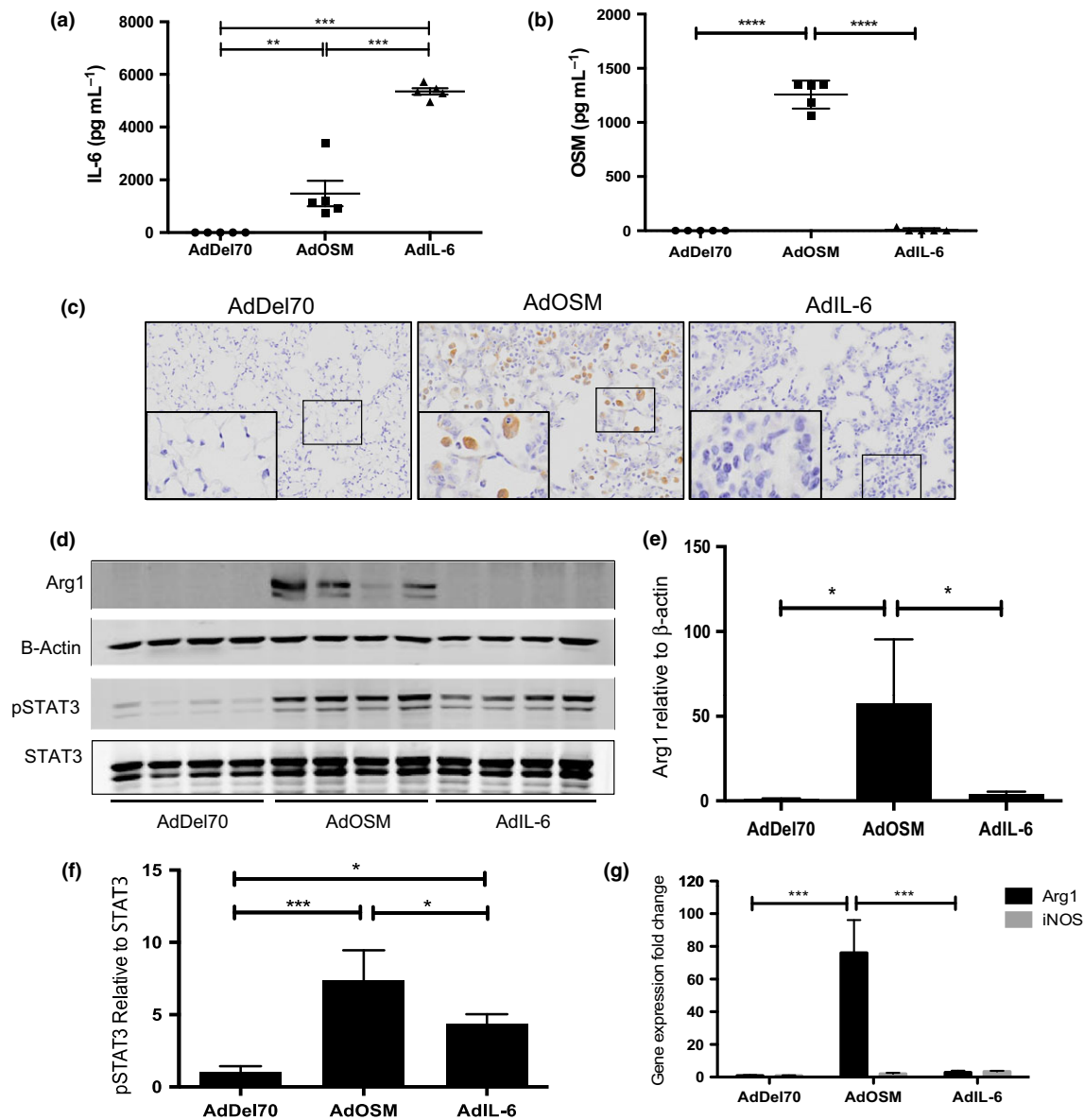


Figure 1. Overexpression of OSM induces Arg-1 protein and mRNA expression. C57Bl/6 mice were endotracheally administered AdDel70 control vector (1×10^8 PFU), AdOSM (5×10^7 PFU), or AdIL-6 (3×10^7 PFU) ($n = 5$ /group) and culled at day 7. IL-6 (**a**) and OSM (**b**) were measured in the bronchoalveolar lavage fluid (BALF) by ELISA (R&D) (AdDel70: circles, AdOSM: squares and AdIL-6: triangles). Representative images of immunohistochemistry for Arg-1 staining of paraffin-embedded formalin-fixed lung tissue (**c**). Western Blot analysis of whole lung homogenates ($n = 4$ /treatment) probed for Arg-1, β -actin, pSTAT3 and STAT3 (**d**). Quantification of Arg1 (**e**) and pSTAT3 (**f**) signal-corrected to β -actin or STAT3, respectively, using Image Studio software analysis of signals in (**d**) and represented as fold-changes relative to AdDel70 control. Gene expression levels of Arg1 and iNOS from total lung RNA ($n = 5$ /treatment) assessed by RT-qPCR (**g**). Western blot images were cropped to improve clarity and conciseness of the presentation. Results shown are from one of two separate experiments showing identical trends. Statistics were completed using a one-way ANOVA for multiple comparisons and subsequent Tukey's post hoc analysis (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

examined whether OSM and/or IL-6 could stimulate increases in M2 macrophages in the lungs of mice. C57Bl/6 mice endotracheally administered AdOSM or AdIL-6 were assessed for the changes in the frequency and total number of pulmonary M2 macrophages after

7 days by flow cytometry. M2 macrophages were identified as being CD45-positive (hematopoietic marker), F4/80-positive (macrophage marker), Gr-1-negative (granulocyte marker) and expressing the M2 markers CD206 and Arginase-1 (Gating strategy and

negative control staining shown in Supplementary figure 3). As shown in Figure 2a, naïve and Ad-infected mice had relatively similar frequencies of macrophages, and AdOSM or AdIL-6 did not significantly increase the number of total CD206⁺ macrophages, as compared to naïve mice. However, consistent with OSM-induced increases in arginase-1 expression in mouse lungs, AdOSM stimulated an approximately fivefold increase in the frequency of CD206⁺ Arg1⁺ macrophages and a 10.6-fold increase in the total number of CD206⁺ Arg1⁺ macrophages (Figure 2b and c), as compared to AdDel70 vector control mice. In contrast, AdIL-6 did not show any significant induction in the frequency or total cell number of CD206⁺ Arg1⁺ macrophages (Figure 2).

Overexpression of OSM and IL-6 induce unique inflammatory microenvironments in the lung

BALF of C57Bl/6 mice treated with AdOSM *versus* AdIL-6 were used to measure levels of various cytokines, chemokines and growth factors at day 7 (Figure 3). Similar to previously published data,¹⁹ overexpression of OSM resulted in elevated levels of IL-4, IL-13, IL-5, eotaxin-1 (CCL11) and eotaxin-2 (CCL24) (Figure 3a–d) as well as IL-6 (shown in Figure 1a). AdOSM-treated mice also demonstrated increased levels of BALF eosinophils, consistent with elevated levels of eotaxin-1 and 2 (Supplementary figure 1d). In contrast, AdIL-6 administration led to increased levels of IFN- γ , monokine-induced by interferon-gamma (MIG/CXCL9), and interferon-gamma inducible protein-10 (IP-10/CXCL10) in BALF compared to mice receiving the control vector (AdDel70) or AdOSM (Figure 3e–g). Both AdOSM- and AdIL-6-treated mice showed increased levels of the neutrophil chemoattractant, KC chemokine (CXCL1) (Figure 2h), consistent with equivalent neutrophil accumulation in BALF (Supplementary figure 1c). These results indicate that OSM overexpression led to the upregulation of Th2-skewed cytokines, while IL-6 overexpression induced a cytokine profile more toward a Th1-skewed inflammatory microenvironment, (although IL-2 or IL-12 were not detectable, data not shown). We next tested whether OSM could directly induce an Arg-1⁺-cell phenotype by assessing mouse macrophage cultures *in vitro*.

IL-6, but not OSM, directly acts on M2-polarized macrophages to increase Arginase-1 activity

To examine whether OSM could directly mediate the polarization of Arg-1⁺ alternatively activated (M2/AAM) macrophage phenotype, we utilized the widely used model of bone marrow-derived macrophages (BMDMs)

differentiated in culture from bone marrow cells for 7 days in M-CSF.²⁰ These cells were then stimulated for 24 h with M1 (IFN γ /LPS) and M2 (IL-4/IL-13) cytokine cocktails (as previous)²¹ to skew M1 and M2 macrophage phenotypes, and tested in combination with varying doses of recombinant OSM, IL-6, and another gp130 cytokine comparator, LIF. After 30 h of cytokine stimulation, we assessed polarization of macrophages toward the Arg-1-expressing M2 phenotype by measuring changes in arginase activity in parallel to a typical M1 macrophage phenotype product nitric oxide (NO). Results indicated increased arginase enzymatic activity in BMDMs stimulated with IL-4/IL-13, confirming an alternatively activated macrophage (AAM) phenotype as previously published²¹ (Figure 4a). OSM, IL-6 or LIF stimulations alone were not sufficient to elevate arginase activity. While neither OSM nor LIF (at 5 or 50 ng ml⁻¹) could modulate IL-4/IL-13 (M2 stimuli) -induced arginase-1, IL-6 further elevated Arg-1 activity in a dose dependent manner. In the same cells, IFN γ /LPS stimulus markedly enhanced NO levels. Neither IL-6, OSM or LIF stimulation alone elevated NO levels, nor did they modulate the response to IFN γ /LPS (Figure 4b).

RT-PCR analysis showed that OSMR β mRNA transcripts were 1000-fold lower and that LIFR α were 100-fold lower than gp130 transcripts (Figure 4c), consistent with low expression of the receptor chains necessary for OSM or LIF responses and the lack of ability of OSM or LIF to directly induce arginase-1 in BMDM *in vitro*. In contrast, IL6R α and IL-4R α mRNA transcripts were at levels consistent with ability of IL-6 and IL-4 to regulate this activity. In addition, IL-6 stimulation elevated IL-4R α transcripts (Figure 4d), consistent with previous results (Mauer *et al.*¹²) identifying IL-4R α expression and elevated levels of responses in these cells when IL-6 was used in combination with IL-4/13. We also tested whether these responses were similar in primary alveolar macrophages by assessing adherent cells retrieved from BALF of naïve mice. In contrast to BMDMs, alveolar macrophages were not responsive to IL-4/IL-13 stimulation with respect to elevation of arginase activity (Supplementary figure 4). In the added presence of IL-6, AMs demonstrated a very small increase in arginase activity; however, the response was markedly lower than that in BMDM (OSM had no effect, data not shown). Alveolar macrophages showed increases in NO levels in response to the M1 cytokine cocktail, although there was no effect of IL-6 on this phenotype, alone or in combination with IFN γ /LPS (Supplementary figure 4). The amount of NO and arginase activity was markedly lower in alveolar macrophages compared to BMDMs on an equal cell number basis.

To assess the activation of cell-signaling pathways upon macrophage polarization, lysates of BMDMs

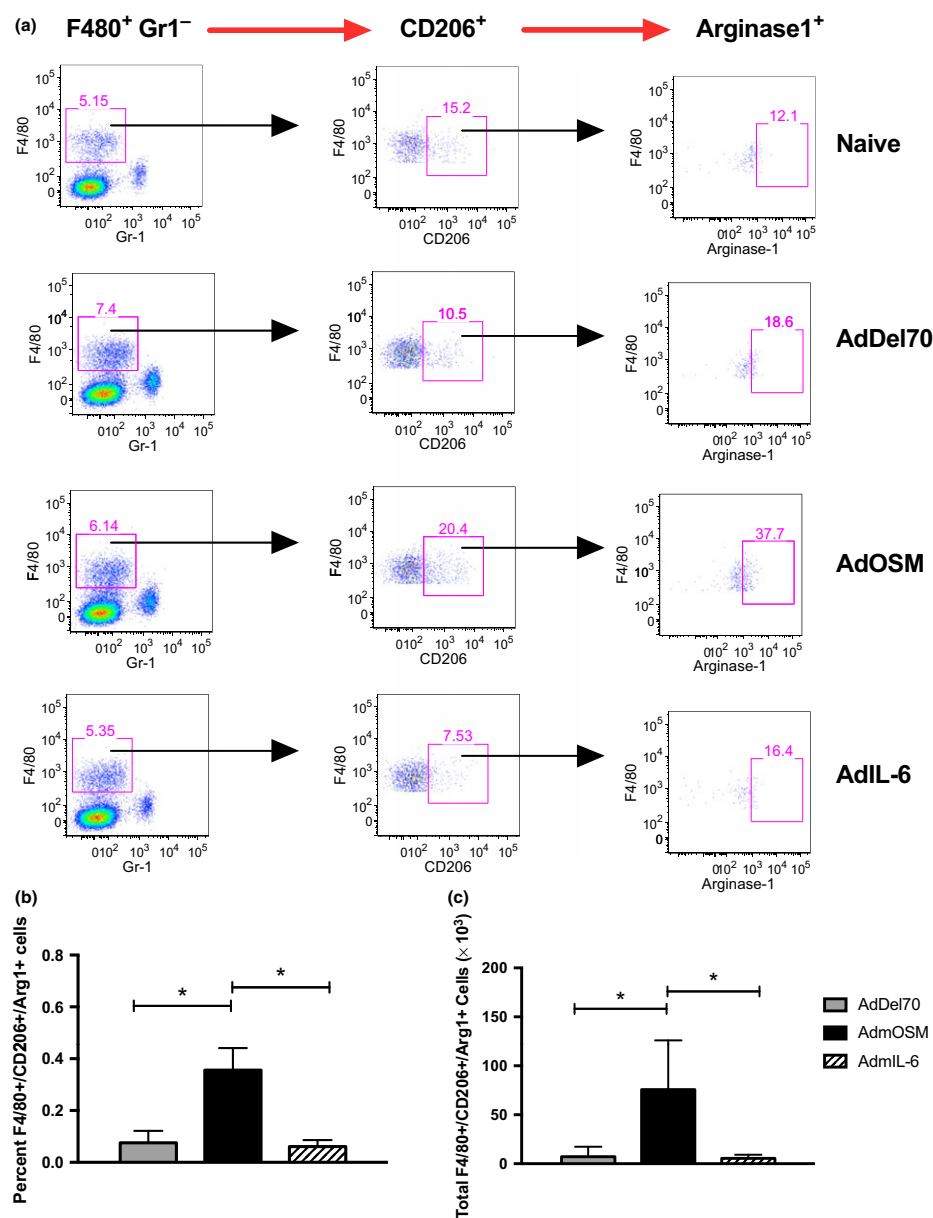


Figure 2. AdOSM, but not AdIL-6, increases M2 macrophages in the mouse lung. C57Bl/6 mice were endotracheally administered AdDel70 (control), AdOSM, or AdIL-6 ($n = 5/\text{group}$) and lungs analyzed after 7 days by flow cytometry for viable/side scatter, Zombie dye-negative) CD45⁺ M2 macrophage ($F4/80^+ Gr1^- CD206^+ Arginase1^+$ (Arg1⁺) cells. Gates are shown for $F4/80^+ Gr1^-$ cells, and $CD206^+$ and $Arginase1^+$ subpopulations cells. Flow cytometry gating scheme for identifying $F4/80^+/CD206^+/Arg1^+$ cells (a) (arrows indicate path to subpopulation). Percent positive (b) and total cell number (c) of $F4/80^+/CD206^+/Arg1^+$ cells in mouse lungs. One-way ANOVA and multiple comparisons test where $*P < 0.01$.

stimulated with M1 and M2 cytokine cocktails, with or without IL-6, were probed by western blot (Figure 5a). As expected, on the basis of previous work,⁶ M1-polarized macrophages demonstrated increased levels of pSTAT1, while pSTAT6 was elevated in M2-polarized macrophages (Figure 5c and e). Additionally, BMDMs stimulated with IL-6, alone or in combination

with the M2 cytokine cocktail, showed upregulation of STAT3 activation (Figure 5d). M1 stimuli inhibited pSTAT3 induction by IL-6. Similar to the results obtained from the Arg-1 activity assay (Figure 4a), BMDMs stimulated with M2 cytokine cocktail show induction of Arg-1 protein signal in cell lysates, which was further enhanced following IL-6 stimulation

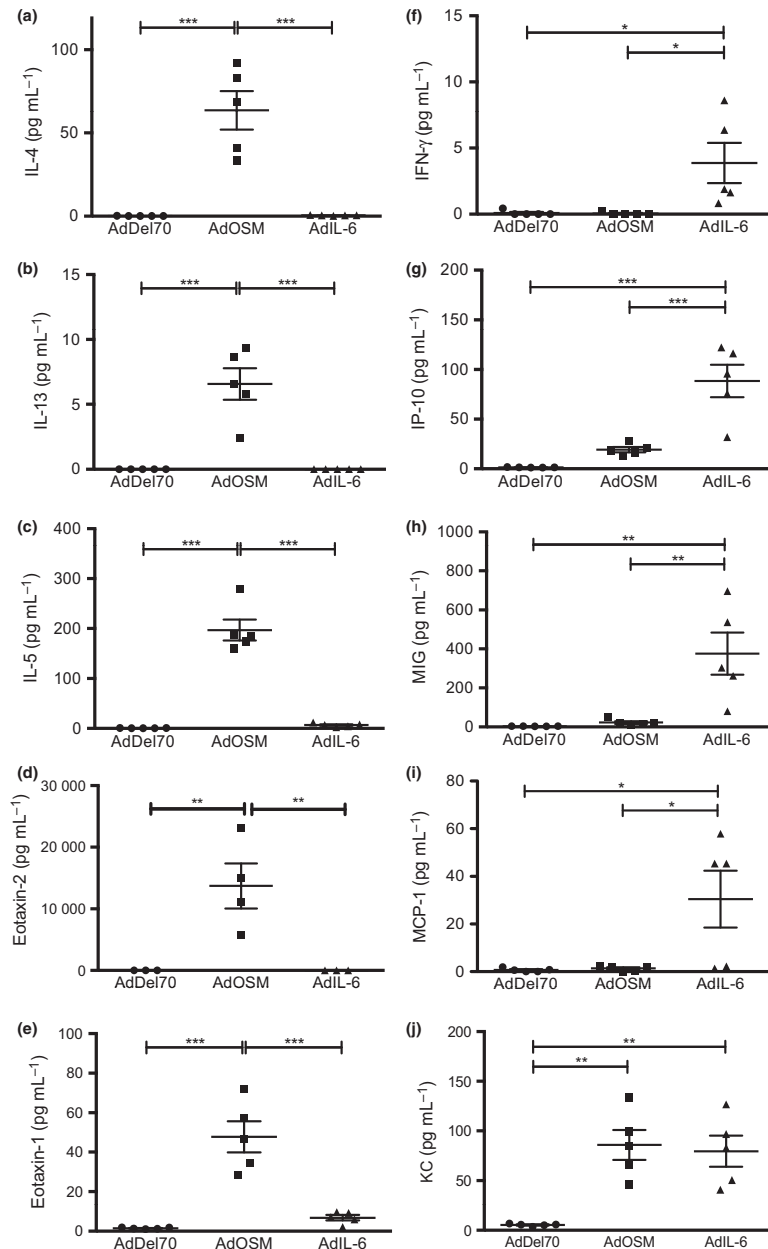


Figure 3. Overexpression of OSM and IL-6 produce unique inflammatory microenvironments in the lung. C57Bl/6 mice were endotracheally administered AdDel70 control vector (1×10^8 PFU), AdOSM (5×10^7 PFU), or AdIL-6 (3×10^7 PFU) ($n = 5$ /group) vectors and culled at day 7. Levels of IL-4 (a), IL-13 (b), IL-5 (c), Eotaxin-2 (d), Eotaxin (e), IFN γ (f), IP-10 (g), MIG (h), MCP-1 (i) and KC (j) were measured in BALF using cytokine-specific Eve Technology Murine Discovery Assay ($n = 5$ /group) or ELISA (Eotaxin-2 only) with remaining available sample ($n = 3-4$ /group) (AdDel70: circles, AdOSM: squares, AdIL-6: triangles). Statistics were completed using a one-way ANOVA for multiple comparisons and subsequent Tukey's post hoc analysis (where $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; indicates a statistically significant difference between indicated groups).

(Figure 5a–b). Together, these results demonstrate a direct effect of IL-6, but not of OSM or LIF, on polarizing macrophages to an alternatively activated phenotype in the presence of M2-polarizing cytokines

in vitro. Thus, OSM overexpression and its induction of Arg-1⁺ M2 macrophage accumulation appear independent of a direct effect of OSM on macrophages.

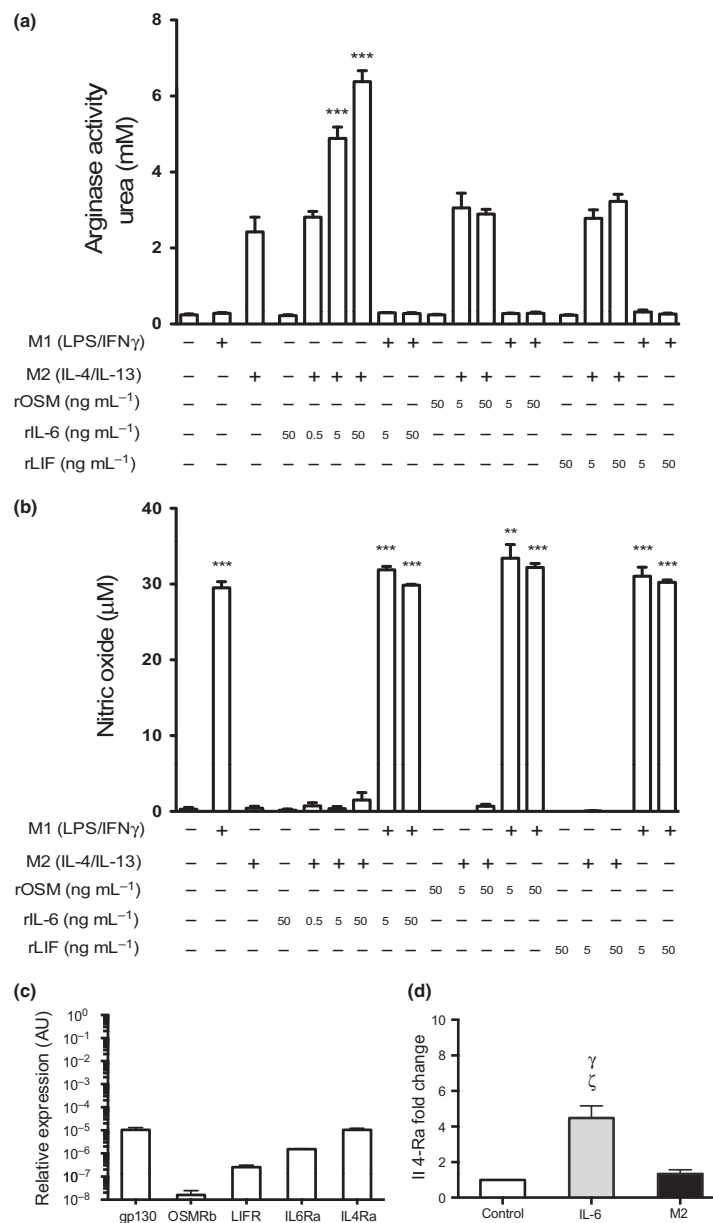


Figure 4. IL-6, but not OSM or LIF, enhances murine macrophage arginase enzymatic activity. BMDMs and primary alveolar macrophages isolated from naïve C57BL6 mice were cultured for 24 h using M2 and M1 cytokine cocktails (M2: IL-4 + IL-13; M1: LPS + IFN- γ) alone or in combination with the indicated gp130 cytokines. Arginase activity from cell lysates **(a)** and Nitric oxide levels are shown in supernatants of BMDMs **(b)**. Bars represent mean $\pm$ SEM from four replicates per group and graph is representative of two independent experiments. Relative expression of gp130, OSMR β , LIFR α , IL-6R α and IL-4R α in untreated BMDMs **(c)** and expression of IL-4R α in BMDMs stimulated with IL-6 or M2 stimuli **(d)**, measured by RT-qPCR. Statistics were completed using a one-way ANOVA and Newman-Keuls post hoc test where $^{**}P < 0.01$; $^{***}P < 0.001$ and $\gamma\zeta$ $P < 0.0001$ represents a statistically significant difference between the indicated conditions and the reference control.

IL-6 and IL-4/IL13 signaling is necessary for OSM-induced Arginase-1 mRNA and protein expression

Since OSM did not directly regulate arginase-1 activity *in vitro* while IL-6 did, and since OSM upregulates IL-6 expression *in vivo*, we next assessed if IL-6 was required

for AdOSM-induced Arg-1+ macrophage accumulation using IL-6 $^{-/-}$ mice. IL-6 $^{-/-}$ and wild-type mice were administered AdDel70 and AdOSM and lung tissue was extracted for mRNA, protein and histological analysis at day 7. Upon overexpression of OSM, IL-6 $^{-/-}$ mice showed decreased levels of Arg1 mRNA, compared to

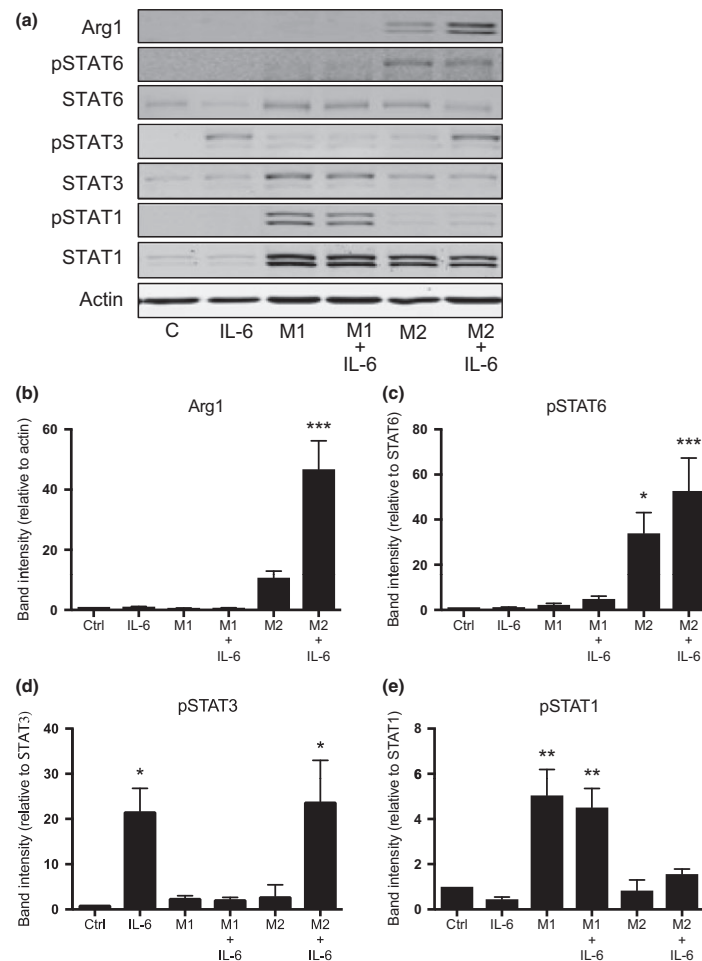


Figure 5. IL-6 directly acts on M2-polarized bone marrow-derived macrophages to upregulate Arginase-1 protein expression. Bone marrow-derived macrophages were stimulated with an M1 (IFN- γ + LPS) or M2 cocktail (IL-4/IL-13) in the presence or absence of IL-6 (5 ng mL⁻¹), for 24 h. Western blot analysis of Arg1, pSTAT1, pSTAT3 and pSTAT6 was performed on cell-culture lysates (a). Quantification of Arg1 (b), pSTAT6 (c), pSTAT3, (d) and pSTAT1 (e) band signals from three separate experiments using Image Studio software and corrected to β -actin, STAT6, STAT3 and STAT1, respectively. Western blot images were cropped to improve clarity and conciseness of the presentation and (a) represents blots from one of three separate experiments showing a similar outcome. Statistics were completed using a one-way ANOVA for multiple comparisons and subsequent Tukey's post hoc analysis (where * P < 0.05; ** P < 0.01; *** P < 0.001; indicates a statistically significant difference between indicated groups).

wild-type mice (Figure 6a and c). In contrast, iNOS gene expression, a marker of M1 macrophage polarization, was similar in all treatment groups (Supplementary figure 2). Arg-1 protein signal in lung homogenates was completely abrogated in IL-6^{-/-} mice treated with AdOSM, whereas wild-type mice showed a 100-fold average increase in Arg-1 protein signal (Figure 6a and b). We also observed that OSM-induced Arg-1 mRNA expression was dependent upon activation of the STAT6 signaling pathway using STAT6^{-/-} mice. Gene expression of Arg-1, but not iNOS, was attenuated in STAT6^{-/-} mice administered AdOSM compared to wild-type mice (Figure 6d and e). These results are consistent with previously published studies showing that STAT6 signaling

is required for an AAM phenotype.^{21,6} Phosphorylation of STAT3 was upregulated in response to OSM overexpression for both wild-type and IL-6^{-/-} mice (Figure 6a), indicating similar gene transduction by AdOSM in both mouse strains. Overall, our results indicate that M2 macrophage accumulation in mouse lung in response to OSM requires IL-6 and functional STAT6 signaling.

Increased tumor burden is associated with OSM-induced arginase-1 expression and is attenuated following OSM overexpression in IL-6^{-/-} mice

We have previously established the effect of AdOSM in inducing ectopic B16 tumor growth in lungs.¹⁴ Since

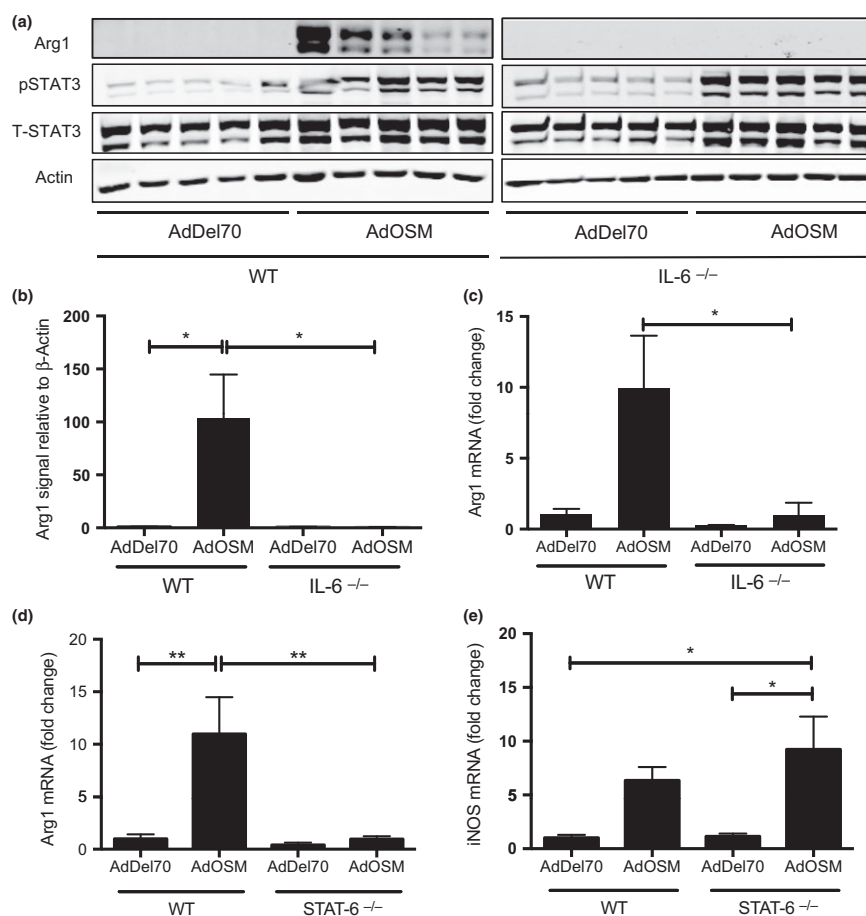


Figure 6. IL-6 and STAT6 activation is necessary for OSM-induced Arg-1 protein expression in mouse lung tissue. C57Bl/6 wild-type or IL-6^{-/-} mice were endotracheally administered AdDel70 (5×10^7 PFU) or AdOSM (5×10^7 PFU) and culled at day 7 ($n = 5$ /group). Western blot analysis of Arg-1, pSTAT3, STAT3 and β -actin from whole lung homogenates (a). Quantification of Arg-1 western blot bands corrected to β -actin using Image Studio Lite, and represented as fold-changes relative to AdDel70 control (b). Arg-1 mRNA levels measured using RT-qPCR and represented as fold-changes relative to AdDel70 control (c). Arg-1 (d) and iNOS (e) gene expression levels using RT-qPCR in wild-type or STAT6^{-/-} mice treated with AdDel70 or AdOSM (7 days) ($n = 5$ /group). Western blot images were cropped to improve clarity and conciseness of the presentation. Statistics were completed using a one-way ANOVA for multiple comparisons and subsequent Tukey's post hoc analysis (* $P < 0.05$; ** $P < 0.01$).

AdOSM induces IL-6 levels in our system and promotes Arg-1⁺ M2 macrophage accumulation *in vivo*, we assessed the effects of IL-6 overexpression on B16-F10 tumor growth compared to OSM. C57Bl/6 mice received 1×10^5 B16-F10 melanoma cells *via* tail vein injection, and were intubated the following day with either 2×10^8 PFU AdDel70, 5×10^7 PFU AdOSM, or 2×10^8 PFU AdIL-6 for 14 days. Representative images of H&E-stained sections of the lungs demonstrated a small increase in number of tumor nodules upon OSM overexpression, compared to the lungs of AdDel70-treated mice, as previously shown¹⁴ (Figure 7a). There was a marked increase in tumor burden upon OSM overexpression within the parenchyma and peribronchiolar space resulting in disruption of alveolar structure. In contrast, IL-6 overexpression did not

significantly modify absolute tumor number or burden (Figure 7b-c). Tumor area quantification using ImageJ indicates an approximately 14-fold increase in tumor burden upon OSM overexpression, compared to the AdDel70 or AdIL-6 treatment (Figure 7c).

Consistent with mRNA and protein data, lung tissue from IL-6^{-/-} mice showed decreased immuno-histochemical staining for Arg-1⁺ cell accumulation compared to wild-type mice in response to OSM overexpression (Figure 7d). M2 macrophages have previously been associated with promoting tumor growth and therefore, we then examined whether IL-6 was also necessary for the AdOSM-induced effects on tumor burden. Wild-type and IL-6^{-/-} C57Bl/6 mice were administered 1×10^5 B16-F10 cells via tail vein injection,

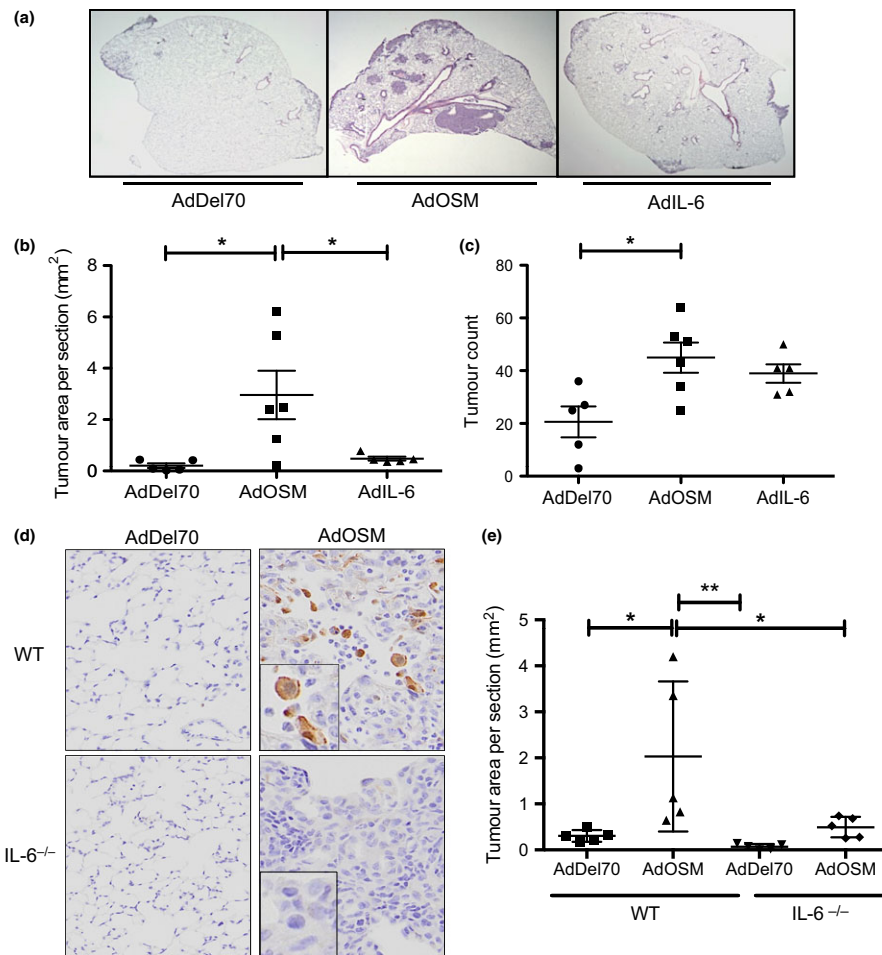


Figure 7. IL-6 is insufficient alone but is necessary for maximal of ectopic B16 melanoma tumor burden following OSM-induced lung inflammation. Representative histological images of lung tissue from wild-type mice injected with B16F10 melanoma cells (1×10^5 cells) in combination with AdDel70 (2×10^8 PFU), AdOSM (5×10^7 PFU), or AdIL-6 (2×10^8 PFU) and culled at day 14 ($n = 5/\text{group}$) (a). Manual quantification of prehistological section tumor burden (b) (as described in the Methods) or macroscopic tumor counts (c). Immunohistochemistry staining for Arg-1 protein expression in wild-type and IL-6^{-/-} mice treated with AdDel70 or AdOSM at 7 days (d). Quantitative analysis of tumor burden following B16F10 melanoma cell injection i.v. and AdDel70 and AdOSM vector endotracheal administration after 14 days in wild-type and IL-6^{-/-} mice (e). Statistics were completed using a one-way ANOVA for multiple comparisons and subsequent Tukey's post hoc analysis (* $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$).

followed by endotracheal intubation of either 5×10^7 PFU AdDel70 or AdOSM 24 h later and lung tissue sections were assessed for tumor burden (Day 14). Quantitative analysis of tumor area using ImageJ software indicated a significant increase in tumor area upon overexpression of OSM in wild-type mice in comparison to the AdDel70 controls (Figure 7e). This increase in tumor burden was attenuated in IL-6^{-/-} mice, suggesting requirement of IL-6 for maximal OSM-induced B16 tumor burden. Together, these results indicate that IL-6 is required for M2 macrophage accumulation as well as B16 melanoma growth following OSM-induced lung inflammation.

DISCUSSION

Several studies have implicated a role for alternatively activated M2 macrophages in enhancing tumorigenesis or pathological wound healing in various models of inflammation.^{5,12,22-25} Superimposed on typical Th2 products IL-4/IL-13 in mediating M2 differentiation, gp130 cytokines are also elevated locally in chronic disease and may participate in the process. Here, we have identified differential functions of IL-6 and OSM on M2 macrophage phenotype and tumor growth. In contrast to OSM overexpression, transient overexpression of IL-6 does not induce pulmonary Arg-1 expression nor Arg-1⁺

cell accumulation. However, elevated Arg-1 expression and Arg-1⁺ macrophage accumulation by OSM in whole lung tissue was abrogated in IL-6^{-/-} and STAT6^{-/-} mice, suggesting IL-6- and STAT6-dependent signaling is required for OSM-mediated effects *in vivo*. This finding was consistent with analysis of BMDMs demonstrating that IL-6, in combination with IL-4/IL-13, directly stimulated Arg-1 activity *in vitro*, while OSM or LIF did not, indicating indirect effects of OSM on Arg-1 induction *in vivo*. Additionally, AdOSM treatment led to enhanced lung tumor burden in comparison to AdDel70 or AdIL-6, which was attenuated in IL-6^{-/-} mice. These findings are consistent with a close association of M2 macrophage elevation and increased tumor growth.

Lung alveolar and interstitial macrophages assume a variety of cellular functions, including phagocytosis, antigen presentation and production of cytokines or chemokines during inflammatory processes.⁶ Functional plasticity of macrophages into M1/M2 subtypes, based on localization signals, enables these cells to participate in multiple stages of injury or wound repair. Murine M1/M2 macrophages can be identified by a unique set of markers. iNOS and CD40 are commonly used to identify M1 macrophages, whereas CD206, FIZZ-1, or Arg-1 are used to identify M2 macrophages.^{14,26-28} *In vivo* macrophages are phenotypically plastic and may simultaneously adopt a range of M1/M2 markers depending on the local cytokine milieu. In this study, we have used Arg-1 as a functional indicator of M2 macrophage activation and accumulation in our model. Arg-1 expression has been associated with lung tissue repair processes and cellular proliferation and collagen synthesis.²⁸ Increased Arg-1⁺ macrophages in AdOSM-exposed lungs may play a role in promoting cellular hyperplasia and thus increase tumor burden. We observed some variability between animals as previously demonstrated in other models of inflammation.¹⁸ This may be due to presence of other Arg-1-expressing cell types within the lung, such as innate lymphoid group 2 cells or fibroblasts.²⁹ In our system, we primarily observed Arg-1 localized to cells resembling macrophage morphology and thus pursued understanding alternative activation of macrophages.

As suggested by others, polarization of macrophages can be characterized as a spectrum of phenotypes with certain markers from classical (M1) phenotypes to alternatively activated phenotypes (M2).³⁰ Similar to previous publications, we have demonstrated here that IL-4/IL-13 signaling is sufficient to induce M2 macrophage polarization *in vitro* using BMDMs.³¹⁻³⁴ Since OSM was unable to induce Arg-1 activity *in vitro*, we speculate that the OSM-induced Th2 cytokine milieu produced *in vivo* sensitizes cells present in mouse lungs

to IL-6-mediated effects. Therefore, IL-6 overexpression, in the absence of a Th2 cytokine milieu, would not induce significant pulmonary Arg-1 expression or M2 macrophage accumulation in mice. Additionally, since IL-6 has a clearly prominent effect on BMDMs compared to alveolar macrophages, we suggest that the reduction in Arg-1⁺ in IL-6^{-/-} mice is due to the lack of newly recruited macrophages to the lung, consistent with a decreased macrophage population in the BALF of AdOSM-treated IL-6^{-/-} mice (as previously shown¹⁹).

Members of the gp130 family, both OSM and IL-6, are cytokines produced from activated lymphocytes and macrophages in the presence of inflammation and participate in similar functions, such as STAT3 activation or acute-phase protein synthesis.³⁵⁻³⁷ However, OSM and IL-6 also exude distinct functions, which we have further delineated within our model. OSM showed ability to upregulate Th2 specific cytokines, whereas IL-6 led to elevation of some cytokines and chemokines associated with Th1-skewed inflammation (Figure 3). Cytokine-specific receptor chains, OSMR β and IL-6R α , are differentially expressed among different cell types, leading to overlapping and distinct roles in inflammatory processes. For instance, OSMR β is well-expressed in stromal cells and can enable signaling and induction of chemokines that participate in inflammatory responses.³⁸ We have observed that IL-6R α mRNA is expressed by BMDMs, whereas OSMR β and LIFR mRNA levels were undetectable/low (Figure 4c and d) and also have found similar results in mouse alveolar macrophages.³⁹ Higher levels of IL-6R α and IL-4R α is consistent with responses to IL-6 and IL-4, respectively, and the further elevation of IL-4R α by IL-6 stimulation is consistent with previously published data¹² and re-enforces IL-4R induction as a mechanism for enhanced arginase in these cells in the response to combinations of IL-4 and IL-6 stimulation. The results showing a lack of significant induction of Arg-1 activity by M2 stimuli, OSM, or IL-6 in cultures of naïve alveolar macrophages suggests that the resident alveolar cells contribute little (if any) to the Arg-1⁺ cell accumulation in the lung due to AdOSM, although IL-4R α and IL-6R α expression is present on these cells.

It is widely recognized that macrophage Arg-1 expression is dependent on IL-4-mediated STAT6 signaling. Others have previously demonstrated that IL-6 can enhance the M2 macrophage phenotype through increased IL-4R α expression and amplification of the IL-4 response in BMDMs.^{12,34} This finding has also been observed in BMDMs stimulated by the anti-inflammatory cytokine, IL-10. Lang *et al.* demonstrated that IL-10 stimulation of BMDM extracted from IL-10-deficient mice, excluding background results from intrinsic IL-10 expression by macrophages, resulted in enhanced IL-4R α

expression. This observation was determined to be STAT3-dependent, similar to IL-6 effects on IL-4R α expression.⁴⁰ The promoter region for the macrophage-specific Arg-1 gene contains binding sites specific for STAT6 and CAAT/Enhancer-binding-protein (C/EBP) transcription factor, which has been shown to be directly activated by pSTAT3 activation.⁴¹ Qualls *et al.* demonstrated that Arg1 expression in BMDMs following mycobacterium infection was dependent upon C/EBP as well as the TLR-adaptor protein MyD88.⁴² Researchers showed that MyD88-dependent induction of soluble mediators, such as IL-6, IL-10 and G-CSF, within BMDMs infected with Mycobacterium activates STAT3 in a autocrine and paracrine manner to induce Arg1 expression.⁴² This was consistent with El Kasmi *et al.*, where TLR-dependent Arg-1 expression in BMDMs of mice infected with tuberculosis was independent of IL-4/IL-13 signaling or STAT6 activation,⁴³ and that IL-6-STAT3 or C/EBP β was critical for fibroblast-mediated alternative activation of macrophages and Arg1 expression in a pulmonary hypertension model of inflammation.⁴⁴ These observations indicate significant roles of STAT3 activation, along with STAT6 and TLR-MyD88 pathways.

Although OSM and IL-6 have been independently implicated in models of inflammation and cancer, their mechanism on cancer progression is not well understood. Yi *et al.* has recently demonstrated upregulation of the IL-6 receptor in lung cancer stem cells compared to noncancer stem cells and significant inhibition of cell growth after transfection with IL-6 siRNA or use of IL-6R monoclonal antibody.⁴⁵ High levels of IL-6 have also been associated with a decreased response to chemotherapy and survival outcome when examined in Stage 3B/4 non-small-cell lung cancer patients.⁴⁶ Similarly, high levels of OSM have also been associated with a shorter survival period for patients with breast cancer, suggesting that OSM may contribute to breast cancer progression.⁴⁷ Populations of tumor-associated macrophages have been described to display an alternatively activated (M2-like) phenotype that can support tumor growth. Potential regulation of TAM/M2 phenotypes by IL-6 or OSM may also contribute to cancer progression in human systems. Our results here suggest that OSM and IL-6 participate in M2 polarization through different roles and together maximize M2 cell accumulation in mouse lungs. Whether similar roles are present in other organs upon inflammation or in human systems including cancer requires further experimentation. M2 macrophages are thought to also participate in chronic inflammation resulting in tissue fibrosis. Thus, determining the roles and consequences of OSM and IL-6 in skewing macrophage phenotype may also identify alternative mechanisms to disease.

METHODS

Animal experiments

Female C57Bl/6 mice (6–8 weeks old) wild type were purchased from Charles River Laboratories (Ottawa, Canada). Female IL-6^{-/-} and STAT6^{-/-} mice (6–8 weeks old) were purchased from the Jackson Laboratory (Maine, USA). Purchased mice were acclimatized 1 week prior to experimental procedures and all mice were housed under specific pathogen-free conditions within the McMaster University Central Animal Facility. All experimental procedures were approved by the McMaster University Animal Research Ethics Board (AUP# 14-08-34).

Adenovirus administration

Adenovirus vectors AdDel70, AdOSM and AdIL-6 were thawed on ice, and diluted in 1 $\times$ PBS to specific concentrations (3 $\times$ 10⁷ PFU/mouse, 5 $\times$ 10⁷ PFU/mouse, 1 $\times$ 10⁸ PFU/mouse or 2 $\times$ 10⁸ PFU/mouse, as indicated in figure legends). AdDel70 is the backbone E1/E3-deleted Adenovirus type 5 vector without any insert in the expression cassette. As previously shown, AdDel70 is used as a control in our approaches, and does not induce any detectable effects (inflammatory cell accumulation, tissue pathology, inflammatory cytokine levels) at the time points examined.^{15,19} Each mouse was endotracheally intubated with 50 μ L of virus solution either alone or 24 h after B16F10 melanoma cell injection. Animals were culled 7 or 14 days following administration.

Ectopic delivery of B16-F10 melanoma cells

B16-F10 melanoma cells were purchased from American Type Culture Collection (CRL-6475) and cultured in DMEM media supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin/Streptomycin. Mice were administered 1 $\times$ 10⁵ B16-F10 cells in 0.2 mL via tail vein injection.

Collection of BALF and lung processing for histology

After mice were culled, lungs were washed once with 0.5 mL 1 $\times$ PBS to collect BALF followed by another three washes to collect the exhaustive BAL. These samples were centrifuged at 12 000 g for 2 min at 4°C, after which supernatants from the first wash were collected as BAL and stored at -80°C. Cells from the first and exhaustive washes were resuspended, combined and counted using Trypan Blue solution (Life Technologies, Burlington, ON). Cells were then cytocentrifuged and stained with Hema 3 Fixative Solutions (ThermoFisher Scientific) for differential cell analysis. After BAL collection, left lobe of lungs were perfused with 7 mL 1 $\times$ PBS to remove the remaining blood, followed by perfusion with 10% formalin fixative for 48 h prior to being transferred and stored in 70% ethanol. For histological sections, lungs were embedded in paraffin, cut into 5 μ m sections and stained with H&E. The right lobes were extracted, immediately frozen in liquid

nitrogen, and stored in -80°C until further processing. Frozen tissue samples were crushed and equal portions were suspended in either radioimmunoprecipitation (RIPA) buffer containing a protease inhibitor cocktail (Aprotinin, PMSF, Na_3VO_4 , DTT) for protein or Trizol (ThermoFisher Scientific) for RNA. Protein samples were homogenized using a syringe/needle or homogenizer and centrifuged at 14,000 rpm for 20 min at 4°C . Supernatants were collected and stored at -80°C . 200 μL of chloroform was added to Trizol samples after homogenization and centrifuged for 20 min. The aqueous phase was extracted and 500 μL of isopropanol was added for 10 min at room temperature and centrifuged again. The supernatant was discarded and the pellet was washed with ethanol. After centrifugation, pellets were resuspended in RNase-free water and stored at -80°C until further analysis.

Tumor counting and quantification

Photographs of lung sections stained with H&E were taken with a microscope at $1.6\times$ magnification, and tumor areas were selected and quantified using ImageJ software. The tumor area for each mouse was then summed, and divided by the number of sections cut by histology.

Immunohistochemistry

Histological sections were first de-paraffinized, then treated with 0.5% peroxide in methanol for 10 min, and blocked with 5% normal goat serum. They were then incubated with an anti-arginase-1 antibody (BD Biosciences Cat#610708) at a 1/100 dilution, followed by incubation with a biotinylated goat anti-mouse antibody (Vector Laboratories) at a 1/500 dilution. Sections were then treated with 1/50 dilution of streptavidin-peroxidase, developed in Nova Red peroxidase substrate (Vector Laboratories) and counterstained with hematoxylin.

Western Blot

Whole lung homogenate or cell lysate samples were loaded onto SDS-PAGE gels (20 μg protein/well) and separated by electrophoresis at 120 V for 1 h and transferred to nitrocellulose membranes at 400 mA for 1 h by standard methods. Following blocking for 1 h (TBS/Odyssey Blocking buffer), primary antibodies Arg-1 (BD Biosciences Cat#610708), pSTAT3 (Cell Signaling Cat#9145), STAT3 (Cell Signaling Cat#4901), pSTAT6 (Cell Signaling Cat#9361), STAT6 (Santa Cruz #sc-981), pSTAT1 (Cell Signaling Cat#9171), STAT1 (Cell Signaling Cat#9172) and Beta-Actin (Santa Cruz Cat#sc-1616) were used to probe membranes overnight at 4°C . Blots were washed the following day with TBS+0.15% Tween-20, and secondary antibodies (Anti-Goat; Mandel Scientific Cat# LIC92668074, Anti-Rabbit; Mandel Scientific Cat# LIC92632213, or Anti-Mouse; Mandel Scientific, Cat#LIC92632212) were added for approximately 1 h. After subsequent washes, blots were imaged using Odyssey LI-COR Imaging technologies. Signals were quantified by densitometry using Image Studio Lite ver. 5.2.

Enzyme-linked immunosorbent assay

Cytokine concentrations were measured in the BALF of mice using commercially available ELISA kits from R&D Systems, following the manufacturer's recommended protocol: mouse IL-6 (R&D Systems Cat# DY406-05) with a limit of detection of 15.65 pg mL^{-1} , mouse OSM (R&D Systems Cat# DY495-05) with a limit of detection of 6.25 pg mL^{-1} and mouse Eotaxin-2/CCL24 (R&D Systems Cat# DY528) with a limit of detection of 15.65 pg mL^{-1} . Thirty-two additional BALF cytokines, chemokines and growth factors were also measured by Eve Technologies Corporation using the Murine Discovery Assay[®].

Reverse-transcription quantitative polymerase chain reaction

Protein-specific mRNA from cells or lung tissues were quantified using Reverse-transcription quantitative polymerase chain reaction (RT-PCR). A volume of 21.5 μL of each sample containing 10 μg of RNA was treated for DNase removal using Life Technologies Ambion DNA-free[™] kit (Life Technologies Cat#AM1906). RNA samples were then quantified and reverse transcribed into complementary DNA (cDNA): 1 μL of 100 mmol L^{-1} dNTP mix and 1 μL of 0.2 $\mu\text{g mL}^{-1}$ Random Hexamer (ThermoFisher Scientific Cat# SO142) was added to 10 μL of RNA sample and incubated at 65°C for 5 min. Then 4 μL of $5\times$ first-strand buffer and 2 μL of 0.1 mol L^{-1} DTT was added to samples and incubated at 25°C for 2 min. RNase-free water (1.5 μL) and 0.5 μL of Superscript II (ThermoFisher Cat# 18064014) was added and samples were then incubated at 25°C for 10 min, 42°C for 50 min, and 70°C for 15 min. Samples were then diluted to a final concentration of 2 ng cDNA/ μL and either immediately analyzed by TaqMan or stored at -20°C . TaqMan reactions were performed using predetermined assay reagents (Universal Master Mix with UNG; ThermoFisher Scientific Cat#4440038) and specific TaqMan probes for mouse Arg-1 (Life Technologies Cat#mm00475988_m1), mouse iNOS (Life Technologies Cat#mm00440485_m1) and mouse 18S (ThermoFisher Scientific Cat#4319413E), as a control housekeeping gene.

Isolation and culture of murine alveolar macrophages

Mice were anesthetized by gaseous isoflurane, ice-cold PBS supplemented with 10 mmol L^{-1} EDTA was injected into the peritoneal cavity. Peritoneal washes were collected and mice were subsequently euthanized by severing the descending aorta. The whole lung was then excised and bronchoalveolar lavage fluid was collected by instilling ice-cold PBS directly into the excised lungs. Collected cells were pelleted and suspended in Dulbecco's modified Eagles medium (nutrient mixture F12) containing 10% fetal bovine serum. After incubation at 37°C , adherent cells were selected by washing all wells with PBS and changing media. Cell morphology was observed under an inverted microscope, confirming the successful macrophage adherence, as well as ensuring the exclusion of other non-adherent cell types. Macrophages were

then subjected to different exogenous stimuli (See below for the detailed M1 and M2 polarizing cocktails).

Isolation and culture of murine bone marrow-derived macrophages

Bone marrow (BM) cells were isolated from the femurs and tibia of naïve female C57BL6 mice aged 10–14 weeks following euthanization, as described above. BM cells were cultured in a medium containing 20 ng mL^{-1} of M-CSF (PeproTech Canada) for 7 days. Following 7 days in culture, non-adherent cells were washed off. Adherent cells constituting the macrophage population, referred to as BMDMs, were replated in 96-well plates at 80 000 cells/well. Cells were then subjected to different exogenous stimuli for 24 h (See below for the detailed M1 and M2 polarizing cocktails).

M1 and M2 macrophage polarization

Following the adherence of macrophages on 96-well plates, cells were treated for 24 h with LPS (100 ng mL^{-1}) and recombinant IFN- γ (10 ng mL^{-1}) to induce classically activated (M1) macrophages and with recombinant IL-4 (20 ng mL^{-1}) and IL-13 (50 ng mL^{-1}) (PeproTech Canada) to induce alternatively programmed (M2) macrophages. Recombinant IL-6 (R&D; 0.5 , 5 and 50 ng mL^{-1}), OSM (R&D; 5 and 50 ng mL^{-1}) and LIF (R&D; 5 and 50 ng mL^{-1}) were also simultaneously added with the M1 and M2 polarization cocktails to test the effect of these cytokines on macrophage polarization. Polarization of macrophages toward the M1 and M2 phenotype was established by assessing nitric oxide in the cell supernatant and arginase activity in the cell lysates, respectively. Nitric oxide was quantified using the griess reagent kit (Life technologies) and arginase activity was determined as described previously.⁴⁸

Isolation of lung mononuclear cells and flow cytometric analysis

Lung mononuclear cell suspensions were generated by mechanical mincing and collagenase digestion. Samples were digested with 15 units of Collagenase D (Roche, Laval, Quebec, Canada) and $250 \mu\text{g}$ of DNaseI (Sigma-Aldrich, Oakville, ON, Canada) in 10 mls of complete RPMI media (supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Oakville, ON, Canada), 1% L-glutamine, and 1% penicillin/streptomycin (Invitrogen, Burlington, ON, Canada)) for 1 h in a 37°C shaker. Debris were removed by passage through 45 micrometer screen size nylon mesh and cells were resuspended in PBS containing 0.3% bovine serum albumin (BSA) (Invitrogen). 1×10^6 lung mononuclear cells were washed once with PBS/0.3% BSA and stained with primary antibodies directly conjugated to fluorochromes for 30 min at 4°C . 10^5 live events were acquired on an LSR II (BD Biosciences, San Jose, California) flow cytometer and the data were analyzed with FlowJo analysis software (TreeStar Inc., Ashland, Oregon). Forward scatter and side scatter parameters

and Zombie-Aqua dye (BioLegend, San Diego, CA) were used to define the live cell gate. Cells were stained with Zombie-Aqua dye (1:200) for 20 min at room temperature prior to surface staining with fluorophore-conjugated antibodies. All antibodies were purchased from BD Biosciences or BioLegend (San Diego, California) unless otherwise stated. The following antibodies were used for surface staining: Brilliant Violet421-conjugated anti-F4/80 (1:100), Pacific Orange-conjugated anti-Gr-1(1:200), and APCcy7-conjugated anti-CD45(1:400). Cells were surface stained for 30 min at 4°C . Following surface staining, cells were fixed with BD Cytfix/CytoPerm (BD Biosciences) for 20 min at 4°C and then washed with BD $1 \times$ Perm/Wash (BD Biosciences) prior to intracellular staining with APC-conjugated Arginase-1 (R&D Systems, Minneapolis, MN) (1:10) and Brilliant Violet650-conjugated anti-CD206 for 30 min and 4°C . Cells were then washed in BD PermWash prior to resuspension in $1 \times$ PBS/0.3% BSA and analysis on an LSR II flow cytometer.

Statistical analysis

All statistical analyses were completed using GraphPad Prism 6.0. Figures indicate mean $\pm$ SEM. A one-way analysis of variance (ANOVA) with Tukey's or Newman-Keuls post hoc test was used to assess statistical differences between means, where $*P < 0.05$ was considered statistically significant.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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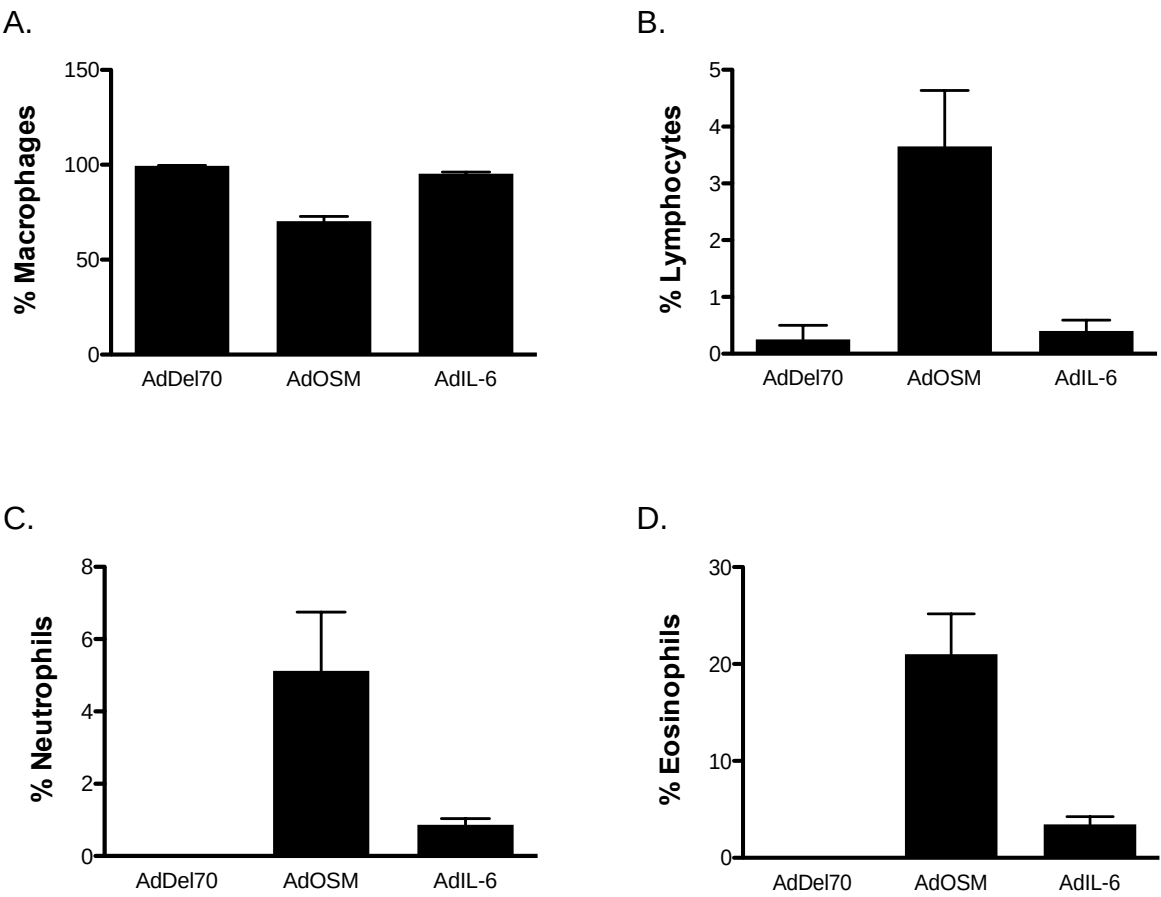
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SUPPORTING INFORMATION

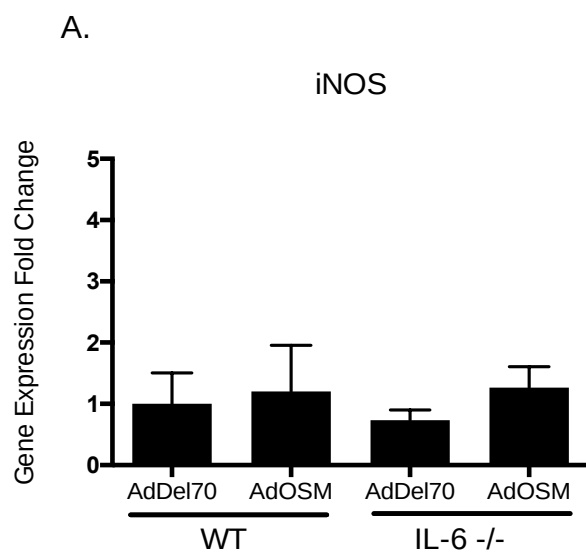
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Supplementary Figure 1



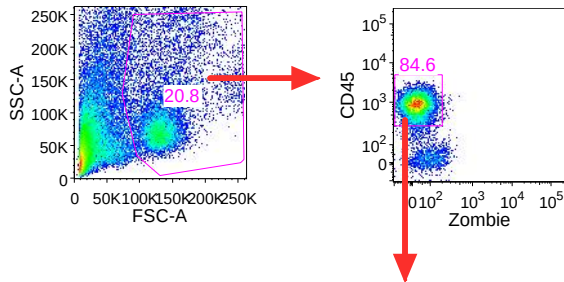
Supplementary Figure 2



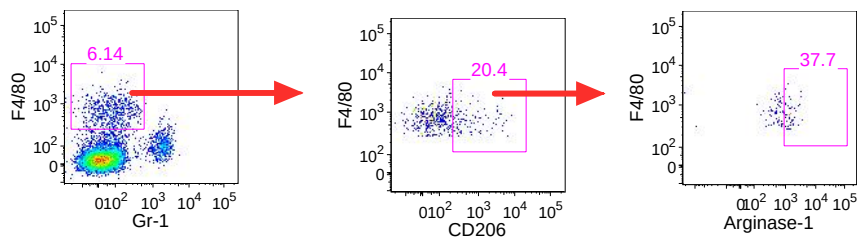
Supplementary Figure 3

(A) M2 cell FACS Gating Scheme

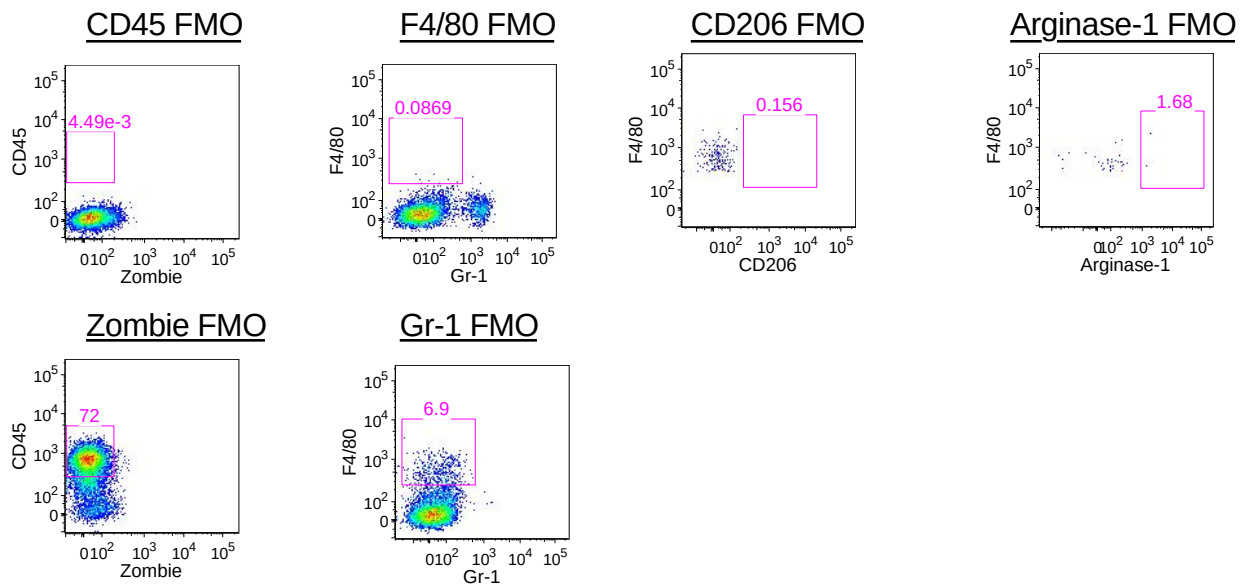
FSC/SSC, CD45+ Live Cells (Zombie-negative):



M2 cell gating (F4/80+ Gr1-, CD206+, Arginase-1+):

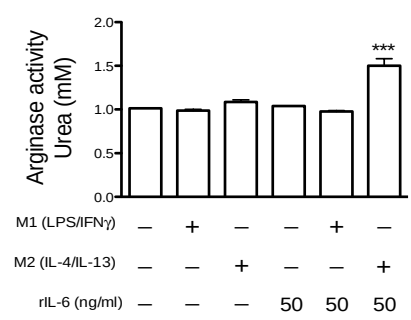


(B) Fluorescence Minus One (FMO) Controls

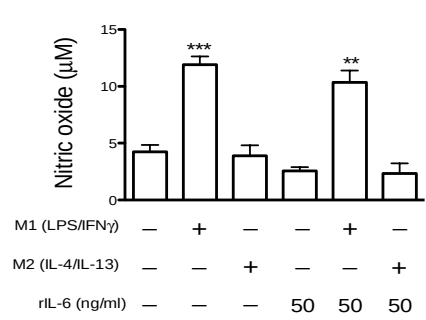


Supplementary Figure 4

A.



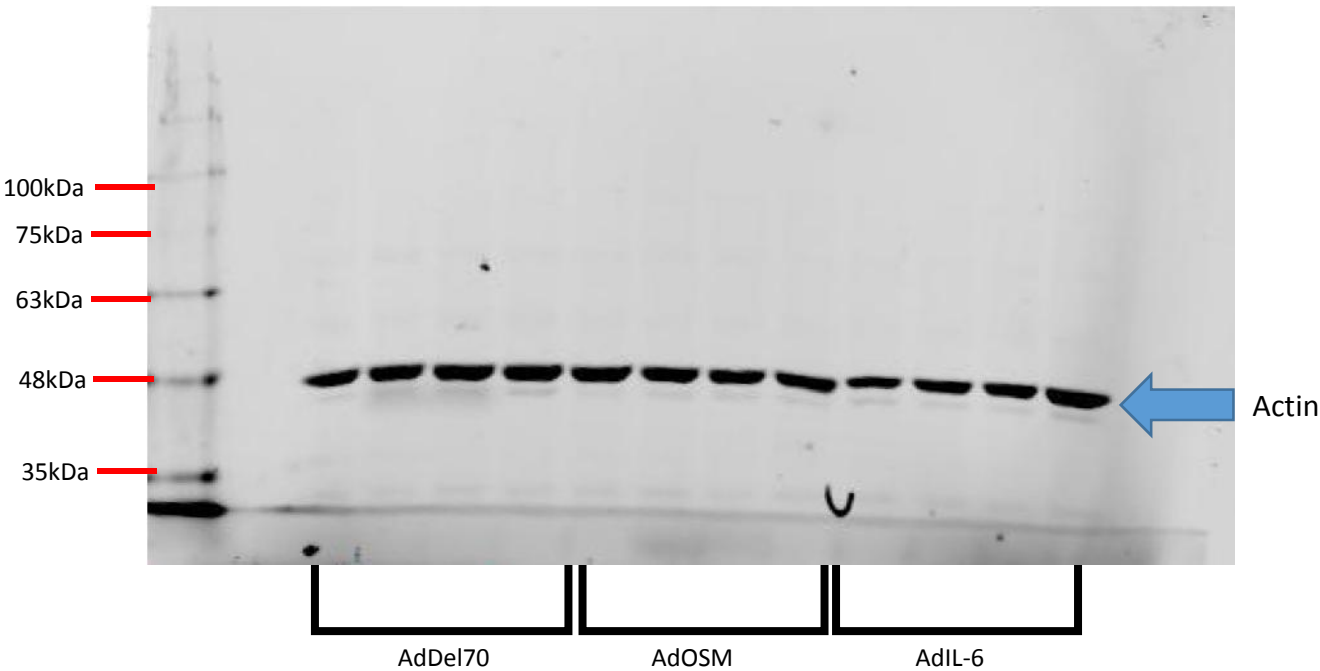
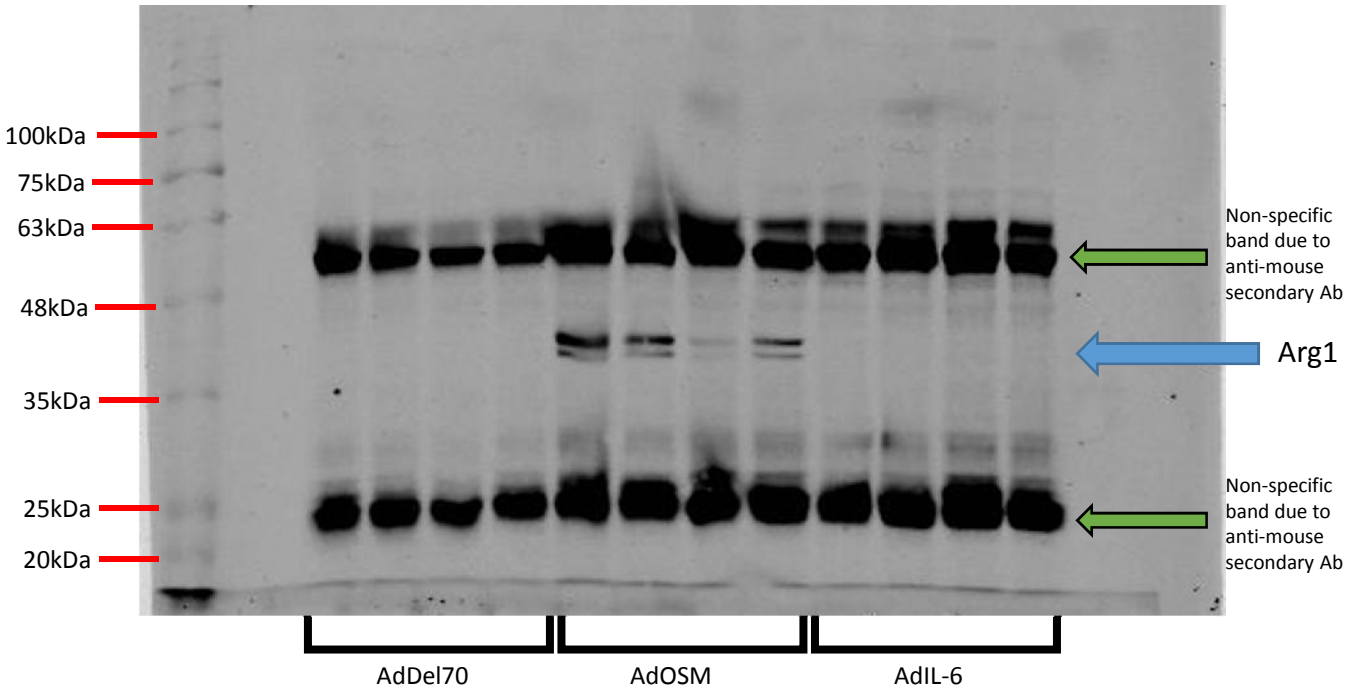
B.



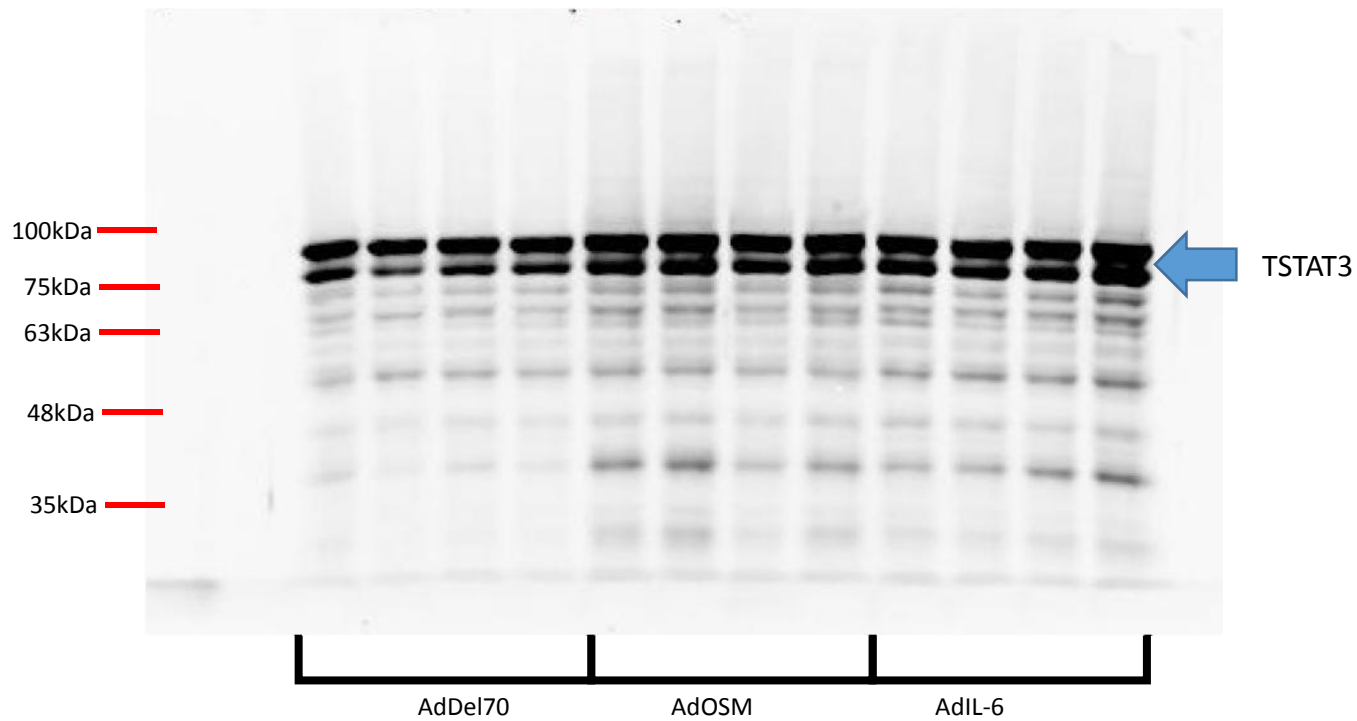
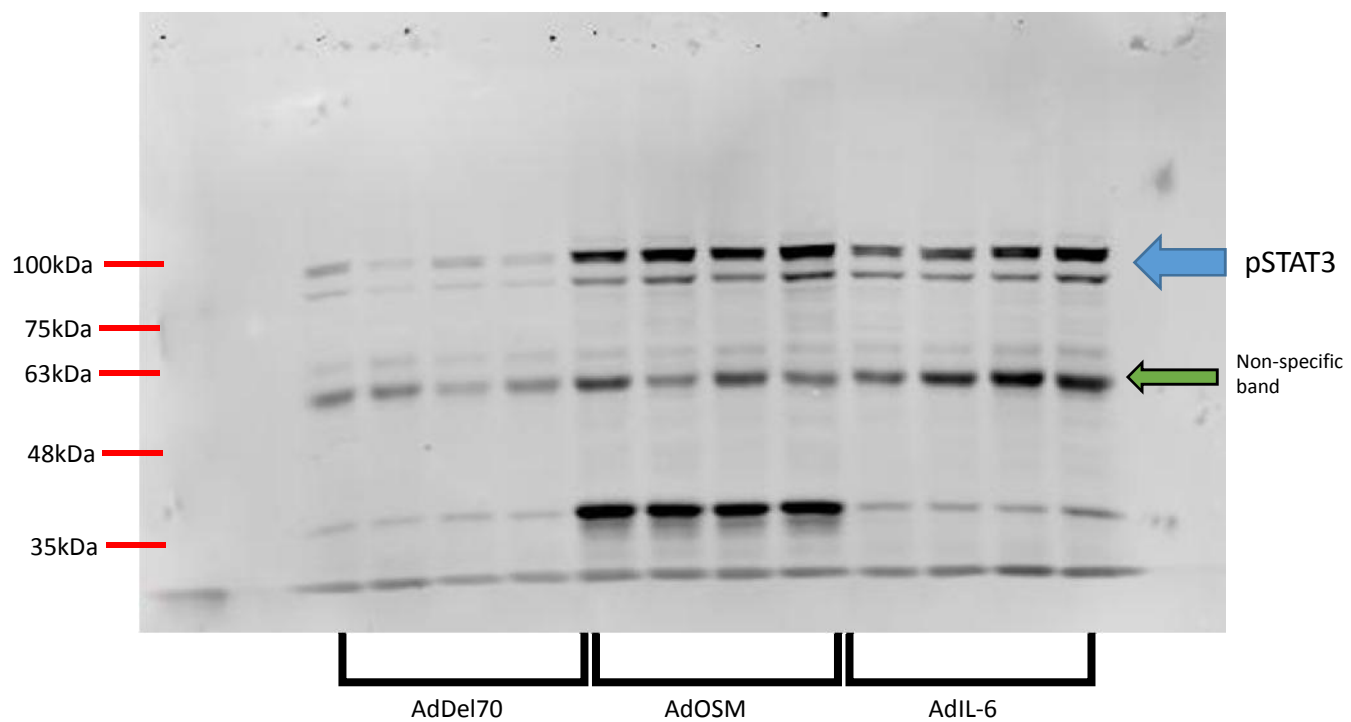
Supplementary Material

Full Western Blot Images

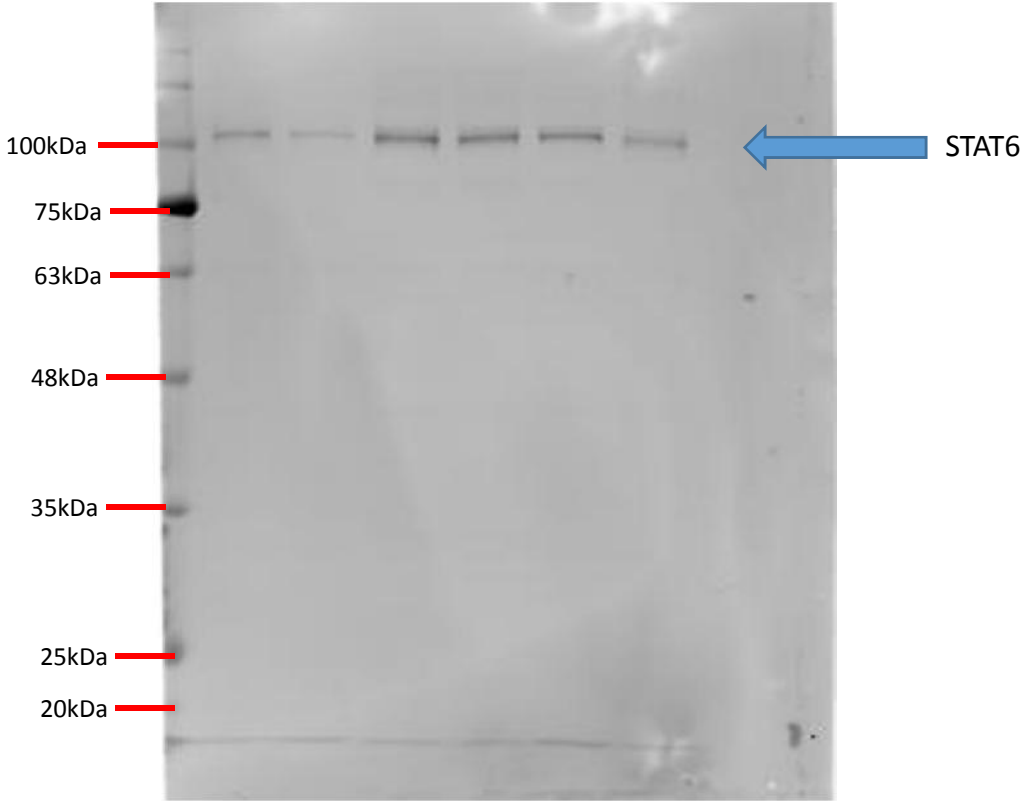
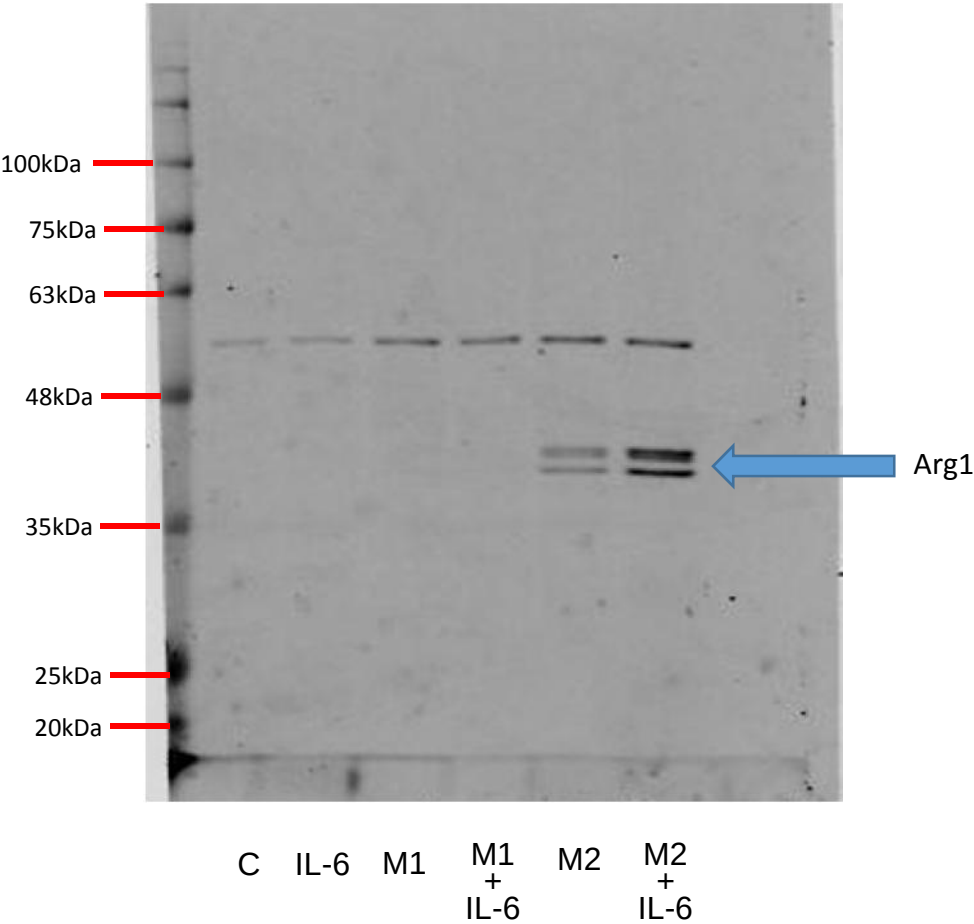
Re: Figure 1D



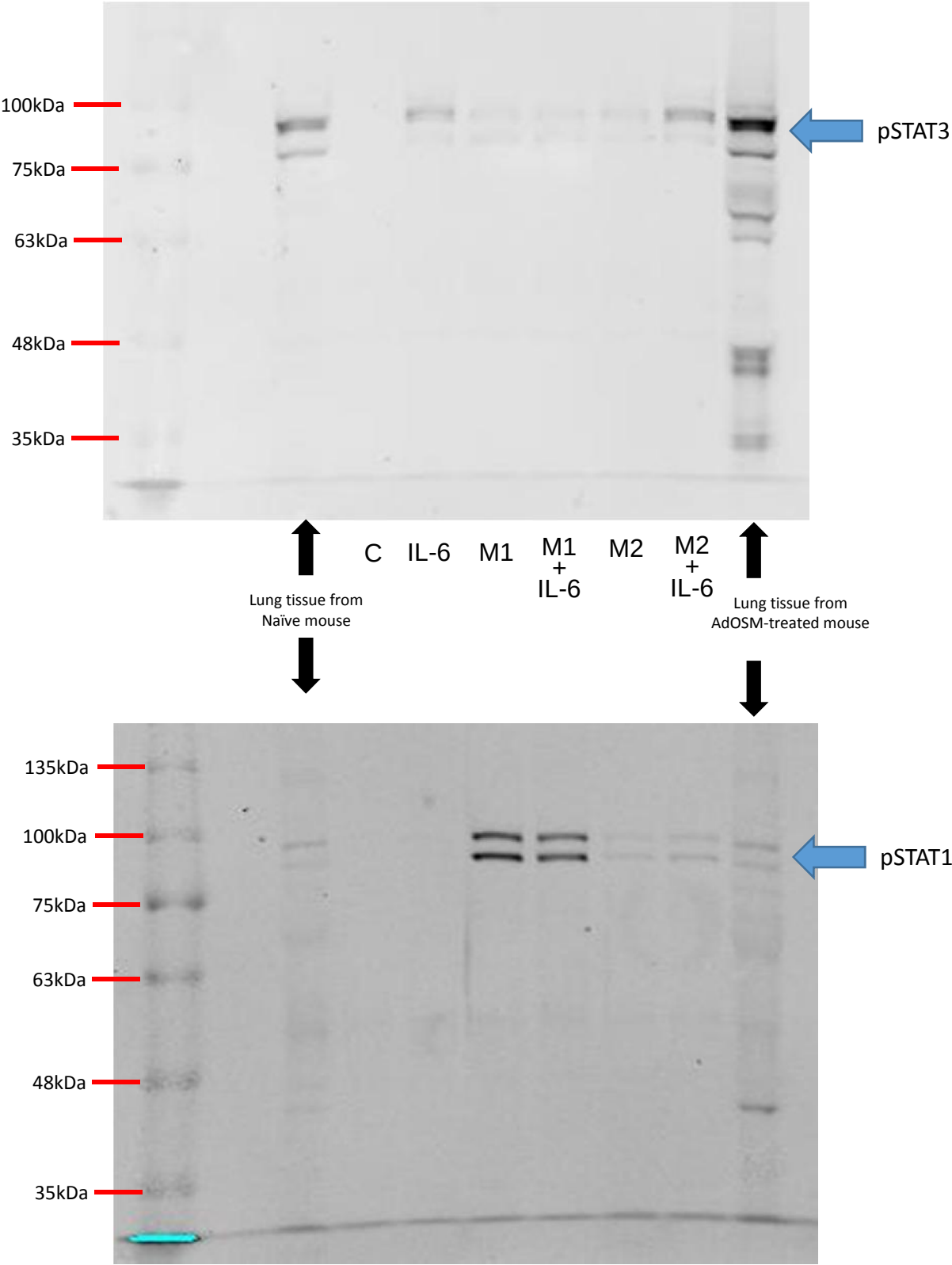
Re: Figure 1D



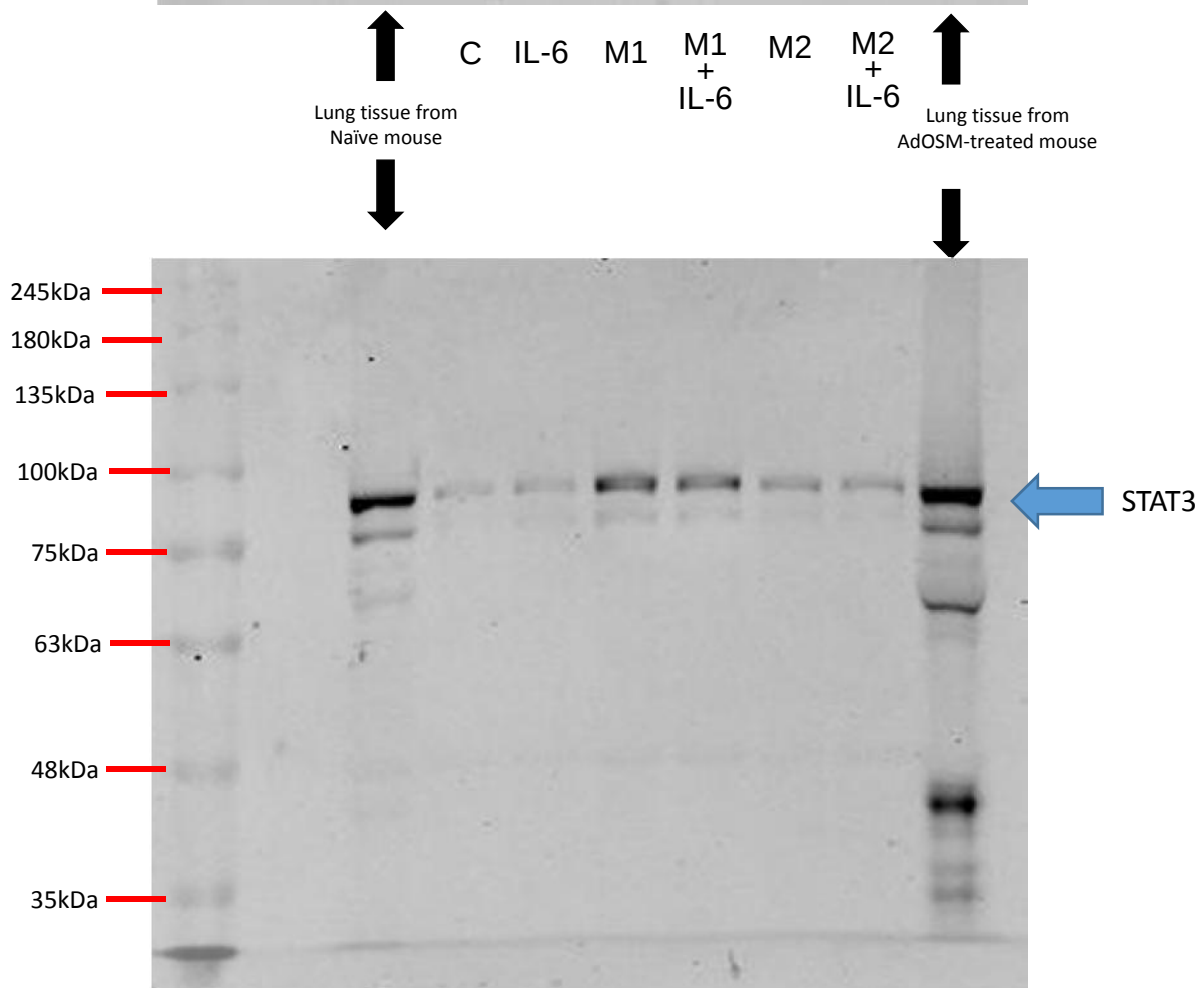
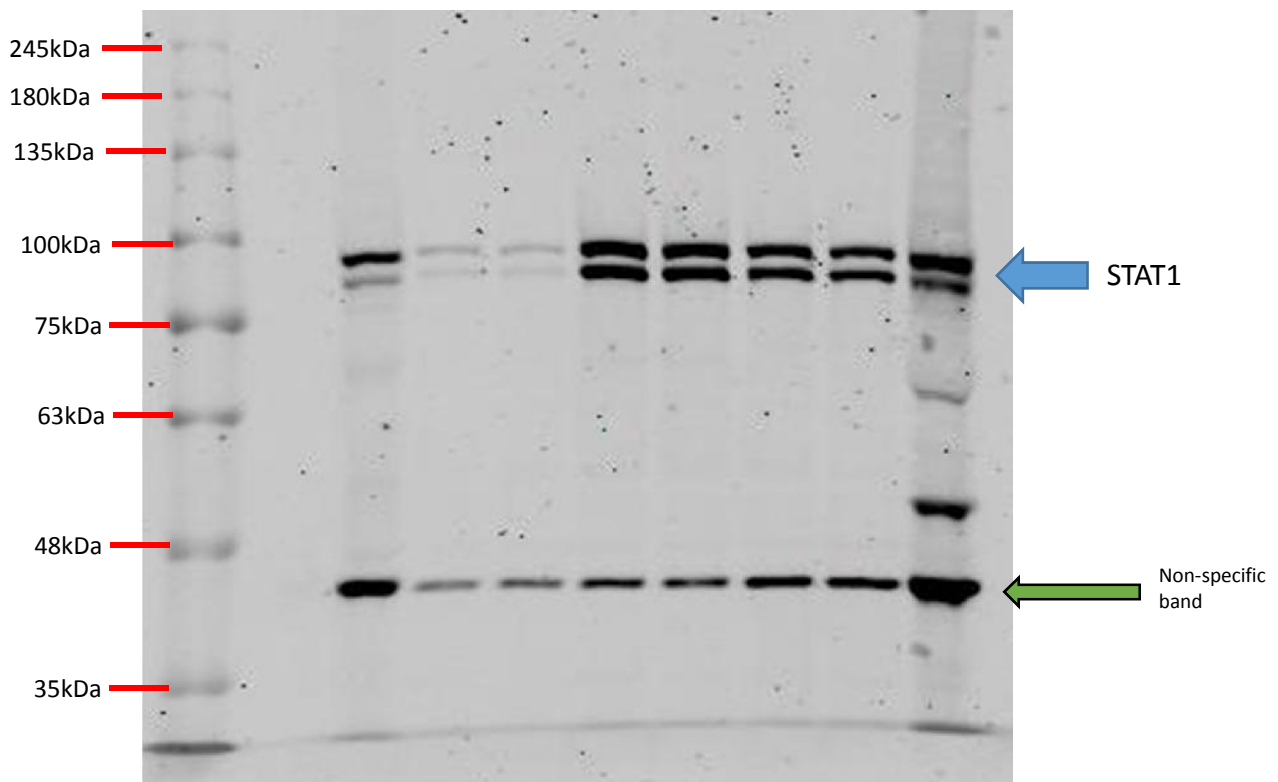
Re: Figure 5A



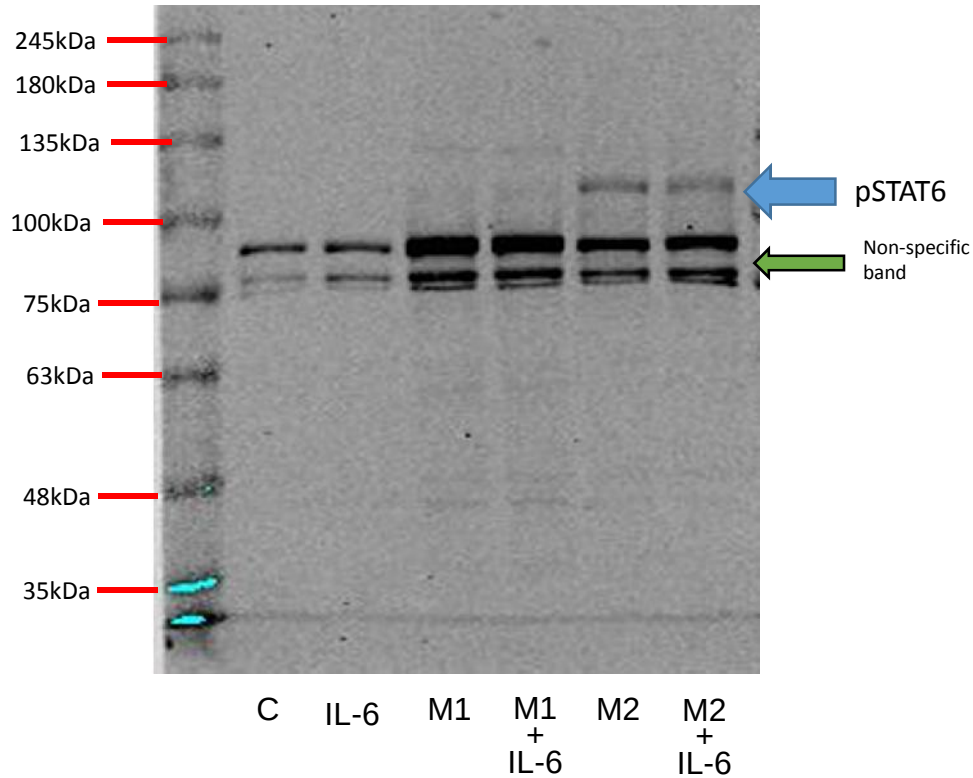
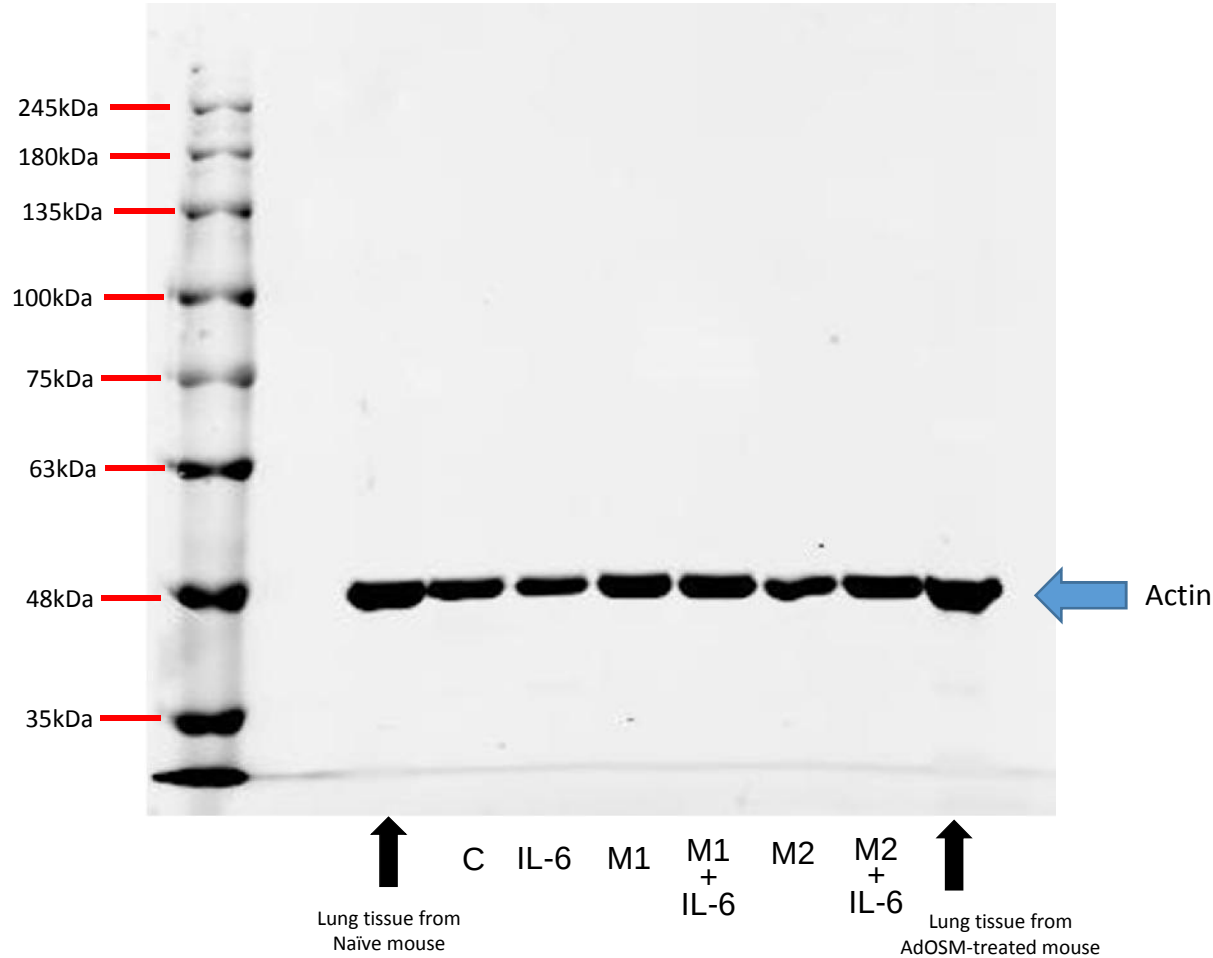
Re: Figure 5A



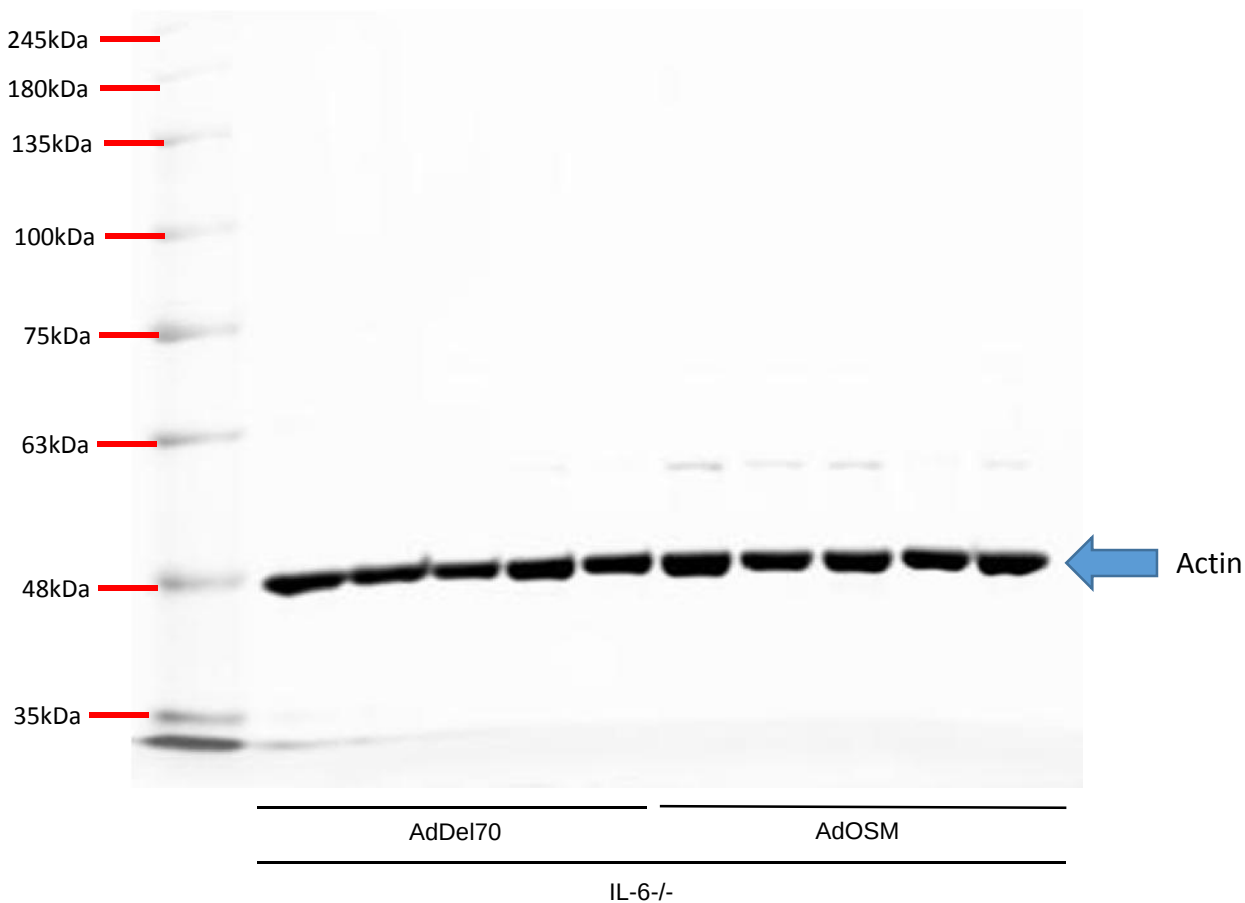
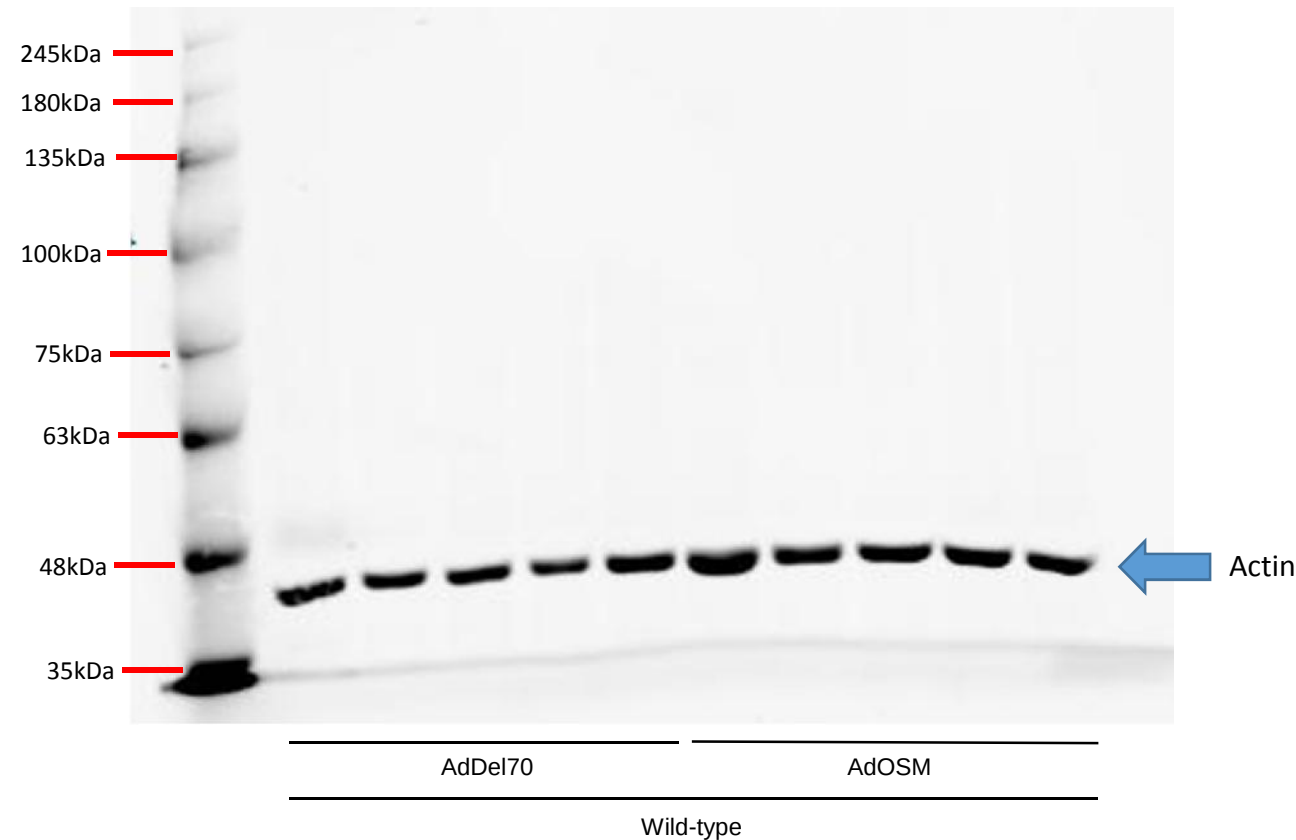
Re: Figure 5A



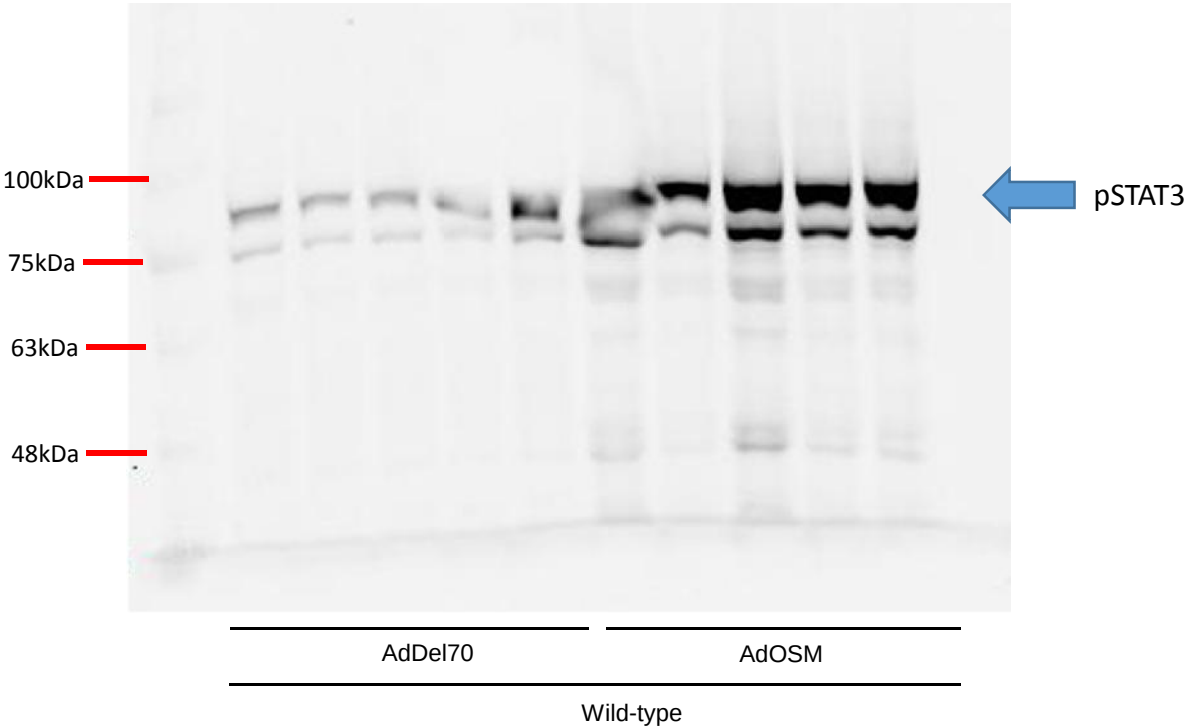
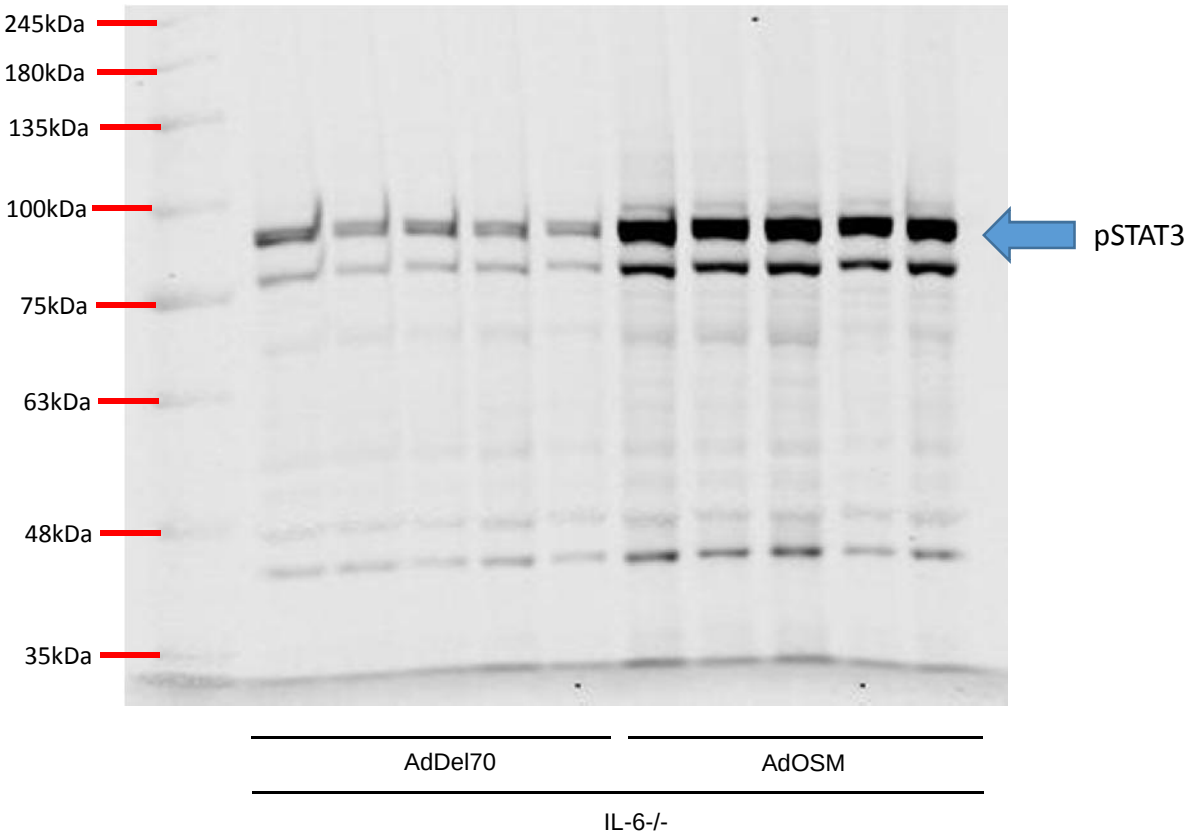
Re: Figure 5A



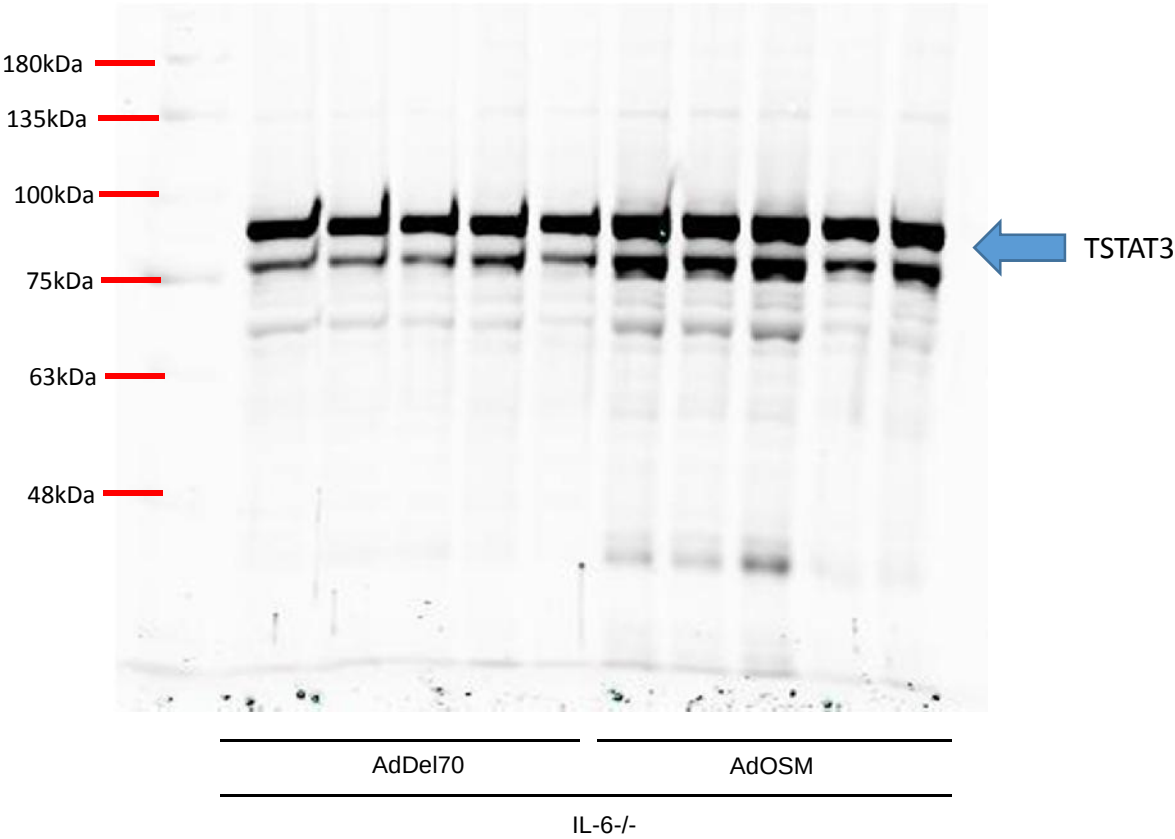
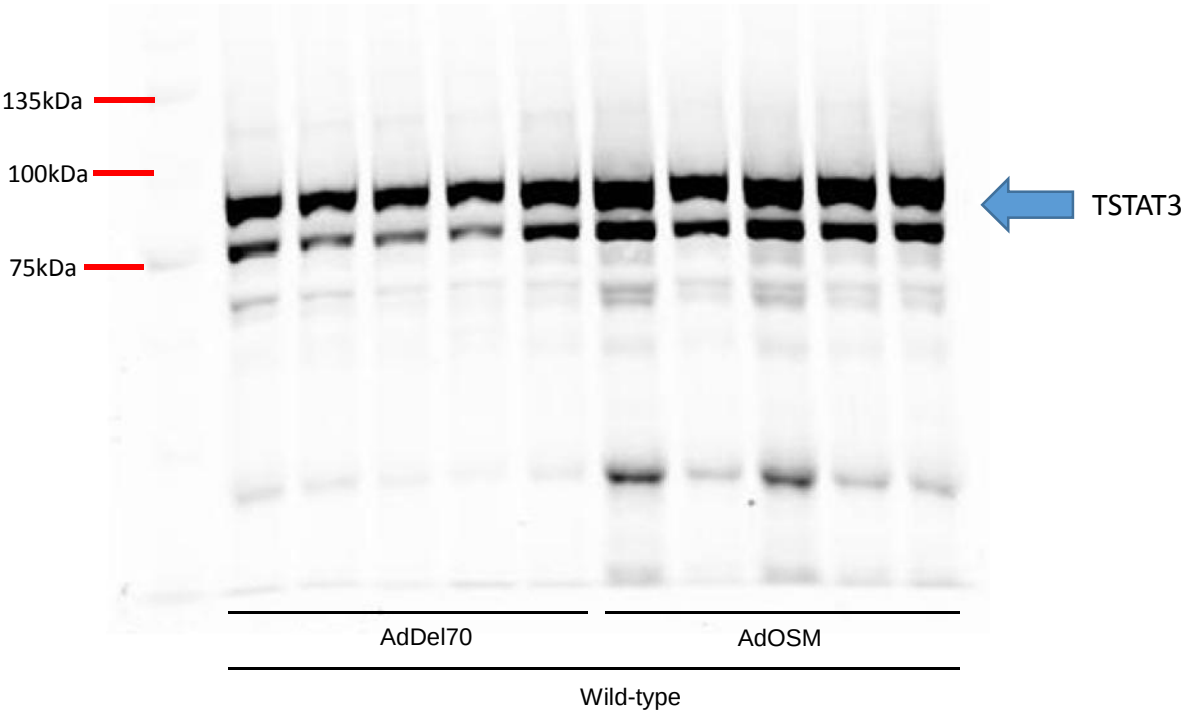
Re: Figure 6A



Re: Figure 6A



Re: Figure 6A



Re: Figure 6A

