Supplementary Figures

Polycomb-mediated loss of microRNA let-7c determines inflammatory macrophage polarization via PAK1-dependent NF-κB pathway

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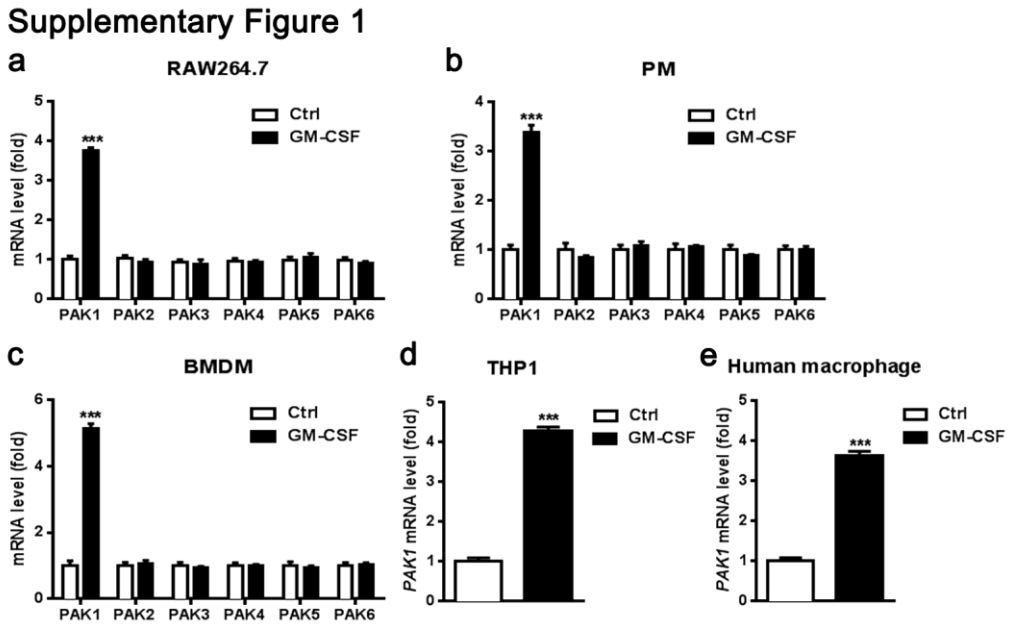
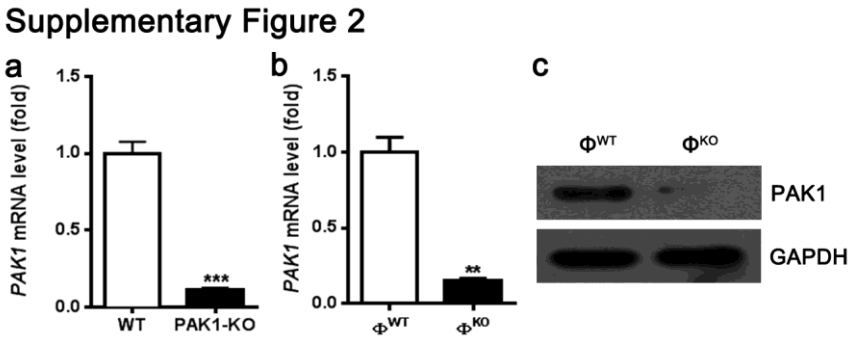
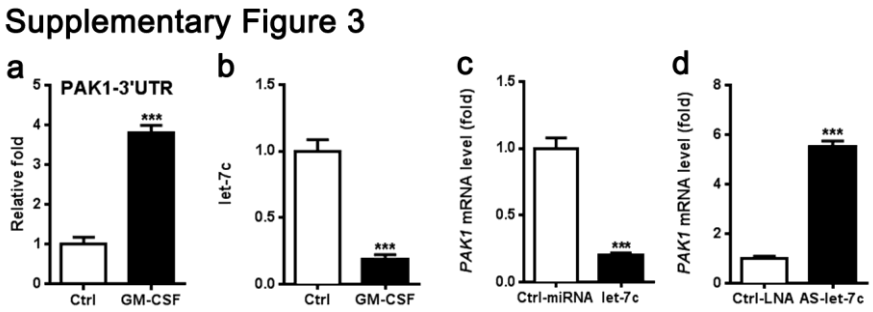
Running Title: PAK1 dictates inflammatory macrophage polarization.



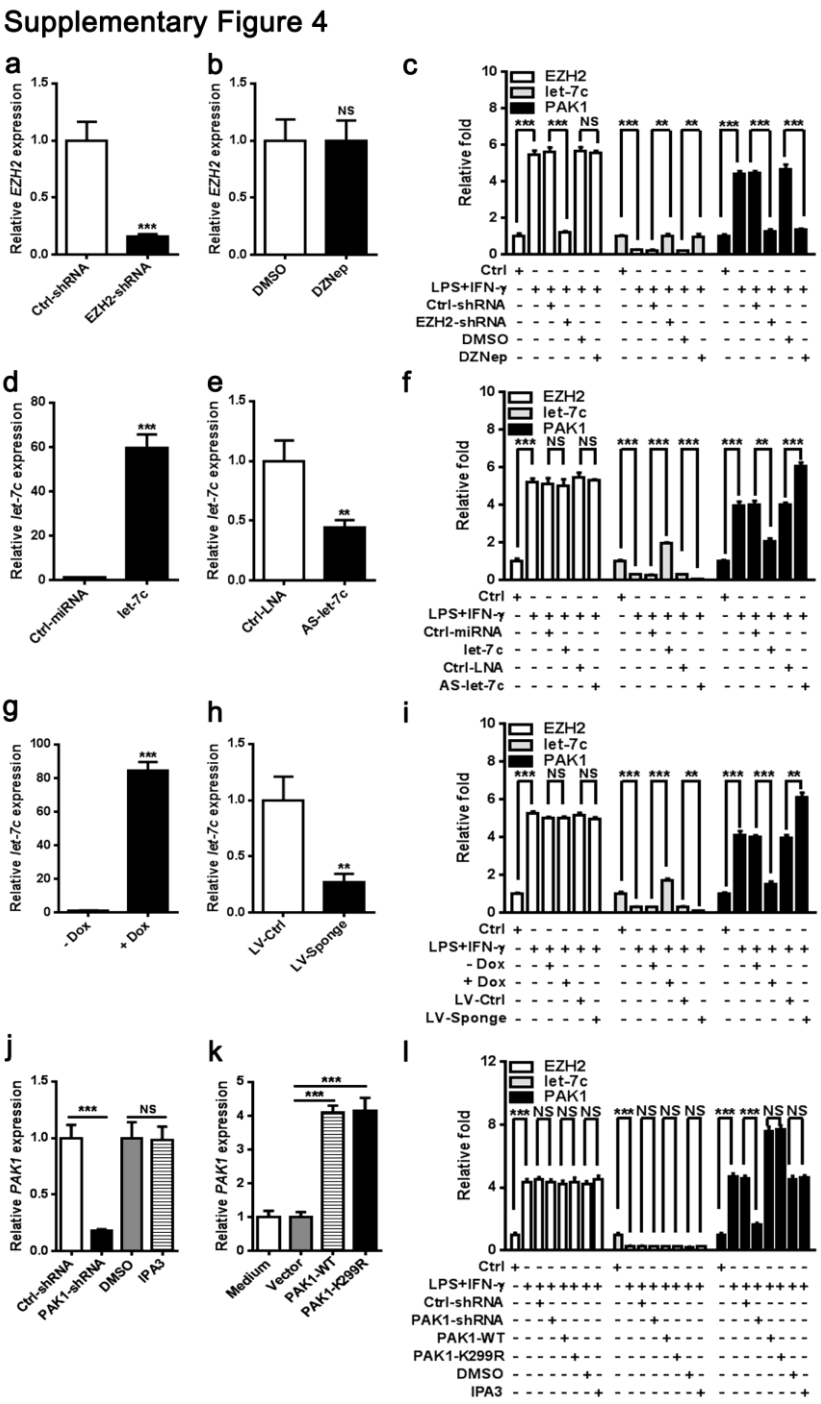
Supplementary Figure 1. PAK1 expression is augmented by inflammatory stimuli in macrophages. (**a-c**) *PAK1* mRNA levels were assessed by real-time PCR and normalized to *GAPDH* in RAW264.7 cells (a), mouse peritoneal macrophages (PMs) (b), and bone marrow-derived macrophages (BMDMs) (c) after 12 hr of stimulation with GM-CSF (50 ng/ml). (**d** and **e**) *PAK1* mRNA levels were quantified by real-time PCR and normalized to *GAPDH* in THP1 cells (d) or GM-CSF–differentiated primary human macrophages (e) stimulated with GM-CSF for 12 hr. Data are from five independent experiments (mean ± SEM). ***$P < 0.001$.



Supplementary Figure 2. Efficient deletion of PAK1 in macrophages. (**a**) Real-time PCR analysis of *PAK1* mRNA expression in BMDMs from *PAK1*$^{fl/fl}$, *Mx1-Cre* (PAK1-KO) mice and *PAK1*$^{+/+}$, *Mx1-Cre* (wild-type; WT) littermate controls. (**b** and **c**) Real-time PCR and immunoblot analysis of PAK1 expression in BMDMs from myeloid-specific PAK1-deficienct (Φ^{KO}) mice and wide type (Φ^{WT}) mice. Results are averaged from six independent experiments (a and b; mean ± SEM) or one experiment representative of three independent experiments with similar results (c). $^{**}P < 0.01$, $^{***}P < 0.001$.

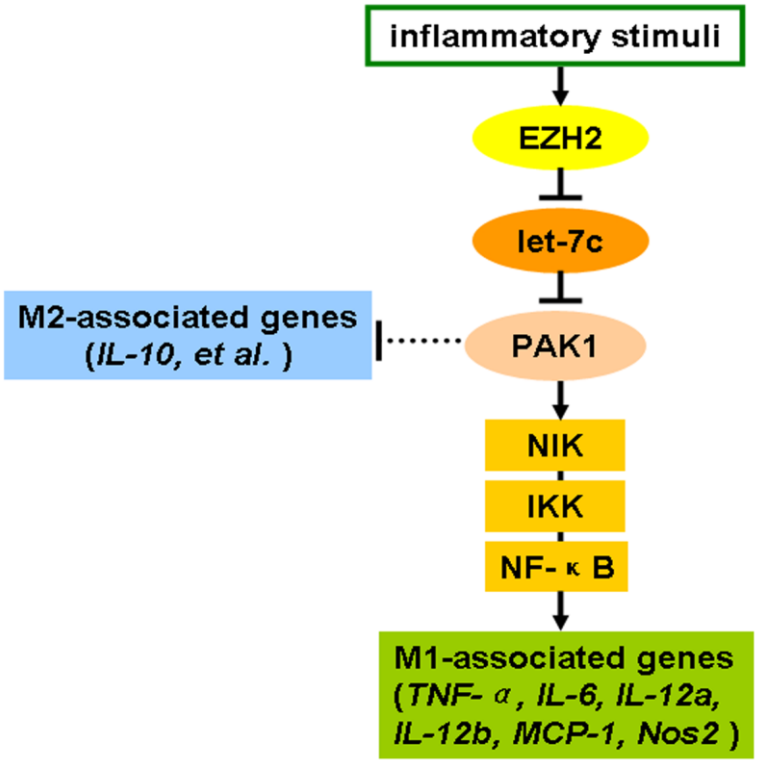


Supplementary Figure 3. *PAK1* mRNA is identified as a *bona fide* target of miR-let-7c in M1 macrophages. (**a**) Luciferase activities for transfected *PAK1* 3'UTR were measured and normalized to *Renilla* luciferase activities in RAW264.7 cells after GM-CSF (50 ng/ml) stimulation. (**b**) Relative levels of let-7c were measured by real-time PCR and normalized to U6 levels in RAW264.7 cells after GM-CSF (50 ng/ml) stimulation. (**c** and **d**) *PAK1* mRNA levels were assessed by real-time PCR and normalized to *GAPDH* in RAW264.7 cells transfected with 40 nM of let-7c mimics (c) and 40 nM of AS-let-7c oligos (d) after stimulated with GM-CSF (50 ng/ml) for 12 hr. Data are from at least four independent experiments (mean ± SEM). ***$P < 0.001$.



Supplementary Figure 4. Regulation of EZH2/let-7c/PAK1 expression in macrophages. Expression levels of EZH2, let-7c, and PAK1 were quantified by real-time PCR and normalized to *GAPDH* and U6, respectively, in LPS plus IFN-γ-treated RAW264.7 cells after EZH2-shRNA introduction or DZNep treatment (**a-c**), LPS plus IFN-γ-treated RAW264.7 cells after let-7c mimics or AS-let-7c oligos introduction (**d-f**), LPS plus IFN-γ-treated RAW264.7 cells after let-7c sponge lentivirus introduction or RAW264.7 Tet-on-let-7c cells with or without Dox treatment (**g-i**), and LPS plus IFN-γ-treated RAW264.7 cells after PAK1-shRNA introduction, PAK1-K299R transfection, or IPA3 treatment (**j-l**). Data are from four independent experiments (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$, NS indicates not significant.

Supplementary Figure 5



Supplementary Figure 5. A model of coordinated regulation of M1 versus M2 transcription programs by PAK1. Epigenetic loss of microRNA let-7c due to EZH2 upregulation determined PAK1 elevation in inflammatory stimuli-induced M1 macrophages. PAK1 signaling via NIK-IKK-NF-κB is essential for inflammatory stimuli-induced expression of a subset of prototypical M1 genes such as *TNF-α*, *IL-6*, *IL-12a*, *IL-12b*, *MCP-1*, and *Nos2*. In addition, PAK1 also suppresses expression of genes essential for M2 polarization such as *IL-10*. Thus, PAK1 regulates the balance between alternative macrophage differentiation programs by supporting the M1 phenotype while blocking M2 polarization.