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RAPID COMMUNICATION

Gp130-dependent STAT3 activation in M—CSF—derived macrophages exaggerates tumor progression



Interleukin-6 (IL-6) is a common pluripotent cytokine that is highly expressed in the tumor microenvironment. The IL-6/ JAK/STAT3 signaling directly promotes tumor progression, severely hampering antitumor immunity. Macrophages, the richest innate immune cells within the tumor microenvironment, manipulate tumor progression via phenotypic plasticity. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are two critical growth factors regulating macrophage differentiation and function. They play different roles during tumor development; while GM-CSF links with enhanced antitumor immunity, M-CSF is associated with M2like phenotypes of tumor-associated macrophages. Nevertheless, the signaling response of differentially differentiated macrophages to IL-6 has not yet been elucidated. Here, we explored IL-6 downstream signaling events in two distinct macrophage populations (M1-like proinflammatory GM-CSF primed and M2-like immunosuppressive M-CSF primed bone marrow-derived macrophages, GM-BMDMs vs. M-BMDMs). We found that IL-6 preferentially induces JAK2/ TYK2/STAT3 activation in M-BMDMs due to their higher glycoprotein 130 (gp130) expression. In vivo, STAT3 phosphorylation is largely present in macrophage populations within the tumor microenvironment. Importantly, the gp130 inhibitor could effectively reverse the tumor-promoting effects of M-BMDMs via IL-6/STAT3 signaling blockage, pointing to its potential therapeutic usage for tumor treatment.

In our current study, STAT3 was highly activated in M-BMDMs post IL-6 treatment (Fig. 1A; Fig. S1A) and the phosphorylation could sustain as long as 48 h (Fig. S1B). Phosphorylation of upstream regulators JAK2 and TYK2, but not JAK1, was responsible (Fig. 1A). No other STAT family members' activation was observed post IL-6 treatment

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(Fig. S1C). Transient activation of the MAPK pathway (ERK, p38, and JNK) was observed post IL-6 challenge but no differences were observed (Fig. S1D). Next, expression levels of JAKs and STATs in these two macrophage populations were determined. No observed overexpression of STAT3 and JAKs in M-BMDMs did account for IL-6-induced STAT3 activation in M-BMDMs (Fig. 1B; Fig. S2A). Classically, IL-6 binds to IL-6R and recruits gp130 to form IL-6/IL-6R/ gp130 heterotrimer to initiate STAT3 signaling. The expression levels of IL-6R (Fig. S2B) and gp130 (Fig. 1C; Fig. S2C) were significantly higher in M-BMDMs, indicating that the insufficient expression of these receptors might account for less STAT3 activation in GM-BMDMs. Interestingly, the exogenous addition of overdose IL-6R failed to induce STAT3 phosphorylation in GM-BMDMs (Fig. S2D). Another IL-6 family cytokine LIF, which depends on gp130 as its signaling receptor β subunit but not IL-6R, could also induce higher levels of STAT3 activation in M-BMDMs (Fig. 1D), pointing to the essential role of gp130 for STAT3 activation. All these data together suggest that the stronger STAT3 activation in M-BMDMs in response to IL-6 family members results from higher gp130 expression.

Next, we determined at which time points of macrophage differentiation the different responses to IL-6 occurred. The freshly isolated bone marrow cells were stimulated with GM-CSF or M-CSF for indicated times followed by vehicle or IL-6 stimulation. The STAT3 phosphorylation levels were not significantly elevated until 4 days of GM-CSF/M-CSF incubation (Fig. 1E; Fig. S3A, B). This suggests that IL-6-induced STAT3 activation requires long-lasting M-CSF priming instead of a transient immediate stimulation. In addition, significantly higher STAT3 phosphorylation levels in the G>M group (6-day incubation with GM-CSF followed by 1-day M-CSF treatment) than in the G>G control group (continuous 7-day GM-CSF incubation) post IL-6 challenge (Fig. S3C), pointing to the direct effects of M-CSF for the strong STAT3 activation. These data

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Figure 1 The IL-6/gp130/STAT3 signaling affected the function of M-BMDMs and significantly promoted tumor growth *in vivo*. **(A–C)** GM-BMDMs and M-BMDMs were treated with IL-6 (20 ng/mL) for indicated times. Whole-cell lysates were processed for Western blot analysis and probed with antibodies against pTyr705-STAT3, pTyr1022/1023-JAK1, pTyr1007/1008-JAK2, and pTyr1054/1055-TYK2 for (A), anti-pTyr705-STAT3, anti-STAT1, anti-STAT2, anti-STAT3, anti-STAT4, anti-STAT5, and anti-STAT6 for

suggested the relatively long-term training by M-CSF is directly responsible for the activated IL-6/gp130/STAT3 cascade in BMDMs.

Transcriptome analysis was performed to systemically elucidate the influence of IL-6-mediated STAT3 activation for M-BMDMs. Twenty-four hours of IL-6 treatment changes numerous gene expressions over 3 h of treatment (Fig. S4A, B). Functional enrichment analysis (M 24 h vs. M 0 h) revealed multiple tumor-suppressive effects of macrophages such as phagosome, antigen processing and presentation, and T cell activation were down-regulated (Fig. 1F). The related genes such as Tnf, Ccl2, Tlr2, and H2-M2 were cored in down-regulated protein-protein interaction networks (Fig. S5A). Besides, the function of the lysosome, the major organelle for phagocytosis, was inhabited as early as 3-h IL-6 treatment (Fig. S5B). To validate these findings, LLC (Lewis lung carcinoma) cells were co-cultured with M-BMDMs, and the phagocytic activity of M-BMDMs towards LLC cells decreased after IL-6 treatment (Fig. S5C, D). Meanwhile, canonical STAT3 downstream signaling pathways such as cell cycle, cell division, and DNA replication were up-regulated by IL-6 treatment (Fig. 1G), which were further verified by ingenuity pathway analysis (Fig. S4C) and protein-protein interaction networks (Fig. S4D).

Survival analysis revealed that cancer patients with higher IL-6 expression had a worse prognosis (Fig. S6A), pointing to the strong relevance of IL-6 signaling with tumor growth. In vitro, STAT3 activation and IL-6 mRNA levels varied greatly among different cancer cell lines (Fig. 1H; Fig. S5B). Interestingly, STAT3 phosphorylation and gp130 expression were generally high in mouse tumor tissues in vivo, regardless of tumor cell types (Fig. 11). Meanwhile, mRNA levels of gp130-dependent IL-6 superfamily cytokines were highly expressed in most subcutaneous tumors (Fig. S6C-E). In particular, IL-6 mRNA levels in subcutaneous tumors were inconsistent with their expression levels in tumor cells (Fig. S6B), indicating that IL-6 expression in vivo is not dependent on cancer cells. Further flow cytometry analysis revealed the presence of a large number of CD11b⁺ myeloid leukocvtes within the LLC tumor microenvironment, of which CD11b⁺ F4/80⁺ CD206⁺ M2 macrophages accounted 3

for approximately 70% (Fig. S6F). Besides, STAT3 phosphorylation was mainly detected in CD11b⁺ cells (Fig. 1J). These data implied that M2-like macrophages could be a significant contributor to high STAT3 activation within the tumor microenvironment *in vivo*.

Therapeutically, gp130 blocker SC144 was added to investigate the contribution of IL-6/STAT3 signaling to tumor progression in subcutaneous LLC and BMDM co-implantation models *in vivo* (Fig. S7A). Consistent with macrophage characteristics, co-injected M-BMDMs provoke and GM-BMDMs suppressed LLC tumor growth (Fig. 1K, L; Fig. S7C). SC144 did significantly reverse M-BMDM-promoted tumor growth with no observed inhibitory effects for LLC alone group (Fig. 1K, L; Fig. S7C), indicating that early SC144 treatment suppresses tumor growth mainly by interfering with IL-6/gp130/STAT3 cascade in M2 macrophages, but not in tumor cells. No obvious drug toxicity or weight loss was observed post SC144 treatment (Fig. S7B).

The hyper-activation of the IL-6/JAK/STAT3 cascade plays a bi-directional role in tumor progression and the related therapeutic strategies to target this pathway failed in a panel of tumor types.^{1,2} To date, studies for IL-6mediated STAT3 activation within the tumor microenvironment mainly focus on tumor cells, T cells, and DC cells. Only limited studies reported STAT3 activation in macrophages and few explored the preference of STAT3 activation in different types of macrophages. The innate antitumor immunity was increased in the hematopoietic conditional STAT3 knockout mice.³ Macrophage conditional STAT3 knockout mice increased expressions of pro-inflammatory mediators.⁴ Blocking STAT3 activation inhibits tumor-associated macrophages' polarization to M2 phenotype.⁵ These findings prompted us to investigate the differences in response to IL-6 of the two distinct types of macrophages. Our research provides new insight into the IL-6/STAT3 signaling regulation in distinct macrophage populations and further proves the therapeutic potential of gp130 blockage for tumor treatment. We acknowledge that our study has limitations as we only investigated one cell line model. Further research involving multiple cell line types would enhance the clarity and robustness of our findings. Since STAT3 is highly activated in macrophages

(B), and gp130 for (C). STAT5 antibodies in (B) recognize endogenous levels of the total STAT5 protein (STAT5a and STAT5b). Anti- α -Tubulin antibodies were used as loading controls. (D) GM-BMDMs and M-BMDMs were cultured and treated with LIF (30 ng/mL) for indicated times. Whole-cell lysates were processed for Western blot analysis and probed with anti-pTyr705-STAT3 antibody. Anti- α -Tubulin antibodies were used as loading controls in all the above-described experiments. (E) The bone marrow cells were isolated and treated with GM-CSF or M-CSF for a series of periods (1 d, 4 d, 8 d), followed by stimulation with or without 20 ng/mL IL-6 for 10 min. The expression level of STAT3 Y705 phosphorylation was detected using Western blot analysis. (F, G) The Gene Ontology analysis of down-regulated (F) or up-regulated DEGs (G) between M 24 h and M 0 h. GM-BMDMs and M-BMDMs were cultured and treated with IL-6 (20 ng/mL) for 3 h (M 3 h) or 24 h (M 24 h) or without IL-6 (M 0 h). Cells were harvested after treatment and their RNA was used for RNA-seq. "Rich factor" represents the proportion of DEGs to all the genes that are annotated in a specific pathway term. The greater the rich factor, the greater the degree of enrichment. (H) Different types of mouse tumor cells were lysed for protein extraction and subjected to Western blot analysis using anti-pTyr705-STAT3, anti-STAT3, and anti-IRF9 antibodies. Anti-a-Tubulin, anti- β -actin, and anti-GAPDH antibodies were used as loading controls. (I) The various types of tumor tissues stripped from subcutaneous tumor-bearing mice were dissociated and collected for protein extraction, followed by Western blot analysis using anti-pTyr705-STAT3, anti-STAT3, and anti-gp130 antibodies. Anti- α -Tubulin and anti-GAPDH antibodies were used as loading controls. (J) Double-color immunofluorescence co-localization of p-STAT3 (green) and CD11b (red) in LLC cell-derived subcutaneous mouse tumor tissue. Original magnification = $200 \times .$ (K, L) LLC subcutaneous tumor-bearing mice injected by macrophages (GM-BMDMs/M-BMDMs) or vehicle were treated with SC144 (5 mg/kg, intraperitoneally, once daily) or vehicle for 3 days. The tumor volumes were calculated (K). Tumors were excised and photographed after sacrificing (L). Statistical significance was calculated by two-way ANOVA for comparisons in (K). Significant differences are indicated as *P < 0.05.

within the tumor microenvironment, combinational treatment of anti-gp130 and anti-PD-1 for T cell re-activation, or targeted drug for tumor cells, could be an optimal regimen for cancer treatment, avoiding the dilemma of insufficient elimination of tumors by STAT3 monotherapy.

Author contributions

All authors participated in interpreting the whole data. Q.S. designed and supervised the study; L.Z., Y.X., X.X., Q.Z., and C.X. performed and evaluated individual experiments; Y.X. and Q.S. performed bioinformatical analyses; Y. X, L.Z., P.L., and C.L. wrote the manuscript with the contributions from all the authors.

Conflict of interests

The authors declare no conflict of interests.

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Appendix A. Supplementary data

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