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Tumor-associated Macrophage-derived Interleukin-23 Interlinks Kidney Cancer Glutamine Addiction with Immune Evasion

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Abstract

Background: Glutamine addiction is a hallmark of clear cell renal cell carcinoma (ccRCC); yet whether glutamine metabolism impacts local immune surveillance is unclear. This knowledge may yield novel immunotherapeutic opportunities.
Objective: To seek a potential therapeutic target in glutamine-addicted ccRCC.
Design, setting, and participants: Tumors from ccRCC patients from a Shanghai cohort and ccRCC tumor data from The Cancer Genome Atlas (TCGA) cohort were analyzed. In vivo and in vitro studies were conducted with fresh human ccRCC tumors and murine tumor cells.
Outcome measurements and statistical analysis: Immune cell numbers and functions were analyzed by flow cytometry. Glutamine and cytokine concentrations were determined. Survival was compared between different subpopulations of patients using Kaplan-Meier and Cox regression analyses.
Results and limitations: We found that in ccRCC, high interleukin (IL)-23 expression was significantly associated with poor survival in both TCGA (overall survival [OS] hazard ratio [HR] = 2.04, cancer-specific survival [CSS] HR = 2.95; all $p < 0.001$) and Shanghai (OS HR = 2.07, CSS HR = 3.92; all $p < 0.001$) cohorts. IL-23 blockade prolongs the survival of tumor-bearing mice, promotes T-cell cytotoxicity in in vitro cultures of human ccRCC tumors, and augments the therapeutic benefits of anti-PD-1 antibodies. Mechanistically, glutamine consumption by ccRCC tumor cells results in the local deprivation of extracellular glutamine, which induces IL-23 secretion by tumor-infiltrating macrophages via the activation of hypoxia-inducible factor 1α (HIF1α). IL-23 activates regulatory T-cell proliferation and promotes IL-10 and transforming growth factor β expression, thereby suppressing tumor cell killing by cytotoxic lymphocytes. The positive correlations

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between glutamine metabolism, IL-23 levels, and Treg responses are confirmed in both TCGA cohort and tumors from Shanghai ccRCC patients. Study limitations include the unclear impacts of glutamine deprivation and IL-23 on other immune cells.
Conclusions: Macrophage-secreted IL-23 enhanced Treg functions in glutamine-addicted tumors; thus, IL-23 is a promising target for immunotherapy in ccRCC.
Patient summary: In this study, we analyzed the immune components in glutamine-addicted clear cell renal cell carcinoma (ccRCC) tumors from two patient cohorts and conducted both in vitro and in vivo studies. We found that ccRCC tumor cell-intrinsic glutamine metabolism orchestrates immune evasion via interleukin (IL)-23, and IL-23–high patients had significantly poorer survival than IL-23–low patients. IL-23 should thus be considered a therapeutic target in ccRCC, either alone or in combination with immune checkpoint inhibitors.

1. Introduction

Clear cell renal cell carcinoma (ccRCC) accounts for up to 4% of all adult malignancies, and its incidence is increasing. Of historical relevance, the only demonstrated complete responses in advanced ccRCC were seen in patients treated with high-dose interleukin (IL)-2 [1]. The observed responses in these patients allowed our understanding of the importance of the immunological milieu in which these tumors develop and accelerated the subsequent development of immune checkpoint inhibitors (ICPIs). ICPIs such as nivolumab and ipilimumab were shown to extend the survival of a subset of ccRCC patients and are licensed therapies for advanced ccRCC [2]. The success of ICPIs in ccRCC has initiated a flurry of preclinical research and clinical trials of other immunotherapeutic strategies as combined therapy with ICPIs. Therefore, identifying the key alteration in the immunosuppressive tumor milieu of ccRCC is of great importance to developing new immunotherapeutic approaches.

The tumor immune microenvironment can be modulated by tumor cell metabolism [3]. Tumor cells take up and catabolize nutrients to generate biomass or energy, and export various metabolic by-products to the stroma. These activities change the species and contents of the metabolites in the tumor microenvironment, which affects the populations and functions of immune cells by shifting the metabolism of these cells and triggering specific signaling related to division, activation, and exhaustion [4,5]. For example, glucose consumption by tumors has been reported to restrict metabolically the effector function of T cells and allow tumor progression, which could be countered by ICPI therapies [6].

One of the metabolic abnormalities in ccRCC is that ccRCC tumor cells are highly addicted to glutamine [7,8] because of the ubiquitous genetic loss of the von Hippel-Lindau tumor suppressor gene [9,10]. Glutamine metabolism not only supports tumor cell proliferation, but also deprives the extracellular stroma of glutamine. Importantly, availability and metabolism of glutamine have been found to play a role in the function of several immune cells, including macrophages and T lymphocytes [3,4]. However, in ccRCC, the influence of tumor-intrinsic glutamine metabolism on the local immune microenvironment is not yet known [11]. In this study, we aim to investigate whether ccRCC tumor-intrinsic glutamine metabolism impacts the formation of the immunosuppressive microenvironment, identify the specific mechanisms or key modulators underlying these connections, and determine whether these connections yield a potentially valuable immunotherapeutic approach for treating ccRCC.

2. Patients and methods

2.1. Clear cell RCC patient cohorts and gene expression data

The basic clinicopathological features of the patient study cohorts are shown in Supplementary Table 1. The Cancer Genome Atlas (TCGA) ccRCC data, including RNA sequencing and clinicopathological data for 533 tumors and 72 paired normal tissues, were downloaded from the Genomic Data Commons Data Portal [7]. The gene signature score was defined as the geometric mean of the normalized expression levels of genes composing the gene signature (Supplementary Table 2).

The Shanghai ccRCC cohort included 1633 ccRCC patients retrospectively obtained from Zhongshan Hospital and Shanghai Cancer Center, Fudan University, Shanghai, China. All patients received nephrectomy between January 1, 2001 and June 23, 2014, and had available formalin-fixed paraffin-embedded samples. This study was approved by the institutional ethical review board of Fudan University. All patients provided informed consent along with telephone follow-up for the use of their tumor tissue for research purposes.

The primary outcomes of survival analyses were overall survival (OS) and cancer-specific survival (CSS). The follow-up time started at the time of diagnosis and ended at the time of last contact. A patient was recorded to have experienced a death event if the patient was dead at the last time of contact. In OS analyses, patients were censored if alive at the last time of contact; in CSS analyses, patients were censored if either alive or dead without any tumors at the last time of contact. For further details of the materials and methods used in this study, see the Supplementary material.

3. Results

3.1. Tumor cell-intrinsic glutamine metabolism is associated with poor survival and an enhanced regulatory T-cell response in ccRCC

First, to define the level of glutamine metabolism in a tumor, we acquired a list of 41 glutamine metabolism-associated genes from the Gene Ontology initiative and from reviews (Supplementary Table 1 and the Supplementary material). We computed a score for the glutamine metabolism signature (Gln signature) by calculating the levels of these 41 genes in 21 ccRCC tumors collected in Shanghai, and we categorized these tumors as Gln signature-high or Gln

signature-low according to the median signature score (Supplementary Fig. 1A). We determined glutamine concentration in these tumors, and found that the Gln signature-high tumors had higher intracellular glutamine concentrations and lower extracellular glutamine concentrations than the Gln signature-low tumors, suggesting increased glutamine consumption in Gln signature-high tumors (Supplementary Fig. 1B). Then, we classified TCGA ccRCC tumors as Gln signature-high or Gln signature-low using the same method. Survival analysis showed that the Gln signature was significantly associated with poor OS and CSS (Fig. 1A) even after adjustment for most clinicopathological risk factors (Supplementary Table 3). These results suggested that ccRCC patients with highly active tumor glutamine metabolism had poorer survival than patients with less active tumor glutamine metabolism.

Then, we tried to investigate whether the ccRCC tumor-intrinsic glutamine metabolism impacts the formation of an immunosuppressive microenvironment. First, we investigated the cytotoxicity of tumor-infiltrating CD8T cells. Flow cytometry showed reduced interferon gamma (IFNγ), granzyme B (GZMB), and perforin (PRF1) expression on CD8T cells from Gln signature-high tumors (Fig. 1B). To confirm that tumor cell-intrinsic glutamine addiction could suppress immune cytotoxicity, we knocked down solute carrier family 1 member 5 (SLC1A5, the main membrane-localized glutamine transporter) or glutaminase (GLS, the rate-limiting enzyme in the conversion of glutamine into glutamate) in the murine renal carcinoma cell line RAG, and injected the knockdown cells into syngeneic mice (Supplementary Fig. 1C). We found that the knockdown of SLC1A5 or GLS reduced the intracellular concentrations of glutamine and glutamate or α-ketoglutarate, suggesting reduced glutaminolysis in tumor cells (Fig. 1C). Importantly, SLC1A5 or GLS knockdown in tumor cells enhanced the proliferation and cytotoxicity of tumor-infiltrating CD8T cells in the murine tumor model (Fig. 1D and Supplementary Fig. 1D). These results confirmed that ccRCC tumor cell-intrinsic glutamine metabolism suppresses cytotoxic T-cell responses in the tumor microenvironment.

As glutamine availability is important for T-cell activation and proliferation [4], we subjected in vitro cultures of CD8T cells to glutamine deprivation. Although glutamine deprivation inhibited the proliferation of the CD8T cells, it did not affect the expression of GZMB and PRF1 (Supplementary Fig. 1E), suggesting an unknown mechanism linking tumor glutamine metabolism to cytotoxicity suppression. Therefore, we compared different immune responses in TCGA tumor data and found an enhanced transforming growth factor β (TGFβ) response in Gln signature-high tumors (Supplementary Fig. 1F). Furthermore, the expression of Treg-associated genes (IL-10, TGFβ, CTLA4, and FOXP3) was higher in Gln signature-high tumors from TCGA (Supplementary Fig. 1G). Finally, flow cytometry showed a higher number of immunosuppressive Treg cells in Gln signature-high tumors from the Shanghai cohort (Supplementary Fig. 1H). These results implied that ccRCC tumor cell-intrinsic glutamine metabolism might activate Treg response, which has been known to extensively inhibit T-cell antitumor cytotoxicity.

To demonstrate our hypothesis, we analyzed tumor-infiltrating Treg cells in the murine tumor model, and found that SLC1A5 or GLS knockdown in tumor cells reduced the number and proliferation of Treg cells and the secretion of IL-10 or TGFβ by these cells (Fig. 1E, and Supplementary Fig. 1I and 1J), while the number of TGFβ–and IL-10–expressing myeloid cells was not affected (Supplementary Fig. 1K). Taken together, our findings demonstrated that ccRCC tumor cell-intrinsic glutamine metabolism could enhance Treg responses in order to suppress T-cell antitumor cytotoxicity.

3.2. *IL-23 is induced by tumor cell-intrinsic glutamine metabolism and associated with poor survival*

The mechanism underlying tumor cell-intrinsic glutamine metabolism and Treg response is unknown; however, a previous report noted that glutamine deprivation induced Treg cell differentiation [11]. We subjected in vitro cultures of Treg cells to glutamine deprivation, but observed decreased proliferation of and IL-10 secretion by Treg cells, suggesting the essentiality of glutamine for T-cell proliferation (Supplementary Fig. 2A). Therefore, we hypothesized that a potential factor may be mediating both tumor cell glutamine metabolism and Treg response. Interestingly, in TCGA ccRCC data, we found significant positive correlations between the IL-23 concentration and both the Gln signature (Spearman rho = 0.312, false discovery rate [FDR] $p < 0.001$) and the Treg signature (spearman rho = 0.346, FDR $p < 0.001$; Fig. 2A). In addition, we found that tumors and peripheral blood from patients with high Gln signatures had higher IL-23 concentrations than tumors and peripheral blood from patients with low Gln signatures (Fig. 2B). Finally, SLC1A5 or GLS knockdown in tumor cells decreased the intratumoral IL-23 concentration (Fig. 2C), which demonstrated that tumor cell-intrinsic glutamine metabolism induced IL-23 secretion in ccRCC cells.

To explore the role of IL-23 in ccRCC, we analyzed IL-23 expression by immunohistochemistry in our Shanghai cohort and found that IL-23 expression was significantly associated with poor OS (hazard ratio [HR] = 2.07, log rank $p < 0.001$) and CSS (HR = 3.92, log rank $p < 0.001$; Fig. 2D) even after adjustment for most clinicopathological risk factors (Supplementary Table 3). Similarly, IL-23 mRNA level was associated with poor OS (HR = 2.04, log rank $p < 0.001$) and CSS (HR = 2.95, log rank $p < 0.001$) in TCGA cohort (Fig. 2E) even in multivariable analyses, confirming the important role of IL-23 in ccRCC development and progression in both cohorts (Supplementary Table 4). Finally, in ccRCC tumors from the Shanghai cohort, we found that the IL-23 concentration was negatively correlated with the extracellular glutamine concentration but positively correlated with the number of tumor-infiltrating Treg cells (Supplementary Fig. 2B and 2C). Similarly, in ccRCC tumors from TCGA, we found that the expression of genes involved in IL-23 signaling was extensively correlated with the expression of genes involved in either glutamine

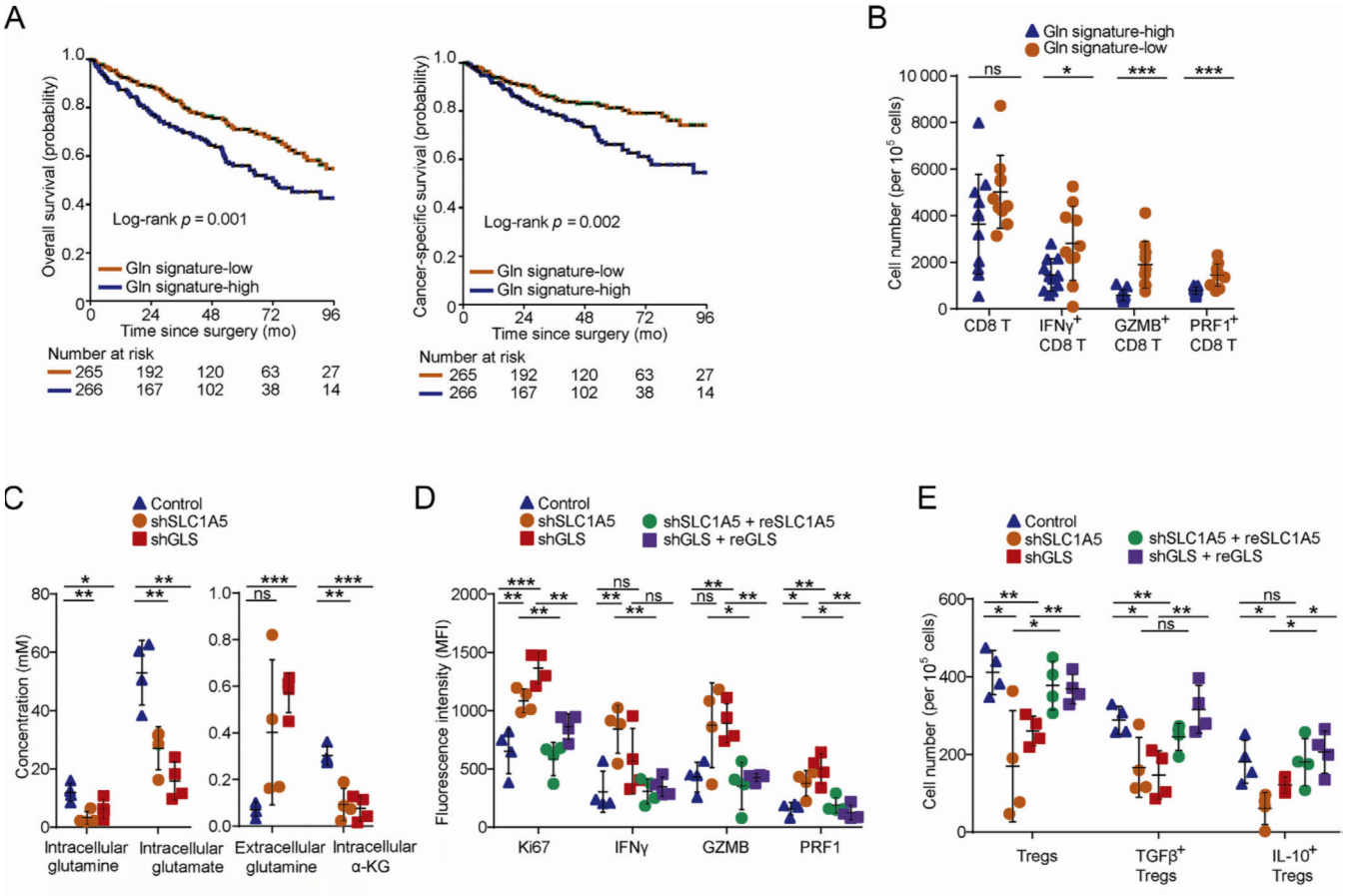

Fig. 1 – Tumor cell-intrinsic glutamine metabolism is associated with poor survival and an enhanced Treg response in ccRCC. (A) OS and CSS curves for TCGA ccRCC patient cohort ($n = 531$) according to the Gln signature. (B) Expression of cytotoxic makers on CD8T cells in Gln signature-high ($n = 11$) or Gln signature-low ($n = 10$) ccRCC tumors, as measured by flow cytometry. (C) Concentration of glutamine or glutamine metabolites in control and SLC1A5-knockdown and GLS-knockdown RAG cells from tumor-bearing mice ($n = 4$ per group). (D) Expression of cytotoxic makers in CD8T cells from control, SLC1A5-knockdown, GLS-knockdown, SLC1A5-rescued, and GLS-rescued RAG tumor-bearing mice, as measured by flow cytometry ($n = 4$ per group). (E) Amount of intratumoral Treg cells in control, SLC1A5-knockdown, GLS-knockdown, SLC1A5-rescued, and GLS-rescued RAG tumor-bearing mice, as measured by flow cytometry ($n = 4$ per group). ccRCC = clear cell renal cell carcinoma; CSS = cancer-specific survival; GZMB = granzyme B; IFNγ = interferon gamma; IL = interleukin; ns = not significant; OS = overall survival; PRF1 = perforin; TCGA = The Cancer Genome Atlas; TGFβ = transforming growth factor β; * indicates a p-value < 0.05, ** indicates a p-value < 0.01, * indicates a p-value < 0.001, ns indicates no significance.**

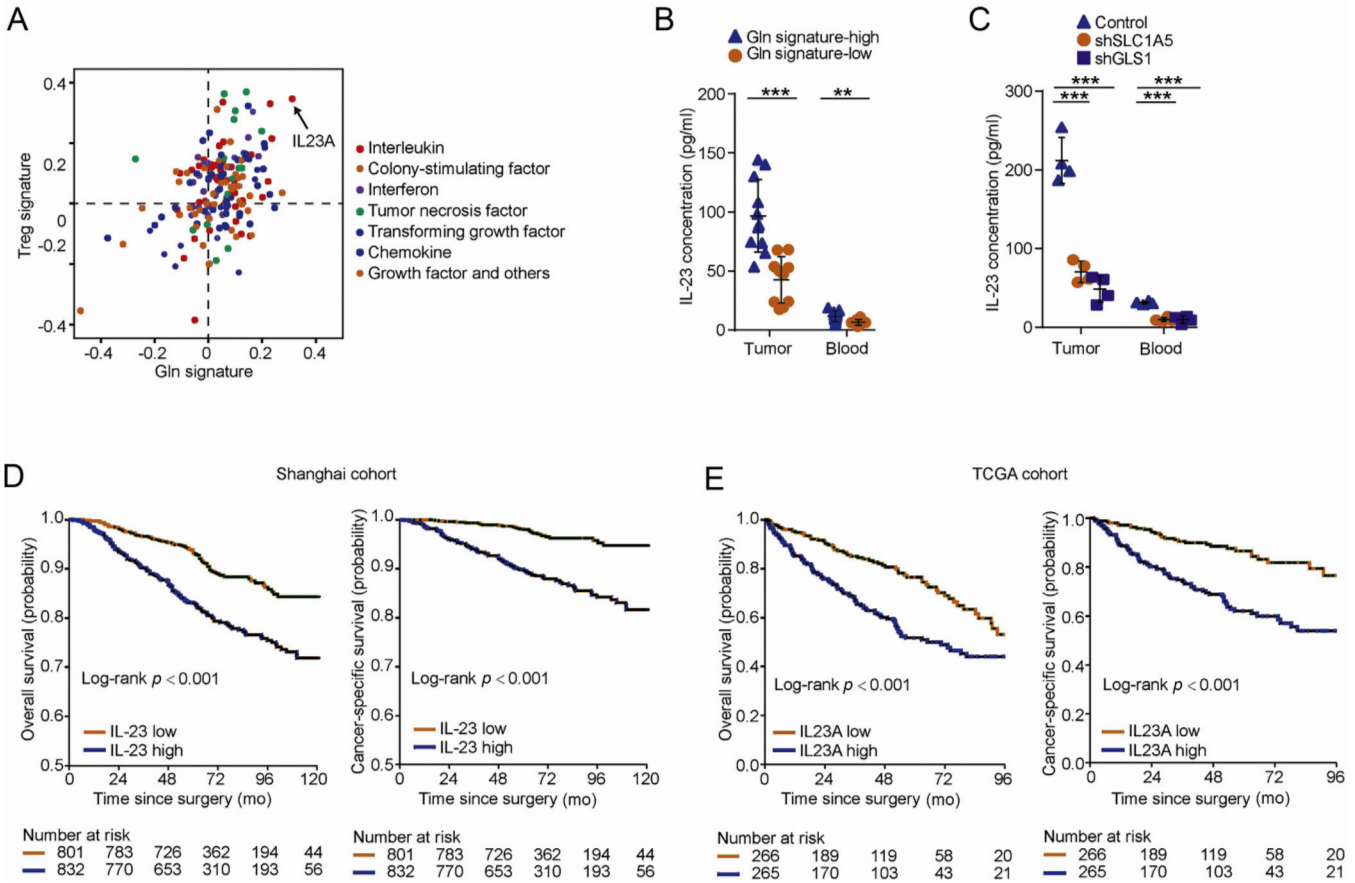

Fig. 2 – IL-23 is induced by tumor cell-intrinsic glutamine metabolism and is associated with poor survival. (A) Correlation between the levels of 179 cytokines and the Gln and Treg signatures in TCGA ccRCC tumors. (B) IL-23 concentration in Gln signature-high ($n = 11$) or Gln signature-low ($n = 10$) human ccRCC tumors and peripheral blood as measured by an ELISA. (C) IL-23 concentration in control, SLC1A5-knockdown, and GLS-knockdown RAG tumor-bearing mice, as measured by ELISA ($n = 4$ per group). (D) OS and CSS curves for ccRCC patients in the Shanghai cohort ($n = 1633$) according to immunohistochemical IL-23 staining intensity. (E) OS and CSS curves for TCGA ccRCC patient cohort ($n = 531$) according to the IL-23A mRNA level. ccRCC = clear cell renal cell carcinoma; CSS = cancer-specific survival; ELISA = enzyme-linked immunosorbent assay; IL = interleukin; OS = overall survival; TCGA = The Cancer Genome Atlas; * indicates a p-value < 0.05, ** indicates a p-value < 0.01, * indicates a p-value < 0.001, ns indicates no significance.**

metabolism or Treg response (Supplementary Fig. 2D). Taken together, our results suggested that IL-23 was associated with tumor cell-intrinsic glutamine metabolism, Treg response, and cancer mortality in ccRCC.

3.3. IL-23 enhances the immunosuppressive function of Treg cells

To explore whether IL-23 affects the functions of Treg cells, we added recombinant human IL-23 (rhIL-23) to in vitro cultures of Treg cells and found upregulation of Ki67, IL-10, and TGFβ staining in the supplemented cells (Fig. 3A); in addition, expression of the activation markers PD-1 and CD69 was upregulated (Supplementary Fig. 3A and 3B). Furthermore, increased secretion of IL-10 and TGFβ was observed in the culture medium after rhIL-23 treatment (Supplementary Fig. 3C). Finally, in vitro assays demonstrated that recombinant IL-23-treated Treg cells had an enhanced capacity to suppress the proliferation of responder CD4T cells and CD8T cells (Fig. 3B and 3C), suggesting that IL-23 enhances the immunosuppressive function of Treg cells. Interestingly, rmIL-23 alone slightly increased the proliferation of responder CD4T cells, emphasizing the immunosuppressive capacity of IL-23 via activation of Treg cells.

We tried to confirm our findings by investigating the IL-23 signaling pathway in Treg cells. IL-23 activates the JAK-STAT pathway, acting mainly on STAT3 [12], and STAT3 signaling is crucial for Treg cell proliferation as well as TGFβ and IL-10 transcription [13]. Consistent with these findings, we found that increased phosphorylation of STAT3 in Treg cells treated with rhIL-23 (Fig. 3D and Supplementary Fig. 3D) and administration of the STAT3 inhibitor S3I-201 abrogated both the elevated IL-10 and TGFβ secretion by and the increased proliferation of rhIL-23-treated Treg cells (Fig. 3E and Supplementary Fig. 3E). Taken together, these results confirmed that IL-23 enhances the immunosuppressive function of Treg cells.

3.4. IL-23 is secreted by glutamine-deprived macrophages via HIF1α

Then, we tried to explore the mechanism by which tumor cell-intrinsic glutamine metabolism induces IL-23 secretion. Flow cytometry showed that all IL-23–secreting cells were CD45+ cells and mostly macrophages (Supplementary Fig. 4A); immunofluorescence showed that IL-23 was mostly colocalized with the macrophage marker CD68 in ccRCC tumors (Fig. 4A). These results demonstrated that IL-23 is predominantly secreted by macrophages. To validate that tumor cell-intrinsic glutamine metabolism induces IL-23 secretion by macrophages, we analyzed tumor-infiltrating macrophages in the murine tumor model and found that SLC1A5 or GLS knockdown in tumor cells reduced IL-23 secretion by macrophages (Fig. 4B, Supplementary Fig. 4B and 4C). Additionally, we found a higher number of IL-23–secreting macrophages in Gln signature-high human tumors than in Gln signature-low tumors (Supplementary Fig. 4D and 4E). Taken together, our results demonstrated that tumor cell-intrinsic glutamine metabolism induced IL-23 secretion by macrophages.

We next sought to determine the detailed mechanism by which tumor cell-intrinsic glutamine metabolism induces IL-23 secretion by macrophages. First, we found that murine peritoneal macrophages produced more IL-23 when cocultured with RAG cells but that this effect was abrogated when SLC1A5 or GLS were knocked down in tumor cells (Fig. 4C and Supplementary Fig. 4F), suggesting that tumor cell-intrinsic glutamine metabolism directly induced IL-23 production by macrophages. Then, we found that the <3 kDa fraction of the tumor cell–conditioned medium, which excluded almost all cytokines, stimulated IL-23 secretion by macrophages (Supplementary Fig. 4G). Previously, lactate was found to induce IL-23 production by macrophages [14], but we found no difference in the lactate concentration when tumor cell glutamine metabolism was inhibited (Supplementary Fig. 4H). Therefore, we focused on the possibility that glutamine deprivation is responsible for IL-23 secretion by macrophages, as we found that tumor cell-intrinsic glutamine metabolism led to low extracellular glutamine concentrations in both human and murine tumors (Fig. 1C and Supplementary Fig. 1B).

Therefore, we cultured murine peritoneal macrophages under glutamine-deficient conditions and found increased IL-23 secretion in the culture medium (Fig. 4D). In addition, we isolated tumor-infiltrating macrophages from ccRCC patients, and found an increased IL-23A level after glutamine deprivation and a reduced IL-23A level after glutamine supplementation (Fig. 4E and Supplementary Fig. 4I). Interestingly, glutamine deprivation led to IL-23 secretion by both M1- and M2-activated macrophages (Supplementary Fig. 4J). Finally, administration of either the SLC1A5 inhibitor GPNA or the GLS inhibitor CB-839 increased IL-23 secretion by tumor-infiltrating macrophages (Fig. 4F). These results demonstrated that the glutamine deficiency resulting from tumor cell glutamine metabolism could directly induce IL-23 secretion by macrophages.

The signaling mechanism underlying the glutamine deficiency–induced secretion of IL-23 is unknown. By Gene Set Enrichment Analysis, we found upregulation of the hypoxia-inducible factor 1α (HIF1α) pathway in murine macrophages cultured in glutamine-deficient medium (Supplementary Fig. 4K). In addition, analysis of a public ChIP-seq database indicated strong binding of HIF1α to the promoter region on the IL-23 transcript (Supplementary Fig. 4L). Flow cytometry showed upregulation of the intracellular HIF1α level in glutamine-deprived macrophages (Fig. 4G and Supplementary Fig. 4M), and administration of the HIF1α inhibitor PX-478 abrogated the elevated secretion of IL-23 by glutamine-deprived macrophages (Fig. 4H). Taken together, these results confirmed that IL-23 is secreted by glutamine-deprived macrophages via HIF1α.

3.5. IL-23 blockade inhibits ccRCC progression and Treg response

We next examined the potential therapeutic effects of IL-23 blockade in ccRCC. We injected an anti-IL-23A antibody in

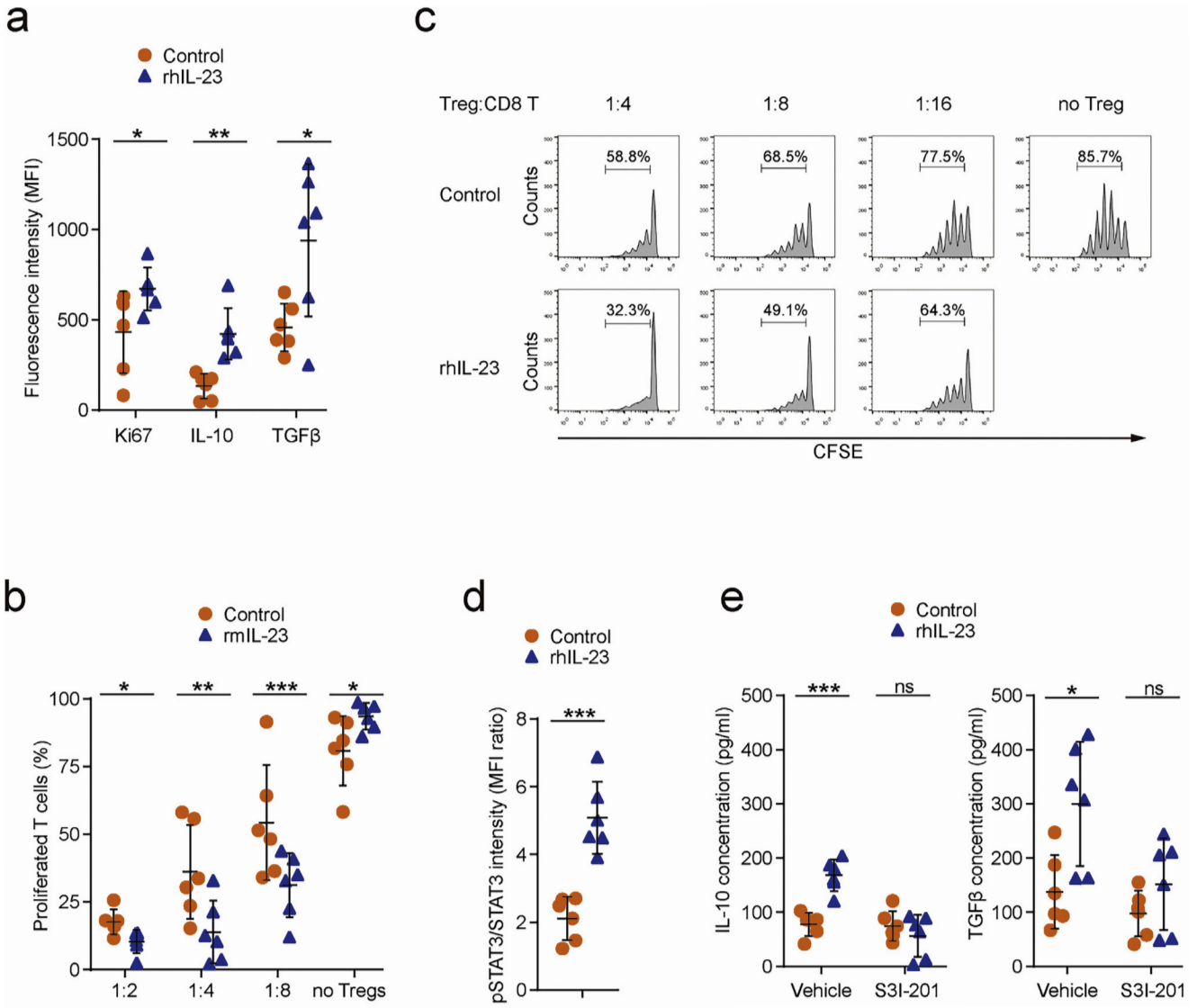

Fig. 3 – IL-23 enhances the immunosuppressive function of Treg cells. (A) Expression of Ki67, IL-10, and TGFb in Treg cells isolated from the peripheral blood of healthy humans and cultured with or without rhIL-23 supplementation, as measured by flow cytometry ($n = 6$ per group). (B) Proportion of proliferating CD4T cells cocultured with Treg cells at the indicated Treg:T cell ratios with or without rmIL-23 supplementation, as measured by CFSE-labeled flow cytometry ($n = 6$ per group). All cells were isolated from mouse spleens. (C) Proliferation of CD8T cells cocultured with Treg cells at the indicated Treg:T cell ratios with or without rhIL-23 supplementation, as measured by CFSE-labeled flow cytometry. All cells were isolated from human peripheral blood. (D) STAT3 phosphorylation in Treg cells isolated from the peripheral blood of healthy humans and cultured with or without rhIL-23 supplementation, as measured by flow cytometry ($n = 6$ per group). (E) Concentration of IL-10 and TGFb in the supernatant of Treg cells isolated from the peripheral blood of healthy humans and cultured with or without rhIL-23 supplementation in the presence of either the STAT3 inhibitor S3I-201 or vehicle, as measured by an ELISA ($n = 6$ per group). ELISA = enzyme-linked immunosorbent assay; IL = interleukin; ns = not significant; TGFβ = transforming growth factor β; * indicates a p-value < 0.05, ** indicates a p-value < 0.01, * indicates a p-value < 0.001, ns indicates no significance.**

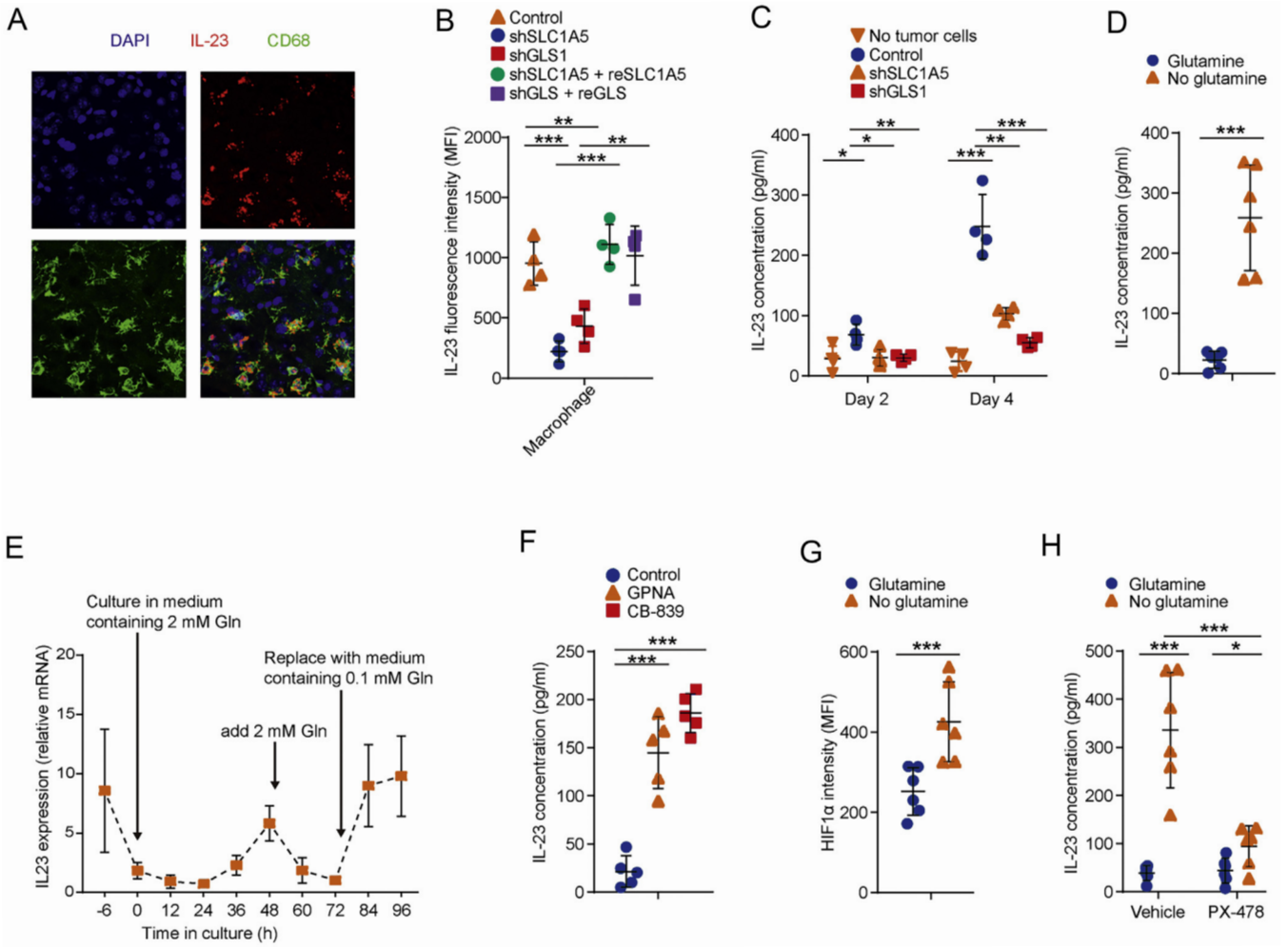

Fig. 4 – IL-23 is secreted by glutamine-deprived macrophages via HIF1α. (A) Representative immunofluorescence staining of IL-23 and CD68 in human ccRCC tumors. (B) Expression of IL-23 in macrophages from control, SLC1A5-knockdown, GLS-knockdown, SLC1A5-rescued, and GLS-rescued RAG tumor-bearing mice, as measured by flow cytometry ($n = 4$ per group). (C) Control, SLC1A5-knockdown, and GLS-knockdown RAG cells were cocultured with murine peritoneal macrophages in complete RPMI 1640 medium (supplemented with L-glutamine), and the IL-23 concentration in the medium at the indicated time points was determined by an ELISA ($n = 4$ per group). (D) Concentration of IL-23 in the supernatant from murine peritoneal macrophages cultured with or without L-glutamine supplementation ($n = 6$ per group). (E) Macrophages were isolated from human ccRCC tumors and cultured in 2 mM glutamine-supplemented medium. Additional medium supplemented with 2 mM glutamine was added after 48 h, and the medium was replaced with 0.1 mM glutamine-supplemented medium after another 24 h. The IL-23 mRNA levels were determined consecutively ($n = 5$). (F) Macrophages isolated from human ccRCC tumors were cultured in complete RPMI 1640 medium in the presence of the SLC1A5 inhibitor GPNA or the GLS inhibitor CB-839, and the concentration of IL-23 in the medium was determined by an ELISA ($n = 5$ per group). (G) HIF1a level in macrophages isolated from human ccRCC tumors and cultured with or without L-glutamine supplementation, as measured by flow cytometry ($n = 6$ per group). (H) HIF1a level in macrophages isolated from human ccRCC tumors and cultured with or without L-glutamine supplementation in the presence of the HIF1a inhibitor PX-478 or vehicle, as measured by flow cytometry ($n = 6$ per group). ccRCC = clear cell renal cell carcinoma; ELISA = enzyme-linked immunosorbent assay; HIF1α = hypoxia-inducible factor 1α; IL = interleukin; * indicates a p-value < 0.05, ** indicates a p-value < 0.01, * indicates a p-value < 0.001, ns indicates no significance.**

orthotopic RAG tumor-bearing mice, and found prolonged survival and a reduced tumor burden as well as a reduced lung metastasis frequency (Fig. 5A and 5B, and Supplementary Fig. 5A and 5B). To confirm that tumor cell glutamine metabolism is required to achieve these benefits, we performed these therapy experiments with SLC1A5- or GLS-knockdown RAG cells, and found that anti-IL-23A therapy resulted in a limited improvement in survival and a reduction in tumor burden (Supplementary Fig. 5C and 5D).

Profiling of tumor-infiltrating lymphocytes from treated mice revealed that anti-IL-23A therapy reduced the infiltration of Treg cells, inhibited the production of IL-10 and TGFβ, and increased the number of intratumoral IFNγ- and GZMB-secreting CD8T cells; these findings suggested an improvement in lymphocyte antitumor cytotoxicity (Fig. 5C and 5D, and Supplementary Fig. 5E and 5F). Similarly, anti-IL-23A had a lesser impact on Treg cells from SLC1A5-knockdown tumor-bearing mice (Supplementary Fig. 5G). Finally, to demonstrate that T lymphocytes are necessary for the therapeutic effects of IL-23 blockade, we repeated these therapy experiments in BALB/c nude mice, which lack mature T lymphocytes, and found that anti-IL-23A therapy resulted in a limited improvement in survival and a reduction in tumor burden (Fig. 5E and Supplementary Fig. 5H).

In addition, we established a novel tumor culture system in vitro to simulate the in vivo tumor immune system of patients. Fresh tumor specimens from ccRCC patients were disaggregated and cultured in vitro, and guselkumab (an FDA-approved anti-IL-23A monoclonal antibody) was added for activity testing (Fig. 5F). In this system, treatment with guselkumab for 24 h decreased the proliferation of and the IL-10 and TGFβ secretion by Treg cells, as well as increased the cytotoxicity of CD8T cells (Fig. 5G and 5H). Furthermore, we found an increased amount of dead tumor cells following guselkumab treatment (Fig. 5I); this increase was abrogated when T lymphocytes were excluded from the experimental system by magnetic isolation (Supplementary Fig. 5I). In addition to observing suppressed Treg functions and enhanced CD8 T-cell cytotoxicity, we observed enhanced natural killer cell cytotoxicity and dendritic cell (DC) antigen presentation capacity, but the Th17 population was not affected (Supplementary Fig. 5J–N). Taken together, these results showed that guselkumab treatment enhanced the tumor-killing capability of the host immune system not only in a murine tumor model, but also in an analogous human tumor model.

As ICPIs such as nivolumab exert therapeutic effects also by motivating CD8 T-lymphocyte cytotoxicity and are licensed therapies for advanced ccRCC, we asked whether IL-23 blockade was synergistic with ICPI treatment. Therefore, we performed a combined treatment experiment utilizing an anti-IL-23A antibody and an anti-PD-1 antibody. Although in both RAG and RENCA cell murine tumor models, the anti-PD-1 antibody had therapeutic effects superior to those of the anti-IL-23A antibody, combination of the anti-IL-23A antibody and the anti-PD-1 antibody was the most beneficial (Fig. 5J and 5K, and Supplementary Fig. 5O and 5P), suggesting that IL-23 blockade could augment the therapeutic effects of currently available ICPIs in ccRCC.

The graphical abstract of this study is shown in Fig. 6.

4. Discussion

Immunotherapies, especially ICPIs (PD-1/PD-L1/CTLA-4 inhibitors), are recently proving to be effective therapeutic approaches in a variety of cancers, emphasizing the importance of normalizing antitumor T-cell dysfunction in cancers [15]. Efficacy of ICPIs highly depends on the presence of both CD4 and CD8T cells in the tumor parenchyma, which is often accompanied by myeloid and monocytic cells. In such “immune-inflamed” tumors, the antitumor capacity of the existing T cells should be suppressed by immunosuppressive cells and factors such as myeloid-derived suppressor cells, Treg cells, and immune checkpoints, and should therefore respond to ICPI therapies [16]. Clear cell RCC ranks highly among tumor types in terms of immune cytolytic activity [17], immune infiltration score [18], and T-cell infiltration score in the tumor microenvironment [19]. These observations led to the rapid development of ICPIs for ccRCC treatment. However, not all ccRCC patients exhibit dramatic clinical responses to ICPIs, which may result from the existence of other immunosuppressive mechanisms, including insufficient tumor antigen priming and exclusion of T lymphocytes from tumors. Hence, improving the effects of ICPIs in ccRCC by developing other immunotherapeutic strategies is clearly within scope [20].

Recently, TGFβ was found to attenuate the tumor response to PD-L1 blockade by contributing to the exclusion of T cells in urothelial [21] and colorectal cancers [22]. In addition, Treg cells, one of the main sources of TGFβ in tumors, strongly suppress the activation and cytotoxicity of T cells. Therefore, targeting TGFβ or TGFβ-secreting Treg cells is becoming an attractive therapeutic approach to improve the effects of ICPIs in cancers [23]. As TGFβ itself participates in many biological immune processes, systemic blockade of TGFβ in vivo may lead to unpredictable toxicities. Coincidently, we found that the immunosuppressive function of Treg cells in ccRCC was supported by a previously poorly characterized cytokine, IL-23. IL-23 directly promotes the proliferation of and the IL-10/TGFβ production by Treg cells, and IL-23 blockade showed therapeutic effects in both murine and human tumor models, either alone or in combination with an anti-PD-1 antibody. Thus, our findings propose IL-23 blockade as a novel therapeutic approach in ccRCC to counter Treg-mediated immunosuppression, elicit T-cell–mediated antitumor immunity, and enhance the immunomodulatory effects of ICPIs.

Another intriguing finding in our research is that ccRCC tumor cell-intrinsic glutamine metabolism drives the production of IL-23 by macrophages. We found that glutamine consumption by ccRCC tumor cells led to the deprivation of stromal glutamine, which constrains glutamine metabolism in tumor-infiltrating macrophages and activates HIF1α to transcribe IL-23 in macrophages. Although the existence and importance of glutaminolysis in ccRCC has been described elsewhere, the impact of

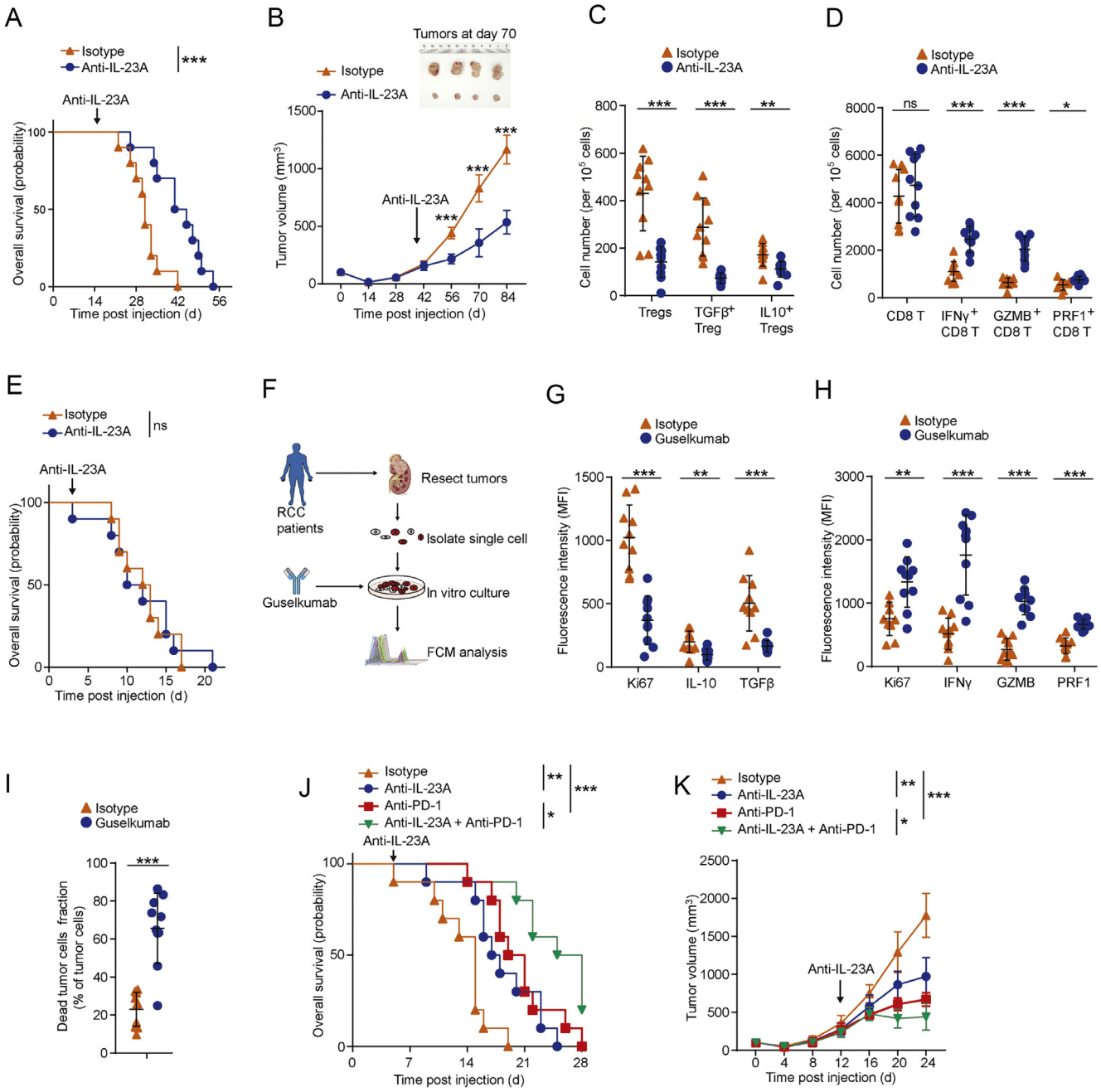

Fig. 5 – IL-23 blockade inhibits ccRCC progression and the Treg response. (A) OS curve for BALB/c mice orthotopically injected with RAG cells and treated with anti-IL-23A or isotype antibodies (n = 10 per group). (B) Tumor volume and representative tumor images from BALB/c mice subcutaneously injected with RAG cells and treated with anti-IL-23A or isotype antibodies (n = 10 per group). (C) Number of intratumoral Treg cells from BALB/c mice orthotopically injected with RAG cells and treated with anti-IL-23A or isotype antibodies, as measured by flow cytometry (n = 10 per group). (D) Amount of intratumoral CD8T cells from BALB/c mice orthotopically injected with RAG cells and treated with anti-IL-23A or isotype antibodies, as measured by flow cytometry (n = 10 per group). (E) OS curve of BALB/c nude mice orthotopically injected with RAG cells and treated with anti-IL-23A or isotype antibodies (n = 10 per group). (F) Flowchart of the in vitro 3D culture experiments. (G) Expression of Ki67, IL-10, and TGFb in Treg cells in cultured human tumors treated with guselkumab or isotype antibody, as measured by flow cytometry (n = 10 per group). (H) Expression of cytotoxic cytokines in CD8T cells from cultured human tumors treated with guselkumab or isotype antibody, as measured by flow cytometry (n = 10 per group). (I) Dead tumor cell fraction in cultured human tumors treated with guselkumab or isotype antibody (n = 10 per group); dead cells were identified as populations that stained positive for the viability dye. (J) OS curve for BALB/c mice orthotopically injected with RAG cells and treated with anti-IL-23A, anti-PD-1, or isotype antibodies (n = 10 per group). (K) Tumor volume from BALB/c mice subcutaneously injected with RAG cells and treated with anti-IL-23A, anti-PD-1 or isotype antibodies (n = 10 per group). ccRCC = clear cell renal cell carcinoma; FCM = flow cytometry; GZMB = granzyme B; IFNγ = interferon gamma; IL = interleukin; ns = not significant; OS = overall survival; PRF1 = perforin; TGFβ = transforming growth factor β; * indicates a p-value < 0.05, ** indicates a p-value < 0.01, * indicates a p-value < 0.001, ns indicates no significance.**

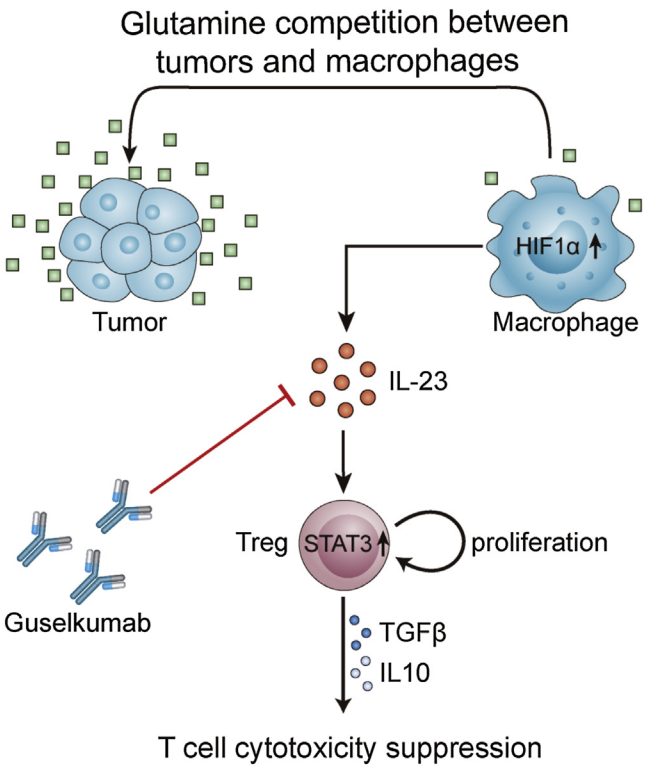

Fig. 6 – Graphical abstract. HIF1α = hypoxia-inducible factor 1α; IL = interleukin; TGFβ = transforming growth factor β.

glutaminolysis on local immunosuppression is not yet known. Our findings identify the basic mechanism by which tumor cells induce the production of immunosuppressive IL-23 by competing with macrophages for glutamine. Notably, almost all available strategies to target glutamine metabolism pharmacologically were stopped at the preclinical stage due to severe side effects [24,25], and our findings suggest that the induction of IL-23 secretion by macrophages via the blockade of glutamine metabolism might explain the side effects of these therapies. If the efficacy of IL-23 blockade could be proved in ccRCC clinical trials, those glutamine metabolism inhibitors could be reintroduced into clinical practice in the future.

This study has some limitations. First, the influence of IL-23 or anti-IL-23 antibodies on the Th17 response was not fully assessed. IL-23 was originally noted for its impacts on the development of the Th17 lineage [12,26], but the biological functions of Th17 and Treg appear to be opposite, according to some studies [27]. In fact, our data showed that the anti-IL-23 antibody did not affect the number of Th17 cells but significantly reduced the number of functional Treg cells in tumor-bearing mice. Second, the mechanism by which deficient glutamine metabolism induces HIF1α signaling in macrophages was not addressed. Several studies have shown that 2-oxoglutarate, the primary metabolite of glutaminolysis, can activate prolyl hydroxylase and thereby destabilize HIF1α [28]; therefore, decreased generation of 2-oxoglutarate might account for HIF1α activation in macrophages. Further this topic needs support from C^{13}-labeled glutamine trace experiments. Finally, glutamine metabolism of tumor cells could affect other tumor-infiltrating immune cells such as DCs, neutrophils, or B cells, which were not assessed in this study. Further investigation of the impact of IL-23 on these cells can contribute to our understanding of the biological functions of IL-23 in tumor immunity.

5. Conclusions

This study highlights previously unknown immunomodulatory functions of IL-23 in glutamine-addicted ccRCC tumors, showing that IL-23 suppresses the cytotoxicity of CD8T cells via enhancing the immunosuppressive function of Treg cells. These findings support IL-23 blockade as an effective therapeutic approach in glutamine-addicted ccRCC, either alone or in combination with ICPIs.

Author contributions: Jiejie Xu had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Analysis and interpretation of data: Q. Fu, L. Xu, Y. Wang, Jiang.
Drafting of the manuscript: Q. Fu, L. Xu, Y. Wang, Jiang.
Critical revision of the manuscript for important intellectual content: Dai, W. Zhang, J. Xu.
Statistical analysis: Q. Fu, L. Xu, Y. Wang, Jiang.
Obtaining funding: Dai, W. Zhang, J. Xu.
Administrative, technical, or material support: Liu, J. Zhang, Q. Zhou, Zeng, Tong, T. Wang, Qi, Hu, H. Fu, Xie, L. Zhou, Chang, Zhu.
Supervision: Dai, W. Zhang, J. Xu.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2018.09.030.

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