

Elevated IgG levels induce an M2-to-M1 phenotypic shift in mucosal macrophages and restrict the growth of invasive sphenoid sinus pituitary adenomas

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Abstract. Although invasive pituitary adenomas (PAs) commonly invade the sellar floor and violate the dura mater, complete penetration of the sphenoid sinus mucosa is uncommon, and thus, mapping of the tumor-mucosa immune landscape is warranted. In the present study, clinical PA specimens were analyzed via H&E staining, Masson's trichrome staining and immunohistochemistry. Spatial immune architecture and activation states were mapped by multiplex immunofluorescence staining. Two models, air-liquid interface culture of mucosal tissue explants and co-culture of dissociated mucosal cells with primary PA cells, were used to test mucosa-derived inhibition. Cytokines were quantified using ELISAs. Tumor growth inhibition and cell cycle changes were assessed by flow cytometry. Intracellular signaling was examined by western blotting. Macrophage phagocytosis of pHrodo™-labeled tumor cells was quantified. The sphenoid sinus mucosa retained structural integrity. Both co-culture systems reduced proliferation (lower Ki-67 labeling) and increased cell death (higher annexin V/PI positivity) in primary PA cells, with effects more pronounced in co-cultures with enzymatically digested mucosa than with intact mucosal

tissue fragments. Macrophages were predominant at the invasive fronts and repolarized from immunoregulatory (M2) to pro-inflammatory (M1) phenotypes. Mucosal macrophages expressed significantly more IFN- γ than their intratumoral counterparts. High IFN- γ levels were associated with lower Ki-67 levels, and exogenous IFN- γ suppressed PA cell proliferation and migration via S-phase arrest and Janus kinase-STAT1 activation. Mucosal B cell-derived IgG levels were higher than IgA levels and were associated with M1-like macrophages rather than M2-like macrophages. IgG treatment increased M2 macrophage pro-inflammatory cytokine levels, particularly IL-6 levels. IL-6 induced G₁-phase arrest. Combined IL-6 and IFN- γ treatment increased STAT1 phosphorylation compared with that observed after IFN- γ treatment alone, without increasing STAT3 activation beyond the activation induced by IL-6 alone, thereby reducing PA cell proliferation and migration. In co-culture experiments, anti-CD47 monoclonal antibody enhanced macrophage-mediated antibody-dependent cellular phagocytosis and was associated with reduced tumor cell proliferation. In conclusion, the sphenoid sinus mucosa establishes an immune barrier centered on M1-polarized macrophages and IgG-high B cells. This network generates an IFN- γ /IL-6 gradient that restricts local PA progression, and highlights macrophage/B cell-directed and CD47-targeted approaches as potential adjuncts to surgery.

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Introduction

Pituitary adenomas (PAs) are among the most common intracranial tumors, accounting for 10-15% of all primary brain neoplasms (1). Previous studies have shown that 30-40% of PAs exhibit varying degrees of invasive behavior (2), most commonly involving invasion of the cavernous sinus, sellar dura mater, osseous structures and sphenoid sinus (3). Invasive growth markedly increases the difficulty of achieving gross-total resection and is closely associated with an increased risk of postoperative tumor residue and recurrence (4). The mechanisms underlying invasive growth

in PAs have been investigated in experimental and clinical studies (5-8). However, despite advances in surgical techniques and adjuvant therapies, effective clinical control of invasive behavior remains suboptimal. Intraoperative and pathological observations suggest a recurrent inferior extension pattern: After breaching the sellar dura and eroding the sellar floor, the tumor may extend toward/into the sphenoid sinus, whereas frank sinonasal epithelial presentation beyond the sphenoid sinus mucosa is uncommon (2). The sphenoid sinus mucosa is now recognized as an immunologically active tissue, in which resident and recruited immune cells cooperatively maintain barrier integrity, immune surveillance, tissue homeostasis and post-injury repair (9-11). Limited clinical evidence also suggests the presence of mucosal immune activation during pituitary apoplexy (12). Despite these observations, little progress has been made in the study of the sphenoid sinus mucosa since its initial description in association with PAs in 1987 (13).

Consequently, a critical gap remains in the understanding of how invasive PA cells interact with the sphenoid sinus microenvironment. The cellular composition, activation states and regulatory programs of mucosal immune populations at the tumor-mucosa interface remain poorly defined. Addressing this gap is therefore essential for refining current models of tumor-microenvironment interactions and for identifying tractable immunological targets to limit invasive progression. The present study characterized the immune microenvironment of sphenoid sinus mucosa adjacent to sphenoid sinus-invasive PAs, compared it with appropriate control mucosa, and evaluated the associations between mucosal immune features and patterns of local invasion. These insights may provide a mechanistic basis for strategies aimed at preventing or mitigating invasive behavior in PAs.

Materials and methods

Definition of invasiveness. PAs are classified into subtypes based on immunohistochemical profiles and clinical manifestations (14). Non-invasive tumors (NITs) were defined using a multimodal approach: i) Radiologically, preoperative sellar MRI with contrast-enhanced coronal T1-weighted imaging confirmed the distinct boundaries between the PA and internal carotid artery (ICA) (15); ii) intraoperatively, direct visualization by an experienced pituitary surgeon revealed intact tumor pseudocapsules without cavernous sinus infiltration or tumor-ICA spatial invasion (3); and iii) routine clinical histopathological examination of the anterior sphenoid sinus dural specimens, performed by the Department of Pathology as part of routine clinical care, showed no evidence of tumor cell infiltration based on H&E staining, indicating the absence of dural invasion at the sampled site (16).

Dural-invasive tumors (DITs) were identified histopathologically, requiring both tumor cell infiltration into the dural collagenous layer and structural disruption of dural continuity. Sphenoid sinus-invasive tumors (SSITs) were defined as follows: i) Preoperative MRI criteria included destruction of the sphenoid sinus structure with tumor filling the sinus cavity; ii) intraoperative criteria involved direct visualization of mucosa displacement and sinus cavity loss after anterior wall resection; and iii) tumor invasion into the sphenoid sinus was

confirmed by H&E staining according to the routine clinical pathology report (17).

Study population. Consecutive patients who underwent endoscopic transsphenoidal surgery for pituitary tumors at Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China) between January 2023 and January 2024 were screened for inclusion. Patients were excluded if they were younger than 18 years of age or had undergone previous pituitary surgery. Following application of these predefined criteria, a total of 63 patients were included in the final statistical analysis. The study cohort consisted of 29 men (mean age, 52.4 ± 12.5 years; age range, 19-77 years) and 34 women (mean age, 40.7 ± 14.4 years; age range, 19-69 years). Following application of the predefined criteria, a total of 63 patients were included in the final statistical analysis. Patients were categorized into NIT (n=32), DIT (n=21) and SSIT (n=10) groups. Clinical imaging and routine pathology information used for classification was retrieved from the medical records, including preoperative MRI (sellar region images and tumor size measurements were extracted from radiology reports) and routine clinical histopathology reports.

H&E staining, immunohistochemistry (IHC) and immunofluorescence staining. Clinical tissues were fixed in 4% formaldehyde at 4°C for 24 h, embedded in paraffin and sectioned into 4- μ m-thick slices. Sections were baked at 60°C for 2 h, deparaffinized in xylene and rehydrated through a descending graded ethanol series (100, 95, 80 and 70% ethanol; 5 min each) to water. For IHC and immunofluorescence staining, antigen retrieval was performed by heating slides in EDTA buffer (G1203; Wuhan Servicebio Technology Co., Ltd.) at 95°C for 20 min.

H&E staining. After deparaffinization and rehydration, sections were exposed to hematoxylin (G1004; Wuhan Servicebio Technology Co., Ltd.) for 5 min at room temperature, briefly differentiated in 1% acid-alcohol for 10 sec, blued under running water for 5 min and counterstained with eosin for 1 min at room temperature. Slides were then dehydrated using ethanol, cleared in xylene and mounted. Images were acquired using a bright-field light microscope (Olympus Corporation) and analyzed using ImageJ (v1.53; National Institutes of Health).

IHC. Following antigen retrieval, endogenous peroxidase was quenched with 3% H_2O_2 for 15 min at room temperature. To minimize non-specific binding, sections were blocked in 5% BSA (GC305006-100 g; Wuhan Servicebio Technology Co., Ltd.) for 60 min at room temperature and then incubated with primary antibodies overnight at 4°C. Biotinylated secondary antibodies were applied for 2 h at room temperature (Table SI), after which the streptavidin-biotin complex was added for 30 min. Signals were developed using a 3,3'-diaminobenzidine kit [GK600510; Gene Technology (Shanghai) Co., Ltd.] and nuclei were counterstained with hematoxylin. Images were acquired using bright-field light microscopy.

Immunofluorescence staining. Following antigen retrieval, sections were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and blocked with 5% BSA (GC305006-100 g; Wuhan Servicebio Technology Co., Ltd.)

for 60 min at room temperature. Sections were then incubated with primary antibodies overnight at 4°C (Table SI), followed by incubation with the appropriate secondary antibodies for 2 h at room temperature (Table SI). Nuclei were labeled with DAPI for 15 min at room temperature and sections were mounted in an anti-fade medium. Images were acquired using a fluorescence microscope and analyzed using ImageJ (v1.53; National Institutes of Health) using threshold-based segmentation or grayscale intensity measurements.

Masson staining. Paraffin-embedded tissue sections (4 μ m) were baked (60°C), deparaffinized in xylene (room temperature) and rehydrated through graded ethanols to water (room temperature). Sections were fixed overnight in Bouin's or Zenker's solution (room temperature), washed with distilled water (room temperature) and stained with Harris hematoxylin (8 min; room temperature). Differentiation in 0.8% acid alcohol (10 sec; room temperature) was followed by bluing in lithium carbonate (30 sec; room temperature). Sections were stained with picosirius red (10 min; room temperature), differentiated in phosphotungstic acid (5 min; room temperature), counterstained with aniline blue (5 min; room temperature), dehydrated (room temperature), cleared (room temperature) and mounted (room temperature). Observations were conducted using bright-field light microscopy (Olympus Corporation), and quantification was performed using ImageJ (v1.53; National Institutes of Health).

Histological quantification. Mucosal thickness was quantified on histological sections using ImageJ (v1.53; National Institutes of Health). Thickness was defined as the perpendicular distance from the epithelial surface (basement membrane) to the outer boundary of the submucosa. For each specimen, measurements were taken at ≥ 5 randomly selected positions per section (avoiding folds/tears), averaged to obtain a specimen-level value and then summarized by group for statistical analysis.

Primary cell isolation and culture. Primary cells were isolated from pituitary tumor tissues obtained from 3 of the aforementioned 63 patients, and samples from each patient were processed and cultured separately without pooling. Fresh mucosal tissue samples from surgical resection were stored on ice, with the digestion process completed within 1.5 h (no longer than 2 h). The tissue blocks were washed three times with pre-chilled PBS (1% penicillin-streptomycin) at 4°C to remove residual blood and necrotic components. Subsequently, the tissue was finely chopped into 1-3-mm³ fragments. For the generation of digested mucosal culture (DMC), the minced mucosal tissue was transferred to a tube containing 10 ml digestion solution consisting of 2 mg/ml collagenase type I (cat. no. 40507ES60; Shanghai Yeasen Biotechnology Co., Ltd.) in complete DMEM [DMEM (G4523; Wuhan Servicebio Technology Co., Ltd.) supplemented with 10% FBS (BMC1021; Abbkine Scientific Co., Ltd.) and 1% penicillin-streptomycin (G4003; Wuhan Servicebio Technology Co., Ltd.; 100 U/ml penicillin and 100 μ g/ml streptomycin)] and incubated at 37°C in a shaking incubator (100 rpm) for 1 h. Every 20 min, gentle pipetting was performed to facilitate tissue dissociation. The digestion was terminated when the tissue became translucent

and the solution became turbid. After digestion, the mixture was filtered through a 40- μ m cell strainer, and cells were collected by centrifugation at 300 x g for 5 min at 4°C. The cell pellet was resuspended in complete DMEM and seeded into collagen-coated culture dishes. Cells were incubated at 37°C with 5% CO₂, with the medium changed after 6 h to remove non-adherent cells and debris. For mucosal tissue culture (MTC), intact sphenoid sinus mucosal tissue fragments were placed onto 0.4- μ m polycarbonate membrane inserts, with the membrane positioned at the same level as the culture medium to establish an air-liquid interface. For primary PA cells, PA tissue was treated as aforementioned, and digestion was carried out with 0.25% trypsin (G4001; Wuhan Servicebio Technology Co., Ltd.) at 37°C for 30 min. Complete DMEM was added to halt digestion. Cells were centrifuged (300 x g; 5 min; 4°C), resuspended in complete DMEM and subsequently seeded. Before co-culture, a pilot experiment was conducted to assess the growth of digested primary PA cells and mucosal cells after plating. Cell morphology and growth were observed under an inverted light microscope on days 1, 3 and 5 after seeding. For the tumor-mucosal co-culture model, the cells (2x10⁵/well) were seeded in the bottom of a 24-well plate, while a non-contact co-culture system was established by seeding mucosal cells (2x10⁵/well) in 0.4- μ m polycarbonate membrane inserts (3470; Corning, Inc.). After incubation for 48 h at 37°C, cells and supernatants were collected for subsequent functional assays.

Cell cycle analysis. TtT/GF cells (~1x10⁶) were treated with the indicated concentrations of IFN- γ (0-100 ng/ml; cat. no. RP01038; ABclonal Biotech Co., Ltd.) or IL-6 (0-100 ng/ml; cat. no. RP00201; ABclonal Biotech Co., Ltd.) alone for 48 h at 37°C. Cells treated with ruxolitinib alone were incubated with ruxolitinib (5 μ M; cat. no. HY-50856; MedChemExpress) for 48 h at 37°C. For combination treatments, cells were pretreated with ruxolitinib for 30 min at 37°C before the simultaneous addition of IFN- γ and/or IL-6, followed by incubation for 48 h at 37°C. Cells (~1x10⁶) were washed with ice-cold PBS, fixed in 70% ethanol at 4°C for 24 h and stained with PI/RNase working solution (BD Pharmingen; BD Biosciences) for 30 min at 37°C in the dark. Data were acquired on a CytoFLEX cytometer (Beckman Coulter, Inc.) and cell cycle fractions (G₀/G₁, S and G₂/M) were modeled in ModFit LT 6.0 (version 6.0; Verity Software House, Inc.) using the Dean-Jett-Fox algorithm.

Apoptosis analysis. Pituitary tumor cells (1x10⁵) were resuspended in 1X binding buffer, labeled using an Annexin V-FITC/PI kit (Shanghai Yeasen Biotechnology Co., Ltd.) for 15 min at room temperature in the dark, diluted with 400 μ l binding buffer and analyzed immediately using the CytoFLEX cytometer. FlowJo v10.8 (version 10.8; BD Biosciences) was used for gating. Annexin V⁺/PI⁺ events were considered as early apoptotic and Annexin V⁺/PI⁺ as late apoptotic/necrotic.

RNA extraction, reverse transcription and quantitative PCR (qPCR). RAW264.7 macrophages in the M0, M1 or M2 state were treated with IgG (10 μ g/ml; cat. no. 14-4714-85; Invitrogen; Thermo Fisher Scientific, Inc.) or anti-CD47 monoclonal antibody (mAb) (10 μ g/ml; cat. no. 16-0479-85; Invitrogen;

Thermo Fisher Scientific, Inc.) at 37°C for 12 h. For polarization, M1 macrophages were induced with lipopolysaccharide (100 ng/ml; cat. no. HY-D1056; MedChemExpress) plus IFN- γ (20 ng/ml; cat. no. RP01070; ABclonal Biotech Co., Ltd.) for 24 h at 37°C, whereas M2 macrophages were induced with IL-4 (20 ng/ml; cat. no. RP01161; ABclonal Biotech Co., Ltd.) for 24 h at 37°C. RAW264.7 mouse macrophages were scraped and immediately lysed in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was purified according to the manufacturer's instructions, including chloroform phase separation, isopropanol precipitation and a 75% ethanol wash, and quantified on a NanoDrop spectrophotometer. Using 500 ng of RNA, first-strand cDNA was generated with the Hifair III 1st Strand cDNA Synthesis Kit (Shanghai Yeasen Biotechnology Co., Ltd.) at 42°C for 15 min. qPCR was carried out with Hifair qPCR SYBR Green Master Mix (Low Rox Plus) (Shanghai Yeasen Biotechnology Co., Ltd.) on an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR thermocycling conditions were as follows: Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 30 sec. GAPDH was used as the internal reference gene (18). Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_q}$ method (18). Primer sequences are listed in Table SII and were designed using the National Center for Biotechnology Information Primer-Basic Local Alignment Search tool (19).

Western blotting. TtT/GF cells were pretreated with ruxolitinib (5 μ M; cat. no. HY-50856; MedChemExpress) at 37°C for 30 min, followed by treatment with IFN- γ (0–100 ng/ml; cat. no. RP01038; ABclonal Biotech Co., Ltd.), IL-6 (100 ng/ml; cat. no. RP00201; ABclonal Biotech Co., Ltd.) or a combination of IFN- γ (50 ng/ml each) and IL-6 (50 ng/ml each) at 37°C for 48 h, as indicated. Cells were lysed in RIPA lysis buffer (Wuhan Servicebio Technology Co., Ltd.) supplemented with protease inhibitor cocktail (G2008; Wuhan Servicebio Technology Co., Ltd.) and phosphatase inhibitor cocktail (G2007; Wuhan Servicebio Technology Co., Ltd.) on ice for 30 min. Protein concentrations were determined using a BCA protein assay kit (Wuhan Servicebio Technology Co., Ltd.). Equal amounts of protein (30 μ g per lane) were separated using 10% SDS-PAGE gels (G2037; Wuhan Servicebio Technology Co., Ltd.) and transferred onto PVDF membranes (IPVH00010; MilliporeSigma). Membranes were blocked with NcmBlot blocking buffer (P30500; Suzhou Xinsaimai Biotechnology Co., Ltd.) for 1 h at room temperature, and then incubated with primary antibodies (Table SI) at 4°C overnight. After washing three times with Tris-buffered saline containing 0.1% Tween-20, membranes were incubated with HRP-conjugated secondary antibodies (SA00001-1/SA00001-2; 1:5,000; Proteintech Group, Inc.) for 2 h at room temperature. Protein bands were visualized using an ECL chemiluminescence kit (P10100; Guangzhou Saiguo Biotech Co., Ltd.), and images were captured using a GeneGnome XRQ imaging system (Syngene Europe). Band intensities were semi-quantified using ImageJ software (version 1.53; National Institutes of Health).

ELISA. Supernatants were collected and clarified by centrifugation (12,000 $\times$ g; 10 min; 4°C). Human IFN- γ , IL-1 β , IL-6, IL-10, TGF- β and TNF- α levels were quantified using commercial

ELISA kits (Human IFN- γ ELISA kit, cat. no. E-EL-H0108; Human IL-1 β ELISA kit, cat. no. E-EL-H0149; Human IL-6 ELISA kit, cat. no. E-EL-H0102; Human IL-10 ELISA kit, cat. no. E-EL-H0103; Human TGF- β ELISA kit, cat. no. E-EL-H0110; Human TNF- α ELISA kit, cat. no. E-EL-H0109; Wuhan Elabscience Biotechnology Co., Ltd.). Briefly, 100 μ l of sample or serially diluted standards, together with a medium-only negative control and a recombinant-protein positive control, were dispensed into each well and samples were incubated for 90 min at room temperature. Plates were then treated sequentially with biotinylated detection antibody and streptavidin-HRP (SA00001 series; 100 μ l/well; Proteintech Group, Inc.), with incubation for 30 min at 37°C in the dark for each step. 3,3',5,5'-Tetramethylbenzidine substrate (E-IR-R307; Wuhan Elabscience Biotechnology Co., Ltd.) was added, color was allowed to develop for 15 min at room temperature in the dark, and the reaction was stopped with 50 μ l of 2 M H₂SO₄. Absorbance at 450 nm was recorded on a Tecan Infinite F50 plate reader (Tecan Group, Ltd.). Cytokine concentrations were calculated from the standard curve and normalized to total protein content determined using a BCA assay.

Green fluorescent protein (GFP)-TtT/GF generation. GFP-expressing TtT/GF cells were generated by lentiviral transduction. Parental TtT/GF cells (CL-0561; Procell Life Science & Technology Co., Ltd.) were used in the present study. Lentiviral particles were produced in 293T cells (ATCC® CRL-3216™; American Type Culture Collection) using a third-generation packaging system by co-transfecting the enhanced green fluorescent protein (EGFP)-expressing lentiviral transfer plasmid pLVX-CMV-EGFP-PGK-Puro together with the packaging plasmids pMDLg/pRRE and pRSV-Rev and the envelope plasmid pMD2.G at a mass ratio of 4:2:1:1, corresponding to 10, 5, 2.5 and 2.5 μ g, respectively, per 10-cm dish. All plasmids (transfer, packaging and envelope plasmids) were constructed in-house by DesignGene Biotechnology. Transfection was performed at 37°C for 6–8 h, followed by replacement with fresh complete medium. Viral supernatants were collected at 48 and 72 h post-transfection, filtered through a 0.45- μ m membrane and used immediately or stored at -80°C. Parental TtT/GF cells were transduced at an MOI of 10 in the presence of polybrene (8 μ g/ml) for 12–16 h, followed by medium replacement. Transduced cells were selected with puromycin (2 μ g/ml) for 5–7 days and maintained in puromycin (1 μ g/ml). Subsequent experimentation was initiated 14 days after the start of lentiviral transduction. This 14-day interval ensured complete antibiotic selection, recovery of cellular homeostasis and the establishment of stable GFP expression prior to functional assays. GFP expression was confirmed by fluorescence microscopy. Cells were cultured at 37°C with 5% CO₂ in complete DMEM as recommended by the supplier.

pHrodo™ phagocytosis assay. RAW264.7 mouse macrophages (ATCC® TIB-71™; American Type Culture Collection) were maintained in complete DMEM at 37°C with 5% CO₂. Macrophages were polarized for 24 h in complete DMEM as follows: M1 polarization was induced by lipopolysaccharide (LPS; 100 ng/ml; L2880; Sigma-Aldrich; Merck KGaA) and IFN- γ (20 ng/ml; 575302; BioLegend, Inc.), and M2 polarization was induced by 20 ng/ml IL-4 (PHC0045;

Thermo Fisher Scientific, Inc.), both for 24 h at 37°C in complete DMEM supplemented with 10% FBS. Polarized macrophages were treated with an anti-CD47 mAb (10 µg/ml; 16-0479-85; Invitrogen; Thermo Fisher Scientific, Inc.) for 12 h at 37°C, followed by incubation for an additional 12 h at 37°C in serum-free DMEM. GFP-labeled TtT/GF cells were pre-stained with pHrodo red dye (1:10,000 in PBS; P35372; Thermo Fisher Scientific, Inc.) for 30 min at 37°C, and then washed three times with PBS containing 1% BSA to remove unbound dye. The co-culture system was established by seeding polarized macrophages and pHrodo-labeled tumor cells at a 1:1 ratio (5×10⁵ cells each) into 6-well plates, followed by incubation at 37°C with 5% CO₂ for 24 h. After incubation, cells were washed with PBS. Phagocytosis of GFP-labeled TtT/GF cells by RAW264.7 macrophages was observed under a fluorescence microscope (Olympus Corporation). Phagocytic events were defined as RAW264.7 cells positive for pHrodo fluorescence (excitation/emission, 560/585 nm), indicating uptake of pHrodo-labeled tumor cells. Phagocytosis was quantified by flow cytometry using a CytoFLEX V5-B5-R3 flow cytometer (Beckman Coulter, Inc.) as the percentage of RAW264.7 cells positive for pHrodo fluorescence, and data were analyzed using FlowJo (v10.8; BD Biosciences) (20).

Wound healing assay. TtT/GF cells were plated in 6-well plates and cultured in complete DMEM at 37°C with 5% CO₂ until 100% confluency. Vertical scratches were created with a 200-µl pipette tip. Cells were maintained in serum-free DMEM and treated with IFN-γ (0-100 ng/ml; cat. no. RP01038; ABclonal Biotech Co., Ltd.), IL-6 (100 ng/ml; cat. no. RP00201; ABclonal Biotech Co., Ltd.) or a combination of IFN-γ and IL-6 (50 ng/ml each) at 37°C for 72 h, as indicated. Where applicable, cells were pretreated with ruxolitinib (5 µM; cat. no. HY-50856; MedChemExpress) for 30 min at 37°C prior to cytokine stimulation. During the 3-day culture period in serum-free DMEM, wound closure was monitored and images were captured every 24 h using an inverted bright-field light microscope. Cell migration distances were quantified using ImageJ software (version 1.53; National Institutes of Health).

5-ethynyl-2'-deoxyuridine (EdU) cell proliferation assay. TtT/GF cells were seeded in 6- or 24-well plates and cultured at 37°C with 5% CO₂ until reaching 50-70% confluency. For direct cytokine treatment experiments, TtT/GF cells were treated with IFN-γ (0-100 ng/ml; cat. no. RP01038; ABclonal Biotech Co., Ltd.) at 37°C for 48 h, as indicated. For co-culture experiments, RAW264.7 macrophages were polarized for 24 h at 37°C with lipopolysaccharide plus IFN-γ or with IL-4, using the same inducing conditions as aforementioned, followed by treatment with anti-CD47 mAb (10 µg/ml; cat. no. 16-0479-85; Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C for 12 h. Anti-CD47 mAb-treated polarized macrophages were then co-cultured with TtT/GF cells for EdU analysis. Cells were then incubated with pre-warmed EdU (50 µM) in complete culture medium for 2 h at 37°C. After incubation, cells were washed with PBS, fixed with freshly prepared 4% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.5% Triton X-100. Cells were then blocked with 3% BSA for 30 min at room temperature. Subsequently, the cells were incubated

with the Click-iT reaction cocktail from the Click-iT™ EdU Cell Proliferation Kit for Imaging (Alexa Fluor™ 594; cat. no. C10339; Invitrogen; Thermo Fisher Scientific, Inc.), prepared according to the manufacturer's instructions, for 30 min at room temperature in the dark. Nuclei were counterstained with DAPI for 5 min at room temperature in the dark, after which cells were washed with PBS and mounted using an antifade mounting medium. EdU-positive cells were visualized using a fluorescence microscope, and proliferation was quantified using ImageJ software (version 1.53; National Institutes of Health).

Statistical analysis. All statistical analyses were performed using GraphPad Prism 6 (Dotmatics). Data are presented as the mean ± SD from ≥3 independent experiments/biological replicates. Where appropriate, measurements were normalized to the relevant control and expressed as the percentage or fold-change. Statistical tests were selected based on the experimental design and underlying assumptions. For comparisons between two independent groups, an unpaired two-sided Student's t-test was used. For paired measurements, a paired two-sided t-test was applied. For one-factor multi-group comparisons, one-way ANOVA followed by Tukey's post hoc multiple comparisons test was performed. For experiments involving two independent factors, two-way ANOVA with Bonferroni's multiple comparisons test was used. For longitudinal/repeated measurements on the same samples, repeated-measures ANOVA followed by Bonferroni's multiple comparisons test was applied. When data did not meet the assumptions for parametric testing, nonparametric tests were used. Specifically, the Mann-Whitney U test was applied for comparisons between two independent groups, the Wilcoxon signed-rank test was used for paired nonparametric data, and the Kruskal-Wallis test followed by Dunn's multiple comparisons test was employed for multi-group comparisons. Bonferroni correction was applied for multiple comparisons where appropriate. Correlations were assessed using Pearson's correlation coefficient. P≤0.05 was considered to indicate a statistically significant difference.

Results

Mucosa prevents tumor breach of the sphenoid sinus. Intraoperative and pathological assessments demonstrated that, in all SSIT cases included in the present study, the lesion occupied the sphenoid sinus lumen but did not breach the sinus mucosal wall to allow further extension (Fig. 1A). For a PA, inferior extension into the sphenoid sinus requires a stepwise traversal of barriers, including penetration of the tumor pseudocapsule, violation of the dura mater and erosion of the sellar floor, before reaching the sinus cavity (Fig. 1B). Consistent with this trajectory, H&E staining demonstrated tumor cell infiltration into the pseudocapsule with focal dural disruption, while there was no infiltration or destruction of the basal layer of the sinus mucosa (Fig. 1C). Masson's trichrome staining revealed densely arranged collagen fibers within the sphenoid sinus mucosa in the SSIT group, and the mucosal thickness was comparable to that observed in the NIT and DIT groups (Fig. 1D). These observations suggested that pre-mucosal anatomical barriers (pseudocapsule, dura

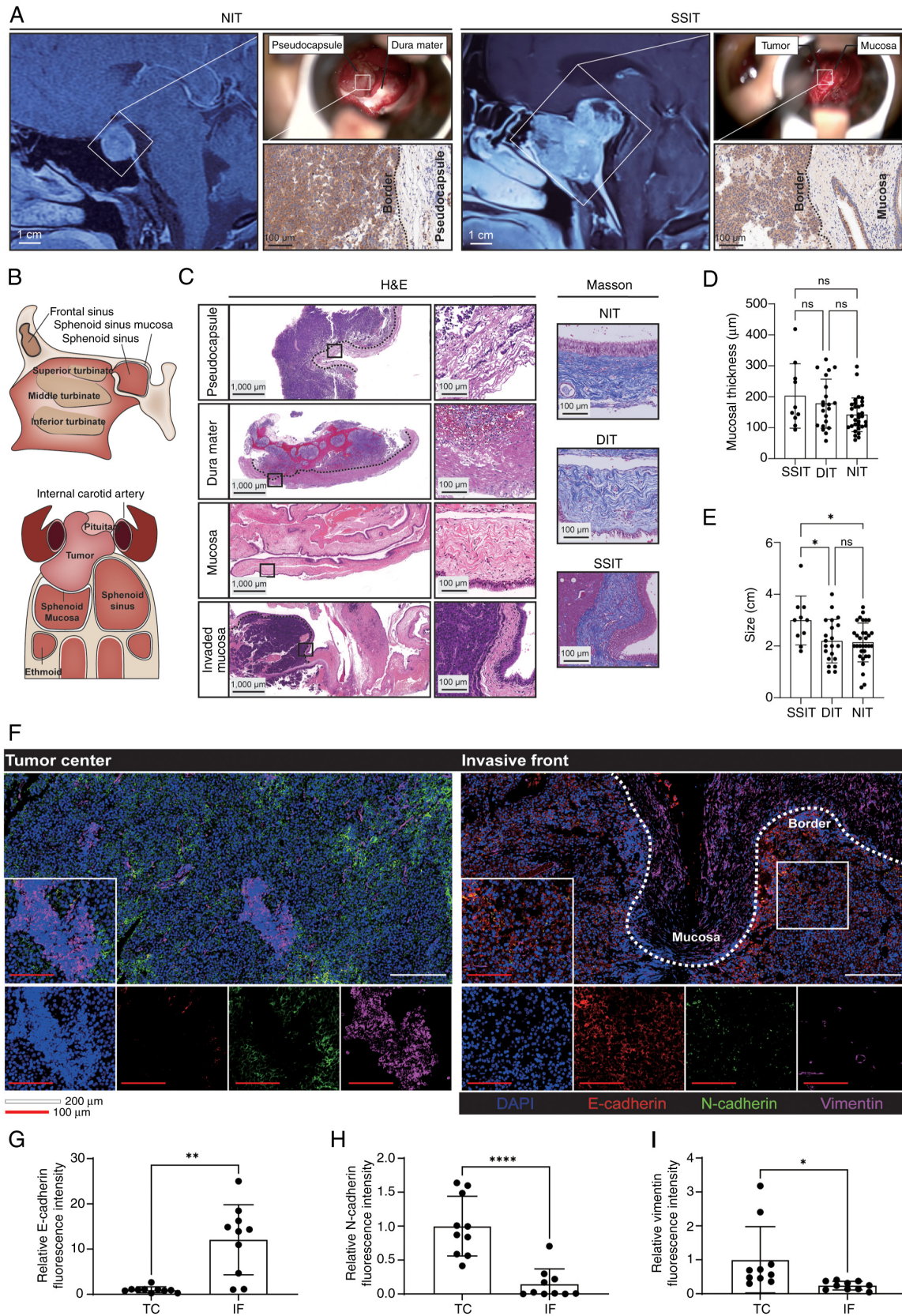


Figure 1. Mucosal integrity at the tumor-sinus interface. (A) Contrast-enhanced sagittal MRI, intraoperative microscope view and prolactin immunohistochemistry staining from 2 representative cases. (B) Schematic of the sellar floor and sphenoid sinus architecture, highlighting sequential barriers encountered during inferior tumor extension. (C) Histopathological characterization of invasion interfaces; H&E and Masson's trichrome staining at the IF of pseudocapsular (n=32), dural (n=21) and sphenoid sinus (n=10) sites, and in non-invaded sphenoid mucosa (n=53), with (D) quantitative analyses of mucosal collagen thickness. (E) Tumor dimension analysis for SSITs (n=10), DITs (n=21) and NITs (n=32). (F) Multiplex immunofluorescence staining of epithelial-mesenchymal transition markers at the IF vs. TC, including E-cadherin (red), N-cadherin (green) and vimentin (magenta). (G-I) Quantitative analysis of (G) E-cadherin, (H) N-cadherin and (I) vimentin levels in paired IF vs. TC samples (n=10). (D-E) One-way ANOVA with Tukey's post hoc multiple comparisons test. (G-I) Two-tailed paired t-test. * $P<0.05$, ** $P<0.01$, **** $P<0.0001$. DIT, dural-invasive tumor; IF, invasive front; NIT, non-invasive tumor; ns, not significant; SSIT, sphenoid sinus-invasive tumor; TC, tumor core.

and sellar floor) alone may be insufficient to restrain tumor progression once erosion occurs, although they constitute relevant physical obstacles (21-23). Despite the larger tumor size in the SSIT group compared with that in the NIT and DIT groups (Fig. 1E), the proliferation index at the SSIT invasive front (IF) was significantly lower than that observed in the DIT group, but not significantly different from the NIT group (Fig. S1A and B).

As invasive behavior is often associated with epithelial-mesenchymal transition (EMT) and stromal remodeling (7,24), the present study subsequently investigated EMT-related phenotypes. Multiplex immunofluorescence staining demonstrated relative suppression of EMT at the SSIT IF versus the tumor core (TC), characterized by higher E-cadherin expression, and lower N-cadherin and vimentin expression (Fig. 1F-I). In parallel, IHC revealed reduced MMP-2/9 expression at the IF compared with the TC in SSIT cases (Fig. S1C and D). Taken together, these findings were consistent with the notion that the sphenoid sinus mucosa may exert an active, context-dependent anti-invasive effect rather than serving solely as a passive physical barrier.

Sphenoid sinus mucosa suppresses growth of primary tumors. To further delineate the contribution of the sphenoid sinus mucosa to PA progression, tumor cell proliferation at the IF versus the TC was compared across SSIT (n=10), NIT (n=32) and DIT (n=21) cases. Ki-67 IHC demonstrated lower proliferative activity at the IF than at the paired TC in SSIT cases (Fig. 2A-F). By contrast, no significant differences between the IF and TC were observed in NIT or DIT cases. Mucosa-derived inhibitory effects on primary PA cells were next evaluated using two complementary *ex vivo* models: i) Air-liquid interface culture of sphenoid sinus mucosal tissue (MTC); and ii) co-culture of enzymatically dissociated mucosal cell preparations (DMC) with primary PA cells (Fig. 2G).

Air-liquid interface culture demonstrated that mucosal tissue viability declined markedly by day 7 (Fig. S2A and B). Following enzymatic digestion of the mucosa, fibroblast outgrowth from DMC preparations became evident from day 5 onward, indicating progressive changes in cellular composition during prolonged culture (Fig. S2C and D). Therefore, co-culture assays were conducted within the validated viability window, and 48-h co-culture with either MTC or DMC significantly reduced tumor cell proliferation (Fig. 2H and I) and increased tumor cell death (Fig. 2J and K) compared with those of tumor cells cultured alone (CTRL).

Mucosal tissues exhibit enrichment of macrophages and B cells. As part of the respiratory mucosa, the sphenoid sinus mucosa harbors abundant resident and recruited immune populations (25). Immune cell-subset distribution was profiled by IHC in pseudocapsule (n=32), dural (n=31) and mucosal (n=63) tissues. Relative to the pseudocapsule and dura mater, mucosal tissues displayed higher infiltration densities of macrophages [ionised calcium binding adaptor molecule 1 (IBA-1)⁺], CD4⁺ T cells, CD8⁺ T cells and B cells (CD19⁺) (Figs. 3A-D and S3A).

To resolve macrophage heterogeneity, multiplex immunofluorescence staining was used to phenotype IBA-1⁺ macrophages into HLA-DR⁺ (M1-like; pro-inflammatory)

and CD206⁺ (M2-like; immunoregulatory) states (26). Macrophages were enriched at the IF of pseudocapsular and dural tissues (Fig. 3E and F). In mucosal tissues, the proportion of HLA-DR⁺ (M1-like) macrophages was higher in tumor-invaded mucosa than in pseudocapsule/dura tissues, whereas non-invaded mucosa remained M2-skewed (Figs. 3G-J and S3B-D). Collectively, these data indicated context-dependent remodeling of mucosal macrophage states adjacent to invasion and were consistent with a microenvironment associated with constraints on local tumor expansion.

IFN- γ suppresses tumor invasion and growth. Cytokine dynamics were profiled using ELISAs in two co-culture systems. ELISA revealed that IFN- γ , IL-6 and IL-10 were increased in both the MTC and DMC group compared with the CTRL group, with higher IFN- γ levels in the DMC group (Fig. 4A). By contrast, TGF- β concentrations were reduced in both the MTC and DMC groups relative to the CTRL group (Fig. 4A). Spatial analyses demonstrated enrichment of TNF- α and IL-1 β at the tumor-mucosa interface, consistent with a pro-inflammatory gradient in regions of invasion (Fig. S4A-D). Immunofluorescence staining indicated that the IFN- γ signal was enriched in mucosal macrophages and exceeded that in intratumoral macrophages, which was in line with the correlation between macrophage density and IFN- γ intensity (Figs. 4B and S4E). IFN- γ expression in SSIT mucosal macrophages also exceeded that in non-invaded mucosa (Fig. 4C and D).

Guided by prior evidence linking IFN- γ to antitumor activity via direct anti-proliferative effects and microenvironmental remodeling (27,28), SSIT cases were stratified into high- and low-IFN- γ groups based on the median IFN- γ expression. Ki-67 IHC showed significantly lower proliferation indices in the high-IFN- γ cohort (Fig. 4E and F). Mechanistic investigation using the TtT/GF PA cell line revealed dose-dependent suppression of proliferation by exogenous IFN- γ (0-100 ng/ml), as indicated by reduced EdU incorporation (Figs. 4G and S4F). Wound healing assays further demonstrated IFN- γ -mediated inhibition of migration (Fig. S4G and H). Flow cytometry cell cycle analysis indicated S-phase arrest following IFN- γ treatment, which was reversed by the Janus kinase (JAK) inhibitor ruxolitinib (5 μ M) (Figs. 4H and I, and S4I-K). Semi-quantitative analysis of the western blot data confirmed that ruxolitinib (5 μ M) significantly reduced IFN- γ -induced STAT1 phosphorylation at concentrations of 0-50 ng/ml, whereas no significant inhibition was observed at 100 ng/ml (Fig. S4L and M). Taken together, these findings support a model in which mucosal macrophages constitute a predominant source of IFN- γ , and where IFN- γ constrains PA growth and invasion through JAK-STAT1 activation.

Elevated IgG levels drive M2-to-M1 macrophage polarization to suppress tumor growth. Tertiary lymphoid structures were observed in selected mucosal regions (Fig. S5A) and have been associated with localized antitumor immunity (29). To investigate mucosal B cell involvement (30,31), SSIT cases were stratified into high- and low-CD19⁺ groups based on the median CD19 expression. Sequential immunofluorescence staining of serial sections for CD19 and macrophage markers

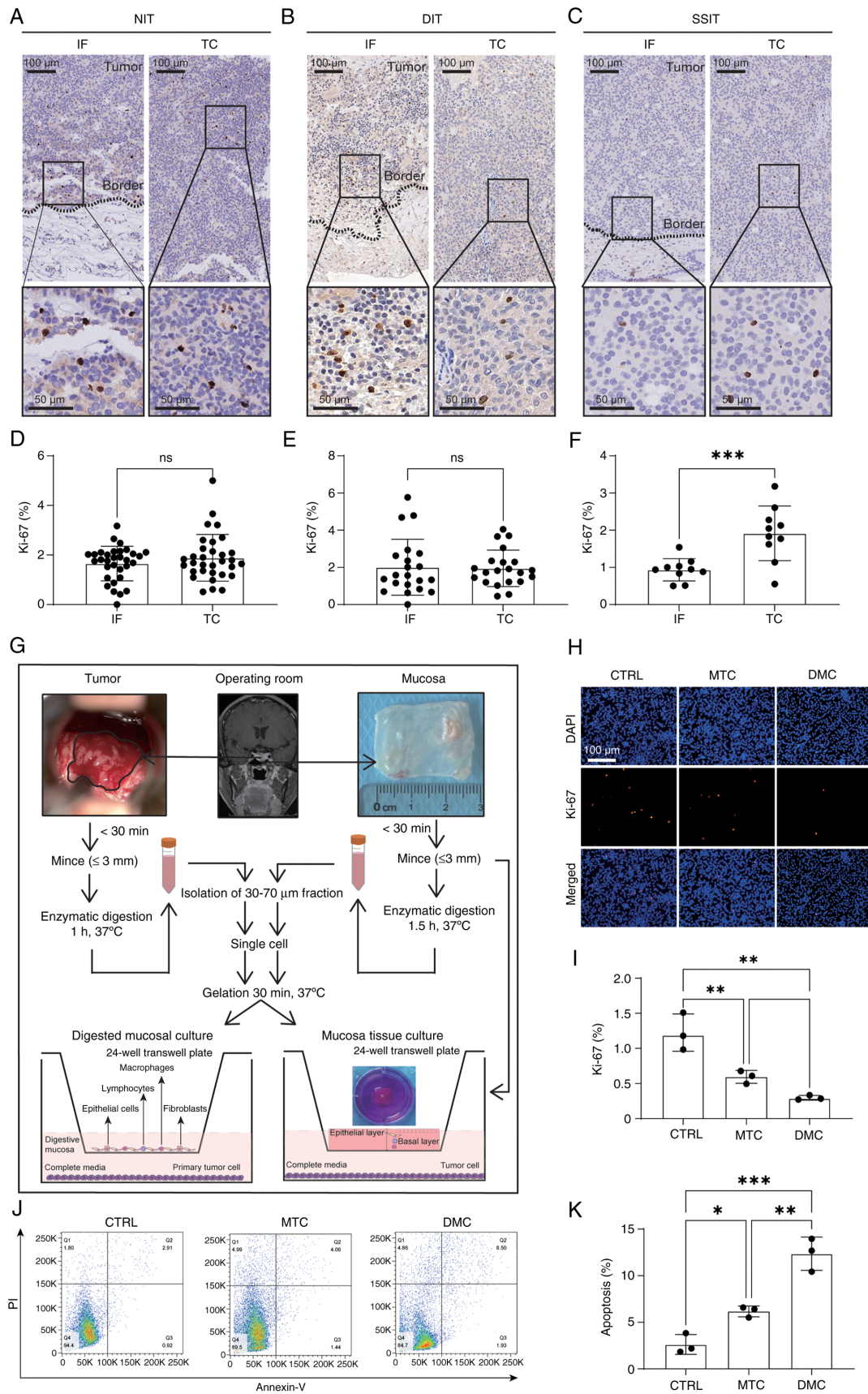


Figure 2. Mucosal suppression of tumor growth. (A-C) Representative immunohistochemistry staining images and (D-F) quantitative analysis of Ki-67 proliferation indices at the IF versus TC in (A and D) 32 non-invasive tumors, (B and E) 21 dural-invasive tumor and (C and F) 10 sphenoid sinus-invasive tumor samples. (G) Schematic showing co-culture models of air-liquid interface MTC or DMC with primary tumor cells. (H) Immunofluorescence images of tumor-mucosa co-culture stained with DAPI (blue) and Ki-67 (red). (I) Quantification of Ki-67-positive tumor cells (n=3). (J) Flow cytometry analysis of annexin V/PI-stained tumor cells in co-culture systems. (K) Quantification of apoptotic tumor cells based on flow cytometry (n=3). (D-F) Two-tailed paired t-test. (I and K) One-way ANOVA with Tukey's post hoc multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. CTRL, control; DMC, digested mucosal culture; IF, invasive front; MTC, mucosal tissue culture; ns, not significant; TC, tumor core.

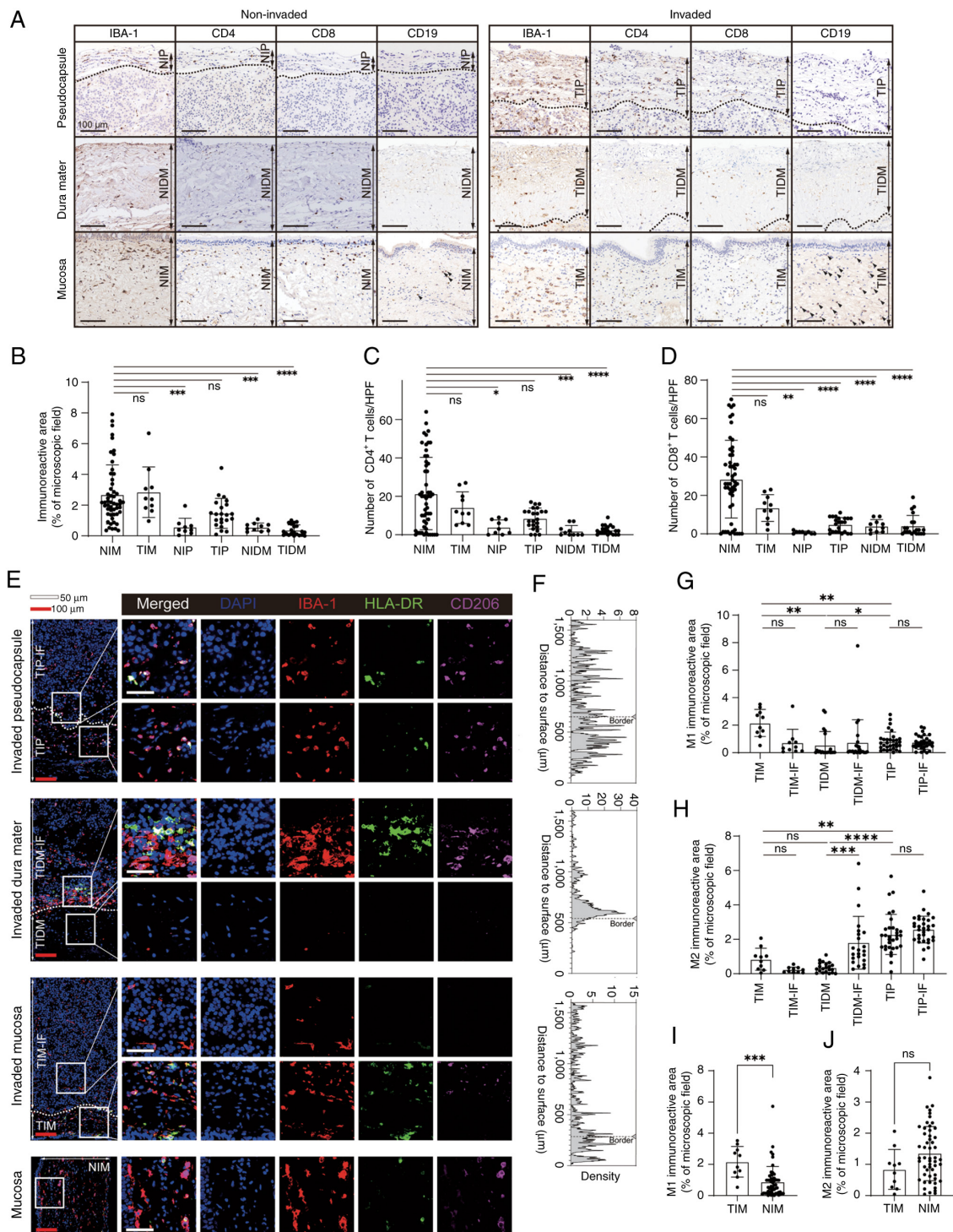


Figure 3. Spatial heterogeneity of immune cells in the tumor microenvironment. Immunophenotypic profiling across invasion states: Non-invaded pseudo-capsule (n=9; pathologically tumor-free), tumor-invaded pseudocapsule (n=23), control dura mater from non-invasive tumor cases (n=10), tumor-invaded dura mater (n=21), tumor-invaded mucosa (n=10) and non-invaded mucosa (n=53). (A) Representative immunohistochemistry images for the detection of macrophages (IBA-1⁺), CD4⁺ T cells, CD8⁺ T cells and CD19⁺ B cells. Arrows indicate CD19-positive cells. (B) Quantification of macrophage burden (IBA-1⁺ immunoreactive area; %). (C) Quantification of CD4⁺ T-cell density (cells per HPF). (D) Quantification of CD8⁺ T-cell density (cells per HPF). (E-J) Spatial heterogeneity of macrophage phenotypes. (E) Multiplex immunofluorescence images showing IBA-1⁺ (red), HLA-DR⁺ (green; M1-like) and CD206⁺ (magenta; M2-like) macrophage distributions at the IF of the pseudocapsule, dura mater and mucosa, and in non-invaded mucosa. (F) Grayscale-intensity distributions for IBA-1 quantified using ImageJ. (G) M1 immunoreactive area (% of microscopic field) in each group (TIM, TIM-IF, TIDM, TIDM-IF, TIP and TIP-IF). (H) M2 immunoreactive area (% of microscopic field) in each group (TIM, TIM-IF, TIDM, TIDM-IF, TIP and TIP-IF). (I) M1 immunoreactive area (% of microscopic field) in the TIM and NIM groups. (J) M2 immunoreactive area (% of microscopic field) in the TIM and NIM groups. (B-D) Kruskal-Wallis test with prespecified Dunn's post hoc planned comparisons (NIM vs. TIM/NIP/TIP/NIDM/TIDM) and Bonferroni correction. (G and H) Unpaired comparisons among TIM, TIDM and TIP, and separately among TIM-IF, TIDM-IF and TIP-IF, were performed using the Kruskal-Wallis test followed by Dunn's multiple-comparisons test, whereas paired comparisons between each tumor region and its matched IF region were performed using the two-tailed Wilcoxon signed-rank test. Bonferroni correction was applied across all nine comparisons performed in this analysis. (I and J) Unpaired comparisons were analyzed using a two-tailed Mann-Whitney U test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. HPF, high-power field; IBA-1, ionised calcium binding adaptor molecule 1; IF, invasive front; NIDM, non-invaded dura mater; NIM, non-invaded mucosa; NIP, non-invaded pseudocapsule; ns, not significant; TIDM, tumor-invaded dura mater; TIM, tumor-invaded mucosa; TIP, tumor-invaded pseudocapsule.

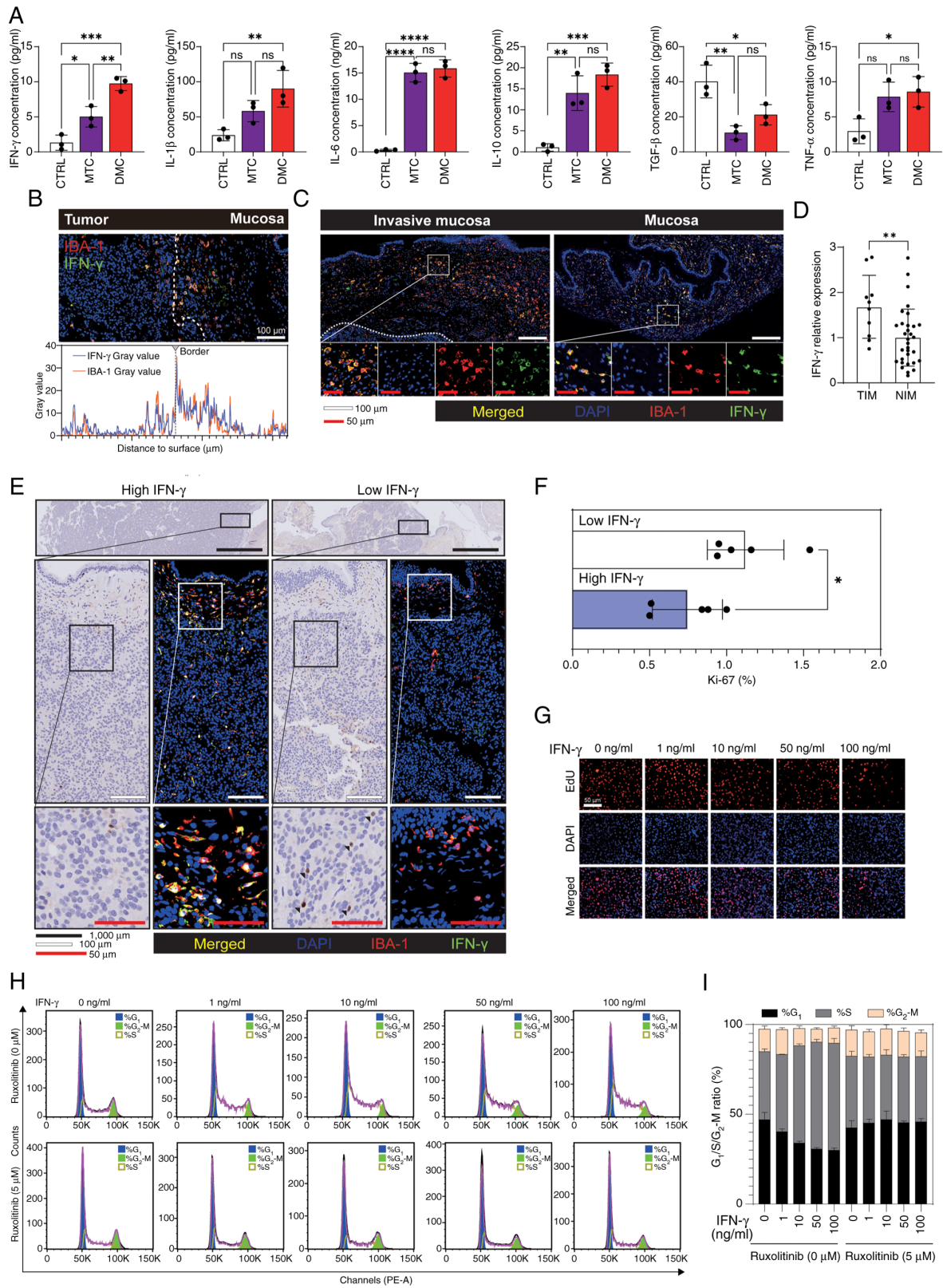


Figure 4. IFN- γ suppresses tumor growth and invasion. (A) Cytokine profiling of co-culture supernatants via ELISAs: IFN- γ , IL-1 β , IL-6, IL-10, TGF- β and TNF- α . (B-D) Spatial expression patterns of IFN- γ . (B) Immunofluorescence imaging of the invasive front in SSIT, showing DAPI (blue), IBA-1⁺ macrophages (red) and IFN- γ ⁺ signals (green), and grayscale intensity distribution. (C) Immunofluorescence imaging of TIM and NIM, showing DAPI (blue), IBA-1⁺ macrophages (red) and IFN- γ ⁺ signals (green). (D) Quantification of relative IFN- γ expression in TIM and NIM. (E) Representative Ki-67 immunohistochemistry images of SSIT cases stratified into IFN- γ -high and IFN- γ -low groups (n=5 each; median split). (F) Quantification of Ki-67 index comparing the two groups. (G) EdU staining demonstrating dose-dependent suppression of Tt/GF pituitary adenoma cell proliferation by IFN- γ (0-100 ng/ml; 48 h). (H) Representative flow cytometry histograms for cell cycle analysis of cells treated with IFN- γ (0-100 ng/ml) in the absence (0 μ M) or presence (5 μ M) of ruxolitinib. (I) Stacked bar plot showing the percentages of cells in the G₁, S and G₂/M phases under the same treatment conditions. (A) One-way ANOVA with Tukey's post hoc multiple comparisons test. (D and F) Unpaired two-tailed Student's t-test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CTRL, control; DMC, digested mucosal culture; EdU, 5-ethynyl-2'-deoxyuridine; IBA-1, ionised calcium binding adaptor molecule 1; MTC, mucosal tissue culture; NIM, non-invaded mucosa; ns, not significant; PE-A, phycoerythrin-area; SSIT, sphenoid sinus-invasive tumor; TIM, tumor-invaded mucosa.

revealed reduced M2 proportions and increased M1 polarization in high-CD19⁺ regions (Fig. 5A and B). IHC revealed strong IgG signal in B cells with undetectable IgA (Fig. S5B). Notably, IgG-high mucosal tissues exhibited significantly greater M1 macrophage proportions than IgG-low counterparts (Fig. 5C and D).

To probe the mechanistic contribution of IgG, RAW264.7 macrophages polarized with IL-4 (20 ng/ml) or LPS (100 ng/ml) plus IFN- γ (20 ng/ml) were treated with IgG (10 μ g/ml) for 12 h. qPCR analysis showed that IgG treatment increased IL-4 expression and reduced IL-18 expression in M0 macrophages, induced upregulation of IL-6, IL-27, TNF- α and IL-10 with concomitant downregulation of IL-18 in M1 macrophages, and increased IL-6, IL-27 and TNF- α while decreasing IL-4 in M2 macrophages, with IL-18 and IL-10 remaining unchanged in the latter (Figs. 5E and F, and S5C).

Consistently, multiplex immunofluorescence staining detected higher IL-6 expression in the high-CD19⁺ group compared with the low-CD19⁺ group (Fig. S5D and E). Given previous evidence indicating that high concentrations of IL-6 can suppress PA progression (32), dose-response assays (0–100 ng/ml) were performed in TtT/GF cells. Flow cytometry cell cycle analysis indicated IL-6-induced G₁-phase arrest (Fig. S5F–J), in contrast to IFN- γ -mediated S-phase arrest (Fig. 4H–I). Combined treatment with IFN- γ (50 ng/ml) and IL-6 (50 ng/ml) altered the cell-cycle phase distribution (Figs. 5G and H, and S5K–M), and reduced migration (scratch wound closure) to a level comparable to the level observed after IFN- γ treatment alone (100 ng/ml) or IL-6 treatment alone (100 ng/ml) (Fig. 5I and J). Western blotting indicated enhanced phosphorylation of STAT1 (phosphorylated-STAT1/STAT1 ratio), whereas STAT3 phosphorylation was attenuated relative to IL-6 monotherapy. This effect was abrogated by the JAK inhibitor ruxolitinib (5 μ M) (Figs. 5K and L, and S5N). Taken together, these data support a model in which elevated mucosal IgG levels reprogram macrophages toward an M1-dominant phenotype and, together with IFN- γ , cooperatively amplify JAK-STAT1 signaling to constrain PA cell proliferation and migration.

Anti-CD47 mAb enhances antibody-dependent cellular phagocytosis (ADCP) to suppress tumor cell proliferation. Immune checkpoint blockade targeting programmed cell death protein-1, programmed death-ligand-1, CD47 or signal regulatory protein- α (SIRP α) has improved outcomes in preclinical murine tumor models (33,34). During progression, tumor cells can evade macrophage phagocytosis via upregulation of CD47, a key component of the ‘don’t-eat-me’ axis with SIRP α . Immunofluorescence staining detected pronounced CD47⁺ signal at the tumor IF (Fig. 6A and B), supporting the rationale for anti-CD47 intervention.

To evaluate the therapeutic potential, M1- and M2-polarized macrophages were exposed to anti-CD47 mAb (10 μ g/ml) for 12 h. Anti-CD47 mAb treatment elicited transcriptional changes in M2 macrophages, marked by increased CD86, IL-1 β and NOS2 expression together with reduced Arg-1, CD206 and TGF- β levels (Fig. 6C). By comparison, anti-CD47 mAb treatment induced only modest effects in M0 (NOS2 upregulation) and M1 macrophages (IL-1 β upregulation) (Fig. 6C). Previous studies have demonstrated that anti-CD47

mAb augments macrophage phagocytosis and immune activation (35,36) (Fig. 6D). In macrophage-TtT/GF co-culture assays, anti-CD47 mAb treatment did not significantly affect tumor cell proliferation or phagocytic activity in the presence of M0 macrophages (Fig. 6F and H). By contrast, anti-CD47 mAb treatment in M1- and M2-polarized macrophages significantly reduced tumor cell proliferation, as determined by EdU incorporation, and significantly increased ADCP compared with isotype controls (Fig. 6F and H).

Collectively, these findings are summarized in the working model shown in Fig. 7.

Discussion

Persistent hormonal hypersecretion and invasion into adjacent critical structures remain major challenges in the management of Pas (37,38). Infiltrative growth into the cavernous sinus, dura mater and bone confers a substantial risk of recurrence (4). Unlike well-characterized IFs in hepatocellular or other solid tumors (39), the tumor-host interface in PAs remains underexplored. Intraoperative and histopathological observations in the present cohort indicated that the sphenoid sinus mucosa could maintain structural integrity even when tumors breached the pseudocapsule and dura, suggesting a potential barrier function analogous to that noted in other tissues (22,40).

Immunohistochemical profiling revealed heterogeneous immune infiltration at the tumor-mucosa interface, with macrophages (IBA-1⁺) constituting the predominant population. Prior studies in colorectal and ovarian cancer have shown that a higher ratio of pro-inflammatory (M1) to immunoregulatory (M2) macrophages at the tumor-stromal interface is associated with a favorable prognosis (41–43). In the present study, multiplex immunofluorescence staining demonstrated an increased M1 proportion in tumor-invaded mucosa relative to both TCs and non-invaded mucosa. In parallel, cytokine analyses delineated a pro-inflammatory gradient at the IF, including increased IFN- γ expression in mucosal macrophages. Given the established role of IFN- γ in constraining tumorigenesis through STAT1 activation (27), functional assays in PA models corroborated JAK-STAT1-dependent anti-proliferative and anti-migratory effects.

In addition to macrophage-mediated immune regulation, the mucosal niche also contained abundant B cells. Accumulating evidence indicates that B cells can mediate antitumor functions within the tumor microenvironment, and that B cell-enriched tertiary lymphoid structures are associated with durable antitumor immunity and favorable clinical outcomes (44,45). In the present study, the mucosal niche also contained abundant B cells, and tertiary lymphoid structures were observed in discrete areas. Within this context, IgG emerged as a candidate immunomodulator. Tissue-level analyses showed an association between IgG-high mucosa and increased M1 macrophage proportions, and cell-based experiments showed that IgG exposure increased macrophage IL-6 expression and was accompanied by enhanced IFN- γ -dependent STAT1 signaling. As PA invasion entails degradation of membranous and stromal barriers (46), strategies that preserve mucosal architecture, potentially by leveraging B cell-IgG axes, merit systematic evaluation.

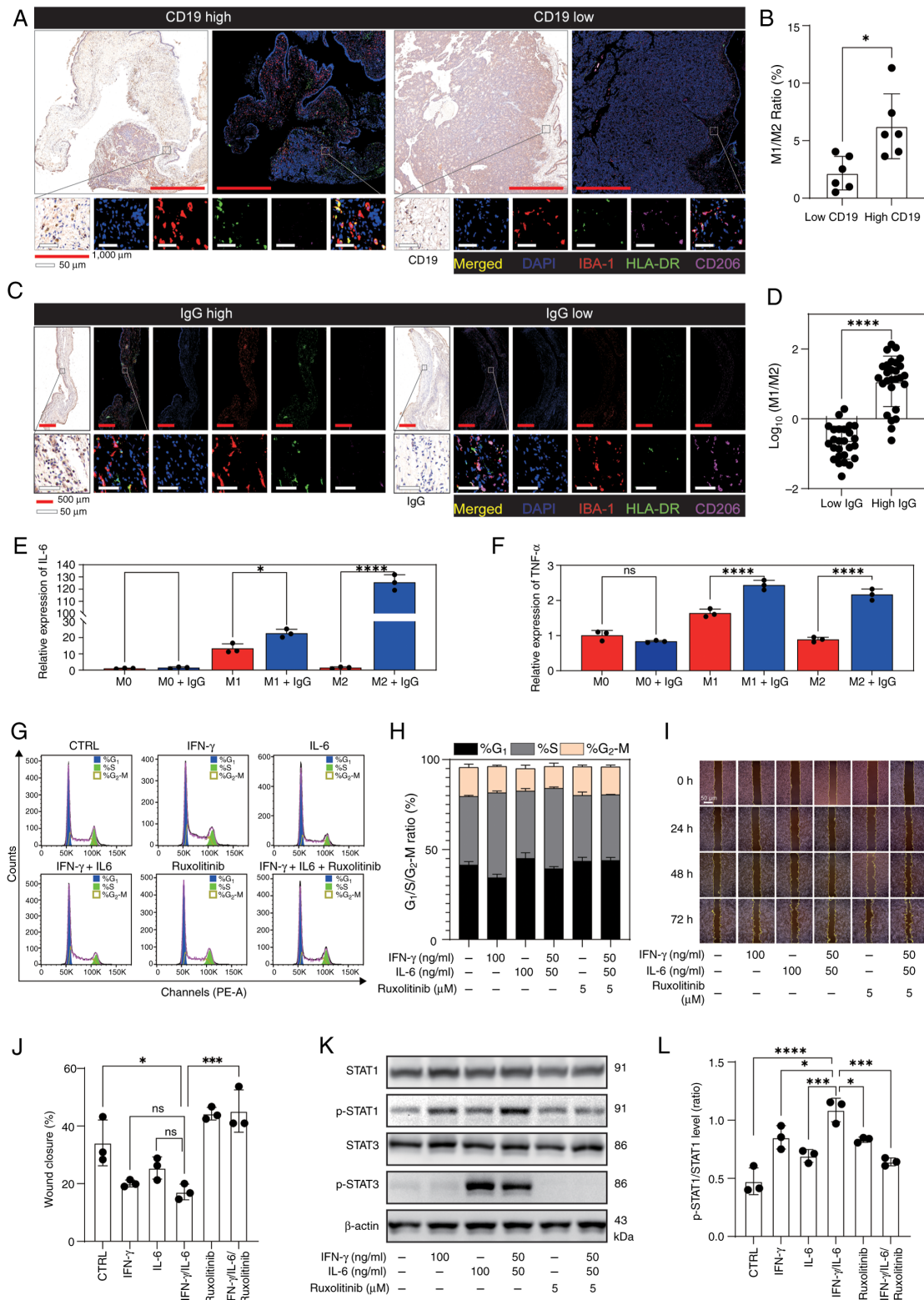


Figure 5. Elevated IgG levels drive macrophage M2-to-M1 reprogramming. (A) Sphenoid sinus-invasive tumor cases stratified into CD19-high (n=5) and CD19-low (n=5) groups based on the cohort median of CD19⁺ B cell density, with (B) quantitative analyses of macrophage polarization (M1-like versus M2-like). (C) Dural-invasive tumor and non-invasive tumor cases stratified into IgG-high (n=27) and IgG-low (n=26) groups based on the cohort median of relative IgG immunohistochemistry staining intensity, with (D) quantitative analyses of M1-like/M2-like macrophage proportions. (E and F) RAW264.7 macrophages were pre-polarized with IL-4 (20 ng/ml) or with lipopolysaccharide (100 ng/ml) plus IFN- γ (20 ng/ml) for 24 h, followed by IgG (10 μ g/ml) exposure. Relative (E) IL-6 and (F) TNF- α mRNA expression in RAW264.7 macrophages pre-polarized to M0, M1 or M2 states. (G) Representative flow cytometric cell-cycle profiles of TtT/GF cells following the indicated treatments. (H) Stacked bar plot summarizing the percentages of cells from (G) in G₁, S and G₂/M phases. (I) Representative images from the scratch wound assay at 0, 24, 48 and 72 h under the indicated treatments. (J) Quantification of scratch wound closure. (K) Representative western blot images showing total STAT1, p-STAT1, total STAT3, p-STAT3 and β -actin levels in cells treated with IFN- γ (100 ng/ml), IL-6 (100 ng/ml), IFN- γ + IL-6 (50 ng/ml each), ruxolitinib (5 μ M) or IFN- γ + IL-6 (50 ng/ml each) plus ruxolitinib (5 μ M), as indicated. (L) Densitometric semi-quantification of p-STAT1/STAT1 (ratio). (B and D) Unpaired two-tailed Student's t-test. (E, F, J and L) One-way ANOVA with Tukey's post hoc multiple comparisons test. *P<0.05, ***P<0.001, ****P<0.0001. CTRL, control; IBA-1, ionised calcium binding adaptor molecule 1; ns, not significant; p-, phosphorylated; PE-A, phycoerythrin-area.

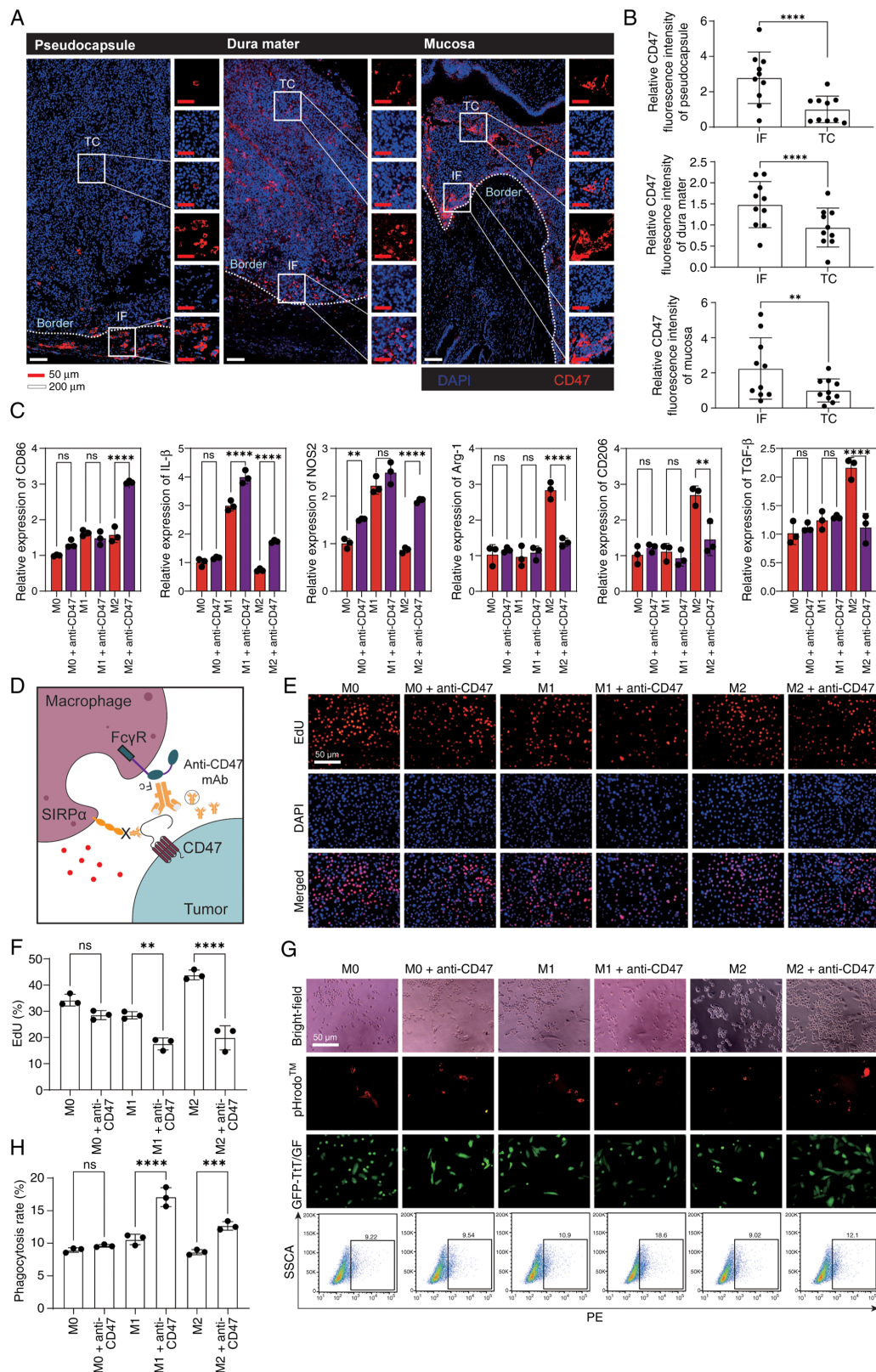


Figure 6. Anti-CD47 mAb enhances ADCP to suppress tumor cell proliferation. (A) Immunofluorescence staining of CD47 (red) and DAPI (blue) in a representative subset of non-invasive tumor, dural-invasive tumor and sphenoid sinus-invasive tumor cases (n=10 per group). (B) Paired comparison of CD47 fluorescence intensity at the IF versus the TC. (C) RAW264.7 macrophages were pre-polarized with IL-4 (20 ng/ml) or with lipopolysaccharide (100 ng/ml) plus IFN-γ (20 ng/ml) for 24 h, followed by anti-CD47 mAb (10 μg/ml) treatment for 12 h. Quantitative PCR was used to analyze polarization/activation markers. (D) Schematic illustrating anti-CD47 mAb-mediated blockade of the CD47-SIRPα axis and enhancement of ADCP. (E) EdU assay of TtT/GF cell proliferation in a Transwell co-culture with anti-CD47 mAb-treated polarized macrophages. (F) Quantification of EdU-positive cells. (G) Representative microscopy images and flow cytometry plots showing macrophage phagocytosis of pHrodo™ Red-labeled GFP-TtT/GF cells. (H) Quantification of phagocytosis (%). (B) Paired two-tailed Student's t-test. (C, F and H) One-way ANOVA with Tukey's post hoc multiple comparisons test. **P<0.01, ***P<0.001, ****P<0.0001. ADCP, antibody-dependent cellular phagocytosis; Arg-1, arginase 1; EdU, 5-ethynyl-2'-deoxyuridine; FcγR, Fcγ receptor; GFP, green fluorescent protein; IF, invasive front; mAb, monoclonal antibody; NOS2, nitric oxide synthase 2; ns, not significant; PE, phycoerythrin; SIRPα, signal regulatory protein-α; SSCA, side scatter area; TC, tumor core.

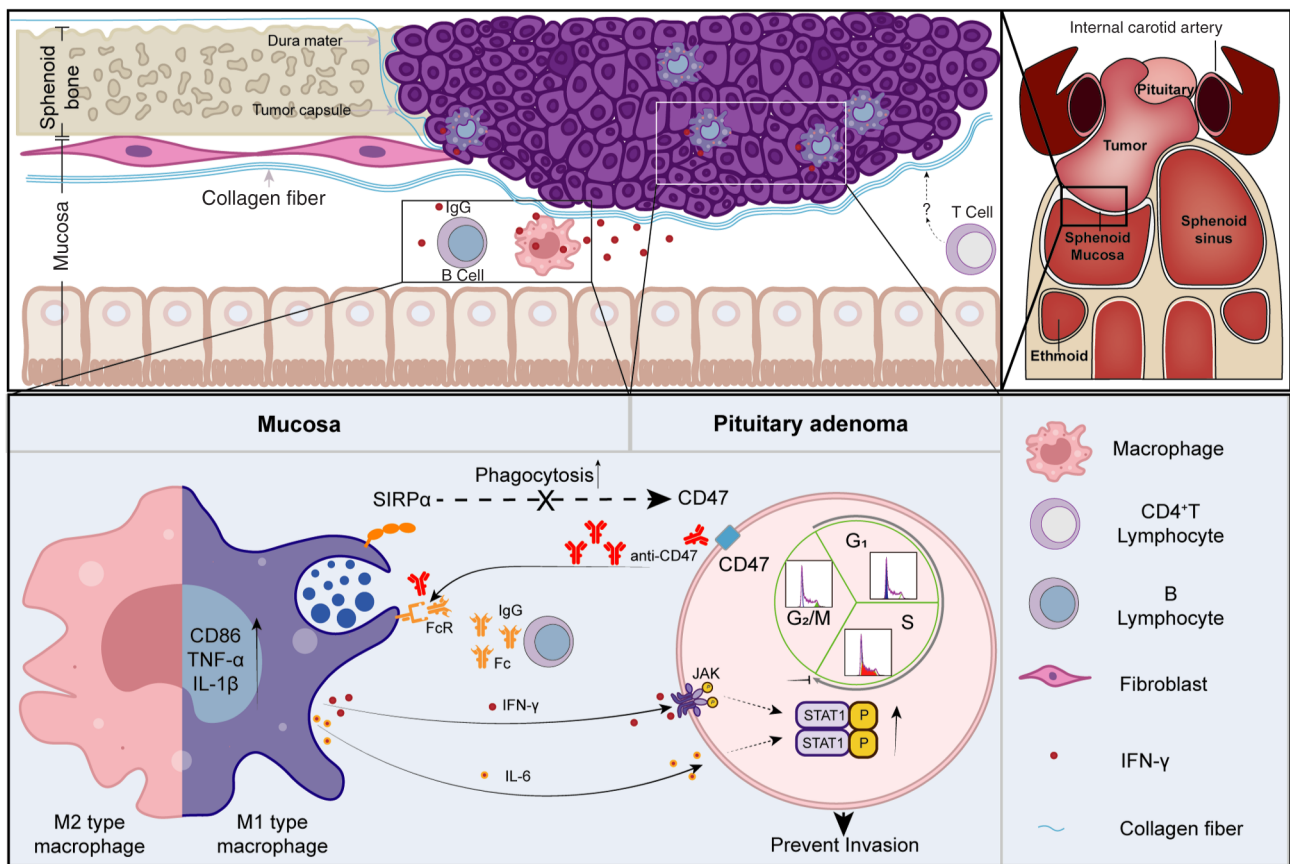


Figure 7. Summary graphic illustration. This illustration summarizes the proposed model during pituitary adenoma invasion. The tumor invasive front abuts an intact sphenoid sinus mucosa, forming a distinct boundary. The mucosal compartment is enriched for ionised calcium binding adaptor molecule 1-positive macrophages with an M1-like predominance and IgG-high B cells. B cell-derived IgG promotes M2-to-M1 macrophage reprogramming, while coordinated IFN- γ and IL-6 production establishes a tumor-suppressive cytokine gradient that decreases from mucosa toward the tumor core, constraining proliferation and migration via JAK-STAT1 activation. Therapeutically, anti-CD47 monoclonal antibody blocks the CD47-SIRP α 'don't-eat-me' axis and augments antibody-dependent cellular phagocytosis, highlighting a strategy for immune checkpoint-targeted therapy that may complement surgical management. FcR, Fc receptor; JAK, Janus kinase; mAb, monoclonal antibody; p-, phosphorylated; SIRP α , signal regulatory protein- α .

Therapeutically, the CD47-SIRP α checkpoint represents a rational target in settings where tumor cells upregulate CD47 levels at the IF. In the present study, anti-CD47 mAb treatment increased activation markers in M2-polarized macrophages, including CD86 and NOS2, reduced PA cell proliferation under co-culture conditions, and enhanced ADCP, in line with prior reports (33,47). Nonetheless, clinical translation is complicated by on-target effects on erythrocytes and attendant anemia (48).

Tissue engineering concepts may offer complementary avenues: Prior work showing growth restraint of MCF7 xenografts by adipose tissue grafts raises the broader hypothesis that perioperative preservation or augmentation of protective mucosal elements, potentially combined with macrophage-directed immunotherapy, could be explored alongside transsphenoidal surgery (49).

The focus of the present study on polarized macrophages overlooks subsets lacking classical M1/M2 markers or exhibiting mixed phenotypes, as reported in other systems (42,43). The roles of T lymphocytes, neutrophils and intercellular crosstalk remain unaddressed. Future studies should isolate fresh mucosal immune cells for single-cell transcriptomic profiling and spatial transcriptomics to elucidate their plasticity within the IF of the tumor microenvironment (50).

Beyond its macrophage-modulating effects, it should be emphasized that IgG exhibits divergent modulatory effects on tumor cell proliferation (30,51), an aspect not systematically addressed in the present study. Constrained by limitations in the *in vitro* passaging of tumor cells and the proliferative dynamics of mucosal tissue, the present study focused on analyzing the inhibitory effect of the mucosa on tumor growth. Future studies will employ functional assays, such as Matrigel invasion assays of primary tumor cells under organoid culture conditions and mucosal explant models, to determine whether the mucosa actively restricts tumor invasion.

In conclusion, the intricate dynamics between sphenoid sinus mucosa and invasive PAs, as revealed in the present study, provide novel insights into tumor-host interactions. Far from a passive barrier, the mucosa orchestrates an active defense system, recruiting macrophages, B cells and cytokines to counteract tumor-driven structural disruption. Understanding and leveraging this natural defense strategy may inspire novel approaches to inhibit tumor invasion.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

YH and TL conceived the study. XL, ZL and LX contributed to experimental design and execution of experimental procedures. XL, ZL, ZW, QW, QJ, LX and TL performed experiments and acquired data. XL performed formal analysis. XL, ZL and SL collected, organized and verified the clinical and experimental data, and ensured the accuracy and completeness of the datasets used for analysis, and SL contributed to data interpretation and figure preparation. XL and ZL were involved in validation. YH, ZL and SL were involved in visualization. XL and LX wrote the original manuscript. TL, SL and HZ and YH reviewed and edited the manuscript. TL and HZ provided resources, and HZ contributed to interpretation of the clinical/pathological data and critically revised the manuscript for important intellectual content. YH and TL supervised the study. TL, XL and YH were involved in project administration. TL acquired funding. XL and YH confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee of Tongji Hospital (Wuhan, China; approval no. TJ-IRB20220325) and conducted in accordance with The Declaration of Helsinki. All participants provided written informed consent before participation.

Patient consent for publication

All patients provided written informed consent for publication of their anonymized data.

Competing interests

The authors declare that they have no competing interests.

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