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Modulation of the IL-6 Receptor α Underlies GLI2-Mediated Regulation of Ig Secretion in Waldenström Macroglobulinemia Cells

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Ig secretion by terminally differentiated B cells is an important component of the humoral immune response. Upon recognition of Ag, B cells undergo a differentiation process into mature plasma cells that ultimately leads to Ig secretion to overcome foreign Ag (1–3). In B cell malignancies, this process is dysregulated and excessive amounts of Ig are often secreted. Several neoplasms including, Waldenström macroglobulinemia (WM), are known for their aberrant production of monoclonal Ig (4–6). This excessive production of a monoclonal Ig protein may lead to renal failure as a result of Bence Jones proteinuria (7) and poor response to chemotherapy (8). Owing to the increased Ig production, patients may present with serum hyperviscosity, a condition responsible for the clinical symptoms and that correlates with aggressiveness of these diseases (8). Despite the clinical relevance of Ig production, little is known about the mechanisms that regulate monoclonal Ig production in these diseases. Therefore, a better understanding of the molecular events regulating Ig secretion by malignant B cells and plasma cells is fundamental for the development of novel targeted therapies for Ig-mediated diseases.

We recently provided evidence that GLI2 expression in the tumor microenvironment plays an indirect role in regulating IgM secretion by WM cells (9). In this study, we define a novel pathway regulated by the oncogene GLI2 controlling IgM secretion in WM cells. GLI2 is a zinc finger transcription factor playing oncogenic roles in several cancers, including basal cell carcinoma, melanoma, colon cancer, and lymphoma among others (10–16). In WM cells pharmacological inhibition of GLI2 reduced IgM secretion without affecting cell proliferation or survival. Characterization of this regulatory pathway shows that an active Hedgehog (HH) pathway, a known modulator of GLI2 protein activity, is not required for GLI2-mediated regulation of IgM secretion. Analysis of the mechanism identified the IL-6Rα (gp80) subunit as a direct target of GLI2. We demonstrate that GLI2 binds to and activates the IL-6Rα promoter in WM cells. Moreover, GLI2 knockdown by RNA interference (RNAi) resulted in a decrease in IgM secretion, which can be rescued by overexpression of IL-6Rα. Taken together, our results identify a novel role for GLI2 in modulating IgM secretion via regulation of the IL-6Rα promoter and expression. Therefore, targeting this axis may provide therapeutic benefit to patients with B cell/plasma cell malignancies associated with increased Ig production.

Materials and Methods

Cell culture and reagents

The IgM-secreting cell line BCWM.1 (17, 18) was a gift from Dr. Steven Treon (Dana Farber Cancer Institute, Boston, MA). MWCL-1 cells (19)
were a gift from Dr. Stephen Ansell (Mayo Clinic, Rochester, MN), and RPCI-WM1 cells (20) were provided by Dr. Asher Chanan-Khan (Mayo Clinic, Jacksonville, FL). All cells were grown in RPMI 1640 supplemented with 10% FBS and penicillin/streptomycin. The GLI1/2 inhibitor (Gant61) and HH inhibitor (cyclopamine) were obtained from EMD Millipore (Billerica, MA). The pan-caspase inhibitor (Q-VD-OPh) and all primers were obtained from Sigma-Aldrich (St. Louis, MO). β-Actin Ab was obtained from Novus Biologicals (Littleton, CO). IL-6R Ab for Western blot was from Santa Cruz Biotechnology (Dallas, TX). FITC-conjugated annexin V was obtained from BD Biosciences (San Jose, CA), and propidium iodide was from Sigma-Aldrich.

**Plasmid constructs and cell transfections**

Short hairpin RNA (shRNA) targeting GLI1 and GLI2 were purchased from Origene Technologies (Rockville, MD) using plasmid vector pGFP-V-RS. The following shRNA targeting sequences were used: shGLI1, 5'-TCCTCGGTGTGACGGCTGCAC-3' (forward) and 5'-AGCTCAGTGGGTTTCCTGAAGCA-3' (reverse); shGLI2(1), 5'-GACCTACATATATTGTCCTGGC-3' (forward) and 5'-AGCTCACTGAGGTTTGCTGAGA-3' (reverse); shGLI2(2), 5'-TCACAGCTGCAGATCTGGAAC-3' (forward) and 5'-CTGTTTGGGACTGAGCTTAATA-3' (reverse); GLI1, 5'-AGTTGCTGATGGGGGACTGGTT-3' (forward) and 5'-CCTGGCTACCTGGACAGGACGCACTGGGATGGA-3' (reverse); GLI2, 5'-GAGGGTGGTTTGAAGCTGAAGG-3' (forward) and 5'-GCTGACAGTATAGGCAGA-3' (reverse); GAPDH, 5'-ACTGTCACTTTGTGGTGAGGAAGTT-3' (forward) and 5'-GGTGGCTACAACTGGGACTT-3' (reverse); IL-6R, 5'-TGTCGTATGACGACAGGACGCACTGGGATGGA-3' (forward) and 5'-GACCTACATATATTGTCCTGGC-3' (reverse); GLI3, 5'-CCAACG-3' (forward) and 5'-GGCTCACAGACA-3' (reverse); TACI, 5'-CAAGGGACAGGCACTGGTCTA-3' (forward) and 5'-CTCGTGTCCTCTCTCTCCATATC-3' (reverse). These experiments were repeated three times with similar results.

**RNA isolation and RT-PCR**

Cells were collected and total RNA was isolated using TRIzol (Bioline, London, U.K.) per the manufacturer’s protocol. Reverse transcription reactions were conducted using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). RT-PCR was performed using the EmeraldAmp GT PCR master mix (Clontech, Mountain View, CA) per the manufacturer’s protocol. PCR products were analyzed by gel electrophoresis using the following forward and reverse primers: GLI1, 5'-GGGACTGAGCTGGCATAGAG-3' (forward) and 5'-CTCAGTGTCACCTGGCAAGA-3' (reverse); GLI2, 5'-ACTGTCACTTTGTGGTGAGGAAGTT-3' (forward) and 5'-GGTGGCTACAACTGGGACTT-3' (reverse); GAPDH, 5'-ACTGTCACTTTGTGGTGAGGAAGTT-3' (forward) and 5'-GGTGGCTACAACTGGGACTT-3' (reverse); BAFFR, 5'-GGGACTGAGCTGGCATAGAG-3' (forward) and 5'-CTCAGTGTCACCTGGCAAGA-3' (reverse); GLI3, 5'-CCAACG-3' (forward) and 5'-GGCTCACAGACA-3' (reverse); TACI, 5'-CAAGGGACAGGCACTGGTCTA-3' (forward) and 5'-CTCGTGTCCTCTCTCTCCATATC-3' (reverse). Quantitative real-time PCR (qPCR) was performed using the ViiA 7 real-time PCR system by Life Technologies (Grand Island, NY). For copy number analysis, 2.0 × 10⁶ cells of BCWM.1, MWCL-1, and RPCI-WM1 were grown in 0.0 ml RPMI 1640 (with 10% FBS and penicillin/streptomycin) for 48 h. Copy number of GLI1, GLI2, and GLI3 was determined in each cell line using the following primers: GLI1, 5'-GCTACATCAAACCTGGGCAAT-3' (forward) and 5'-GGCCGTTACAGAACGAGACTGG-3' (reverse); GLI2, 5'-CAGTCCAGGACCATCAAGACCAC-3' (forward) and 5'-ACTGCTACCTGGGGGACATC-3' (reverse); GAPDH, 5'-GAGGGTGGTTTGAAGCTGAAGG-3' (forward) and 5'-GCTGACAGTATAGGCAGA-3' (reverse); and GLI3, 5'-CCAACG-3' (forward) and 5'-GGCTCACAGACA-3' (reverse). A standard curve was generated using a plasmid with the coding region of the gene of interest incorporated into it. For expression of IL-6R or β-actin was used to detect protein, and β-actin Ab was used as a reference. Densitometry was performed using ImageJ software, normalized to control, and presented as an average of three independent experiments.

**Cell viability**

Cells (0.25 × 10⁶) treated with Gant61 or cyclopamine were incubated in 48-well plates in RPMI 1640 containing 10% FBS for 48 h. Cells were washed with annexin V binding buffer and stained with 1 μg Annexin V (BD Biosciences) for 20 min at 4°C. The cells were again washed with annexin V binding buffer and then stained with 0.5 μg propidium iodide. Cells were immediately processed using a FACSCalibur (BD Biosciences) and data were analyzed using FlowJo software.

**Proliferation assay**

Cells were suspended in RPMI 1640 (without phenol red, 5% FBS plus penicillin/streptomycin) at a concentration of 0.25 × 10⁶ cells/ml. Cell suspensions (100 μl) were plated in triplicate in a 96-well plate and treated...
with 20 μM Gant61 or DMSO as a control. Final volume was brought to 200 μl/well. After 3 d, 50 μl XTT working solution (Trevegin, Gaithersburg, MD) was added to each well and incubated at 37˚C for 1 h and then analyzed on an Epoch plate reader (BioTek).

Luciferase reporter assay

Cells (4.0 × 10^6) were transfected, as described above, plated in triplicate in 24-well plates, and incubated in RPMI 1640 supplemented with 10% FBS. After 48 h, cells were harvested and assayed for luciferase activity per the manufacturer’s protocol (Promega). To control for variations due to transfection efficiency, total protein for each sample was quantified using a BCA protein assay kit (Thermo Fisher Scientific). Luciferase readouts were normalized to total protein content and relative luciferase represents luciferase readouts/protein concentration relative to control samples in each experiment.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was conducted using a modification of the Magna ChIP kit protocol (EMD Millipore, Bedford, MA) as previously described (21). Chromatin was digested with micrococcal nuclease (2 gel units/μl; New England Biolabs, Ipswich, MA) for 20 min at 37˚C. Chromatin was further sheared by sonication for 15 cycles of 30 s on/30 s off at 4˚C (Bioruptor; Diagenode, Denville, NJ). Aliquots of the sheared chromatin were subjected to immunoprecipitation using anti-GLI2 Ab (Novus Biologicals) or normal rabbit IgG (EMD Millipore). qPCR of the ChIP products and genomic input DNA was performed using primers that amplify an area of the IL-6Rα promoter containing consensus GLI binding sites. The sequences of the primers are the following: 5’-CCAATCACGGCTCAGTGA-3’ (forward) and 5’-TTACAGGTCGAGCCAC-3’ (reverse). Quantitative SYBR PCR was performed at 59˚C in triplicate for each sample or control using the C1000 Thermal Cycler (Bio-Rad), and the results are presented as percentage of input.

Peritoneal cell isolation and culture

Normal B cells were isolated from female BALB/cByJ mice (5–8 wk) that were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were cared for and handled in accordance with institutional and National Institutes of Health guidelines. Peritoneal washout cells were obtained as previously described (22). B-1a cells were enriched by a B220 negative selection process followed by CD5 positive selection using mAb-coupled MicroBeads (Miltenyi Biotec, Auburn, CA) following the manufacturer’s instructions. Purity was determined as ≥95% by FACS. Total peritoneal cell isolation and culture

FIGURE 2. Treatment with Gant61 affects IgM secretion and GLI mRNA expression in WM cells. (A) Relative IgM secretion measured by ELISA for BCWM.1, MWCL-1, and RPCI-WM1 cells treated with 20 μM Gant61 for 48 h when compared with cells treated with only DMSO. (B) mRNA expression of GLI1 and GLI2 in BCWM.1, MWCL-1, and RPCI-WM1 cells treated with 20 μM Gant61 or DMSO for 48 h determined by RT-PCR. (C) Effect of Gant61 (20 μM, 48 h) treatment on relative proliferation of BCWM.1, MWCL-1, and RPCI-WM1 cells when compared with cells treated with DMSO measured by XTT assay. (D) Effect of Gant61 (20 μM, 48 h) treatment on relative viability of BCWM.1, MWCL-1, and RPCI-WM1 cells when compared with control. Cells (0.25 × 10^6) were stained with annexin V and propidium iodide and analyzed by flow cytometry as described in Materials and Methods. Supernatants were used to quantify IgM secretion by ELISA and (F) cells were used to determine cell viability by annexin V/propidium iodide staining. Bars represent mean ± SEM. Each experiment was repeated at least three times and the bars represent the average of three to four pooled biological replicates. *p < 0.05, **p < 0.0001.
cells or purified B-1a cells were cultured at 1 × 10^6 cells/ml in 6- or 12-well plates in the presence or absence of the indicated treatments.

**Flow cytometry**

Fluorochrome-conjugated Abs used for flow cytometry were obtained from BD Biosciences. For GLI detection, 0.5 × 10^6 WM B cells were collected in FACS tubes. The cells were then permeabilized and stained with the BD Cytofix/Cytoperm Plus kit (BD Biosciences, catalog no. 555028) according to the manufacturer’s recommendations with some modifications. Briefly, cells were spun down and resuspended in 100 µl Fix/Perm solution and incubated at 4°C for 30 min. Cells were then washed twice with Fix/Perm wash buffer and resuspended in 50 µl Fix/Perm solution. Four micrograms primary Ab from Novus for GLI1 (NB600-600), GLI2 (NB600-874), GLI3 (NBp2-29627), and isotype control (NBp2-36463) was added and the tubes were incubated at 4°C for 1 h. Cells were washed twice with Fix/Perm wash buffer and resuspended in 50 µl Fix/Perm solution. Anti-rabbit IgG (PE conjugated; 10 µl) secondary Ab from BD Biosciences (catalog no. 558416) was added. Tubes were incubated at 4°C for 45 min and then washed twice with Fix/Perm wash buffer. Cells were resuspended in 500 µl FACS buffer and analyzed via flow cytometry. Cells were analyzed on a FACS Calibur instrument and analyzed using FlowJo software.

**Statistical analysis**

A two-tailed Student t test was used to determine statistical significance between two data sets or ANOVA between more than two data sets. A p value <0.05 was considered significant. Analysis was carried out using Prism software (GraphPad Software, La Jolla, CA).

**Results**

**GLI inhibition reduces IgM secretion**

Because of the oncogenic role of GLI proteins described in several malignancies, we initially screened for the expression of GLI family members and found that WM cell lines MWCL-1 and RPCI-WM1 and the IgM-secreting cell line BCWM.1 express GLI1, GLI2, and GLI3 (Fig. 1). To determine whether GLI played a role in WM biology, we used the GLI pharmacological inhibitor Gant61 and found a significant reduction in IgM secretion in response to Gant61 treatment in BCWM.1 (p < 0.0001), MWCL-1 (p < 0.0001), and RPCI-WM1 (p < 0.0001) cells (Fig. 2A). As previously described (23), Gant61 treatment reduced the expression of GLI1 and GLI2 (Fig. 2B) in our model cells. Investigation of the effect of GLI inhibition on cell growth and survival indicated that GLI inhibition had a modest effect on the proliferation of BCWM.1 (20% reduction) (p = 0.0032) and RPCI-WM1 (15% reduction) (p = 0.0381) cells whereas it had no effect on MWCL-1 cells (Fig. 2C). GLI inhibition also resulted in a modest (14%) reduction in cell viability in BCWM.1 cells (p < 0.0001) and a 15% reduction in MWCL-1 cells (p < 0.01) (Fig. 2D). This modest reduction in growth and survival is likely not responsible for the significant reduction in IgM secretion observed in cells treated with Gant61 as pretreatment of cells with the pan-caspase inhibitor Q-VD-OPh had no effect on IgM secretion by cells treated with Gant61 (Fig. 2E), although it did restore cell viability lost by GLI inhibition in MWCL-1 cells (p < 0.0001) (Fig. 2F). Taken together, these results suggest that GLI inhibition results in a reduction in IgM secretion that is independent of cell viability.

**HH-independent regulation of IgM**

GLI transcription factors were initially described as the effectors of the HH signaling pathway (24, 25). Therefore, we screened for the expression of the HH receptors patched (PTCH)1 and smoothened (SMO). Using PCR, we found that WM cells express PTCH and SMO (Fig. 3A), suggesting that HH signaling may play a role in GLI-mediated regulation of IgM secretion. Using the pharmacological inhibitor of HH signaling cyclopamine, we found that WM cells treated with Gant61 as pretreatment of cells with the pan-caspase inhibitor Q-VD-OPh had no effect on IgM secretion by cells treated with Gant61 (Fig. 2E), although it did restore cell viability lost by GLI inhibition in MWCL-1 cells (p < 0.0001) (Fig. 2F). Taken together, these results suggest that GLI inhibition results in a reduction in IgM secretion that is independent of cell viability.
pathway, was used to confirm the effect of cyclopamine in our cell line models. As expected, the inhibitor lowered the levels of GLI1, demonstrating the efficacy of the treatment (Fig. 3B). Interestingly, cyclopamine treatment had no effect on GLI2 or GLI3 expression in these cells (Supplemental Fig. 1A). Further investigation indicated that BCWM.1 cells treated with cyclopamine had a reduction in cell growth ($p = 0.0055$) (Fig. 3C) and cell viability ($p = 0.0007$) (Fig. 3D), with no change in MWCL-1 or RPCI-WM1 cells. Taken together, these results suggest that signaling mediated through GLI but not SMO is likely responsible for the regulation of IgM secretion.

**IL-6Ra is a direct target of GLI2**

To characterize the mechanism by which GLI inhibition reduces IgM secretion, we hypothesized that GLI transcription factors may modulate expression of cytokines or cytokine receptor genes known to modulate IgM secretion (26–33). We therefore screened for the expression of receptors for IL-2, IL-6, IL-21, and B lymphocyte stimulator (BAFF). We found that GLI inhibition using Gant61 reduced IL-6Ra mRNA expression, but it had no effect on other cytokine receptors investigated in MWCL-1 cells. We confirmed these findings using qPCR in RPCI-WM1 cells, where we found that GLI inhibition significantly reduced IL-6Ra expression ($p < 0.0001$) (Fig. 4B). Because Gant61 reduces GLI1 and GLI2 mRNA expression (13, 23, 34) (Fig. 2B), we next targeted GLI1 and GLI2 using RNAi to determine the involvement of each in regulating IL-6Ra. We found that GLI2 knockdown but not GLI1 reduced IL-6Ra mRNA (Fig. 4C) and protein expression (Fig. 4D). Consistent with expression data, GLI2 knockdown reduced the surface expression of IL-6Ra (Fig. 4E). Finally, to confirm the specificity of the RNAi targeting, we used two different shRNA sequences targeting GLI2 and found a reduction in IL-6Ra expression and IgM secretion (Fig. 4F, Supplemental Fig. 1B). A control showing a reduction in GLI2 protein expression was also performed in all three cell lines (Supplemental Fig. 1C). Taken together, these experiments show

**FIGURE 4.** GLI2 inhibition affects expression of IL-6Ra. (A) mRNA expression of cytokine receptors in MWCL-1 cells treated with Gant61 determined by semiquantitative RT-PCR. (B) Relative expression of IL-6Ra in cells treated with Gant61 (20 μM) by qPCR ($p = 0.0007$ for MWCL-1 and $p < 0.0001$ for RPCI-WM1 cells). (C) Relative mRNA expression of IL-6Ra, GLI1, or GLI2 in MWCL-1 and RPCI-WM1 cells transfected with shGLI1 or shGLI2 by qPCR. GAPDH was used as a housekeeping gene. (D) IL-6Ra protein expression in cells transfected with shGLI2 determined by immunoblot. β-Actin was used as a loading control. Densitometry on analyzed immunoblot images from three pooled independent experiments shows a reduction in IL-6Ra protein expression. (E) Cells ($4 \times 10^5$) were transfected with either scramble shRNA (shScr) or GLI2 shRNA (shGLI2) for 2 d followed by staining for surface IL-6Ra expression by FACS. (F) qPCR for GLI2 expression, IL-6Ra expression, and relative IgM secretion in MWCL-1 cells transfected with two different shRNAs targeting GLI2 (shGLI2(1), shGLI2(2)). Bars represent means ± SEM. Each experiment was repeated at least three times. *$p < 0.05$, **$p < 0.0001$. 

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that GLI inhibition reduces IL-6Rα expression and that GLI2, but not GLI1, reduces IL-6Rα expression and ultimately IgM secretion.

Using bioinformatics analysis, we found several candidate GLI binding sites on the IL-6Rα promoter region suggesting that GLI2 may modulate IL-6Rα expression by modulating its promoter (Fig. 5A). We therefore cloned the IL-6Rα promoter into a luciferase reporter construct and used that to determine the ability of GLI2 to modulate this regulatory sequence. We initially tested this reporter construct in our cell lines compared with empty vector (Supplemental Fig. 2). Consistent with our expression data, Ganet61 treatment significantly reduces IL-6Rα promoter activity in BCWM.1 (p = 0.0027), MWCL-1 (p = 0.0017), and RPCI-WM1 (p = 0.0004) cells (Fig. 5B). Furthermore, GLI2 knockdown significantly decreased IL-6Rα promoter activity (p < 0.0001 for each cell line) (Fig. 5C). Using a ChIP assay followed by quantitative PCR (ChIP-PCR), we found direct binding to the IL-6Rα promoter in the distal region in BCWM.1, MWCL-1, and RPCI-WM1 cells (Fig. 5D). Next, we performed a ChIP assay on MWCL-1 cells treated with either Ganet61 or vehicle control and found a significant reduction in GLI2 binding to the IL-6Rα promoter in cells treated with the GLI inhibitor (p = 0.0174) (Fig. 5E). Similarly, in cells transfected with shGLI2, there was a significant reduction in GLI2 binding to the IL-6Rα promoter compared with control cells transfected with the control scramble shRNA (p < 0.0001) (Fig. 5F).

FIGURE 5. GLI2 affects IL-6Rα promoter activity and directly binds to the IL-6Rα promoter. (A) Schematic representing the IL-6Rα promoter with candidate GLI (G) binding sites as determined through bioinformatics analysis. GLI2 box represents the region where GLI2 binds IL-6Rα and the transcription start site (TSS). (B) Relative luciferase activity in cells transfected with IL-6Rα promoter–luciferase reporter and treated with either 20 μM Ganet61 (+) or DMSO control (−). Cells were harvested after 48 h, and changes in luciferase activity were assayed as described in Materials and Methods. (C) Relative luciferase activity in cells transfected with IL-6Rα promoter–luciferase reporter and either scramble shRNA control (Scr) or one of two different shRNA targeting GLI2, shGLI2(1) and shGLI2(2). Cells were lysed 48 h after transfection and used to determine changes in luciferase activity. (D) A ChIP assay was performed on cell lysates using Abs specific for GLI2 or IgG control. qPCR was performed and binding was determined in the IL-6Rα promoter region (−1065/−1294) containing the three candidate GLI binding sites indicated in the schematic above (A). (E) A ChIP assay was performed on MWCL-1 cell lysates from cells treated with either 20 μM Ganet61 or DMSO for 2 d. qPCR was performed to determine the effect of treatment on GLI2 binding to the IL-6Rα promoter. (F) A ChIP assay was performed on cell lysates from MWCL-1 cells transfected with either scramble shRNA (shScr) or GLI2 shRNA (shGLI2) for 2 d. qPCR was performed to determine the effect of treatment on GLI2 binding to the IL-6Rα promoter. Each experiment was repeated at least 3 times. **p < 0.0001.
Finally, to confirm the biological role of GLI2–IL-6Rα axis, we overexpressed IL-6Rα in cells transfected with shRNA targeting GLI2 and determined the effect on IgM secretion by ELISA. We found that overexpression of IL-6Rα can rescue the reduction in IgM secretion that is observed with GLI2 knockdown (p < 0.0001) (Fig. 6A). These data suggest that GLI2 modulates IgM secretion by regulating an important cytokine receptor, the IL-6Rα.

**GLI2/IL-6Rα/IgM axis is active in natural IgM-secreting cells**

Our data so far were from B cell lines derived from patients with WM. To confirm the role of GLI2 in modulating IL-6Rα and IgM secretion in primary cells, we used mouse B-1 cells, which secrete natural IgM. Because these cells exist in high numbers in the peritoneal cavity (35, 36), we treated total peritoneal cells with or without Gant61. After 2 d of culture, cells were harvested and stained with B220 and CD23 to distinguish B-1/B-2 cells (22). We found that B-1 cells treated with Gant61 had reduced surface expression of IL-6Rα (Fig. 7A). We also found a significant reduction in IgM secretion in culture supernatant harvested from cells treated with Gant61 (p < 0.0001) (Fig. 7B). Next, we purified B-1a cells and used them to determine the effect of Gant61 on IgM secretion. Similar to the results obtained with total peritoneal cells, treatment of B-1a cells with Gant61 results in a significant reduction in natural IgM in culture supernatants by ELISA (p < 0.0001) (Fig. 7C). We also confirmed that GLI2, GLI1, and GLI3 results in a positive feed-forward loop. Together, these data confirm that the GLI2 regulatory mechanism controlling IL-6Rα and ultimately IgM secretion is conserved beyond WM cells and may have important physiological functions under non–disease conditions.

**Discussion**

Our findings elucidate a novel role for GLI2 transcription factor in regulating IgM secretion by WM B cells. Several studies have investigated the role of GLI proteins in cancer, particularly focusing on their role as mediators of HH signaling (37–42). Until recently, GLI proteins were only described as mediators of HH signaling (reviewed in Ref. 10). In the present study, we show that HH signaling has very minimal effect on GLI-mediated IgM secretion (Fig. 3). Furthermore, we show that the IL-6Rα (gp80) subunit is a novel transcriptional target of GLI2 (Figs. 4, 5). The finding that GLI can target cytokine or cytokine receptor genes has been shown recently by other studies. In CD4+ T cells, GLI2 has been shown to activate the TGF-β1 gene promoter (43). Our group has shown that GLI2 can directly target the IL-6 promoter in bone marrow stromal cells and lead to increased IL-6 expression and secretion (9). However, to our knowledge, a direct role of GLI in WM and its role in regulating IgM secretion had not been addressed until this study.

Our data identify the IL-6Rα as a novel transcriptional target of GLI2 in IgM-secreting malignant B cells (Figs. 4–6) and primary B cells secreting natural IgM (Fig. 7). This suggests that this signaling axis is active in both normal and malignant B cells. This novel mechanism of regulation of IL-6Rα is important, as very few studies have addressed the transcriptional regulation of this cytokine receptor, although some studies have examined the regulation of IL-6Rα by a variety of receptor/ligand interactions. Early studies have shown that steroid hormones can regulate IL-6Rα (44–46). Other studies have suggested that IL-6 can regulate IL-6Rα expression in a positive feed-forward loop; however, conflicting data exist in support of this finding and the regulation of IL-6Rα by IL-6 appears to be cell type specific (47, 48). We investigated this mechanism of regulation of IL-6Rα by IL-6 in our WM B cells and found that IL-6 induces IL-6Rα expression (Supplemental Fig. 3A). We also activated IL-6 signaling using a gp130 expression construct and found an increase in IL-6Rα promoter activity (Supplemental Fig. 3B). This suggests that IL-6 can modulate IL-6Rα in our model. Other molecules such as IL-1β have been shown to increase the expression of IL-6Rα in hepatocytes and bronchial epithelial cells (47, 49). Additionally, IL-2 has been shown to negatively regulate IL-6Rα mRNA expression (50). This suggests that several STATs may be involved in the transcriptional regulation of IL-6Rα. Indeed, in CD8+ T cells, STAT5 appears to negatively regulate IL-6Rα expression (51). Furthermore, the transcription factor E2F3 has been shown to directly target IL-6Rα (52). In WM, elevated IL-6 levels (53–55) and soluble IL-6Rα (55) have been reported. Although IL-6Rα levels in the serum did not correlate with disease severity, the limited number of patient samples examined may have contributed to this finding. Regardless, the finding that soluble IL-6Rα levels are elevated warrants further studies into the mechanisms that regulate the expression of this cytokine receptor. We investigated the potential role of these factors on IL-6Rα expression in our WM B cells and found none to induce increases in IL-6Rα expression or promoter activity (Supplemental Fig. 3C, 3D). Therefore, further exploration of the mechanism by which IL-6 regulates IL-6Rα expression in our model will be important to further elucidate the different signaling events involved in the regulation of IgM in disease and normal conditions. Despite this, our studies identify a novel mechanism of regulation of IL-6Rα by the transcription factor GLI2. We demonstrate that GLI2 modulates IgM secretion by regulating the expression of IL-6Rα. Additionally, we show that this mechanism occurs independent of HH signaling (Fig. 3). Finally, we show that IL-6Rα is able to rescue the reduction in IgM secretion that is observed in response to GLI2 knockdown (Fig. 6A). Therefore, this work expands our knowledge of the mechanisms regulating IL-6Rα and confirms the biological role of this receptor in modulating IgM secretion.

**FIGURE 6.** IL-6Rα expression rescues GLI2-mediated reduction in IgM secretion. Relative IgM secretion in cells transfected with GLI2 shRNA (shGLI2) or scramble (Scr) and either empty vector (Ctrl) or IL-6Rα expression construct (shGLI2 plus IL-6Rα). Supernatants were harvested 48 h after transfection and relative IgM secretion was determined by ELISA. Bars represent means ± SEM of pooled data from three independent experiments. Each experiment was repeated at least three times. **p < 0.0001.
We have previously shown that elevated levels of CCL5 in the tumor microenvironment can regulate GLI2/IL-6/IgM (9). To enable targeting GLI from a therapeutic perspective, an investigation of the role of GLI in malignant cells was needed. In this study, we show that GLI1, GLI2, and GLI3 are expressed in IgM-secreting cell lines from WM patients (Fig. 1). This suggested that GLI proteins may play a role in WM biology. Indeed, inhibition of GLI using Gant61 resulted in a reduction in IgM secretion while having no effect or minimal effect on cell growth or survival (Fig. 2). Although ideally, targeting malignant cell growth and survival is optimal in cancer therapy, WM is an indolent lymphoma with many patients only requiring treatment initiation when they become symptomatic (8, 56–59). Because elevated IgM is associated with disease symptoms in WM, targeting IgM secretion or the signaling pathways leading to increased IgM secretion may provide therapeutic benefit to patients by alleviating disease-associated symptoms.

In summary, our studies demonstrate a novel mechanism of modulation of IgM secretion by GLI2 via transcriptional regulation of IL-6Rα. These studies provide insight into the molecular mechanisms regulating IL-6Rα and IgM secretion. Therefore, further investigation of the utility of the GLI2/IL-6Rα has great potential in increasing our understanding of the mechanisms that regulate IgM secretion in malignant B cells.

Disclosures
The authors have no financial conflicts of interest.

References