Molecular analyses reveal inflammatory mediators in the solid component and cyst fluid of human adamantinomatous craniopharyngioma

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Abstract

Pediatric adamantinomatous craniopharyngioma (ACP) is a highly solid and cystic tumor, often causing substantial damage to critical neuroendocrine structures such as the hypothalamus, pituitary gland, and optic apparatus. Paracrine signaling mechanisms driving tumor behavior have been hypothesized, with IL-6R overexpression identified as a potential therapeutic target. To identify potential novel therapies, we characterized inflammatory and immunomodulatory factors in ACP cyst fluid and solid tumor components. Cytometric bead analysis revealed a highly pro-inflammatory cytokine pattern in fluid from ACP compared to fluids from another cystic pediatric brain tumor, pilocytic astrocytoma (PA). Cytokines and chemokines with particularly elevated concentrations in ACPs were IL-6, CXCL1 (GRO), CXCL8 (IL-8) and the immunosuppressive cytokine IL-10. These data were concordant with solid tumor compartment transcriptomic data from a larger cohort of ACPs, other pediatric brain tumors and normal brain. The majority of receptors for these cytokines and chemokines were also over-expressed in ACPs. In addition to IL-10, the established immunosuppressive factor IDO-1 was overexpressed by ACPs at the mRNA and protein levels. These data indicate that ACP cyst fluids and solid tumor components are characterized by an inflammatory cytokine and chemokine expression pattern. Further study regarding selective cytokine blockade may inform novel therapeutic interventions.

Keywords: Adamantinomatous Craniopharyngioma; Craniopharyngioma Cyst; Cytokine; Inflammatory; Immunomodulation; IL-6
Introduction:

Adamantinomatous Craniopharyngioma (ACPs) is an uncommon brain neoplasm accounting for approximately 4% of pediatric brain tumors(1). It is associated with significant neurological and endocrine morbidity despite being a World Health Organization (WHO) grade I tumor. The most severe damage occurs in patients who suffer hypothalamic injury(2), which may occur as a result of finger-like tumor infiltration(3) or therapeutic efforts to surgically remove the tumor(4). ACP is among a small number of pediatric brain tumors characterized by the combination of cystic and solid tumor components and the other most common such entity is Pilocytic Astrocytoma (PA). ACP cysts often exert substantial mass effect on critical structures, including the hypothalamus, optic pathway, Circle of Willis vessels, and the third ventricle. This phenomenon, combined with the slow growth of solid components of ACP, has prompted efforts to control this tumor using cyst-directed therapies(5-7). Most cyst-directed therapies require direct surgical access, and may be associated with significant complications(5,7).

Systemically administered anti-tumor therapies for ACP could control cyst expansion without the risk associated with operative intra-cystic catheter placement and subsequent care, or the need for repeated transcutaneous device access to deliver therapy. In addition, reduction of cyst volume might decrease the risk of hypothalamic injury associated with radical resection of ACP(8), by facilitating separation of the cyst wall from the hypothalamus.

Emerging evidence suggests that ACP pathology may involve inflammatory mechanisms. In 2004, Mori and colleagues described elevated IL-6 expression in ACP(9). Subsequent analyses of ACP cyst fluid demonstrated inflammatory activity(6,10). In a recent study of pediatric ACP,
our laboratory described elevated levels of mRNA transcripts associated with \textit{IL-6R} in ACP tumor tissue, when compared to other pediatric brain tumors and normal tissue\cite{11}. In the clinical setting, a review of intra-cystic therapies in childhood ACP concluded that interferon (IFN) has the highest benefit to risk ratio, versus Bleomycin or radioisotopes\cite{12}. Yueng and colleagues\cite{13} described 5 children with recurrent ACP managed using subcutaneously administered pegylated IFN-\textit{α}-2b. Further support for the assertion that ACP growth uses paracrine mediators comes from genetic tracing experiments using a novel mouse model\cite{14,15}. This work additionally demonstrated 2-fold enrichment of \textit{Cxcl1}, 30-fold enrichment of \textit{Cxcl2}, and 29-fold enrichment of \textit{Cxcl3} gene expression in clusters of β-catenin mutated cells (the only known genetic mutation in ACP\cite{16}), relative to non-cluster regions\cite{15}. To date, however, there has been no detailed description of immunomodulatory pathways in ACP.

The clinical relevance of investigating inflammatory drivers in ACP is bolstered by the successful utilization of directed therapies against inflammatory mediators in other tumor types and other diseases in both adults and children, notably systemic juvenile idiopathic arthritis and Castleman’s disease\cite{17,18}. Monoclonal antibodies directed against human IL-6R (tocilizumab) and IL-6 (siltuximab) both have regulatory approval in the United States and Europe. Small molecule antagonists of other pro-inflammatory cytokines, such as CXCL1 and CXCL8 (IL-8) have been suggested to have efficacy in preclinical models of human cancers\cite{19}, and are generally well tolerated in humans\cite{20}.

We sought to determine whether comprehensive analysis of cytokine and chemokine levels in pediatric ACP cyst fluids and solid tumor tissue would implicate inflammatory mechanisms of
tumor pathogenesis, and whether these mechanisms may be susceptible to available directed therapies.
Materials and Methods

Cytometric Bead Analysis

Cyst fluids from 5 histologically confirmed pediatric ACPs and 5 pediatric PAs were analyzed using multiplexed cytometric bead analysis (Milliplex Map kit; Millipore, Temecula, CA) to measure the concentration of 24 cytokines and 11 chomokines (listed in Supplementary Table 1) per manufacturer’s instructions. The specimens were obtained with IRB approval (COMIRB 14-0426 and 95-500). ACP samples were obtained through the Advancing Treatment for Pediatric Craniopharyngioma Consortium and PAs were from the clinical service at Children’s Hospital Colorado. ACPs were compared to PAs as another histologically benign pediatric brain tumor that is characterized by cyst formation. For comparative studies, measured analyte concentrations were transformed from raw values to \( \log_2 \) to better express biological relationships.

Transcriptomic Analysis

Transcriptome assays were completed using 23 ACP samples, processed as previously described(11). Three of these specimens were also analyzed in the above cyst fluid analysis. Samples were collected at surgery and snap frozen. The proportion of tumor tissue and of brain tissue with glial reaction was estimated histologically. The reference panel included 148 samples of primary tumors and non-neoplastic (“normal”) cerebral tissues. This included 10 atypical teratoid/rhabdoid tumors (AT/RT), 29 ependymomas (EPN), 10 glioblastomas (GBM), 15 medulloblastomas (MED), 10 pilocytic astrocytomas (PA), 11 embryonal tumors (EmT), 23 specimens of non-neoplastic pituitary and 27 specimens of non-neoplastic cerebrum. Tumors were classified according to the WHO international histological tumor classification. Non-neoplastic brain samples were obtained during epilepsy surgery or autopsy at Children’s Hospital Colorado.
Colorado.

Tumor samples were analyzed using the Human Genome U133plus2 Array (Affymetrix) platform as previously described (11, 21). Data analysis was performed in R (http://www.r-project.org), using publicly available packages (http://www.bioconductor.org). Microarray .CEL datafiles were background corrected and normalized using the guanine cytosine robust multi-array average (gcRMA) algorithm resulting in log2 expression values (22). To reduce error associated with multiple testing, a filtered list was created containing the highest expressed probe across all samples for each gene with multiple probesets. This list was further filtered to remove probe sets that were expressed below a threshold level denoting absence of expression in any sample. These data are available in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database (23) and are publicly accessible through GEO Series accession number GSE94349 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE94349).

Transcripts corresponding to cytokine and chemokine analytes included on the 35-plex cytometric bead assay, cognate receptors of key cytokines and chemokines and established soluble immunosuppressive factors were identified and extracted from the microarray dataset for further analysis (Supplementary Tables 1-3). Genes with significant differential expression were defined as those in ACP compared to other tissues by pair-wise comparisons (p < 0.05).

**Immunohistochemistry**

Immunohistochemistry assessing the presence of IL-6Rα was performed on archival paraffin embedded sections of human ACPs and normal non-cerebral tissues from the Childhood Cancer and Leukaemia Group and Department of Histopathology at Great Ormond Street Hospital for Children using mouse monoclonal H7 anti-IL-6Rα antibody (Santa Cruz Biotechnologies) at a
dilution of 1:300 on the Leica Bond-Max automated immunostainer.

Immunohistochemistry assessing the presence of IDO-1 was completed in specimens of ACP and normal brain tissue using the 4.16H1 anti-IDO-1 antibody, as previously described (gift from Dr. Benoit Van den Eynde, Ludwig Cancer Inst. for Cancer Research, Brussels, Belgium). The antibody was diluted at 1:1000 after DAKO pH9 antigen retrieval(24).

**Immunofluorescence**

Immunostaining for pSTAT3 and in situ hybridisation for CXCR2 were performed on archival paraffin embedded sections of human ACPs from the Department of Histopathology at Great Ormond Street Hospital for Children. Primary antibodies (Rabbit anti-pSTAT3 at 1:200 and Mouse anti-β-catenin at 1:300) were incubated together overnight at 4°C. The next day after washing the primaries with PBS (0.1% Triton-X), we incubated the slides with Goat anti-rabbit biotinylated antibody at 1:250 dilution for 1 hour at room temperature. After washing, the slides were incubated 1 hour at room temperature with a Streptavidin-AlexaFluor 555 conjugate (1:500 dilution) as well as Goat anti-mouse AlexaFluor 488 (1:250 dilution). pSTAT3 was thereby labelled with S555, and β-catenin with AlexaFluor 488. Sections were controlled for autofluorescence using Sudan Black. They were counterstained with DAPI and mounted.

**In Situ Hybridization**

In situ hybridization on paraffin sections was performed as previously described(25). The CXCR2 probe was generated by amplification of a 633bp fragment from genomic DNA, using forward and reverse primers: 5’- GCCTCGAGATGTGGCATCCTGCTACTG -3’ and 5’-GCGAATTCTGATCAAGCCATGTACTG-3’, respectively. PCR product was cloned into
the pBlueScript SK vector (XhoI 5′, EcoRI 3′) and antisense riboprobes synthesized with T3 RNA Polymerase.

**Statistical analyses**

Statistical analyses were performed using R bioinformatics, Prism (GraphPad), and Excel (Microsoft) software. For all tests, statistical significance was defined as $P < 0.05$. 
Results

ACP cyst fluid is characterized by high levels of cytokines and chemokines

Cyst formation is associated with multiple pediatric brain tumors, most commonly ACPs and PAs, but also occasionally EPNs, Gangliogliomas and high-grade neoplasms, among others. In the present study, cytokine and chemokine levels in ACP cysts were only compared to PA cyst levels due to the number of samples available.

Of the 35 analytes measured by the multiplex assay, 16 chemo-/cytokines were detected at significantly (p<0.05) higher levels in fluid from ACPs (n=5) versus PAs (n=5). Six cytokines demonstrated differential levels at a significance level of p<0.001, and 4 at a significance level of <0.0001 (figure 1). These included IL-6, IL-10, CXCL8 (IL-8), and CXCL1 (GRO). The full list of analyte levels in ACPs compared to PAs is in Supplementary Table 1.

IL-6 levels showed the greatest statistical difference between the 2 tumor types (ACP/PA ratio 7.64, \( p=5.97 \times 10^{-8} \)). The mean concentration in ACP cyst fluids was 8.9 ng/ml. CXCL1 (GRO) demonstrated the greatest absolute concentration difference (ACP/PA ratio 1311.3, \( p=0.000285 \)) with a mean concentration in ACPs of 8.6 ng/ml. CXCL8 (IL-8), which shares receptor CXCR2 with CXCL1, also demonstrated significantly higher levels in ACPs (ACP/PA ratio 4.18, \( p=9.44 \times 10^{-5} \)) with a mean concentration of 6.1 ng/ml. IL-10 levels were higher in ACPs compared to PAs (ACP/PA ratio 15.0, \( p=6.79 \times 10^{-5} \)) at a concentration of 221 pg/ml in ACPs. Each of these cytokines: IL-6, IL-10, CXCL1, and CXCL8 and/or their receptors, has 1 or more clinically available medications that target their function (Table 1).
Transcript expression of several cytokines and chemokines is elevated in the solid tumor component of ACPs.

Given the potential for rapid clinical translation, we further investigated these molecules by examination of gene expression levels in the solid tumor component of ACPs. We extracted cytokine and chemokine data from a transcriptomic microarray dataset containing a large cohort of surgical specimens of ACP, PA and a variety of other pediatric brain tumors as well as normal brain tissue(11). This furthered our study of these molecules in ACPs by (i) validating and expanding the findings of our cytometric bead analysis, (ii) exploring the relationship between the presence of key cytokines and chemokines in ACP cyst fluid and corresponding solid ACP tumor components, and (iii) examining expression patterns of associated cytokine and chemokine receptors and key soluble immunosuppressive factors.

**Pro-inflammatory Mediators**

**IL-6 and IL-6 receptors**

IL-6 transcript levels were, as anticipated from cytometric bead analysis, higher in solid tumor components of ACPs than PAs (ratio=3.25; p=0.018). Furthermore, transcript expression was higher in ACP tumors than any other brain tumor or normal brain tissues (figure 2a). This difference was statistically significant (p<0.05) when compared to AT/RTs, MEDs, EmTs, and normal brain samples, but not when compared to EPNs, GBMs, GGs or normal pituitaries. The IL-6 receptor is a heterodimeric complex of two proteins, IL-6R and IL-6ST (gp130). Transcript levels of these components in ACP solid tumors were examined. *IL-6R* transcript expression was statistically significantly higher in ACPs relative to any other tumor/tissue type.
(figure 2b) except GGs. *IL-6ST* was present at similar levels in all tumor/tissue types, including ACPs (figure 2c). Details of cytokine and chemokine receptor transcript probesets are in Supplementary Table 2.

We further examined the pattern of IL6-Rα expression at the protein level in ACP tumor sections by immunohistochemistry. Using a monoclonal antibody for IL6-Rα we identified immunoreactivity that was predominantly limited to the stellate reticulum and occasional reactive cells, and was not present in the palisading epithelium (figure 2d-f). As expected, there was light immunoreactivity in control tissues including human kidney, palatine tonsil and placenta (data not shown).

IL-6 drives phosphorylation and activation of STAT3. Persistent signaling through this pathway in cancer can result in a chronic inflammatory phenotype and suppression of antitumor immunity(26). To determine the activation status of STAT3 in ACPs, we performed immunofluorescence for phospho-STAT3 in ACP tumor sections. Phospho-STAT3 expression, indicating pathway activation, was similarly observed in tumor cells and some surrounding areas of reactive tissue (figure 2g-i) consistent with the IL6-Rα immunostaining.

**CXCL1, IL-8 and CXCR2**

*CXCL1* (*GRO-α*) transcript levels were significantly higher in ACP solid tumors than in PAs (ratio=19.7; p=3.61x10⁻⁹), in concordance with its overexpression in ACP cyst fluids (figure 3a). *CXCL1* was significantly higher in ACP tumors than any other brain tumor or normal brain tissue type. Similar to *CXCL1*, levels of *IL-8 (CXCL8)* transcript were higher in ACPs than in
PAs, consistent with overexpression of IL-8 protein in cyst fluid. IL-8 transcript levels were higher in ACP than other tissue types examined, apart from EPNs and GBMs (figure 3b). CXCL1 and IL-8 both bind the chemokine receptor CXCR2. Transcript levels of CXCR2 were higher in ACP tumors than in any other tissue type, except PAs, however this difference only reached statistical significance when ACPs were compared to AT/RTs, MEDs and normal tissues (figure 3c).

To investigate the distribution of CXCR2 within the ACP tumor microenvironment, we performed in-situ hybridization (ISH) for CXCR2 in ACP tissue sections. There was strong hybridization to CXCR2 mRNA in the epithelial compartments of the tumor and less in reactive glial areas (figure 3b-d). Collectively, these findings suggest that the effects of pro-inflammatory factors CXCL1 and IL-8 are predominantly focused on the epithelial component of ACPs.

Immunosuppressive Factors

**IL-10, IL10RA, IL10RB**

IL-10 was expressed at relatively high levels in ACP cyst fluids. This cytokine is significant due to its well-established immunosuppressive role in the brain and in systemic tumor models(27,28). *IL-10* transcript expression in ACP solid tumors was marginally higher than in any other tumor type, but this difference reached statistical significance only when ACPs were compared to EmTs (figure 3b). This is likely due to transcript levels for *IL-10*, as is the case for many other interleukin transcripts, being detected at very low levels. The uniformly low levels seen in all tumor samples in this dataset make comparisons with protein levels, as measured in corresponding cyst fluid components of the same tumor, less reliable than for more abundant transcripts.
The IL-10 receptor is a heterodimer of proteins IL-10RA and IL-10RB. Levels of *IL-10RA* and *IL-10RB* transcript were both higher in ACP solid tumors than in any other brain tumor or normal tissue examined, with this difference reaching statistical significance for all comparisons except ACPs relative to PAs and GGs regarding levels of *IL-10RA* (figure 3b).

**IDO-1**

Given that IL-10 is a well-established soluble immunosuppressive factor in brain and other tumor types, we examined the transcript expression of a panel of other established soluble immunosuppressive factors in ACP solid tumors(29) (see Supplementary Table 3). This analysis identified significantly elevated levels of *IDO-1* mRNA in ACPs relative to any other tissue type (figure 4a, fold change 9.43 versus all other tumor types combined, \( p=1.4\times10^{-28} \)). As IDO-1 is a therapeutic target undergoing early phase clinical trials, we examined its expression at the protein level in ACP tumor sections by immunohistochemistry. Using the monoclonal antibody 4.16H1(24) we identified immunopositivity of superficial epithelial cells in specimens of ACP (figure 4c-f) while there is no immunoreactivity in specimens of normal frontal lobe (figure 4b). Of the other candidate soluble immunosuppressive factors, only *galectin-1* transcript levels were significantly elevated in ACP tumors relative to other tissues (fold change 3.27, \( p=2.12\times10^{-7} \)).
**Discussion**

Children afflicted with ACPs have not yet benefited from biologically guided antitumor therapies. As a histologically benign and heterogeneous tumor, ACPs present challenges for biological investigations, especially given the paucity of experimental models in particular the lack of any established ACP cell lines. The cystic component of ACPs can present unique clinical challenges, such as rapid enlargement and unpredictable response to radiation therapy. This work presents novel insights regarding the biological characteristics of ACP cyst fluid and tumor tissue, indicating that immune system modulators may play a critical role in tumor behavior. As with systemic inflammatory processes, it is possible that high levels of chemokines drive fluid secretion in a process that allows for greater infiltration of immune cells. Therapies that diminish or reverse this process may offer the opportunity to control the local effect of ACP without, or in conjunction with, surgical and/or radiation therapy, but further preclinical investigation is needed.

This work is also the first to describe immunosuppressive factors, such as IL-10 and IDO-1, in ACPs. Our findings build on previous work in human and mouse models (11,15) indicating that pro-inflammatory mediators such as IL-6, CXCL1 and CXCL8 are present at unique levels in ACPs, both at protein and transcript levels. The consistency of our findings across different compartments of ACP tissue (cyst fluid and tumor), and within human and animal contexts, strengthens the findings.

The only confirmed genetic alteration in ACP is a mutation lies within exon 3 of the CNTTB1 gene, resulting in accumulation of β-Catenin. While pro-inflammatory mediators have been
identified in clusters of β-catenin mutated cells in mouse ACPs(15), the cellular origin of these mediators in human tumors remains unconfirmed. Mechanistic links between β-catenin mutations and inflammatory or immunomodulatory mediators in human ACP have also yet to be defined, and should be the focus of future studies. A number of studies in other tumors, including colon cancer, which is also associated with β-catenin mutation, have identified stimulation of the key inflammatory mediator NF-κB in response to upregulated Wnt/β-catenin activation(30).

Oncogenic β-catenin has also been shown to have a significant inflammatory impact on the crosstalk of tumor cells with tumor associated immune cells in colon cancer(31). Such crosstalk, mediated by cytokines and chemokines, may occur between tumor cells, associated immune cells and reactive glia in ACP.

**IL-6**

Concerning rapid translation into clinical application, our findings regarding IL-6 may be the most immediately relevant. The above referenced animal models of ACP(14,32,33) combined with the clinical success of therapeutic monoclonal antibodies against IL-6R and IL-6, such as Tocilizumab and Siltuximab in other clinical contexts (17,18) offers the opportunity to explore IL-6 activity in preclinical models. Evidence of pituitary origin of ACPs(15) and a preliminary report using systemically administered IFN-α-2b(13) support the notion that ACPs do not dwell within an immunoprivileged space, which may allow these large molecules to affect ACP tissues following systemic administration.

We identified uniquely elevated levels of IL-6 protein in ACP cyst fluids; and of IL-6R and, to a lesser extent, IL-6 transcripts in tumor tissue. Although levels of IL-6ST (gp130) transcripts were
not uniquely elevated in ACP, the presence of pSTAT3 is consistent with IL-6 active signaling. These findings taken together are consistent with the assertion that IL-6 plays a critical role in the pathogenesis of ACPs. As a ubiquitous cytokine that demonstrates context-dependent inflammatory activity(34,35), IL-6 has myriad effects in oncologic systems, including brain tumors such as EPNs(36) and GBMs(37), both of which demonstrated high levels of IL-6 transcripts in our analysis. While this work suggests that IL-6 participates in ACP pathogenesis, its precise role and intratumoral cell of origin remain unknown. The observation that IL-6R expression and STAT3 phosphorylation are observed in the stellate reticulum and, to a much lesser extent, in the adjacent reactive glial regions, suggest that the effects of IL-6 may be widespread throughout the tumor microenvironment. In the context of posterior fossa EPN, subgroup A, IL-6 induces the expression of CXCL8 (IL-8) from cells of myeloid lineage through STAT3, in a process that can be reversed using the IL-6R antibody, tocilizumab(36). Interestingly, herein we also describe high levels of IL-8 (CXCL8) in ACP cyst fluids and tissues (discussed further below). While IL-6 has been demonstrated to mediate cell proliferative activity through a paracrine mechanism, it also contributes to autocrine mediated oncogene-induced cellular senescence(35).

Other ACP-associated Pro-inflammatory Mediators

CXCL1 (GRO) is a soluble growth factor that, like IL-8 (CXCL8), signals through CXCR2. Each of these chemokines is tightly linked with neutrophil chemoattraction and activation, and has been extensively investigated in the context of inflammatory airway diseases. As with IL-6, CXCL1 is associated with tumor pathogenesis in multiple contexts(19,38,39), but their roles in ACPs remain to be described. Interestingly, IL-8 has been shown to induce tumor invasive
behavior through a mechanism that includes matrix metallopeptidase-9 (MMP-9) in normal tissue and neoplasia models (40,41). Our findings, combined with previous data (11) demonstrate elevations in both IL-8 and MMP-9 in ACPs, potentially implying a mechanism of hypothalamic tumor invasion. The finding that CXCR2 expression is largely restricted to epithelial tumor regions in ACPs suggest that IL-8 and CXCL1 exert a targeted effect in tumor pathogenesis. This may have implications for the efficacy of therapeutically targeting IL-8/CXCL1/CXCR2 versus IL-6 and/or it’s receptors.

The orally active combined small molecule inhibitor of the CXCR1 and CXCR2 receptors, Navarixin (SCH527123/MK7123), inhibited CXCL1 and IL-8 mediated neutrophil migration in humans (20). Preclinical studies have further demonstrated antitumor activity of SCH527123 in models of human cancer (19). Selective CXCR2 antagonists that have demonstrated acceptable risk profiles in humans include Danirixin (42) and AZD5069 (43).

*Immunosuppressive Factors*

This is the first work to demonstrate elevated levels of immunosuppressive factors in ACPs. Specifically, we present evidence of IL-10 and IDO-1 expression at both the protein and gene expression levels. The presence of these molecules may facilitate tumor escape from host immune surveillance, or represent a response to the pro-inflammatory conditions described above. Given the recent clinical successes of therapies that target immunosuppressive factors, such as CTLA-4, in other tumors, identification of immunosuppressive mechanisms in ACPs may allow us to select similarly effective therapies.
IL-10, formerly known as Cytokine Synthesis Inhibitory Factor, is recognized as a pleiotropic cytokine with primarily immunosuppressive effects(29,44), although it has also been demonstrated to play an immunostimulatory role in certain contexts(27,45,46). It is produced by a host of cell types, including monocytes/macrophages, B cells, NK cells, and T cells(47). In experimental systems, IL-10 effectively limits IL-6 and IL-8 production at the transcriptional level, in addition to downregulating its own mRNA production in an autoregulatory manner(48). As with IL-6ST (gp130), the Rβ subunit of the IL-10 receptor serves as a component in a multitude of cytokine receptor complexes(49). However, there is evidence that IL-10Rα expression plays a critical role in the balance of IL-6 and IL-10 driven signaling from T cells(50). Further studies regarding the cells of origin of the cytokines in ACPs may help determine whether a disordered balance of these 2 cascades (IL-6 versus IL-10 mediated) contributes to a pathological inflammatory environment. Although clinical trials of recombinant human IL-10 in autoimmune diseases have demonstrated mixed degrees of therapeutic efficacy, there have not been concerns with regard to medication safety(51).

IDO-1 is a well-described immunosuppressive metabolic enzyme that is believed to play roles in immune tolerance and defense against pathogens. It is commonly expressed by antigen presenting cells in the human gut, thymus, lymph nodes, lung and placenta(24,29). The enzyme works through the oxidation of tryptophan to kynurenine, thereby restraining the proliferation and activation of both innate and adaptive immune actors, including NK cells and T-lymphocytes. It is believed to mediate a locally immunosuppressive milieu(24). IDO-1 may also suppress intratumoral IFN levels. This mechanism may underlie the pilot findings of Yeung and colleagues(13), who described the successful treatment of 5 children with multiply recurrent
ACPs using pegylated IFN-α-2b, which may overcome IDO-1 mediated suppression. Our data indicate that levels of IDO-1 transcripts are uniquely elevated in ACPs, and that the protein is localized to the superficial epithelium of the tumor tissue. Localization of IDO-1 in epithelial cells is consistent with previous findings by both Sedlmayr and Théate, who identified IDO-1 expression in squamous epithelium of the human female reproductive tract (24,52). Presently, orally available direct inhibitors of IDO-1, Epacadostat (INCB24360) and GDC-0919 are in multiple clinical trials for adult malignancies (https://clinicaltrials.gov/ct2/results?term=IDO-1&type=&rslt=&recr=Open&age_v=&gndr=&cond=&intr=&titles=&outc=&spons=&lead=&id =&state1=&cntry1=&state2=&cntry2=&state3=&cntry3=&locn=&rcv_s=&rcv_e=&lup_s=&lup _e=).

The Inflammatory Milieu in Adamantinomatous Craniopharyngiomas

While this work demonstrates that inflammatory processes are highly active in pediatric ACP, the nature and balance of pro-inflammatory and immunomodulatory activity require further exploration before safe and effective anti-tumor regimens can be introduced into human trials. For example, the context-dependent action of cytokines such as IL-6 and IL-10 prevents us from elucidating their roles in ACP at this time. Further insight regarding the cells of origin of the inflammatory actors that we have identified may allow inferences regarding their activity, thereby informing preclinical trials that could include directed therapeutics, such as those described above.

For example, elevated IL-6 expression may represent a mechanism of mitogenesis. Alternatively, IL-6 may be serving to limit tumor stem cell growth. The presence of immunomodulators such as
IDO-1 and IL-10 may facilitate tumor cell escape from a previous immunoediting equilibrium, or reflect inadequate control of tumor-induced inflammation, mediated by IL-6, CXCL1 and IL-8. In addition, the link between IL-8 and MMP-9 may help explain the mechanism through which ACP invades the hypothalamus, eliciting brisk reactive astrocytosis and Rosenthal fiber formation, a characteristic that is responsible for a considerable proportion of ACP-associated morbidity.

Efficient translation of these findings may be facilitated by the current availability of medications that target these pathways in combination with the recent development of mouse models of ACP(14,32).

**Conclusions**

This work provides novel insights regarding the activity of inflammatory mediators in pediatric ACP cyst fluids and corresponding tumor tissue. Through analysis on the transcript and protein levels, we describe elevated levels of IL-6, CXCL1, IL-8, IL-10 and their receptors in ACPs, relative to other pediatric brain tumors and normal cerebral tissue. These findings are also consistent with previous data from an embryonic mouse model of ACP. We additionally describe high levels of the immunomodulatory enzyme IDO-1. These findings, taken within the context of recent advances in ACP animal models and successful directed therapies in humans, provide a robust platform for multiple preclinical studies, with the potential for rapid translation towards novel directed therapies against pediatric ACPs.
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Figure Captions

**Figure 1:** Differential Levels of Cytokines and Chemokines in ACPs relative to PAs based on Cytometric Bead Analysis of Tumor Cyst Fluid. Significant differences in protein levels were identified in 16 of 41 analytes tested. The greatest statistical differences between ACP and PA levels were identified in IL-6, IL-10, IL-8 and CXCL1.

**Figure 2:** (a-c) Gene expression of IL-6 and IL-6 receptor components in ACPs and a panel of 148 other brain tumors and normal brain specimens including Pilocytic Astrocytomas (PAs), Atypical Teratoid/Rhabdoid Tumors (AT/RTs), Ependymomas (EPNs), Glioblastomas (GBMs), Gangliogliomas (GGs), Medulloblastomas (MEDs), and Embryonal Tumors (EmTs). Values are expressed as log2 gene expression. Horizontal red bars represent the mean, and error bars represent standard error of the mean (SEM). (d-f) Immunohistochemical stains of human ACP tissues using anti-IL-6Rα antibody (Santa Cruz). (d, 10x magnification) ACP tissue demonstrates strong staining throughout the stellate reticulum, with sparse staining in the adjacent reactive gliotic region and epithelial regions. (e, 20x magnification) Redemonstration of IL-6Rα staining in the stellate reticulum. (f, 10x magnification) Sparse IL-6Rα staining in the reactive glial tumor region adjacent to the epithelium and stellate reticulum. (g-i) Double immunofluorescence staining revealing the expression of pSTAT3 (red) and β-catenin (green) in both epithelial and surrounding glial tumor components. Labels in panel e include whorl-like epithelial regions (EW); palisading epithelium (PE); reactive/glial supporting tissue (GS). Cell nuclei are counterstained with DAPI (blue in panel f).

**Figure 3:** Gene expression of (a) CXCL1, IL-8 and CXCR2 in ACPs and a panel of 148 other
brain tumors and normal brain specimens including Pilocytic Astrocytomas (PAs), Atypical Teratoid/Rhabdoid Tumors (AT/RTs), Ependymomas (EPNs), Glioblastomas (GBMs), Gangliogliomas (GGs), Medulloblastomas (MEDs), and Embryonal Tumors (EmTs). Values are expressed as log₂ gene expression. Horizontal red bars represent the mean, and error bars represent standard error of the mean (SEM). (b-d) in situ hybridization in histological sections of human ACPs demonstrating the expression of CXCR2 throughout the tumor tissue. Panel b demonstrates a low magnification view. Panel c demonstrates a high magnification view with expression within an epithelial whorl (EW) as well as within the stellate reticulum (SR). Panel d demonstrates intermittent expression within the reactive/glial supporting tissue (GS) as well as palisading epithelium (PE).

**Figure 4:** Gene expression of IL-10 and IL-10 receptor components in ACPs and a panel of 148 other brain tumors and normal brain specimens including Pilocytic Astrocytomas (PAs), Atypical Teratoid/Rhabdoid Tumors (AT/RTs), Ependymomas (EPNs), Glioblastomas (GBMs), Gangliogliomas (GGs), Medulloblastomas (MEDs), and Embryonal Tumors (EmTs). Values are expressed as log₂ gene expression. Horizontal red bars represent the mean, and error bars represent standard error of the mean (SEM)

**Figure 5:** IDO-1 Gene Expression and immunohistochemistry. (a) Gene expression of IDO-1 in ACPs and a panel of 148 other brain tumors and normal brain specimens including Pilocytic Astrocytomas (PAs), Atypical Teratoid/Rhabdoid Tumors (AT/RTs), Ependymomas (EPNs), Glioblastomas (GBMs), Gangliogliomas (GGs), Medulloblastomas (MEDs), and Embryonal
Tumors (EmTs). Values are expressed as log2 gene expression. Horizontal red bars represent the mean, and error bars represent standard error of the mean (SEM) (b) immunohistochemical stain of normal frontal cortex using 4.16H1 anti-IDO-1 antibody. (c-f) immunohistochemical stains of ACP using 4.16H1 anti-IDO-1 antibody at 4x(c,e) and 20x(d,f) magnification