

Article type : Letter to the Editor

Anti- β_c mAb CSL311 inhibits human nasal polyp pathophysiology in a humanized mouse xenograft model

To the Editor,

Chronic rhinosinusitis with nasal polyps (CRSwNP) is an inflammatory disease of the nose and paranasal sinuses characterised by eosinophilia, elevated levels of local IgE and Type 2 (T2) inflammatory cytokines(1) and mucus production. Interleukin (IL)-5, together with the other T2 beta common (β_c) cytokines IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), support the survival, and enhance the differentiation and activation of myeloid cells, which play a key role in the pathogenesis of this disease(2, 3).

We have developed a fully-human therapeutic monoclonal antibody (mAb), CSL311, with specificity for the common cytokine binding site of the human β_c receptor(4). The ability of CSL311 to block IL-3, IL-5 and GM-CSF signals simultaneously may offer distinct advantages in the treatment of inflammatory diseases compared to mAbs targeting single cytokines. Preclinical testing of CSL311 is limited in animal models due to the species-specificity of CSL311. To overcome this constraint we modified the human nasal polyp (NP) xenograft model described previously(5), and utilised *Rag2^{-/-}Il2rg^{-/-}*hIL-3/GM-CSF knock-in mice, which express human IL-3 and GM-CSF to support longer-term survival, growth and differentiation of human myeloid cells to evaluate, for the first time, the *in vivo* efficacy of CSL311, on NP progression in a preclinical proof-of-concept study.

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NPs from 12 patients, who were withdrawn from any medication one month prior to polypectomy, were used in this study. The most frequent comorbidities in these patients were allergic rhinitis (50%) and asthma (41.7%) (Table S1); consistent with atopy, histological analysis revealed that the NPs were highly eosinophilic (11/12), demonstrating characteristic T2 inflammation (data not shown). High levels of β_c cytokines were detected in the NP compared to normal sinus tissues (Figure S1), suggesting a role of β_c cytokines in contributing to the pathogenesis of CRSwNP. NP from each patient was engrafted into 4–10 mice with all treatment groups included in each independent experiment (Table S2). CSL311 significantly suppressed NP progression in recipient mice after 5 weeks compared with isotype control mAb (Figure 1A, Figure S3). We found that treatment with CSL311 for one week resulted in an immediate reduction of NP volume measured externally, which was not observed with positive control prednisolone treatment. After 5 weeks treatment, CSL311 significantly suppressed NP volume in recipient mice when compared with isotype control mAb.

To understand how CSL311 treatment inhibited NP growth, we examined the immune cell profiles in the xenografted NPs collected at the end of the 5-week engraftment. The percentage of eosinophils, neutrophils and plasma B cells (Figure 1B, Figure S2, Table S3), as well as the number of mast cells (Figure 1C) were significantly reduced in the presence of CSL311 when compared with isotype control mAb treatment. These cells are known to express β_c receptor and when activated by β_c cytokines produce chemical mediators that directly promote tissue remodelling and oedema. Next, we assessed the effect of CSL311 on mucous gland hyperplasia, a general feature of NPs(6). Histological examination revealed a significant decrease in mucous gland number and mucus production in NPs treated with

CSL311 that was not observed in those treated with isotype control mAb (Figure 1D, Figure S3). The stroma of NPs contains increased levels of fibroblasts(7). Fibroblast-specific protein 1(FSP-1) is expressed in fibroblasts in different organs that undergo tissue remodelling and is commonly used as a marker to identify fibroblasts(8). Using immunofluorescence staining, we found that CSL311 significantly decreased the percentage of FSP-1 positive fibroblasts compared to isotype control mAb (Figure 1E, Figure S3).

We also performed transcriptome analysis by RNA sequencing to understand the impact of CSL311 on the global gene expression profile in the xenografted NPs. Treatment with CSL311 resulted in differential expression of 29 genes with a fold change >2 ($|\log_2FC| >1$) and false discovery rate < 0.05 . The top 25 differentially expressed (DE) genes (Figure 2A) were used to calculate a gene score based on the average counts per gene, which confirmed that CSL311 significantly reduces expression of this gene set compared to isotype control mAb; no significant effect of prednisolone was observed on expression of these genes (Figure 2B). The average log fold change ($\log_2FC$) in gene expression, induced by CSL311 vs isotype control mAb and prednisolone vs saline, is shown in Figure 2C. These DE genes encode: i) surface markers associated with macrophage/dendritic cells e.g. *CD14*, *CD206*, *CD209* and granulocytic cell subsets e.g. *CLEC4A*, *CD32*, *FCERG1*, ii) chemokines that recruit T2 cells (basophils, eosinophils, TH2 cells) e.g. *CCL7*, *CCL13*, *CCL18* *CCL23* and iii) inflammatory response proteins including complement components *C1QA*, *C1QB* and *C1QC*. Ingenuity Pathway Analysis (IPA) upstream regulator tool, which predicts the most likely regulators of the DE genes, identified GM-CSF (Figure 2D) and IL-3 (Table S4) as regulators of genes down-regulated by CSL311. In addition, this analysis identified other cytokines that may contribute to airway inflammation, such as IL-4, IL-13, IFN γ , IL-17A and IL1 β as contributing to gene expression down-regulated by CSL311(Figure 2D and Table S4). This is

consistent with reduced IL-4 and IL-13 protein production observed in allergic $\beta_c^{-/-}$ mice and point to a potentially important link between the β_c -signaling cytokines and the IL-4/IL-13 axis in T2 cytokine-mediated diseases(9).

mAbs that target T2 inflammation have emerged as important alternative therapeutics for patients with CRSwNP. Clinical trials using omalizumab (anti-IgE), mepolizumab (anti-IL-5), and dupilumab (anti-IL-4 receptor α) have demonstrated that these mAbs shrink NPs and improve clinical symptoms (10). The key mechanisms of these drugs on blocking the IgE-dependent mast cell activation, IL-5-driven eosinophil activities, and IL-4/IL-13-mediated inflammation were replicated by CSL311 treatment in our xenograft model. Our study provides the first demonstration that targeting β_c with a therapeutic mAb may be an effective strategy for treating CRSwNP. Intrapolyp local drug delivery, as has been utilised in this model, has been reported to be an effective and safe method for treatment of NPs(11). Systemic efficacy of CSL311 treatment in this mouse model, as well as clinical trials will be required to assess the safety and therapeutic potential of CSL311 in improving clinical outcomes of patients with nasal polyposis.

AUTHOR CONTRIBUTIONS

K.H.Y, N.J.W, M.A.G. and C.M.O were involved in study concept and design. K.H.Y, N.J.W, C.L.B, H.P., M.S., S.B., M.N, M.A. and N.W. contributed to the acquisition, analysis or interpretation of data. G.V., A.F.L., A.D.N. D.J.T. and M.J.W. provided intellectual input; K.H.Y, N.J.W and C.M.O drafted the manuscript. All authors approved the manuscript for submission

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CONFLICTS OF INTEREST

K.H.Y., H.P., C.L.B., M.S. D.J.T declare no conflict of interest. N.J.W., M.N., M.A., N.W., G.V., A.D.N, M.J.W. and C.M.O. are employees of CSL Limited. S.B. is a former employee of CSL Limited and declares no conflict of interest. A.F.L. and M.A.G. receive funding from CSL Limited.

Kwok Ho Yip^{1*}

Nicholas J. Wilson^{2*}

Harshita Pant^{1,3}

Christopher L. Brown⁴

Samantha Busfield²

Milica Ng²

Monther Alhamdoosh²

Naomi Woodman²

Mark Schembri⁵

Damon J. Tumes¹

Gino Vairo²

Angel F Lopez¹

Andrew D. Nash²

Michael J. Wilson²

Michele A. Grimbaldeston^{1#}

Catherine M. Owczarek^{2#&}

* Kwok Ho Yip and Nicholas J. Wilson should be considered joint first authors

Michele A. Grimbaldeston and Catherine M. Owczarek should be considered joint senior authors.

¹ Centre for Cancer Biology, SA Pathology and the University of South Australia, UniSA CRI, Building HB, North Terrace, Adelaide, South Australia 5001, Australia.

² Research and Development, CSL Limited, Bio21 Institute, 30 Flemington Road, Parkville Victoria 3010, Australia.

³ Department of Otolaryngology, Head and Neck Surgery, University of Adelaide, Adelaide, South Australia 5005, Australia.

⁴ Rhinology Clinic, Royal Victorian Eye and Ear Hospital, 32 Gisborne Street, East Melbourne, Victoria 3002, Australia.

⁵ Department of Otolaryngology and Head & Neck Surgery, Women's and Children's Hospital, Adelaide, South Australia 5005, Australia

Correspondence

Catherine M. Owczarek, CSL Limited, Bio21 Institute, 30 Flemington Road, Parkville, Victoria 3010, Australia.

Email: catherine.owczarek@csll.com.au;

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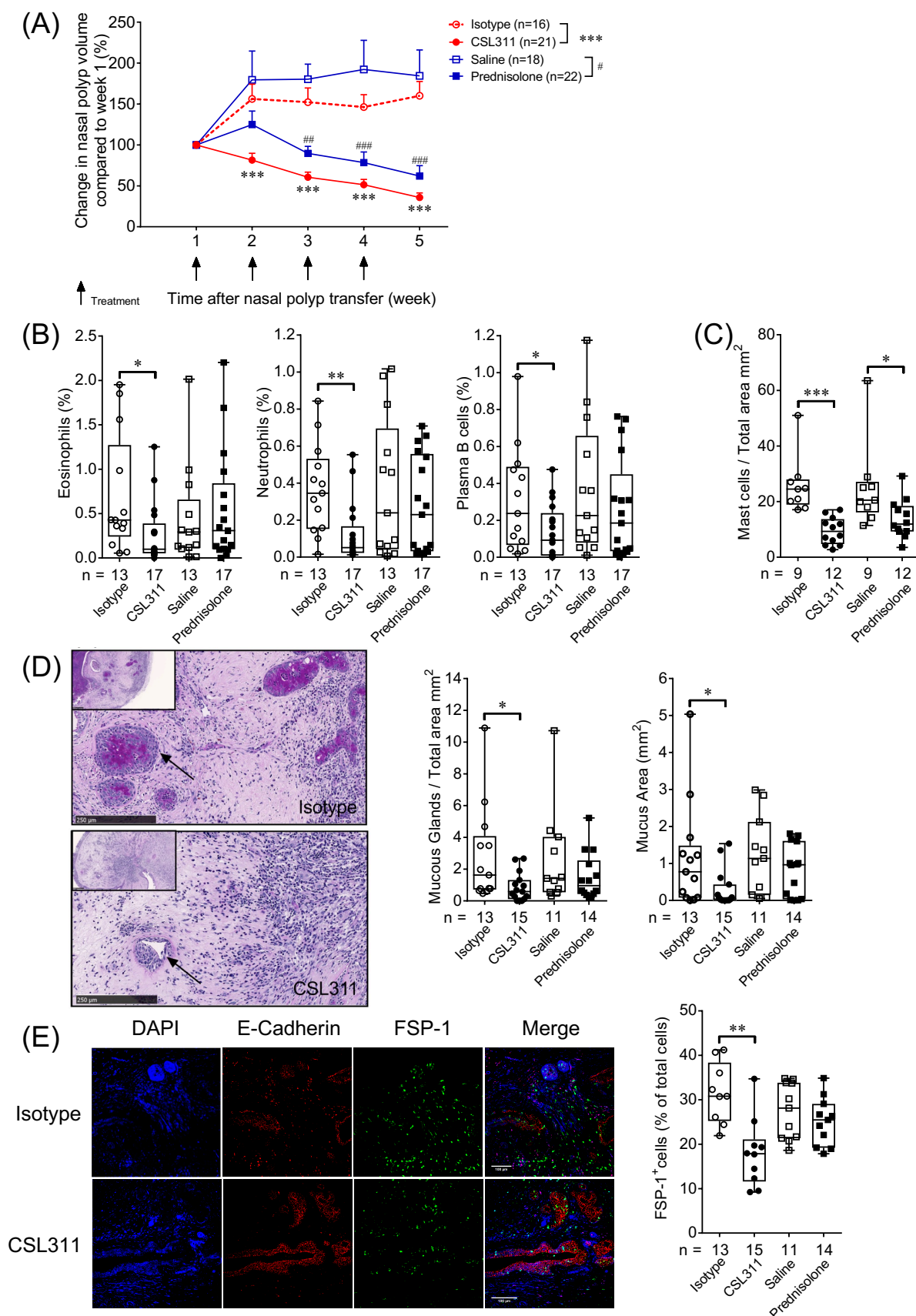


FIGURE 1. CSL311 restrains human nasal polyp xenograft progression and pathophysiology *in vivo*. CSL311 mAb treatment reduces (A) volume of nasal polyp measured externally every week, (B) granulocytes and plasma B cell populations, (C) mast cell numbers, (D) mucous gland numbers and mucus production and (E) fibroblast numbers in xenografted nasal polyps compared to isotype control mAb treatment. #/* $p < 0.01$, ##/** $p < 0.01$, ###/*** $p < 0.001$, (A) data are mean $\pm$ S.E.M of 12 independent experiments with $n = 16$ to 22 mice for each group. Two-way ANOVA with Bonferroni post-test for comparison on indicated time points between isotype control mAb vs CSL311 and saline vs prednisolone treatments; (B – E) data: median $\pm$ range, with the number of mice in each group indicated below x-axis. Mann-Whitney U test for indicated comparisons.

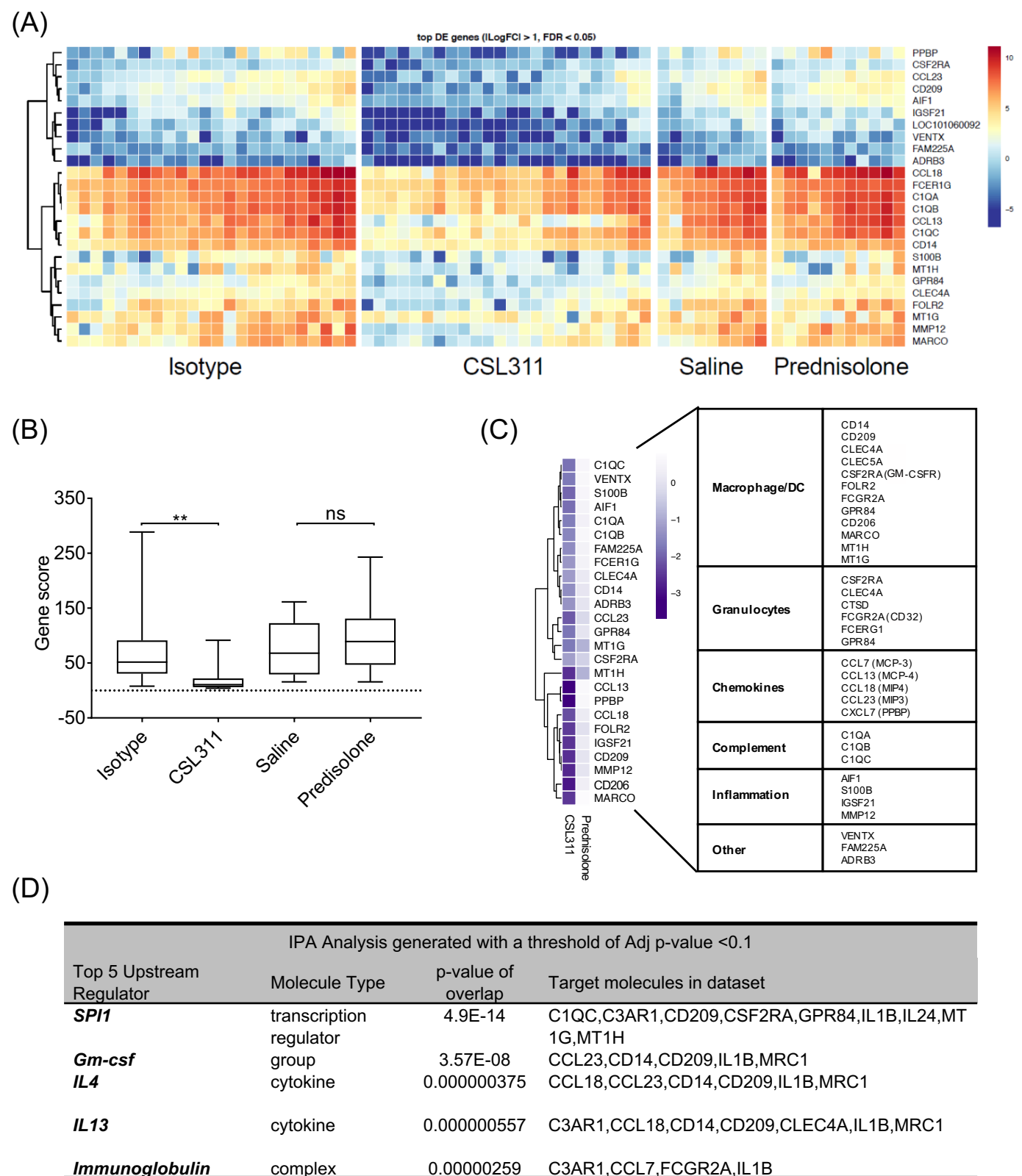
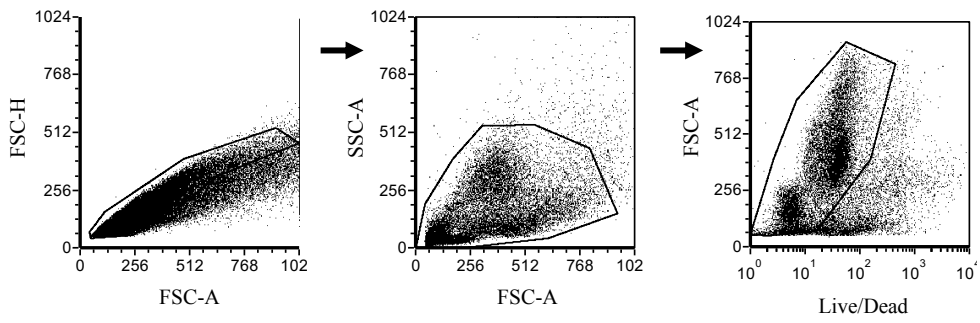


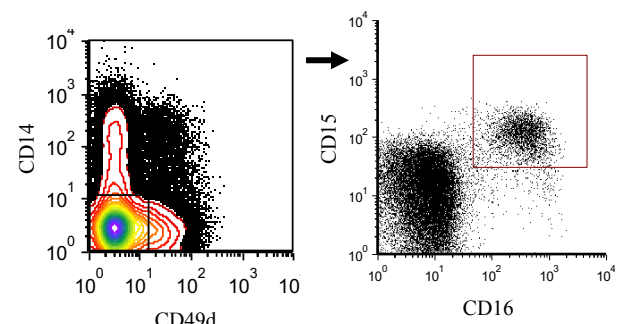
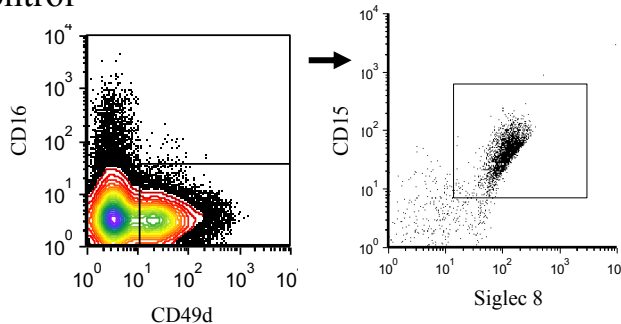
FIGURE 2. RNA sequencing and Ingenuity Pathway Analysis (IPA) reveal CSL311 targets type 2 inflammation-associated genes in xenografted human NPs. (A) Hierarchical clustering of top 25 differentially expressed (DE) genes. (B) The average expression of the top 25 DE genes was calculated for each treatment and expressed as a “gene score”. The difference in gene score between isotype control and CSL311 mAb and between saline control and prednisolone was determined (** $p < 0.01$, one-way ANOVA with Tukey’s multiple comparison tests). (C) The heat map shows average fold change in DE genes (isotype control mAb vs CSL311 and saline control vs prednisolone) and genes annotated for cellular expression or function. (D) IPA Upstream Regulator Analysis reveals top 5 targeted upstream regulators by CSL311 treatment in xenografted NPs.



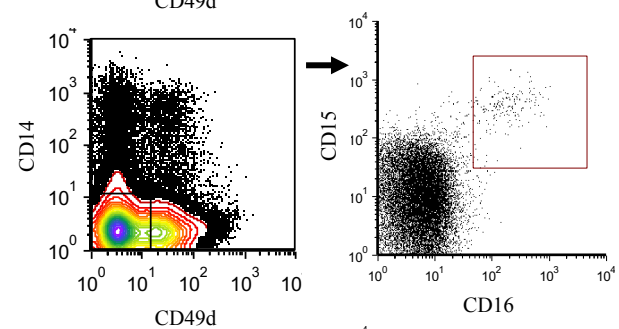
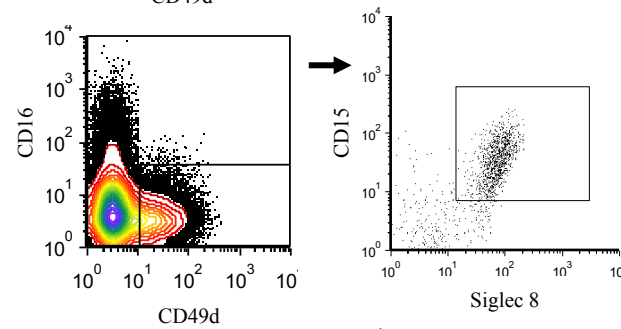
(A) Eosinophils

(B) Neutrophils

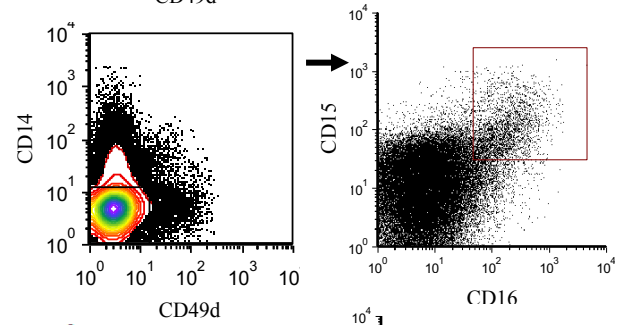
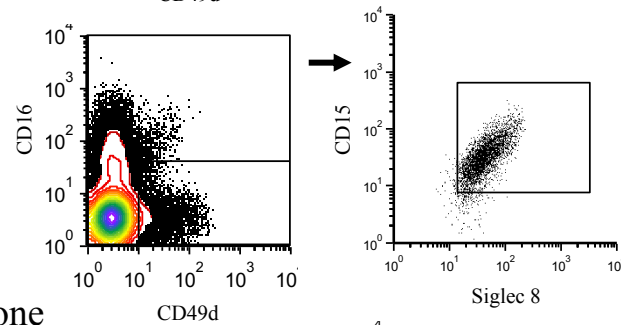
Isotype control



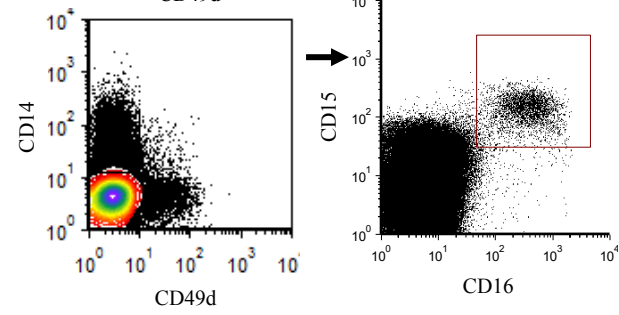
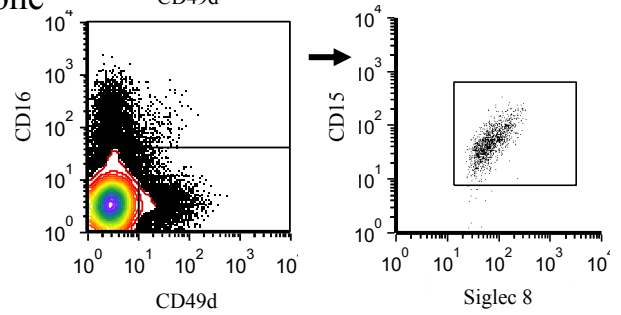
CSL311



Saline

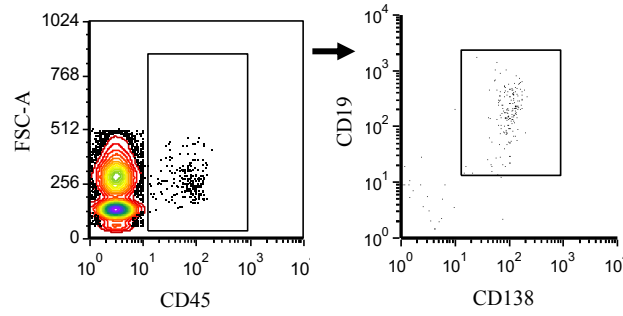


Prednisolone

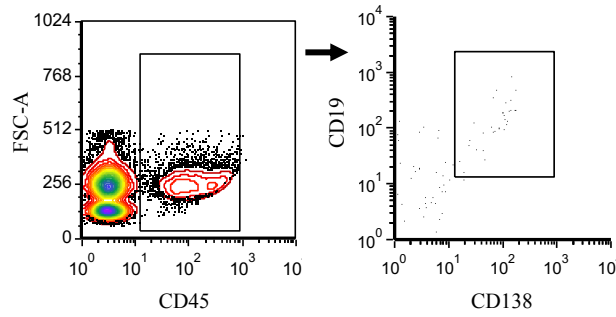


(C) Plasma B cells

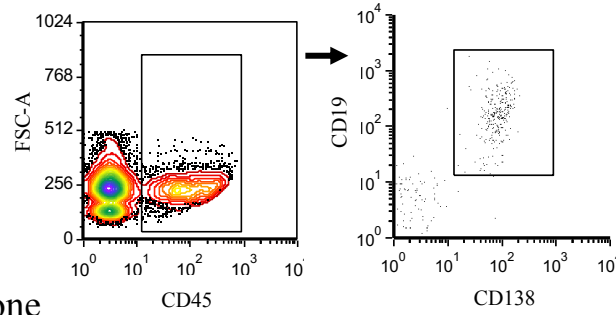
Isotype control



CSL311



Saline



Prednisolone

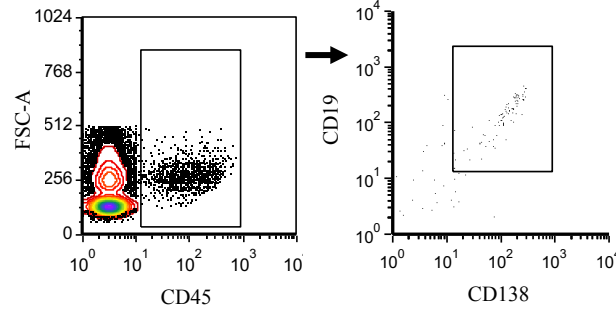
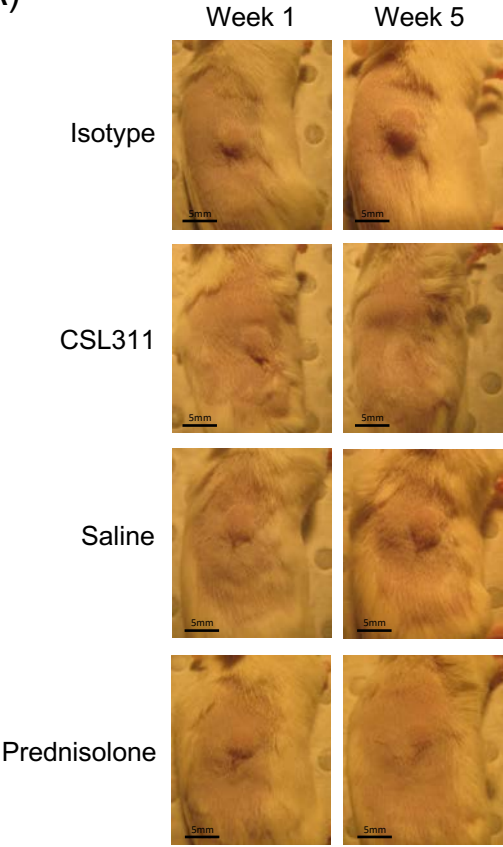
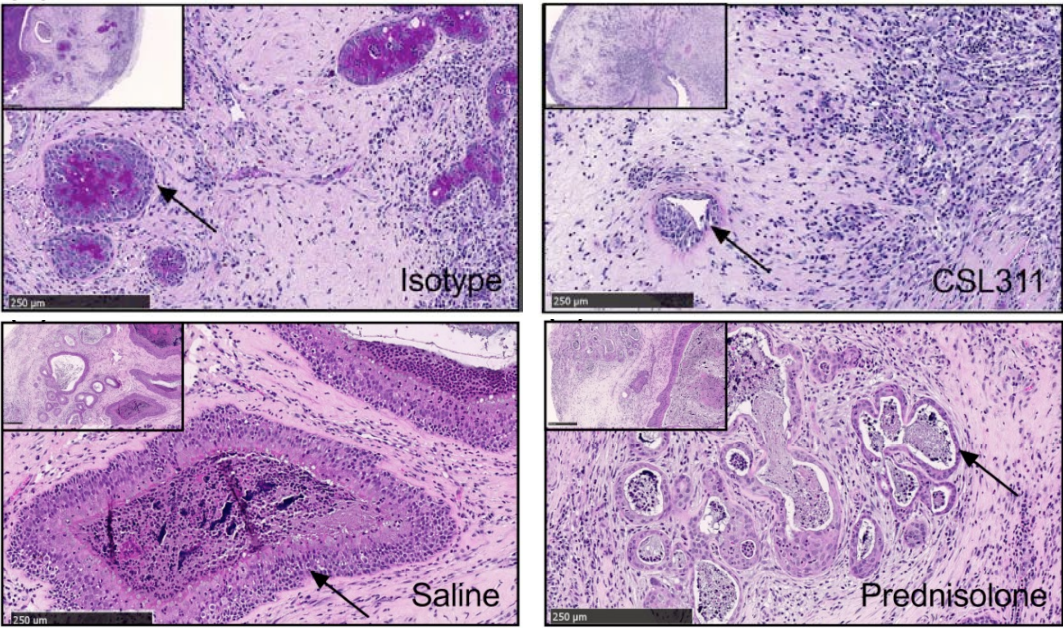


Figure S2. Gating strategies of (A) eosinophils (B) neutrophils and (C) plasma B cells in Figure 1B.

(A)



(B)



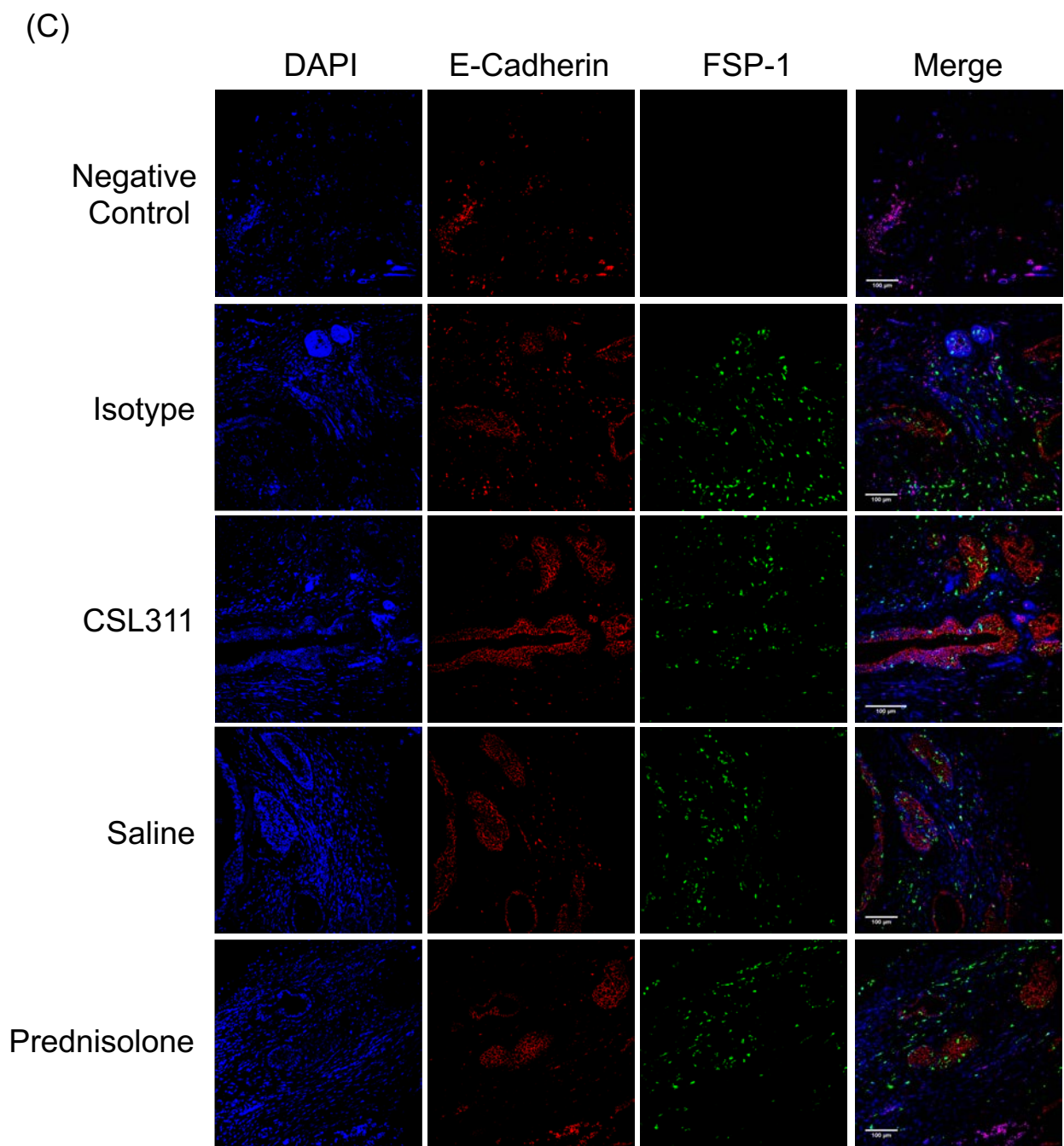


Figure S3. Representative images for (A) Figure 1A (B) Figure 1D and (C) Figure 1E.

ONLINE SUPPLEMENTARY

Anti- ϵ mAb CSL311 inhibits human nasal polyp pathophysiology in a humanized mouse xenograft model.

METHODS

Animals

Immunodeficient 129S4-*Rag2*^{tm1.1Flv}*Csf2/Il3*^{tm1.1(CSF2,IL3)Flv}*Il2rg*^{tm1.1Flv}/J mice(1) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). Three to four-month-old female mice were used for *in vivo* xenograft experiments. Mouse experiments were performed in compliance with the ethical guidelines of the National Health and Medical Research Council of Australia and approval from the Institute of Medical and SA Pathology Animal Ethics Committee (Animal Ethics Committee Project numbers: 127/13).

Nasal polyp (NP) xenograft studies

This study was approved by the VPSC Medical Advisory Committee and the Royal Adelaide Hospital Human Research Ethics Committee (HREC/13/RAH/603, RAH Protocol no: 131203). Human NP tissues were obtained from patients who had undergone endoscopic sinus surgery with informed written consent. They were withdrawn from any medication one month prior to polypectomy. Fresh NP specimens obtained were dissected into 4 x 4 mm pieces and weighed. Under sterile conditions, mice were first weighed, their right flanks shaved and then anaesthetised with isoflurane. A small midline incision through the skin to expose the peritoneal cavity was then made on the upper right flank and with blunt forceps, a pocket formed subcutaneously (s.c.) to house two of the 4 × 4 mm NP samples. Following insertion of the tissues, the wound was closed with a clip (Stoelting Co.) and mice injected subcutaneously in the scruff at the back of the head with 0.05 ml Buprenorphine analgesic (Merck, 1 mg/ml) and monitored closely until they regained consciousness. No deaths were

associated with the surgery. One week after surgical transfer, administration of either CSL311 antibody (5 mg/kg), isotype control antibody (5 mg/kg), prednisolone (1 mg/kg) or saline control (0.9%) commenced. All treatments groups (CSL311, isotype control antibody, prednisolone and saline) were included in each independent experiment and NP pieces from the same patients were used. Briefly, isoflurane anaesthetized mice were first weighed, and the external dimension (length and width) of the implanted NPs determined with a calliper. The treatments for each group of mice were then injected directly into the implanted NP (maximum injection volume of 20 μ l) once a week for a total of 4 weeks. At the end of 5 weeks, the mice were humanely killed and NPs excised.

Flow Cytometric Analysis

NPs were incubated with Liberase TL (Roche, 500 μ g/ml) in RPMI at 37°C for 90 min before passing through a 70 μ m nylon cell strainer to obtain single cell suspension. Cells were incubation with Human Fc Receptor Binding Inhibitor (eBioscience) for 15 min prior to incubation with fluorochrome-conjugated antibodies. For detection of human T cell subsets, cells were incubated for 30 min with the following antibodies: anti-human CD45-FITC (eBioscience, 2D1, 0.5 μ g/ml), anti-human CD4-PE (eBioscience, OKT4, 0.24 μ g/ml), anti-human CD3-PerCP-Cy5.5 (eBioscience, OKT3, 1 μ g/ml) and anti-human CD8-PE-Cy7 (eBioscience, SK1, 0.06 μ g/ml). For detection of human B cell subsets, anti-human CD45-FITC (2D1, 0.5 μ g/ml), anti-human CD19-PE-Cy7 (eBioscience, HIB19, 0.25 μ g/ml), anti-human CD20-APC (eBioscience, 2H7, 0.06 μ g/ml) and anti-human CD138-PE (eBioscience, DL-101, 0.5 μ g/ml) were used. For the detection of neutrophils, eosinophils and macrophages, anti-human CD14-APC-Cy7 (BD Pharmingen, M ϕ P9, 2 μ g/ml), anti-human CD15-FITC (BD Pharmingen, HI98, 2 μ g/ml), anti-human CD16-PE-Cy7 (BD Pharmingen, 3G8, 2 μ g/ml), anti-human CD49d-PE (BD Pharmingen, 9F10, 0.5 μ g/ml) and

anti-human Siglec-8-APC (Miltenyi, 7C9, 1:200 dilution) was used. Dead cells were excluded by Live/Dead Aqua stain (Life Technologies) Data were collected on a GalliosTM flow cytometer (Beckman Coulter) and analysed using FCS Express 6 (De Novo Software).

Histology

NP tissues were fixed in 10% buffered formalin, processed and embedded in paraffin, 4- μ m sections cut and stained with 0.1% Toluidine Blue (pH 1.0, Sigma-Aldrich) for the detection of mast cells (cytoplasmic granules appear purple). Periodic acid-Schiff (PAS) staining was performed by the Tissue Pathology Department, SA Pathology. An image of the entire nasal polyp section was captured using a Hamamatsu NanoZoomer 2.0 HT (40 $\times$ magnification, Adelaide Microscopy Facility). Mast cells per total nasal polyp area (mm²), mucus area (mm²) and the number of mucous glands per total nasal polyp area (mm²) were evaluated using Fuji Image J software (1.50e, National Institute of Health).

Immunofluorescence staining

Antigen retrieval was performed on NP tissues sections by boiling slides in a pressure cooker in 10 mM citrate buffer (pH 6). Sections were blocked in normal goat serum in CAS-Block (blocking buffer, Thermo Fisher Scientific) for at least 20 min in a humidified chamber at RT then rabbit anti-FSP-1 antibodies (Sigma, 1:200) were added overnight in blocking buffer at 4°C. Sections were washed in distilled water and Alexa Fluor®-488 goat anti-rabbit secondary antibody (Thermo Fisher Scientific, 1:400) were added for 1 h at RT in a darkened humidified chamber. Sections were washed in distilled water and incubated with Alexa Fluor® 555 mouse anti-E-Cadherin (BD Pharmingen, 1:200) for 2 h at RT. Section without the anti-FSP-1 antibodies (1^o Ab) was used as negative control. After a final wash with distilled water and the sections were mounted in ProLong® Gold Antifade Mountant with

DAPI (Thermo Fisher Scientific). Images were acquired using 20x (PlanApo 20x/0.8 DICII) objectives at RT on a Zeiss LSM 700 confocal system and recorded using a ZEN 2011 (Black Edition) software (Carl Zeiss Microscopy). All quantification was performed using ImageJ (National Institutes of Health (NIH)), thresholding on the corresponding isotype control, and a minimum of two fields of view were averaged for every nasal polyp tissue.

RNAseq data analysis

RNA from excised human NP tissue was extracted from the tissue homogenate using the Qiagen RNeasy® Fibrous Tissue Mini Kit (Qiagen) as per manufacturer's protocol. RNA sequencing (RNAseq) was performed on HiSeq (Illumina, California, USA) at the Australian Genome Research Facility and >10 million 100bp single-end, stranded reads were analyzed per sample with the bcl2fastq 1.8.4 pipeline (Illumina, California, USA). Sequenced reads were mapped to reference genome GRCh37.p11 using *bowtie2* (v2.2.5) and *TopHat2* (v2.1.0) with default parameters, except for the *--b2-very-sensitive* option, and *--library-type fr-firststrand* setting. Reads were summarised at the gene level using *featureCounts* function from the *Rsubread* package (v1.26.0), in a strand-specific manner. Differential expression was performed via the *limma* (v3.32.2) Bioconductor package.

Ingenuity Pathway Analysis (IPA)

Differentially expressed genes between NP xenografts treated with CSL311 and NP xenografts treated with isotope control were selected using 2-fold change and FDR 0.05 cut-off. The gene list was annotated using Ensembl IDs, and logFC values and FDR values were uploaded into IPA (Qiagen Inc., <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>). The Ensembl IDs were then mapped onto IPA internal entity IDs. The Core Analysis was performed, and

the Comparison Analysis was conducted on the results of the four core analytics to identify top-ranked gene sets. The core analyses were performed using the default settings while *Ingenuity Knowledge Base (genes only)* was selected as a reference dataset, direct and indirect relationships were included, evidence predicted with *moderate confidence* was excluded, and only evidence from *Human species* was considered. No cut-offs were applied to the logFC or FDR values. Finally, the top-ranked canonical pathways, diseases and functions, and upstream regulators were selected from the comparative analysis and their calculated statistical p-value scores were exported.

ELISA on NP tissue

Normal sinus tissue and NP samples were homogenised in PBS with protease inhibitor (Roche Life Science). Levels of cytokine in supernatants were determined using human IL-3 Quantikine ELISA Kit (R&D system), human IL-5 ELISA Ready-SET-Go! Kit (eBioscience) and human GM-CSF ELISA Ready-SET-Go! Kit (eBioscience). Total protein levels in the supernatants were measured by a Bio-Rad D_c protein assay, according to the manufacturer's instructions (Bio-Rad Laboratories).

Statistics

The difference between groups was analyzed using 2-way ANOVA with Bonferroni post-test, or Mann Whitney U test. Asterisks between indicated comparison show the level of significance, *p<0.05; **p<0.01; ***p<0.001. GraphPad Prism Version 5.0d was used.

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SUPPLEMENTARY TABLES

Table S1. Baseline characteristics of subjects: Data = median with interquartile range.

Aspirin-Exacerbated Respiratory Disease (AERD); Allergic fungal rhinosinusitis (AFRS)

	Patients with CRSwNP (n=12)
Age (y), median (IQR)	62.5 (38.5-64.5)
Men : women (n)	6 : 6
Allergy, no. (%)	6 (50.0%)
Asthma, no. (%)	5 (41.7%)
Allergic rhinitis, no. (%)	6 (50.0%)
AERD, no. (%)	0 (0%)
AFRS, no. (%)	1 (8.3%)
Previous sinus surgery, no. (%)	4 (33.3%)
Duration of condition (y), median (IQR)	9 (0.9 – 21.8)
Medication (Patients were withdrawn from medication one month prior to polypectomy)	
Prednisolone alone, no. (%)	6 (50.0%)
Prednisolone + Antibiotics, no. (%)	3 (25.0%)
Prednisolone + Budesonide, no. (%)	1 (8.3%)
Nasal decongestant, no. (%)	2 (16.7%)
Nil, , no. (%)	2 (16.7%)

Table S2. Number of mice used for different treatments in each xenograft experiment .

	Number of mice per treatment group			
	Isotype	CSL311	Saline	Prednisolone
Patient 1	1	2	1	2
Patient 2	2	2	2	2
Patient 3	1	2	1	2
Patient 4	2	2	2	2
Patient 5	1	1	1	1
Patient 6	1	2	1	2
Patient 7	1	1	1	1
Patient 8	2	3	2	3
Patient 9	2	2	2	2
Patient 10	1	2	2	2
Patient 11	1	1	2	2
Patient 12	1	1	1	1

Table S3. CSL311 did not affect macrophages, B cells, CD4⁺ & CD8⁺ T cell population in xenografted NPs

	Treatment group			
	Isotype	CSL311	Saline	Prednisolone
Flow cytometric analysis (n = 13 – 17 mice per group)				
Macrophages (%)	0.35 (0.19 – 0.57)	0.21 (0.12 – 0.43)	0.19 (0.13 – 0.46)	0.29 (0.17 – 0.45)
B cells (%)	0.05 (0.02 – 0.24)	0.10 (0.04 – 0.61)	0.07 (0.03 – 0.14)	0.20 (0.02 – 0.37)
CD4 ⁺ T cells (%)	1.21 (0.24 – 2.70)	0.93 (0.10 – 4.11)	0.89 (0.26 – 2.04)	1.49 (0.29 – 4.03)
CD8 ⁺ T cells (%)	1.05 (0.33 – 3.73)	1.95 (0.55 – 4.11)	1.02 (0.36 – 2.68)	1.99 (0.52 – 3.36)

Table S4. Full list of IPA Upstream Regulator Analysis on upstream regulators by CSL311 treatment in xenografted NPs.

IPA Analysis generated with a threshold of Adj p-value <0.1			
Upstream Regulator	Molecule Type	p-value of overlap	Target molecules in dataset
<i>SPI1</i>	transcription regulator	4.9E-14	C1QC,C3AR1,CD209,CSF2RA,GPR84,IL1B,IL24,MT1G,MT1H
<i>Gm-csf</i>	group	3.57E-08	CCL23,CD14,CD209,IL1B,MRC1
<i>IL4</i>	cytokine	0.000000375	CCL18,CCL23,CD14,CD209,IL1B,MRC1
<i>IL13</i>	cytokine	0.000000557	C3AR1,CCL18,CD14,CD209,CLEC4A,IL1B,MRC1
<i>Immunoglobulin</i>	complex	0.00000259	C3AR1,CCL7,FCGR2A,IL1B
<i>ACVR1</i>	kinase	0.000003	CD14,CLEC5A,FOLR2
<i>IL27</i>	cytokine	0.0000155	CCL7,CD14,IL1B,MRC1
<i>IFNG</i>	cytokine	0.000022	AIF1,C1QA,C1QB,C1QC,CCL18,FCER1G,IL1B
<i>RNASE2</i>	enzyme	0.0000515	CCL23,CCL7,PPBP
<i>mir-155</i>	Micro RNA	0.0000515	CCL18,CD209,IL1B
<i>miR-155-5p (miRNAs w/seed UAAUGCU)</i>	mature micro RNA	0.0000576	CCL18,CD209,IL1B
<i>IL10</i>	cytokine	0.0000577	CCL18,CD14,IL1B,IL24
<i>PSMD4</i>	other	0.0000708	CD14,MARCO
<i>IL3</i>	cytokine	0.0000786	CD14,FCGR2A,IL1B
<i>PI3K (family)</i>	group	0.000105	CTSD,IL1B,MMP12,PTGS1
<i>IL1B</i>	cytokine	0.000186	CCL13,CCL7,IL1B,IL24,MMP12
<i>FAT1</i>	other	0.000253	IL1B,PLAU
<i>IL17A</i>	cytokine	0.000371	CD14,IL1B,MRC1
<i>FCGR2A</i>	transmembrane receptor	0.000385	CCL18,IL1B
<i>Pde4</i>	group	0.000544	CD14,PPBP