

Autotaxin is an inflammatory mediator and therapeutic target in thyroid cancer

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Abstract

Autotaxin is a secreted enzyme that converts extracellular lysophosphatidylcholine to lysophosphatidate (LPA). In cancers, LPA increases tumour growth, metastasis and chemoresistance by activating six G-protein coupled receptors. We examined > 200 human thyroid biopsies. Autotaxin expression in metastatic deposits and primary carcinomas was four- to tenfold higher than in benign neoplasms or normal thyroid tissue. Autotaxin immunohistochemical staining was also increased in benign neoplasms with leukocytic infiltrations. Malignant tumours were distinguished from benign tumours by high tumour autotaxin, LPA levels and inflammatory mediators including IL1 β , IL6, IL8, GM-CSF, TNF α , CCL2, CXCL10 and platelet-derived growth factor (PDGF)-AA. We determined the mechanistic explanation for these results and revealed a vicious regulatory cycle in which LPA increased the secretion of 16 inflammatory modulators in papillary thyroid cancer cultures. Conversely, treating cancer cells with ten inflammatory cytokines and chemokines or PDGF-AA and PDGF-BB increased autotaxin secretion. We confirmed that this autotaxin/inflammatory cycle occurs in two SCID mouse models of papillary thyroid cancer by blocking LPA signalling using the autotaxin inhibitor ONO-8430506. This decreased the levels of 16 inflammatory mediators in the tumours and was accompanied by a 50–60% decrease in tumour volume. This resulted from a decreased mitotic index for the cancer cells and decreased levels of vascular endothelial growth factor and angiogenesis in the tumours. Our results demonstrate that the autotaxin/inflammatory cycle is a focal point for driving malignant thyroid tumour progression and possibly treatment resistance. Inhibiting autotaxin activity provides an effective and novel strategy for decreasing the inflammatory phenotype in thyroid carcinomas, which should complement other treatment modalities.

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Introduction

Worldwide, the incidence of papillary thyroid cancer has doubled in the past decade and is expected to overtake colorectal cancer as the fourth leading cancer diagnosis by 2030 (Pellegriti *et al.* 2013, Jung *et al.* 2014, Rahib *et al.* 2014). Although overall survival rates are excellent, thyroid cancer has a very high propensity to metastasize, and between 25 and 40% of patients require either surgery and/or radioactive iodine to control the disease (Hughes *et al.* 2012, Jung *et al.* 2014). Of this group ~20% demonstrate metastatic disease that is resistant to radioactive iodine (Hughes *et al.* 2012, Jung *et al.* 2014). These patients suffer a disproportionate burden because they are often subject to repeated attempts at surgical extirpation of metastatic disease as well as repeat, high-dose application of radioactive iodine (Hughes *et al.* 2012). The introduction of tyrosine receptor kinase inhibitors in clinical trials has generated mixed results with response rates between 15 and 50%, and few patients derive a lasting benefit from these adjuvant treatments (Anderson *et al.* 2013). Moreover, there are no clinically utilized markers for metastatic disease, and morphologic predictors of papillary thyroid cancer behaviour are imperfect. Although new molecular diagnostics based on the presence of *RET/PTC*, *RAS*, *BRAF*, and other mutations may aid clinicians in identifying malignant nodules, treatment plans rely primarily on morphologic characteristics such as tumour size. At present, personalized therapy remains out of reach (Anderson *et al.* 2013). Predicting occurrence of radioactive-iodine resistant and recurrent disease requires a better understanding of the pathways that drive thyroid tumour growth and metastases.

Aggressive malignancy is driven by a migratory phenotype that shares common pathways with inflammation (Colotta *et al.* 2009, Mantovani 2010). We propose that autotaxin, and its production of lysophosphatidate (LPA), is an important component of this inflammatory axis (Benesch *et al.* 2014a). LPA drives cancer cell division, survival and migration by activation of six G-protein coupled receptors (Brindley *et al.* 2013, Benesch *et al.* 2014a). The autotaxin gene is among the top 40 upregulated genes in metastatic colon, breast and prostate cancers (Euer *et al.* 2002). LPA contributes significantly to the resistance of effective cancer therapy (Brindley *et al.* 2013). LPA protects cancer cells from radiation-induced cell death by depleting cells of siva-1, a pro-apoptotic signalling protein, and by stimulating pro-survival kinase pathways (Brindley *et al.* 2013). LPA also decreases the efficacy of chemotherapy by increasing the expression of

antioxidant genes and multi-drug resistant transporters (Venkatraman *et al.* 2015).

Autotaxin is normally produced in response to injury and inflammation and its levels fall with the resolution of tissue repair (Benesch *et al.* 2014a, 2015). Uncontrolled inflammation disrupts the healing cycle and high autotaxin levels lead to a variety of pathological conditions such as arthritis and inflammatory bowel disease (Nikitopoulou *et al.* 2012, Hozumi *et al.* 2013, Benesch *et al.* 2014a). Moreover, persistently high autotaxin levels are linked to tumorigenesis and tumour progression in animal models of breast cancer, melanoma and glioblastoma (Jonkers & Moolenaar 2009, Altman *et al.* 2010, Bhave *et al.* 2013, Benesch *et al.* 2014b, Venkatraman *et al.* 2015). Cancers have been described as 'wounds that do not heal' (Dvorak 1986, Schafer & Werner 2008, Hanahan & Weinberg 2011), and inflammation is a hallmark of cancer progression (Allavena *et al.* 2008, Germano *et al.* 2008, Wu *et al.* 2010, Chopra *et al.* 2013, Grivennikov 2013). The role of individual cytokines such as IL1 α , IL6 and IL17 has been documented in the growth of solid tumours such as pancreatic, gastric and colorectal cancers (Tjomsland *et al.* 2013, Wang *et al.* 2014, Li *et al.* 2015). Despite an increasing appreciation for the role of inflammation in thyroid cancer progression, the connection between particular inflammatory signals and the potential for locally aggressive and metastatic papillary thyroid cancer variants is poorly understood (Cunha *et al.* 2014a,b, Ehlers & Schott 2014, Marcello *et al.* 2014, Provatopoulou *et al.* 2014, Tafani *et al.* 2014, Ward 2014).

In the present study we examined >200 human thyroid biopsies and demonstrated that malignant tumours are distinguished from benign tumours and normal thyroid tissue by high autotaxin activity and LPA concentrations coupled with a four- to tenfold increase in inflammatory chemokine and cytokine levels. The only exception to this was that samples from patients with thyroiditis in the absence of cancer also had high autotaxin expression. We established the mechanistic explanation for these results by demonstrating that autotaxin-mediated LPA formation is a central component of a vicious cycle of inflammatory chemokine and cytokine production, which stimulates further autotaxin secretion. Ten of the inflammatory mediators that we tested, and platelet-derived growth factor (PDGF), markedly increased autotaxin secretion by thyroid cancer cells. LPA in turn increased the secretion of 14 inflammatory chemokines and cytokines, PDGF and vascular endothelial

growth factor (VEGF). The relevance of these findings was demonstrated *in vivo*. Inhibition of autotaxin activity disrupted the cycle of inflammation in two mouse models of papillary thyroid cancer consequently decreasing tumour growth, proliferative index and angiogenesis. Our work provides a novel demonstration that blocking autotaxin activity provides a unique strategy for decreasing inflammatory chemokine and cytokine expression, thyroid cancer cell growth and tumour angiogenesis.

Materials and methods

Patient specimens and cell culture

Patient consent, tissue isolation and banking protocols were assessed and approved by the Alberta Research Tumour Bank and the Northern Alberta Research Ethics Board Pro00018758. All of the patients with papillary thyroid cancer who were identified on initial fine needle aspiration cytology and ultimately confirmed histologically on the final pathologic specimens were included. Patients with other concurrent malignancies, anaplastic or other poorly differentiated follicular carcinomas of the thyroid and previous radiation exposure were excluded. Two pathologists confirmed the diagnosis on core sections and validated all of the histologic specimens prepared for the tissue arrays. Specimens prepared for primary culture or tissue banking were placed in culture medium or optimal cutting temperature compound or were flash frozen, respectively, within 10 min of devascularization. A representative frozen section determination was used to ensure that >90% of the banked specimens represented the thyroid neoplasm, benign or malignant, or histologically normal thyroid tissue. Benign specimens with leukocytic infiltrations were excluded. Primary culture specimens were prepared and purified using the Cancer Cell Isolation kit (CI0010, Affymetrix, Santa Clara, CA, USA) according to its protocol. Cells were cultured in high-glucose DMEM containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 10 mU/ml thyroid stimulation hormone, 10 µg/ml insulin, 5 µg/ml transferrin, 10 nM hydrocortisone, 10 ng/ml somatostatin and 10 ng/ml glycyl-L-histidyl-L-lysine acetate (Ambesi-Impimbato *et al.* 1980) and incubated at 37 °C, 5% CO₂ and 95% humidity. Experiments were performed within 3–4 passage numbers of isolation and purification. 8305C (ACC-133) thyroid cancer cells were obtained from DSMZ (Braunschweig, Germany), and SW-579 (HTB-107) thyroid cancer cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), where cell lines

were authenticated by short tandem repeat analysis. All of the experiments were performed on cells 3–8 passage numbers from the time they were received. Cell lines were proven to be mycoplasma free using the MycoAlert Mycoplasma Detection kit (Lonza, Allendale, NJ, USA). Low passage number cells were cultured in RPMI 1640 media with 10% FBS and 1% P/S.

Mouse studies

Animal procedures were conducted in accordance with the Canadian Council of Animal Care as approved by the University of Alberta Welfare Committee. Eight- to 10-week-old female Fox Chase-SCID beige (CB17.Cg-Prkdc^{scid}Lyst^{bg-J}/Crl) mice (18–20 g) were obtained from Charles River (Kingston, ON, Canada). Mice were maintained at 21 ± 2 °C, 55 ± 5% humidity and a standard 12 h light:12 h darkness cycle with free access to food and water. 5 × 10⁵ 8305C or 1 × 10⁶ SW-579 cells were prepared (Benesch *et al.* 2014b) and injected subcutaneously into the flank with a 27-gauge needle while mice were anesthetized with 5% isoflurane. Tumours became palpable after 7 days when mice were gavaged daily with water or 20 mg/kg ONO-8430506 (Ono Pharmaceuticals, Osaka, Japan; patent WO2012005227) (Benesch *et al.* 2014b, Saga *et al.* 2014, Venkatraman *et al.* 2015) and tumour volume was measured (Benesch *et al.* 2014b).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, 5 µm paraffin-embedded tissue sections (Zhang *et al.* 2012, Benesch *et al.* 2014b) using the Dako LSA+Universal kit (K0679) and staining was visualized using Dako Envision+ rabbit HRP (K4002) (Burlington, ON, Canada). A rabbit IgG isotype control (bs-0295P, Bioss Antibodies; Woburn, MA, USA) was used as the control for staining. Tissue arrays were incubated with anti-autotaxin rabbit antibody (1:1000 dilution; ATX-102) from Dr Tim Clair (NCI, Bethesda, MA, USA) (Murata *et al.* 1994). Two independent pathologists reviewed and quantified the level of antibody staining from 0 (minimal), 1+, 2+, to 3+ (strong) without knowledge of the clinical outcome of the patients. At least two of the three tissue array cores needed to be intact for analysis and cores that were fragmented or incomplete were not scored. Autotaxin staining was assessed for each case, in triplicate. For xenograft tumours, staining was performed with anti-Ki67 (1:200 dilution; D3B5) rabbit antibody from Cell Signaling (Danvers, MA, USA) and anti-CD-31 rabbit antibody

Table 1 Patient demographics of immunohistochemistry specimens

	Normal (n=33)	Benign neoplasm (n=64)	PTC (LN-) (n=123)	PTC (LN+) (n=71)	LN (n=15)
Mean age	49.4±13.4	51.9±13.7	48.6±15.3	46.9±16.6	51.2±18.3
Female (%)	85	59	72	70	73
Mean tumor size (cm)	–	3.6±1.9	2.3±1.8	2.6±1.5	4.1±1.6

PTC, papillary thyroid cancer; LN, metastatic lymph node status. Results shown are means±s.d.

(1:50 dilution; ab28364) from Abcam (Toronto, ON, Canada) and quantified from at least three random fields per specimen. Images were acquired at 10× and 40× using a Zeiss Axioskop 2 imaging system (Carl Zeiss Canada, Toronto, ON, Canada).

Multiplex measurements

Eve Technologies Corp. (Calgary, AB, Canada) analysed 64 human cytokines, chemokines and growth factors. This analysis used a MILLIPLEX Human Cytokine/Chemokine 41-plex kit and a MILLIPLEX Human Cytokine/Chemokine 23-plex kit (Millipore, St Charles, MO, USA), according to the manufacturer's protocol on a Luminex 100 system (Luminex, Austin, TX, USA) (Supplementary Table 1, see section on supplementary data given at the end of this article for a list of analytes). Tissue specimens (5–10 mg) were homogenized in 200 µl of 20 mM Tris HCl (pH 7.5) buffer with 0.5% Tween 20, 150 mM NaCl and protease inhibitor, centrifuged for 10 min at 4 °C and then the supernatant was transferred to a fresh tube. Protein content was measured using the BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA) and adjusted to 5 µg/µl. For supernatant analysis, cancer cells were seeded in 12-well plates, grown to ~70% confluence and then washed and pre-incubated for 6 h in 1 ml phenol red-free RPMI 1640 medium containing delipidated FBS (FBSC) that was treated with 1% charcoal to remove lipids (Gaetano et al.

2009, Samadi et al. 2009). The media were then changed with or without an addition of 5 µM oleoyl-LPA and incubated for 24 h. Conditioned media were centrifuged prior to multiplex analysis. Measurements were normalized to both volume of medium and cell protein.

Real-time quantitative RT-PCR

RNA was isolated from Trizol (Invitrogen Life Technologies) using the Direct-zol RNA MiniPrep kit (R2052; Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. RT and quantitative RT-PCR (qRT-PCR) was then performed (Gaetano et al. 2009). Gene expression was normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) and essentially the same results were obtained relative to cyclophilin A. All primers are from Integrated DNA Technologies (Coralville, IA, USA) and used at a final concentration of 250 nM: *CSF2*: F: 5'-ATGATGGCCAGCCACTACAA-3', R: 5'-CTGGCTCC-AGCAGTCAAAG-3'; *CXCL10*: F: 5'-AGCAGAGGAACC-TCCAGTCT-3', R: 5'-ATGCAGGTACAGCGTACAGT-3'; *ENPP2* (autotaxin): F: 5'-CATTTATTGGTGAACGCAGA-3', R: 5'-ACTTTGTCAAGCTCATTTC-3'; *GAPDH*: F: 5'-ACTTTGTCAAGCTCATTTC-3', R: 5'-TCTTACTCCTGGAGGC-CAT-3'; *IL1A*: F: 5'-CTTCTGGGAAACTCACGGCA-3', R: 5'-AGCACACCCAGTAGTCTTGC-3', *IL1B*: F: 5'-AACAGG-CTGCTCTGGGATTC-3', R: 5'-AGTCATCCTCATTGCCAC-TGT-3'; *IL6*: F: 5'-AGTTCCTGCAGAAAAAGGCAAAG-3',

Table 2 Percentage (%) of specimens staining for autotaxin. Results are shown for normal thyroid, benign neoplastic tumours, papillary thyroid cancer primary tumours with and without metastases and papillary thyroid cancer metastases in lymph node deposits. Benign neoplastic tumours were further subdivided into patients with Hashimoto's thyroiditis or otherwise significant lymphocytic infiltration as defined on the final pathological report

Scoring	0 (%)	1+ (%)	2+ (%)	3+ (%)
Normal thyroid* (n=33)	81	8	11	0
Benign neoplastic tumours† (n=64)	26	34	20	18
No lymphocytic infiltration‡ (n=41)	24	34	24	17
Lymphocytic infiltration‡ (n=23)	4	13	17	65
Node-negative primary tumours*† (n=123)	8	9	30	52
Node-positive primary tumours*† (n=71)	8	8	31	53
Lymph node metastasis*† (n=15)	13	27	40	20

* $P < 0.0001$; † $P < 0.0001$; ‡ $P < 0.005$.

R: 5'-AAAGCTGCGCAGAATGAGATG-3'; *IL8*: F: 5'-CCACCGGAAGGAACCATCTC-3', R: 5'-CTCCTTGGCAAACTGCACC-3'; *PDGFA*: F: 5'-AAGCAGCCAACCAGATGTGA-3', R: 5'-GGAGGAGAACAAGACCGCA-3'; *PDGFB*: F: 5'-CTTCTGTCTCTGCTGCTA-3', R: 5'-CATCTTCTCTCCGGGGTC-3'; *TNFA*: F: 5'-CCC ATGTTGTAGCAAACCCTC-3', R: 5'-CCCATGTTGTAGCAAACCCTC-3'.

Measurement of lysophospholipid concentrations

Tissue specimens and plasma were extracted and quantified by mass spectrometry (Benesch *et al.* 2014b).

Measurement of autotaxin activity

Conditioned media (phenol red-free RPMI 1640, 1% FBSC) were prepared by incubating cells at ~70% confluency in six-well plates with 2 ml medium for 48 h following a 6-h preincubation with 1% FBSC. Control medium was not exposed to cells. Collected conditioned media were centrifuged and concentrated ~25-fold using an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane (UFC501096) (EMD Millipore, Billerica, MA, USA). Autotaxin activity was measured by the FS-3 assay (Gaetano *et al.* 2009, Benesch *et al.* 2015) by mixing 20 μ l

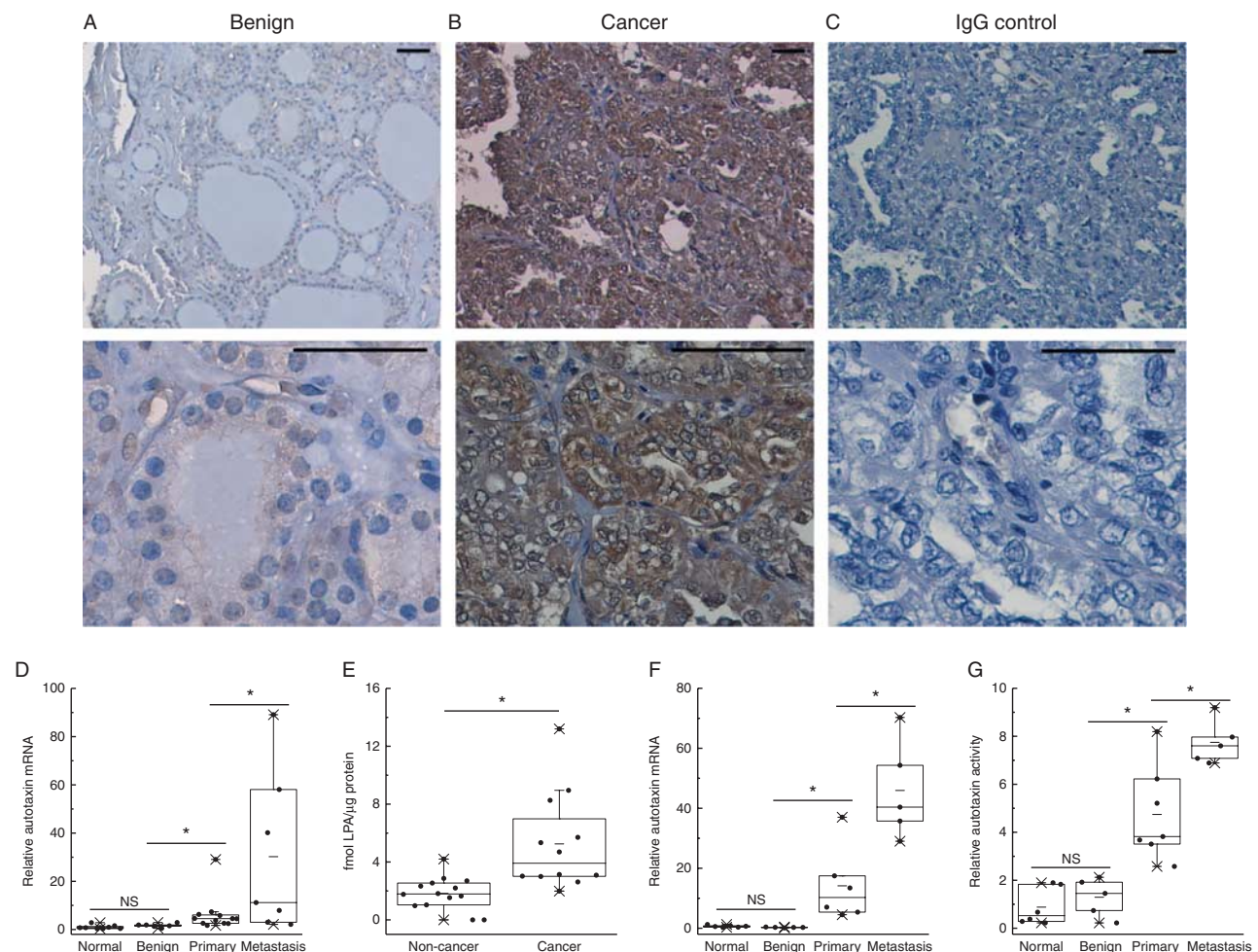
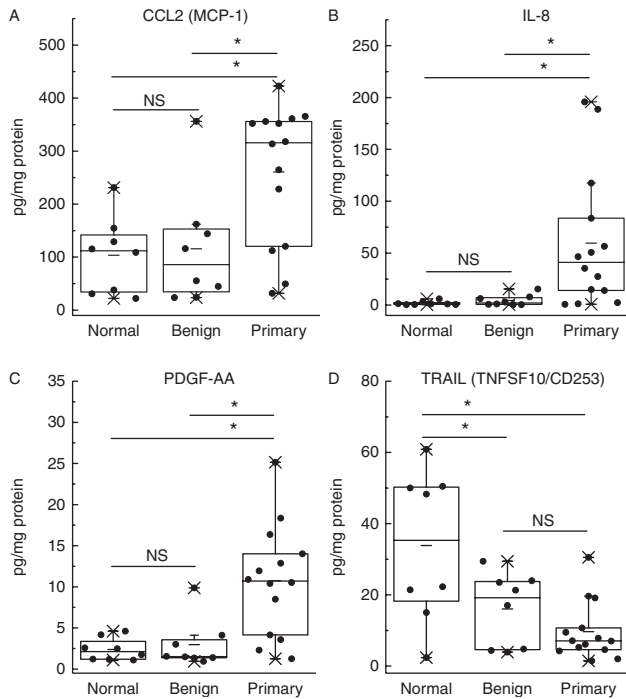


Figure 1

Autotaxin is highly expressed in papillary thyroid cancer but not in benign specimens. Representative immunohistochemical stains of autotaxin at low power (10 \times ; top) and high power (40 \times ; bottom) are illustrated in (A) benign thyroid adenomas ($n=64$), (B) primary tumours ($n=152$) and (C) rabbit IgG isotype control on primary tumours. (D) Autotaxin mRNA measurements in patient samples normalized to GAPDH. $n=7-12$ per group. (E) Non-cancer specimens (normal or benign nodules) have lower LPA levels than cancer (primary or metastasis) specimens (sum of LPA species

C16:0, C18:0, C18:1 and C20:4, normalized to total extracted protein) $n=12-14$ per group. (F) Autotaxin mRNA measurements and (G) activity measurements in conditioned media from primary cultures established from patient tissues $n=5-7$ per group. Box plots show minimum, mean and maximum values (—), 25th, 50th and 75th percentiles (box) and 1st and 99th percentiles (x). * $P<0.05$ by ANOVA with *post hoc* test; NS, not significant. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0045>.

**Figure 2**

(A) Chemokines, (B) cytokines and (C) growth factors are significantly elevated in patient primary thyroid cancer specimens compared to benign nodules and normal thyroid tissue. (D) TRAIL, a driver of apoptosis, is decreased in primary tumors. Multiplex measurement results are normalized to total protein content. Box plots show minimum, mean and maximum values (–), 25th, 50th, and 75th percentiles (box) and 1st and 99th percentiles (x). $n = 8-14$ specimens per group; $*P < 0.05$ by ANOVA with *post hoc* test. NS, not significant. Results for additional inflammatory mediators are presented in [Supplementary Figure 3](#).

of concentrated conditioned medium with 70 μ l of 3 μ M FS-3 substrate, and fluorescence intensity was normalized to cell protein content. All of the agonists used to induce autotaxin expression were purchased from Peprotech (Rocky Hill, NJ, USA). Autotaxin activity was measured in mouse plasma by the choline released from 3 mM C14:0-lysophosphatidylcholine (LPC) (Benesch et al. 2014b).

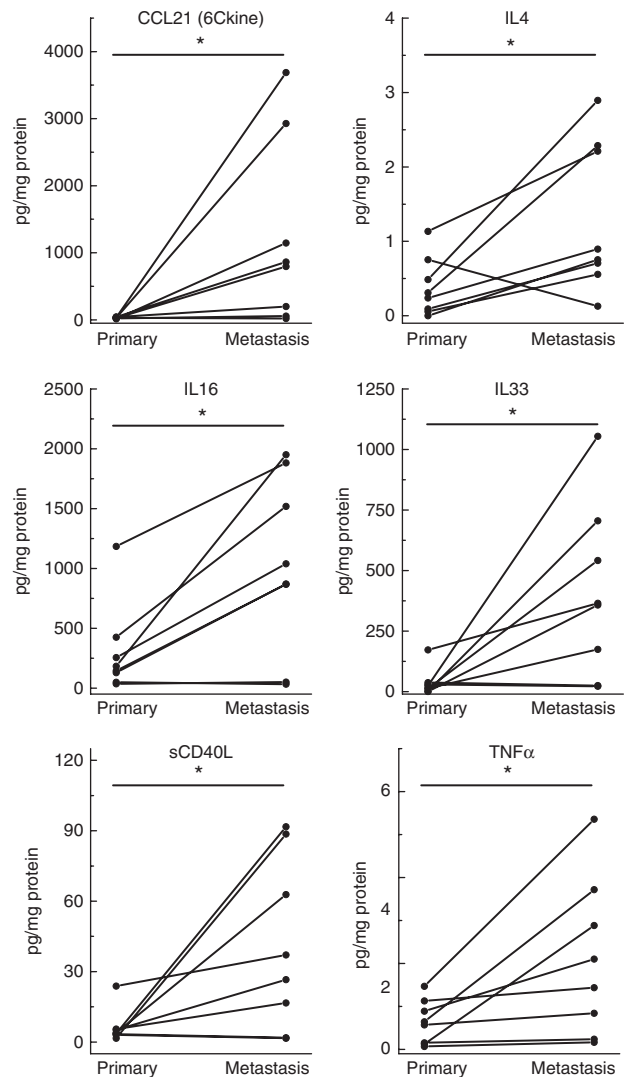
Statistics

Results are means \pm S.E.M. Two-tailed Student's *t*-test, a paired *t*-test and one-way ANOVA with a Bonferonni *post hoc* test were used to test significance as appropriate. Statistics were calculated and results plotted using Origin Pro 9.1 (OriginLab Corporation, Northampton, MA, USA). Correlations for staining were assessed using Fisher's exact test for tables and the Spearman rank correlation for continuous variables.

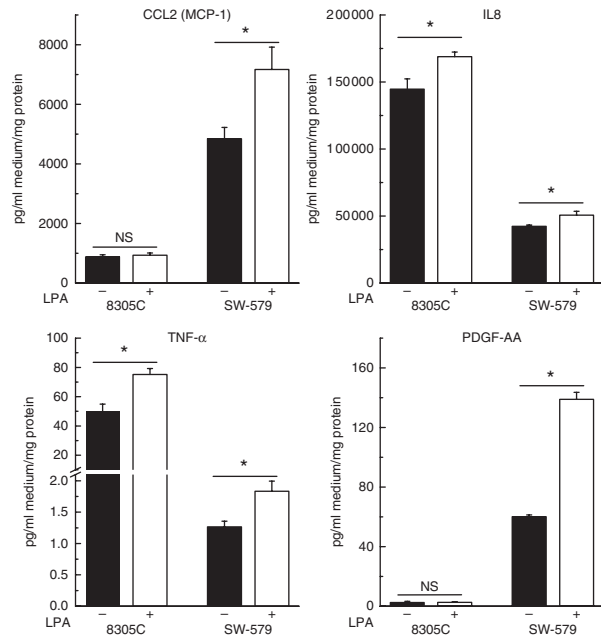
Results

Expression of autotaxin and inflammatory chemokines, cytokines and growth factors is characteristic of malignant thyroid tumours and metastases

[Table 1](#) summarizes the demographic results for tissue arrays comprising thyroid cancer patients with and without metastases. We determined autotaxin expression in primary tumour specimens with ($n = 71$) and without ($n = 123$) nodal metastases as well as in nodal metastatic sections ($n = 15$), benign follicular adenomas ($n = 64$) and

**Figure 3**

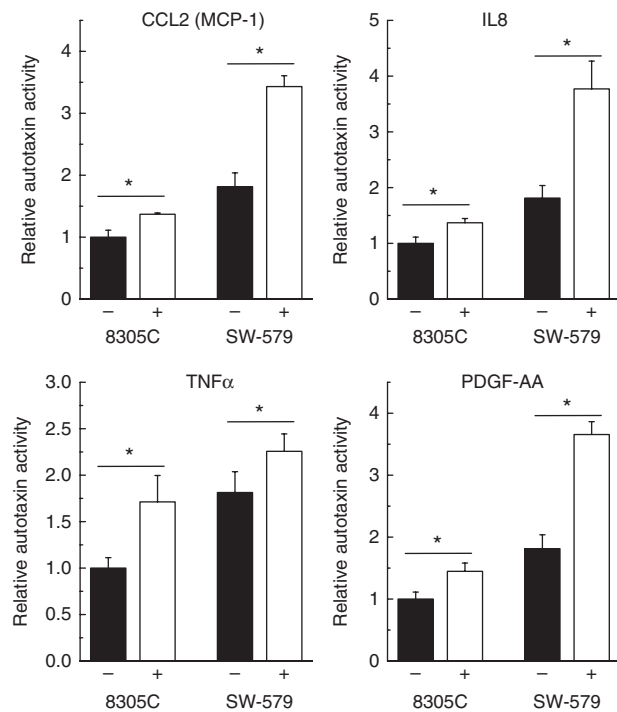
Chemokines and cytokines are significantly elevated in patient-matched thyroid metastatic lymph node nodules (metastasis) compared to primary thyroid tumours (primary). Multiplex measurement results are normalized to total protein content. $n = 8$ matched specimens, $*P < 0.05$ by paired *t*-test.

**Figure 4**

Chemokine, cytokine and growth factor secretion in 8305C and SW-579 thyroid cancer cells is significantly increased by LPA treatment. 8305C and SW-579 cancer cells were treated with 5 μ M LPA for 24 h in 1% FBSC medium and medium was analysed by multiplex measurements. Results are normalized to both total volume of medium and cell protein content. Results are means \pm s.e.m. from four independent experiments, * P <0.05 compared to no treatment; NS, not significant. Results for additional inflammatory mediators are presented in Supplementary Figure 4.

neighbouring normal thyroid tissue ($n=33$) (Table 2). In accordance with clinical standards, patients in this cohort with biopsy-proven papillary thyroid cancer had ipsilateral or bilateral level 6 lymph node dissections to address the possibility of lymphatic metastases. Autotaxin staining was typically cytoplasmic/extracellular (Fig. 1A, B and C). Autotaxin protein expression was undetectable, or minimally so, in about 90% of the non-neoplastic thyroid tissue. In benign neoplastic tissue comprising follicular adenomas, autotaxin expression was typically low, but a small fraction did stain positive for autotaxin (Table 2). On further analysis, autotaxin staining in benign neoplasms correlated strongly with diffuse lymphocytic infiltration or Hashimoto's thyroiditis (P <0.005) (Table 2). More than 80% of the primary papillary thyroid tumours exhibited strong autotaxin staining regardless of age, gender, tumour size, nodal status and multifocality. There was no correlation between *BRAF* status, when known, and autotaxin staining (not shown). Primary tumours were much more likely to demonstrate moderate or strong staining compared to normal thyroid tissue (P <0.001) or

benign neoplasms (P <0.001) (Table 2). Nodal metastases also showed stronger autotaxin staining compared to normal thyroid tissue and benign thyroid neoplasms (P <0.001) (Table 2). mRNA levels obtained from snap frozen specimens further outlined the strong preference for autotaxin expression in malignancy, consistent with previous reports (Barden *et al.* 2003, Kehlen *et al.* 2004, Seifert *et al.* 2008, Hassan *et al.* 2013). Primary papillary thyroid tumours and, even more so, lymph node metastases exhibited mRNA levels between three- and 30-fold higher than normal thyroid tissue or benign tumours (Fig. 1D) (P <0.05). Increased autotaxin mRNA expression in primary tumours compared to normal tissue was confirmed in matching specimens from individual patients, and this increase was even greater in metastatic samples compared to matched primary tumours (P <0.05) (Supplementary Figure 1, see section on supplementary data given at the end of this article). High autotaxin expression in thyroid tumours also correlated with greater

**Figure 5**

Chemokines, cytokines and growth factors increase autotaxin secretion from thyroid cancer cells. 8305C and SW-579 cancer cells were treated with 10 ng/ml CCL2 (MCP-1), IL8 or TNF α , or 50 ng/ml PDGF-AA for 48 h in 1% FBSC. Autotaxin activity in concentrated conditioned medium was normalized to cell protein content. Results are from at least three independent experiments. * P <0.05 compared to no treatment. Results for additional inflammatory mediators are presented in Supplementary Figure 6.

than twofold increases in LPA concentrations compared to the patients without thyroid cancer ($P < 0.01$) (Fig. 1E, Supplementary Figure 2).

To quantify the levels of autotaxin production and to exclude other sources of production, we determined autotaxin expression in purified cells cultured from freshly isolated human tumours. The expression of autotaxin mRNA was tenfold higher in cancer cells from primary tumours and 40-fold higher in cells from metastatic nodules compared to cells from normal or benign thyroid samples (Fig. 1F). Furthermore, significantly higher levels of secreted autotaxin activity (Fig. 1G) were obtained in metastatic specimens compared to primary tumours ($P < 0.01$), benign tumours ($P < 0.01$) or normal thyroid tissue ($P < 0.01$). These results correlated closely with mRNA concentrations identified in corresponding tissues and define a clear role for autotaxin in primary and metastatic papillary thyroid cancer.

We also characterized 64 analytes encompassing regulators of communication between cells and their extracellular environment (chemokines, chemokines, lymphokines, interferons, colony stimulating factors and growth factors) in the human thyroid tissue isolates to define the inflammatory phenotype (Supplementary Table 1 for list of analytes and abbreviations); 15 of these were significantly higher in the papillary thyroid cancer

tumours than in normal thyroid tissue or benign disease (Fig. 2, Supplementary Figure 3, see section on supplementary data given at the end of this article). None of these mediators were increased in benign neoplasms (without lymphocytic infiltrations) compared to normal tissue. In the primary tumours, we observed increases in inflammatory chemokines (CCL family and CXCL1) and a marked elevation of cytokines (Fig. 2, Supplementary Figure 3) involved with immune cell activation and migration (Turner *et al.* 2014). PDGF-AA levels are higher in malignancy (Fig. 2C) indicating probable activation of the PDGFR α cascade and its signalling pathways that are strongly associated with cell migration and angiogenesis as shown for papillary thyroid cancer (Xue *et al.* 2012, Zhang *et al.* 2012). Conversely, the level of TRAIL ligand (CD253), a central driver of apoptosis (Oikonomou & Pintzas 2013), was decreased by 75% in primary tumours compared to normal tissue (Fig. 2D). The levels of all analytes are given in Supplementary Table 2. The metastatic phenotype was emphasized further by elevation of immune cell recruiters and modulators, CCL21, TNF α , sCD40L, IL4, IL16 and IL33 in metastatic specimens matched with primary tumours (Fig. 3). The complete list of inflammatory modulators and the levels identified in primary tumour tissue and patient-matched metastatic deposits of papillary thyroid cancer is shown in Supplementary Table 3.

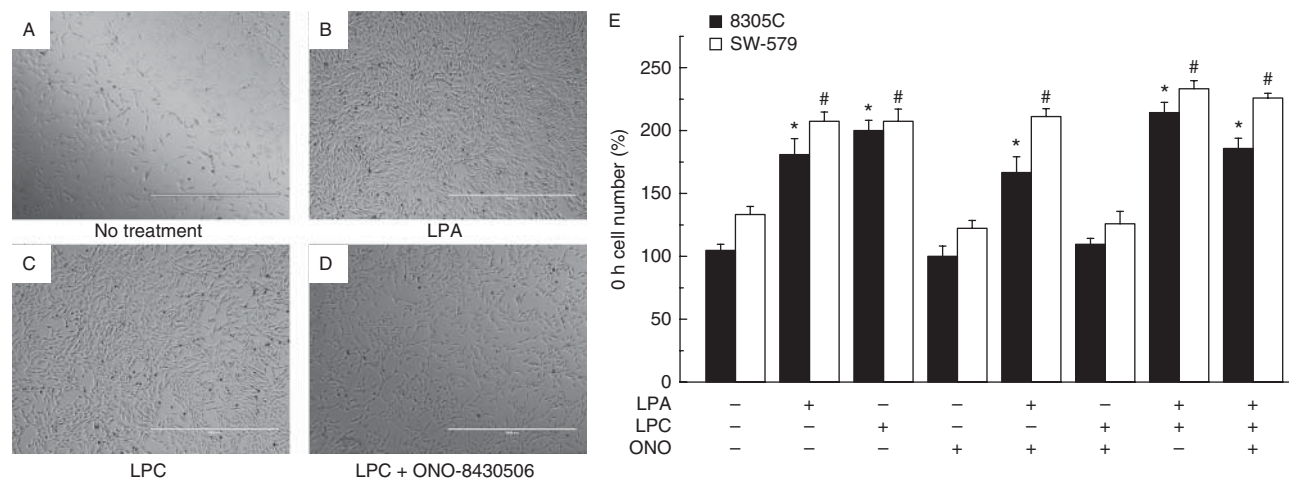


Figure 6

LPA increases the growth rate of thyroid cancer cells, and autotaxin inhibition blocks LPC-mediated cell growth. 8305C and SW-579 cells were grown in serum-free medium supplemented with 1% bovine serum albumin for 36 h and treated with or without 5 μ M LPA, 100 μ M LPC or 10 μ M of the autotaxin inhibitor ONO-8430506. (A, B, C and D)

Representative photographs of SW-579 cells after 36-h treatment. Scale bar = 1000 μ m. (E) Counts of cells at the end of the 36-h treatment. Each sample represents three independent experiments, and results are expressed as means \pm s.e.m. * $P < 0.05$ compared to no treatment for 8305C cells; # $P < 0.05$ compared to no treatment for SW-579 cells.

Autotaxin through LPA formation initiates a vicious cycle of inflammatory chemokine, cytokine and growth factor expression in human thyroid cancer cells

We next explored the mechanistic relationship between the elevated expression of autotaxin and inflammatory mediators using 8305C and SW-579 thyroid cancer cells. Incubation with LPA for 24 h increased the secretion of 16 chemokines, cytokines and growth factors with the exception of CCL2 and IL1 α in 8305C cells (Fig. 4, Supplementary Figure 4, see section on supplementary data given at the end of this article). These changes were accompanied by increased concentrations of the corresponding mRNAs (Supplementary Figure 5). Conversely, incubating the cancer cell lines with ten inflammatory chemokines and cytokines and the growth factors PDGF-AA and BB markedly increased autotaxin mRNA expression and secretion (Fig. 5, Supplementary Figures 6 and 7). These results establish the importance of autotaxin in mediating a broad activation of the inflammatory cycle.

Thyroid cancer cell division was stimulated by LPC, and this depended on the catalytic activity of autotaxin since the autotaxin inhibitor ONO-8430506 completely blocked the effect (Fig. 6). As controls, we demonstrate that ONO-8430506 itself does not block LPA-induced cell division. Furthermore, the effects of LPC on cell division were additive with those of CCL2, IL1 β , IL6, IL8, GCSF, PDGF-AA, sCD40L and TNF α . The autotaxin inhibitor ONO-8430506 blocked only the LPC-dependent component of this growth (Fig. 7, Supplementary Figure 8).

Inhibition of autotaxin in mouse models of papillary thyroid cancer decreases tumour growth and abrogates the malignant inflammatory phenotype

We hypothesized from the results described above that blocking LPA signalling would have two effects in thyroid tumour growth: it would attenuate both the direct effects of LPA and the indirect actions of the increased production of inflammatory mediators. This hypothesis was tested using a SCID mice model with the human thyroid cancer cell lines 8305C and SW-579, which are also highly tumorigenic. Daily dosing with 20 mg/kg of the autotaxin inhibitor ONO-8430506 at 7 days after establishing the tumours decreased tumour growth by nearly 60% (Fig. 8A and B). The inhibitor was well tolerated in mice, since no differences were seen in body weights between vehicle and ONO-8430506-treated groups (Supplementary Figure 9, see section on supplementary data given at the end of this article). This highly potent and selective autotaxin

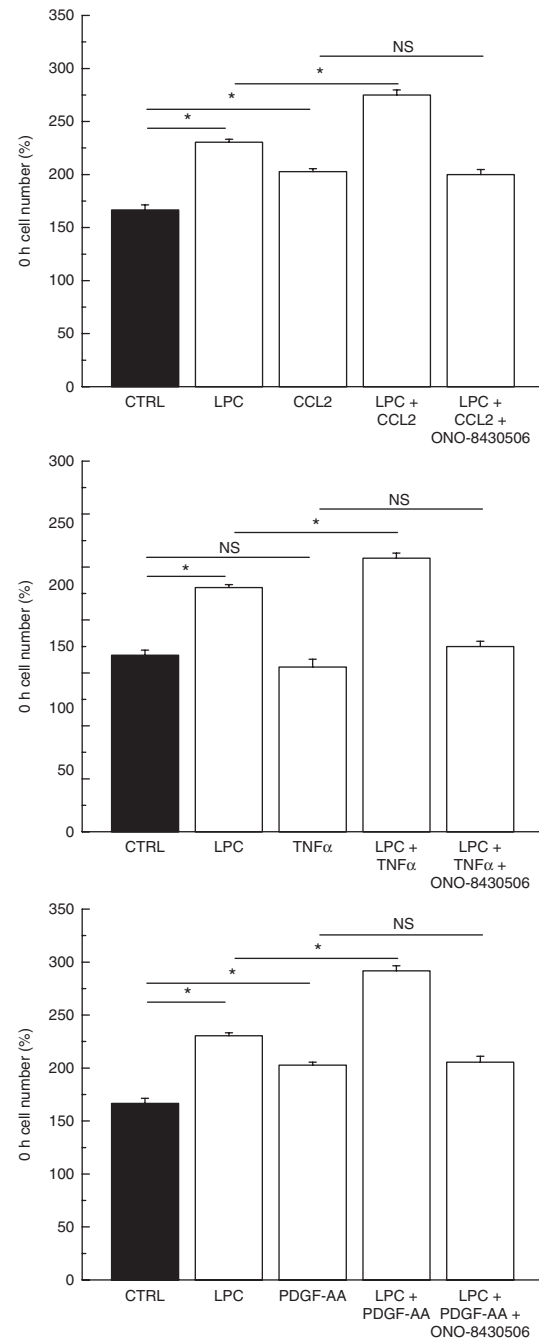


Figure 7

Effects of incubating SW-579 thyroid cancer cells with LPC and chemokines, cytokines or growth factors. SW-579 cells were grown in serum-free medium supplemented with 1% bovine serum albumin for 36 h and treated with or without 100 μ M LPC, 10 ng/ml CCL2 or TNF α , or 50 ng/ml PDGF-AA and 10 μ M of the autotaxin inhibitor ONO-8430506. Cells were then counted at the end of the 36-h treatment. ONO-8430506 blocked LPC-dependent cell growth. Each sample represents three independent experiments and results are expressed as means \pm s.e.m. CTRL, no treatment (control); * P < 0.05; NS, not significant. Results for additional inflammatory mediators are presented in Supplementary Figure 8.

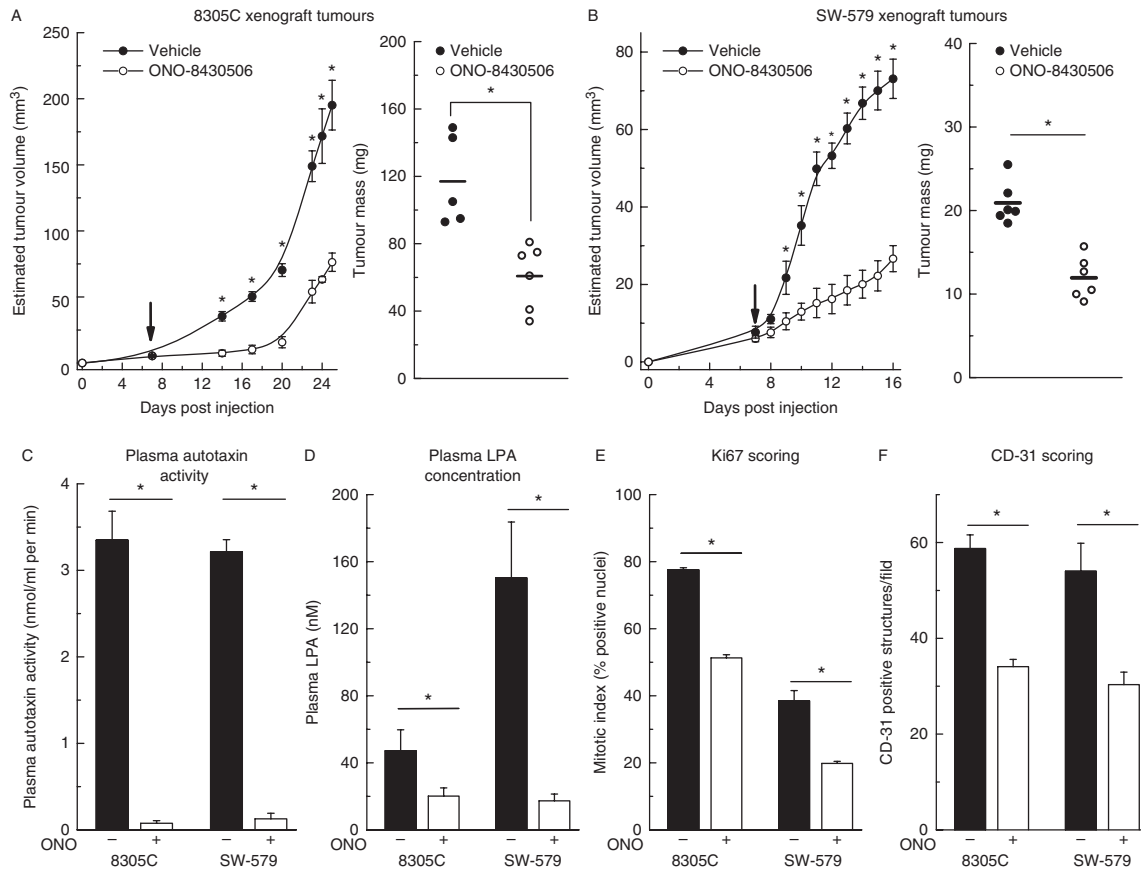


Figure 8

Autotaxin inhibition with ONO-8430506 slows the growth of xenograft thyroid tumours in SCID mice and reduces plasma autotaxin activity, LPA concentrations, tumour Ki67 and CD-31 staining. (A) Estimated tumour size and excised tumour mass from mice injected with 8305C cancer cells. $n=5-6$ per group. (B) Estimated tumour size and excised tumour mass from mice injected with SW-579 cancer cells. $n=6$ per group. In both experiments mice were gavaged daily with either vehicle or 20 mg/kg

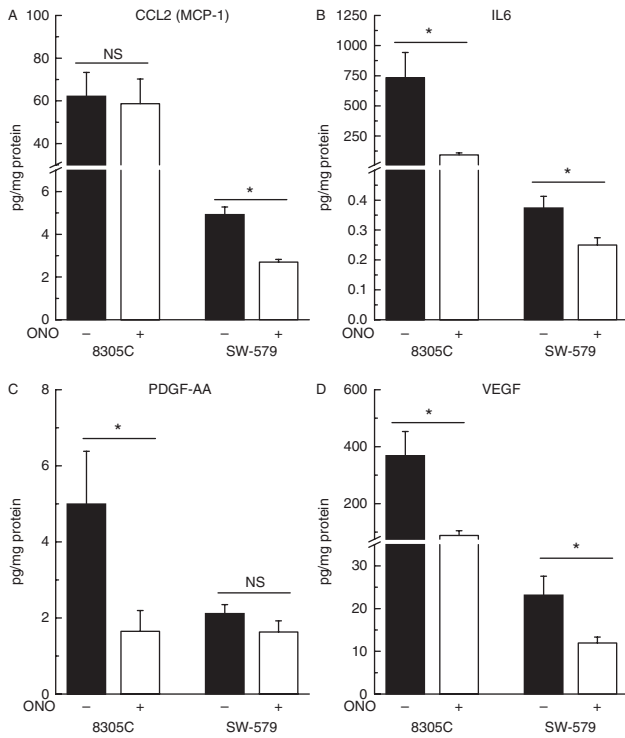
ONO-8430506 after 7 days of tumour growth (arrow). (C) Autotaxin activity and (D) LPA concentrations (sum of LPA species C16:0, C18:0, C18:1 and C20:4) in plasma collected 6 h after last treatment. $n=5-6$ per group. (E) Quantification of nuclear Ki67 staining and (F) quantification of CD-31 positive structures. $n=3-4$ per group. Representative photographs are in [Supplementary Figure 10](#). All results are means \pm s.e.m. * $P < 0.05$ compared to vehicle treatment.

(ENPP2) inhibitor does not inhibit the activity of other ENPP family members nor does it affect ligand binding to 60 tested pharmacological targets (Saga *et al.* 2014). Effective autotaxin blockade with ONO-8430506 was demonstrated by the decreased plasma autotaxin activity (>90%) and LPA concentrations (>70%) (Fig. 8C and D). This was associated with a decreased mitotic rate and vascularity of the tumours as quantified by Ki67 (Fig. 8E, [Supplementary Figure 10A](#)) and CD-31 staining (Fig. 8F, [Supplementary Figure 10B](#)) respectively. Inhibition of autotaxin also decreased the concentrations of 14 inflammatory chemokines and cytokines within the tumours (Fig. 9A and B, [Supplementary Figure 11](#)). The effects on angiogenesis are compatible with the decreased PDGF-AA and VEGF concentrations caused by ONO-8430506 (Fig. 9C and D). Staining for the inhibited autotaxin in

tumours showed that it was confined to the cancer cells ([Supplementary Figure 10C and D](#)). Autotaxin inhibition did not alter the plasma concentrations of sphingosine-1-phosphate and sphinganine-1-phosphate, sphingolipid analogs of LPA, which are not produced significantly by autotaxin (results not shown) (31–33).

Discussion

The present study provides a new paradigm for understanding the role of inflammation in papillary thyroid cancers. First, we show that autotaxin is a potent inflammatory mediator. High autotaxin expression and the resulting elevation in LPA concentrations are associated with an increased expression of inflammatory chemokines, cytokines and growth factors. This phenotype

**Figure 9**

(A) Chemokines, (B) cytokines and (C–D) growth factors in 8305C and SW-579 xenograft thyroid tumours are significantly suppressed by ONO-8430506 treatment compared to vehicle treatment. Mice were treated daily with 20 mg/kg of the autotaxin inhibitor ONO-8430506, and vehicle mice were gavaged with water. Tumours were collected 6 h after last treatment. Multiplex measurement results are normalized to total protein content. Results are means \pm s.e.m. for 4–6 mice per group. * $P < 0.05$ compared to vehicle treatment; NS, not significant. Results for additional inflammatory mediators are presented in Supplementary Figure 11.

distinguishes malignant nodules and inflamed neoplasms from normal tissues and benign disease without leukocytic infiltration. Second, we document that the progressive inflammatory response persists in metastatic tissue where similar or even higher levels of inflammatory cytokines are observed compared to primary tumours. The inflammatory response leads to larger tumours with an increased mitotic rate and vascularity. This is similar to what is observed in aggressive disease where tumour size, mitotic rate and vascularity are linked with decreased survival (Chou *et al.* 2014). Third, treatment with ONO-8430506 decreases plasma LPA concentrations by $>70\%$ as well as significantly decreasing the concentrations of several inflammatory modulators in our mouse models of thyroid cancer. These effects were accompanied by decreased tumour growth, which was associated with a decreased proliferative index and vascularity of these tumours.

The increased LPA concentrations in thyroid tumours relative to benign growths could result from increased

glycerolipid synthesis associated with cancer cell growth. However, LPA from the biosynthetic pathway should not be released by cancer cells and thereby should not stimulate external LPA receptors. Furthermore, the concentration of C20:4-LPA, which is not produced in the de novo pathway, was also increased. Although polyunsaturated LPA species can be produced by phospholipase A₁ activities, autotaxin is responsible for the majority of LPA production (Benesch *et al.* 2014a). This conclusion is also supported by the observation that autotaxin inhibition decreases the concentrations of polyunsaturated LPA species in mouse breast tumours (Benesch *et al.* 2014b).

Despite the availability of autotaxin inhibitors over the last decade, most do not have good bioavailability (Federico *et al.* 2008, Albers & Ovaia 2012, Barbayianni *et al.* 2013, 2015, Benesch *et al.* 2014a, 2015). By contrast, a daily dosage of mice with ONO-8430506 substantially decreased plasma autotaxin activity and LPA concentrations, and it produced a 50–60% decrease in breast tumour (Benesch *et al.* 2014b) and thyroid tumour growth. We propose that ONO-8430506, as a selective agent disrupting autotaxin activity, represents a new option for therapy for aggressive, metastatic papillary thyroid carcinoma. Autotaxin blockade also decreases the production of inflammatory mediators, which in other tumours promotes tumorigenesis, immune evasion and treatment resistance (Brindley *et al.* 2013, Tjomsland *et al.* 2013, Benesch *et al.* 2014a, Wang *et al.* 2014, Li *et al.* 2015).

Sustained inflammatory signalling and high autotaxin expression are correlated with the progression of hepatitis and inflammatory bowel diseases into cancer (Benesch *et al.* 2014a). In this study, autotaxin immunohistochemical staining was significantly elevated in benign adenomas with lymphocytic infiltration and Hashimoto's thyroiditis compared to those adenomas without infiltration. Progression of Hashimoto's thyroiditis to thyroid cancer has been proposed by several studies (Bozec *et al.* 2010, Azizi *et al.* 2014, Ehlers & Schott 2014). Our results demonstrate that autotaxin inhibition decreases the inflammatory phenotype of thyroid tissue, possibly disrupting the potential for progression to malignant thyroid disease.

Our work with cultured thyroid cancer cells explains the connection between LPA signalling and inflammation in thyroid cancer that was inferred by previous studies (38–41). Autotaxin activity and LPA formation stimulated the production of inflammatory mediators, which resulted in a vicious cycle of more autotaxin secretion (Fig. 10). This inflammatory phenotype increased the division of thyroid cancer cells, and the component that depends

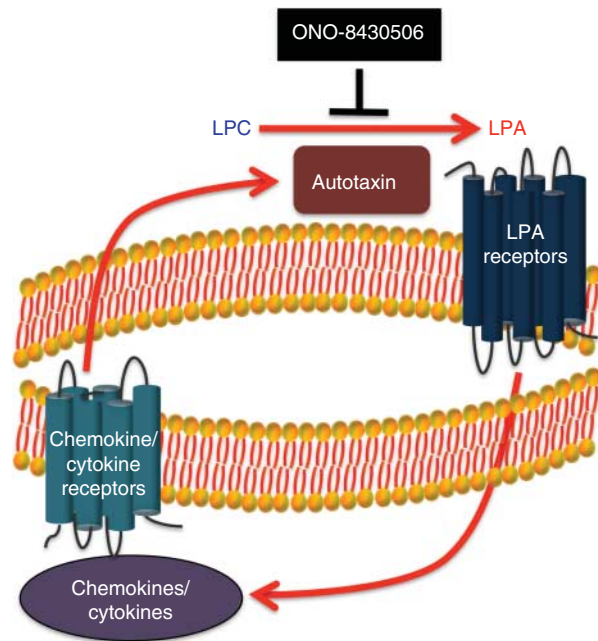


Figure 10

Model of the relationship between autotaxin/LPA and inflammatory chemokine and cytokine production and signalling in thyroid cancer cells. Autotaxin produces LPA by hydrolysing the choline headgroup from LPC. LPA signalling through its receptors leads to an increase in chemokine and cytokine production. These inflammatory mediators signal through their own receptors to further induce autotaxin production, establishing a vicious cycle that drives thyroid cancer progression. This cycle can be broken by inhibiting autotaxin catalytic activity with ONO-8430506, which in turn reduces both LPA and chemokine/cytokine concentrations and ultimately slows thyroid tumour growth. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-15-0045>.

directly on LPA formation was blocked by autotaxin inhibition. LPA also had an indirect effect on tumour growth by stimulating the production of inflammatory mediators, which also promotes growth. An interesting concept is that autotaxin may provide a singular focus for initiating the global inflammatory response in tumours, although the inflammatory mediators most important to particular cancer types may vary. For example, IL1 α , IL6 and IL17 are involved in pancreatic, gastric and colorectal cancers respectively (Tjomsland *et al.* 2013, Wang *et al.* 2014, Li *et al.* 2015). In general, autotaxin/LPA signalling and the subsequently upregulated inflammatory programs drive tumour growth, immune evasion, metastasis, resistance to chemotherapy and decreased efficacy of radiotherapy in major cancers. These include melanomas, neuroblastomas, glioblastoma multiforme and breast, lung, colon, ovarian, renal cell and hepatic cancers (Gaetano *et al.* 2009, Samadi *et al.* 2009, Brindley *et al.* 2013, Hozumi *et al.* 2013, Benesch *et al.* 2014a,b, Venkatraman *et al.* 2015).

We used two SCID mouse models with human thyroid cancer cells to demonstrate the existence of the autotaxin-LPA-inflammatory cycle *in vivo*. These mice have severe decreases in T and B lymphocyte expression, which prevents immune rejection of heterologous cancer cells. However, we could still determine the effects of the autotaxin inhibitor ONO-8430506 on the production of inflammatory mediators by the cancer cells themselves. ONO-8430506 had a long-acting effect in decreasing autotaxin activity and LPA production. As a result it effectively broke the inflammatory cycle by decreasing the concentrations of inflammatory cytokines in the thyroid tumours, resulting in decreased tumour growth. Our mouse models of thyroid cancer, which employ flank injections of cancer cells, do not lead to significant metastasis. However, inhibiting autotaxin activity and LPA signalling does decrease metastasis in other mouse models (Zhang *et al.* 2009, Schleicher *et al.* 2011, Benesch *et al.* 2014b, Venkatraman *et al.* 2015).

Tumours have been likened to 'wounds that do not heal' (Dvorak 1986, Schafer & Werner 2008, Hanahan & Weinberg 2011) and autotaxin is part of the defence mechanism used by the body to facilitate wound healing (Nakanaga *et al.* 2010, Okudaira *et al.* 2010). However, once wound repair is complete, autotaxin production levels should decrease. We recently demonstrated that LPA suppresses autotaxin production in a feedback regulatory loop. This feedback regulation is overcome by inflammatory mediators, such that high LPA and autotaxin levels can coexist in unresolved inflammation (Benesch *et al.* 2015).

In conclusion, we show that increased expression of autotaxin, LPA and inflammatory mediators is characteristic of malignant thyroid disease. Thus, autotaxin is an integral component of tumorigenesis in thyroid follicular cells. This includes driving tumour growth and promoting markers of more aggressive tumour phenotypes including proliferation, vascularity, metastasis, immune evasion and treatment resistance (Britschgi *et al.* 2012, Pylayeva-Gupta *et al.* 2012, Brindley *et al.* 2013, Chung *et al.* 2013, Benesch *et al.* 2014a). The inflammatory process mediated by autotaxin in thyroid cells is an autocrine cycle. This paradigm probably extends to other cancers such as glioblastomas and melanomas where autotaxin is secreted by the cancer cells (Kishi *et al.* 2006, Jankowski 2011). By contrast, breast cancer cells do not normally make significant quantities of autotaxin. Instead, the inflammatory milieu produced by the breast tumour stimulates autotaxin secretion by adjacent adipose tissue (Benesch *et al.* 2014b). In both scenarios, breaking the inflammatory

cycle with a long-acting autotaxin inhibitor decreases tumour growth in mice. Therefore, regardless of where autotaxin is produced, autotaxin inhibition deprives cancer cells of LPA and the consequent secretion of a large variety of inflammatory chemokines and cytokines, which drive cancer progression. So far, no autotaxin inhibitor has been introduced into clinical practice. Our results support the use of autotaxin inhibition as a potentially new strategy for counteracting the autotaxin-LPA-inflammatory axis, which drives the progression of thyroid and other cancers.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0045>.

Declaration of interest

D N Brindley and T P W McMullen received consulting fees from Ono Pharmaceuticals Ltd, the developers of the autotaxin inhibitor ONO-8430506 used in this study. The other authors declare no conflicts of interest.

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Author contribution statement

M G K Benesch, D N Brindley and T P W McMullen conceived and designed the study. D N Brindley and T P W McMullen were responsible for financial support. M G K Benesch, Y M Ko, X Tang, J Dewald and A Lopez-Campistrous performed the experiments. R Lai and T P W McMullen provided study specimens and completed immunohistochemical scoring. Y Y Zhao and J M Curtis completed mass spectrometry measurements. M G K Benesch, R Lai, D N Brindley and T P W McMullen analysed and interpreted the results. D N Brindley and T P W McMullen supervised the project. M G K Benesch, D N Brindley and T P W McMullen wrote the manuscript, and all authors gave final approval to the manuscript.

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