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Cancer Therapy: Preclinical

Clinical Cancer Research

Human Anaplastic Thyroid Carcinoma Cells Are Sensitive to NK Cell–Mediated Lysis via ULBP2/5/6 and Chemoattract NK Cells

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Abstract

Purpose: Anaplastic thyroid carcinoma (ATC) is one of the most aggressive forms of cancer with no curative therapies available. To date, strategies to target ATC by immunotherapy have not been evaluated. We investigated whether ATC would be a suitable target for natural killer (NK) cell–based immunotherapy.

Experimental Design: We first established seven new cell lines from ATC tumors, three from papillary thyroid carcinoma tumors and analyzed them together with eight additional ATC cell lines. Cells were analyzed for sensitivity to lysis by NK cells and their ability to chemoattract and regulate the activity of NK cells. In addition, fresh tumor samples and peripheral blood from six patients with ATC were analyzed for NK cell infiltration and phenotype.

Results: We observed that ATC cell lines are sensitive to lysis by *ex vivo* expanded NK cells and that the lysis was abrogated upon blockade of NKG2D. Sensitivity of thyroid cancer cell lines to NK cell–mediated lysis correlated with surface expression of UL16-binding protein 2 on tumor cells. Moreover, ATC cell lines produced high levels of CXCL10 and stimulated migration of expanded NK cells and ATC tumors were enriched for NK cells expressing the cognate chemokine receptor CXCR3. However, compared with NK cells in peripheral blood, ATC tumor–derived NK cells displayed a suppressed phenotype with a downregulated expression of NKG2D. *In vitro*, suppression of NK cell–mediated lysis and NKG2D expression by ATC cells was restored upon neutralization of prostaglandin-E2.

Conclusions: ATC cell lines are sensitive to NK cell–mediated lysis via ULBP2/5/6 and chemoattract CXCR3-positive NK cells. Patients with ATC may benefit from NK cell–based immunotherapy. *Clin Cancer Res; 20(22); 5733–44. ©2014 AACR.*

Introduction

With a rapid proliferation, resistance to apoptosis, and highly invasive and metastatic properties, anaplastic thyroid carcinoma (ATC) is one of the most aggressive types of human cancer (1). ATC cells can arise either *de novo* or from a dedifferentiation of papillary thyroid carcinoma (PTC) or follicular thyroid carcinoma (FTC) cells, rendering them highly mitotic and metastatic. The 5-year survival rate of ATC is less than 7% and current therapies, comprising radical thyroidectomy, radiosensitizing chemotherapy, and external beam radiotherapy, are rarely curative (2). Few efforts have been made at targeting thyroid cancer by immunotherapy. Dendritic cell (DC) vaccination trials against medullary thyroid cancer (MTC) have been performed using full-length calcitonin-primed DCs, which have shown antitumor activity (3). In contrast to PTC and FTC, where several tumor-associated antigens (TAA) have been identified, there are no immunogenic proteins described for ATC (4). Natural killer (NK) cells are innate lymphocytes that can kill tumor cells without prior sensitization to an antigen. They can be divided into 2 main subsets on the basis of their surface expression of CD56. The $CD56^{dim}$ NK cells have potent cytotoxic capacity, whereas the $CD56^{bright}$ NK cells have an immunoregulatory role through secretion of cytokines. The use of NK cells in adoptive cell therapy has been successful in a limited number of patients with acute myeloid leukemia (AML) and other hematologic malignancies (5). However, few clinical responses have been observed in patients with solid

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Translational Relevance

There are currently no curative treatments available for anaplastic thyroid carcinoma (ATC), which is considered to be one of the most aggressive cancer types in humans. There are few tumor types that are responsive to natural killer (NK) cell therapy, particularly in solid tumors. This is attributable to resistance of tumor cells to NK cell lysis, poor homing, and intratumoral infiltration of NK cells as well as immunosuppression of NK cells in the tumor site. We find that ATC cell lines are sensitive to lysis by NK cells correlating with their surface expression of ULBP2/5/6, indicating that ULBP2/5/6 could be used as a predictive marker for NK cell therapy. The clinical relevance is further strengthened by our finding that ATC tumors are able to attract CXCR3-positive NK cells. On the basis of our findings, NK cell therapy could prove to be a promising novel treatment strategy for patients with ATC.

tumors (6). In an ongoing clinical study, we observed that 7 of 14 patients with advanced solid tumors had stable disease after infusions with expanded NK cells. Two of the patients developed acute thyroiditis (7). The activity of NK cells is regulated through a balance of activating and inhibitory signals emanating from a repertoire of receptors expressed on the cell surface. One of the most studied receptors is the natural killer group 2, member D (NKG2D) receptor, which is constitutively expressed on NK cells. Expression of NKG2D is not necessary for NK cell development but is critical for immunosurveillance of cancer (8). Upon binding of the NKG2D receptor with its ligands, MHC class I chain–related chain A/B (MICA/B) or the UL16-binding proteins (ULBP 1–6), NK cells release perforin and granzyme to induce apoptosis of the target cells (9). It has previously been shown that tumors overexpressing ULBP2 through activation of wild-type p53 are more sensitive to lysis by NK cells (10).

Intratumoral infiltration of NK cells has been reported to correlate with good prognosis (11, 12). Tumor cells can be stimulated to secrete the chemokines CXCL9, CXCL10, and CXCL11, which attract NK cells expressing the chemokine receptor CXCR3 (13). In mice, the expression of CXCR3 on NK cells is essential for homing toward CXCL10-producing solid tumors, and in humans, CXCL10 secretion from tumors is found to be a strong prognostic marker for infiltration of cytotoxic T cells, which in turn correlates with a favorable prognosis (14, 15). Although NK cells do infiltrate tumor cells, it is well known that tumor cells are immunosuppressive and limit the activity of NK cells. Among the more studied NK cell immunosuppressive factors are TFGβ, arginase-1, indoleamine 2,3-dioxygenase (IDO), inducible nitric oxide (iNOS), and prostaglandin-E2 (PGE2), which have been shown to downregulate activating receptors, including NKG2D, as well as suppress NK cell cytotoxicity (16). Thus, tumors that exert little immunosuppression, secrete high amounts of CXCL10, and express high levels of NKG2D ligands are suitable for cell therapy using *ex vivo* expanded NK cells, which we previously have described to be more cytotoxic than resting NK cells (17). In the present study, we sought to investigate whether ATC is a suitable target for NK cell therapy. We found that low-passage ATC cell lines express high levels of the NKG2D ligands ULBP2/5/6 and produce high amounts of CXCL10 in response to low-dose IFNγ. As a consequence, ATC cell lines were highly susceptible to NK cell lysis and effectively chemoattracted NK cells, respectively. We further found that in patients with ATC, intratumoral NK cells are positive for CXCR3 but display a suppressed phenotype compared with peripheral blood NK cells. The direct suppressive effect of ATC cells was pronounced in COX2-positive cell lines and blockade of PGE2 reduced the suppressive effect of ATC on NK cells. On the basis of our findings, we propose that ATC represents a good candidate target for NK cell–based adoptive therapy.

Materials and Methods

Patient samples

ATC cells were obtained using conventional fine-needle aspiration (FNA) technique under approved ethical permits according to the Declaration of Helsinki (#522/2008, #KS 91:86/9104). The thyroid nodules were punctured using a 0.6 or 0.7×30 mm^2 needle. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using density centrifugation. Cells (FNA and PBMC) were stained with fluorescence-conjugated monoclonal antibodies against human CD3, CD56, NKG2D, CD69, CXCR3, CD11c, MICA/MICB, IgG1-PE, IgG1-APC, 7AAD (Biolegend); ULBP1, ULBP2/5/6, ULBP3 (R&D Systems); and LIVE/DEAD marker (Invitrogen) and analyzed by flow cytometry. All flow cytometric data were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

Cell lines

All thyroid cancer cell lines are listed in Supplementary Table S1 except the LUTC-5 (PTC) and LUTC-17 (ATC) cell lines. The LUTC cell lines were established from FNA taken from thyroid tumors before initiating cancer treatment. Single tandem repeat analysis was performed on early passages (4–10 passages) of the cell lines LUTC-1, LUTC-2, LUTC-8, LUTC-10, LUTC-12, and LUTC-14, as well as the K562 cell line (Supplementary Table S2). The U-Hth cell lines and C643 and SW1736 were kindly provided by Dr. Nils-Erik Heldin (Uppsala University, Uppsala, Sweden). The K562, MOLT-4, and EST112 cell lines were purchased from ATCC or ESTDAB (http://www.ebi.ac.uk/ipd/estdab/). The remaining cell lines were not verified within 6 months of manuscript submission. All cell lines were maintained in RPMI-1640 supplemented with 10% FBS. Cell lines were stained with fluorescence-conjugated monoclonal antibodies against HLA-ABC, MICA/B (Biolegend); ULBP1, ULBP-2/5/6, and ULBP3 (R&D Systems). Intracellular flow cytometric staining for COX2 (Biolegend) was performed

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using a detergent-based permeabilization protocol (BD Biosciences). Tumor cells were seeded at 2×10^5 cells per well in a 24-well plate and incubated at 37°C overnight. The following day, recombinant human IFNγ (PeproTech) was added to the cells and incubated overnight. Supernatants were collected and analyzed for CXCL10 by ELISA according to the manufacturer's instructions (R&D Systems).

Isolation and expansion of NK cells

NK cells were isolated from PBMC by negative selection (NK cell isolation kit, Miltenyi Biotech). NK cells that were assayed directly after purification without any cytokine stimulation were termed resting NK cells. Purified NK cells were cocultured with irradiated (100 Gy) Epstein–Barr virus–transformed lymphoblastic cells (EBV-LCLs) at a 10:1 (feeder:NK) cell ratio in X-vivo 20 medium (Lonza) supplemented with 10% AB serum (Karolinska Hospital) and 1000 U/ml IL2 (Novartis Pharma) at 37°C. Medium was replenished with 500 U/ml IL2 on days 5, 8, and 11. NK cells assayed on days 11 to 14 of expansion were termed expanded NK cells. The phenotype of NK cells was analyzed by flow cytometry by staining with fluorescence-conjugated monoclonal antibodies directed against human CD3, CD56, NKG2D, NKp30, CD16, CD69, DNAM-1, TRAIL, CXCR3, FasL, or CD14 (Biolegend). Where indicated, NK cells were treated with ATC cell supernatant for 24 hours before assaying for cytotoxicity and phenotype.

Cytotoxicity assay

Target cells were labeled with ^{51}Cr (30 μCi; PerkinElmer) for 45 minutes at 37°C. Expanded NK cells were seeded in a 96-well U-bottom plate (Corning Lifesciences) together with target cells (10,000 cells per well) and incubated for 4 to 24 hours at 37°C. Supernatants were thereafter transferred to 96-well LUMA plates (Perkin Elmer) and analyzed for chromium release in a TRILUX 1450 Microbeta scintillation counter (Perkin Elmer). Where indicated, NK or ATC cells were pretreated with blocking antibodies against TRAIL (RIK-2, 10 μg/mL), Fas ligand (NOK-1, 2.5 μg/mL), NKG2D (1D11, 10 μg/mL), DNAM-1 (TX25, 5 μg/mL; Biolegend); ULPB2/5/6 (polyclonal goat IgG, AF1298, R&D Systems); or concanamycin A (100 nmol/L; Sigma-Aldrich).

Migration assay

Tumor cells were cultured in a 24-well plate to 80% confluency and then treated with IFNγ (5 ng/mL) for 24 hours. Thereafter, tumor supernatant (600 μL) was transferred to a 24-well plate. Expanded NK cells (2.5×10^5) were added in 100 μL medium to Transwell inserts (5-μm pore size, Cell Biolabs) and incubated for 2 hours at 37°C. Inserts were thereafter blinded, washed in PBS, fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes, and stained with hematoxylin (Histolab Products AB). Images of Transwell membranes were acquired with an Olympus CKX41 microscope using the CellsenseEntry Software (Olympus). The number of stained cells per membrane was enumerated using the ImageJ software (NIH, Bethesda). Where indicated, human CXCL10 capture antibody (840420, R&D Systems) was present in the lower wells during the migration assay.

Immunohistochemistry

Paraffin-embedded tissues were sectioned and stained with polyclonal goat anti-human ULBP2/5/6 (polyclonal goat IgG, AF1298, R&D Systems) or polyclonal rabbit anti-human CXCL10, PAB19527, Abnova). Slides were deparaffinized, rehydrated, and subsequently boiled in citrate buffer (pH 6). Slides were blocked with 0.5% hydrogen peroxide (30 minutes) followed by 5% horse serum (30 minutes). Primary antibodies diluted in 1% BSA were added and incubated overnight at 4°C. Horse anti-goat secondary antibody diluted in 1% BSA was added to the slides and incubated at room temperature for 30 minutes. ABC peroxidase solution (Vector Laboratories) was added to the slides and incubated for 30 minutes at room temperature followed by staining with 3,3′-diaminobenzidine (DAB; Vector Laboratories) according to the manufacturer's instructions. Slides were stained with hematoxylin for 1 minute and dehydrated using EtOH and xylene and were subsequently mounted using permanent mounting media (Pertex, Histolab products AB).

Results

ATC cell lines are sensitive to lysis by expanded NK cells

In vitro, the majority of ATC cell lines were sensitive to lysis by expanded NK cells, and several of the cell lines were equally or more sensitive to NK cell lysis than the NK cell sensitive cell line K562. In contrast, the PTC cell lines were less sensitive to NK cell lysis than the ATC cell lines (Fig. 1A and B and Supplementary Table S1) ATC cells from the NK cell–sensitive ATC cell lines LUTC-10, U-Hth 83, and U-Hth 104 as well as nonmalignant thyroid cells isolated from atoxic goiter tissue were positive for MHC class I (Fig. 1C). Furthermore, the expression of MHC class I was slightly higher in PTC cell lines than in the ATC cell lines. However, there was no significant correlation observed between the level of NK cell–mediated lysis and MHC class I expression in a linear regression analysis of 7 ATC cell lines and 2 PTC cell lines ($R^2 = 0.35$, $P = 0.1$; data not shown). Importantly, expanded NK cells did not display any significant cytotoxicity against nonmalignant thyroid cells, immortalized BJ fibroblast cells, or autologous B and T cells (Supplementary Fig. S1A).

Blockade of NKG2D abrogates NK cell–mediated killing of ATC cell lines

In vitro, resting NK cells were ineffective at lysing ATC cells compared with expanded NK cells, whereas the levels of lysis of the NK cell sensitive cell line MOLT-4 by resting and expanded NK cells were comparable (Fig. 2A). The experiment was repeated with the LUTC-1, LUTC-10, and LUTC-12 cell lines with similar results (1.6% ± 3.6% vs. 50.4% ± 19.0% specific lysis, $P = 0.0023$, $n = 4$; data not shown). The NKG2D receptor is ubiquitously expressed on NK cells, although we show that NKG2D expression is increased

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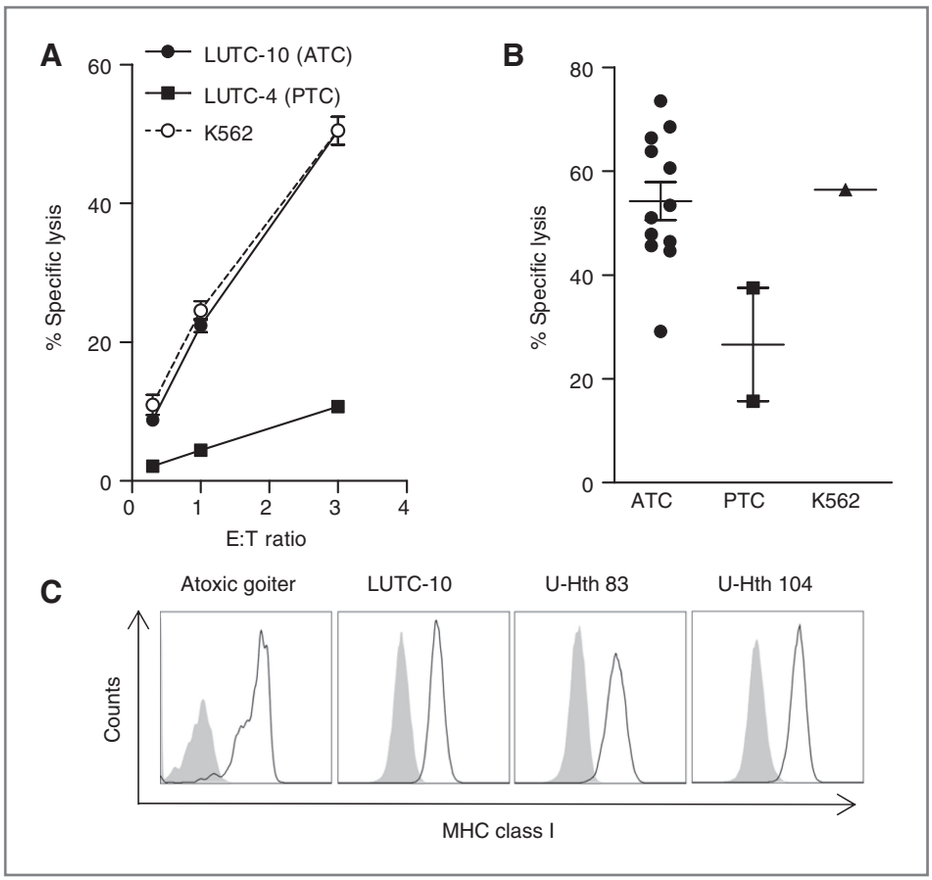

Figure 1. A, specific lysis of the ATC cell line LUTC-10 and the PTC cell line LUTC-4 by allogeneic NK cells in a 4-hour cytotoxicity assay. K562 was used as a positive control for NK cell killing. B, average lysis of 12 ATC cell lines, 2 PTC cell lines, and K562 by expanded allogeneic NK cells isolated from 5 healthy donors. Effector:target ratio = 3:1, 18-hour coculture. C, surface expression of MHC class I on nonmalignant thyroid cells isolated from an atoxic goiter and on the ATC cell lines LUTC-10, U-Hth 83, and U-Hth 104. Solid gray histograms represent staining with isotype control antibody, and open histograms represent staining with pan-MHC class I antibody.

after *ex vivo* expansion (Fig. 2B). The mean fluorescence intensity (MFI) of NKG2D is increased from 85.0 ± 8.2 on resting NK cells to 1,491 ± 785 after 11 days of *ex vivo* expansion ($P = 0.037$, $n = 4$; data not shown). To investigate the mechanism of NK cell–mediated killing of ATC cells, the activating ligands TRAIL, FasL, NKG2D, and DNAM-1 as well as perforin/granzyme were blocked on expanded NK cells. Blockade of the NKG2D receptor or inhibition of perforin/granzyme-based cytotoxicity significantly reduced the NK cell–mediated killing of the ATC cell lines U-Hth 104, U-Hth 83, LUTC-2, and LUTC-10 from 32.3% ± 16.9% by unblocked NK cells to 13% ± 12.4% ($P < 0.05$) and 4.3% ± 1.1% ($P < 0.05$), respectively. NK cells blocked with antibodies targeting TRAIL, FasL, or DNAM-1 did not significantly reduce NK cell–mediated lysis of ATC cells (Fig. 2C). No significant changes in lysis of ATC cells in presence of isotypes and blocking antibodies targeting CD16 were observed, thus excluding the potential contribution of antibody-dependent cell-mediated cytotoxicity (ADCC; data not shown).

NK cell–mediated lysis of ATC and PTC cell lines is dependent on the expression level of ULBP2/5/6 on tumor cells

To identify which NKG2D ligands were responsible for the increased killing by NK cells, the ATC cell lines were analyzed for the expression of NKG2D ligands. We found that ULBP2/5/6 was expressed on all of the ATC cell lines, although there was little or no expression of ULBP1, ULBP3, MICA, or MICB on the ATC cell lines. We did not observe expression of any NKG2D ligands on nonmalignant thyroid tissue including atoxic goiter and follicular adenoma. Although there was great variability in expression of NKG2D ligands in FNA from patients with ATC, we also detected expression of NKG2D ligands including ULBP2/5/6 on tumor cells in all patients (Fig. 3A and Supplementary Fig. S1B). In formalin-fixed, paraffin-embedded tumor material from untreated patients with ATC, we observe ULBP2/5/6 expression in ATC cells, whereas there was no expression in normal thyroid tissue (Fig. 3B). Expression of ULBP2/5/6 was significantly higher on ATC cell lines ($n = 14$) than on PTC cell lines ($n = 3$; $P = 0.047$, data not shown). In a regression analysis, the MFI expression of ULBP2/5/6 on ATC ($n = 13$) and PTC ($n = 2$) cell lines correlated with their susceptibility to NK cell lysis ($R^2 = 0.595$, $P = 0.002$; Fig. 3C). No correlation between lysis and surface expression of the NKG2D ligands MICA, MICB, ULBP-1, or ULBP-3 was observed (data not shown). In addition, ATC cell lines expressed the DNAM-1 ligands poliovirus receptor (PVR; $n = 2$) and Nectin-2 ($n = 2$), as well as the death receptors Fas ($n = 2$) and TRAIL receptors ($n = 2$). However, expression of these ligands and death

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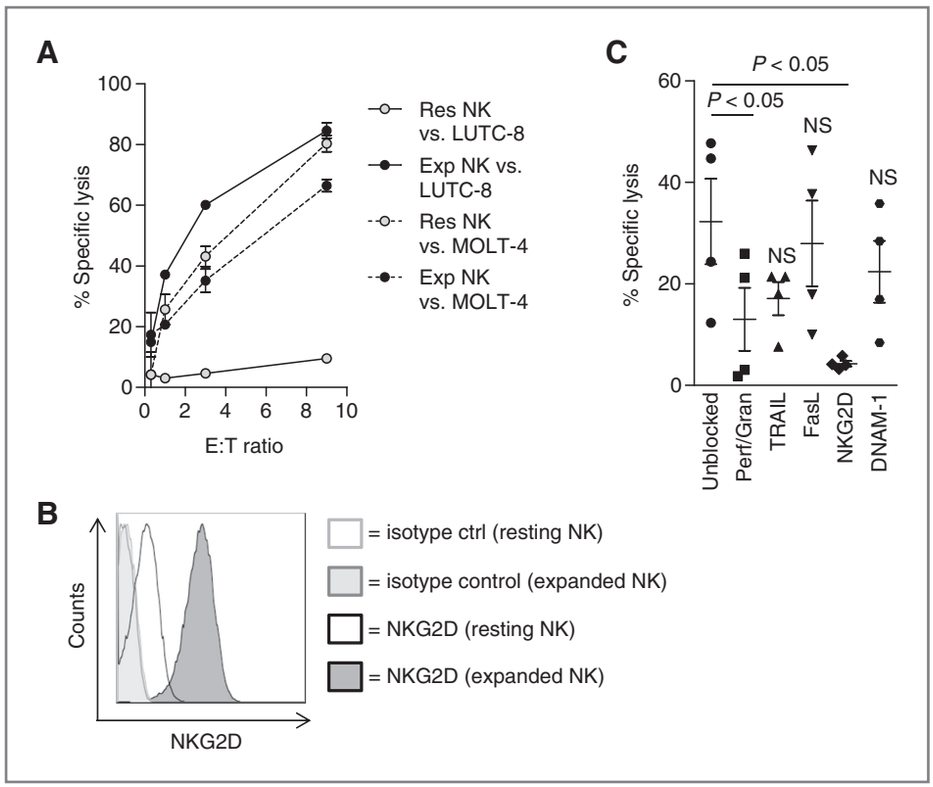

Figure 2. A, specific lysis of the LUTC-8 and MOLT-4 cell lines by resting (Res) and expanded (Exp) NK cells in an 18-hour cytotoxicity assay. One of 4 representative experiments is shown. B, surface expression of NKG2D on resting NK cells and expanded NK cells on day 11 of expansion. One of 4 representative experiments is shown. C, NK cell–specific lysis of LUTC-2, LUTC-10, U-Hth 83, and U-Hth 104 cell lines. NK cells were treated with concanamycin A (CMA, 100 nmol/L) to block perforin/granzyme-based cytotoxicity or blocking antibodies against TRAIL (RIK-2, 10 μg/mL), FasL (NOK-1,2, 5 μg/mL), NKG2D (1D11, 10 μg/mL), or DNAM-1 (TX25, 5 μg/mL) 30 minutes before and during tumor cell coculture. Effector:target ratio = 3:1. NK cells were cocultured with ATC cell lines for 5 hours except for the U-Hth 104 cell line where the coculture period was 18 hours. Paired 2-tailed *t* test was used to calculate statistical significance between the blocking conditions. NS, not significant compared with unblocked.

receptors did not correlate with the sensitivity to NK cell lysis *in vitro* (data not shown). Blocking of ULBP2/5/6 on ATC cells from 4 different ATC cell lines in coculture with expanded NK cells resulted in a significantly reduced NK cell–mediated lysis of the ATC cells (average reduction of lysis 38.2% ± 9.5%, $P = 0.033$; Fig. 3D). Furthermore, ATC cell lines silenced for ULBP2 was significantly less susceptible to NK cell–mediated lysis (data not shown). Thus, the ULBP2 receptor may represent the major NKG2D ligand responsible for the sensitivity of ATC cell lines. We were able to isolate and expand NK cells from 1 patient with ATC from whose tumor we were also able to establish a cell line. In an autologous cytotoxicity assay against the LUTC-17 cell line, we found that the NK cells killed the LUTC-17 cell lines and upon receptor blockade with ULBP2/5/6-antibody, the lysis was reduced by 47.8%, whereas blocking of NKG2D on NK cells resulted in 80.4% reduced lysis (Supplementary Fig. S1C). Importantly, blockade of MHC class I on tumor cells did not increase the lysis significantly. The NK cell–mediated lysis of autologous ATC cells in absence or presence of MHC class I blocking antibody was 30.7% or 37.0%, respectively (data not shown).

ATC intratumoral NK cells express CXCR3 and ATC cell lines secrete CXCL10 resulting in chemoattraction of expanded NK cells

To assess the NK cell chemoattracting properties of ATC cells, we analyzed the secretion of CXCL10 by ATC cells. The CXCL10 secretion was significantly higher in the ATC cell lines LUTC-2, LUTC-10, and U-Hth 104 (1,833 ± 80 pg/mL) than in the PTC cell lines LUTC-4, LUTC-5, and LUTC-13 (682 ± 160 pg/mL) after stimulation with 3 ng/mL IFNγ ($P = 0.0004$; Fig. 4A). In comparison, the melanoma cell line EST112 produced similar levels of CXCL10 as the PTC cell lines. Although no significant differences in Transwell assays were observed, expanded NK cells showed a higher migratory capacity toward supernatant from LUTC-10 tumor cells treated with 5 ng/mL IFNγ compared with supernatant from untreated LUTC-10 tumor cells (4.5-fold increased migration) or medium containing IFNγ (2.7-fold increased migration). The NK cell migration was attenuated in presence of anti-CXCL10 antibodies (Fig. 4B). We next analyzed the expression of CXCR3 on NK cells in FNA specimens and PBMC from patients with ATC and found that the expression of CXCR3 was higher on intratumoral NK cells than on blood-derived NK cells (Fig. 4C). An analysis of CXCR3 expression on NK cells from 3 patients with ATC revealed that on average, 56.1% ± 18.2% of NK cells from FNA were positive for CXCR3 compared with 4.6% ± 3.0% on NK cells from PBMC ($P = 0.03$; data not shown). To confirm that expression was not limited to cell lines *in vitro*, formalin-fixed, paraffin-embedded ATC tissue as well as normal thyroid tissue (parathyroid adenoma) was stained for CXCL10. Tumor sections from an untreated patient with ATC and normal thyroid tissue stained positive for CXCL10 (Fig. 4D).

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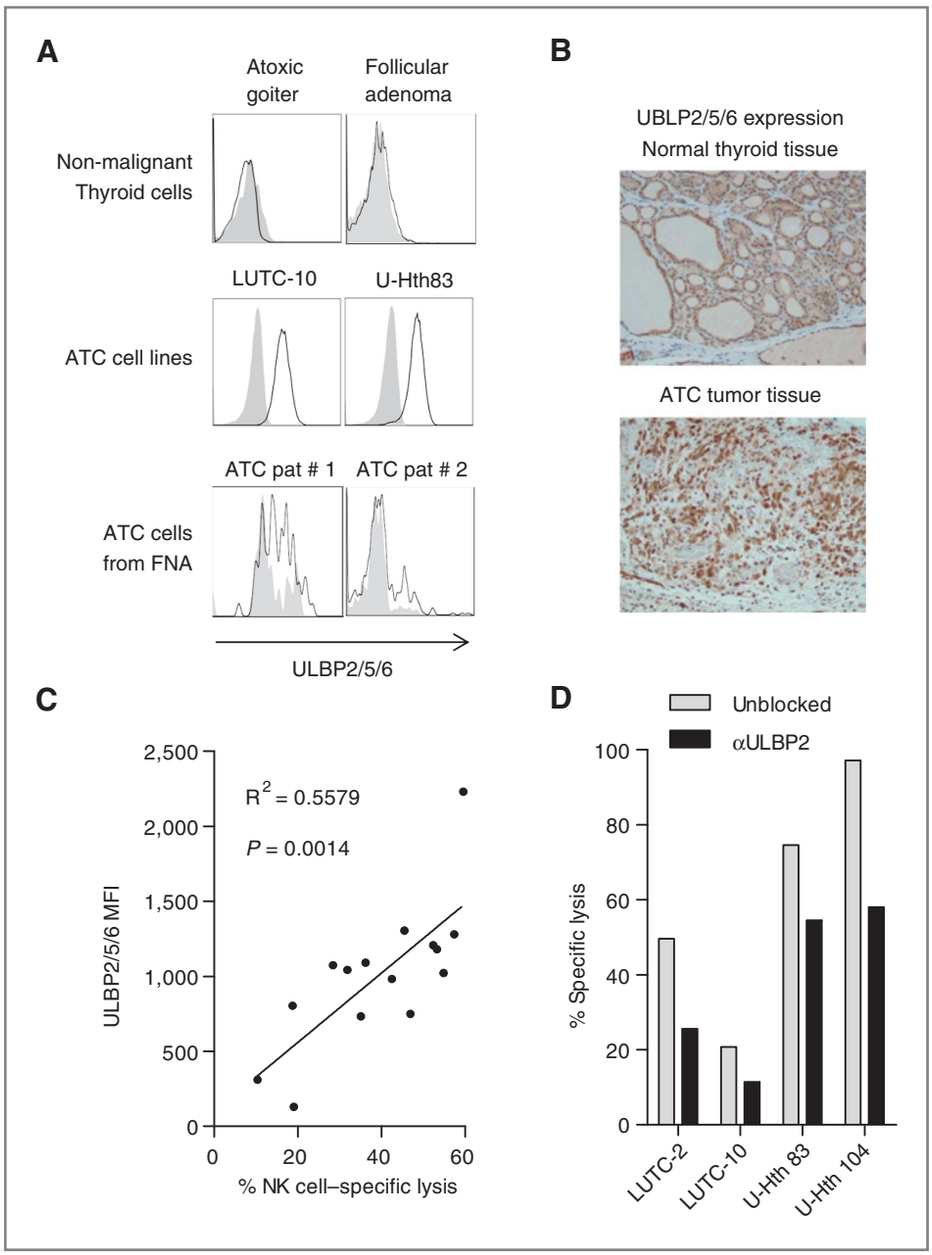

Figure 3. A, surface expression of ULBP2/5/6 on nonmalignant thyroid cells (isolated from an atoxic goiter and a follicular thyroid adenoma), cells from the ATC cell lines LUTC-10 and U-Hth 83, and cells in FNA from 2 untreated patients with ATC. Solid gray histograms represent staining with isotype control antibody, and open histograms represent staining with ULBP2/5/6 antibody. B, immunohistochemical analysis of ULBP2/5/6 protein expression (brown) in an angioinvasive tumor thrombus analyzed on paraffin-embedded tumor material from an untreated patient with ATC and from normal thyroid tissue (20× magnification). C, linear regression (Spearman rank analysis) of ULBP2/5/6 MFI and NK cell–specific lysis of 13 ATC and 2 PTC cell lines. D, NK cell–specific lysis of the ATC cell lines LUTC-2, LUTC-10, U-Hth 83, and U-Hth 104 in an 18-hour cytotoxicity assay. Tumor cells were pretreated with ULBP2/5/6-specific blocking antibody (1 μg/mL) 30 minutes prior and during the cytotoxicity assay. Effector:target ratio was 3:1 except for LUTC-10 where it was 0.3:1. Paired 2-tailed *t* test was used to calculate statistical significance.

ATC-infiltrating NK cells display a suppressed phenotype compared with peripheral blood NK cells

To further analyze the phenotype of ATC tumor–derived NK cells, FNA and peripheral blood from 6 patients with ATC was stained for NK cell markers. FNA samples were also stained for expression of NKG2D ligands on tumor cells. In 6 patients with ATC, the percentage of NK cells in the lymphocyte population was significantly lower in FNA than in PBMCs ($P = 0.006$; Fig. 5A). The frequencies of $CD3^+CD56^-$ in the lymphocyte population were 49.8% ± 16.3% and 57.5% ± 21.2% in PBMC and FNA, respectively ($P = 0.44$), and the frequencies of $CD3^+CD56^+$ cells in the lymphocyte population were 9.3% ± 5.3% and 4.2% ± 2.9% in PBMC and FNA, respectively ($P = 0.03$; data not shown). Furthermore, a decreased proportion of $CD56^{dim}$ NK cells in the NK cell population was found in the FNA compared with PBMCs ($P = 0.015$, Fig. 5B). We analyzed the CXCR3 expression on $CD56^{dim}$ and $CD56^{bright}$ NK cells in 2 patients with ATC. The percentage of CXCR3-positive cells in the $CD56^{bright}$ NK cell population was on average 91.6% compared with 50.7% in the $CD56^{dim}$ NK cell population. The third patient was not analyzed because of insufficient number of cells in the $CD56^{bright}$ population. Moreover, intratumoral NK cells expressed higher levels of the activation marker CD69 compared with NK cells in PBMCs ($P = 0.025$, Fig. 5C). Importantly, a reduced

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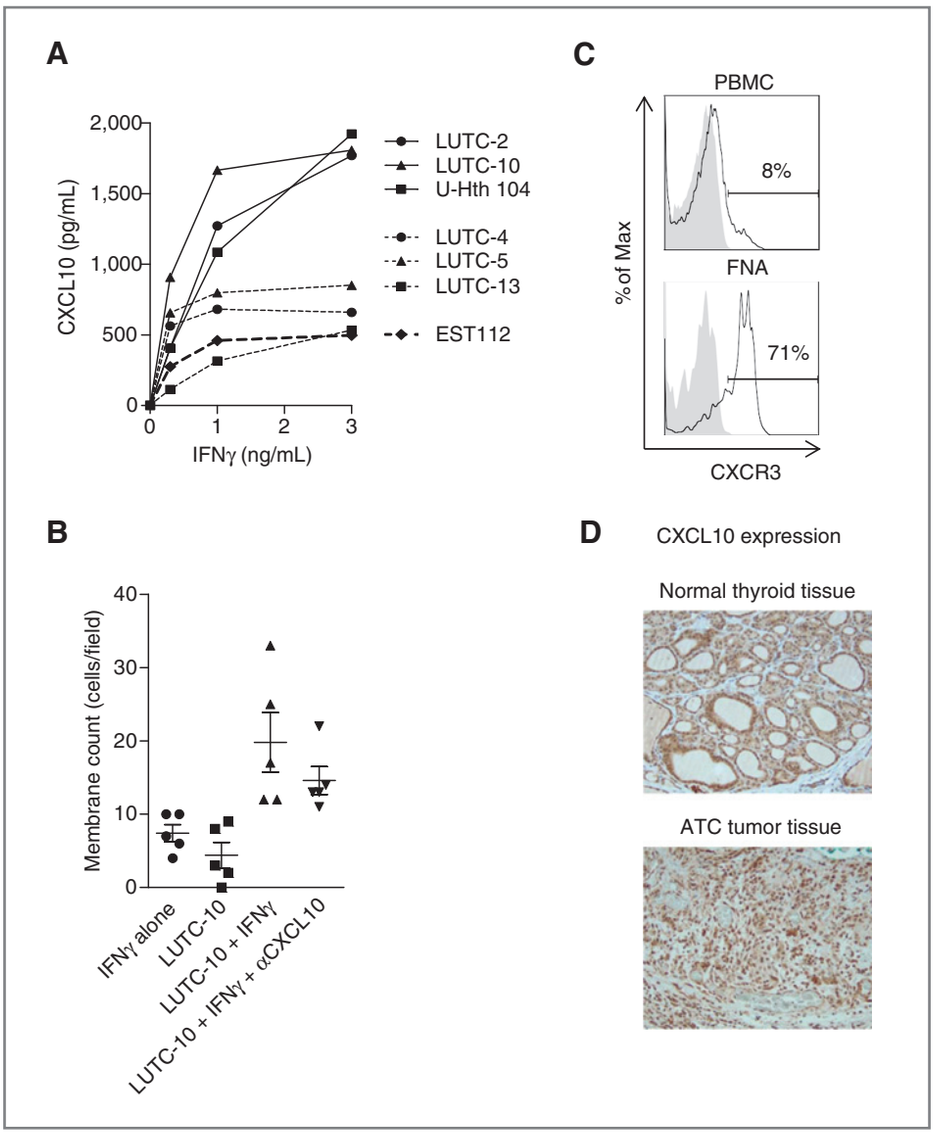

Figure 4. A, CXCL10 ELISA of tumor supernatants from 3 ATC cell lines (LUTC-2, LUTC-10, U-Hth 104), 3 PTC cell lines (LUTC-4, LUTC-5, LUTC-13), and 1 melanoma cell line (EST112) after 24-hour stimulation with increasing doses of IFNγ. B, 2-hour Transwell chemotaxis assay of expanded NK cells migrating toward medium containing 5 ng/mL IFNγ, supernatant from untreated LUTC-10 cells, or supernatant from LUTC-10 cells treated with 5 ng/mL IFNγ for 24 hours. The NK cell migration toward IFNγ-treated LUTC-10 supernatant was also performed in the presence of blocking antibodies targeting CXCL10. The graph indicates enumeration of NK cells in 5 randomly chosen fields of each Transwell membrane. The experiment is representative of 3 independent experiments. C, CXCR3 expression (open histogram) on NK cells in PBMCs (CXCR3 MFI: 33) or FNA (CXCR3 MFI: 460) from a patient with ATC. Light gray histograms represent staining with isotype control antibody. Percentage of $CXCR3^+$ cells is indicated in the graph. D, immunohistochemical analysis of CXCL10 protein expression (brown) in an angioinvasive tumor thrombus analyzed on paraffin-embedded tumor material from an untreated patient with ATC and from normal thyroid tissue (20× magnification).

expression of NKG2D on NK cells was observed in FNA compared with PBMC in 5 of 6, patients although the difference was not statistically significant. In one patient, the NKG2D expression was elevated on intratumoral NK cells ($P = 0.35$; Fig. 5D).

ATC cell lines expressing COX2 suppress NK cells via PGE2

Given the suppressed phenotype of ATC-infiltrating NK cells, we sought to elucidate the mechanism of NK cell suppression by ATC cells. Four of 8 ATC cell lines stained positive for expression of COX2, the enzyme that catalyzes the synthesis of PGE2. Coculture with supernatants from COX2-positive ATC cell lines resulted in significantly lower expression of NKG2D on NK cells than when cocultured with COX2-negative ATC cell lines ($P = 0.02$; Fig. 6A). We proceeded to coculture expanded NK cells with supernatant from the COX2-positive cell line LUTC-17, which reduced the expression of NKG2D on NK cells. However, NKG2D expression was restored to baseline in presence of neutralizing antibodies against PGE2, whereas neutralization of TGFβ did not restore the NKG2D expression (Fig. 6B). Neutralization of IL10 or IL6 in NK cells cultured with LUTC-17 supernatant did not affect the NKG2D levels (data not shown). Moreover, the cytotoxic capacity of NK cells was reduced after coculture with supernatant from LUTC-17 but was restored after neutralization of PGE2 (Fig. 6C). Of note, no difference in susceptibility to NK cell killing was observed between COX2-positive and -negative ATC cell lines.

Discussion

We show that ATC cells express high levels of ULBP2/5/6 and are sensitive to NKG2D-mediated lysis by NK cells.

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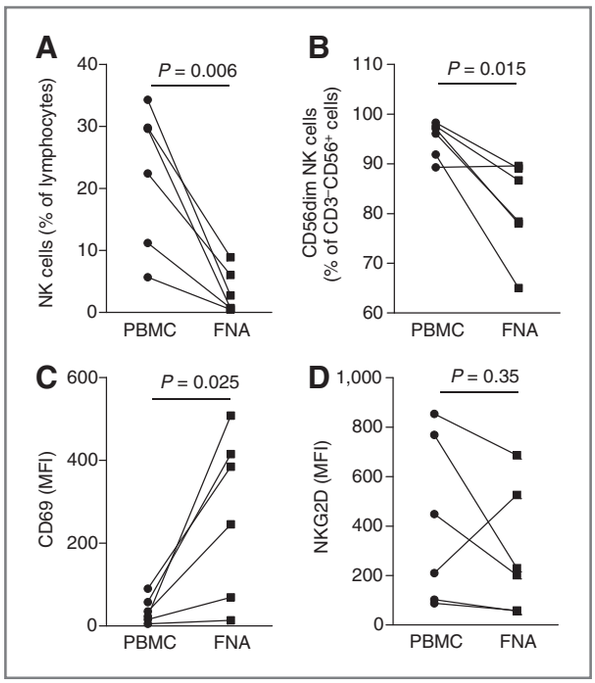

Figure 5. A, percentage of viable (7AAD$^-$) NK cells (CD3$^-$CD56$^+$ cells) in the lymphocyte population in PBMCs and in FNA from patients with ATC. B, percentage of CD56^{dim} NK cells of the NK cell population in PBMC and FNA from patients with ATC. CD69 (C) and NKG2D (D) MFI on NK cells in PBMC and FNA from patients with ATC. Paired 2-tailed *t* tests were used to calculate statistical significance between FNA and PBMC samples.

Furthermore, ATC tumors chemoattract CXCR3-positive NK cells *in vitro* and *in vivo*. Intratumoral NK cells display a suppressed phenotype, and *in vitro*, COX2-positive ATC cell lines inhibit NKG2D expression and cytotoxicity of NK cells via production of PGE2.

Adoptive transfer of *ex vivo* expanded NK cells is being increasingly used to treat different forms of malignancies and technical improvements for large-scale GMP-grade production of NK cells for adoptive transfer has escalated over the past decades (18). However, the clinical success of NK cell adoptive cell transfer against solid tumors has been limited because of several factors such as resistance of tumor cells to NK cell lysis, poor migration of infused NK cells toward tumors, and suppression of NK cell activity in the tumor microenvironment.

Little is known about immune responses in thyroid cancer, and the role of NK cells has not been studied in the context of ATC. However, toxic reactions in the thyroid gland have been reported in several clinical trials involving either administration of activated immune cells or immunostimulatory cytokines such as IL2 or IFNα, indicating that the thyroid gland may be particularly sensitive to lysis by activated immune cells (19, 20). Studies investigating the clinical effect of the anti-CTLA4 monoclonal antibody ipilimumab have reported cases of hypothyroidism and thyroiditis (21, 22). Furthermore, patients suffering from Grave disease and Hashimoto thyroiditis have increased activity of peripheral NK cells (23).

We demonstrate that NKG2D/ULBP2/5/6 signaling is the major mechanism of sensitivity of ATC cells to lysis by expanded NK cells. Upon binding of the NKG2D receptor, NK cells release perforin and granzyme to induce apoptosis of the target cells (9). In the present study, we did not analyze for correlation of apoptotic signaling pathways and the expression of ULBP2/5/6 in targeted tumor cells. In agreement with previous studies, showing that insufficient release of perforin/granzyme by resting NK cells contributes to the low cytotoxic capacity of resting NK cells, we demonstrate that resting NK cells do not but expanded NK cells do kill ATC cell *in vitro* and that neutralization of perforin by expanded NK cells results in reduced lysis of ATC cells by expanded NK cells (24). These findings provide an explanation as to why expanded NK cells display potent

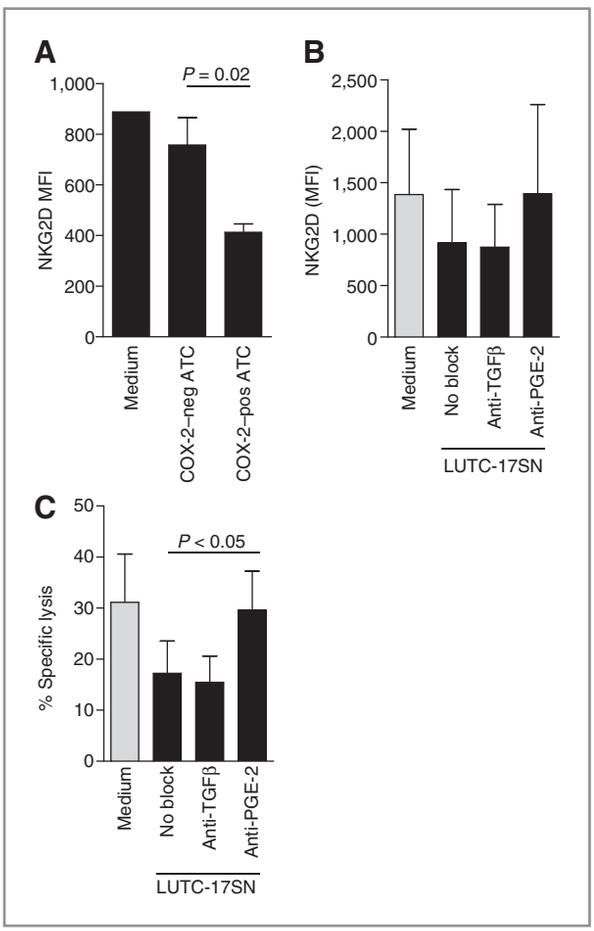

Figure 6. A, MFI of NKG2D on expanded NK cells cultured for 24 hours with culture medium or with supernatant (SN) from ATC cells that are either negative ($n = 4$) or positive ($n = 4$) for COX2 expression. NKG2D MFI (B) and specific lysis of K562 cells (C) by NK cells cultured with either medium or with LUTC-17 SN for 24 hours in the presence of neutralizing antibodies targeting TGFβ or PGE2. In the cytotoxicity assays, the effector:target ratio was 3:1, and the NK cells were cocultured with target cells for 5 hours. The experiment was performed 3 times, and statistical significance was calculated using paired 2-tailed *t* test. neg, negative; pos, positive.

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cytotoxicity against ATC cells expressing high levels of ULBP2/5/6, whereas NKG2D-low resting NK cells are ineffective at killing ATC cells. We previously showed that expansion of NK cells results in increased expression of NKG2D, TRAIL, FasL, CD56, CD48, and CD25 and these NK cells displayed significantly higher cytotoxicity against tumor cell *in vitro*. Furthermore, the expression of NKG2D on NK cells varied significantly between donors, and maintenance of NKG2D expression on expanded NK cells is highly dependent on sustained IL2 stimulation (17). This highlights the importance of monitoring the expression levels of NKG2D in NK cells for clinical use. When blocking ULBP2/5/6 on tumor cells or NKG2D on NK cells, we found an increased resistance of ATC cells to lysis by both allogeneic and autologous NK cells. We also confirmed the expression of ULBP2/5/6 on ATC cells in FNA as well as in tumor sections from untreated patients with ATC. We could not detect ULBP2/5/6 expression in nonmalignant thyroid tissue and observed only minimal killing of thyroid goiter cells *in vitro*. It has previously been shown that ULBP2 as well as other NKG2D ligands are overexpressed in several human cancers (25, 26) and that ectopic expression of NKG2D ligands in mice results in NK cells overcoming MHC class I–induced inhibition, thus promoting tumor rejection (27). ULBP2 has characteristics that distinguish it from ULBP1 and ULBP3. The ULBP1–3 molecules are all anchored to the plasma membrane via a glycosylphophatidylinositol (GPI) domain. However, it has been shown that ULBP2, but not ULBP1 and 3, can relocate to the cell surface in the absence of a GPI moiety as a transmembrane protein that allows for more stable interaction with NKG2D receptors on NK cells (28). Moreover, soluble levels of ULBP2 have been correlated with reduced survival in patients with cancer, which has not been shown for other NKG2D ligands (29).

In tumor material from patients with ATC, we observed a significant enrichment of CXCR3-posititve NK cells compared with circulating NK cells in the same patients where only a fraction of NK cells expressed the CXCR3 receptor. This finding, together with our observation that ATC cell lines were prone to produce high levels of CXCL10, which could chemoattract NK cells *in vitro*, indicates that in the patients with ATC, CXCR3-expressing NK cells may have migrated to the tumor via CXCL10-induced chemoattraction. Stimulation of thyroid follicle cells by pattern-associated molecular patterns (PAMP) induces cytokine production including the chemokines CXCL9, CXCL10, and CXCL11 (30). Antonelli and colleagues have reported that patients with autoimmune thyroiditis and Grave disease have elevated serum levels of CXCR3 ligands and that primary thyrocytes can be stimulated to secrete CXCL11 after treatment with IFNα, β, and γ (31, 32). Moreover, it has been shown that PTC cells are more prone to secrete CXCL10 than normal thyroid follicular cells upon stimulation with IFNγ (33). We show that ATC cells secrete significantly higher levels of CXCL10 than PTC cells after stimulation with minute doses of IFNγ. In the microenvironment of an inflamed gland or a tumor, the local infiltration of immune cells such as NK cells, NKT cells γδ T cells, T_H1, and T_H2 cells contributes to a sustained secretion of IFNγ as well as other cytokines that can synergize with IFNγ to trigger a release of IFNγ-inducible cytokines, such as CXCL10 (34–37). ATC cells cultured in presence of expanded NK cells resulted in secretion of high levels of CXCL10 by the ATC cell lines (data not shown). Furthermore, while resting NK cells express low levels of CXCR3, the CXCR3 expression on NK cells is highly upregulated after *ex vivo* expansion (data not shown). Thus, adoptively infused CXCR3-expressing NK cells would be recruited to the tumor via CXCL10-induced chemoattraction and further reinforce the secretion of CXCL10 from the tumor by production of IFNγ. *In vitro*, expanded NK cells actively migrated toward supernatant from IFNγ-treated ATC cells. Neutralization of CXCL10 in the supernatant resulted in a partial reduction of NK cell migration, indicating that other IFNγ-inducible CXCR3 ligands may be responsible for the chemoattraction of NK cells.

In our analysis of patient material from patients with ATC, we found that the NK cell population constituted a lower percentage of the total lymphocyte population in the FNA than in PBMCs. Also, the percentage of $CD56^{dim}$ NK cells was lower in the tumor than in in peripheral blood. Gogali and colleagues have recently reported that in patients with PTC, $CD56^{dim}$ NK cells were lower in PTC tissue than in peripheral blood, although the ratio of $CD56^{dim}$ NK cells in the PTC-infiltrating NK cell population correlated positively with disease stage (38). There are several possible explanations for the skewed ratio between $CD56^{bright}$ and $CD56^{dim}$ NK cells in tumors. Studies have shown that intratumoral $CD56^{dim}$ NK cells are preferentially eliminated either by susceptibility to apoptosis or to suppression by reactive oxygen species present in the tumor microenvironment (39, 40). We observed that in PBMC from patients with ATC, CXCR3 is predominantly expressed on $CD56^{bright}$ NK cells compared with $CD56^{dim}$ NK cells. This distribution of CXCR3 on NK cells has previously been described in healthy individuals and in patients with hepatitis C (41, 42). We also found that the percentage of CXCR3-positive cells in FNA was higher in the $CD56^{bright}$ NK cell population than in the $CD56^{dim}$ NK cell population, indicating that the $CD56^{bright}$ NK cells may have been preferentially recruited to the tumor possibly explaining the skewed ratio of $CD56^{dim}$ NK cells. However, we see that the CXCR3 expression on $CD56^{dim}$ NK cells is higher in FNA than in PBMC, indicating that $CD56^{dim}$ NK cells are also enriched in in ATC tumors. In 5 of 6 patients with ATC, we found a lower expression of NKG2D on intratumoral NK cells compared with NK cells derived from peripheral blood. Interestingly, we observed an increased expression of NKG2D on the intratumoral NK cells in one of the patients with ATC. We also observed that NK cells in ATC FNA expressed elevated levels of CD69 compared with NK cells in peripheral blood. This finding suggest that intratumoral NK cells are in an activated state, although previous studies have shown that elevated CD69 expression on NK cells is detrimental to antitumor activity of NK cells due to

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induced TGFβ synthesis (43, 44).There are several mechanisms that may suppress NK cells in the tumor microenvironment. Activated NK cells and NK cells from patients with cancer can express the inhibitory receptor programmed death 1 (PD-1), which delivers an inhibitory signal to NK cells upon ligation with tumor cells expressing PD-L1 or PD-L2 (45). We found that ATC cell lines express PD-L1 but did not suppress NK cell cytotoxicity (data not shown). It is known that COX2, which is the key regulator of PGE2 synthesis (46), is expressed in several tumor types (47, 48) and can suppress the cytotoxicity and NKG2D expression on NK cells (49). *In vitro*, we found that ATC cell lines with high expression of COX2 downregulated the expression of NKG2D on expanded NK cells more than ATC cell lines with low expression of COX2. Furthermore, expression of NKG2D and cytotoxic activity of NK cells, which was downregulated upon coculture with supernatant from a COX2-positive ATC cell line, was restored to normal levels upon neutralization of PGE2 in the coculture. Although NKG2D expression is downmodulated on expanded NK cells after exposure to COX2-positive ATC cells, the NKG2D levels remain significantly (15-fold) higher than NKG2D levels on resting NK cells (data not shown), indicating that they may still be more effective against COX2-positive ATC tumors than endogenous NK cells. Shedding of NKG2D ligands from tumors cells due to overexpression of metalloproteases has also been shown to inhibit NK cells by downregulating surface expression of NKG2D (50). We detected varying levels of soluble ULBP2 in ATC cultures *in vitro*. However, we did not observe any restoration of NKG2D levels on NK cells after coculturing NK cells with the ULBP2-secreting ATC cell line LUTC-17 in the presence of ULBP2/5/6 blocking antibodies (data not shown). In an attempt to treat ATC xenografts, we only observed a minor delay in the tumor progression in mice treated with NK cells compared with untreated mice (data not shown). Although we did not investigate the activity of tumor-infiltrating NK cells, we speculate that the immunosuppressive nature and aggressiveness of ATC tumors may have contributed to the ineffectiveness of NK cell infusion.

In summary, few tumor types are responsive to NK cell therapy, either due to resistance to lysis or due to ineffective recruitment and infiltration of NK cells. Our findings collectively show that ATC may be a promising target for NK cell–based adoptive cell therapy. We describe ATC to be sensitive to lysis by expanded NKG2D-positive NK cells and able to chemoattract adoptively transferred expanded CXCR3-positive NK cells. Our findings also indicate that PGE2 may be the predominant factor for suppression of NK cells induced by COX2-positive ATC cells. Therefore, screening for ULBP2/5/6, CXCL10, and COX2 in cytologic examination of FNA from patients with ATC could be used as a predictive marker for treatment with adoptive NK cell therapy and efforts to reduce the effects of PGE2 adjunct to NK cell infusion are warranted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Wennerberg, A. Pfefferle, L. Ekblad, Y. Yoshimoto, V. Kremer, C.C. Juhlin, A. Höög, V. Svjatoha, C. Larsson, J. Zedenius, J. Wennerberg, A. Lundqvist

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Wennerberg, A. Pfefferle, Y. Yoshimoto, A. Höög, J. Zedenius, J. Wennerberg, A. Lundqvist

Writing, review, and/or revision of the manuscript: E. Wennerberg, A. Pfefferle, L. Ekblad, Y. Yoshimoto, C.C. Juhlin, A. Höög, V. Svjatoha, C. Larsson, J. Zedenius, J. Wennerberg, A. Lundqvist

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): V.O. Kaminskyy, C.C. Juhlin, I. Bodin, J. Zedenius, A. Lundqvist

Study supervision: A. Lundqvist

Other (technical support): I. Bodin

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Clinical Cancer Research

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