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A dynamic inflammatory cytokine network in the human ovarian cancer microenvironment

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Abstract

Constitutive production of inflammatory cytokines is a characteristic of many human malignant cell lines, however, the *in vitro* and *in vivo* interdependence of these cytokines, and their significance to the human cancer microenvironment, are both poorly understood. Here, we describe for the first time how three key cytokine/chemokine mediators of cancer-related inflammation, TNF, CXCL12 and IL6, are involved in an autocrine cytokine network, the 'TNF network', in human ovarian cancer. We show that this network has paracrine actions on angiogenesis, infiltration of myeloid cells and NOTCH signalling in both murine xenografts and human ovarian tumor biopsies. Neutralising antibodies or siRNA to individual members of this TNF network reduced angiogenesis, myeloid cell infiltration and experimental peritoneal ovarian tumor growth. The dependency of network genes on TNF was demonstrated by their down regulation in tumor cells from patients with advanced ovarian cancer following the infusion of anti-TNF antibodies. Together, the findings define a network of inflammatory cytokine interactions that are crucial to tumor growth and validate this network as a key therapeutic target in ovarian cancer.

Keywords

GSEA; autocrine cytokine networks; ovarian cancer; microenvironment; therapeutics

Introduction

A majority of human malignant cell lines constitutively secrete cytokines and chemokines as a consequence of oncogenic mutations and dis-regulated signalling pathways (1). However,

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it is not clear whether this cytokine/chemokine expression has relevance to the regulation of complex human tumor microenvironments.

We previously reported that the cytokines TNF and IL6, the chemokine CXCL12 and its CXCR4 receptor were constitutively expressed and co-regulated in ovarian cancer cell lines in tissue culture (2, 3). Stable knockdown of TNF mRNA in one of these cell lines provided evidence for an autocrine cytokine network with paracrine actions on blood vessel development in a peritoneal xenograft model (3).

The aim of the current study was to investigate if this autocrine cytokine network was relevant to the human tumor microenvironment of ovarian cancer. We present evidence, for the first time, that the cytokine network exists in human cancer biopsies. TNF, CXCL12 and IL6 are co-expressed and co-regulated in human ovarian cancer biopsies in what we describe as the 'TNF network'. We show that high TNF network pathway gene expression in the tumor microenvironment associates with genes involved in angiogenesis, inflammation, leukocyte infiltrates and NOTCH signalling. This was confirmed by inhibition of the TNF network in experimental ovarian cancer models, and in tumor cells from ovarian cancer patients after infusion of an anti-TNF antibody.

Materials and Methods

Ovarian cancer cells

IGROV-1 high grade serous ovarian cancer cells and TOV21G clear cell cancer cells and were cultured as described (3). All cell lines have undergone 16 loci STR authentication (LGC Standards, London, UK) and were most recently authenticated in September 2011. The cells were cultured in RPMI 1640 10% FCS.

Immunohistochemistry

Paraffin embedded biopsies, tissue arrays and xenografts were stained with antibodies for CXCR4 (MAB173, R&D Systems), CXCL12 (MAB350, R&D Systems), TNF (MAB 610; R&D systems), IL6 (SC-7920; Santa Cruz Biotechnology), F4/80 (MCA497, Serotec, Oxford, UK) CD68 (Dako) and HES1 (Sc-25392, Santa Cruz Biotechnology Inc). Negative controls were isotype matched.

Western blotting

Cell extract (15µg) was run on an SDS 10% acrylamide gel and transferred to a nylon membrane. The membrane was blocked overnight (4°C in PBS with 0.1% Tween and 10% milk powder) and probed using the anti-JAG1 antibody (AF1272, R&D Systems, UK). A horseradish peroxidase-conjugated secondary antibody was used for detection (1:5,000) dilution at room temperature for 1 hour. Protein concentration equivalence was confirmed by anti-β-actin antibody

Flow cytometry

Cells were counterstained with FITC-conjugated secondary antibody (Sigma, Poole, UK) and analysed on a FACScan® flow cytometer using Cellquest software (BD Pharmingen, Oxford, UK).

Cytokine ELISA

Cells were plated at 3×10^5 cells/well, cell culture supernatants removed after 48 hours of culture and cytokine concentrations measured using Quantikine® ELISA kits (R&D Systems). Frozen tissue was ground in liquid nitrogen and lysed in Tris-HCl, pH 8/50 mM/

NaCl 150 mM/1% Triton X-100/DTT 1 mM/1x inhibitor mixture mix (Calbiochem, San Diego, CA)/100 µg/ml PMSF. Protein concentrations were determined by Bradford assay (Rad Laboratories, Hercules, CA, USA).

Transfection of IGROV-1 cells

IGROV-1 cells were transfected with SUPER RNAi™ plasmids containing two different shRNA sequences targeting CXCR4, or a control plasmid containing scrambled RNA (IGROV-Mock) and isolated according to (4). Lipofectamin was used for transfection as described previously (3). For transient knockdown the ON-TARGET plus SMART pool of oligos targeting CXCR4 gene expression was transfected using Dharmafect1 transfection reagent (Dharmacon, Chicago, IL). SiCONTROL non-targeting siRNA pool served as control. Lentiviral vectors containing the luciferase reporter construct were as described (3).

RNA extraction and Real time Quantitative RT-PCR

RNA was extracted using Tri Reagent (Sigma), and treated with 10 U DNase (Pharmacia, Milton Keynes, UK). DNase treated RNA (2 µg) was reverse transcribed with M-MLV reverse transcriptase (Promega, Southampton, UK). Multiplex real-time RT-PCR analysis was performed using pre-made TaqMan® probes (FAM) and 18s rRNA (VIC) specific primers and probes with the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Warrington, UK). Expression values were normalised (Δ Ct) to 18s rRNA by subtracting the cycle threshold (Ct) value of 18s rRNA from the Ct value of the experimental value.

Peritoneal xenografts

SPF female nude mice (Cancer Research UK, Clare Hall Laboratories, South Mimms, UK) 6-8 weeks of age, housed in sterile individually ventilated cages (IVC) were injected i.p. with 5×10^6 cells. Twice weekly treatment (5 mg/kg i.p.) was with monoclonal antibody infliximab or non-specific, Gamimune N polyclonal human IgG control. Bioluminescence imaging was carried out and blood vessels quantified as before (3).

Microarray experiments

Total RNA was isolated using the standard Trizol protocol and purified further with the RNeasy kit (Qiagen). RNA was quantified using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Expression profiles of all specimens were compared with a commercial universal reference RNA (Clontech). The Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays were used. Probe synthesis and microarray hybridization were performed according to standard Affymetrix (Santa Clara, CA) protocols.

Microarray analysis

Three Affymetrix data sets were obtained in triplicate. The NCBI GEO accession number for the arrays is GSE 13763. Data were analysed using Bioconductor 1.9 running on R 2.6.0. Probeset expression measures (5, 6); were calculated using the Affymetrix package's Robust Multichip Average (RMA) default method (7). Differential gene expression was assessed between control (IGROV-1 and IGROV-Mock) and shRNAi (I) and (II) replicate groups, using an empirical Bayes' t-test (limma package) (6); p values were adjusted for multiple testing using the Benjamini-Hochberg method (8). Any probe sets that exhibited an adjusted p value of 0.05 were called differentially expressed. In addition, any probe sets that exhibited an absolute fold change of greater than 2 were used to generate a heatmap. Two-dimensional (2D) hierarchical clustering of expression data using differentially expressed genes across control and shCXCR4 (I) and (II) samples was performed. Samples were clustered using a 1 - Pearson correlation distance matrix and average linkage clustering.

Genes were clustered using a Euclidean distance matrix and average linkage clustering. The NCBI GEO accession number for the cell lines is GSE 13763. The NCBI accession number for datasets from the ascites cells GSE18681.

Gene Set Enrichment Analysis (GSEA)

Differentially expressed probes were selected based on meeting criteria of $FDR < 0.05$. Probes were divided into positive and negative fold change lists and used to determine enrichment using Genego processes within MetaCore™ pathway tool [GeneGo, Inc, St. Joseph, MI]. The analysis employs a hypergeometric distribution to determine the most enriched gene-set

The microarray datasets GSE6008, GSE3149 and GSE9899 were downloaded from the GEO website. Datasets GSE6008 and GSE3149 were merged using Bioconductor 1.9 running on R 2.6.0. Probeset expression measures were calculated using the Affymetrix package's Robust Multichip Average (RMA) default method. The microarray dataset from The Cancer Genome Atlas Network (TCGA) comprising 590 biopsies of high-grade serous ovarian cancer was downloaded from the TCGA data portal site (9).

The function GeneSetTest from the limma package was used to assess whether each sample had a tendency to be associated with an up or down regulation of members of the e.g. TNF pathway. The individual genes in the TNF, CXCL12 and IL-6 pathways, as defined by MetaCore™, are listed in Supplementary Table 1. 14% of the genes are shared among the three pathways. The function employs a Wilcoxon t-test to generate p-values. All samples were ranked on this enrichment, from the most significant to the least significant. The top and bottom 50 samples were extracted from the dataset as "high- TNF" and "low- TNF". The same process was used to identify samples that were enriched with members of the CXCL12 and IL6 pathway. We also used immune cell-specific signatures (10).

Patients and clinical trial design

The phase I/II trial was a single centre, open-label, study of infliximab at two dose levels (5 mg/kg and 10 mg/kg) in patients with advanced epithelial ovarian cancer; full details in (11).

Tissue Microarray, Image Analysis and cell counting in biopsies

Fifty-three cases of stage III/IV high-grade serous tumors were used for tissue microarray (TMA), construction as described in (12), four cores per patient. The Aperio ScanScope XT Slide Scanner (Aperio Technologies, Vista, CA) system was used to capture whole slide digital images with a 20X objective. Slides were de-arrayed to visualize individual cores, using Spectrum (Aperio). Genie™ histology pattern recognition software (Aperio) was used to identify tumor from stroma in individual cores and a colour deconvolution algorithm (Aperio) was used to quantify TNF, CXCL12 and IL6. For assay of CD68+ cells in the AOCS ovarian cancer biopsies, microscope fields that contained tumor and adjacent stroma were counted blind at x40 magnification.

Ethics

The clinical study was approved by North East London and the City Health Authority Research Ethics Committee (LREC P/02/150) and Lothian Research Ethics Committee (LREC2000/4/60, LREC/2002/8/31) and conducted according to the declaration of Helsinki. All patients gave voluntary, written informed consent. The TMA study was approved by the Research Ethics Committee at the National Maternity Hospital, Dublin, Ireland. Access to human biopsy samples satisfied requirements of the East London and City Health Authority Research Ethics Subcommittee (LREC number 07/Q0604/25).

Statistical analysis

Statistical analysis of *in vitro* and animal experiments used one-way ANOVA, Chi-square test or unpaired T-test with Welch correction (GraphPad Prism version 4 Software, San Diego, CA).

Results

Evidence for a cytokine network in ovarian cancer biopsies

Using gene expression microarray datasets from ovarian cancer biopsies where >90% of samples were Stage III/IV high-grade serous cancer, we first looked for associations between gene expression levels of the TNF, CXCL12 and IL6 signalling pathways. Biopsies from the Australian Ovarian Cancer Study (AOCS) were ranked by expression levels of genes in the TNF, CXCL12 and IL6 signaling pathways in each sample relative to the mean levels of gene expression in all samples (Method, Supplementary Figure 1; individual genes in TNF, CXCL12 and IL-6 pathways, Supplementary Table 1). The top 50 samples with the highest levels of gene expression were compared to the bottom 50 samples with the lowest levels of gene expression in the TNF, CXCL12 and IL6 pathways, so that each sample was associated with three p values. Using a binomial distribution we found that samples that were highly enriched in one pathway, i.e. ranked in the top 50, were highly likely to have high expression levels of genes in the other two pathways (Table 1). We then merged two additional ovarian cancer gene expression microarray datasets to give another 245 ovarian cancer biopsy samples and also conducted the same analysis on the 590 biopsies of high-grade serous cancer from the TCGA dataset, this time analysing those samples that were in the highest 25% and lowest 25% of samples. The associations between high CXCL12, TNF and IL6 signalling pathway gene expression were fully validated (Table 1). The same significant associations were found if expression levels of individual receptor/ligand pairs, rather than pathways, were tested (data not shown). Hence there was a three-way interdependency of the cytokines in the human tumor biopsy samples, with the expression of each ligand and its signalling pathway related to the other.

TNF, CXCL12 and IL6 proteins co-localise in ovarian cancer biopsies

We used automated immunohistochemistry (IHC) to localise TNF, CXCL12 and IL6 and determine whether the proteins were co-expressed at cellular level using a tissue microarray (TMA) of fifty-three cases of Stage III/IV high-grade serous ovarian cancer (12). Using an automated algorithm, staining was expressed as a score that combined both the intensity and density of positive pixels and partitioned into epithelial and stromal fractions using image analysis software. In the malignant cell compartment we found a significant association between expression of TNF and CXCL12 ($P<0.004$), and between TNF and IL6 ($p<0.05$). There was also significant correlation between TNF levels in the stromal compartment and CXCL12 ($P<0.004$), and between TNF and IL6 (<0.001). We named the co-expression of these three mediators the TNF network.

Further confirmation of the TNF network in cell lines

We previously reported that stable expression of short hairpin RNA (shRNA) to TNF inhibited CXCL12 and IL-6 production and CXCR4 expression in IGROV-1 cells (3). To look for further *in vitro* evidence of the TNF network we stably expressed shRNA to CXCR4 in these cells (Figure 1A). This also reduced constitutive production of TNF, CXCL12 and IL6 (Figure 1B), but did not affect production of TGF- β 1 or bFGF. Transient transfection of RNAi to CXCR4 in IL-6 and TNF-producing TOV21G clear cell carcinoma cells also attenuated TNF and IL6 expression (Supplementary Figure 2).

Functional interdependence of TNF network cytokines

We then compared gene expression patterns in high and low TNF network biopsies and the IGROV-1 cells in order to obtain information on the actions of the TNF network. We prepared in triplicate microarrays from the mock transfected and shCXCR4 IGROV-1 cell lines described in Fig1A and B in which the TNF network was inhibited. An eBayes t-test was used to determine a list of differentially expressed probes between IGROV1 cells treated with shCXCR4 versus IGROV-1 controls. The heatmap in figure 1C shows a hierarchical clustering using probes differentially expressed in shCXCR4 IGROV-1 cells compared to control/mock transfected IGROV-1 cells. We next selected gene expression data from biopsies that were in the highest or lowest 50 for gene expression levels for all three signalling pathways (n=28 high TNF network biopsies, n=25 low TNF network biopsies) from the AOCS dataset.

We then identified, using gene set enrichment analysis, GSEA, (13), pathways and processes significantly increased in the high TNF network biopsies and cell lines compared to low TNF biopsies and cell lines, pathways and processes that were also significantly reduced in the cell lines when the network was inhibited by shRNA to CXCR4. We validated the results using the TCGA dataset, this time comparing 52 high TNF network samples versus 45 low TNF network samples defined as above for AOCS samples.

GSEA revealed a strong association between high TNF network expression and angiogenesis, cell adhesion, cell cycle and inflammation signaling (Table 2). Strikingly, there was also a significant association with NOTCH signaling. As the biopsies would also contain infiltrating leukocytes, we looked for associations with immune cell signatures (10). High TNF network biopsy samples had significant enhancement of gene expression profiles for T-cells, neutrophils, myeloid cells, monocytes, dendritic and B-cells ($P<0.0001$) but not NK-cells ($P=0.053$) compared to the low TNF network group (Table 2 column 1). The complete gene list associated with high TNF network in the IGROV-1 cell lines and biopsies is available in Supplementary Tables 2 and 3 respectively.

Knock down of TNF network *in vivo*

Stable knockdown of CXCR4 in the IGROV-1 cells was maintained when they were grown as intra-peritoneal xenografts (Supplementary Figure 3A). Levels of CXCL12 and TNF protein were significantly decreased in tumors derived from shCXCR4 transfected cells (Supplementary Figure 3B) and IL6 levels were also significantly reduced in the tumors ($16 \text{ pg} \pm 13 \text{ pg} / 100 \mu\text{g}$ protein compared with mean $846 \text{ pg} \pm 70 \text{ pg} / 100 \mu\text{g}$ protein in mock transfected tumors).

Stable knockdown of CXCR4 inhibited growth of peritoneal ovarian cancer xenografts measured by bioluminescence imaging (Figure 2A) although it had no effect on cell growth *in vitro* (data not shown). Median survival of mice injected with IGROV-Mock cells was 46 days in contrast to 92 and 79 days in mice bearing tumors derived from two different clones of IGROV-shCXCR4 cells ($P<0.0001$) (Figure 2B). The number of tumor deposits and the extent of organ involvement was significantly reduced in mice injected with IGROV-shCXCR4 cells ($P<0.0001$ for both parameters) after 42 days (data not shown). F4/80+ macrophages were significantly reduced comparing size-matched shCXCR4 and mock-transfected tumors (Figure 2C). The vascular area of size-matched tumor deposits was significantly reduced in IGROV-shCXCR4 compared to IGROV-Mock tumors ($p<0.0001$) (Figure 2D).

Antibodies to TNF also inhibit angiogenesis and a myeloid cell infiltrate

The anti-human TNF antibody infliximab had similar actions to CXCR4 knockdown. After four weeks intraperitoneal growth and treatment *in vivo*, there was a reduction in tumor growth as evidenced by reduced luciferase expression in the anti-TNF treated mice. (Mean RLU of 1.1×10^7 and 1.1×10^7 for PBS and control IgG groups respectively compared to mean RLU 0.3×10^7 for anti-TNF treated mice). The vascular area and the F4/80+ infiltrate were significantly reduced following anti-TNF treatment (Figure 3A, B). There was also a reduction in IL6 protein as assessed by IHC in the tumors treated with anti-TNF antibodies (Figure 3C).

TNF network and the myeloid cell infiltrate in human biopsies

As the TNF network associated with the myeloid cell infiltrate, we asked if TNF network gene expression correlated with myeloid cells in human tumor biopsies. We obtained twenty-one high-grade serous ovarian cancer biopsies from the AOCS series that matched those used in the analyses of Tables 1 and 2; seven were from high and fourteen from low TNF network biopsies. There was a striking association between CD68+ macrophages in the stromal areas and high TNF network expression ($p=0.01$) (Figure 3D). In 10/14 of the low TNF network biopsies, no CD68+ cells could be detected in either tumor or stromal areas at all whereas 7/7 high TNF network biopsies had visible CD68+ cells.

NOTCH signaling is correlated with the TNF network

To further investigate the association between the TNF network and NOTCH signaling (Table 2), we focused on NOTCH3 and JAG1 as both have been implicated in ovarian cancer (14, 15).

In the AOCS dataset, high TNF, CXCL12 and IL6 signalling pathway expression significantly associated with JAG1 ($P=0.03$; $P=7.7 \times 10^{-7}$; $P=0.00053$ respectively) and high CXCL12 and IL6 with NOTCH3 ($P=0.00016$; $P=0.0044$ respectively). In IGROV-1 cells, NOTCH3 and JAG1 mRNA expression was significantly diminished when the TNF network was inhibited by shRNA to CXCR4 (Figure 4A). Anti-IL6 antibodies reduced constitutive JAG1 expression in IGROV-1 as well as in the TOV21G cells. Exogenous IL6 stimulated JAG1 mRNA expression in a STAT3 dependent manner and the effects of TNF could be inhibited by anti-IL6 (Figure 4B). NOTCH3 and JAG1 mRNA levels were also reduced in the tumor lysates from IGROV-1 shCXCR4 tumors. The % of cell nuclei that stained positive for the HES1 transcription factor, downstream of NOTCH signaling, was also significantly inhibited in sections from the shCXCR4 tumors compared to controls (Figure 4C, Supplementary Figure 3C). Treatment of tumors with the anti-TNF antibody also significantly reduced nuclear HES1 protein staining (Supplementary Figure 4). We conclude that key members of the TNF network regulate NOTCH3, JAG1 and HES1.

Anti-TNF treatment of patients with ovarian cancer

We then sought evidence that the TNF network could be inhibited in ovarian cancer patients. Serial samples of ovarian cancer ascites were obtained from nine patients with advanced disease who had been treated with the anti-human TNF antibody infliximab (11). We compared ascites cell TNF network gene expression levels pre-treatment, 24 and 48 hours after infliximab infusion. The results are shown as a heatmap (Figure 5A), with green indicating lower and red indicating higher expression relative to pretreatment. Four patients showed a significant down-regulation of TNF network gene expression 24 and/or 48 hours after anti-TNF antibody infusion. We compared the global gene expression patterns in pre-treatment tumors from patients who showed robust down regulation of the TNF network versus those with little attenuation (patients 1-4 versus patients 5-9 Figure 5A). An empirical

Bayes t-test (6) identified 280 probes that were significantly different between the groups (with an FDR of $p < 0.001$). Probes and samples were clustered with the 280 genes, using average linkage clustering to generate a hierarchical clustering heatmap (Figure 5B). We tested whether the gene sets representing the TNF, CXCL12 and IL6 pathways were enriched in either group. TNF ($P = 2.68 \times 10^{-9}$), CXCL12 ($P = 3.83 \times 10^{-9}$) and IL6 ($P = 5.37 \times 10^{-7}$) pathways were statistically enriched in patients 1-4 compared to patients 5-9, as was NOTCH signaling ($P = 1.64 \times 10^{-3}$) and enhanced gene expression profiles of T cells, neutrophils, myeloid, monocytes, dendritic and B cells ($p < 0.0001$). Patients who had the highest level of TNF network expression showed the most intense down-regulation of the network following infliximab anti-TNF antibody.

Discussion

We have used a combination of molecular biology, bioinformatics and cancer biology techniques to show that key pathways in cancer-related inflammation and Notch signalling are part of an autocrine malignant cell network in human ovarian cancer, a network with paracrine actions on angiogenesis and myeloid cell infiltration into tumors. The starting point of the current work was an observation in ovarian cancer cell lines (3). Here we have found that information obtained from these cell lines is relevant to biopsies advanced human high-grade serous ovarian cancer. Our data also suggest that malignant cells regulate the inflammatory cytokine network in the human ovarian cancer microenvironment.

The factors co-regulated in the TNF network have been individually considered as targets for cancer treatment. In two clinical studies of TNF antagonists in women with advanced ovarian cancer (11, 16), there was some evidence of transient disease stabilization and biological effects consistent with our knowledge of the actions of TNF. In a Phase II trial of the anti-IL6 antibody siltuximab in 18 patients with relapsed progressing ovarian cancer, there was one partial response and seven patients achieved periods of disease stabilisation (17). In the context of the TNF network data described here, patients receiving siltuximab for six months had a significant decline in plasma levels of CXCL12.

The most common and lethal form of ovarian cancer is high-grade serous ovarian cancer (18). Over 95% of all the biopsies investigated (mRNA samples and biopsy sections) in our work were of this subtype. The IGROV-1 cell line is derived from a high-grade serous case. Clear cell carcinoma of the ovary is characterised by over-expression of the IL6-STAT3-HIF pathway (19) and has distinct genetic drivers compared to high grade serous cancers (20, 21). TOV21G cells produced the highest levels of IL6 but also produced other TNF network members. We recently found that one other high-grade serous and six clear cell carcinoma cell lines constitutively co-produce TNF, CXCL12 and IL-6 (unpublished data). There are many examples in the literature of human tumor cell lines that produce TNF, IL-6 or CXCL12 but to our knowledge, few studies have assessed all three of these cytokines together. We believe it is likely that the TNF network is active in tumors with distinct genetic lesions.

Our data show that targeting cytokines such as TNF and IL6 is more likely to influence the tumor microenvironment than to kill malignant cells directly. Therefore anti-cytokine treatments, which are generally well tolerated in patients with inflammatory or malignant disease, are likely to be most useful in combination with conventional chemotherapy or treatments that target the malignant cell directly.

The TNF network defined here has features of a robust network as described by Yarden and colleagues for EGF-ERBB (22, 23). Further characterisation of the TNF network using a systems biology approach may suggest new ways of treating the high grade serous and clear

cell carcinoma. This approach may also help determine the best treatments to combine with anti-cytokine/chemokine agents. Because other malignant cell types produce TNF, IL-6 and CXCL12, these therapeutic strategies may be widely applicable in other cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. *Nature*. 2008; 454:436–44. [PubMed: 18650914]
2. Szlosarek PW, Grimshaw MJ, Kulbe H, Wilson JL, Wilbanks GD, Burke F, et al. Expression and regulation of tumor necrosis factor- α in normal and malignant ovarian epithelium. *Mol Cancer Therapy*. 2006; 5:382–90.
3. Kulbe H, Thompson RT, Wilson J, Robinson SC, Hagemann T, Fatah R, et al. The inflammatory cytokine TNF- α generates an autocrine tumour-promoting network in epithelial ovarian cancer cells. *Cancer Res*. 2007; 67:585–92. [PubMed: 17234767]
4. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science*. 2002; 296:550–3. [PubMed: 11910072]
5. Team RDC. R: A language and environment for statistical computing. R Foundation for Statistical computing; 2008. ISBN 3-900051-07-0: Available from: <http://www.R-project.org>
6. Smyth GK. Limma: linear models for microarray data. *Bioinformatics and Computational Biology Solutions using R and Bioconductor*. 2005:397–420.
7. Gautier L, Cope L, Bolstad BM, Irizarry RA. Affy-analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics*. 2004; 20:307–15. Available from: <http://bioconductor.org>. [PubMed: 14960456]
8. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J Roy Stat Soc, Ser B*. 57:289–300.
9. Network TCGAR. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011; 474:609–15. Available from <http://tcgadata.nci.nih.gov/tcga/>. [PubMed: 21720365]
10. Abbas AR, Baldwin D, Ma Y, Ouyang W, Gurney A, Martin F, et al. Immune response in silico (IRIS): immune-specific genes identified from a compendium of microarray expression data. *Genes Immun*. 2005; 6:319–31. [PubMed: 15789058]
11. Charles KA, Kulbe H, Soper R, Escorcio-Correia M, Lawrence T, Schultheis A, et al. The tumor-promoting actions of TNF- α involve TNFR1 and IL-17 in ovarian cancer in mice and humans. *J Clin Invest*. 2009; 119:3011–23. [PubMed: 19741298]
12. Brennan DJ, Brandstedt J, Rexhepaj E, Foley M, Ponten F, Uhlen M, et al. Tumour-specific HMG-CoAR is an independent predictor of recurrence free survival in epithelial ovarian cancer. *BMC Cancer*. 2010; 10:125. [PubMed: 20359358]
13. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *PNAS*. 2005; 102:8554–8. [PubMed: 16199517]
14. Park JT, Li M, Nakayama K, Mao TL, Davidson B, Zhang Z, et al. Notch3 gene amplification in ovarian cancer. *Cancer Res*. 2006; 66:6312–8. [PubMed: 16778208]

15. Choi J-H, Park JT, Davidson B, Morin PJ, Shih I-M, Wang T-L. Jagged-1 and Notch3 juxtacrine loop regulates ovarian tumor growth and adhesion. *Cancer Res.* 2008; 68:5716–23. [PubMed: 18632624]
16. Madhusudan S, Muthuramalingam SR, Braybrooke JP, Wilner S, Kaur K, Han C, et al. A phase II study of Ethenarecept (ENBREX) a tumour necrosis factor-alpha inhibitor in recurrent ovarian cancer. *J Clin Oncol.* 2005; 23:5950–9. [PubMed: 16135466]
17. Coward J, Kulbe H, Chakravarty P, Leader D, Vassileva V, Leinster DA, et al. Interleukin-6 as a Therapeutic Target in Human Ovarian Cancer. *Clinical Cancer Research.* 2011; 17:6083–96. [PubMed: 21795409]
18. Vaughan S, Coward J, Bast RC Jr, Berchuck A, Berek JS, Brenton JD, et al. Rethinking ovarian cancer: recommendations for improving outcomes. *Nature Reviews Cancer.* 2011; 11
19. Anglesio MS, George J, Kulbe H, Friedlander M, Rischin D, Lemech C, et al. IL6-STAT3-HIF signaling and therapeutic response to the angiogenesis inhibitor sunitinib in ovarian clear cell cancer. *Clinical Cancer Research.* 2011; 17:2538–48. [PubMed: 21343371]
20. Bowtell DD. The genesis and evolution of high-grade serous ovarian cancer. *Nat Rev Cancer.* 2010; 10:803–8. [PubMed: 20944665]
21. Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T, et al. ARID1A mutations in endometriosis-associated ovarian carcinomas. *The New England Journal of Medicine.* 2010; 363:1532–43. [PubMed: 20942669]
22. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. *Nature Reviews Mol Cell Biol.* 2006; 7:505–16.
23. Bubil EM, Yarden Y. The EGF receptor family: spearheading a merger of signaling and therapeutics. *Curr Opin in Cell Biol.* 2007; 19:124–34. [PubMed: 17314037]

Precis

Key pathways involved in cancer-associated inflammation and Notch signalling appear to contribute to an autocrine cell network in ovarian cancer, with implications for new therapeutic approaches.

Figure 1. The TNF network and gene expression *in vitro*

A) Cell surface expression of CXCR4 in IGROV-1 cells; IgG2 α isotype control (black line), CXCR4 (red line). B) Effects of CXCR4 knockdown on cytokine secretion by IGROV-1 ovarian cancer cells mean values ($\pm$ SD) from triplicate wells (**, $P < 0.01$ as compared with IGROV-Mock cells). A and B Representative of three separate experiments. C) Affymetrix cDNA array analysis was performed on IGROV-1 and IGROV-Mock transfected versus two independent IGROV-1 clones stably transfected with CXCR4 shRNA (I and II). Statistically different increases in gene expression are shown as pseudocolour red and green as decreases in expression.

Figure 2. TNF network inhibition by shCXCR4 in ovarian cancer xenografts

A) Bioluminescence imaging 42days after i.p. injection of IGROV-Mock, and IGROV-shCXCR4 luciferase expressing cells: Red highest photon flux; blue, lowest photon flux. Quantification of bioluminescence from primary tumors (n=6 mice per group) (*, $P < 0.05$ and **, $P < 0.01$). B) Survival of mice injected with ovarian cancer xenografts. Ten mice per group were injected with IGROV-Mock (squares), IGROV-shCXCR4 (I) (triangles) or IGROV-shCXCR4 (II) (inverted triangles) cells. Combined data from two separate experiments, $P < 0.0001$, both lines of IGROV-shCXCR4 compared with mice injected with IGROV-Mock cells. C) F4/80+ macrophages in the tumors. Graph represents number of macrophages quantified in 10 randomly selected areas (high power field $\times 40$) of tumor sections (n=5 each group) at 42 days (***, $P < 0.001$). D) Angiogenesis evaluated 42 days after tumor cell injection. Confocal images (magnification, $\times 20$) shown here are representative sections from tumors following injection of FITC-conjugated lectin and quantification of vascular area. Columns, mean vascular area in each group quantified in 10 randomly selected areas of tumor sections (mean $\pm$ SEM, **, $P < 0.01$).

Figure 3. The TNF network, angiogenesis and the myeloid cell infiltrate

A) Treatment with anti-human TNF- α antibody infliximab influences tumor angiogenesis 28 days after tumor cell injection. Confocal images (magnification, x20) of representative sections from tumors after injection of TRITC-conjugated lectin and B) quantification of the vascular area. Columns, mean vascular area in each group quantified in 10 randomly selected areas of tumor sections (mean $\pm$ SEM, **, $P < 0.01$) and number of macrophages in each group quantified in 10 randomly selected areas (high power field x40) of tumor sections (n=5 each group) at 28 days. C) IL6 protein after treatment with infliximab. D) Correlation between high TNF network gene expression in human high-grade serous ovarian cancer biopsies and CD68+ cells.

Figure 4. NOTCH signaling and the TNF network in ovarian cancer cells

A) Levels of NOTCH3 mRNA in IGROV-Mock or IGROV-shCXCR4 cells and validation of the microarray gene expression analysis of JAG1 by real time RT-PCR. Three samples in each group were used and in vitro mRNA expression levels in shCXCR4 cells compared with mock transfected cells (*, $P < 0.05$, **, $P < 0.01$) B) IL6 dependent expression of Jagged1 in ovarian cancer cell lines assessed by real time RT-PCR after stimulation with either 20ng/ml IL-6 (with or without 1 μ M Stat3 inhibitor WP1066), 20ng/ml TNF or TNF with 10ug/ml anti-IL6 antibody for 48 hours. Data representative of three independent experiments. C) real time RT-PCR measurement of mRNA levels of NOTCH3, JAG1 (n=5 each group, *, $P < 0.05$, **, $P < 0.01$) and number of tumor cell nuclei showing positive staining for HES1 in 10 randomly selected areas per tumor section (n=3, **, $P < 0.01$).

Figure 5. Effects on ascites cell gene expression during infliximab treatment

Serial ascites cell samples pre- and during treatment were obtained from nine patients. Patients 1,2,5,6,8 received 10mg/kg and 3,4,7,9 received 5mg/kg infliximab. A) RT-PCR results for the member of the TNF network shown in heatmap format. Red indicates higher expression and green indicates low expression relative to the mean expression of the gene across all samples. A Pearson's correlation was used to determine how similar the expression levels of the genes were to the expression profile of TNF. A correlation coefficient of 1 indicates an exact match. (CXCR4, $cc=0.70$; CXCL12, $cc=0.77$; IL6, $cc=0.72$; JAG1, $cc=0.95$ and NOTCH3, $cc=0.79$). B) Differential gene expression of the same nine patient samples before treatment was assessed using an empirical Bayes t-test. 280 probes were found to be statistically significant. At a P value threshold of 0.001. These were used to draw a hierarchical cluster heatmap using Cluster.

Table 1

Co-expression of TNF network signalling pathways in ovarian cancer biopsies

Pathways enrichment	Pathways linked	AOCS dataset	Merged dataset	TCGA dataset
TNF	CXCL12	6.6×10^{-32}	3.6×10^{-8}	1.6×10^{-11}
	IL-6	1.5×10^{-25}	3.8×10^{-6}	4.5×10^{-13}
CXCL12	TNF	1.3×10^{-20}	1.1×10^{-4}	2.0×10^{-9}
	IL-6	2.1×10^{-25}	1.3×10^{-5}	1.5×10^{-16}
IL-6	CXCL12	7.6×10^{-33}	6.7×10^{-16}	3.3×10^{-17}
	TNF	4.1×10^{-25}	3.0×10^{-16}	1.0×10^{-9}

Comparison of the samples with the 50 'highest' versus the 50 'lowest' levels reveals an association between expression levels of genes in the TNF, CXCL12 and IL-6 signalling pathways in the AOCS (n=285) and merged datasets (n=245) and highest and lowest 25% of the TCGA samples (n=590).

Table 2

Pathways and processes that correlate with the TNF network

Pathways and processes	High versus low TNF network		
	AOCS dataset	TCGA dataset	shCXCR4
Development regulation of angiogenesis	0.0007	<0.0001	<0.0001
Cell adhesion	<0.0001	<0.0001	<0.0001
NOTCH signaling	0.0003	0.007	0.0003
Cytoskeleton actin filaments	0.0051	<0.0001	0.0001
Inflammation MIF signaling	0.0007	<0.0001	0.0021
Development blood vessel morphogenesis	0.0004	<0.0001	0.0026
Muscle contraction	0.0002	0.0002	0.004
Androgen receptor signaling	0.003	0.003	0.004
Inflammation protein C signaling	0.007	<0.0001	0.0081
Cell adhesion cell junctions	<0.0001	0.018	0.0087
Cell cycle	<0.0001	<0.0001	0.009
Immune cell signatures*	<0.0001	<0.0001	N/A

The most significant pathways and processes were determined by the criteria of p value $p < 0.01$. Column 1 = high TNF network versus low TNF network samples from AOCS dataset; Column 2 = high TNF network versus low TNF network TCGA dataset; column 3 = IGROV-1 wild type and IGROV-1 mock versus two clones of shCXCR4 knockdown cells each parameter analysed in triplicate.