Published in final edited form as:

Cancer Res. 2020 August 01; 80(15): 3088–3100. doi:10.1158/0008-5472.CAN-18-3825.

IL-26, a non-canonical mediator of DNA inflammatory stimulation, promotes TNBC engraftment and progression in association with neutrophils.

Timothy N. Trotter[*,1], **Casey W. Shuptrine**[*,1], **Li-Chung Tsao**[1], **Robert D. Marek**[2], **Chaitanya Acharya**[1], **Jun-ping Wei**[1], **Xiao-Yi Yang**[1], **Gangjun Lei**[1], **Tao Wang**[1], **Herbert Kim Lyerly**[1], **Zachary C. Hartman**[1,2]

[1]Department of Surgery, Duke University, Durham NC

[2]Department of Pathology/Immunology, Duke University, Durham NC

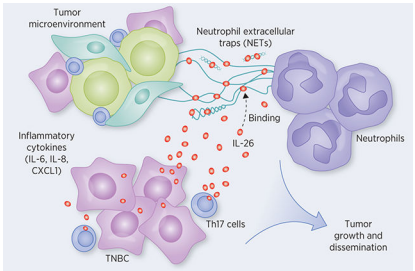
Abstract

Interleukin-26 (IL-26) is a unique amphipathic member of the IL-10 family of cytokines that participates in inflammatory signaling through a canonical receptor pathway. It also directly binds DNA to facilitate cellular transduction and intracellular inflammatory signaling. While IL-26 has almost no described role in cancer, our in vivo screen of inflammatory and cytokine pathway genes revealed IL-26 to be one of the most significant inflammatory mediators of mammary engraftment and lung metastatic growth in triple-negative breast cancer (TNBC). Examination of human breast cancers demonstrated elevated IL-26 transcripts in TNBC specimens, specifically in tumor cells as well as in Th17 CD4+ T-cells within clinical TNBC specimens. IL-26 did not have an autocrine effect on human TNBC cells, but rather its effect on engraftment and growth in vivo required neutrophils. IL-26 enhanced mouse-derived DNA induction of inflammatory cytokines, which were collectively important for mammary and metastatic lung engraftment. To neutralize this effect, we developed a novel IL-26 vaccine to stimulate antibody production and suppress IL-26 enhanced engraftment in vivo, suggesting that targeting this inflammatory amplifier could be a unique means to control cancer-promoting inflammation in TNBC and other autoimmune diseases. Thus, we identified IL-26 as a novel key modulator of TNBC metastasis and a potential therapeutic target in TNBC as well as other diseases reliant upon IL-26-mediated inflammatory stimulation.

Graphical Abstract

Corresponding Author: Zachary C. Hartman, MSRBI Room 414 Box 2606, Durham NC 27710, Phone- 919-613-9110, zachary.hartman@duke.edu.
*Authors contributed equally to this manuscript

The authors declare no potential conflicts of interest.



TNBC and Th17 cells express IL-26 which binds neutrophil-derived NETs in the TME. NET DNA-bound IL-26 induces inflammatory signaling in the TME resulting in tumor progression

Keywords

Breast cancer; triple-negative breast cancer; inflammation; IL-26; genomic screen

Introduction

Triple-Negative Breast Cancers (TNBC) are a heterogeneous group of cancers that lack activating mutations in typical proto-oncogenes, making targeted therapy difficult compared to other breast cancers (BC)(1,2). However, many TNBC tumors display an inflammatory signature composed of cytokines and chemokines that directly stimulate tumor growth at the primary site, protect tumor initiating cells, enhance dissemination and engraftment in new niches, and ultimately promote overt metastasis (3–6). Cytokines and chemokines produced by TNBC cells also recruit tumor-enhancing innate immune cells to the primary site while systemically mobilizing innate immune cells to distant niches. We and others have previously shown that various cytokine networks are essential for tumor progression, both clinically and in animal models, particularly through a combination of TNBC cell derived Interleukin (IL) −6 and IL-8 (3). Furthermore, animal studies have concluded that inflammation from infiltrating innate immune cells plays a key role in enhancing tumor growth and metastasis (4). Once in the tumor microenvironment (TME), these immune cells are activated to produce even greater inflammation which directly supports tumor growth, invasion, and subsequent metastasis (4,6–9). These data are backed by numerous clinical studies of human TNBC that describe them as highly infiltrated with immune cells – including T cells, neutrophils, and monocyte/macrophage lineage cells – especially when compared to other sub-types of BC (10–12). Nevertheless, which cytokine networks are most important for overall regulation of TNBC progression, or if there are any central regulators that have an outsized impact on patient survival, remain unknown.

In order to investigate the role of inflammation in TNBC and narrow down the list of candidates involved in TNBC progression, dissemination, and engraftment, we conducted a focused inflammatory screen of TNBC in vivo, similar to our previous in vivo screen of immune modulator genes (13). Specifically, we modeled multiple stages of BC progression by direct injection into the mammary fat pad (MFP) for engraftment and growth and intra-venous (i.v.) injection via lateral tail vein for dissemination, engraftment, and growth at a metastatic site (lung). These screens revealed interleukin-26 (IL-26) as a significant and novel mediator of progression in both primary and disseminated niches and was validated in multiple TNBC cell lines as well as in BC samples.

IL-26 is a 19-kDa α-helical cytokine that belongs to the IL-10 cytokine family (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26) and has no known homolog in mice (14). IL-26 mRNA has most widely been detected in activated lymphoid cells including Th17 and NK cells (15), but reports of expression in a variety of other cell types are emerging, including monocytes, bronchial epithelial cells, fibroblast-like synoviocytes, and smooth muscle cells (14). IL-26 canonically activates the Jak-Stat3 pathway through binding a heterodimeric complex of IL10RB and IL20RA (16,17). This triggers expression of pro-inflammatory cytokines IL-1β, IL-6, GM-CSF, and TNFα in human monocytes and activates type I (β) and type II (γ) interferons (IFN), as well as induces IL-8 and IL-10 expression, in human epithelial cells (14). Recent evidence also suggests that the highly cationic amphipathic properties of IL-26 results in novel functions, such as direct bactericidal activity through pore formation and bacterial-membrane disruption (18). In addition, the amphipathic nature of IL-26 allows it to act as a cell penetrating carrier molecule for DNA, particularly Neutrophil Extracellular Traps (NETs), giving extracellular DNA access to intracellular receptors such as STING and TLR9 (14,18,19). Though the lack of a mouse homolog has made mechanistic studies difficult, mounting clinical data suggests IL-26 is involved in a host of human diseases. High serum levels of IL-26 are observed in patients with contact dermatitis, rheumatoid arthritis, Crohn's disease, chronic hepatitis C infection, and severe pediatric asthma (14,20). Finally, emerging evidence implicates a role of IL-26 in various cancers. IL-26 mRNA is elevated in biopsies of cutaneous T-cell lymphomas, IL-26 directly promotes proliferation and survival of gastric cancer cells, and elevated IL-26 expression is a poor prognostic indicator of both recurrence-free survival and overall survival of hepatocellular carcinoma after surgical resection (21–23).

The appearance of IL-26 as a significant target in both shRNA screens, along with the apparent ability of human IL-26 to modulate tumor progression in mice with no native homolog, warranted further investigation. Herein, we demonstrate the role of IL-26 in TNBC engraftment and progression in mice and the conserved ability of IL-26 to elicit DNA-mediated inflammation in mouse cells. Further, our study implicates the effect primarily depends upon neutrophils in the TME, potentially serving as an microenvironmental regulator in orchestrating tumor inflammation by enhancing neutrophil NET DNA stimulation of multiple inflammatory factors, which are collectively important for TNBC. Due to this critical role, we also investigated its therapeutic potential through IL-26-specific vaccine-induced antibodies. In sum, our studies reveal a potentially significant role for IL-26 in TNBC and suggest it as an actionable therapeutic target to suppress inflammation and inhibit TNBC progression.

Materials and Methods

Cell Lines and reagents:

MDA-MB-231, HEK293TW, SUM159, SUM149, MDA-MB-468 and 32DC3 cells were acquired from ATCC and through the Duke Cell Culture Facility. Mouse E0771 cells were purchased from CH3 Biosystems (940001). MDA-MB-231-LM2 cells were a kind gift from Joan Massague (24). All cells were cultured according to ATCC and vendor specifications and tested to be free of Mycoplasma and other rodent pathogens (RADIL IMPACT III Test).

For human cell lines, Short Tandem Repeat (STR) DNA profiling was performed on parental stocks to verify their identity through the Duke DNA Analysis Sequencing Facility. Individual lines were expanded upon receipt for frozen stocks, and cells were used for experiments at ≤ 20 passages. Recombinant human IL-26 (rhIL-26) was acquired from either R&D Systems (cat 1870-IL) or produced and validated in collaboration with Genscript (Piscataway, NJ).

Library Deconvolution and analysis:

DNA from MDA-MB-231 shRNA containing library cells was isolated using QIAamp DNA mini kit and sent to Sigma for shRNA quantification. Log transformed expression values were compared between lung tumors, MFP tumors, and initial (pre-engraftment) tumor cells using LIMMA version 3.14.4 on the software R. shRNAs with an absolute Log2 fold change greater than 1 and FDR values for empirically Bayes moderated statistics below 0.05 were considered to have statistically significant differential expression.

Lentiviral and Adenoviral techniques:

All lentiviral vectors were produced in 293T cells, using 2nd generation packaging plasmids and using our previously described techniques (3). Viral stocks were concentrated by ultracentrifugation and utilized with 5ug/ml polybrene to generate stable cell lines. Inducible shRNA vectors were generated as previously described (3) and CRISPR lentiviral vectors were produced using the plentiCRISPRv2 backbone (25) (obtained from Addgene) and produced using standard methods. The chemokine-cytokine shRNA library was generated by combining a human cytokine-chemokine pLKO.1 library (Sigma SH0811, 106 genes targeted by 528 shRNA constructs) with a custom second library (78 genes targeted by 642 shRNA constructs) to cover all genes listed in the KEGG Cytokine-Chemokine set. Viral shRNA library was tittered on MDA-MB-231 cells and utilized at a MOI of 1 with puromycin selection beginning 48 hrs post-infection. After 5 more days, MDA-MB-231 library cells were utilized in the in vivo screen. The OVA and IL-26 adenoviral vectors were generated using Gateway cloning techniques. We first generated OVA and IL-26 ENTRY plasmid clones, then recombined them with pAd-CMV5 vectors (Invitrogen) using LR clonase II. Vectors were linearized using PacI and adenoviral stock amplified using previously described techniques (26).

NET isolation:

NETs were isolated from SCID-beige mouse neutrophils after enriching with the EasySep Mouse Neutrophil Enrichment Kit (StemCell Technologies, Vacouver, Canada). Purity of isolated neutrophils were >90% as analyzed by LY6G+ and CD11b+ FACS staining. NETosis was induced via 100nM PMA (Sigma) for 4 hours at 37°C. Plates were gently washed with PBS followed by strong pipetting to remove NETs attached to the plate, and cellular debris were removed by centrifugation.

PBMC/Splenocyte/Neutrophil Stimulation:

Human PBMCs isolated from healthy donors, mouse SCID-Beige splenocytes, or mouse 32DC3 neutrophil cells were plated in a 96 well plate with RPMI-1640 medium (Invitrogen)

at 37°C with 5% CO_2. rhIL-26 (50 ng/mL) was incubated with 1 or 10 μg/ml of sheared DNA or isolated NETs in nuclease-free H_2O for 30 minutes at 37°C for the IL-26 to bind to the DNA. In some conditions DNase (30 IU/mL) was pre-incubated with IL-26 and/or DNA/NETs. DNA was isolated from MDA-MB-231, Sum159, and MDA-MB-468 cultured cells, combined, and sheared using a 30 second sonication pulse. The resulting solutions were added to cells in a 96 well plate and allowed to incubate for 24 hours after which conditioned medium was collected for ELISA.

ELISPOT Assay:

Elispot assays were performed using our previously described technique (27). Briefly, splenocytes (500,000 cells/well) were plated in RPMI-1640 medium with 10% heat inactivated fetal bovine serum and stimulated with OVA peptide (SIINFEKL; 1 ug/ml; Sigma), lysed E0771-WT cells (10k/well), or lysed E0771-IL26+ cells (10k/well). PMA (50ng/ml) and Ionomycin (1 ug/ml) (Sigma) were used as positive controls. Irrelevant HIV-gag peptide mix (2.6 ug/ml; JPT, Germany) was used as a negative control.

RNA in situ hybridization (RNAscope):

In situ detection of IL26 mRNA transcripts in was performed on formalin-fixed, paraffin-embedded Cancer Diagnosis Program (CDP) Breast Cancer Progression Tissue Microarray sets using the RNAscope Multiplex Fluorescent kit (323100; Advanced Cell Diagnostics, Newark, CA)(28) following the manufacturer's protocol. A more detailed description can be found in the Supplemental Methods.

qRT-PCR Breast Cancer cDNA Arrays:

Commercially available TissueScan Breast Cancer cDNA arrays were obtained from OriGene (Rockville, MD, United States). Each array (BCRT101, BCRT102, BCRT103, BCRT104) contains cDNA from 48 samples comprised of normal breast tissue or Stage I, IIA, IIB, IIIA, IIIB, IIIC, and IV BC tissue, along with designations of tumor molecular subtype. Reactions were performed with iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA) and normalized to β-Actin using primers provided by the manufacturer.

In vivo tumor growth and metastasis:

Female, 6–8 week old SCID-beige (Taconic Biosciences, Rensselaer, NY) or C57Bl/6J (Jackson Labs, Bar Harbor, MA) mice were implanted with into the 4^{th} inguinal MFP [MDA-MB-231 = 10^6 cells in PBS; MDA-MB-468 = 3×10^6 cells in 1:1 PBS with matrigel (Corning, Tewksbury, MA); SUM159 = 5×10^6 cells in 1:1 PBS with matrigel; SUM149 = 4×10^6 cells 1:1 PBS with matrigel]. For E0771 cells, animals were either injected with 10^5 cells in the MFP or with 10^5 cells in 100 μL PBS directly into the lateral tail vein. Tumor growth was monitored bi-weekly by caliper to track tumor growth for up to 6 weeks or when reaching a terminal endpoint of 2 cm^3, and volume was calculated as (length × $width^2$)/2. For bioluminescence imaging, tumor-bearing animals were injected intraperitoneally with D-luciferin (100 mg/kg) and live, whole-body bioluminescence intensity was measured using an IVIS Kinetic (Perkin Elmer, Waltham, MA). Mice were then immediately sacrificed via CO_2 inhalation and lungs were isolated for further ex vivo imaging. For

vaccine experiments, animals were vaccinated with 5×10^{10} viral particles of described adenoviral vectors via foot pad as described previously (28). To deplete neutrophils in vivo, 300 μg of anti-Ly6G (1A8) or isotype control antibodies (2A3; BioXCell, West Labanon, NH) were injected i.p. into mice 24 hours before engraftment of tumor cells and again twice weekly i.p. for a total of 6 treatments. All studies with animals were approved by the Duke University Institutional Animal Care and Use Committee.

Single-cell RNA seq Analyses:

FACS-sorted CD3+ single cell (scRNAseq) data as unique molecular identifier (UMI) count matrix from two individual triple negative BC primary tumor samples (project approval number is 'SEGMENT' 13/123) was obtained from Gene Expression Omnibus (GEO) repository under the accession id GSE110686. The UMI count matrix was previously generated by the authors using Cell Ranger software (version 1.3.1) as provided by the 10xGenomics pipeline. A more detailed description of data processing and analysis can be found in the Supplemental Methods.

Human Breast Cancer dataset analysis:

METABRIC and TCGA data was accessed and queried using the web-based cBioPortal software (29–32) and data was visualized and statistics were performed using Graphpad Prism software. According to cBioPortal, copy number data is derived by algorithms such as GISTIC or RAE and the copy number level per gene is reported as: −2 = deep deletion/ possible homozygous deletion; −1 = shallow deletion/possible heterozygous deletion; 0 = diploid; +1 = low-level gain/few additional copies; and +2 = amplification.

Statistical Analyses:

All statistical analysis, unless otherwise noted, was performed using GraphPad Prism version 8 (GraphPad Software; La Jolla, CA). Marks for significance include: * for p-value ≤ 0.05; ** for p-value ≤ 0.01; *** for p-value ≤ 0.001; and **** for p-value ≤ 0.0001. Error bars represent SEM, tests between two groups were performed via t-test, and tests between 3 or more groups were performed via ANOVA with Tukey post-hoc correction. Survival analysis for all mouse xenograft studies was performed using Prism 8 (GraphPad). Survival plots were considered significantly different if the log-rank (MantelCox) test resulted in a p ≤ 0.05.

Results

An in vivo functional genomics screen using a custom designed shRNA library comprised of 1,170 shRNAs (targeting all annotated human cytokines, chemokines, and their receptors) was initially used to identify inflammatory genes that contribute to TNBC cell engraftment and experimental metastasis in vivo using a well-defined metastatic human TNBC cell line (186 genes, Table S1) (33). Cells were infected with the library at an MOI=1 to limit multiple integrations and selected using puromycin (1 week) to generate a stable library. MDA-MB-231 shRNA library infected cells (1×10^6 to ensure ~800x coverage of the library) were either implanted orthotopically into the MFP or injected intravenously into female SCID-beige mice and allowed to form tumors (Fig. 1A). Excised tumors from MFP (3

weeks) and lungs (4 weeks) revealed significantly selected shRNAs compared to the parental library (Fig. 1B); 120 shRNAs impacting TNBC cell progression in the MFP and 166 shRNAs in the lung (Fig. 1C). Further analysis established 35 shRNAs that were significant in both microenvironments (Fig. 1D, Table S2, S3). In comparing shRNA candidates between the lung and MFP environments, multiple significant shRNAs targeting interleukin 26 (IL-26) were identified which was determined to be one of the most significant genes influencing tumor cell engraftment and colonization in both niches. IL-26 is a human specific gene without a homolog in mice and has no reported connection with BC. Therefore, to verify that IL-26 is a clinically relevant hit, the METABRIC dataset was analyzed (31) which revealed that IL-26 DNA is amplified in approximately 3% of BCs (60 of 1981 profiled), and amplification of IL-26 is a poor prognostic indicator of BC as a whole (Fig. 1E). This data suggested that IL-26 could play a role in BC progression and prompted further investigation.

The relative protein expression in multiple human BC cell lines was also assessed for IL-26 expression based on molecular subtype (Supplemental Fig. S1A). IL-26 protein was detectable in TNBC lines compared to to HER2+ BT474, ER+ MCF-7 cell lines, and a 293T control indicating that IL-26 expression may be most abundant in TNBC. Next, mRNA expression in the TCGA data set (n=981) was interrogated by the clinically relevant molecular subtypes (32), which revealed IL-26 was most highly expressed in Basal-like BC (which is highly enriched for TNBC) (34). This was statistically significant compared to both Luminal A and Luminal B subtypes, and narrowly missed the $p \leq 0.05$ cutoff against Normal-like (n=981 samples profiled, Fig 2A). While not significant, HER2-enriched tumors appeared to also contain elevated IL-26 expression. To validate these findings an independent panel of BC mRNAs was interrogated for IL-26 expression by qRT-PCR. Significantly elevated IL-26 was observed in all BCs (n=160, Fig S1B), although unlike the TCGA results the expression was not specifically higher in TNBC compared to ER+ or HER2+ BC (Supplemental Fig. S1B,C). As these and TCGA data are comprised of bulk sequencing data, tumor specific expression of IL-26 in formalin-fixed, paraffin-embedded Cancer Diagnosis Program (CDP) BC Progression Tissue Microarrays was analyzed. Critically, these histologically defined assessments revealed significant upregulation of IL-26 in TNBC tumors compared to other BC tumors (Fig. 2B), which was observed directly in tumor cells (Non-TNBC n=106, TNBC n=23, Fig 2C). The microarrays also contained a small number of matched, tumor-adjacent normal mammary epithelium, pairwise assessment indicated a specific and significant increase of IL-26 in tumor cells compared to the normal cells (Supplemental Fig. S1D). To identify other cells expressing IL-26 in TNBC, single-cell RNA sequencing of CD45+ immune cells from two patient TNBCs was utilized. The analyses revealed that IL-26 was expressed in a CD3+CD4+IL17+ T-cell cluster in TNBC (Fig. 2D, E; Supplemental Fig. S2), directly consistent with recently published reports that associate Th17 T-cells with IL-26, neutrophils and metastasis (18,35). Collectively, these data confirmed IL-26 expression in BC cells specifically, indicate that TNBC expresses significantly more IL-26 than other molecular subtypes, and implicate Th17 cells within tumors as another source of IL-26 in TNBC.

After confirming that the shRNAs selected in the screen could all significantly suppress IL-26 expression (Supplemental Fig. S3), IL-26 knock-down (KD) MDA-MB-231 cells

were generated to determine the functional effect of IL-26 in TNBC (16,22). While IL-26 suppression did not alter proliferation or in anchorage-independent growth rates in vitro (Supplemental Fig. S4A,B), it did significantly delay tumor formation and growth in the MFP (Fig. 3A, B). Additionally, mice injected with control cells had a greater proportion of lungs positive for luciferase (4/10) compared to mice injected with IL-26 KD cells (1/10) (Supplemental Fig. S4C), though this did not reach statistical significance. In addition, i.v. injections of control or IL-26 KD cells into SCID-beige mice revealed that this effect was not niche specific as IL-26 KD reduced experimental metastasis to the lungs (Supplemental Fig. S4D, E). While some measure of IL-26 suppression persisted in vivo, IL-26 expression was strongly selected for in both control and shRNA KD tumors compared to cell lines before injection, further suggesting that IL-26 is a tumor-promoting cytokine (Supplemental Fig. S5A, B). Notably, IL-26 KD tumors expressed significantly more inflammatory cytokine mRNAs known to be downstream of IL-26, such as IL-6 and IL-8 (Supplemental Fig. S5C). Whether this reflects compensatory selection for inflammatory cytokines downstream of IL-26 or the temporal result of IL-26 expression returning by the end of the experiment due to the limitations of shRNA KD, or possibly both, is unclear. IL-26 KD cells were also generated for two additional TNBC lines (SUM159 and MDA-MB-468 cells) and one TNBC Inflammatory BC (IBC) cell line (SUM149). As before, suppression of IL-26 did not alter proliferation in vitro (Supplemental Fig. S6) but did suppress engraftment and growth of all types of TNBC tumors in vivo (Fig. 3C–E). Finally, the effect of IL-26 in murine tumors was assessed by overexpression in mouse TNBC E0771 cells (Supplemental Fig. S7). In this setting, IL-26 conferred a significant growth advantage in both SCID-beige (Fig. 3F) and immune competent mice (Supplemental Fig. S8), strongly suggesting that IL-26 was acting as a paracrine factor through a mechanism conserved in mice.

The reported canonical signaling pathways for IL-26 were initially investigated to determine the possible mechanism behind the effects of IL-26 in vivo. Surprisingly, canonical receptors (IL20RA and IL10RB) were not detectable on MDA-MB-231 cells by flow cytometry and only minimal expression was observed by qRT-PCR (Supplemental Fig. S9A) (16). However, artificial expression of IL20RA and IL10RB in MDA-MB-231 cells resulted in Stat3 signaling upon stimulation with rhIL-26, thus demonstrating that this pathway is functionally intact and that the lack of canonical signaling is likely due to a lack of receptor expression (Supplemental Fig. S9B). Furthermore, Stat3 activity was undetectable when mouse IL10RB and IL20RA counterparts were artificially expressed in 293T cells (Supplemental Fig. S9C, D) suggesting the in vivo effects observed were not likely due to interaction with mouse IL10RB/IL20RA receptors.

Recent studies suggest that IL-26 is an amphipathic, cationic cytokine that directly binds extracellular DNA (and human NET DNA) and acts as a carrier molecule to mediate DNA entry into cells for intracellular DNA-stimulation of inflammation (18,19). Because this function of IL-26 would not necessarily be dependent on species-specific receptors, we hypothesized that IL-26 could be functioning as a mediator of DNA-induced inflammatory signaling in the TME. To test this hypothesis, primary murine monocytes/granulocytes (SCID-beige splenocytes, Supplemental Fig. S10A) or mouse 32DC3 neutrophil cells were stimulated overnight with rhIL-26, DNA, or DNA + rhIL-26. In these experiments rhIL-26 or DNA alone were unable to induce significant cytokine production, however the

combination of rhIL-26 and DNA significantly induced IL-6, CXCL1, and IL1β in mouse splenocytes (Fig. 4A) and IL-6 and CXCL1 in mouse neutrophils (Fig. 4B). Similar effects were seen in human PBMCs, although there was some stimulatory effect of IL-26 alone (Supplemental Fig. S10B). Importantly, IL-26 DNA binding was confirmed similarly to previous groups (Supplemental Fig. S10C) and pre-incubation of DNase to IL-26+DNA tempered mouse splenocyte stimulation, indicating that these effects are due to DNA-bound IL-26 (Supplemental Fig. S10D)(19).

Extracellular DNA alone is not known to induce inflammatory responses in vivo, but DNA NETs have recently been described to promote TNBC metastasis (7,36,37). Binding of rhIL-26 to NETs produced by mouse neutrophils (Supplemental Fig. S11) was first confirmed by confocal microscopy (Fig. 4C). Additionally, stimulation of mouse splenocytes with rhIL-26 alone, rhIL-26+NETs, NETs alone, or rhIL-26+NETs pre-treated with DNAse demonstrated that IL-26 induces a strong cytokine response (IL-6, Cxcl1) when combined with NETs which could be diminished with pre-treatment of DNase (Fig. 4D). These data reveal that human IL-26 interacts with mouse NETs, and that IL-26+NETs are a potent inducer of inflammatory signaling in mouse cells. Finally, to confirm that neutrophils (and thereby NETs) mediate the effects of IL-26 in vivo, neutrophils were depleted by anti-Ly6G antibody treatment of SCID-beige mice harboring MDA-MB-231 tumors. Antibody treatment significantly depleted neutrophils (Supplemental Fig. S12) and significantly suppressed tumor growth relative to isotype treated mice (Fig. 4E). Notably, this treatment did not alter the growth of shIL-26 cells (Fig. 4E) suggesting that neutrophils have little effect in the absence of IL-26. Collectively, these data suggest that tumor-derived IL-26 binds DNA to stimulate pro-inflammatory cytokines in innate immune cells to promote an inflammatory TME.

The ability of IL-26/DNA to elicit inflammatory cytokines in vitro, along with the selection of these cytokines/chemokines over time in tumors in vivo (especially when IL26 was suppressed), suggested that these IL-26 induced cytokines could play a key role in early engraftment of TNBC (14). Notably, the highly metastatic MDA-MB-231 LM2 subclone (24) expressed significantly elevated levels of these IL-26 inducible cytokines (IL-6, IL-8, and CXCL1), supporting this hypothesis (Supplemental Fig. S13A–C). To formally test this, several of these cytokines/chemokines (IL-6/IL-8/CXCL1) were directly suppressed using inducible shRNA vectors in TNBC cells which we have previously published (Supplemental Fig. S13D–F). While single gene suppression did not alter engraftment to the lung after i.v. injection, tandem suppression significantly reduced TNBC dissemination and growth in the lung (Fig. 5A–B). This effect was temporary, however, and tumors with coordinately suppressed cytokines eventually expanded in the lungs, potentially indicating insufficient gene suppression. These limitations led to generation of CRISPR-based knock-outs (KOs) completely deficient in expression of IL-6, IL-8, and CXCL1 (Crispr3x) (Supplemental Fig. S14A). Again, mammary engraftment and experimental metastasis was suppressed (Fig. 5C–D) and was comparable to the single suppression of IL-26. Importantly, isolation of these outgrowths revealed that tumor cells did not re-acquire expression of these genes (Supplemental Fig. S14B). To determine if IL-26 expression and stimulation of other non-tumor cells could rescue this deficit, IL-26 was overexpressed in MDA-MB-231 Crispr3x cells. Notably, IL-26 overexpressing Crispr3x tumors grew significantly faster in the MFP

compared to both control cell lines (Fig. 5E). Furthermore, lungs from mice bearing Crispr3x + IL-26 tumors had significantly more de novo metastases compared to controls, and the level of metastasis was not correlated with the size of the tumor (Fig. 5F,G; Supplemental Fig. S14C). Collectively, these data indicate that the impact of IL-26 is not limited to these specific cytokines produced by tumor cells and suggests that its enhancement of tumor engraftment and dissemination may be mediated through other cell types in the TME.

Assessment of inflammatory signaling was also expanded to patient samples by analyzing the METABRIC dataset for IL-26 and associated cytokines/chemokines (IL-6, IL-8, and CXCL1). This IL-26 signature, consisting of the IL26, IL6, IL8, and CXCL1 genes, was significantly elevated in TNBC, elevated in HER2+/ER-BC, and low in ER+ and HER2+ BC (Fig. 6A). Because neutrophils were critical for IL-26 function in vivo, if an established neutrophil signature consisting of 23 documented neutrophil selective genes (38–41) would also be elevated in TNBC and associate with this IL-26 network in human BC (METABRIC) was determined. These analyses revealed that the neutrophil gene signature was also significantly enriched in TNBC (Fig. 6B), and that these signatures are significantly correlated in patients (Supplemental Fig. S15). Furthermore, there was an inverse relationship between 10-year survival and expression of both the IL-26 network ($p=0.033$) and neutrophil signature ($p<0.00038$), suggesting that these networks may contribute to metastasis and poor survival in human TNBC (Fig. 6C, D). In sum, these data demonstrate that an IL-26 inflammatory network is associated with TNBC, that a neutrophil signature is enriched in TNBC in association with the IL-26 network, and that both correlate with BC survival.

Finally, the therapeutic potential of targeting IL-26 in TNBC was tested. We were unable to identify a specific inhibitor or antibody to block IL-26 in vivo, therefore we generated an adenoviral vaccine to immunologically target IL-26, as done previously (28). To determine the immunologic potential of this vaccine, C57/Bl6 mice were vaccinated and assessed for IL-26-specific antibody and T-cell responses. While Ad-IL-26 vaccination elicited significant IL-26-specific antibody responses compared to control vaccination (Fig. 7A), only modest (non-significant) T-cell responses to IL-26+ cells was observed (Supplemental Fig. S16A). Importantly, both groups of mice produced equivalent anti-Ad antibody responses, indicating equivalent injection (Supplemental Fig. S16B). Based on these results, vaccine-induced anti-tumor responses to IL-26 versus a control Ad vector (Ad-OVA) were tested. In this study, one cohort of mice was vaccinated two weeks prior to tumor implantation (Prevention group) and another cohort was vaccinated post-implantation (Treatment group). Both prevention and treatment administration of the Ad-IL-26 vaccine significantly suppressed the engraftment and growth of IL-26 + E0771 cells compared to control vector (Fig. 7B). Notably, IL-26 expression was elevated after implantation in vivo (Fig. 7C, S6) compared to in vitro passaged controls, again suggesting selection for IL-26 expression or the stimulation of IL-26 from factors in the TME as with MDA-MB-231 cells. Additionally, modest IL-26 expression from the largest Ad-IL26 vaccinated tumor that yielded sufficient protein to analyze was noted by western blot (Fig. 7C, S6). Collectively, these studies suggest that antibodies and/or vaccines targeting IL-26 may have a therapeutic benefit in TNBC treatment.

Discussion

While inflammation is known to play a key role in the progression and growth of TNBC, the identities of the most critical cytokines and chemokines remain unclear (3,42,43). To decipher the impact of inflammatory genes important for TNBC progression in different native and metastatic microenvironments, we utilized an in vivo focused shRNA screen of cytokine/chemokine pathways that identified IL-26 as one of the most significant tumor-expressed cytokines in both orthotopic and disseminated tumors. As a unique human gene without a murine homolog and little association with cancer, we first confirmed the clinical relevance and expression of IL-26 in TNBC by multiple means, including analysis of TCGA and METABRIC datasets, qRT-PCR of BC tissue and through RNAscope analysis of human BC tissue microarrays. These studies revealed that human BC cells directly express IL-26, which is elevated in TNBC compared to other subtypes. Additionally using scRNAseq data, we also identified significant IL-26 expression in a Th17 sub-population based on human BC single-cell RNA sequencing, thus replicating similar results from other groups (18). To our knowledge this is the first report demonstrating IL-26 expression in BC or TNBC and suggests that this cytokine may also play a role in prompting other types of breast cancer.

The conserved ability of IL-26 to promote tumor progression using multiple TNBC and IBC cell lines in vivo was in some regards surprising, especially in light of these cells lacking canonical IL-26 receptors and no discernable direct effect of IL-26 in vitro (based on Stat3 reporter assays). While we found that IL-26 did not elicit Stat3 signaling through the mouse homologs of IL-26 receptors (IL20RA and IL10RB), it is possible that IL-26 is acting in part through other non-canonical receptors. Alternatively, the ability of IL-26 to bind DNA/NETs and induce inflammation was intriguing, especially because NETs have been shown to promote BC metastasis and exit from dormancy (7,44). We confirmed that this mechanism is indeed conserved, as human IL-26 and mouse NETs stimulated the secretion of multiple inflammatory cytokines from mouse (and human) immune cells. Using a depletion strategy, we also found that neutrophils mediate the IL-26 effect in vivo and that their depletion only suppresses tumor growth of human TNBC tumors in the presence of IL-26, while growth was unaffected in tumors with suppressed IL-26. These data support that IL-26 is acting through NETs in vivo to promote an inflammatory TME, and future studies to define the exact mechanisms of this process are ongoing.

Our observation that IL-26 is associated with TNBC, typified by inflammation in the TME, along with the induction of inflammatory cytokines in mouse cells by IL-26, led us to hypothesize that these downstream cytokines also contribute to tumor dissemination and growth. In vivo experiments revealed that IL-26 induced cytokines (such as IL-6, IL-8, and CXCL1), collectively contributed to TNBC metastatic dissemination to the lungs, comparable to IL-26 suppression alone. Moreover, IL-26 overexpression was able to rescue (and even promote metastasis of) IL-6, IL-8, and CXCL1 triple KOs cells, indicating that IL-26 plays a more central, individual role in mediating local inflammation through the stimulation of multiple inflammatory genes. This could suggest that IL-26 may be more significant in human TNBC, where multiple cells express IL-26, such as Th17 CD4+ T-cells. Notably, we found that elevated expression of an IL-26 inflammatory axis or neutrophil signature inversely correlates with survival in BC and were positively associated, supporting

a potential link with clinical metastatic progression. Furthermore, both the IL-26 inflammatory axis and neutrophil signature are highest in TNBC, which matches the TCGA data for IL-26 mRNA expression in TNBC and to a lesser extent, in HER2+ BC. As several studies have demonstrated that IL-26 is highly expressed in inflammatory environments (such as those found in primary and metastatic tumor lesions), as well as in leukemias where it was originally cloned (15,45,46), it may play a significant role in dissemination, survival, and growth in metastatic niches of other cancers as well.

While many studies have implicated neutrophils in BC metastasis and progression (8,47–49), it is unclear how these effects are mediated. The elegant study by Park and colleagues demonstrated that DNA NETs from neutrophils served as the dominant mechanism through which neutrophils exerted their pro-metastatic effect on tumors (7). However, it was not clear how these NETs elicited changes in tumor metastasis, although the authors speculated that a chemotactic factor, such as HMGB1 (which binds DNA), may be associated with the NETs. Our work suggests that IL-26 produced by tumors cells (as well as possibly Th17 T-cells) may be the critical factor through which DNA NETs, as well as other DNA in the TME, stimulate inflammatory cytokines/chemokines (such as IL6, IL8, CXCL1 and others). This is supported by other studies of IL-26 in infection, where IL-26 was demonstrated to be a principal mediator of inflammation and innate immune recruitment in the lungs. Collectively, these data support a role for IL-26 as a major regulator of DNA-elicited inflammation and may explain why suppression of this single cytokine had a significant effect in TNBC engraftment screens in two different and distinct microenvironments.

Therapeutically, our studies suggest a key role for IL-26 in tumor progression through its amplification and stimulation of multiple cytokines, in comparison to the contribution of other individual inflammatory cytokines. As TNBC cannot be treated with conventional targeted therapy, we investigated the potential of blocking IL-26 through vaccination to suppress metastasis and tumor growth. Ad-IL-26 vaccination was well-tolerated and elicited significant levels of anti-IL-26 antibodies, which significantly reduced IL-26+ tumor growth and prolonged survival. Our findings, together with previous reports demonstrating that neutrophil NETs enhance metastatic seeding and awakening of dormant cells in TNBC (7,44), raise the intriguing possibility that targeting IL-26 could be a safe and effective means of reducing metastasis and controlling outgrowth of TNBC without compromising the essential functions of neutrophils in patients. This identification of a conserved IL-26-DNA/NET axis that regulates inflammation could offer new potential approaches to combat oncogenic inflammation in cancer, as well as inflammation responsible for autoimmunity and other diseases. Few pharmacologic means exist to prevent DNA deposition by neutrophils, especially as their depletion is problematic in chemotherapy treatments where G-CSF is often administered to combat infections resulting from neutropenia. In comparison to systemic reduction of DNA via i.v. delivery of DNAse enzymes, targeting IL-26 (a highly regulated non-enzymatic protein) for transient blockade/neutralization may be a more tractable approach. Indeed, if blockade of IL-26 can be achieved, it may also be possible to limit inflammation in other solid cancers, leukemias, and in various autoimmune diseases, where IL-26 has been implicated (35,45).

In conclusion, our studies reveal that IL-26 is highly expressed in TNBC and mediates a pro-inflammatory TME, at least partially through binding extracellular DNA (particularly NETs) to stimulate the expression of multiple pro-inflammatory cytokines (IL6, IL8, and CXCL1). This phenotype appears to be dependent upon neutrophils, supporting and providing mechanistic evidence for how DNA excreted from these cells supports tumor growth and metastasis (7,8,47–49). Interestingly, our data also suggests that individual inflammatory cytokines are not essential for growth and metastasis of TNBC cells, but would rather be collectively targeted through IL-26 as a more manageable method of extinguishing pro-inflammatory responses in tumors. Thus, this unique, non-canonical avenue of cytokine driven inflammation presents a distinct co-factor target that may be exploited therapeutically for multiple types of cancer, as well as in autoimmune disease settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by grants from the National Institutes of Health (NIH) (T32-CA009111 to T. Trotter; 5K12CA100639-09 and R01 CA238217-01A1 to Z. Hartman), Department of Defense (DOD) (BC113107 to H.K. Lyerly), and Susan G. Komen (CCR14299200 to Z. Hartman).

We would like to especially thank the members of the Duke Surgery Center for Applied Therapeutics for their technical assistance and stimulating discussion regarding this project. We also thank Dr. Josh Snyder and Josh Ginzel for their assistance in performing RNA scope experiments and Lauren Halligan, MSMI, who created the illustration in Figure 1 and edited the graphical abstract.

References

1. Bianchini G, Balko JM, Mayer IA, Sanders ME, Gianni L. Triple-negative breast cancer: challenges and opportunities of a heterogeneous disease. Nat Rev Clin Oncol 2016;13:674–90 [PubMed: 27184417]
2. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486:346–52 [PubMed: 22522925]
3. Hartman ZC, Poage GM, den Hollander P, Tsimelzon A, Hill J, Panupinthu N, et al. Growth of triple-negative breast cancer cells relies upon coordinate autocrine expression of the proinflammatory cytokines IL-6 and IL-8. Cancer research 2013;73:3470–80 [PubMed: 23633491]
4. Shalapour S, Karin M. Immunity, inflammation, and cancer: an eternal fight between good and evil. J Clin Invest 2015;125:3347–55 [PubMed: 26325032]
5. Hartman ZC, Yang XY, Glass O, Lei G, Osada T, Dave SS, et al. HER2 overexpression elicits a proinflammatory IL-6 autocrine signaling loop that is critical for tumorigenesis. Cancer Res 2011;71:4380–91 [PubMed: 21518778]
6. Korkaya H, Liu S, Wicha MS. Breast cancer stem cells, cytokine networks, and the tumor microenvironment. J Clin Invest 2011;121:3804–9 [PubMed: 21965337]
7. Park J, Wysocki RW, Amoozgar Z, Maiorino L, Fein MR, Jorns J, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med 2016;8:361ra138
8. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. Nature 2015;528:413–7 [PubMed: 26649828]
9. Su S, Liu Q, Chen J, Chen J, Chen F, He C, et al. A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. Cancer Cell 2014;25:605–20 [PubMed: 24823638]

10. Pistelli M, De Lisa M, Ballatore Z, Caramanti M, Pagliacci A, Battelli N, et al. Pre-treatment neutrophil to lymphocyte ratio may be a useful tool in predicting survival in early triple negative breast cancer patients. BMC Cancer 2015;15:195 [PubMed: 25884918]
11. Wei B, Yao M, Xing C, Wang W, Yao J, Hong Y, et al. The neutrophil lymphocyte ratio is associated with breast cancer prognosis: an updated systematic review and meta-analysis. Onco Targets Ther 2016;9:5567–75 [PubMed: 27660475]
12. Matsumoto H, Koo SL, Dent R, Tan PH, Iqbal J. Role of inflammatory infiltrates in triple negative breast cancer. J Clin Pathol 2015;68:506–10 [PubMed: 25750267]
13. Shuptrine CW, Ajina R, Fertig EJ, Jablonski SA, Kim Lyerly H, Hartman ZC, et al. An unbiased in vivo functional genomics screening approach in mice identifies novel tumor cell-based regulators of immune rejection. Cancer Immunol Immunother 2017
14. Larochette V, Miot C, Poli C, Beaumont E, Roingeard P, Fickenscher H, et al. IL-26, a Cytokine With Roles in Extracellular DNA-Induced Inflammation and Microbial Defense. Front Immunol 2019;10:204 [PubMed: 30809226]
15. Donnelly RP, Sheikh F, Dickensheets H, Savan R, Young HA, Walter MR. Interleukin-26: an IL-10-related cytokine produced by Th17 cells. Cytokine Growth Factor Rev 2010;21:393–401 [PubMed: 20947410]
16. Sheikh F, Baurin VV, Lewis-Antes A, Shah NK, Smirnov SV, Anantha S, et al. Cutting edge: IL-26 signals through a novel receptor complex composed of IL-20 receptor 1 and IL-10 receptor 2. J Immunol 2004;172:2006–10 [PubMed: 14764663]
17. Hor S, Pirzer H, Dumoutier L, Bauer F, Wittmann S, Sticht H, et al. The T-cell lymphokine interleukin-26 targets epithelial cells through the interleukin-20 receptor 1 and interleukin-10 receptor 2 chains. J Biol Chem 2004;279:33343–51 [PubMed: 15178681]
18. Meller S, Di Domizio J, Voo KS, Friedrich HC, Chamilos G, Ganguly D, et al. T(H)17 cells promote microbial killing and innate immune sensing of DNA via interleukin 26. Nat Immunol 2015;16:970–9 [PubMed: 26168081]
19. Poli C, Augusto JF, Dauve J, Adam C, Preisser L, Larochette V, et al. IL-26 Confers Proinflammatory Properties to Extracellular DNA. J Immunol 2017;198:3650–61 [PubMed: 28356384]
20. Konradsen JR, Nordlund B, Levanen B, Hedlin G, Linden A. The cytokine interleukin-26 as a biomarker in pediatric asthma. Respir Res 2016;17:32 [PubMed: 27029915]
21. Xi ZF, Jeong S, Wang CC, Li HJ, Guo H, Cai J, et al. Expression of IL-26 predicts prognosis of patients with hepatocellular carcinoma after surgical resection. Hepatobiliary Pancreat Dis Int 2019;18:242–8 [PubMed: 30956053]
22. You W, Tang Q, Zhang C, Wu J, Gu C, Wu Z, et al. IL-26 promotes the proliferation and survival of human gastric cancer cells by regulating the balance of STAT1 and STAT3 activation. PLoS One 2013;8:e63588 [PubMed: 23704922]
23. Wolk K, Mitsui H, Witte K, Gellrich S, Gulati N, Humme D, et al. Deficient cutaneous antibacterial competence in cutaneous T-cell lymphomas: role of Th2-mediated biased Th17 function. Clin Cancer Res 2014;20:5507–16 [PubMed: 25212608]
24. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. Nature 2005;436:518–24 [PubMed: 16049480]
25. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. Nat Methods 2014;11:783–4 [PubMed: 25075903]
26. Hartman ZC, Black EP, Amalfitano A. Adenoviral infection induces a multi-faceted innate cellular immune response that is mediated by the toll-like receptor pathway in A549 cells. Virology 2006
27. Crosby EJ, Wei J, Yang XY, Lei G, Wang T, Liu CX, et al. Complimentary mechanisms of dual checkpoint blockade expand unique T-cell repertoires and activate adaptive anti-tumor immunity in triple-negative breast tumors. Oncoimmunology 2018;7:e1421891 [PubMed: 29721371]
28. Hartman ZC, Wei J, Osada T, Glass O, Lei G, Yang XY, et al. An adenoviral vaccine encoding full-length inactivated human Her2 exhibits potent immunogenicty and enhanced therapeutic efficacy without oncogenicity. ClinCancer Res 2010;16:1466–77

29. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401–4 [PubMed: 22588877]
30. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013;6:pl1 [PubMed: 23550210]
31. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 2012;486:346–52 [PubMed: 22522925]
32. Cancer Genome Atlas N Comprehensive molecular portraits of human breast tumours. Nature 2012;490:61–70 [PubMed: 23000897]
33. Minn AJ, Kang Y, Serganova I, Gupta GP, Giri DD, Doubrovin M, et al. Distinct organ-specific metastatic potential of individual breast cancer cells and primary tumors. J Clin Invest 2005;115:44–55 [PubMed: 15630443]
34. Badve S, Dabbs DJ, Schnitt SJ, Baehner FL, Decker T, Eusebi V, et al. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. Mod Pathol 2011;24:157–67 [PubMed: 21076464]
35. Kaabachi W, Bouali E, Berraies A, Dhifallh IB, Hamdi B, Hamzaoui K, et al. Interleukin-26 is overexpressed in Behcet's disease and enhances Th17 related -cytokines. Immunol Lett 2017;190:177–84 [PubMed: 28811236]
36. Yasuda K, Yu P, Kirschning CJ, Schlatter B, Schmitz F, Heit A, et al. Endosomal translocation of vertebrate DNA activates dendritic cells via TLR9-dependent and -independent pathways. J Immunol 2005;174:6129–36 [PubMed: 15879108]
37. Barton GM, Kagan JC, Medzhitov R. Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA. Nat Immunol 2006;7:49–56 [PubMed: 16341217]
38. Itoh K, Okubo K, Utiyama H, Hirano T, Yoshii J, Matsubara K. Expression profile of active genes in granulocytes. Blood 1998;92:1432–41 [PubMed: 9694733]
39. Hanzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. BMC Bioinformatics 2013;14:7 [PubMed: 23323831]
40. Bertrand G, Coste J, Segarra C, Schved JF, Commes T, Marti J. Use of serial analysis of gene expression (SAGE) technology reveals new granulocytic markers. J Immunol Methods 2004;292:43–58 [PubMed: 15350511]
41. Palmer C, Diehn M, Alizadeh AA, Brown PO. Cell-type specific gene expression profiles of leukocytes in human peripheral blood. BMC Genomics 2006;7:115 [PubMed: 16704732]
42. McDaniel JM, Varley KE, Gertz J, Savic DS, Roberts BS, Bailey SK, et al. Genomic regulation of invasion by STAT3 in triple negative breast cancer. Oncotarget 2017;8:8226–38 [PubMed: 28030809]
43. Rody A, Karn T, Liedtke C, Pusztai L, Ruckhaeberle E, Hanker L, et al. A clinically relevant gene signature in triple negative and basal-like breast cancer. Breast Cancer Res 2011;13:R97 [PubMed: 21978456]
44. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. Science 2018;361
45. Knappe A, Hor S, Wittmann S, Fickenscher H. Induction of a novel cellular homolog of interleukin-10, AK155, by transformation of T lymphocytes with herpesvirus saimiri. J Virol 2000;74:3881–7 [PubMed: 10729163]
46. Tengvall S, Che KF, Linden A. Interleukin-26: An Emerging Player in Host Defense and Inflammation. J Innate Immun 2016;8:15–22 [PubMed: 26202572]
47. Fridlender ZG, Albelda SM, Granot Z. Promoting metastasis: neutrophils and T cells join forces. Cell Res 2015;25:765–6 [PubMed: 26138787]
48. Acharyya S, Massague J. Arresting supporters: targeting neutrophils in metastasis. Cell Res 2016;26:273–4 [PubMed: 26823207]

49. Wculek SK, Malanchi I. Neutrophils fan cancer's flames. EMBO J 2015;34:2211–2 [PubMed: 26194723]

Significance:

Findings identify IL-26 as a unique, clinically relevant, inflammatory amplifier that enhances triple-negative breast cancer engraftment and dissemination in association with neutrophils, which has potential as a therapeutic target.

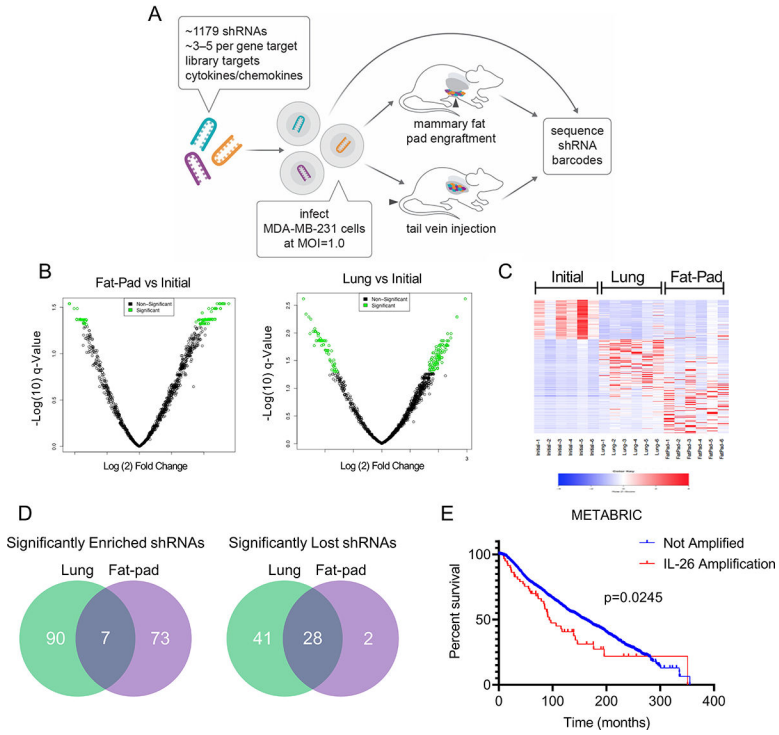


Figure 1. In vivo shRNA focused cytokine screen identifies IL-26 as a functional mediator of TNBC progression in orthotopic and metastatic sites.

A) Lentivirally packaged library with 1179 shRNAs targeting 186 unique genes was exposed to MDA-MB-231 cells at an MOI of 1. 10^6 MDA-231-library cells were injected into SCID-Beige mice through either MFP or tail vein injection and resulting tumors were sequenced and analyzed for shRNA representation. B) Volcano plots of the fold change and statistical significance of each shRNA between mammary fat-pad and lung tumors compared to the initial library. Green shRNAs represent those considered statistically significant. C) Heat map of raw shRNA counts for statistically significant shRNAs for each tumor. D) Venn diagram showing the overlap of significant shRNAs between each independent screen. E) Analysis of the METABRIC dataset reveals that IL-26 DNA amplification in BC results in significantly reduced survival compared to all other BCs (n=1981 profiled samples).

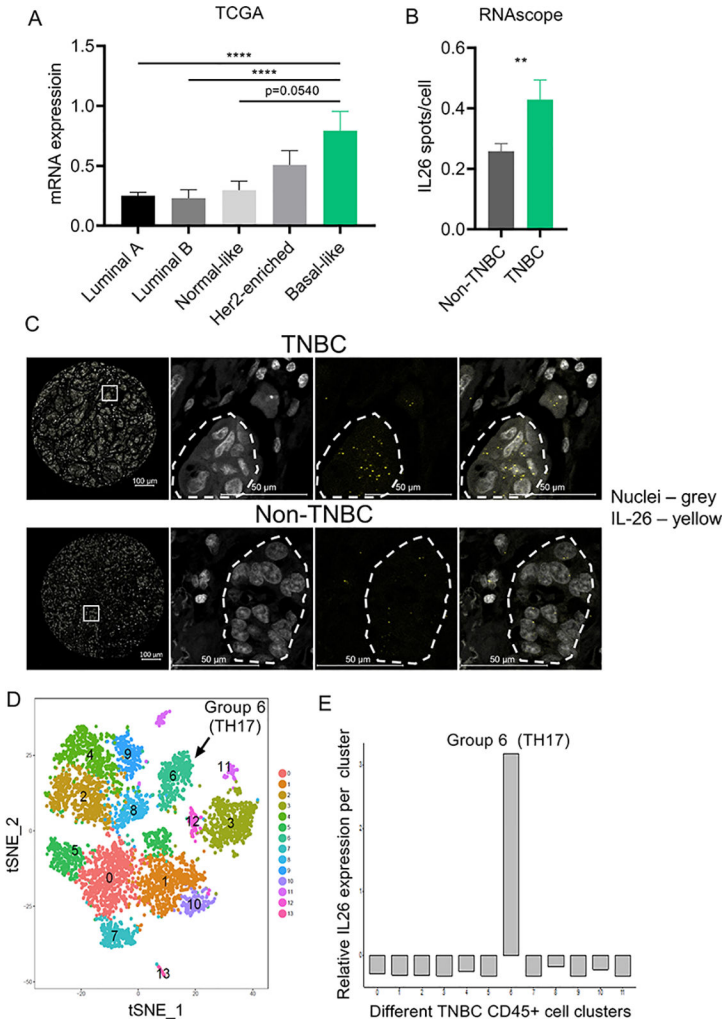


Figure 2. IL-26 is overexpressed in TNBC and is directly produced by tumor cells.

A) Stratifying TCGA BC dataset by molecular subtype reveals that Basal-like tumors are significantly enriched for IL-26 compared to other molecular subtypes (Total= 981 BCs; Luminal A n=499; Luminal B n=197; Her2-enriched n=78; Normal-like n=36; Basal-like n=171). B) RNAscope analysis of a BC Progression Tissue Microarray for IL-26 mRNA detected significantly more IL-26 transcripts compared to all other BCs (Non-TNBC n=107; TNBC n=23). C) Representative images of RNAscope results on tissue microarrays for both TNBC and Non-TNBC tumors. Tumor cell clusters are indicated with dashed lines. Nuclei (Dapi) are indicated in grey and IL-26 is indicated by yellow. D) Single-cell RNA profiling of TNBC patients (GSE110686), Group 6 indicated. E) Expression profile of IL-26 among different CD45+ groups identified in patient TNBC samples. Bars indicate SEM and ** $p < 0.01$, **** $p < 0.0001$.

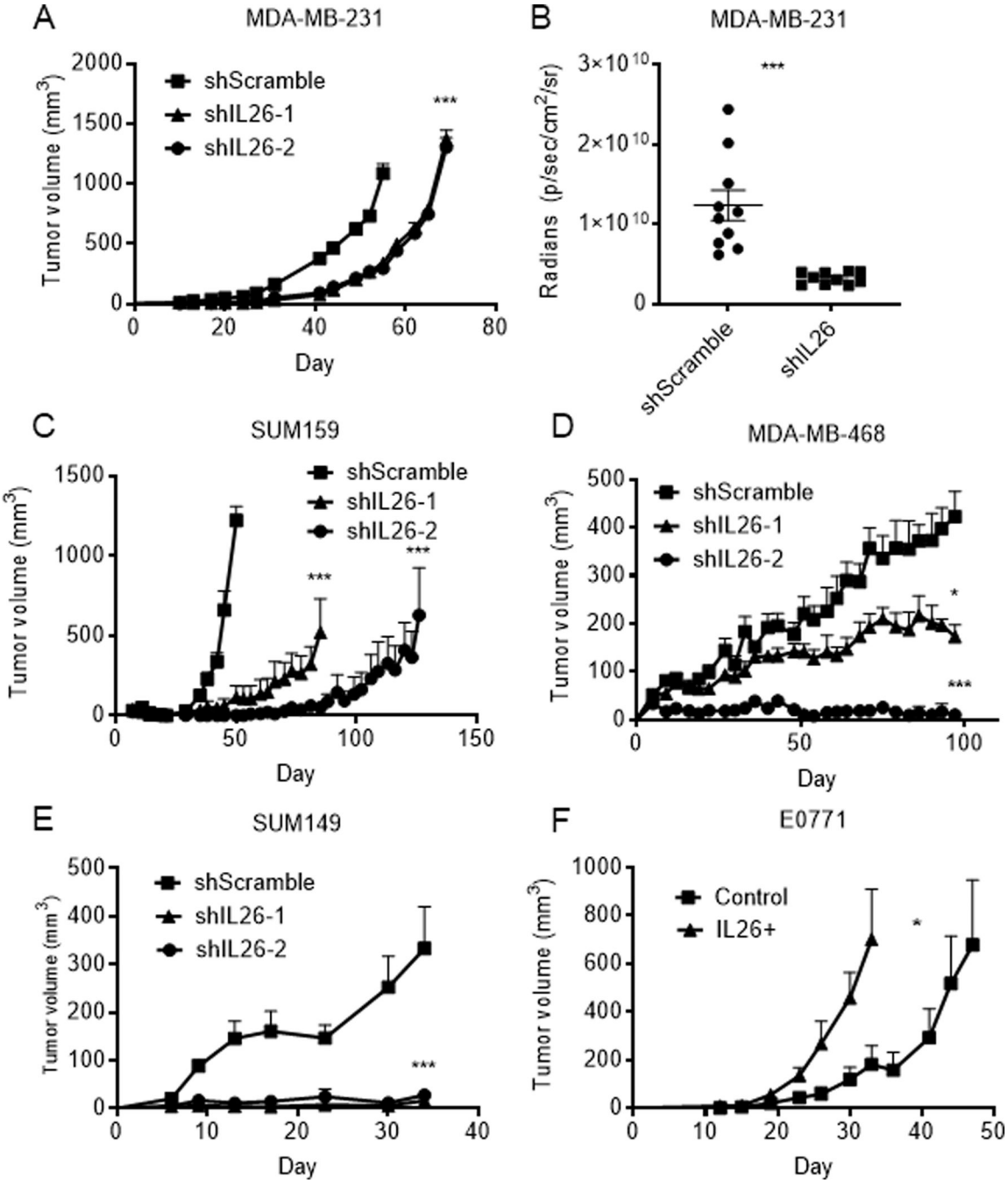


Figure 3. IL-26 is critical for engraftment and growth of TNBC cells.

A) IL-26 knockdown (k/d) reduces the growth of MDA-MB-231 tumors after mammary engraftment in female SCID-Beige mice. B) IL-26 k/d also suppresses overall tumor burden in the fat pad (Luminescence measured from tumors upon sacrifice of mice at week 6, n=10 each). C-E) IL-26 knockdown reduces the growth of SUM159, MDA-MB-468, and SUM149 cells in the MFP of SCID-Beige mice. F) Forced expression of IL-26 in mouse E0771 TNBC cells results in enhanced tumor growth in SCID-Beige mice. In all panels except B, n=5, Bars indicate SEM and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

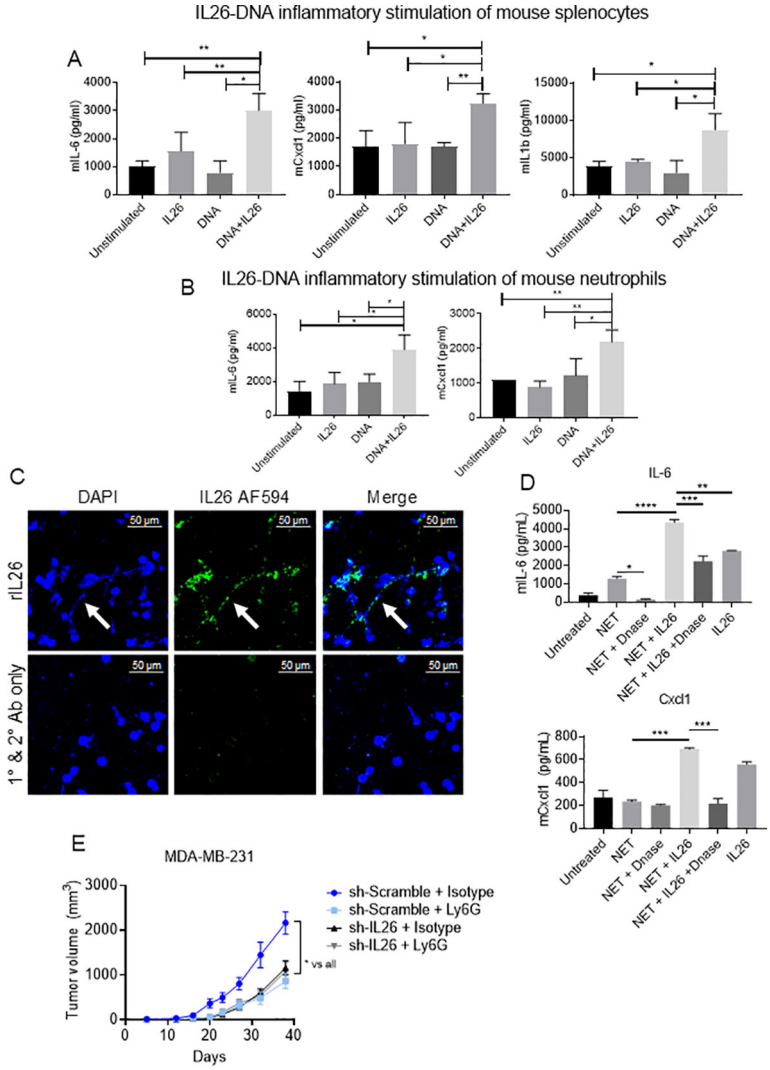


Figure 4. IL-26 enhances DNA/NET stimulation of pro-inflammatory cytokines in mouse cells.

A) Detection of cytokines by ELISA after treatment of primary mouse splenocytes (n=4) and B) a mouse neutrophil cell line 32DC3 (n=4). C) Recombinant IL-26 binding to mouse neutrophil-derived DNA NETs. Arrow indicates co-localization of IL-26 to expulsed DNA. D) Cytokine expression of primary mouse splenocytes after incubation with a combination of mouse NETs and/or IL-26 (n=3 each). E) In vivo antibody depletion of Ly6G+ neutrophils reduces the in vivo growth of MDA-231 cells only in the presence of IL-26 (n=5 each except for shScramble+Ly6G n=4). In all panels Bars indicate SEM and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and $p < 0.0001$.

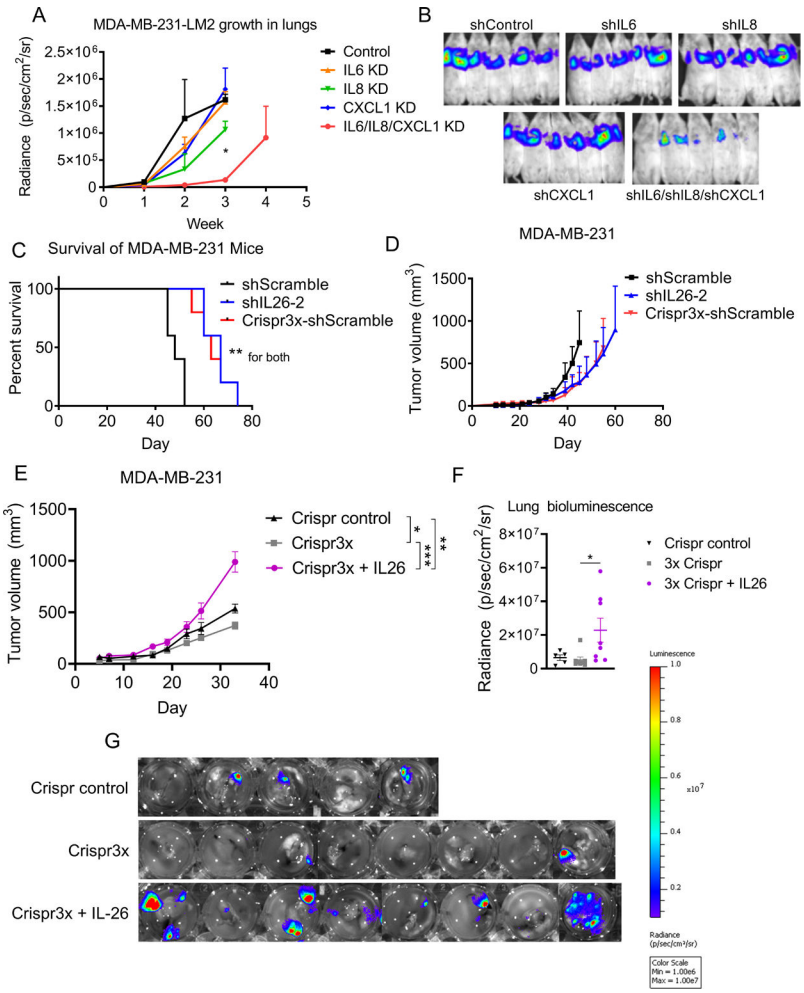


Figure 5. IL-26 inflammatory network is critical for engraftment and metastasis of TNBC but can be rescued by IL-26.

A) In vivo luciferase imaging growth of MDA-MB-231-LM2 cells with IL6, IL8, and CXCL1 knockdown (n=5 each). B) Representative in vivo luciferase imaging of SCID-Beige mice i.v. injected with MDA-MB-231-LM2 knockdown cells. KD of IL-26 and triple k/o of IL6, IL8, and CXCL1 in MDA-MB-231-LM2 cells results in the equivalent survival of (C) and tumor growth in SCID Beige mice (D) (n=5 each). E) Overexpression of IL-26 in MDA-MB-231 cells lacking IL6, IL8, and CXCL1 (Crispr3x) significantly promotes tumor growth in the MFP (Crispr control n=5; Crispr3x +/− IL-26 n=8). F) Crispr3x cells have significantly increased de novo lung metastasis when overexpressing IL-26 compared to controls. G) Bioluminescent images of harvested lungs from E & F. Bars indicate SEM and * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

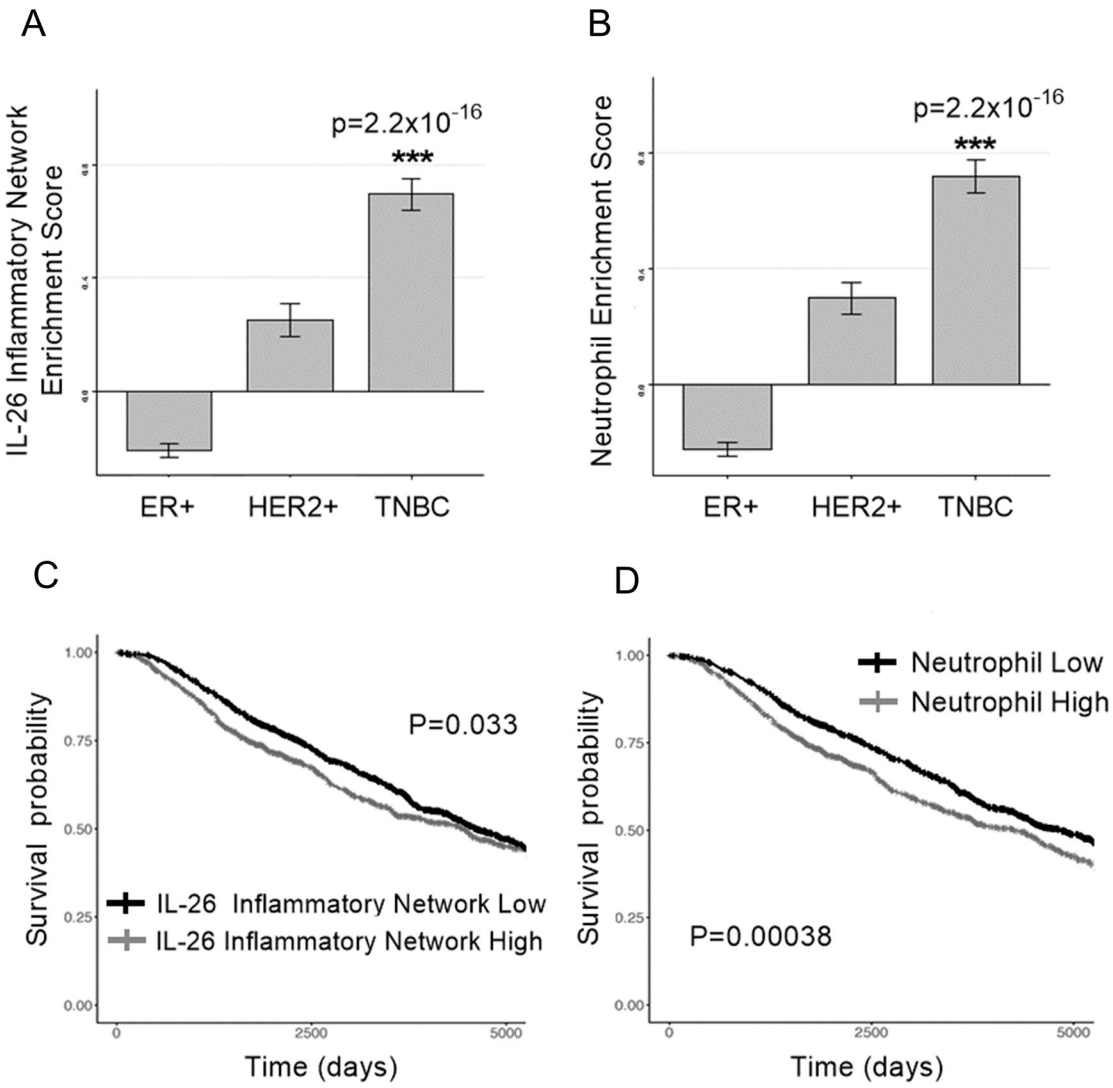


Figure 6: IL-26 inflammatory network expression and neutrophil infiltration is significantly enriched in clinical TNBC and correlates with poor overall survival.

A) Expression of IL26/IL6/IL8/CXCL1 gene network and B) neutrophil network (based on a 23 neutrophil gene signature) in METABRIC dataset. Kaplan-Meier survival curve of BC patients with high or low expression of C) IL-26 inflammatory network or D) neutrophil signature in METABRIC Dataset.

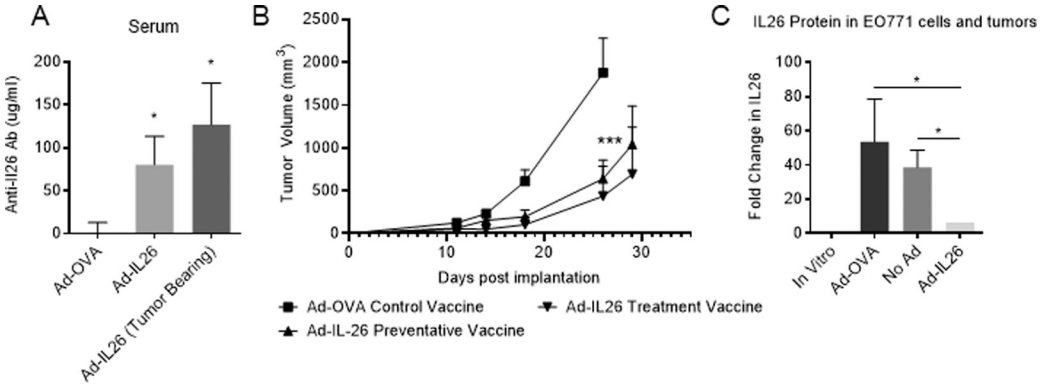


Figure 7. Vaccination targeting IL-26 elicits IL-26-specific antibodies and suppresses IL-26-mediated engraftment and growth of TNBC.

A) Induction of anti-IL-26 antibodies in mice vaccinated with Ad-OVA versus Ad-IL-26 and in Ad-IL26 tumor bearing animals (normalized with control mice; n=5 each). B) In vivo growth of C57Bl/6 mice implanted with 10^5 E0771 tumor cells and treated with Ad-OVA, or Ad-IL-26 (n=5 each). C) Protein expression of IL-26 from bulk E0771 tumors (with tumor cells as a fraction of total cells) after growth in C57Bl/6 mice (densitometry of western blot bands, normalized to IL-26 expressing cells in vitro). In all panels bars indicate SEM and * $p<0.05$, *** $p<0.001$.