Co-Expression and Functional Interactions of Death Receptor 3 and E-Selectin in Clear Cell Renal Cell Carcinoma

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Renal cell carcinoma (RCC) is the most common type of kidney cancer, with the clear cell subtype (ccRCC) representing approximately 70% of cases.1 Prognosis for high-grade RCC is poor, and new approaches to treat this tumor are needed. In this study, we report a new autocrine/paracrine signaling pathway in ccRCC cells involving death receptor 3 (DR3) and E-selectin that could be a target for new therapies.

DR3, also designated as TNFRSF25, TRAMP, LARD, WSL-1, Apo-3, or TR3, is one of eight death domain (DD)—containing receptors of the tumor necrosis factor (TNF) superfamily.2 On binding of its ligand, known as tumor necrosis factor—like 1A (TL1A or TNFSF15), the ligand for DR3, activates NF-κB and mitogen-activated protein kinases, induces both DR3 and E-selectin expression in an NF-κB—dependent manner, and promotes cell cycle entry. DR3 immunoprecipitated from ccRCC tissue contains sialyl Lewis X moieties (the ligand recognized by E-selectin), proximity ligation assays reveal DR3, and E-selectin interacts on ccRCC cells. Like the addition of TL1A, the addition of soluble E-selectin to ccRCC organ cultures activates NF-κB and mitogen-activated protein kinases in ccRCC cells and increases both DR3 and E-selectin expression and cell-cycle entry. In contrast, normal renal tubular epithelium, which poorly expresses DR3, is minimally responsive to either of these ligands. These data suggest a functional role for autocrine/paracrine DR3/E-selectin interactions in ccRCC and its progression, revealing a potential new target for therapeutic intervention. (Am J Pathol 2022, 190: 1–15; https://doi.org/10.1016/j.ajpath.2021.12.010)

Similar to the behavior of inflamed tubular epithelial cells, clear cell renal cell carcinoma (ccRCC) cells express death receptor 3 (DR3 or TNFRSF25) in situ, and expression increases with tumor grade. Surprisingly, E-selectin, which can be induced in endothelial cells by DR3 signaling, is also expressed by ccRCC cells and increases with tumor grade. In ccRCC organ cultures, addition of tumor necrosis factor—like 1A (TL1A or TNFSF15), the ligand for DR3, activates NF-κB and mitogen-activated protein kinases, induces both DR3 and E-selectin expression in an NF-κB—dependent manner, and promotes cell cycle entry. DR3 immunoprecipitated from ccRCC tissue contains sialyl Lewis X moieties (the ligand recognized by E-selectin), proximity ligation assays reveal DR3, and E-selectin interacts on ccRCC cells. Like the addition of TL1A, the addition of soluble E-selectin to ccRCC organ cultures activates NF-κB and mitogen-activated protein kinases in ccRCC cells and increases both DR3 and E-selectin expression and cell-cycle entry. In contrast, normal renal tubular epithelium, which poorly expresses DR3, is minimally responsive to either of these ligands. These data suggest a functional role for autocrine/paracrine DR3/E-selectin interactions in ccRCC and its progression, revealing a potential new target for therapeutic intervention. (Am J Pathol 2022, 190: 1–15; https://doi.org/10.1016/j.ajpath.2021.12.010)

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tissue and colon cancer. Recent studies have reported DR3 expression by some tumors, but its role in tumorigenesis is controversial; it has been implicated as both an anticancer and proancer molecule.

E-selectin (CD62E, also known as endothelial leukocyte adhesion molecule 1), is a type I transmembrane protein that contains lectin-like and epithelial growth factor–like domains, followed by short cysteine-rich repeats. It is primarily expressed by activated but not resting postcapillary venular ECs, but rare epithelial cell expression of E-selectin has been previously reported. E-selectin is transcriptionally induced by cytokines, such as TNF or IL-1, mediated by NF-kB and a variant of activator protein 1. These are the same signaling pathways activated by TL1A binding to DR3. In humans, E-selectin plays a critical and nonredundant role in tethering and rolling of leukocytes to ECs, allowing their subsequent extravasation into inflamed tissues. Expression of E-selectin has been reported on some tumor vasculatures, where it has been proposed as a target for metastasis of tumor cells bearing E-selectin ligands, typically cell surface proteins or lipids bearing sialyl Lewis X (sLeX) or sialyl Lewis A. sLeX may be detected using a monoclonal antibody that binds to human skin–homing T cells where the antigenic determinant is designated as cutaneous lymphocyte antigen (CLA). sLeX expression increases from normal tissue to early-stage bladder or renal cancers to metastatic disease. In RCC, sLeX expression increases from normal tissue to early-stage bladder cancer, tumor stage, and metastases in breast cancer. In addition to adhesion to ECs, one report has suggested that in colon carcinoma, E-selectin binds to and can serve as a functional ligand for DR3, triggering activation of p38 and ERK mitogen-activated protein kinases (MAPKs) and conferring metastatic and survival advantages.

Because DR3 can be induced on normal kidney TECs by inflammation, we hypothesized that it might also play a role in their malignant counterparts, namely, ccRCC cells. In this article, we report that both DR3 and, unexpectedly, E-selectin are expressed on ccRCC cells and that expression of both molecules increases with tumor grade. DR3 extracted from ccRCC tumors bears sLeX moieties and tumor cell E-selectin appears to be complexed with tumor cell DR3 in situ as assessed by proximity ligation assay. In ccRCC, organ culture exogenous E-selectin, like exogenous TL1A, acts as a DR3 ligand, leading to activation of NF-kB and MAPKs, increased expression of both DR3 and E-selectin, and increased cell cycle entry of ccRCC cells.

Materials and Methods
Reagents and Antibodies

Goat anti-human DR3 (catalog number sc6175), rabbit anti-human DR3 (H-300) (catalog number sc7907), mouse anti-human E-selectin (catalog number sc8419), mouse anti-human phosphorylated NF-kB65 (catalog number sc8008), mouse pancytokeratin (C11) (catalog number sc8018), rabbit anti-pancytokeratin (catalog number sc15367), and mouse anti-human phosphorylated ERK (E-4; Tyr^204) (catalog number sc7383) antibodies are from Santa Cruz (Heidelberg, Germany). Rabbit anti-human DR3 (catalog number GTX105713), mouse anti-human E-selectin (catalog number GTX52484), and mouse anti-human sLeX (catalog number GTX39137) antibodies are from Gentex (Taiwan, Republic of China). Mouse anti-sLeX antibody (catalog number AGM-209YJ) is from Creative Biolabs (London, U.K.). Purified mouse anti-human CLA-1 antibody (catalog number 610882) is from BD Biosciences (Berkshire, UK), and rabbit anti-human SCARBI (anti-CLA-1) (catalog number HPA072449) antibody is from Atlas Antibodies AB (Bromma, Sweden). Goat anti-human E-selectin antibody (catalog number BBA18) is from Merck Life Science UK Limited (Dorset, UK). Rabbit anti-human E-selectin (catalog number ab18981) and rabbit anti-human CD31 (platelet endothelial cell adhesion molecule) (catalog number 166994) antibodies are from Abcam (Cambridge, UK). Rabbit anti-human phosphorylated p38 MAPK (Thr180/Tyr183) (3D7) (catalog number 9215s), rabbit anti-human phosphorylated NF-kB65Ser^276 (catalog number cs-3037), and mouse anti-human CD31 (catalog number cs-3528) antibodies are from Cell Signaling Technology (London, UK). Rabbit anti-human phosphorylated p38 MAPK (Thr180, Tyr183) (catalog number -684G), rabbit anti-human CD15/Fut4/SSEA-1 polyclonal antibody (catalog number BS-1702R), rabbit anti-human CD68 (catalog number PA5-32330), and 3,3'-diaminobenzidine tetrahydrochloride substrate kit (catalog number 34,002) are from Thermo Fisher Scientific (Milton KeynesUK). Rabbit anti-phosphorylated JNK (Thr183, Tyr185) antibody (clone 3F7, ZooMab; catalog number ZRB1173) is from Merck Life Science UK Ltd. Monoclonal mouse anti-human cytokertatin (close AE1/AE3) (catalog number 3515) and mouse anti-human CD45 (leukocyte common antigen, clone 2B11 + PD7/26; catalog number GA751) are from Agilent Technologies LDA UK Ltd. (Cheshire, UK). Mouse anti-human DR3/TNFRSF25 antibody (catalog number MAB943), goat anti-human TL1A/TNFSF15 antibodies (catalog number AF744), human recombinant (rh) TL1A protein, anti-goat NL493 (catalog number NL003), anti-goat NL-557 (catalog number NL001), and anti-rabbit NL493 (catalog number NL006), anti-rabbit NL557 (catalog number NL004), anti-mouse NL493 (catalog number NL007), anti-mouse NL493 (catalog number NL009), and sheep anti-human CD14 (catalog number AB383) antibodies are all from R&D Systems (Abingdon, UK). Soluble E-selectin (carrier-free) (catalog number 21-7178) was purchased from Tonbo Biosciences (San Diego, CA). Human selectin-E antisense and sense digoxigenin-labeled DNA oligonucleotides probes were purchased from Eurofins Genomics Limited (Wolverhampton, UK). Horseradish peroxidase--conjugated anti-goat and anti-rabbit horseradish peroxidase--conjugated anti-DR3 antibodies were purchased from Cell Signaling Technology (Berkshire, UK). Goat anti-rabbit horseradish peroxidase--conjugated anti-CD68 antibodies were purchased from Abcam (Cambridge, UK). A rabbit anti-p38 MAPK (Thr180, Tyr183) (3D7) antibody was purchased from Cell Signaling Technology (London, UK).

Anti-human DR3 and E-selectin primary antibodies were first tested on RCC tissue arrays using an automated immunohistochemistry system (Ventana/Highland, CA). The slides were then counterstained with hematoxylin. The antibody reaction was visualized by diaminobenzidine tetrahydrochloride peroxidase staining. Immunohistochemical analysis was performed by a pathologist (A.L.) who was blinded to the clinical status of the tissues. Immunohistochemical sections were scored using a semi-quantitative system.

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antibodies andVectashield Mounting Media were purchased from Vector Laboratories Ltd. (Peterborough, UK). Chicken anti-rabbit AlexaFluor 488, goat anti-mouse AlexaFluor 568, and anti-rabbit AlexaFluor405 were purchased from Invitrogen Ltd. (Paisley, UK). The ECL system was purchased from Amersham Pharmacia Biotech UK Ltd. (distributed by Merck) and Duolink in situ reagents and probes anti-mouse minus (catalog number DUO92004) and anti-rabbit plus (catalog number DUO92002) were purchased from Merck.

Tissue Samples

Experiments usinghuman tissue were performed with informed consent of patients and approval of the local ethics committee and Cambridge University Hospitals Tissue Bank. RCC tissue obtained from radical nephrectomy specimens was immediately excised from tumors that grossly appeared to be ccRCC. This tumor classification was later verified by routine histologic assessment of paraffin-wax embedded samples. Non-clear cell histologic tumor types (eg, papillary, chromophobe, and collecting duct) were excluded, and only ccRCC, graded according to the four-tiered Fuhrman nuclear grading system and pathologically staged based on the TNM classification, was used. Tissue samples from 40 patients were collected and scored as Fuhrman grade 1 (n = 10), Fuhrman grade 2 (n = 10), Fuhrman grade 3 (n = 10), and Fuhrman grade 4 (n = 10). In parallel, adjacent non–tumor kidney (NK) (n = 40), categorized histologically as normal kidney cortex, was collected in sites remote from the tumor. All samples were fixed overnight at 4°C in 4% formaldehyde in 0.1 mol/L phosphate buffer (pH 7.6) and paraffin wax embedded for immunofluorescence or snap frozen in isopentane cooled in liquid nitrogen. Parallel unfixed fresh samples were processed for organ culture experiments. Sections 5 μm/L thick were prepared for subsequent experiments, and hematoxylin and eosin (H&E) staining was performed for morphologic studies.

Kidney Organ Cultures

As previously described, a duplicate <1-mm^3 fragments of ccRCC (grade 1/2) and adjacent NK (n = 5 per study group) were obtained immediately from surgically excised specimens. Fragments of tissue were placed in a Corning flat-bottomed, 96-well tissue culture plate (Appleton Woods Limited, Birmingham, UK) and immediately immersed in Medium 199 containing 10% heat inactivated fetal calf serum (TCS, Bucks, UK) and 2.2 mmol/L glutamine. Multiple randomized samples from each patient were used to obtain parallel-group comparisons and to assess the reliability and reproducibility of these assays. Tissue was left in media alone (untreated [UT] controls) or pretreated with rhTL1A (0.2 μg/mL) (R&D Systems, Oxford, UK) and soluble E-selectin (sE-selectin) (5 μg/mL) (Tonbo Biosciences) for 3 hours at 37°C. In parallel, some organ cultures were pretreated with 5 μmol/L of the NF-κB inhibitor BAY11-7082 for 1 hour at 37°C before treatment with TL1A or sE-selectin for 3 hours at 37°C. All cultures were harvested, half of the samples were fixed in 4% formaldehyde for 1.5 hours at 4°C and processed for paraffin wax embedding, and half were snap frozen and stored at −70°C for cryosectioning. H&E was performed formalin-fixed, paraffin-embedded (FFPE) sections for morphologic studies.

IF and IHC

The 5 μmol/L FFPE sections or cryosections of ccRCC, NK, and corresponding organ cultures were subjected to immunofluorescence (IF) and immunohistochemistry (IHC) as previously described. Briefly, some sections were incubated with antibodies to DR3 and/or sE-selectin in combination with anti-CD31, anti-cytokeratin, anti-CLA-1, anti-αSLeX, antiphosphorylated p38(Thr180/Tyr182), anti-ERK(Tyr20) or anti-JNK (Thr183/Tyr185). To determine the presence of leukocytes, parallel sections of ccRCC and NK were incubated with anti-CD45 alone or with CD15, CD14, or CD68. The intensity of fluorescence was calculated as the corrected total cell fluorescence (CTCF) using ImageJ software version 1.53a (NIH, Bethesda, MD; http://imagej.nih.gov/ij) to control for local background fluorescence. The following formula was used to calculate CTCF: integrated density – (area of selected cell × mean fluorescence of background readings). Antibody-binding sites were visualized with fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 or Alexa Fluor 568 or Alexa Fluor 635) plus Hoechst 333,342 for nuclei detection. The brightest fluorophores were used for low-abundant proteins to maximize sensitivity and with narrow emission spectra to avoid bleed-through. The indirect IF amplification technique was used, which permits simultaneous detection of two antibodies raised in the same species. After IF, all sections were washed in phosphate-buffered saline, mounted in Vectashield mounting media, and imaged with a Leica SPE confocal laser scanning microscope (Leica Microsystems Ltd., Milton Keynes, UK).

In addition, IHC was used on organ cultures treated with TL1A or sE-selectin with or without Bay 11-7089 to examine expression of NF-κBp65Ser276 and phosphorylated H3 Ser10 (pH3-Ser10). Endogenous peroxidase was blocked using 30% H2O2 in absolute methanol for 30 minutes at room temperature before incubation with blocking buffer (10% fetal calf serum in phosphate-buffered saline) for 1 hour in room temperature followed by 1:100 dilution of primary antibodies overnight at 4°C. After rinses in phosphate-buffered saline, sections were incubated with 1:200 dilution of secondary antibodies for 1 hour in room temperature and antibody binding sites visualized using 3,3′-diaminobenzidine tetrahydrochloride (Thermo Fisher Scientific) plus 0.01% H2O2 and viewed using a Nikon Optiphot-2 microscope. To quantify the intensity of immunostaining, we imported images of 3,3′-


diaminobenzidine tetrahydrochloride-stained sections into ImageJ, and the mean intensity of staining was determined. Negative controls included replacement of the primary antibody with an isotype-specific serum.

Immunoprecipitation and Immunoblotting

Total proteins were extracted from clinical samples of ccRCC grades 1 to 4 and adjacent NK with radioimmunoprecipitation assay lysis buffer containing protease and phosphatase inhibitors (Merck Life Sciences UK Ltd.), and protein was quantified using a bicinchoninic acid kit (Merck). DR3 epitope was pulled down from tissue extracts using rabbit anti-DR3 antibody and with Magnetic Dynabeads-Protein G (catalog number 10007D; Fischer Scientific UK Ltd., Loughborough, UK) following the manufacturer’s instructions. Briefly, Dynabeads—Protein G was suspended in antibody binding and washing buffer containing anti-DR3 antibody and incubated for 10 minutes at room temperature. The mixture was then placed on the magnet and supernatant removed. The Dynabeads—Protein G antibody complex was resuspended in binding and washing buffer and antigen-containing samples (typically 100 to 1000 μL) were added and incubated on a rotor for 10 minutes at room temperature. The mixture was then placed on the magnet and supernatant removed. The Dynabeads—Protein G antibody antigen complex was rinsed in washing buffer and resuspended in elution buffer for 2 minutes at room temperature. The tube was placed on the magnet and the supernatant/sample transferred to a clean tube. Eluates were then resuspended in the SDS sample buffer and incubated for 10 minutes at 70°C and subjected to immunoblotting. Then 50 μg of total protein per sample was separated by SDS-PAGE. After transfer to a nitrocellulose membrane, proteins were probed with 1:10000 dilution mouse anti—CLA-1 overnight at 4°C, and the signal was detected using Super Signal West Pico Chemiluminescent Substrate according to the manufacturer’s instructions (Fisher Scientific UK Ltd.).

In Situ PLA

FFPE sections of ccRCC and adjacent NK organ cultures treated with TL1A or sE-selectin or left UT in media alone (negative controls) were subjected to proximity ligation assay (PLA) as previously described. In brief, sections were incubated with rabbit anti-DR3 and mouse anti—E-selectin or anti—CLA-1 or anti—sLeX antibodies overnight at 4°C. This procedure was followed by a mixture of 1:5 PLA probes (anti-mouse and anti-rabbit antibodies attached with different oligonucleotides, which can ligate through the addition of two other oligonucleotides in ligation solution) for 1 hour. After rinses in 1x wash buffer A (provided with the kit), sections were incubated in a ligation solution for 30 minutes at 37°C and then rinsed in 1x wash buffer A and incubated in amplification solution in a dark humidity incubator for 100 minutes at 37°C. Slide were then washed in 1x and 0.01x wash buffer B (provided with the kit) and mounted in VectaMount mounting medium with DAPI. For negative controls, the primary antibody was replaced with isotype-specific serum. Sections were examined on confocal laser scanning microscope, and red fluorescence dots, indicative for protein localization, were counted using ImageJ and quantified with GraphPad Prism version 9.0 software (GraphPad, La Jolla, CA).

In Situ Hybridization

Nonradioactive in situ hybridization was performed on 5-μm FFPE sections of ccRCC and adjacent NK organ cultures as previously described. Briefly, sections were incubated overnight at 37°C with hybridization solution that contained single-stranded antisense DNA oligonucleotide probes 5’ end labeled with digoxigenin specific for human E-selectin 4 μg/mL (5’-ATTGTCCCCTAGCAAGGCAT-3’) and for human DR3 (5’-AGTCTAGGCA-TGC TGGCAG-TAGAAGGTG-AACTGCTGACATTGGCT G-ACCTGGCAG-3’) (MWG Biotech, Milton Keynes, UK; and Eurofins Genomics Limited, Wolverhampton, UK) followed by alkaline phosphatase—conjugated sheep antidigoxigenin antibody (Roche Diagnostics Ltd., West Sussex, UK) for 2 hours at room temperature and visualized with alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride solution (Merck). Negative controls included incubation of sections with a sense probe to DR3 or E-selectin. The sections were visualized on Leitz Laborlux 12 microscope equipped with an Infinity 2 camera.

Statistical Analysis

The mean number of tumor cells and normal TECs positive for NF-κBp65p-Ser276 and pH3-Ser10 and positive cells for CD45 and CD15, CD14, or CD68 were counted in 10 random high-power fields. We then examined DR3 protein expression in clinical samples of ccRCC grades 1 to 4 and adjacent NK. Sections of NK and low-grade ccRCC (grades 1 and 2) demonstrated only a rare signal for DR3 on resident leukocytes in some glomeruli and in ECs of occasional interlobular arteries.

Results

DR3 and E-Selectin Are Highly Expressed in Tumor Cells in ccRCC

We first examined DR3 protein expression in clinical samples of ccRCC grades 1 to 4 and adjacent NK. Sections of NK and low-grade ccRCC (grades 1 and 2) demonstrated only a rare signal for DR3 on resident leukocytes in some glomeruli and in ECs of occasional interlobular arteries,
with the latter also positive for expression of CD31 (an EC marker) but rarely on TECs and positive for expression of cytokeratin (an epithelial cell marker). These findings are concordant with our earlier report of expression in non-inflamed kidney. A similar pattern of staining for E-selectin was detected on parallel sections. In comparison, staining for both proteins was elevated in higher-grade ccRCC (grades 3 and 4) mainly confined to cytokeratin-positive tumor cells and CD31+ microvascular ECs (Figure 1, A and B, quantified in Figure 1C and Supplemental Figure S1, A–D). Parallel sections stained with H&E showed pleiomorphic nuclei in tumor cells in ccRCC (Figure 2, A–E, quantified in Figure 2F). These patterns of expression were similar across multiple different ccRCC samples of the same histologic grade, and a similar staining pattern was seen using different DR3 or E-selectin antibodies.

CLA-1 and E-Selectin Binding Carbohydrate Moiety sLeX Are Highly Expressed in Tumor Cells and Are Associated with DR3 in ccRCC

Epitopes recognized by anti-CLA-1 antibodies and other E-selectin–binding carbohydrate moiety sLeX-bearing molecules are highly expressed in tumor cells and are associated with DR3 in ccRCC. A relationship among CLA-1 molecules, sLeX+ determinants, and E-selectin has been previously reported. E-selectin binds to proteins that bear sLeX oligosaccharides. We, therefore, examined whether CLA-1 is associated with DR3 in ccRCC by immunoprecipitating DR3 from extracts of ccRCC and adjacent NK, separating the
immunoprecipitates by SDS-PAGE and immunoblotting with anti-CLA-1 antibody. Co-immunoprecipitation of CLA-1 with DR3 was observed in all ccRCC extracts and to a much less extent in NK extracts (Figure 3A). We next analyzed FFPE sections from corresponding tissue for expression of DR3 and CLA-1 or sLeX by dual IF and found a strong signal for CLA-1 or sLeX but not DR3 in normal TECs. In comparison, co-signal for DR3 and CLA-1 or sLeX was accentuated in tumor cells in ccRCC and more pronounced in high-grade tumors (grades 3 and 4) (Figure 3, B and C, Supplemental Figures 3, A and B). These data suggest that DR3 expressed by ccRCC cells contains CLA-1 and sLeX and together these molecules could be ligands for E-selectin.

**DR3** ccRCC Cells Are Responsive to TL1A

We previously reported that TL1A, the principal ligand for DR3, is up-regulated in TECs and vascular ECs in kidney inflammation and injury and that signaling through DR3 can activate NF-κB signaling in organ culture of human kidney.\(^7\)

Moreover, various MAPKs, including ERKs, JNKs, and p38 MAPK, contribute to tumor cell growth and survival, and phosphorylation of JNK is increased in ccRCC.\(^47\) We assessed whether DR3 in ccRCC cells is functional using a human organ culture model established in our laboratory.\(^7\) Organ cultures were treated with TL1A or left untreated and analyzed for the presence of phosphorylated p65 subunit of NF-κB (NF-κBp65Ser276), indicative of induction of gene transcription, for expression of various activated MAPKs, namely, p38, ERK, and JNK, using antibodies specific for the activated phosphorylated forms of these enzymes and for induction of cell-cycle entry comparing the expression of pH3 (Ser10), an indicator of cell proliferation. The effect of treatment on morphology was determined on H&E-stained sections from all the cultures. Compared with UT controls, both the treatments induced distortion of the cell architecture, some areas of fibrosis, and thickened wall in some vessels (Supplemental Figure S4). IHC revealed a statistically significant increase in nuclear expression of NF-κBp65Ser276 in tumor cells in TL1A-treated cultures (approximately

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**Figure 2** Hematoxylin and eosin (H&E) and representative confocal images of dual immunofluorescence staining for death receptor 3 (DR3) or E-selectin and CD31 or cytokeratin (CK) in clear cell renal cell carcinoma (ccRCC) Fuhrman grades 1 to 4 and adjacent normal kidney (NK). **A**: Fuhrman grade 1 (B); Fuhrman grade 2 (C); Fuhrman grade 3 (D); and Fuhrman grade 4 (E) (red circles indicate pleiotropic nuclei in grade 3 and 4 tumors). A rare signal for DR3 but not E-selectin is seen in interlobular capillaries (PCs) in NK. In comparison, all grades of ccRCC show co-localization of the proteins in tumor cells (white arrows) and in vascular endothelium (ECs), more pronounced in high-grade tumors (grades 3 and 4). A few vascular ECs in high-grade ccRCC were positive for E-selectin but negative for DR3 (green arrows). **F**: Quantification of the percentage of tumor cells in ccRCC and normal tubular epithelial cells (t) in NK. Nuclei were counterstained with Hoechst 33342 (blue). Data are expressed as means ± SEM. n = 10 per group with similar results. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Scale bars: 75 μmol/L (A, merged), 25 μM (B and D, merged); 50 μM (C and E, merged); 100 μM (E, H&E). Original magnification, ×100 (light micrographs); ×40 (confocal images).
42% ± 0.2%) and in vascular ECs in ccRCC compared with UT controls (approximately 4.5% ± 0.6%) (Figure 4A). TL1A also induced NF-κBp65Ser276 expression in NK, but this was less frequent by approximately 2.2-fold (approximately 19% ± 0.8%), with staining mainly confined to normal TECs and vascular ECs (Figure 4B). Tissue pretreated with Bay11-7082, an inhibitor of the enzyme IKKβ, which is required for canonical NF-κB signaling, before the addition of TL1A showed weakened green fluorescence nuclei staining of p65 in both study groups with pronounced effects in ccRCC compared with NK (by approximately threefold) and staining mainly localized to tumor cells, vascular ECs, and infiltrating cells (Figure 4C). Interestingly, TL1A also induced a statistically significant increase in the level of activation of MAPKs with much pronounced signal for p38 and phosphorylated JNK than for phosphorylated ERK P < 0.001 versus P < 0.05) in ccRCC compared with NK (by approximately threefold) and UT controls and staining mainly localized to tumor cells, vascular ECs, and infiltrating cells (Figure 4D, quantified as CTCF in Figure 4E). Tumor cells (approximately 35.1% ± 0.4%) and vascular ECs in treated ccRCC also showed an increased expression of nuclear pH3Ser10 compared with UT controls (approximately 4.7% ± 0.1%), and treated cultures of NK showed a much reduced effect (by approximately twofold) with signal detected mainly in normal TECs (Figure 4F, quantified in Figure 4G). Cumulatively, these data demonstrate that DR3 in ccRCC tumor cells is functional, can activate NF-κB and MAPKs, and promotes tumor cell cycle entry, indicative of mitogenesis.

TL1A Signaling Induces the Expression of E-Selectin in ccRCC Cells

The expression of E-selectin on ccRCC cells was unexpected and could arise from adsorption of shed E-selectin from ECs or de novo synthesis by ccRCC cells. To address this, we analyzed the effect of TL1A on regulation of E-selectin mRNA and protein expression using organ cultures of low-grade ccRCC (grades 1 and 2). Signal for E-selectin protein and mRNA was seen in a few scattered tumor cells in UT controls, whereas TL1A-treated cultures showed an enhanced signal in tumor cells (approximately 26% ± 0.6%), vascular ECs, and infiltrating leukocytes (Figure 5A). In comparison, treatment induced a much reduced effect (approximately 7.0-fold less) in NK cultures with signal for both protein and mRNA seen in normal TECs and glomerular ECs. E-selectin protein was absent in...
Figure 4  A: Light micrographs of the effect of tumor necrosis factor–like 1A (TL1A) on activation of NF-κB, mitogen-activated protein kinase (MAPK), and cell cycle entry in organ cultures of clear cellular renal cell carcinoma (ccRCC) and adjacent normal kidney (NK). B: Untreated (UT) controls of ccRCC show a rare nuclei signal for NF-κBp65Ser276 in vascular endothelial cells (ECs), which is remarkably increased in TL1A-treated cultures with signal mainly in tumor cells (green arrows) and in ECs and significantly attenuated by pretreatment with Bay11-7082. C: Treated cultures of NK show a similar pattern of staining with signal mainly confined to normal tubular epithelial cells (t) (red arrows) and in vascular ECs, attenuated by Bay11-7082. D: Quantification of the percentage of positive tumor cells and in ccRCC and NK. D: TL1A also induced expression for all the three kinases in both study groups with a strong signal localized in tumor cells (white arrows), ECs, infiltrating mononuclear cells (MNCs), t in NK compared with UT controls. E: Quantification of immunofluorescence presented as corrected total cell fluorescence (CTCF) in tumor cells and in normal t in ccRCC and NK. F: Nuclear pH3Ser10 is rarely detected in UT controls, but a much-reduced level with signal seen in t (red arrow) and a rare signal detected in UT controls. G: Quantification of the percentage of positive pH3Ser10 in tumor cells and t. Nuclear were counterstained with Hoechst 33342 (blue). Data are expressed as means ± SEM. n = 5 per group with similar results. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Scale bars: 100 μM (A and F); 25 μM (D). Original magnification: ×400 (A, D, and F; confocal images). Glom, glomeruli; hpf, high-power field.

UT controls of NK and signal for mRNA detected only in interlobular capillaries (Figure 5B). Fluorescence intensity in both study groups is presented as CTCF in Figure 5C. In addition, no signal was detected on sections hybridized with corresponding sense probes (data not shown). These data suggest that the TL1A can induce E-selectin mRNA and protein synthesis in both tumor cells and in tumor vascular ECs in ccRCC and that this response is much more pronounced in ccRCC compared with adjacent NK.

We further assessed whether TL1A promotes interaction of DR3 and E-selectin or CLA-1 or sLeX using PLA on organ cultures of ccRCC and adjacent NK. Stimulation with TL1A enhanced the DR3–E-selectin, DR3–CLA-1, and DR3–sLeX interaction by approximately 4.6-fold (approximately 2.1-fold and 2.2-fold in organ cultures of ccRCC compared with NK), evidenced by numerous red fluorescent PLA spots within the cytoplasm of tumor cells (approximately 67 PLA spots per cell) compared with UT cultures (approximately 6 PLA spots per cell) for DR3–E-selectin (Figure 6A, C, and E, quantified in Figure 6B, D, and F).

These data indicate the ability of TL1A to enhance interaction of DR3–E-selectin, DR3–CLA-1, and DR3–sLeX in tumor cells in ccRCC. A summary of DR3 and E-selectin protein and mRNA expression and their localization in ccRCC and adjacent NK is presented in Table 1.

DR3⁺ccRCC Cells Are Responsive to Soluble E-Selectin

In a final series of experiments, we wondered whether the interactions demonstrated by PLA between DR3 and E-selectin expressed on ccRCC cells could involve signaling through DR3. To explore this hypothesis, we treated grade 1
ccRCC organ culture and adjacent NK with exogenous sE-selectin and analyzed induction of DR3 and E-selectin protein and mRNA expression by dual IF and in situ hybridization. A strong co-signal for DR3 and E-selectin protein was demonstrated in tumor cells (approximately 28% ± 0.8%) and in interlobular capillaries in treated cultures of ccRCC compared with UT controls (approximately 5.1% ± 0.4%), which showed co-signal in vascular ECs and in a few isolated tumor cells. Treatment also induced increased mRNA signal for both proteins in tumor cells, interlobular capillaries, and vascular ECs compared with UT controls, which showed a positive signal in vascular ECs and DR3 mRNA in isolated leukocytes (Figure 7A). A similar but much reduced effect of the treatment was evident in cultures of NK with co-signal for protein detected in normal TECs (approximately 12% ± 0.7%) and in vascular ECs but not in UT controls (Figure 7B). Treatment also induced mRNA signal for both epitopes in organ cultures of NK (mainly consisting of normal TECs, interlobular capillaries, vascular ECs, and glomerular ECs) at lower levels than in ccRCC cells and was absent in UT NK controls (Figure 7B). In addition, no signal was detected in parallel sections hybridized with sense probes (data not shown). Taken together, these data indicate TL1A-inducible up-regulation of DR3/E-selectin expression in renal TECs at low levels and a marked increase in the magnitude of this response in ccRCC tumor cells.

Having established that DR3 signaling events activated by TL1A in tumor cells result in the induction of both DR3 and E-selectin expression and that induced E-selectin interacts with DR3, we then analyzed downstream cellular responses to determine whether sE-selectin can function as a ligand for DR3 in ccRCC. For this, low-grade ccRCC and adjacent NK cultures were treated with sE-selectin and left UT and examined for the presence of NF-κBp65Ser276, activation of MAPKs, and induction of cell cycle entry. Nuclear NF-κBp65Ser276 expression was induced in tumor cells (approximately 22.82% ± 0.5%) and in vascular ECs.
Figure 6  Proximity ligation assay (PLA) of organ culture of clear cell renal cell carcinoma (ccRCCoC) grade 1 and adjacent normal kidney (NKoC) for detection of death receptor 3 (DR3) and E-selectin or cutaneous lymphocyte antigen (CLA)-1 or sialyl Lewis X (sLeX). A, C, and E: In comparison with untreated (UT) controls, tumor necrosis factor–like 1A (TL1A) treatment induced a strong interaction of DR3–E-selectin (A), DR3–CLA-1 (C), and DR3–sLeX (E), appearing as strong red fluorescent spots mainly in cytoplasm of tumor cells and in normal tubular epithelial cells (t) (arrows). B, D, and F: Quantification of the number of PLA spots per cell in tumor cells and t show a statistically significant difference, more pronounced in ccRCCoC. Nuclei were counterstained with Hoechst 33342 (blue). n = 5 per group with similar results. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Scale bars = 75 μM. Original magnification, ×40.

Table 1  DR3 and E-Selectin Protein and mRNA Expression and Localization in Clinical Samples of ccRCC and NK

<table>
<thead>
<tr>
<th>Variable</th>
<th>NK EPCs</th>
<th>NK VECs</th>
<th>ccRCC EPCs</th>
<th>ccRCC VECs</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR3</td>
<td>− mRNA</td>
<td>− mRNA</td>
<td>++++ mRNA</td>
<td>++++ mRNA</td>
</tr>
<tr>
<td></td>
<td>± protein</td>
<td>± protein</td>
<td>++++ protein</td>
<td>++++ protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(† with tumor grade); contains sLeX</td>
<td>(† with tumor grade)</td>
</tr>
<tr>
<td>E-selectin</td>
<td>−</td>
<td>−</td>
<td>++++/+++</td>
<td>++++/+++</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(† with tumor grade)</td>
<td>(† with tumor grade)</td>
</tr>
</tbody>
</table>

Scores are as follows: −, no labeling; ±, occasional positive labeling (>2%); ++, strong positive labeling (>20%); ++++, very strong labeling (>30%); †, increased.

cCRCC, clear cell renal cell carcinoma; DR3, death receptor 3; EPCs, epithelial cells; NK, normal kidney; sLeX, sialyl Lewis X; VECs, vascular endothelial cells.

in treated cultures of ccRCC (Figure 8A, quantified in Figure 8B) compared with UT controls (approximately 4.63% ± 0.3%). In comparison, cultures of NK showed a less marked effect (by approximately 2.7-fold) with signal detected mainly in normal TECs (approximately 8.42% ± 0.1%) and vascular ECs compared with UT controls, which showed a rare signal (approximately 2.1% ± 0.2%). Pretreatment of parallel cultures with Bay11-7082 resulted in a significant reduction in NF-κBp65-Ser276 signal (by approximately 4.3-fold) in ccRCC compared with NK (by less than approximately twofold). Activation of MAPKs was noticeably increased in treated cultures of ccRCC with signal mainly confined to tumor cells and vascular ECs compared with UT controls, which...
showed no signal (Figure 8C). Fluorescence intensity calculated as CTCF is presented in Figure 8D. A much reduced intensity of staining (by approximately twofold) in normal TECs was seen in NK. sE-selectin also induced a strong nuclear signal for pH3-Ser10 in tumor cells and in vascular ECs in ccRCC (approximately 23.5% ± 0.3% versus approximately 5.2% ± 0.1% in UT controls) compared with NK (approximately 10.2% ± 0.2% versus <2% in UT controls), with signal localized to normal TECs and vascular ECs (Figure 8E, quantified in Figure 8F). These data are consistent with the interpretation that sE-selectin activates NF-κB, MAPKs, and cell cycle in tumor cells through DR3 in ccRCC and suggest that the same process could be occurring by interactions of endogenously induced DR3 and E-selectin. A summary of TL1A- and sE-selectin–mediated responses in organ cultures of ccRCC and NK is presented Table 2.

**Discussion**

We previously reported that DR3 is induced in renal TECs and ECs in kidney injury associated with transplant rejection. We extended our studies to examine the expression of DR3 in a clinical cohort of excised ccRCC tumors because these cells are the malignant counterpart of tubular epithelium. We found that DR3 is expressed in tumor cells and vascular ECs in ccRCC, and its expression increases with tumor grade. In endothelium, signaling through DR3 results in expression of E-selectin, thought to be a specific response of this cell type. Surprisingly DR3+ tumor cells also show expression of E-selectin that increases with tumor grade. We found that DR3 on ccRCC cells also contains sLeX and that DR3 and E-selectin appear to form complexes in ccRCC cells that can be detected by PLA. Stimulation of DR3+ tumor cells in ccRCC organ culture with exogenous TL1A

![Figure 7 Representative confocal images and light micrographs of the effect of soluble E-selection (sE-selectin) on protein and mRNA expression for death receptor 3 (DR3) and E-selectin in organ cultures of clear cell renal cell carcinoma (ccRCCoC) and adjacent normal kidney (NKoC). A: Untreated (UT) controls of ccRCC show co-signal for DR3 and E-selectin protein in a few isolated tumor cells (white arrows) and in vascular endothelial cells (ECs). mRNA signal for both DR3 and E-selectin is seen ECs and DR3 mRNA in scattered infiltrating mononuclear cells (MNCs). A similar pattern with a much more pronounced signal is detected in tumor cells (green arrows) and in interlobular capillaries (PCs) in treated cultures. B: In contrast, UT controls of NK show an absence of signal, but a strong signal is detected in treated cultures mainly confined in normal tubular epithelial cells (t) (red arrows), vascular ECs, and glomeruli (Glom). n = 5 per group with similar results. Scale bars: 100 μM (photomicrographs); 25 μM (confocal images). Original magnification: ×400 (photomicrographs); ×40 (confocal images).](https://www.amjpathol.org/article/S0002-9440(21)30900-1/pdf)
induces NF-κB activation, increases phosphorylation of p38, ERK, and JNK at sites indicative of enzyme activation, and promotes cell cycle entry (indicated by H3<sup>Ser10</sup> expression). Unexpectedly, TL1A also induces upregulation of E-selectin protein/mRNA expression and promotes interaction of DR3 and E-selectin protein in tumor cells in ccRCC. Perhaps most surprisingly, exogenous sE-selectin also induces NF-κB activation, increases phosphorylation of p38, ERK, and JNK MAPKs, and promotes cell cycle entry in DR3<sup>T</sup> tumor cells. Exogenous

![Figure 8](https://example.com/figure8.png)

**Figure 8** Light micrographs of the effect of soluble E-selectin (sE-selectin) on activation of NF-κB, mitogen-activated kinases (MAPKs), and cell cycle entry on organ cultures of clear cell renal cell carcinoma (ccRCCoC) and adjacent normal kidney (NKoC). **A**: Untreated (UT) cultures of ccRCC show a rare nuclei signal for NF-κBp65<sup>Ser276</sup> in vascular endothelial cells (ECs), noticeably increased in treated cultures with signal mainly confined to tumor cells (green arrows) and in ECs, and significantly attenuated in cultures pretreated with Bay11-7082. Similarly, UT cultures of NK show a negligible signal, which is increased in treated cultures mainly confined to tubular epithelial cells (t) (red arrows), in vascular ECs, and within glomerular (Glom), with a diminished signal in cultures pretreated with Bay11-7082. **B**: Quantiﬁcation of the percentage of positive tumor cells and t. **C**: Both treated study groups show induction of expression for all the three kinases, with signal mainly confined to tumor cells (white arrows) and in vascular ECs and inﬁltrating mononuclear cells (MNCs) in cultures of ccRCC and in t in cultures of NK. **D**: Quantiﬁcation of immunofluorescence presented as calculated total cell fluorescence (CTCF) in tumor cells and t in cultures of ccRCC and NK. **E**: UT organ cultures of ccRCC show a negligible level of signal for pH3<sup>Ser10</sup> in nuclei of tumor cells, signiﬁcantly increases in cultures treated with sE-selectin (green arrow), and detected in vascular ECs. Similar induction of signal is detected in cultures of NK but less pronounced with signal mainly conﬁned to normal t (red arrow). **F**: Quantiﬁcation of the percentages of positive tumor cells and normal t. Data are presented as means ± SEM. n = 5 per group with similar results. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Scale bars = 100 μM (A and E); 50 μM (C). Original magniﬁcation: ×400 (photomicrographs); ×40 (confocal images).

### Table 2 TL1A and E-Selectin—Mediated Responses in Organ Cultures of ccRCC and NK

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ccRCC NF-κBp65&lt;sup&gt;Ser276&lt;/sup&gt;</th>
<th>pH3-Ser&lt;sup&gt;10&lt;/sup&gt;</th>
<th>MAPKs</th>
<th>NK NF-κBp65&lt;sup&gt;Ser276&lt;/sup&gt;</th>
<th>pH3-Ser&lt;sup&gt;10&lt;/sup&gt;</th>
<th>MAPKs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL1A</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>E-selectin</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
</tbody>
</table>

Scores as follows: ++, intermediate labeling (>10%); ++++, strong labeling, ++++, very strong labeling (>30%).

ccRCC, clear cell renal cell carcinoma; MAPKs, mitogen-activated protein kinases; NF-κBp65<sup>Ser276</sup>; NF-κB phosphorylated at 65 subunits at serine 276; NK, normal kidney; pH3<sup>Ser10</sup>; phosphorylated histone H3 at Serine10; TL1A, tumor necrosis factor–like ligand A1.
sE-selectin-induced responses appear less pronounced than those induced by TL1A. This difference may be ascribed to an artifact of differences in the concentrations of ligands use or in affinity of sE-selectin and TL1A for DR3 or that many sLeX groups on DR3 are already engaged by endogenous E-selectin and unavailable for binding and hence DR3 clustering. TL1A and sE-selectin induced similar effects in organ cultures of NK but to a much lower extent.

Currently, little is known about the role(s) of DR3 in cancer, particularly its potential as an antitumor target. Increased levels of DR3 expression have been reported in various cancer cell lines and tumors compared with normal counterparts. DR3 mRNA encoding the membrane and soluble receptors has been reported in colorectal cancer, and DR3 transcript expression levels are increased in high-grade breast tumors. As noted above, a potential cancer migratory role for DR3 has also been previously highlighted, and DR3 has been reported to be a new receptor for E-selectin that confers metastatic and survival advantages to colon cancer via MAPKs and phosphatidylinositol 3-kinase/NF-κB axis. Our observation that DR3 in tumor cells is induced by TL1A and sE-selectin suggests that it is a functional receptor in ccRCC with the ability to activate downstream pathways that promote survival/growth signals. Interestingly, DR3 is labeled as a death receptor, but its activation by sE-selectin does not induce apoptosis in colon cancer cells, except when ERK is inhibited. A previous study by Wen et al reported that the binding of TL1A to DR3 activated the ERK pathway, whereas JNK and apoptosis-inhibiting protein c-IAP2 prevented DR3-mediated apoptosis in TF-1 cells. Because DR3 uses the same signaling apparatus as TNFR1 for both protein induction and apoptosis initiation, it is also possible that TNF-inducible antiapoptotic proteins, such as cellular FADD-like IL-1 converting enzyme— inhibitory protein, are induced and then play a protective role.

Accumulating evidence suggests that E-selectin facilitates metastasis in various cancers. The mechanism entails adhesion cascade governed by cell-cell interactions between circulating tumor cells and vascular ECs through a sequential affinity interaction between adhesion molecules and the counterreceptor ligand. We document E-selectin expression in tumor cells in ccRCC, which is unexpected. However, epithelial cell expression of E-selectin has been previously reported in human colonic epithelium. The factors that result in preferential expression of E-selectin in ccRCC cells and its pathogenic importance are at present unclear. Although the major role of E-selectin expression in ccRCC tumor cells is likely to be autocrine/paracrine signaling, it also could function to retain leukocytes within the tumor microenvironment.

Our findings on E-selectin/DR3 interaction in cancer cells and their growth-promoting properties suggest that disruption of this signaling axis could be a new approach to treating high-grade ccRCC. Specifically, small-molecule inhibitors that mimic CLA epitopes could prevent E-selectin from engaging DR3, although it will be important to assess whether these moieties induce signaling. Such potential inhibitors may already have been developed and tested for inhibiting E-selectin-mediated recruitment of leukocytes.

Acknowledgments

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Supplemental Data

Supplemental material for this article can be found at http://doi.org/10.1016/j.ajpath.2021.12.010.

Author Contributions

R.S.L., J.S.P., J.R.B. designed the experiment; R.S.L. and J.W. performed experiments and analyzed the data; J.R.B. secured funding.

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