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Endoglin and the immune system: immunomodulation and therapeutic opportunities for cancer

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ENDOGLIN AND THE IMMUNE SYSTEM

IMMUNOMODULATION AND THERAPEUTIC OPPORTUNITIES FOR CANCER

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Endoglin and the immune system

Immunomodulation and therapeutic opportunities for cancer

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Contents

1

General introduction

CANCER

Cancer results from the accumulation of genetic [1] and epigenetic changes [2] in cells over time, which converts healthy cells into cancerous cells. These changes enable the cells to grow out of control and become invasive. Cancer cells can ignore signals that are generally used to stop dividing or that initiate the process known as programmed cell death or apoptosis, which eventually results in the formation of the tumor [3]. Malignant cells can influence and transform surrounding normal cells like fibroblasts [4], immune cells, [5] and endothelial cells [6]. These cells combined form the tumor microenvironment (TME).

Colorectal cancer

Colorectal cancer (CRC) is also known as bowel cancer and refers to all malignancies in the colon or rectal area (large intestine) [7]. CRC is the 3rd most common cause of cancer in the Netherlands with a 5-year survival of only 65% [8]. CRC starts with the formation of benign polyps in the large intestine. Some of these polyps can grow out to form invasive cancer [9]. The process from polyp to invasive cancer is a process that can take up to 15 years due to the slow accumulation of mutations. Fearon and Vogelstein proposed a model known as the Vogelgram in which normal epithelium acquires mutations in the Adenomatous Polyposis Coli (APC), Kirsten (K)RAS, Deleted in Colorectal Carcinoma (DCC), and *P53* genes in sequential order leading to cancer progression [10]. However, they emphasized that the number of accumulated mutations rather than the order in which they are acquired is most important during carcinogenesis [11]. The most commonly (34-70%) mutated gene in all CRC is the *APC* gene, which produces the APC protein and is involved in Wnt signaling [12]. Beyond defects in Wnt signaling, other mutations must occur for the cell to become cancerous [13]. Approximately 30%-40% of CRC carry an activating mutation in the *KRAS* gene, driving cell proliferation. Patients with a *KRAS* mutation are unlikely to benefit from therapies that target the Epithelial Growth Factor (EGF) pathway since the mutation is associated with resistance to the EGFR tyrosine kinase inhibitors. Furthermore, other proteins responsible for programmed cell death and differentiation are commonly mutated in CRC like the P53 protein [14] and members of the transforming growth factor- ß (TGF-ß) pathway [15]. The TGF-ß pathway displays inactivating mutations in at least half of CRCs, mostly in a downstream protein called SMAD4. SMAD4 is the central mediator of the TGF-ß, Bone morphogenic protein (BMP) and Activin signaling pathways, by forming a heterotrimeric complex with receptor-regulated SMADs, enabling translocation to the nucleus, where the complex binds Deoxyribonucleic acid (DNA) and regulates gene expression. Metastasis is the major cause of death in CRC patients. The most common side of metastasis are the liver and peritoneum [16]. Once metastasized the life expectancy declines dramatically.

Pancreatic cancer

Pancreatic cancer has a five-year survival rate of only 4-7%, which makes it one of the deadliest types of cancer known in humans [17]. The most common mutations in pancreatic cancer are in *KRAS* (95%), *P53* (75%), and *SMAD4* (55%) [18]. Pancreatic cancers are characterized by a high proportion of non-epithelial, stromal cells [19]. These consist mainly of fibroblasts which are known as "cancer-associated fibroblasts (CAFs)". These are found in high numbers within the tumor, forming a barrier that prevents immune cells and therapeutic agents from entering the tumor [20]. Multiple cellular and molecular levels underlay the therapeutic resistance in pancreatic cancer, including stromal proliferation, reduced vascular density and immune suppression contributing to therapeutic resistance [21].

The tumor microenvironment

Tumors consist not only of a heterogeneous population of cancer cells but also a variety of resident and infiltrating cells known as the tumor microenvironment (TME) [22]. The TME consists of extracellular matrix (ECM), stromal cells (including fibroblasts, pericytes, adipocytes, endothelial cells forming blood- and lymphatic vessels) and immune cells (such as T and B cells, natural killer cells and tumor-associated macrophages and neutrophils). Both CRC and pancreatic cancer are known for their high influx of stromal cells. High accumulation of stromal cells is a predictor for worse survival in both CRC and pancreatic cancer [23]. Furthermore, the TME can also shape therapeutic responses and resistance, justifying the recent interest in targeting components of the TME as a novel therapeutic strategy [24]. One of the best examples of successfully targeting the TME are the immune checkpoint inhibitors [25] of which inhibitors targeting programmed cell death protein 1 (PD-1) and its ligand, programmed cell death ligand 1 (PD-L1) are established examples. These inhibitors are widely used in the clinic and lead to lasting disease response in several cancer types [26]. Although this all sounds very promising, only a minority of patients currently respond to these immunomodulatory therapies. Therefore, multiple therapeutic combinations are being developed to target both the tumor cells and the TME to increase therapeutic responses.

Immune responses against cancer

The immune system consists of a network of multiple organs, tissues, and specialized cells that protect the body from infections and other conditions like cancer. Although these immune cells typically remove damaged or abnormal cells from the body, some cancer cells can evade the immune system [27]. Immune cells continuously scan the body for the occurrence of any molecules that are considered to be 'non-self'. Cancer cells acquire mutations that lead to antigen formation that is recognized as 'non-self', the so-called neo-antigens. Once the immune system recognizes these

cancer cells, a specific immune response is generated that results in the proliferation of antigen-specific lymphocytes. These T-cells can recognize the tumor cells by the binding of the T-cell receptor to the major histocompatibility complex (MHC)-1, presenting the antigen on the cell surface of the tumor. After recognition, the T-cell secrete cytotoxic granules which can kill the tumor cell. However, multiple escape mechanisms enable the tumor to evade the immune response against the tumor. Many of these escape mechanisms can be targeted by immunoregulatory antibodies. Currently, numerous different immunoregulatory antibodies are approved to treat multiple different cancers and many more are being tested pre-clinically or clinically [28]. These antibodies are directed against molecules on immune cells that inhibit or activate the immune system. One of these antibodies is directed against programmed cell death protein 1 (PD-1), which prevents the binding of PD-1 to its ligand PD-L1 [29]. PD-1 and PD-L1 regularly interact with each other preventing the overactivation of the immune system. However, in tumors, cancer cells can overexpress PD-L1, thereby inhibiting the T-cell responses against the tumor and preventing the killing of cancer cells.

Table 1. T-cell subsets

Cell type	Cytokines produced	Markers	Role
Cytotoxic T-cell	IL-2, INFγ	CD3, CD8	Kill virus-infected cells and tumor cells
T helper cell type 1	INFγ	CD3, CD4, CCR5, T-bet	Induce inflammatory response key for defense against viruses and cancer
T helper cell type 2	IL-4	CD3, CD4, CCR3, GATA-3	Induce differentiation and antibody production by B-cells
Regulatory T-cell	IL-10, TGF-ß	CD3, CD4, CD25, Foxp3	Inhibit T-cell mediated immunity

T-cells (characterized by CD3 expression) are usually grouped into subsets based on their function. These can be identified by their expression of various cell surface markers [30]. While T-cell subsets were initially defined by function, they can also be defined by their associated gene or protein expression patterns. Table 1 shows the subsets of T-cells that are described in this thesis. Besides, many more T-cell subsets have been described, which are not discussed and therefore not included here.

CD8 positive, cytotoxic T-cells can kill virus-infected cells and tumor cells [31]. They recognize their target by binding to short peptides presented on MHC class I molecules on the surface of all nucleated cells. Cytotoxic T-cells also produce key

cytokines like Interleukin (IL)-2 and INFγ, which influence the effector function of other immune cells, particularly macrophages and Natural Killer (NK) cells.
T helper cells (Th-cells) assist other lymphocytes, including stimulating the maturation of B-cells into plasma cells and memory B-cells, and the activation of cytotoxic T-cells and macrophages. These Th-cells express CD4 on their surface and s become activated once an antigen is presented by antigen-presenting cells (APCs), in association with MHC class II molecules. After activation, they divide rapidly and secrete cytokines that regulate or assist the immune response [32].
Regulatory T-cells (T-regs) are crucial for the maintenance of immunological tolerance. Their primary role is to shut down T-cell mediated immunity at the end of an immune reaction and suppress autoreactive T-cells that have escaped the process of negative selection in the thymus. T-regs can develop either during normal development in the thymus or can be induced peripherally and are called peripherally derived T-regs. Both subsets require the expression of transcription factor Foxp3, which can be used to identify these cells [33].

Anti-tumor responses mostly rely on adaptive immunity, as described above. However, innate immune cells are also present in the TME [34]. Neutrophils are one of the most abundant cells within the circulation and also found in the tumor. Neutrophils have been described as having both pro-tumor and anti-tumor effects [35]. Two distinct subsets are found within the tumor, the N1 and N2 neutrophils. N2 neutrophils induce cancer growth, metastasis, and immune suppression, whereas N1 neutrophils can induce a cytotoxic response, induce T-cell activation, and antibody-dependent cellular cytotoxicity (ADCC).

ADCC can be induced by cells that express the Fcγ Receptor (FcγR) like macrophages, monocytes, neutrophils, and NK-cells [36]. These cells express FcγRIIA and FcγRIIIA, which are the activating receptors. However, FcγRIIB is an inhibitory FcγR expressed by B-cells, macrophages, monocytes, neutrophils, eosinophils, and basophils. This inhibitory receptor reduces ADCC activity. FcγRs can bind the Fc tail of an antibody, and in this way induce an ADCC response. Many subclasses of Fc tails are known in humans. For example, the IgG1 Fc tail is known for its high-affinity binding to FcγRIIA. Once an antibody has bound its target via its antigen-binding variable region, the effector cell expressing the FcγR can bind the Fc tail of the antibody and thus induce lysis or phagocytosis of the cell.

Tumor vascular system

Blood vessel formation is vital for tumor development and metastasis. Once the tumor grows beyond 2-3mm^3, the lack of nutrients and oxygen promotes the generation of tumor-associated neovasculature [37]. This process is known as the

angiogenic switch and is regulated directly and indirectly by the tumor using pro- and anti-angiogenic signaling molecules, including vascular endothelial growth factor (VEGF) [38], platelet-derived growth factor (PDGF) [39], TGF-ß [40] and BMP9 [41], among others. These newly formed blood vessels are characterized by their immature phenotype. The first cancer therapy that specifically targeted blood vessels was FDA approved in 2004 (bevacizumab), and neutralizes vascular endothelial growth factor (VEGF) [42]. However, therapy resistance has been an enormous setback in targeting the tumor vasculature, and many mechanisms have been described in which both tumor and stromal cells induce resistance [43]. These mechanisms include the activation of alternative angiogenic signaling pathways [44]. Furthermore, host-derived cells such as myeloid cells, pericytes, and CAFs can contribute to therapy resistance by various mechanisms. Myeloid cells can secrete angiogenic and lymphangiogenic cytokines [45], pericytes can increase vessel stabilization [46], which mediates resistance to VEGF(R) therapy and CAFs can secrete proangiogenic cytokines [47].

Cancer-associated fibroblasts

CAFs provide the structural framework of the tumor [4]. They form a vital component of the tumor microenvironment in multiple solid tumors. CAFs have diverse functions, including matrix deposition and remodeling, extensive reciprocal signaling interactions with cancer cells, and crosstalk with infiltrating leukocytes [48]. The precise origin of CAFs is still under debate, but the consensus is that most CAFs likely result from the activation of local fibroblasts or recruitment of precursor cells, although alternative sources have been proposed [49]. Previously CAFs were seen as one group, however, it is becoming increasingly clear that multiple subtypes of CAFs exist. These include myCAFs, with a high TGF-ß driven α-Smooth Muscle Actin (SMA) expression and contractile phenotype, and iCAFs which are known for their high secretion of IL-6. In the future, multiple subtypes will probably be defined, since the function of CAFs ranges from matrix remodeling and the secretion of growth factors to metabolic functions and immune crosstalk. In **Chapter 3** of this thesis, we describe a subset of Endoglin-expressing CAFs responsible for the migration and metastasis of CRC tumors [50].

Endoglin

Endoglin (CD105) is a homodimeric transmembrane protein with a short cytoplasmic domain, which reflects its co-receptor function for the ligands of the transforming growth factor (TGF-ß) superfamily. Endoglin is predominantly expressed by activated endothelial cells and plays a crucial role in angiogenesis. Endoglin expression is regulated by TGF-ß, bone morphogenic protein (BMP)-9, and hypoxia. Since Endoglin is highly expressed by newly formed endothelial cells, therapies targeting Endoglin

have been evaluated as potential new anti-angiogenic therapies [51]. TRC105 is one of these therapies, targeting Endoglin with a Human IgG1 antibody capable of inducing ADCC and successfully passed multiple phase 1 and 2 clinical studies [52-59]. However, TRC105 showed no additional clinical effects over the standard of care in a phase 3 study at the interim analysis, eventually resulting in discontinuation of its clinical development for oncology. However, more evidence is arising that TRC105 targets not only endothelial cells but also CAFs, T-regs and other cells in the TME.

THESIS AIM AND OUTLINE

The TME has increasingly been recognized as an important player in tumor progression and metastasis and a possible target for therapy. The TME consists of multiple cell types secreting growth factors and cytokines that exert either pro- or anti-tumor effects. This thesis mainly focusses on studies of the TME, especially the effects of Endoglin, on several cell types within the TME, including endothelial cells, fibroblasts, and immune cells.

This thesis **aims** to unravel the role of Endoglin as a possible target on various cell types within the TME of solid tumors. Endoglin is known for its role during angiogenesis, however, an increasing number of studies have shown the importance of Endoglin expression on several other cell types (e.g., immune cells, CAFs, tumor cells). Therefore, in **Chapter 2,** the studies on Endoglin beyond endothelial cells are summarized and discussed. CAFs are a major component of the TME and causally involved in tumor progression and metastasis. Multiple subsets of CAFs, with either pro- or anti-tumor effects, are being identified in different tumor types. In **Chapter 3**, we report the presence of an Endoglin-expressing subset of CAFs, localized at the invasive borders of CRC. The presence of these cells is associated with the formation of metastases in stage-II CRC patients. This chapter furthermore shows that Endoglin plays a role in CAF invasion *in-vitro* and appears to be involved in CRC metastasis *in-vivo*. To further investigate fibroblast-specific Endoglin expression and especially in early stages of carcinogenesis, we generated a fibroblast-specific Endoglin knockout mouse in **Chapter 4**. Fibroblast-specific Endoglin deletion resulted in enhanced tumorigenesis in a model for colitis-associated cancer, accompanied by an expansion of stromal cells, with a possible role for myeloid cells. To further investigate the effects of Endoglin targeting in a model that is characterized by a high influx of CAFs, we employed a murine pancreatic cancer model in **Chapter 5**. Although increased immune activation was observed in both fibroblast-specific Endoglin knockout mice and mice treated with an Endoglin neutralizing antibody, no effect on tumor growth was seen. Since increased immune activation was observed, we combined anti-Endoglin therapy with anti-PD-1 treatment to enhance

these effects in multiple colorectal cancer models, as described in **Chapter 6**. Here we describe that anti-Endoglin therapy is effective in reducing tumor volume/progression and reducing the percentage of T-regs within these tumors. Furthermore, we show a subset of Endoglin expressing T-regs in both mouse and human CRC samples. Since the immune system plays a vital role during immunotherapy and therapeutic responses to both TRC105 and PD-1, we were curious to explore the extent to which tumor-draining lymph nodes are involved. In **Chapter 7** we have investigated the effects of the tumor-draining lymph nodes during PD-1/PD-L1 checkpoint therapy. We show that removal of these tumor-draining lymph nodes resulted in a dramatic decline in therapeutic responses, suggesting a pivotal role of local draining lymph nodes during PD-1/PD-L1 checkpoint therapy. In **Chapter 8** the data from the various studies are summarized and discussed.

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Endoglin, the endothelium and beyond

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ABSTRACT

Endoglin, a type-III accessory receptor for the Transforming Growth Factor (TGF)-β superfamily pathway, is known for its crucial role during angiogenesis. Extensive work has shown the important roles endoglin plays in balancing the TGF-β signalling pathway, thereby regulating endothelial cell proliferation and migration. However, recent work indicates a far more widespread role for endoglin beyond the endothelial cells. In this review we will provide a summary of recent publications on endoglin expression on epithelial (cancer) cells, cancer-associated fibroblasts and mesenchymal stem cells. Next to that we discuss the role of endoglin in innate and adaptive immunity. Finally, we discuss the results of clinical trials using the endoglin targeting antibody (TRC105), focusing on the effects observed beyond the endothelium. In conclusion, although endoglin was initially identified as an endothelial marker, additional roles for endoglin on other cell types has been shown, although the number of studies is still limited with sometimes conflicting data. Future studies will further establish the roles of endoglin beyond the endothelium.

Keywords
Endoglin; CD105 TGF-β; BMP9; ALK-1; TRC105; tumor microenvironment

INTRODUCTION

Endoglin is a 180kDa, type-I transmembrane glycoprotein and functions as a coreceptor for ligands of the Transforming Growth Factor (TGF)-β superfamily. Endoglin is predominantly expressed by activated endothelial cells [1] and plays a crucial role in (developmental) angiogenesis. In mice, a complete loss of endoglin is embryonic lethal around embryonic day 10.5, primarily due to impaired development of the vascular plexus into a mature vascular network, causing hampered flow and osmotic imbalance, disturbing normal cardiac development. Part of the cardiac abnormalities is caused by pericardial effusion due to disturbed osmotic balance [2]. This indicates the pivotal role endoglin plays in developmental angiogenesis. Early work has shown that endoglin contributes to angiogenesis by regulating proliferation [3] and migration [4] of endothelial cells. This work has been extended with multiple studies showing an important role for endoglin in tumor angiogenesis and followed by strategies to inhibit tumor angiogenesis by targeting endoglin.
The role of endoglin in developmental- and tumor angiogenesis has been extensively reviewed elsewhere [5-8]. However, more recent studies have reported novel roles for endoglin signalling in (cancer-associated) fibroblasts (CAFs), Mesenchymal Stromal Cells (MSCs), epithelial cancer cells, and various immune cell subpopulations. This review highlights the current knowledge on endoglin expression and function on non-endothelial cells and what implications this might have.

Endoglin structure and function

Endoglin (CD105) is a homodimeric transmembrane receptor composed of disulphate bond-linked subunits of 95kDa [9] and is highly homologous between species [10,11]. In humans, the endoglin gene is located on chromosome 9 [12] and composed of exons 1 to 8, 9A and 9B and 11 to 14 [13,14]. Endoglin has a short cytoplasmic domain which reflects its co-receptor function rather modulating the response than initiating the signalling cascade [15]. Therefore, it requires additional receptors to induce signalling. In both human and mouse tissues two spliced isoforms, long- (L) and short-(S) endoglin, have been reported [16]. S-endoglin and L-endoglin proteins vary from each other in their cytoplasmic tails, that contain 14 and 47 amino acids respectively [17,18]. L-endoglin is the predominantly expressed isoform and signals via the ALK1 pathway, while S-endoglin seems to promote the ALK5 pathway [16]. Activation of the activin receptor-like kinas (ALK)1 and ALK5 pathway leads to downstream activation of respectively the smad2/3 or smad1/5/8 pathway (see below), resulting in the transcription of different target genes. About the exact role for S-endoglin not much is known. It has been reported that transgenic mice with endothelial specific Intercellular Adhesion Molecule 2 (ICAM-2) S-endoglin overexpression show hypertension and decreased response to nitric oxide (NO)

inhibition, which was associated with hypertensive response. Furthermore, decreased TGF-β1 responses were detected in these endothelial cells supporting that upregulation of S-endoglin is part of the senescent program of endothelial cells [19]. Endothelial endoglin expression is regulated by TGF-β, bone morphogenetic protein (BMP)-9 and hypoxia [20]. A hypoxia responsive element was identified downstream of the endoglin promoter, which can bind the hypoxia-inducible factor (HIF)-1a, resulting in increased endoglin transcription [20]. Furthermore, stimulation of endothelial cells shows ligand depending upregulation of endoglin expression. Endoglin, however, is not only regulated on the transcriptional level. Cell surface endoglin expression is also regulated via receptor shedding. Our previous work showed that the membrane bound protease Matrix Metalloproteinase-14 (MMP-14, also known as Membrane Type-1 MMP) is able to cleave endoglin in the extracellular domain close to the cell membrane, generating a soluble form of endoglin (sol-eng) [21]. Sol-eng can disturb vascular remodelling and maintenance resulting in vascular abnormalities. High levels of sol-eng have been measured in the circulation of women developing preeclampsia, a disease characterised by high blood pressure and vascular abnormalities during pregnancy [22]. In cancer, different studies have reported conflicting data about the levels of sol-eng [23-25]. The anti-angiogenic function described for sol-eng suggests a tumor suppressor role in cancer, which is in contrast to studies reporting that high levels of sol-eng correlate to poor patient prognosis [24].

Sol-eng was originally described to inhibit angiogenesis by acting as a ligand trap for the endoglin ligands TGF-β [26] or, as has been more commonly reported, BMP-9 [27,28]. Interestingly, recent data indicate that in addition to being an inhibitory ligand trap, increased circulating monomeric sol-eng might stimulate BMP-9 signalling via binding to endothelial endoglin. The authors have demonstrated that binding of monomeric sol-eng to BMP-9 does not inhibit BMP-9 signalling in endothelial cells [29], but rather potentiates it. Furthermore, they show that sol-eng in plasma from preeclampsia patients consist primarily of a monomeric sol-eng form. Suggesting that sol-eng is this case would not act as an inhibitory ligand trap for BMP9. For cancer this has yet to be investigated. Taken together, these data show that the role of sol-eng in regulating angiogenesis might be more complex as originally anticipated.

Endoglin signalling pathways and ligands

Members of the TGF-β family exert their cellular effects by binding to a complex of type-I and type-II transmembrane receptors. Seven type-I receptors, also known as ALKs, and five, ligand binding type II receptors have been identified [30,31]. Upon ligand binding a heterotetrameric receptor complex is formed, resulting in transphosphorylation of the type I receptor on specific serine and threonine residues

in the intracellular region by the constitutively active type-II receptor. Endoglin (a type-III receptor) is reported to play an important role in balancing the TGF-β signal in endothelial cells, by regulating the recruitment of different type I receptors. Next to the TGFβ type I receptor ALK5, endoglin can induce TGFβ signalling via ALK1 in endothelial cells [32,33]). Endoglin interacts with TGF-β1 and TGF-β3, but only when it is associated with TGF-βRII. Furthermore, the endoglin extracellular and intracellular domains interact with both TGF-βRII and ALK5 [7]. ALK5 then phosphorylates smad2/3 and translocate to the nucleus together with the common smad4. In the presence of endoglin ALK1 is recruited into this complex, shifting pathway activation towards the ALK1 kinase. Next to TGF-β, endoglin is able to bind BMPs directly via a complex with ALK1. This complex is then able to bind BMP-9 with much higher affinity than to TGF-β1, up on receptor complex activation. ALK1 then phosphorylates smad1/5/8 together with smad4 this complex translocate to the nucleus where it increases proliferative signals in endothelial cells [34] [35] [36]. However it must be noted that contradicting results on the role of BMP-9 in inducing angiogenesis have been reported, some also showing a potential inhibitory role for BMP-9 in angiogenesis [37,38]. The contradictions might be due to the use of different cell types, receptors and concentrations of the ligands.

Next to endoglin betaglycan is also an accessory type-III receptor for the TGF-β signalling pathway [39]. The main function of betaglycan is presenting ligands to the TGF-β signalling receptors [40]. Betaglycan is important (especially in vivo) for TGFβ2 to exert an effect. TGF-β2 binds poorly to the TGF-β type II receptor in the absence of betaglycan [41]. Furthermore, betaglycan is crucial during reproduction [42] and foetal development [43] and acts as a potent tumor suppressor in many different types of tumors. [44-46]. The interaction of betaglycan with GIPC is required for TGF-β type III mediated suppression of the TGF-β signalling and invasion [44].

Endoglin and developmental/tumor angiogenesis

Hereditary Haemorrhagic telangiectasia (HHT), also known as Rendu-Osler–Weber syndrome, is a rare genetic disease, which is characterized by mutations in the endoglin (HHT-1) or ALK-1 (HHT-2) [47] genes. HHT-1 is more severe compared to HHT-2 and is associated with vascular abnormalities in the lungs and brain. HHT occurs in 1 in 5.000-8.000 people in North America and is found more frequently in people form the Netherlands Antilles (Bonaire and Curaçao). Although patients suffer from frequent nose bleeds and arteriovenous malformations in the brain, lung and liver, most HHT patients have a normal lifespan. Mechanistic studies on HHT have been performed in endoglin heterozygote mice, which in contrast to endoglin knockout mice, are viable and show signs of HHT such as telangiectasias and nosebleeds after prolonged time [48]. Endoglin-Knockout mice have defects in endothelial cell dependent smooth muscle cell recruitment (19705428). Studies in

HHT patients have shown that they display a decreased number of lymphocytes compared to healthy controls [49]. Furthermore, HHT patients have an increased risk of severe bacterial infections due to defects in both polymorphonuclear and monocytic cells [50], stressing out the importance of endoglin, as further discussed below. Endoglin heterozygote mice, and HHT patients can reveal important information on the role of endoglin, both in- and beyond angiogenesis.

Non-ligand dependent interactions (integrins/leukocyte trafficking)

As described above endoglin dependent signalling can directly influence endothelial cell migration and proliferation in a TGF-β/BMP-9-dependent manner. In addition, several ligand-independent interactions of endoglin have been reported. After the original identification of endoglin, it was already discovered in 1992 that endoglin can bind to integrins on leukocytes [51,52] allowing them to extravasate in a process called Trans Endothelial Migration (TEM). Integrins are ubiquitous cell surface receptors involved in cell-cell and cell matrix interactions [53]. The functional role of endothelial endoglin as a receptor for integrins on leukocytes has been reported by Rossie et al. [54]. An interesting observation they made was the ability of sol-eng to inhibit leukocyte adhesion to endothelial cells [54], suggesting that sol-eng binds to integrins on leukocytes, thereby blocking their extravasation.

Although most studies have focused on myeloid cells, T-cells have a major contribution to immune responses during viral infections and anti-tumor immunity. Therefore, it might be of great interest to investigate the endoglin dependent TEM of T-cells in cancer patients. Taken together, sol-eng might inhibit TEM of pro-inflammatory cells and/or anti-inflammatory cells, which might be of great interest and a possible therapeutic target. An excellent review on the interaction of endoglin with integrins has recently been published [55].

Endoglin beyond the endothelium

As discussed above endoglin plays a crucial role in angiogenesis and leukocyte trafficking via ligand dependent- and independent interactions. For a long time endoglin was considered a marker for angiogenic endothelial cells and exclusively expressed by endothelial cell. However, more recent work has shown endoglin expression on a variety of other cells, with distinct roles in their behaviour. Below we will discuss the various studies where non-endothelial endoglin expression has been investigated.

Endoglin expression on epithelial cells

In normal epithelial cells endoglin expression has been studied during wound healing [56], where enhanced endoglin expression was found in mouse epidermal keratinocytes. *In vivo* endoglin was associated with hyperproliferation [57]. Endoglin

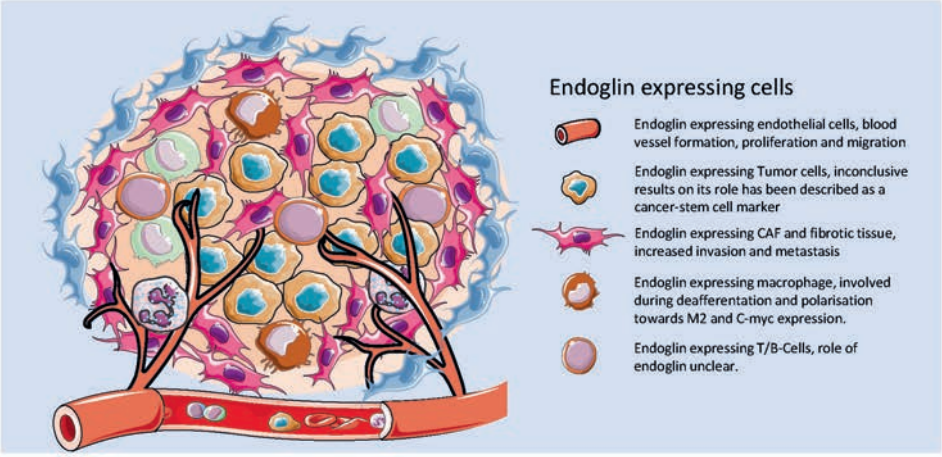

Figure 1. The role of endoglin on different cell types within the tumor microenvironment.

expression on epithelial cells has been a subject of debate for quite some time. In prostate cancer, loss of epithelial endoglin expression has been associated with increased metastatic behaviour, both *in vitro* as well as *in vivo* in orthotopic mouse models for prostate cancer [58,59]. In breast cancer, endoglin expression has been investigated in a subset of invasive breast cancer cell lines. Expression of endoglin in MDA-MB-231 cells blocks TGF-β enhanced cell motility and invasion and reduces lung colonization in a murine metastasis model [60]. Furthermore, in a large breast cancer patient cohort, it was shown that lack of endoglin expression on tumor cells correlates with poor clinical outcome [60]. Similar findings have been reported in oesophageal squamous cell carcinoma, where lack of endoglin expression was associated with decreased migration and colony formation *in-vitro* [61]. These data suggest that endoglin might act as a tumor suppressor in both breast cancer as oesophageal squamous cell carcinoma.

In contrast other reports have described a pro-tumorigenic role for endoglin expression on epithelial cancer cells. In hepatocellular carcinomas (HCC) it has been described that that endoglin expression on HCC cells promotes metastasis in a vascular endothelial growth factor (VEGF)-dependent manner [62]. Next to HCC, endoglin expression in ovarian cancer and renal cell carcinoma has been linked to be stem-cell like phenotype, accompanied by higher invasion in transwell migration assays [63]. Furthermore, it has been described that endoglin induces epithelial to mesenchymal transition (EMT), but not metastasis in clear cell renal cell carcinoma [64].

Taken together the collective data on epithelial endoglin expression revealed a tumor type-specific and not yet fully understood role for endoglin in epithelial cancer cell behaviour. To elucidate this furthermore mechanistic studies, supported by protein expression data in clinical samples are needed to draw firm conclusions.

Endoglin expressing on innate immune cells

The immune system is divided into innate- and adaptive immunity. The innate immune system is composed of several cell types including the neutrophils, eosinophils, basophils, mast cells and monocytes/macrophages, of which the latter have been reported to express endoglin. Monocytes are derived from hematopoietic stem cells in the bone marrow and spleen [65], before they enter the circulation. Upon entering the blood stream two subsets of monocytes can be distinguished. One subset is being recruited into the tissue throughout the entire body [66], while the second subset has endothelial cell-supporting functions [67]. During monocyte differentiation endoglin is highly expressed [52,68]. Interestingly, endoglin seems to be involved in the differentiation from monocytes into both M1 and M2 macrophages in the tissue. M1 macrophages are characterised by their pro-inflammatory and anti-tumor functions and secretion of inflammatory cytokines, whereas M2 macrophages are known for their anti-inflammatory and pro-tumor functions. Furthermore, M2 macrophages are characterised by the expression of c-myc [69]. Little is known about the regulation and function of endoglin on these cells [70]. Endoglin expression on M2 macrophages leads to the downregulation of c-myc, which implies that endoglin might be responsible for the polarisation of these M2 macrophages towards a M1 phenotype. Interestingly, TGF-β is one of the drivers of c-myc expression in a macrophage cell line U937 [71]. Blocking endoglin on macrophages might therefore skew the TGF-β pathway towards smad2/3 signalling, causing the differentiation of macrophages towards an M2 phenotype. This would generate an anti-inflammatory response and thereby would act pro-tumerogenic. Mouse studies on the role of macrophage specific endoglin expression have been performed. Mice with a floxed endoglin gene were crossed with a macrophage (and neutrophil) specific CRE (Engfl/fl-LysMCre). Endoglin deletion changed the differentiation and function of macrophages. The authors showed that phagocytic activity by peritoneal macrophages was reduced in the absence of endoglin leading to sustained infections. Furthermore, altered TGF-β1 expression was found in endoglin negative peritoneal macrophages suggesting an M2 phenotype [72]. These studies all suggest that endoglin is important during the polarisation to M1 macrophages.

To study the role of endoglin in a tissue injury model, a study was performed using $ENG^{+/-}$ mice which received kidney irradiation, after which macrophage function was studied. These results showed impaired IL-1b and IL-6 secretion by macrophages [73] in endoglin heterozygote mice. This again suggests impaired polarisation towards M1 macrophages which are known to secrete IL-1b. As described above, patients with HTT have an increased risk of severe bacterial infections possibly due to defects of monocyte oxidative burst and phagocytosis [50]. Furthermore, increased levels of Dipeptidyl peptidase-4 (DPP4) were found in patients with HHT

which showed impaired homing towards damaged tissue. An excellent review on mononuclear cells and vascular repair in HHT has been published[74]. In cancer, endoglin is highly expressed by acute myeloid leukaemia (AML) subsets. In this study the authors suggest that endoglin can possibly be used as a potential therapeutic target in AML [75].

Interestingly, all the studies described above indicate that the endoglin is involved during the polarisation of macrophages. Most studies on endoglin and macrophage function do not discriminate between M1 and M2 phenotypes and the cytokines produced by the macrophages, which hampers exact interpretations of the endoglin function on macrophages.

Endoglin on cells of the adaptive immune system

Although the majority of endoglin studies have focussed on cells of the innate immune system, more recent work also shows a role for endoglin expression on cells of the adaptive immune system. The adaptive immune system is triggered when a pathogen evades the innate immune system, and consists of B-cells, T-cells and NK cells. The adaptive immunity works closely together with the innate immune system. Within the adaptive immunity, there is a key role for the TGF-β ligands, as recently reviewed in [76]. TGF-β plays an important role in hampering the adaptive immunity by both inhibiting the proliferation and effector functions of T-cell. Furthermore TGF-β induces the differentiation of CD4+ T-cells into T-regulatory cells and TH17 cells inhibiting the immune response even further. Recent papers describe endoglin expression on lymphocytes, mainly the CD4+ T-cells. Endoglin surface expression seem to be regulated by T cell receptor activation. Cross-linking of endoglin enhanced CD4+ T-cell proliferation via smad independent ERK phosphorylation. This study showed that endoglin is expressed by activated CD4+ T-cells and that endoglin is able to counteract the suppressive signal induced by TGF-β [77]. Additionally, more recent work from our group has shown that a subset of FOXP3 expressing, endoglin positive regulatory T cells (Treg) exist. These cells were detected in preclinical mouse models for cancer, as well as in human colorectal tumors. Interestingly an antibody against endoglin (TRC105/Carotuximab) significantly decreased their number within a mouse MC38 tumor. Although the number of studies is limited, the high abundance and immunosuppressive role of Tregs, warrants further investigations into endoglin and regulatory T cells. Since Tregs play an important role in generating an immunosuppressed environment targeting them might alleviate this. The role for endoglin on Tregs is currently unknown, but might have to do with counteracting the canonical TGF-βRII/ALK5 dependent TGF-β responses, similarly as shown for macrophages.

Endoglin expression on fibroblasts

Fibroblasts are cells of mesenchymal origin and are the main producers of extracellular matrix components. Fibroblast play an important role in organ development [78], regulating cell differentiation [79] and tissue repair [80]. In healthy tissue, fibroblasts are quiescent and hardly proliferate. Upon tissue injury a massive expansion of the fibroblast population with an activated phenotype is observed [81] These activated fibroblasts disappear when the wound is repaired [82]. Under pathologic conditions this process seems disturbed, leading to sustained fibroblast activation and accumulation, resulting in fibrosis. Activated fibroblasts are characterised by high TGF-β signalling and recent studies show a role for endoglin in this process. Below we discuss the current knowledge on endoglin expression of fibroblasts and mesenchymal stem cells, since they show high phenotypic similarities.

Endoglin on Mesenchymal stem cells (MSCs)

Mesenchymal Stem Cells (MSC) are multipotent cells, which are present in virtually all tissues and organs [83,84]. *In vivo*, MSCs are thought to be quiescent cells at a perivascular location which are mobilized upon injury in order to promote tissue repair [85]. MSCs suppress overactivation of the immune system, but how they act is still under debate [86]. MSCs are characterized by the expression of CD73, CD90, endoglin, and absence of CD45, CD34, CD14, HLA class II. Endoglin has been reported as an important MSC marker [87,88], as reflected by the fact that for clinical applications, MSCs should always express endoglin. The role of endoglin expression on MSCs has not been elucidated yet, but studies have revealed that absence of endoglin expression on mouse and human MSCs leads to a more differentiated MSC phenotype, with increased osteogenic gene expression [89,90]. Interestingly, when endoglin-negative mouse MSCs were sorted they showed to be more efficient in inhibiting T cell proliferation, compared to their endoglin expressing counterparts [91]. In addition to healthy MSC, endoglin expression has also been reported on sarcomas, which are tumors that arise from transformed mesenchymal cells. Endoglin was associated with worse survival of Ewing sarcoma patients and played a role in a process called vascular mimicry. Moreover, endoglin knockdown in these tumor cells reduces invasiveness and growth [92,93].

Endoglin expressing fibroblasts in fibrosis

Besides their crucial role in wound healing, sustained activation and accumulation of fibroblasts can cause tissue damage and fibrosis. Prolonged exposure to inflammatory conditions, induced by tissue damaging agents, seems the underlying cause of most fibrotic diseases [94]. Chemokines, cytokines and other factors excreted by immune cells lead to sustained activation of local fibroblasts [95]. One of the key inducers of fibroblast activation is TGF-β [96,97]. TGF-β activates fibroblasts

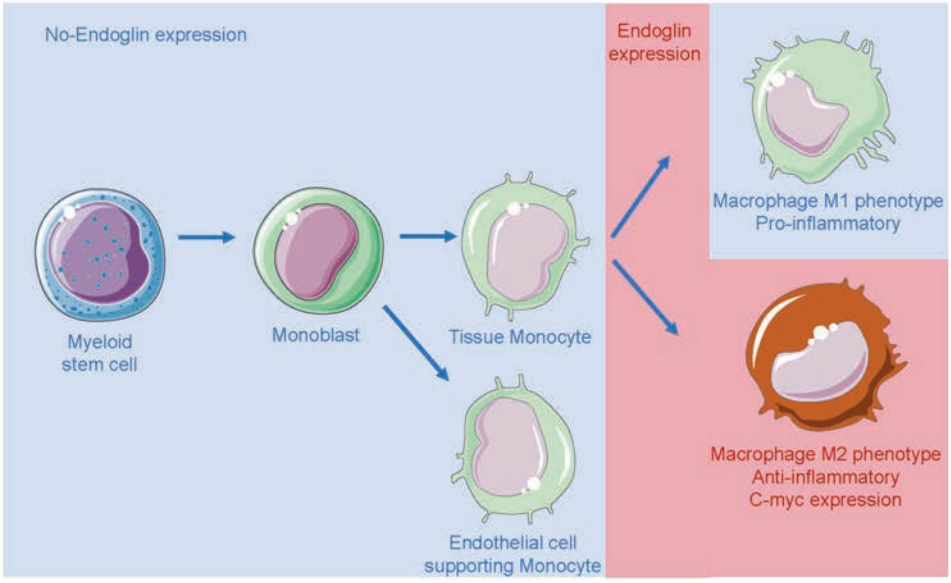

Figure 2. Monocyte to macrophage differencation in red the endoglin expression during differencation and M2 like phenotype.

which on their turn start to produce excessive amounts of extracellular matrix (ECM) and proteins involved in the degradation and remodelling of the ECM, like Matrix metalloproteinases (MMPs). TGF-β can exert its profibrotic effects directly via TGF-βRII/ALK5 mediated signalling, but there also seems a role for endoglin in the profibrotic effects of TGF-β, although this has been less well established. Several reviews highlight the role of endoglin in liver fibrosis [98,99], myocardial fibrosis [100] and kidney fibrosis [101]. Endoglin expression has been described on profibrotic cells such as renal fibroblasts [102], myofibroblasts [103], mesangial cells [104], scleroderma fibroblasts [105] and hepatic stellate cells (HSCs) [106]. In liver fibrosis, HSCs upregulate endoglin during trans differentiation both *in vitro* and rat model for liver fibrosis [106]. Furthermore, endoglin overexpression in hepatic stellate cells has associated with enhanced TGF-β driven smad1/5/8 phosphorylation and the upregulation of α-smooth muscle actin (α-SMA). Other studies on the other hand, show that endoglin might also be protective during fibrosis. In a murine model for liver fibrosis endoglin deficiency enhances the expression of pro-fibrotic factors such as α-SMA and fibronectin [107]. The authors suggest that that endoglin might work protective by modulating ALK1 versus ALK5 dependent TGF-β signalling.

In addition to endoglin expression on stellate cells, several studies have also shown increased levels of sol-eng in the circulation during liver fibrosis [108-110]. These data suggest that a substantial part of endoglin in fibrotic liver tissues is cleaved

and subsequently released into the circulation. This might be organ-specific, since in kidney fibrosis (chronic kidney disease and end stage kidney disease) no changes in sol-eng were observed [111]. Although sol-eng was not elevated in patients in mouse models for kidney fibrosis unilateral ureteral obstruction (UUO) significantly elevated the mRNA expression of endoglin within the kidney. When the authors next investigated if heterozygote mice would develop less fibrosis, they observed no changes in severity of the fibrosis compared to wildtype mice [112]. Another interesting finding in this model was that overexpression of L-endoglin seem to increase kidney fibroses after UUO in mice [102], whereas the overexpression of s-endoglin seems to reduce kidney fibrosis and inflammation [113]. Although overexpression was not fibroblast-specific, the authors showed that L-endoglin increased both the smad1/5/8 and smad2/3 pathways while s-endoglin showed decreased phosphorylation of both smad1/5/8 and smad2/3 pathways. These data indicate that the effects are dependent on the cytoplasmic domain.
Finally, endoglin expression has also been studied in cardiac fibrosis [100]. Endoglin expression on cardiac fibroblasts was highly upregulated upon TGF-β1 stimulation [114] and mediates the profibrotic effects of angiotensin II on cardiac fibroblasts [115,116]. Furthermore, sol-eng limits TGF-β1 signalling in cardiac fibroblasts. Interestingly treatment with sol-eng limits cardiac fibrosis in an *in vivo* model for heart failure [117].
Taken together, there is no consensus yet about the pro- or anti-fibrotic role of endoglin. The role of endoglin might be cell- and tissue type specific. Interestingly many studies show that endoglin might restore the balance between the smad2/3 pathway and smad1/5/8 pathway balancing the TGF-β induced signalling.

Endoglin expression in cancer-associated fibroblasts (CAFs)

In various solid tumors high accumulation of fibroblasts with an activated phenotype, called CAFs, are observed and their abundance seems to predict patient survival [118]. CAFs can stimulate cancer progression via stimulating growth, secretion of pro-invasive, pro-metastatic and pro angiogenic factors. The origin of CAFs most probably heterogeneous and composed of activated local fibroblasts, bone marrow cells [119-122] or resulting from epithelial to mesenchymal transition (EMT) [123] and endothelial to mesenchymal transition (EndMT) [124,125]. These various sources might also lead to various CAF subsets, all with distinct roles in immune regulation, tumor progression and metastasis [126,127]. Similar to its role in fibrosis, TGF-β is a main driver of CAF activation, mostly via the ALK5 signalling pathway. In addition, recent studies show an additional role for endoglin. The striking phenotypical resemblance between CAFs and MSCs, makes it in literature hard to distinguish CAFs from MSCs in tumors both subsets are described to express endoglin.

Romero et al. were the first to describe endoglin on CAFs in prostate cancer [128]. They show in the Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP) mice on an endoglin heterozygote background that tumors are less fibrotic and less prone to form metastasis. Furthermore, endoglin expressing CAFS were able to promote neovascularization and tumor growth, suggesting that endoglin on CAFs in prostate tumor mediate metastasis and tumor growth. Our study in colorectal cancer (CRC) shows that endoglin expressing αSMA+ CAFs at the invasive front of CRC, are related to metastasis-free survival. Furthermore, when we targeted endoglin on these CAFs in a mouse model for experimental liver metastasis a reduction in the number of metastasis was detected [129], in line with the earlier results reported in prostate cancer. Although the number of studies describing endoglin on CAFs is still limited, there seems to be a tumor promoting role for endoglin expressing CAFs.

Single cell RNA sequencing studies in breast cancer identified a subpopulation of so called vascular CAFs (vCAFs), which are characterised by their expression of endoglin [130]. In pancreatic cancer endoglin is only expressed in cluster 12 which was defined as an endothelial (non-CAF) cell cluster [131]. In contrast to that our unpublished data showing strong expression of most of the CAFs in human pancreatic ductal adenocarcinoma (PDAC) tissues cancer tissues. In gastric- and breast cancer a strong association between endoglin expressing CAFs/MSCs and a poor prognosis was reported [132,133].

These studies suggest that there seems a pro-tumerogenic/pro-metastatic role for endoglin expression on CAFs, potentially via regulating/balancing ALK1 versus ALK5 pathways. Identification of CAF subsets, using multiomics data is rapidly increasing and should reveal the potential for endoglin
targeting on these CAF subsets.

Targeting endoglin in diseases

Because of the high endothelial endoglin expression, therapies targeting endoglin have been evaluated [6,8,134], mostly focussed on its endothelial expression. With increasing knowledge on endoglin expression beyond the endothelium it might be that endoglin targeting directly targets other cell types. In cancer TRC105 has been clinically tested and although encouraging results have been published [135-139]. However, a recent phase-III trail in angiosarcomas did not show clinical efficacy at the interim analysis.

Based on the data aboveTRC105 might not only target endothelial cells, but also other cells. This has been shown in pre-clinical models for breast cancer, where a decrease in the amount of a-SMA positive cells was reported by our group upon TRC105 treatment. Interestingly in the reported clinical studies with TRC105, like in a phase II study for advanced metastatic urothelial carcinoma, a decrease in circulating T-regs was observed [140]. Our own unpublished data might provide an

explanation for this phenomenon, since we have detected a subset of endoglin expressing T-regs in CRC, which can be depleted using TRC105. Further validation of these findings should show if this is an additional target of endoglin therapy.
Next to regulatory T cells, and endoglin is highly expressed on some tumor cells direct targeting of endoglin expressing tumor cells might induce a direct anti-tumor response. In urothelial carcinoma patients treated with TRC105 a decreased number of circulating tumor cells were observed [140], although the authors have not shown that this is a direct effect of the targeting of CTCs by TRC105. This also correlates with data that endoglin targeting inhibits metastatic spread in pre-clinical models for breast [141] and colorectal cancer [129]. These first data open op many new possibilities to look back at valuable data obtained from clinical studies involving TRC105 and its effects on non-endothelial cells.

Concluding remarks

Originally identified on endothelial cells, more recent work showed additional and not yet defined role for endoglin on other cell types. Although the numbers of studies investigating endoglin on non-endothelial cells has increased, much is still unknown. Endoglin expression seems to be upregulated strongly in a multitude of cells upon in vitro cell culture possibly due to activation status or stress of the cells, hampering thorough mechanistic studies. Next to that there seem to be opposing roles for endoglin in different tissues/diseases. It is clear that multiple cells can express endoglin mainly in a TGF-β environment such as cancer in which both CAFs as some immune cells express endoglin. The exact role of endoglin expression beyond the endothelium is studied but still unclear, nevertheless represent an exciting new area of research

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3

Endoglin expression on cancer-associated fibroblasts regulates invasion and stimulates colorectal cancer metastasis

CLIN CANCER RES. 2018 DEC

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ABSTRACT

Purpose

Cancer-associated fibroblasts (CAF) are a major component of the colorectal cancer tumor microenvironment. CAFs play an important role in tumor progression and metastasis, partly through TGF-β signaling pathway. We investigated whether the TGF-β family coreceptor endoglin is involved in CAF-mediated invasion and metastasis.

Experimental Design

CAF-specific endoglin expression was studied in colorectal cancer resection specimens using IHC and related to metastases-free survival. Endoglin-mediated invasion was assessed in vitro by transwell invasion, using primary colorectal cancer–derived CAFs. Effects of CAF-specific endoglin expression on tumor cell invasion were investigated in a colorectal cancer zebrafish model, whereas liver metastases were assessed in a mouse model.

Results

CAFs specifically at invasive borders of colorectal cancer express endoglin and increased expression intensity correlated with increased disease stage. Endoglin-expressing CAFs were also detected in lymph node and liver metastases, suggesting a role in colorectal cancer metastasis formation. In stage II colorectal cancer, CAF-specific endoglin expression at invasive borders correlated with poor metastasis-free survival. In vitro experiments revealed that endoglin is indispensable for bone morphogenetic protein (BMP)-9–induced signaling and CAF survival. Targeting endoglin using the neutralizing antibody TRC105 inhibited CAF invasion in vitro. In zebrafish, endoglin-expressing fibroblasts enhanced colorectal tumor cell infiltration into the liver and decreased survival. Finally, CAF-specific endoglin targeting with TRC105 decreased metastatic spread of colorectal cancer cells to the mouse liver.

Conclusions

Endoglin-expressing CAFs contribute to colorectal cancer progression and metastasis. TRC105 treatment inhibits CAF invasion and tumor metastasis, indicating an additional target beyond the angiogenic endothelium, possibly contributing to beneficial effects reported during clinical evaluations.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and accounts for 8.5% of all cancer-related deaths [1]. Ninety percent of all patients with cancer die from metastatic disease. Therefore, patients at risk to develop metastatic disease (stage III/IV) are eligible for (neo)adjuvant (chemo)-radiotherapy [2]. However, a significant proportion of patients with localized disease will still develop metastases, emphasizing that a better understanding of the mechanism underlying tumor metastasis is needed.

The tumor stroma can account for ≥50% of the tumor mass, and its extent is predictive for worse patient survival in patients with colorectal cancer [3]. The tumor stroma, or tumor microenvironment (TME), is composed of endothelial cells, pericytes, immune cells, and cancer-associated fibroblasts (CAF; ref. 4). CAFs interact with all other cells in the TME via direct cell–cell contact and secretion of cytokines [5, 6], thereby stimulating tumor progression and ultimately metastasis [7]. Therefore, CAFs are considered a potential novel target for cancer therapy.

TGF-β mediates the transdifferentiation of resident fibroblasts into CAFs [8, 9], as indicated by increased expression of α-smooth muscle actin (αSMA) and fibroblast activation protein (FAP; [ref. 10]). Endoglin is a coreceptor for TGF-β and bone morphogenetic protein (BMP)-9 that, upon ligand binding, can facilitate Smad1 phosphorylation [11]. Endoglin is highly expressed on the surface of activated endothelial cells and indispensable for developmental angiogenesis [12–14]. Furthermore, endoglin microvessel density is correlated with tumor progression and metastases in colorectal cancer [15, 16]. Different mutations in the endoglin gene have been reported in The Cancer Genome Atlas (TCGA) for colorectal cancer, although the affected cell types were not specified. The endoglin-neutralizing antibody TRC105 binds human endoglin with high affinity, competitively inhibits BMP-9 binding [17], and induces antibody-dependent cell-mediated cytotoxicity [18, 19]. Currently, TRC105 is in phase III clinical trials in patients with advanced cancer as antiangiogenic therapy.

Although endoglin expression on endothelial cells has been extensively studied and is the focus of targeted cancer therapy, endoglin is also expressed on other cells in the TME. Therefore, in this study, we investigated the role of endoglin expression on CAFs. We show that CAFs, located specifically at invasive borders of colorectal tumors and in metastatic lesions, express endoglin. We further demonstrate that targeting endoglin on CAFs with TRC105 modulates CAF function and that endoglin regulates tumor invasiveness in zebrafish and liver metastases in vivo. Taken together, our data suggest an additional working mechanism for endoglin-targeted therapy on CAFs, besides targeting the endothelium and highlights its therapeutic potential.

MATERIALS AND METHODS

Patient samples

Paraffin-embedded tissue samples were obtained from the Department of Pathology, Leiden University Medical Center (LUMC, Leiden, the Netherlands), used according to the guidelines of the Medical Ethical Committee of the LUMC, and conducted in accordance to the Declaration of Helsinki and the Code of Conduct for responsible use of Human Tissue and Medical Research as drawn up by the Federation of Dutch Medical Societies in 2011. This Code permits the further use of coded residual (historical) tissue and data from the diagnostic process for scientific purposes. Permission is granted by implementing an opt-out procedure for the patients; written informed consent in that case is not needed. The first cohort consisted of 25 adenomas, 140 stage II, and 94 stage III tumors from treatment-naïve patients with colorectal cancer and the same number of adjacent normal tissue samples. Patient characteristics have been included in Supplementary Table S1. The second cohort consisted of 31 patients, of which resection specimens of the primary tumor, lymph node, and liver metastases were available. Patient characteristics and >10-year follow-up were recorded. TCGA databases “COAD - TCGA Colonadenocarcinoma – June 2016" (350 patients) and “COADREAD – TCGA Colon and Rectum adenocarcinoma June 2016" (466 patients) were analyzed using SurvExpress (http://bioinformatica.mty.itesm.mx:8080/Biomatec/SurvivaX.jsp) to analyze potential correlations between patient survival, risk classification, and endoglin expression in colorectal tumors.

Tissue analysis

IHC and fluorescent staining were performed as described before (20), using antibodies as shown in Supplementary Table S2. CAF-specific endoglin expression was scored on a scale of 1 to 4 (≤10%; 10%–25%; 25%–50%, and ≥50% positive) in a blinded manner by two independent observers. Pictures were obtained using a Leitz Diaplan microscope (Leitz). Quantitative PCR analyses were performed as described before [6] using primer sequences shown in Supplementary Table S3.

Cell culture and signaling assays

Human CAFs, the human colorectal cancer cell lines HCT116 and HT29 (obtained from ATCC), and the mouse colorectal cancer cell line MC38 [21] (obtained from Kerafast) were cultured in DMEM/F12, supplemented with 10% FCS, 10 mmol/L HEPES, 50 μg/mL gentamycin, 100 IU/mL penicillin and 100 μg/mL streptomycin (all Thermo Fisher Scientific). The immortalized HUVEC cell line ECRF [22] was cultured as described before [23]. Human embryonic kidney (HEK293T) cells were obtained from ATCC and maintained in DMEM, supplemented with 10% FCS and penicillin/streptomycin (all

from Thermo Fisher Scientific). Primary human CAFs were isolated from nonnecrotic parts of the tumors and normal fibroblasts (NF) from adjacent healthy tissue as described before [6]. Tissues were cultured in DMEM/F12 as described above supplemented with 2.5 µg/mL Fungizone (Thermo Fisher Scientific). Mouse CAFs were isolated from colorectal cancer tissue by culturing 5 × 5 mm pieces of tumor in DMEM/F12 as described above. For both human and mouse isolations, fibroblast-like cell outgrowth was observed after 7 to 10 days. Murine embryonic fibroblasts (MEF) were obtained from E12.5 embryos as described before [24] from endoglin floxed mice [25]. MEFs were maintained in DMEM, supplemented with 10% FCS and penicillin/streptomycin (all Thermo Fisher Scientific). Cell lines were used for 20 passages, and all cell cultures were tested monthly for Mycoplasma contamination.

Constructs expressing human endoglin [26], Cre recombinase (pLV.mPGK.iCRE.IRES.PuroR, kindly provided by Dr. M. Gonçalves, LUMC), or endoglin short hairpin RNA (shRNA, Sigma Mission shRNA library, constructs SHC001, TRCN0000083138, TRCN0000083139, TRCN0000083140, TRCN0000083141 and TRCN0000083142) were delivered by lentiviral transduction to 80% confluent fibroblasts or endothelial cells. After 48 hours, transduced cells were selected using 1.5 µg/mL puromycin (Sigma). HEK293T cells were grown to 80% confluency and transfected with endoglin-expressing plasmids using 1 mg/mL polyethylenimine (PEI; Polysciences Inc.).

For signaling assays, fibroblasts were seeded in 6-well plates. Upon 90% confluency, cells were serum-starved overnight in medium containing 40 µg/mL TRC105 (TRACON Pharmaceuticals) or 40 µg/mL human IgG (Bio X Cell) for human cells. Mouse fibroblasts were incubated in the presence of 40 µg/mL M1043 (anti-mouse endoglin, Abzena) or 40 µg/mL Rat IgG (Bio X Cell). Next day, cells were stimulated with either 5 ng/mL TGF-β3 [27], 0.1 ng/mL BMP-9 (R&D Systems), or 100 ng/mL BMP-6 (PeproTech) for one hour. Cells were lysed in RIPA buffer, protein content was determined, and Western blot analysis was performed as described before [28]. Membranes were incubated overnight with primary antibodies against endoglin, phosphorylated (p)Smad1 or pSmad2 (Supplementary Table S2). Blots were stripped and reprobed with mouse anti-GAPDH or anti-actin antibodies as loading control. Blots were developed using the Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad).

TGF-β and BMP-9 ELISA

BMP-9 levels were measured by ELISA as described before [29]. TGF-β1 levels were analyzed using commercially available duo-set ELISA (R&D Systems) as described before [30, 31].

Invasion assays

A total of 1,000 HEK293T cells or 2,500 CAFs were seeded on top of 0.6% agarose (Sigma) coated 96-well plates and left to form spheroids for 48 hours. Spheroids

were collected and embedded in 1 mg/mL collagen-I matrix (Advanced BioMatrix) containing 10% FCS. At 0, 24, and 48 hours, pictures were taken using a Zeiss Axiovert 200M microscope (Carl Zeiss BV). Quantification of the invaded area was performed using Adobe Photoshop CC2014 software (Adobe Systems).

For transwell invasion assays, the upper surface of 8.0-μm pore size ThinCert (Greiner Bio-One) was coated with 200 μg/mL collagen-I in culture medium containing 0.5% FCS. The lower compartment of the transwell system contained medium with 0.5% FCS and 0.1 ng/mL BMP-9 or 5 ng/mL TGF-β3. When invasion toward colorectal cancer cells was assessed, 2 × 105 HT29, HCT116, or MC38 cells were seeded in the lower well. A total of 2.5 × 104 fibroblasts were seeded on the coated inserts in medium containing 0.5% FCS and left to invade for 24 hours, in the presence of ligands and inhibitors as described above. After 24 hours, invaded cells were fixed with 4% paraformaldehyde and stained with crystal violet. Using an Olympus BX51TF microscope (Olympus Life Science Solutions), five pictures per insert were obtained at ×20 magnification. Cell invasion was quantified in at least three independent experiments by counting the number of invaded cells or percentage of positive stained area using ImageJ software (NIH, Bethesda, MD).

Zebrafish

Zebrafish were maintained according to standard methods approved by the Leiden University animal welfare committee. Two-day-old Tg(fli1:GFP) [32] dechorionated zebrafish embryos were injected with 400 cells in the heart cavity: either 400 dTomato-labeled MC38 cells or 200 dTomato-labeled MC38 combined with 200 unlabeled MEFs. After injection, embryos were maintained at 33°C. Fluorescent imaging was performed using a Leica SP5 STED confocal microscope (Leica Microsystems) under sedation with 0.003% tricaine (Sigma). Confocal stacks were processed using Leica software. For survival analysis, embryos were injected and transferred to a 24-well culture plate, and viability was monitored daily for 6 to 12 days. For IHC, zebrafish were fixed, embedded in paraffin, and processed as described above.

Experimental metastasis model

Animal experiments were approved by the animal welfare committee of the LUMC. Twenty-week-old Crl:CD-1Fox1nu male mice (Charles River Laboratories) were injected intrasplenically with 5 × 105 HT29 cells expressing firefly luciferase under isoflurane anesthesia, either alone or combined with 105 human CAFs. CAFs were pretreated with 40 μg/mL TRC105 or 40 μg/mL human IgG. Mice were treated twice weekly, with 15 mg/kg TRC105 or 15 mg/kg human IgG, intraperitoneally. Metastatic spread was monitored twice weekly using bioluminescent imaging on the IVIS Lumina-II (Caliper Life Sciences). Twenty-five days after tumor cell injection, mice were sacrificed, and blood and tissue samples were collected.

Statistical analysis

Differences between two groups were calculated using Student t test; for multiple groups, one-way ANOVA analysis was used. Survival curves were generated using Kaplan–Meier analysis and log-rank test. Differences in bioluminescent signals over time were calculated using two-way ANOVA analysis. P values of ≤0.05 were considered statistically significant. Error bars represent either SEM or SD, depending on appropriateness, as described in figure legends.

RESULTS

Endoglin expression on CAFs correlates with metastasis-free survival in stage II colorectal cancer

To investigate endoglin expression in colorectal cancer, sequential sections of colorectal cancer tissues were stained for cytokeratin (epithelium), CD31 (endothelial cells), αSMA (CAFs), and endoglin. As previously described, endoglin is highly expressed on endothelial cells in the tumor, as shown by the overlap between CD31 and endoglin staining (Fig. 1A, white arrowheads). However, we also observed endoglin expression on CAFs at the invasive borders, as indicated by an overlap in endoglin and αSMA, a marker for CAF activation (Fig. 1A, black arrowheads; [ref. 33]). Endoglin expression by CAFs was further confirmed using immunofluorescent double staining (Fig. 1B; Supplementary Fig. S1A and S1B). Notably, fibroblasts in adjacent normal colonic tissue or CAFs in the tumor core did not express endoglin (Fig. 1C). This specific localization of endoglin-expressing CAFs at the invasive border and their absence in the tumor core suggest that endoglin on CAFs plays a role in colorectal cancer invasion and metastasis. Exploring this hypothesis, we stained primary colorectal cancer, lymph node, and liver metastases from the same patients for endoglin and αSMA. Endoglin expression was present on CAFs at the invasive border of primary tumors, while staining intensity was remarkably higher on CAFs in both lymph node and liver metastases (Fig. 2A). To assess CAF-specific endoglin expression at different colorectal cancer stages, we stained normal colonic tissue, polyps, and stage II and III primary colorectal cancer tissues for αSMA and endoglin. Average scores for endoglin-expressing CAFs increased significantly with tumor stage (Fig. 2B). To determine whether CAF-specific endoglin expression predicted the development of metastatic disease, we assessed the relation between endoglin expression and metastasis-free survival. In stage II colorectal cancer, high CAF-specific endoglin expression significantly correlated with poor metastasis-free patient survival (Fig. 2C). In stage III colorectal cancer, no relation between metastases-free survival and CAF-specific endoglin expression was observed (Supplementary Fig. S1C). Analyses of two colorectal cancer TCGA databases showed a correlation between

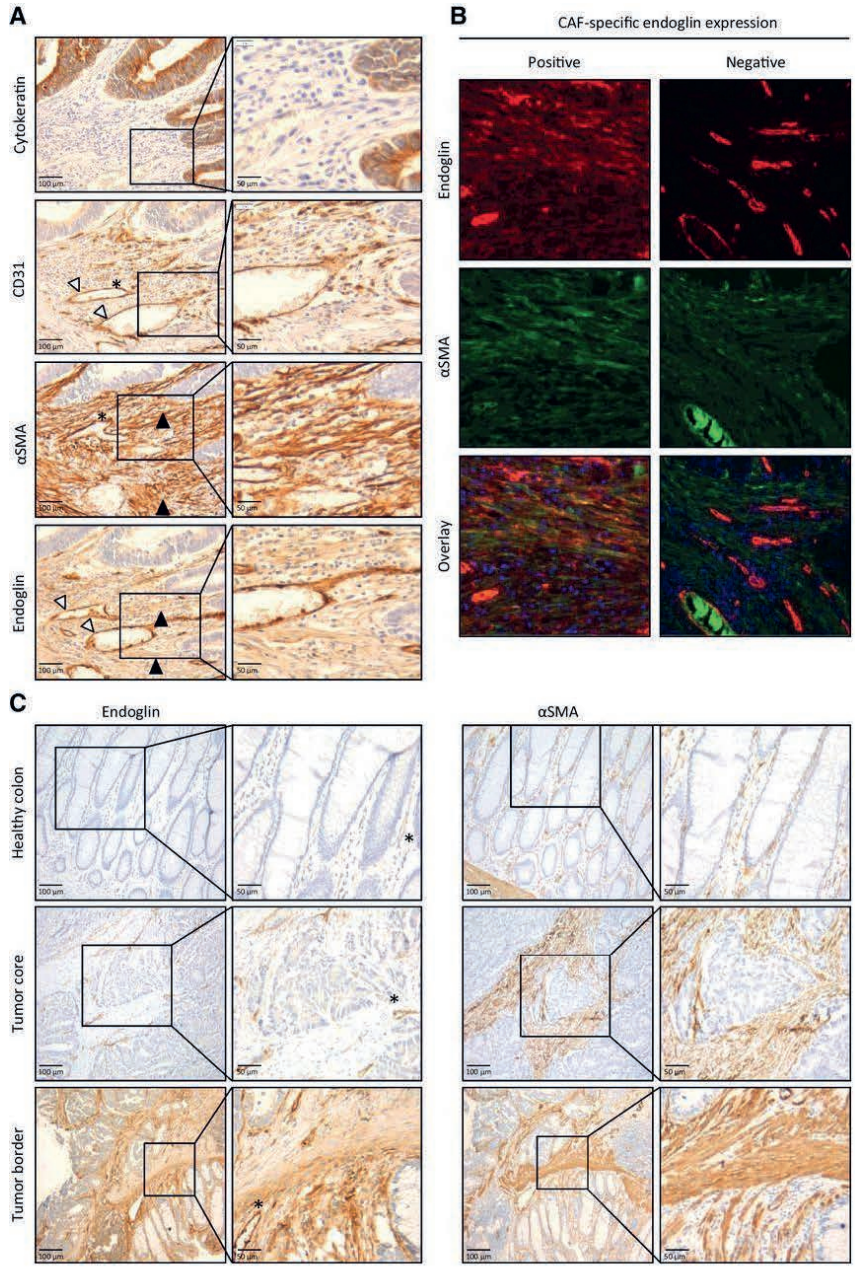

Figure 1. CAF-specific endoglin expression in colorectal cancer. A, IHC staining of colorectal cancer tissue for cytokeratin, CD31, αSMA, and endoglin. White arrowheads, high endoglin expression on the vasculature (CD31+/endoglin+); black arrowheads, endoglin-expressing CAFs (αSMA+/endoglin+); asterisks, αSMA+ smooth muscle cells surrounding vasculature. **B**, Immunofluorescent staining for endoglin (red) and αSMA (green) indicates high vascular endoglin expression and colocalization of αSMA and endoglin on CAFs (yellow, left). Colorectal cancer tissue containing endoglin-negative CAFs was used to show staining specificity (right). **C**, IHC staining for endoglin (left) and αSMA (right) in healthy colonic mucosa, tumor core, and invasive tumor border of the same patient with colorectal cancer. Asterisk indicates endoglin+ blood vessel.

endoglin expression and patient risk classification. Overall patient survival was not significantly different between the groups (Supplementary Fig. S1D), most probably due to the involvement of other factors besides endoglin expression, for example tumor stage, age, and sex. These data indicate that CAF-specific endoglin expression is predictive for metastasis-free survival in stage II colorectal cancer and could pose to be a relevant marker in selecting patients for adjuvant treatment.

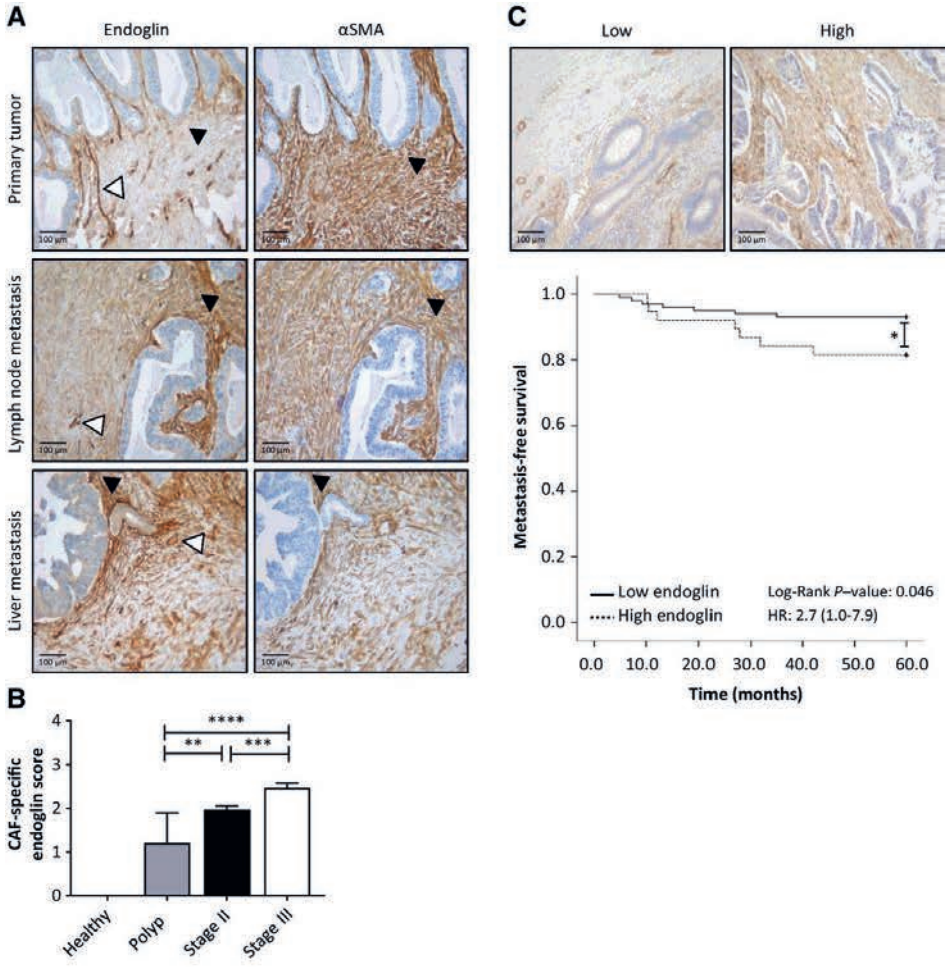

Figure 2. CAF-specific endoglin expression at the invasive border correlates to metastasis-free survival in stage II colorectal cancer. A, Primary tumor, lymph node, and liver metastases from the same patient with colorectal cancer show endoglin-expressing CAFs (black arrowheads). White arrowheads, endothelial endoglin expression. **B**, Average score of CAF-specific endoglin expression in healthy tissue, polyps, and stage II and III colorectal cancer (mean + SEM). **C**, Endoglin expression on CAFs at the invasive border of colorectal cancer correlates to metastasis-free survival in patients with stage II colorectal cancer (n = 140), analyzed as either high or low (exemplified in top boxes). Log-rank P value: 0.046; HR: 2.7 (1.0–7.9). *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

NFs and CAFs display similar receptor expression profiles in vitro

To characterize TGF-β signaling in CAFs, we isolated NFs and CAFs from patients with colorectal cancer. CAFs were isolated from colorectal cancer tissues and NFs from adjacent normal mucosa (>10 cm from primary tumor) from four different patients and gene expression was assessed. Although NFs in tissue do not express endoglin (Supplementary Fig. S2A), endoglin expression is highly upregulated during in vitro culture. Therefore, endoglin mRNA expression did not differ between NFs and CAFs from the same patient, or between patients (Supplementary Table S4). Because endoglin can bind multiple TGF-β family members and mediate downstream signaling, expression of various TGF-β/BMP receptors was determined (Supplementary table S4). No differences in expression levels were observed between patients or between NFs and CAFs from the same patient after in vitro culturing, which might be due to the fact that in vitro culturing of NFs results in fibroblast activation, potentially influencing gene expression and therefore an accurate comparison between NFs and CAFs. To confirm CAF phenotype, protein expression of CAF markers αSMA and vimentin was confirmed by Western blot analysis and expression of the epithelial marker cytokeratin was excluded (Supplementary Fig. S2B). Platelet-derived growth factor receptor (PDGFR) expression can be used to distinguish certain CAF subpopulations [34]. However, no clear distinction in PDGFR expression was observed in NFs or CAFs from these patients in vitro (Supplementary table S4).

Fibroblast signaling in response to endoglin ligands

Canonical TGF-β signaling is regulated through different type-I receptors. Recruitment of ALK1, in the presence of endoglin, results in Smad1/5/8 phosphorylation [5, 36], whereas ALK5 directs phosphorylation of Smad2/3 [11]. Therefore, we determined endoglin signaling and downstream transcriptional regulation after TGF-β or BMP-9 stimulation. TGF-β stimulation of high endoglin-expressing human CAFs resulted in increased expression of the Smad2/3 target gene PAI-1, whereas this was unaffected by BMP-9 stimulation (Fig. 3A). BMP-9 induced expression of the Smad1 target gene inhibitor of differentiation-1 (ID-1). TGF-β also induced ID-1 expression, probably via ALK1 (Fig. 3A). Endoglin mRNA expression was not affected by TGF-β or BMP-9 stimulation (Fig. 3A). BMP-9 stimulation resulted in rapid and strong Smad1 phosphorylation, whereas TGF-β stimulation only slightly increased Smad1 phosphorylation (Fig. 3A, right). Next, the experiment was repeated in CAFs expressing very low levels of endoglin. In these CAFs, TGF-β–mediated effects were similar as observed for high endoglin-expressing CAFs (Fig. 3B). BMP-9 stimulation, however, did not induce ID-1 gene expression (Fig. 3B), nor Smad1 phosphorylation (Fig. 3B, right), confirming endoglin importance. Endoglin expression was unaffected by ligand stimulation (Fig. 3B). Mouse CAFs, which highly express endoglin, showed

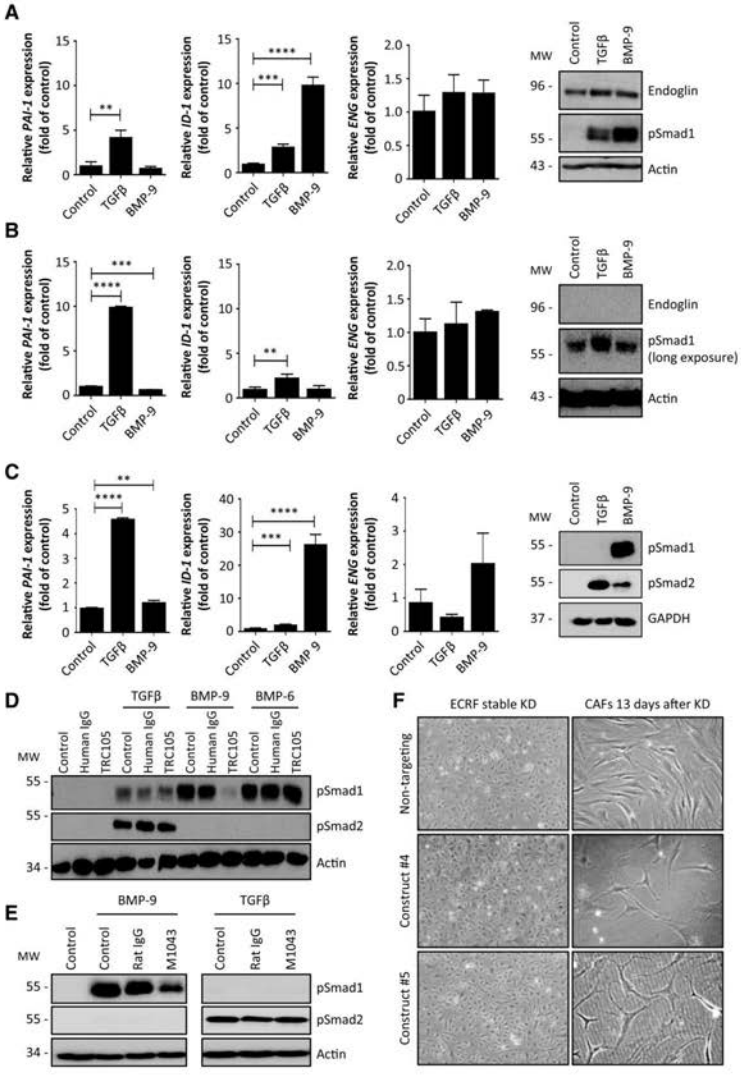

Figure 3. BMP-9–induced signaling in CAFs is endoglin dependent. A, BMP-9 stimulation of high endoglin–expressing human CAFs did not affect expression of PAI-1, but increased ID-1 expression. TGF-β slightly increased ID-1 expression and highly stimulated PAI-1. Both ligands did not affect endoglin expression. BMP-9 stimulation strongly induced pSmad1, whereas TGF-β showed slight pSmad1. Endoglin protein expression was unaffected upon stimulation. **B**, Low endoglin–expressing human CAFs showed a similar response to TGF-β and BMP-9 for PAI-1, whereas BMP-9 stimulation failed to induce ID-1 expression. Endoglin expression remained unaffected. No BMP-9–induced pSmad1 in low endoglin–expressing human CAFs. **C**, Mouse CAFs showed high induction of PAI-1 and ID-1 after stimulation with TGF-β or BMP-9, respectively. Endoglin expression was unaffected. BMP-9–induced pSmad1 in mouse CAFs; pSmad2 was strongly increased by TGF-β. **D**, TGF-β–induced pSmad2 and pSmad1 were unaffected by TRC105 in human CAFs. BMP-9–induced pSmad1 was abrogated by TRC105. Endoglin-independent pSmad1 induction by BMP-6 was unaffected by TRC105 treatment. **E**, In mouse CAFs, M1043 strongly decreased BMP-9–induced pSmad1. TGF-β increased pSmad2, which was unaffected by M1043. **F**, shRNA-mediated endoglin knockdown (KD) led to cell death in human CAFs, whereas endothelial ECRF cells remained viable. **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. pSmad1, phosphorylated Smad1; pSmad2, phosphorylated Smad2. Graphs are representative of at least three independent experiments and show mean + SD.

similar gene expression results to those observed in high endoglin-expressing human CAFs (Fig. 3C). Furthermore, BMP-9 induced strong Smad1 phosphorylation, whereas TGF-β increased Smad2 phosphorylation in these cells (Fig. 3C, right). Ligand stimulation did not affect expression levels of total Smad2 in CAFs (Supplementary Fig. S2C). ELISA analysis was used to confirm endoglin protein expression on mouse CAFs (Supplementary Fig. S2D). To demonstrate that BMP-9 signals through endoglin to induce Smad1 phosphorylation in CAFs, we used the endoglin-neutralizing antibody TRC105, which competitively inhibits BMP-9 binding to endoglin. Human CAFs were stimulated with TGF-β, BMP-9, or BMP-6 in the presence or absence of TRC105. Stimulation with TGF-β increased Smad2 and, to a lesser extent, Smad1 phosphorylation, independent of TRC105 (Fig. 3D; Supplementary Fig. S3A). BMP-9 stimulation strongly increased Smad1 phosphorylation, which was abrogated by TRC105. BMP-6 induced endoglin-independent Smad1 phosphorylation and was unaffected by TRC105 (Fig. 3D; Supplementary Fig. S3A). In mouse CAFs, stimulation with BMP-9 strongly induced Smad1 phosphorylation (Fig. 3E; Supplementary Fig. S3B). This was efficiently blocked by the mouse endoglin-neutralizing antibody M1043, whereas TGF-β–induced Smad2 phosphorylation was unaffected (Fig. 3E; Supplementary Fig. S3B). TRC105 also inhibited Smad1 phosphorylation in mouse CAFs, although to a lesser extent (Supplementary Fig. S3B). Therefore, subsequent experiments using mouse cells were performed using M1043. Together, these results confirm that BMP-9–induced Smad1 phosphorylation is endoglin-dependent in CAFs, and this can be inhibited using endoglin-neutralizing antibodies.

Endoglin is required for CAF survival in vitro

After characterizing endoglin-mediated signaling in CAFs, its functional role was further evaluated. Short hairpin RNA (shRNA) constructs targeting endoglin were introduced using lentiviral transduction, and knockdown efficiency of the constructs at RNA and protein level was confirmed in endothelial cells (Supplementary Fig. S3C). Endoglin expression was reduced by 40% to 90% compared with non-targeting control. This degree of endoglin knockdown did not affect endothelial cell morphology or survival (Fig. 3F). However, CAFs transduced with endoglin shRNA constructs ceased to proliferate and cells adopted a senescence-resembling phenotype (Fig. 3F), progressing to cell detachment and death. The phenotype was confirmed in different CAFs and NFs, indicating that endoglin is indispensable for CAF survival in vitro.

Endoglin regulates CAF invasion in vitro

Different roles for endoglin in cell migration have been reported for endothelial and nonendothelial cells. Ectopic expression of endoglin in HEK293T cells enhanced cell invasion into a collagen-I matrix (Supplementary Fig. S4A and S4B). Spheroid diameters remained similar, suggesting that invasion rather than proliferation is the main

determinant of this effect. Next, we examined the role of endoglin in CAF invasion. High endoglin-expressing CAFs (F2) were compared with CAFs expressing 200-fold lower levels of endoglin (F1, Fig. 4A). After 48 hours, F2 CAFs invaded a collagen-I matrix to a higher extent than F1 CAFs (Fig. 4B), suggesting a role for endoglin in CAF invasion. Because 3-dimensional invasion assays with CAFs are difficult to accurately quantify, we overexpressed endoglin in F1 CAFs (Fig. 4C), and invasive capacity was determined using transwell invasion assays through collagen-I–coated inserts. Quantification of the number of invaded cells after 24 hours showed that endoglin overexpression significantly increased basal CAF invasion (Fig. 4D). Because endoglin knockdown is not possible in CAFs, we used MEFs isolated from endoglin fl/fl mouse embryos (25). To induce endoglin deletion (KO), MEFs were transduced with Cre recombinase or an empty vector. Endoglin KO did not affect MEF proliferation and cells remained viable for up to three passages after transduction, possibly due to their embryonic nature. Endoglin mRNA levels were reduced by 90% (Fig. 4E), and endoglin KO MEFs showed significantly reduced invasion in transwell invasion assays (Fig. 4F). These data demonstrate the importance of endoglin in CAF invasion.

Endoglin targeting reduces invasive capacity of fibroblasts

To confirm that CAF invasion is dependent on ligand binding to endoglin, and not merely on its presence, the invasive capacity of mouse CAF was assessed in the presence of M1043. M1043 treatment reduced basal mouse CAF invasion, without affecting cell morphology (Fig. 5A). Because both colorectal cancer cells and CAFs express the endoglin ligands TGF-β [6, 37] and BMP-9 (Supplementary Fig. S4C and S4D), these factors were used to determine their individual contributions to CAF invasion. TGF-β stimulation did not increase BMP-9 expression in CAFs (Supplementary Fig. S4E), excluding indirect effects on CAF invasion by TGF-β, through BMP-9 signaling. BMP-9– and TGF-β–induced invasion were inhibited by M1043 to a similar level, albeit not statistically significant (Supplementary Fig. S4F). Because interactions in the TME can be mediated by paracrine signaling, we assessed CAF invasion toward murine MC38 colorectal cancer cells. MC38 cells stimulated CAF invasion to a similar extent as BMP-9, which could not be further enhanced by combining MC38 cells with BMP-9 (Fig. 5B). M1043 treatment significantly decreased MC38-induced CAF invasion, compared with IgG control (Fig. 5C). In human CAFs, TRC105 decreased basal human fibroblast invasion (Fig. 5D), while cell morphology remained similar and proliferation was unaffected (Supplementary Fig. S4G). BMP-9 and TGF-β only marginally affected CAF invasion in this case, resulting in limited inhibitory effects of TRC105 on invasion (Supplementary Fig. S4H). In coculture experiments, treatment with TRC105 inhibited HCT116- and HT29-induced CAF invasion (Fig. 5E and F, respectively). Taken together, these experiments imply a substantial role for endoglin/BMP-9 signaling in CAF invasion in vitro.

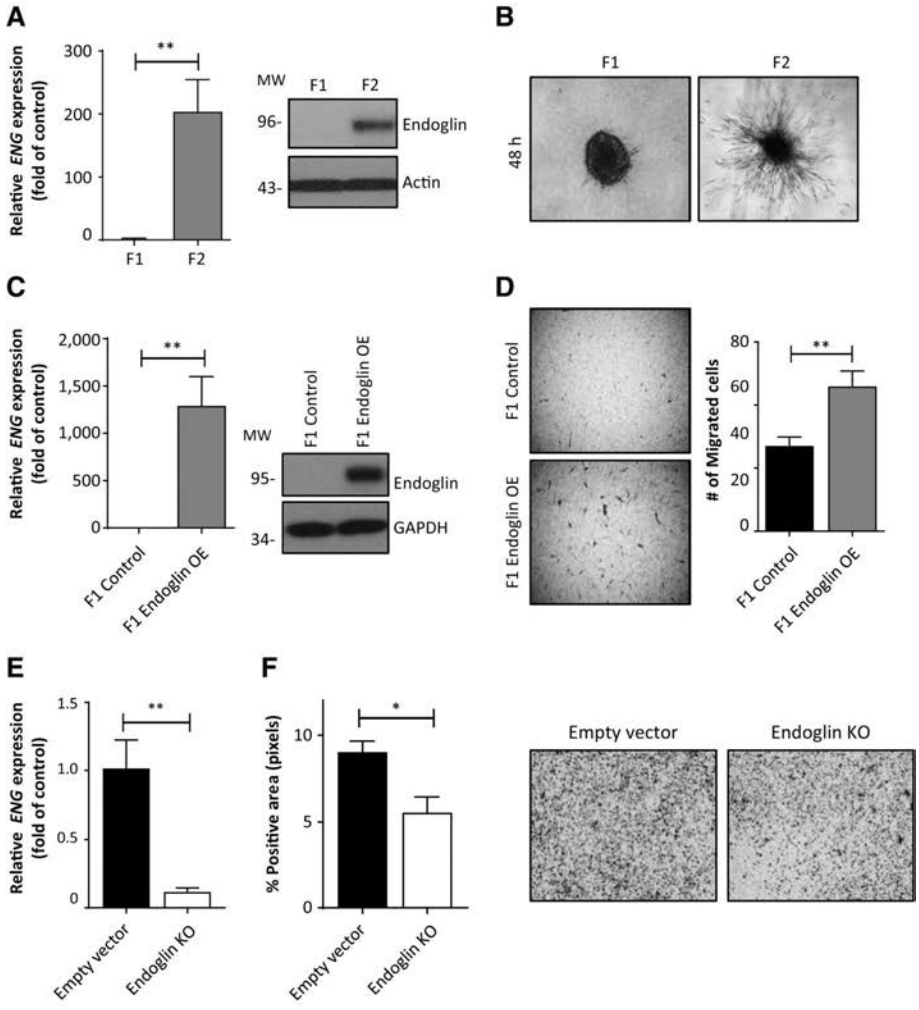

Figure 4. Endoglin regulates CAF invasive capacity in vitro. Low (F1) and high (F2) endoglin–expressing CAFs (**A**) were assessed for invasive properties. **B**, High endoglin–expressing F2 CAFs invaded collagen-I matrix more extensively than low endoglin–expressing F1 CAFs. Representative pictures of two experiments performed in triplicate. h, hours. **C**, Confirmation of endoglin overexpression (OE) in F1 CAFs. Representative graph from three independent experiments. **D,** Endoglin OE significantly enhanced F1 CAF invasion after 24 hours in a transwell invasion assay. Data represent mean of three independent experiments performed in triplicate. **E**, Reduced endoglin expression in endoglin knockout MEFs (endoglin KO) upon Cre expression. **F**, Endoglin KO significantly reduced MEF invasion in transwell invasion assays. Data represent mean of three independent experiments performed in triplicate. *, $P \leq 0.05$; **, $P \leq 0.01$. Quantification of expression data shown as representative mean + SD, invasion as mean + SEM of at least three independent experiments.

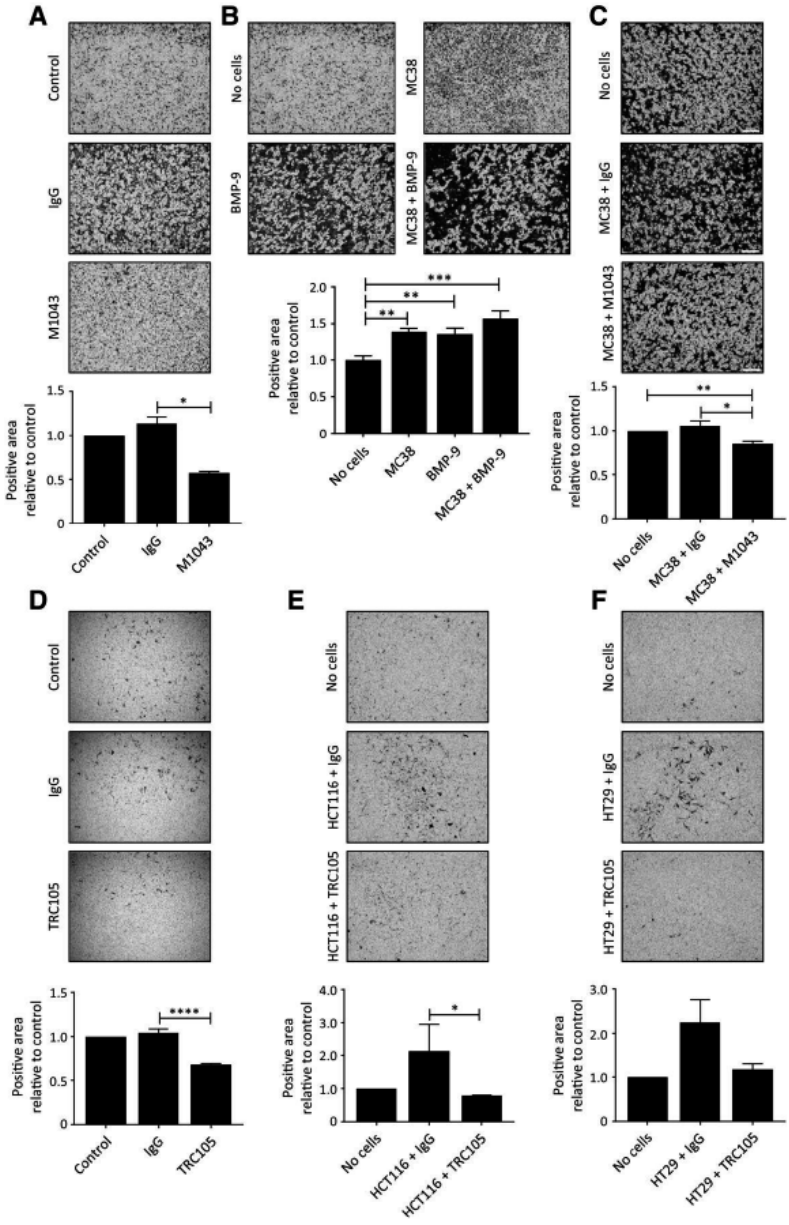

Figure 5. Endoglin targeting inhibits CAF invasion in vitro without affecting CAF morphology. A, M1043 significantly inhibited basal mouse CAF invasion. **B**, The presence of MC38 cells in the lower transwell compartment increased mouse CAF invasion to a similar extent as observed for BMP-9 stimulation. Addition of BMP-9 in the presence of MC38 did not further increase CAF invasion. **C**, Mouse CAF invasion toward MC38 mouse colorectal cancer cells was slightly, but significantly, reduced by M1043 when compared with IgG control. **D**, TRC105 significantly inhibited basal human CAF invasion. Human CAF invasion toward the human colorectal cancer cell lines HCT116 (**E**) or HT29 (**F**) was inhibited by TRC105 when compared with IgG control. All data represent mean of at least three independent experiments performed in triplicate. No cells, no tumor cells present in lower compartment. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001. All graphs show mean + SEM of at least three independent experiments. Representative pictures for all conditions are shown at ×20 magnification.

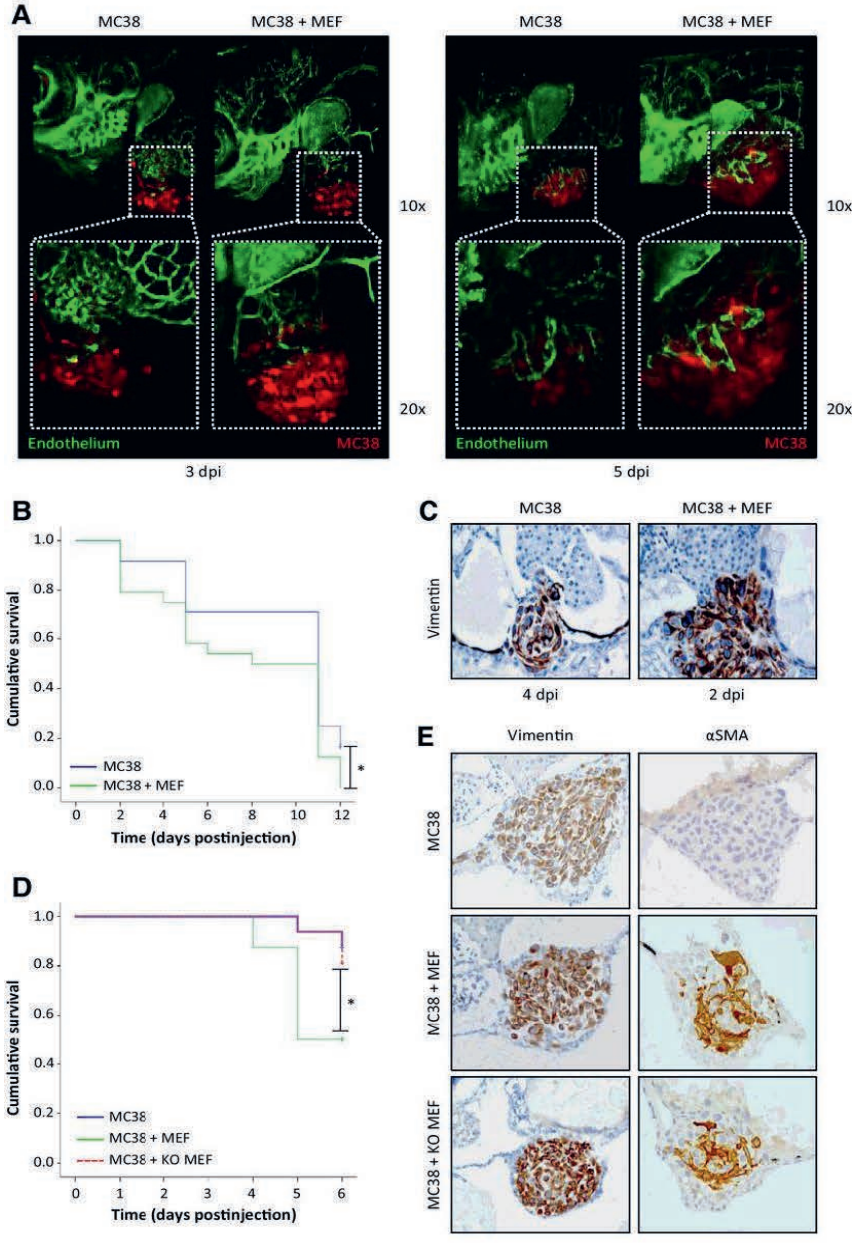

Figure 6. Endoglin-expressing CAFs reduce survival in a zebrafish model for colorectal cancer.
A, Injection of dTomato MC38 cells in the absence or presence of MEFs (MC38 + MEF) induced formation of solid tumors (red) and recruitment of vasculature (green) in zebrafish embryos. **B**, Coinjection of MC38 cells with MEFs decreased survival in zebrafish embryos when compared with MC38 alone (n = 24/group). **C**, IHC staining showed vimentin-expressing MC38 cells invading the zebrafish liver. Coinjection with MEFs accelerated liver invasion (2 dpi, right) compared with MC38 alone (4 dpi, left). **D**, Coinjection of MC38 with endoglin knockout (KO) MEFs (MC38 + KO MEF) resulted in similar fish survival when compared with injection of MC38 alone (n = 23/group). **E**, IHC staining of MC38 tumors for vimentin (MC38 cells) and αSMA (MEFs). *, P ≤ 0.05. dpi, days post injection.

Endoglin expression on fibroblasts promotes colorectal cancer liver invasion in zebrafish

To study the role of CAF-specific endoglin in tumor metastasis in a multicellular model, we developed a zebrafish model for colorectal cancer. Fluorescently labeled MC38 cells, in the presence or absence of MEFs (because endoglin deletion can be established in these cells, in contrast to CAFs), were injected in the heart cavity of zebrafish embryos and zebrafish were followed over time. Solid tumor-like structures were formed and induced angiogenesis (Fig. 6A). Coinjection of MC38 with MEFs significantly decreased fish survival (Fig. 6B), probably due to compromised liver function caused by tumor cell invasion. Histologic analysis revealed invasion of the vimentin-positive MC38 cells (MC38 cells do not express epithelial markers, like cytokeratin) into the liver in both experimental groups. However, coinjection with MEFs resulted in liver invasion as early as 2 days after injection, versus 4 days after injection when MC38 cells were injected alone, suggesting that the presence of MEFs accelerates this invasive process (Fig. 6C). To investigate endoglin dependency, MC38 cells were injected in combination with normal or endoglin KO MEFs. Tumor formation and angiogenesis were not affected (Supplementary Fig. S5). However, zebrafish survival markedly improved when endoglin KO MEFs were coinjected and resembled survival of zebrafish injected with MC38 alone (Fig. 6D). Integration of MEFs in the tumors of both coinjected groups was confirmed by staining for mouse-specific αSMA expression (Fig. 6E). These data indicate that endoglin expression on MEFs affects colorectal cancer cell invasion in zebrafish.

Endoglin targeting inhibits colorectal cancer liver metastasis in mice

Finally, we assessed therapeutic targeting of endoglin in an experimental mouse model for colorectal cancer liver metastasis. HT29 cells were injected in the spleen, alone or in combination with human CAFs. Mice were treated (Fig. 7A) and metastatic spread was monitored using bioluminescent imaging (BLI). HT29 cells express very low levels of endoglin, and in vitro proliferation of HT29 cells was not affected by TRC105 (Supplementary Fig. S6A and S6B). Therefore, as expected, TRC105 treatment did not affect metastatic spread in mice injected with HT29 cells alone (Supplementary Fig. S6C and S6D). In mice coinjected with HT29 and CAFs, however, TRC105 significantly reduced BLI signal from the liver (Fig. 7B). This indicates that TRC105 affects metastasis formation by directly targeting human CAFs. Metastatic lesions in the liver were visualized using ex vivo BLI upon termination of the experiment (Fig. 7C). IHC staining revealed no morphologic differences in liver metastases between groups (Fig. 7D). These data show that targeting endoglin on CAFs inhibits metastatic spread of HT29 colorectal cancer cells and imply CAFs as an additional target cell for TRC105 therapy.

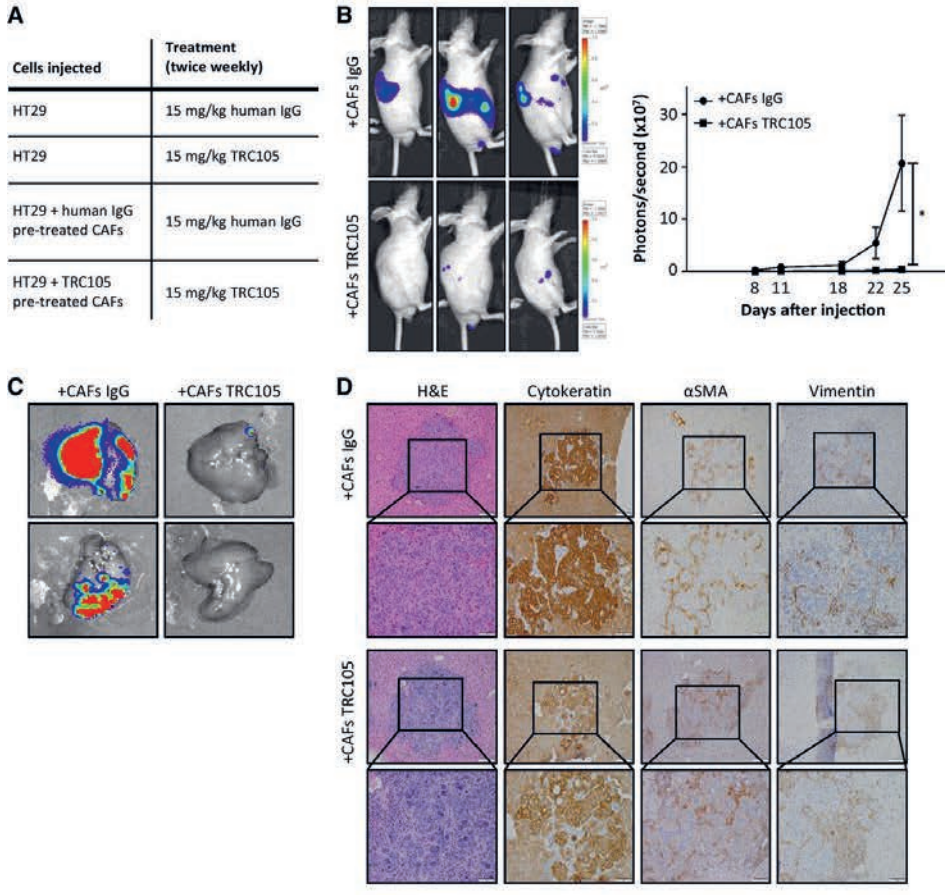

Figure 7. TRC105 inhibits CAF-mediated metastatic spread in a mouse model for liver metastasis. **A**, Experimental setup. Mice were injected with HT29 cells alone or in combination with human CAFs. Two days after injection, treatment with human IgG or TRC105 started. **B**, TRC105 treatment reduced metastatic spread to the liver in mice injected with HT29 and CAFs (+CAFs TRC105) as quantified by in vivo bioluminescence. Graph represents two independent experiments, 15 mice/group in total, showing mean ± SEM. **C**, TRC105 treatment decreased ex vivo bioluminescent signal in livers of mice coinjected with HT29 and CAFs. **D**, (Immuno)histologic analysis of liver metastasis with H&E and staining for cytokeratin (HT29), αSMA, and vimentin (CAFs). Magnification: ×100 and ×200. *, P ≤ 0.05.

DISCUSSION

In this study, we show that CAF-specific endoglin expression correlates with the development of metastatic disease in stage II colorectal cancer and endoglin-expressing CAFs are detected in metastatic lesions of patients with colorectal cancer. CAF-specific endoglin expression stimulates CAF invasion in vitro and tumor cell invasion and metastasis in a novel zebrafish model and in a murine model for colorectal cancer.

Endoglin is crucial for vascular development as underlined by embryonic lethality of endoglin knockout mice [12–14]. Our current study shows the importance of endoglin for fibroblast survival in vitro. Romero and colleagues reported the inability to culture primary prostate CAFs isolated from endoglin heterozygous mice, whereas CAFs from endoglin wild-type mice could easily be propagated in vitro (38). We observed that although NFs in vivo do not express endoglin, its expression is highly upregulated during cell culture. Because of its indispensability in vitro, neutralizing antibodies pose a useful tool to study the role of endoglin in culture, especially because CAF proliferation remains unaffected.

In endothelial cells, endoglin has been shown to be important for proliferation [39] and migration [40], and deletion of endoglin results in decreased Smad1 phosphorylation [41]. Recently, the crystal structure of BMP-9 bound to endoglin has been reported, revealing that endoglin is required to efficiently present BMP-9 to ALK1 [17] and induce downstream signaling. In our in vitro assays, endoglin was essential for BMP-9–induced Smad1 phosphorylation in CAFs and important for CAF invasion through collagen-I. Inhibition of ALK5 signaling by the ALK1/endoglin complex has been described in endothelial cells [42]. In accordance with this, we have recently reported increased Smad2 phosphorylation in endothelial cells upon TRC105 treatment [28], which alleviates the inhibitory function of this complex. Activation of ALK5 signaling results in decreased endothelial cell migration [42], which could pose an additional mechanism by which cell migration is decreased next to inhibition of endoglin signaling. However, increased Smad2 phosphorylation was not observed in CAFs upon TRC105 treatment, suggesting differences in TGF-β/BMP signaling between CAFs and endothelial cells, possibly by receptor abundance or expression of different type I receptors.

Colorectal cancer cells produce high levels of TGF-β [6], which could affect endoglin signaling by indirectly increasing BMP-9 expression in CAFs. In this article, we show that TGF-β stimulation does not affect BMP-9 expression in CAFs. In addition, Nolan-Stevaux and colleagues [19] and unpublished observations from our group showed that TRC105 does not inhibit binding of TGF-β to endoglin, further rendering observed effects on CAF invasion to be BMP-9 and most likely not TGF-β endoglin dependent.

In addition to signaling through ALK1, endoglin interacts with integrins, crucial for adhesion and migration of ECs [43]. In fibroblasts, interactions between ECM and integrins were shown to be important for cellular migration [44], but the role of endoglin in this interaction was not investigated. In our in vitro experiments, inhibiting endoglin function reduced CAF invasion through a collagen-I matrix, suggesting involvement of endoglin in cell–ECM interaction, although the underlying mechanism is yet unresolved.

The specific localization of endoglin-expressing CAFs at the invasive border of colorectal tumors suggests a role in tumor metastasis, a suggestion that is further strengthened by their detection in lymph node and liver metastases from patients with colorectal cancer. Recently, Labernadie and colleagues revealed that CAFs use heterotypic cadherin interactions to interact with tumor cells and physically pull these cells out of the tumor mass in order to induce tumor invasion [45]. Interactions of endoglin with VE-cadherin were reported in endothelial cells, in which VE-cadherin regulates cell migration [46]. Interactions of endoglin with other cadherins have not yet been reported but might be involved in CAF-mediated tumor invasion. The physical interaction of CAFs with tumor cells implies that CAFs could travel in a complex with tumor cells to metastatic sites. In accordance with this hypothesis, results from an experimental model for colorectal cancer metastasis showed that GFP-expressing CAFs were localized in liver metastases and increased the formation of these lesions [47]. In vivo experiments in a lung cancer model also showed stromal cells derived from the primary tumor in metastatic lesions [48]. These data combined with our observation that TRC105 inhibits liver metastasis by targeting endoglin on CAFs in vivo imply that metastatic spread could, at least in part, be regulated by endoglin.

Previously, in prostate cancer models, it was shown that although endoglin heterozygosity increased primary tumor growth, the number of metastases was lower than in wild-type mice [38]. In contrast, increased metastatic spread of pancreatic tumors and subcutaneous implanted lung cancer cells has been reported in, respectively, endoglin heterozygous or endothelial-specific endoglin KO mice [49]. Although these data contradict our findings, these studies investigated endoglin expression on endothelium and did not consider fibroblast-specific endoglin expression. Heterozygous endoglin deletion in vivo results in a phenotype resembling hereditary hemorrhagic telangiectasia, including increased vascular permeability [50, 51], possibly facilitating tumor cell intra- and extravasation and subsequent metastasis. Our current experiments specifically assessed the role of endoglin on CAFs. Moreover, unpublished data from our group showed that TRC105 treatment does not affect endothelial cell integrity in vitro. More in-depth studies using cell type-specific endoglin knockout mice have to be performed in order to unravel the exact contribution of endoglin on individual cell types in metastatic spread.

The fibrotic response is an important regulator of tumor progression and metastasis [52] and has been proposed as a prognostic factor in colorectal cancer [53]. Endoglin expression on fibroblasts has been reported during cardiac fibrosis and reduction of endoglin expression or endoglin targeting prevented cardiac fibrosis in vivo [54]. Interestingly, prostate tumors grown in endoglin heterozygous mice are not fibrotic and lack αSMA-expressing cells [38]. In line with these data, we have previously shown that TRC105 treatment reduced metastatic breast cancer spread in vivo,

which was accompanied by a decreased αSMA-positive stromal content [28]. Even though the αSMA content in the liver metastasis in vivo in this article was not different between groups, metastatic spread was reduced after TRC105 treatment, suggesting that these residual lesions managed to escape treatment. Moreover, we show here a correlation between high CAF-specific endoglin expression at the invasive tumor borders and worse metastasis-free survival in patients with stage II colorectal cancer, implying the involvement of this CAF subset in tumor invasion and metastasis. Analyses of different TCGA databases for colorectal cancer showed that endoglin is correlated to risk classification. Despite the fact that these databases provide valuable information, they do not distinguish between cell type-specific endoglin expression and specific localization, as reported in this study. Although we did not assess the relation between CAF-specific endoglin expression and fibrosis in this study, these data could imply that endoglin on CAFs contributes to peritumoral fibrosis as an adverse prognostic factor in colorectal cancer. In addition, it might suggest another potential therapeutic field for TRC105 for the treatment of fibrotic diseases.

In summary, the data presented here point to a crucial involvement of endoglin-expressing CAFs in colorectal cancer invasion and metastasis and could therefore be a potential therapeutic target. In addition, CAF-specific endoglin expression might be a novel prognostic factor in early-stage colorectal cancer. In a phase I study, TRC105 showed clinical efficacy on preexisting metastases in 2 patients [18]. Combined with our recently published data that adjuvant TRC105 treatment decreased metastatic spread in breast cancer [28], targeting endoglin on CAFs, in addition to the endothelium, could be a potent approach in preventing metastasis formation and underlines the potential of TRC105 being more than a classic antiangiogenic drug.

Disclosure of Potential Conflicts of Interest

C.P. Theuer has ownership interest (including patents) in TRACON Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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4

Fibroblast-specific endoglin knock out changes the colonic immune infiltrate and increases formation of colitis associated intestinal adenomas

MANUSCRIPT IN PREPARATION

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ABSTRACT

In addition to high endoglin expression on endothelial cells, endoglin expression has been reported on Cancer Associated Fibroblasts (CAFs) in solid tumors, including colorectal cancer (CRC). Although data indicate an important role in later stages of tumor development, the role of endoglin-expressing fibroblasts in early cancer development and its interaction with the immune system has hardly been investigated. Therefore, in this study we have generated a tamoxifen-inducible fibroblast-specific (col1a1 and col1a2) endoglin knock out mouse ($ENG^{Fib-/-}$). Tumor formation was studied using the azoxymethane/dextran sodium sulphate (DSS) model for colitis-associated cancer. Interestingly, a significant increase in the number of adenomas was observed in $ENG^{Fib-/-}$ mice compared with control mice. Analysis of infiltrating immune cells revealed that deletion of endoglin in fibroblasts resulted in enhanced recruitment of macrophages and neutrophils to AOM/DSS-induced adenomas. However, these neutrophils did not cause the increased tumorigenesis observed in the $ENG^{Fib-/-}$mice. To investigate the tumor initiation stage, a short DSS was performed, revealing in contrast to late stages, a decrease in the number of myeloid cells in both the colon as well as the blood of these mice. These data suggest a delayed immune response, which was reflected in the severe weight loss of the $ENG^{Fib-/-}$ mice. Together, these data suggest that endoglin expression on fibroblasts plays a role in the initiation of colitis induced CRC, potentially by altered or delayed inflammatory responses.

INTRODUCTION

Paracrine interactions between malignant epithelial cells and their tumor microenvironment (TME) play a crucial role in cancer progression [1, 2]. The TME of solid tumors, such as Colorectal Cancer (CRC), is composed of non-malignant cells, including tumor infiltrating inflammatory cells, endothelial cells forming the tumor vasculature system and cancer-associated fibroblasts (CAFs) [3]. CAFs compose the major part of the tumor stroma. CAFs are a diverse group of fibroblasts expressing vimentin, fibroblast activating protein (FAP) and α-smooth muscle Actin (αSMA) [4]. CAFs in solid tumors interact with other cells via several cytokines and direct cell-cell contact (reviewed in [5]). Members of the Transforming growth factor β (TGF-β) superfamily seem to play a central role in the generation of CAFs [6-8]. TGF-β is an extensively studied family of cytokines, both important in tissue homeostasis and cancer. Signaling is regulated via the expression of a distinct set of receptors and co-receptors. One of the TGF-β coreceptors is endoglin, which can interact with TGF-β and Bone Morphogenetic Protein 9 (BMP9) and regulate angiogenesis [9-13]. TGF-β can interact with endoglin and activate the ALK5-SMAD2,3 pathway, which causes blood vessel maturation. BMP9 can signal through the endoglin- ALK1-SMAD1/5/8 pathway, which causes migration and proliferation of endothelial cells [14-17]. In addition to very high endoglin expression on angiogenic endothelial cells, we and others have observed endoglin expression on cancer associated fibroblasts (CAFs) in both Prostate [18] and CRC [19]. In CRC, endoglin on CAFs seems to play a role in the development of CRC derived liver metastasis in experimental models. Although the role of endoglin on fibroblasts has been studied using endoglin heterozygous mice and targeted antibodies, these results poorly reflect the specific role of endoglin on fibroblasts.

Therefore, in this project, we aim to investigate the role of endoglin on fibroblasts during CRC initiation using the azoxymethane/dextran sodium sulphate (AOM/DSS) model [20]. This most commonly used model for colitis associated cancer generates tumors that resemble (early stage) human CRC [21]. To investigate the endoglin specific role on fibroblasts in tumorigenesis we generated a tamoxifen-inducible fibroblast-specific (col1a1 and col1a2) endoglin knock out mouse ($ENG^{Fib-/-}$). Tumor initiation and the role of infiltrating immune cells was further investigated in these KO mice.

MATERIALS AND METHODS

Cell culture, preparation of CM and signaling assays

Mouse fibroblasts and the mouse CRC cell line MC38 [22] were cultured in DMEM/F12, supplemented with 10% fetal calf serum (FCS), 10 mM HEPES, 50 µg/mL

gentamycin, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFisher, Waltham, MA, USA). The mouse CRC cell line CT26 [23] was maintained in RPMI 1640, supplemented with 10% FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoFisher). Murine embryonic fibroblasts (MEFs) were obtained from E12.5 embryos as described before [24], from an endoglin flox/flox mouse strain in which exons 5 and 6 are flanked by LoxP sites [25]. MEFs and the mouse myoblast cell line C2C12 were maintained in DMEM, supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin and 100 µg/mL streptomycin (all ThermoScientific).
Constructs expressing Cre recombinase (pLV.mPGK.iCRE.IRES.PuroR, kindly provided by Dr. M. Goncalves, Dept.of Cell and Chemical Biology, Leiden University Medical Center) or empty vector control were delivered using lentiviral transduction using polybrene (4 µg/mL, Hexadimethrine bromide, Sigma Aldrich) to 80% confluent MEFs and after 48 hours, transduced cells were selected by 1.5 µg/mL puromycin (Sigma Aldrich).
Conditioned medium (CM) from MEFs was prepared by serum starving subconfluent cells for four days. CM used for proliferation assays was two-fold diluted with culture medium, containing 5% FCS.

MTS proliferation assay

5000 CT26 or MC38 cells were seeded in 96-well plates in triplicate. After 16 hours, the medium was replaced with 100 µL CM, from either control or endoglin knock out MEFs or with non-conditioned medium. At indicated time points 20 µL MTS substrate (Promega, Madison, WI, USA) was added to each well and absorbance was measured at 490 nm using a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA).

Mice

The Dutch animal ethics committee approved all animal experiments. Collagen1α1-Cre/ERT2 and Collagen1α2-CRE/ERT,-ALPP mice were purchased from Jackson Laboratory (strain B6.Cg-Tg(Col1a1-cre/ERT2)1Crm and Tg(Col1a2-cre/ERT,-ALPP)7Cpd, Jackson laboratory Bar Harbor, ME, USA). ENG*fl/fl* mice in which exons 5 and 6 of the endoglin gene are flanked by LoxP sites were generated by Allinson et al. [25]. Before tamoxifen induction, mice were divided into two groups, based on sex and body weight. Cre-mediated recombination was induced at eight weeks of age by oral administration of 50 uL tamoxifen (100 µg/mL, Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in sunflower oil, on three consecutive days. Control mice had the same genotype but were treated with sunflower oil only. Collagen1α1-CreERT2.ENG*fl/fl* or Collagen1α2-Cre/ERT,-ALPP.ENG*fl/fl* and ENG*fl/fl* mice were used for control experiments as described. Mice were genotyped by PCR for the presence of the Cre recombinase and endoglin LoxP gene as described before [19].

AOM/DSS model

Two days after tamoxifen induction male and female mice received one intraperitoneal (i.p.) injection with 10 mg/mL azoxymethane (AOM; Sigma-Aldrich, Zwijndrecht, The Netherlands) dissolved in saline. Two days later, the first 7-day cycle with 1.5% dextran sodium sulphate (DSS; MP Biomedicals, Santa Ana, CA, USA) dissolved in drinking water, supplemented with artificial sweetener (Natrena, Utrecht, the Netherlands), was started. After seven days, drinking water was changed to normal conditions for 14 days. This three-week cycle was repeated twice more during the experiment. During DSS cycles, mice weight and overall health were monitored daily, while during the "off" period animals were weighted and checked every other day. Two weeks after the third DSS cycle, mice were sacrificed, and blood and tissue samples were collected. Colons were partially snap-frozen in liquid nitrogen for RNA analysis and partially fixed in 4% formaldehyde and photographed by using a NIKON D750 camera equipped with a TAMRON 24-70mm f2.8 lens. Lesion volume was measured in photographed colons by ImageJ (National Institute of Health), and tumor volume was calculated (tumor volume = (width^2 × length)/2). For the short DSS induced colitis mice were sacrificed after the first cycle of DSS, blood and tissue samples were collected and possessed for histology and flow cytometry analysis.

Neutrophil (Ly6G) depletion

Ly6G depleting antibody was given either on the day of the start the first DSS cycle or the second DSS cycle followed by a twice weekly i.p. injection of 0.2mg antibody 1A8 or IgG control dissolved in PBS (BioxCell, West Lebanon, USA). Blood was monitored weekly during the time of depletion. Depletion was stopped once the neutrophils (GR-1+ Cells) repopulated the blood of the mice (after +/-20 days of depletion).

RT-qPCR

Tissue samples were disrupted using a TissueLyser (Qiagen, Hilden, Germany) and RNA was isolated using Nucleospin RNA kit (Bioké, Leiden, The Netherlands), according to manufacturers' instructions. For *in vitro* experiments, MEFs were grown to confluency, harvested and RNA was isolated according to manufacturers' instructions. RNA concentration and purity were determined using NanoDrop 3300 (Thermo Scientific, Breda, The Netherlands). Complementary DNA synthesis was performed using 1 µg RNA using RevertAid First Strand cDNA synthesis kit, according to manufacturers' instructions (ThermoScientific). Quantitative PCR analyses were performed as described before [19], using primers as described in supplementary table 1 (Invitrogen). All values were normalized by *GAPDH* expression.

Table 1. Assessment of inflammation by means of clinical and macroscopic score.

Score	Diarrhea score	Visible fecal blood	Inflammatory Score
0	Normal Pallets	Normal	Normal
1	Slightly loose feces	Slightly bloody	Slight inflammation
2	Loos feces	Bloody	Moderate inflammation and or edema
3	Watery diarrhea	Blood in the whole colon	Heavy inflammation and or ulcerations and or edema

Flow cytometry

Tumor material was minced with scalpels and digested with Liberase (Roche, Basel, Switzerland) (Sigma-Aldrich, Zwijndrecht, NL) 375 µg/ml DMEM/F12 containing 10% FCS for 30 minutes at 37°C. To obtain single cells, the suspension was filtered through Falcon™ Cell Strainers 70 µm pore size (Fisher Scientific, Landsmeer, NL, 352350) and both tumor as blood was stained with antibodies against CD45, CD11b (both eBioscience, Vienna, Austria), F4/80, Ly6C GR-1 and Ly6G (all BioLegend, San Diego, CA, USA). FACS analysis was performed on the LSR II system (Becton Dickinson, Breda, The Netherlands). Data were analyzed using FlowJo data analysis software (FlowJo, Ashland, OR, USA).

Tissue analysis

Immunohistochemical stainings were performed as described previously [26], using primary antibodies against vimentin, cleaved caspase 3 (both Cell Signaling Technologies, Danvers, MA, USA), αSMA (Progen, Heidelberg, Germany), Ki67 (Millipore, Amsterdam, The Netherlands), Ly6G (BioLegend), F4/80 (eBioscience) and endoglin (R&D systems, Abington, UK). For quantification of total collagen, tumor sections were stained with Sirius red (Klinipath, Duiven, The Netherlands). In short, paraffin sections were deparaffinized, stained with 0.1% Sirius red in picric acid, washed in 0.01M HCl and subsequently dehydrated and mounted in entellan. Three to five representative pictures per mouse were taken with an Olympus BX51TF microscope (Olympus Life Science Solutions, Zoeterwoude, The Netherlands) and staining was quantified using ImageJ software (National Institutes of Health). Quantification of macrophage infiltration was scored based on F4/80 staining. Score 1; ≤5% stroma positive, score 2; 5-25% stroma positive, score 3; 25-50% stroma positive, score 4; ≥50% stroma positive.

Macroscopic disease score

Macroscopic disease score (Table 1) was calculated by scores reflecting degree of diarrhea, visible fecal blood, and inflammatory score on the day of termination. The scores are characterized on a scale of 0-3 including half points. An experienced

technician conducted the score, the inflammatory score was judged by degree of inflammation and presence of ulcerations and or edema in the tissue. The total score was calculated by adding up the three individual scores and illustrated in the graph.

Statistical analysis

Data indicate mean ± SD, as indicated in figure legends. Differences between groups were calculated using Students' *t*-test, Mann-Whitney or ANOVA analysis when appropriate. P-values ≤0.05 were considered statistically significant.

RESULTS

Fibroblast-specific endoglin knock out enhances AOM/DSS-induced adenoma formation

To assess the effect of fibroblast-specific endoglin deletion in tumorigenesis, collagen1α1-Cre-ERT2 mice were crossbred with ENG*fl/fl* mice, generating collagen1α1-CreERT2.ENG*fl/fl* mice. Cre-mediated recombination was induced in 8-week-old animals by oral administration of tamoxifen, generating ENG$^{Fib-/-}$ mice. Animals were exposed to the AOM/DSS protocol, as described in figure 1A. During the first DSS administration mice from the ENG$^{Fib-/-}$ lost significantly more weight compared to the control mice suggesting enhanced colonic inflammation Figure 1 B. In the ENG$^{Fib-/-}$ group rectal blood loss, rectal prolapse and substantial weight loss, were more often observed, reflecting the more severe phenotype.

At the end of the experiment, the number of lesions in the colorectum was quantified. The occurrence of colonic lesions was significantly increased in the ENG$^{Fib-/-}$ group compared with control mice (Fig. 1C and D). Although the number of lesions differed significantly, the average lesion size was similar in both groups (Fig. 1E). Lesions were analyzed by H&E staining and adenomas with high-grade dysplasia were found in both groups (Fig. 1F). In addition, the number of adenomas was not dependent on the sex of the mice (Suppl. Fig. S1B). To exclude the possibility that endoglin deletion in fibroblasts results in spontaneous neoplastic growth, mice were induced with tamoxifen to obtain ENG$^{Fib-/-}$. Animals were kept for 13 weeks, identical to the experiment's time course, and did not receive AOM or DSS. At the end of the experiment, adenoma formation in the colorectum was assessed. In ENG$^{Fib-/-}$, no lesions in the colorectum were observed (Suppl. Fig. S1C). Additional histological analysis did not reveal any morphological changes in the colorectum. This suggests that fibroblast-specific endoglin knock out does not result in spontaneous neoplastic growth during our experiments.

Additionally, to assess the effect of tamoxifen administration on tumor induction, ENG*fl/fl* mice without the Cre recombinase, as a negative control, received oral

tamoxifen and were subsequently exposed to the AOM/DSS regimen as described in figure 1A. After 13 weeks, the number of adenomas in the colorectum of tamoxifen-induced ENG*fl/fl* mice was similar to non-induced Collagen1α1-CreERT2. ENG*fl/fl* mice treated with AOM and DSS (Suppl. Fig. S1C). This suggests that tamoxifen administration does not affect AOM/DSS-induced adenomas formation.

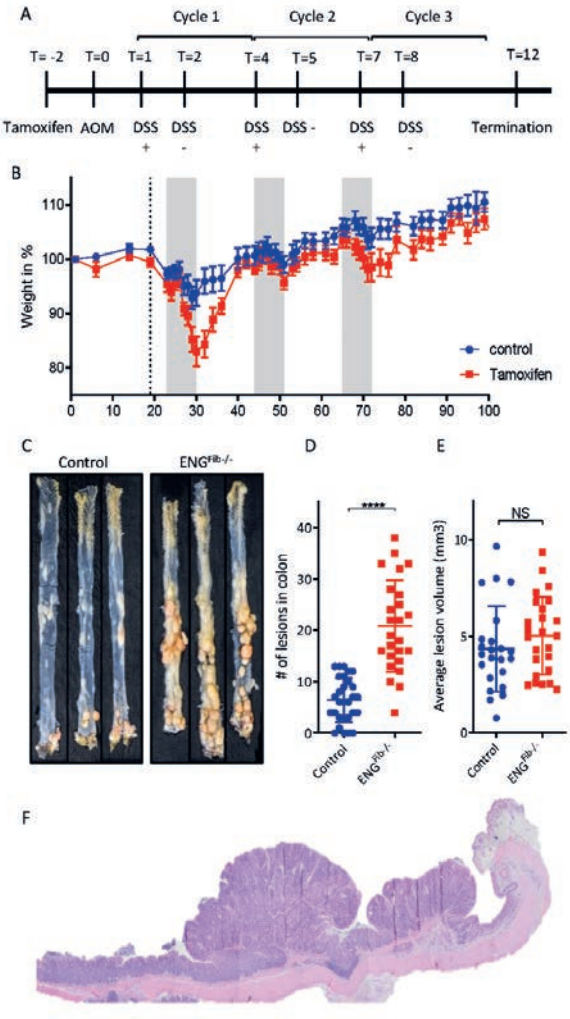

Figure 1. Fibroblast-specific endoglin knock out enhances AOM/DSS-induced neoplastic growth.
A. Experimental set-up. At 8 weeks of age, mice were induced with tamoxifen. After two weeks, AOM was injected, followed by three 21-day DSS cycles. Two weeks after the last DSS cycle, experiments were terminated. **B**. Representative pictures of colons obtained from control and fibroblast-specific endoglin knock out (ENGFibfl/fl) mice. **C**. Neoplastic growth was highly increased in ENGFibfl/fl mice, although size of the lesions was not different between the groups (**D**). Representative histological picture of a lesion qualified as high grate dysplasia by an independent pathologist. Graphs represent mean of 29-25 mice/group from two independent experiments. ****P≤0.0001.

These data imply that loss of endoglin on fibroblasts enhances colonic adenoma formation in chemically induced inflammation-driven colorectal cancer model.

Fibroblast-specific endoglin knock out does not affect epithelial proliferation

Since fibroblasts play an important role in intestinal homeostasis, we analyzed adenomas from control and ENG$^{Fib-/-}$ mice to assess changes. First, we determined the number of proliferating cells in AOM/DSS-induced adenomas using Ki67 as a marker for proliferating cells upon quantification, similar numbers of proliferating cells were observed in both groups (Fig. 2A). Next, we stained for the apoptotic marker cleaved caspase 3 to assess the number of apoptotic cells in the adenomas, apoptotic rates were similar in both control and ENG$^{Fib-/-}$ mice (Fig. 2B).

To confirm our *in vivo* observations the effects of endoglin deletion in fibroblasts on epithelial cell proliferation was assessed. Since knockdown of endoglin in primary fibroblasts results in a lethal phenotype *in vitro [19]*, murine embryonic fibroblasts (MEFs) from ENG*fl/fl* mice were used. Cre was introduced in these cells using lentiviral transduction, resulting in genetic deletion of endoglin (Fig. 2C). Conditioned medium (CM) from empty vector and endoglin knock out MEFs was prepared to assess paracrine signaling to mouse epithelial cells. Mouse CRC cells CT26 and MC38 were stimulated with CM from either empty vector control or endoglin knock out MEFs and proliferation was measured. Over the course of three days using an MTS assay, proliferation rates between non-stimulated cells, control CM or endoglin knock out CM stimulated cells were similar in both CT26 (Fig. 2D) and MC38 cells (Fig. 2E). This suggests that endoglin expression on fibroblasts does not directly affect epithelial tumor cell proliferation in a paracrine manner, confirming our *in vivo* findings.

Fibroblast-specific endoglin knock out increases stromal content

Since CRC tumors are generally characterized by an abundant stromal compartment, the effect of fibroblast-specific endoglin deletion on the adenomas' total stroma was assessed by staining for the mesenchymal marker vimentin. Adenomas from ENG$^{Fib-/-}$ mice showed a significant increase in vimentin-positive cells when compared with control mice (Fig. 3C). To assess the number of activated fibroblasts αSMA staining was used. As observed for vimentin, the percentage of αSMA-positive content was increased in ENG$^{Fib-/-}$ adenomass compared with the control (Fig. 3D). Although more αSMA-positive fibroblasts were observed in ENG$^{Fib-/-}$ adenomas, total collagen production determined by a Sirius red staining, did not differ between the two groups (Fig. 3E). Together these data imply that fibroblast-specific Endoglin deletion leads to expansion of the stromal compartment without affecting the balance between epithelial proliferation and apoptosis in CRC.

Enhanced myeloid infiltration in fibroblast-specific endoglin knock out adenomas

Since our model depends on DSS induced inflammation to enhance AOM induced carcinogenesis, we investigated the composition of immune infiltrates in the AOM/DSS-induced adenomas. At the end of the experiment, adenomas of three mice per group were analyzed by flow cytometry. Total immune infiltrates, based on the percentage of CD45 positive cells, was not significantly altered between the control and ENG$^{Fib-/-}$ group, although a lower percentage of CD45+ cells was observed in the ENG$^{Fib-/-}$ group (Fig.4A). Interestingly, when we determined the percentage of CD11b-expressing myeloid cells in the CD45+ population, increased infiltration was observed in ENG$^{Fib-/-}$ adenomas (Fig.4B). To further specify which cells of the myeloid population were increased, the abundance of macrophages was determined by assessing the percentage of F4-80 and Ly6C positive and negative cells . Although Ly6C+ monocytes

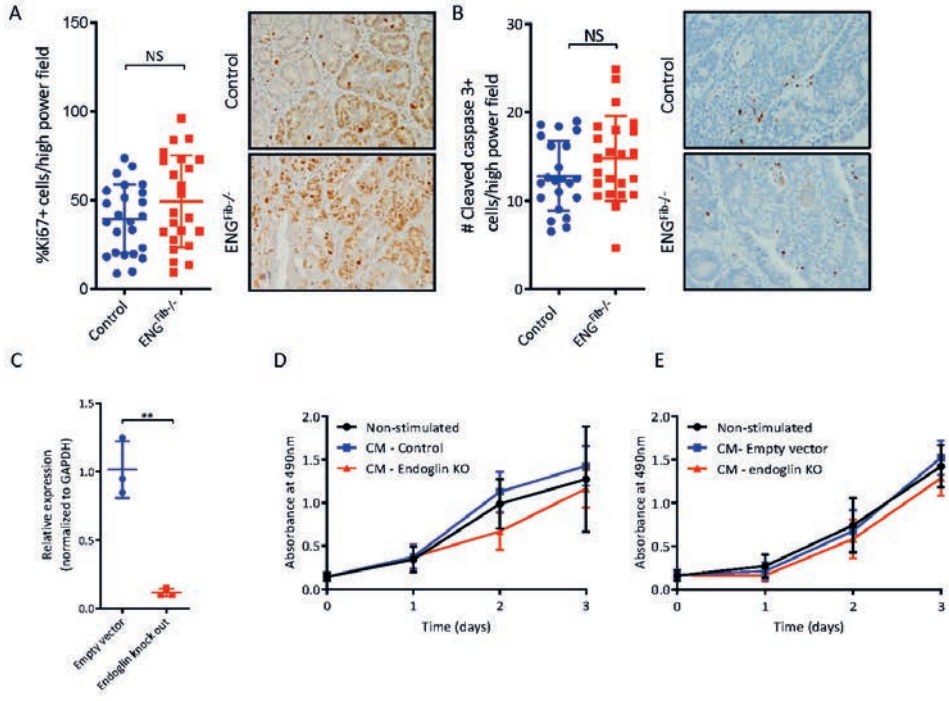

Figure 2. Proliferation in adenomas is not affected by endoglin deletion in fibroblasts. **A** Cellular proliferation in tumors was assessed by Ki67 staining and was similar in the control and ENGFibfl/fl group. **B** Cleaved caspase 3 was assessed to investigate the number of apoptotic cells which was similar in controls and ENGFibfl/fl group. n=24/23 tumors per group from two independent experiments. **C** MEFs were transduced with a Cre expressing lentivirus or empty vector control and endoglin expression was significantly reduced after transduction with Cre recombinase. Graph represents mean of three independent experiments. Stimulation with conditioned medium from MEFs, either empty vector or Cre recombinase transduced, did not affect tumor cell proliferation in CT26 (**D**) or MC38 cells (**E**). Graphs represent mean of three independent experiments performed in triplicate.

appeared to be increased in the ENG$^{Fib-/-}$ group, this did not reach statistical significance (Fig.4C). The percentage of Ly6C- macrophages, was significantly increased in ENG$^{Fib-/-}$ adenomas when compared with controls (Fig.4D). Indeed, increased macrophage recruitment to ENG$^{Fib-/-}$ adenomas was confirmed by quantifying immunohistochemical staining for the macrophage marker F4/80 (Fig.4E). Additionally, we assessed the percentage of neutrophils in adenomas using Ly6G stainings. Flow cytometry analysis showed that the percentage of Ly6G+ cells in the CD45+ population was strongly increased in the ENG$^{Fib-/-}$ group when compared with control mice, respectively 6% to 15% (Fig.4F). The number of Ly6G+ cells in adenomas was also assessed by IHC, and this analysis confirmed higher neutrophil infiltrate upon fibroblast-specific endoglin knock out (Fig. 4G). These data suggest that fibroblast-specific deletion of endoglin results in enhanced recruitment of myeloid cells, especially in neoplastic adenomas.

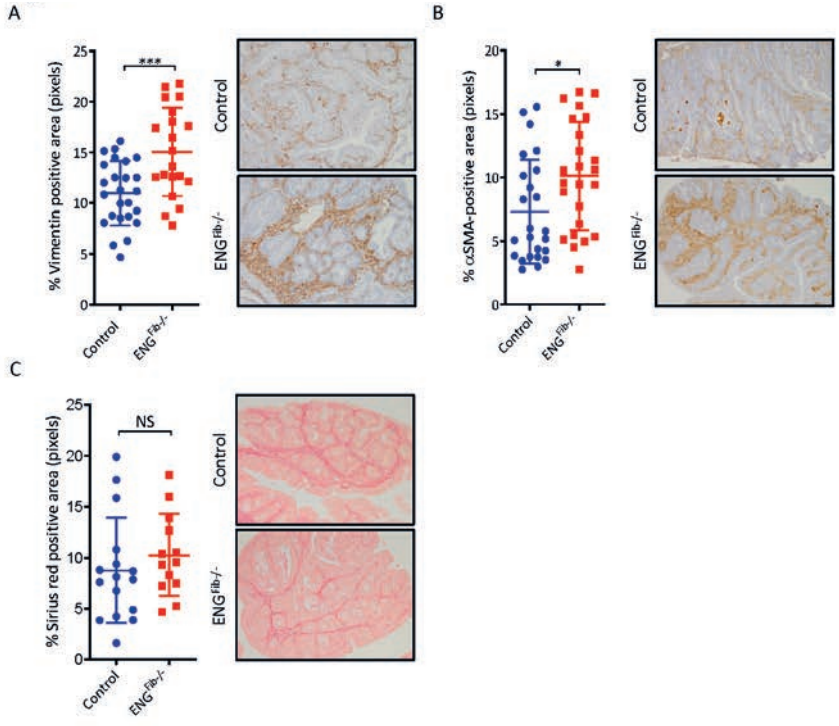

Figure 3. Endoglin deletion in fibroblasts increases stromal component in AOM/DSS-induced lesions. **A**. Lesions derived from control and ENGFibfl/fl mice were morphologically similar and were characterized as adenomas with high grade dysplasia. Total stroma content (**B**) and the abundance of activated fibroblasts (**C**) was assessed and proved to be increased after fibroblast-specific endoglin deletion. **D**. Total collagen deposition was not affected by endoglin knock out in fibroblasts. Data represent 29-25 mice/group from two independent experiments, average number of positive pixels. *P≤0.05, ***P≤0.001.

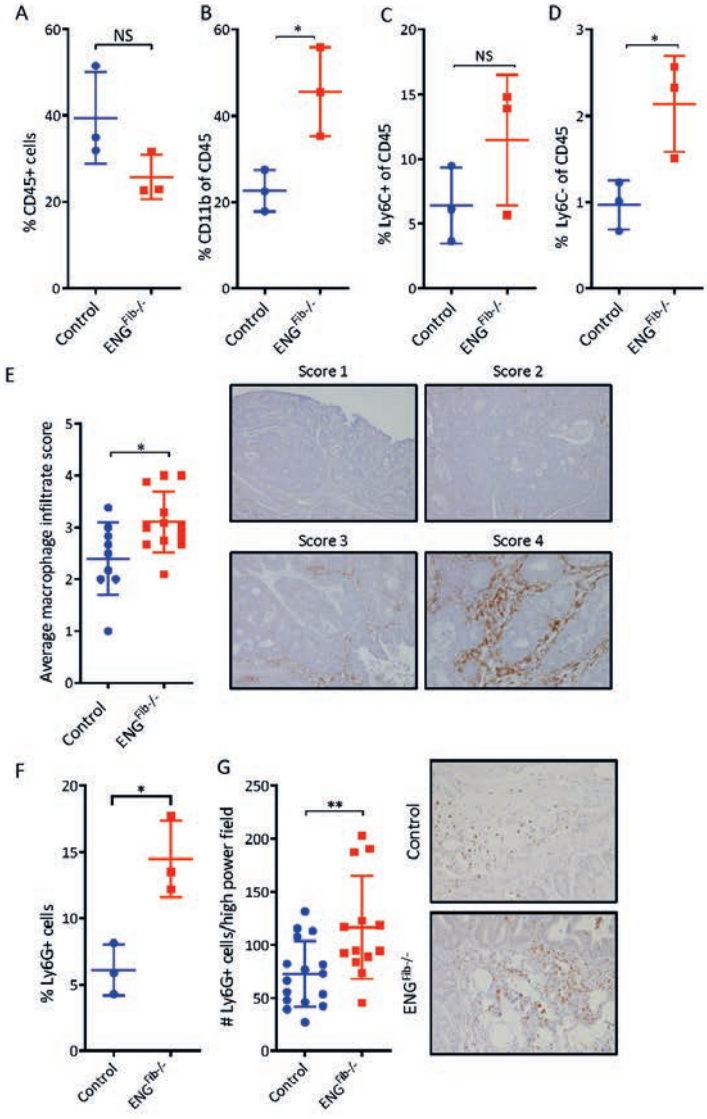

Figure 4. Increased macrophage recruitment to ENGFibfl/fl lesions. Immune cell infiltrate in the lesions was determined using flow cytometry. CD45+ cells were gated out of the life cell population (**A**). Subsequently, CD11b+ cells were gated out of CD45+ (**B**). Next, F4/80 expressing cells were selected from the CD11b+ population. Using Ly6C expression, subdivision between Ly6C+ monocytes (**C**) and Ly6C- macrophages (**D**) cells was made. (n=3 tumors/group) **E**. The extent of macrophage infiltration was scored based on F4/80 IHC. Score 1; ≤5% stroma positive, score 2; 5-25% stroma positive, score 3; 25-50% stroma positive, score 4; ≥50% stroma positive. (n= 11-12 tumors/group). *P≤0.05. Fibroblast-specific endoglin deletion increases neutrophil recruitment. **F**. Flow cytometry showed increased neutrophil infiltrate in ENGFibfl/fl lesions. Neutrophils were selected by gating for Ly6G in the CD45+/CD11b+ population. (n=3 tumors/group) **G**. IHC for Ly6G confirmed increased neutrophil influx in ENGFibfl/fl lesions. Graph represents 29/25 mice per group from two independent experiments, average number of Ly6G+ cells. *P≤0.05, **P≤0.01.

Neutrophils not responsible for the increased adenoma formation in the ENG$^{Fib-/-}$

Since the AOM DSS model is largely dependent on the presence of neutrophils and an increase in the number of neutrophils was observed in the adenomas, we investigated their role. First, we investigated the role of neutrophils during the formation of adenomas in the second DSS cycle, as indicated in [Fig. 5A]. Successful depletion of neutrophils was confirmed by flowcytometric analysis of the blood (Fig. 5B). The depletion was successful for three weeks and after that period, neutrophils started to re-appear, . At the end of the experiment mice were sacrificed, and intestinal adenomas were counted. As expected, a significant difference was seen between the ENG$^{Fib-/-}$ and the control samples. However, no differences were detected between the ENG$^{Fib-/-}$ and the ENG$^{Fib-/-}$ ly6G depleted group and the appropriate controls (Fig. 5C). Suggesting that neutrophils did not play a crucial role in the formation of the adenomas in this phase.

To investigate the possibility that neutrophils play a more crucial role in earlier stages of carcinogenesis in this model, they were depleted in the first DSS cycle in a follow-up experiment (Fig. 5D). Neutrophil depletion remained effective for 3 weeks (Fig. 5E). When mice were sacrificed after 13 weeks, a decrease in the number of adenomas within the ly6G depleted group [Fig. 5F]. However, once we calculated the relative number of adenomas, there was no difference between the controls and the ly6G depleted samples [Fig. 5G]. These data suggest that Ly6G+ neutrophils are important for the formation of adenomas in the AOM DSS model but do not seem to contribute to the increased lesion formation in the ENG$^{Fib-/-}$mice.

Loss of myeloid and Ly6C positive immune cells in blood and intestine from the ENG$^{Fib-/-}$ mice

Since ENG$^{Fib-/-}$ mice lost significantly more weight especially during the first DSS cycle we hypothesized that this could be due to increased inflammation, thereby enhancing lesion formation. To test this hypothesis, we performed a short-term DSS experiment to assess inflammation in the ENG$^{Fib-/-}$ *mice* versus control mice. During DSS treatment mice from the ENG$^{Fib-/-}$ lost significantly more weight than the controls (Fig. 6A). When the mice were sacrificed, a macroscopic disease score was performed [27, 28] to analyze the severity of the intestines' inflammation, stool, and general colonic conditions, which revealed a significant increase in the macroscopic disease score suggesting more tissue damage and inflammation (Fig. 6B). However, this was not reflected in the relative weight of the intestine which was significantly decreased in the ENG$^{Fib-/-}$ *mice* (Fig. 6C).

To further explore the composition of the intestinal immune infiltrate, colons were processed for flow cytometry. The number of CD45+ cells within the colon showed no differences, as shown in figure 6D, suggesting that the inflammation might not be different within the colon. However, the composition of the immune cells was changed. Interestingly, as observed in the blood, a decrease in CD11B+ myeloid cells

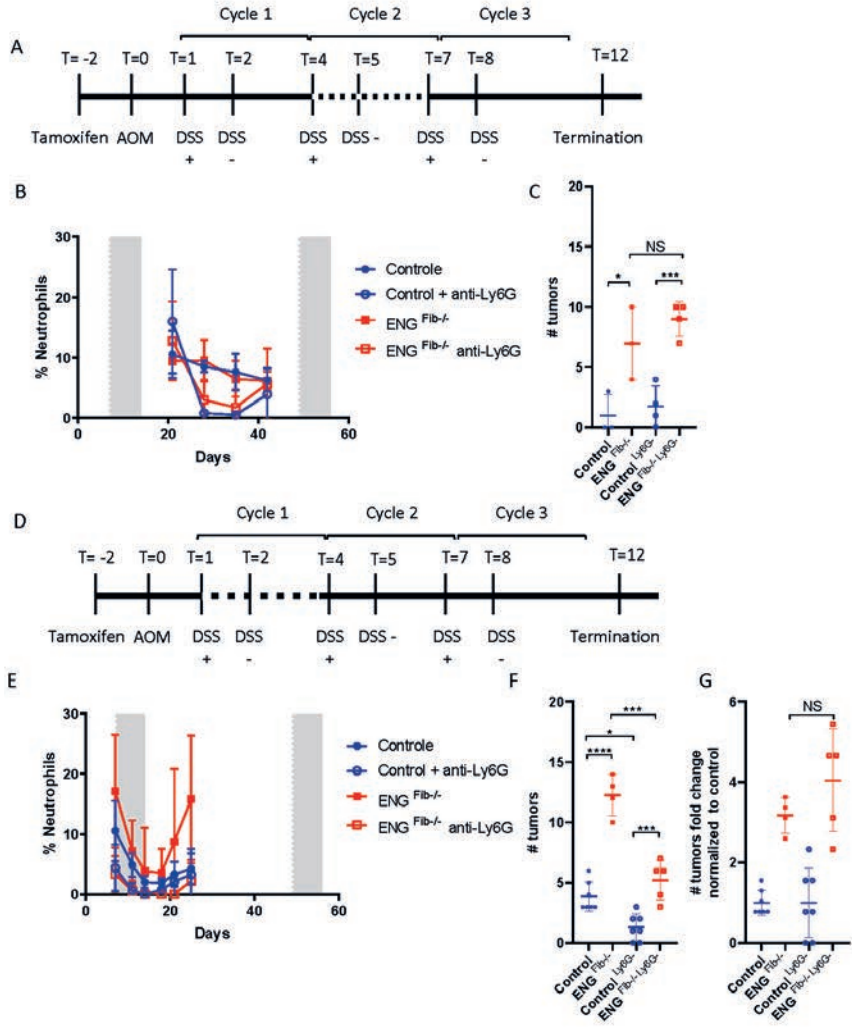

Figure 5. Neutrophils not responsible for the increased adenoma formation in the ENGFib-/-.
A experimental setup of the results presented in **B** and **C**. **B** decrease un the % of neutrophils up on depletion with a neutrophil depleting antibody. C Number of lesions which showed no difference between the neutrophil depleted group ENGFib-/- and ENGFib+/+. **D** experimental setup for the results presented in **E** and **F**. **E** decrease un the % of neutrophils up on depletion with a neutrophil depleting antibody. **F** number of lesions showing no difference between the neutrophil depleted groups ENGFib-/- and ENGFib+/+ data normalized to control (**G**).

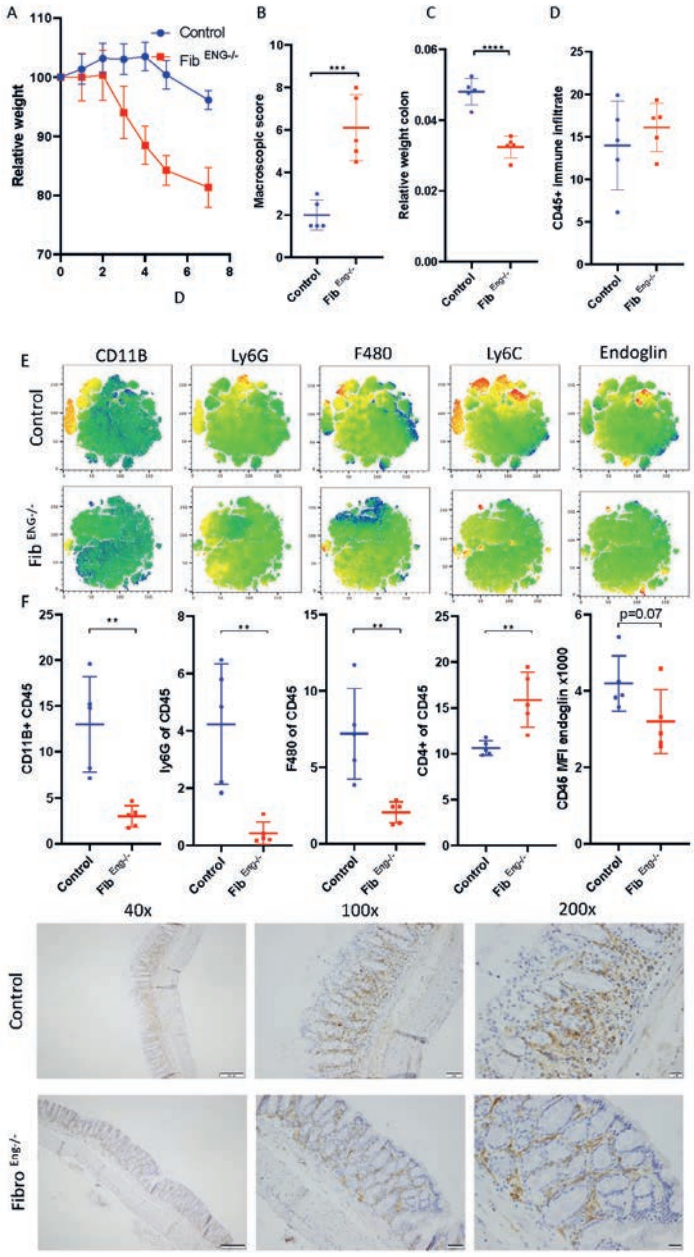

Figure 6. ***Loss of myeloid and Ly6C positive immune cells in blood and intestine from the ENGFib-/- mice.*** **A** short-term DSS experiment to assess inflammation in the ENGFib-/- *mice* versus control mice. ENGFib-/- lost significantly more weight than the controls (**A**). increase in the macroscopic disease score (**B**) no difference in relative weight of the intestine (**C**) the ENGFib-/- *mice*. (**D**) no difference in the total number of infiltrating immune cells (CD45+). Flow-cytometic analysis revealed a loss of myeloid and ly6C positive immune cells in the intestine from the ENGFib-/- mice presented as a TSME plot (RED high expression Bleu low expression) in **F** these results were plotted and showed decreased myeloid cells. Endoglin expression was assessed on CD45+ cells which showed to be decreased in the ENGFib-/- mice.

were observed in the intestines (Fig 6E and quantified in Fig. 6F). This is the same immune profile as seen in the blood of the ENG$^{Fib-/-}$ mice. Interestingly, a decrease endoglin (p=0.07) mean fluorescent intensity on CD45+ cells was observed in the ENG$^{Fib-/-}$ mice. This might indicate a decrease in endoglin expressing CD45+ cells or reduced recruitment of endoglin expressing CD45 cells.
Similar findings were observed in collagen1α2-CreERT2.ENG*fl/fl* mice (supplementary figure 2 and 3). These results might indicate hampered recruitment or proliferation/ differentiation of monocytes in both blood and intestine but this needs to be further evaluated.

DISCUSSION

This study shows that fibroblast-specific endoglin deletion increases tumorigenesis in a mouse model for colitis-associated CRC. Fibroblast specific endoglin deletion did not affect epithelial cell proliferation in adenomas but resulted in stromal expansion and increased influx of macrophages and neutrophils into the adenomas. However, this influx of neutrophils was not responsible for the increased tumorigenesis as shown by a ly6G depletion. Although increased numbers of myeloid cells were observed in late stages of intestinal lesion formation in the ENG$^{Fib-/-}$ mice, the opposite was observed in both the intestines as well as the blood in DSS induced colitis. Therefore, the underlining cause of the increased tumorigenesis is still unclear.
The immune system plays an important role during tumor development and progression. Neutrophils (ly6G positive) have been reported to be essential for the formation of tumors in the AOM DSS model [29, 30]. We show indeed that these cells play an important role in the AOM DSS tumor model. However, the increased tumorigenesis caused by the fibroblast specific endoglin deletion did not seem to be provoked by the influx of Ly6G positive cells, since depletion of the neutrophils did not abrogate the increased lesion formation.
Previously it was shown that adoptive transfer of ly6C high monocytes limits bacterial translocation and intestinal damage [31]. We found a decreased ly6C high profile during a short DSS experiment both in the blood of the ENG$^{Fib-/-}$ mice as in the intestines of the ENG$^{Fib-/-}$ mice, suggesting that loss of these cells increases intestinal adenoma formation by increased bacterial translocation, which might partially be reflected in the increased intestinal macroscopic score. Changes in immune composition were observed and the hampered intestinal ly6C high influx might be explained by altering the cytokine expression by the fibroblast specific endoglin knockout in the intestines or the bone marrow. However, this has not been examined and needs to be further explored.

During inflammation pro-inflammatory cytokines such as IFNγ, IL1β and TNFα activate progenitors in the bone marrow to differentiate into myeloid effector cells. These progenitor cells or hematopoietic stem/progenitor cells (HSPC) express both Col1a1 and Col1a2 [32], which might suggest that we have also performed an endoglin knockout on HSPCs. Interestingly, we found no evidence of endoglin knockout on myeloid cells since there was no difference in endoglin expression on CD45+ CD11B+ cells when compared to the controls. However, CD45+ Ly6C+ CD11B- cells have been reported to be monocyte-macrophage progenitors cells which showed a reduction (p=0.051) of endoglin in the blood of mice up on fibroblast (Col1a1 and Col1a2) specific endoglin deletion. Mice lacking endoglin in macrophages show an impaired phagocytic activity. The altered immune activity of endoglin deficient subsets might explain the higher rate of infectious disease seen in HHT1 patients [33] and partially the phenotypic differences in the $ENG^{Fib-/-}$ mice.

The interplay between stroma and epithelium is crucial during intestinal homeostasis. Imbalance in this paracrine interaction can lead to decreased epithelial apoptosis or an increase in stem-cell proliferation [34], both resulting in spontaneous polyp formation. Inactivation of the BMP pathway by knocking out the BMP receptor type II (BMPRII) in intestinal stromal cells [35], increased epithelial cell proliferation and resulted in local polyp formation in mouse intestine [35]. Similar effects were observed for the TGF-β receptor type II (TGF-βRII) deletion in fibroblasts [36]. Inactivation of BMPRII or TGF-βRII leads to tumor formation within seven weeks in the colorectum or stomach, respectively [35, 36]. Our study did not observe spontaneous tumor formation after endoglin deletion in fibroblasts during the 13-week experimental period. Our *in vitro* proliferation data supported this observation, where stimulation with conditioned medium from endoglin knock out MEFs did not affect proliferation in two mouse CRC cell lines. Although no spontaneous tumors developed, fibroblast-specific endoglin deletion enhanced chemically-induced adenoma formation. One of the differences between adenomas from the control and $ENG^{Fib-/-}$ group, was the increase of activated fibroblasts upon endoglin knock out. In CRC patients, the abundance of tumor stroma has been reported to be prognostic for both overall and metastasis-free survival [37]. This could imply that $ENG^{Fib-/-}$ adenomas would be more aggressive and could have a higher malignant potential. However, the severity of animal discomfort in our model does not allow for a prolonged experimental period, therefore tumor progression and metastatic spread could not be evaluated.

Our data regarding the SMA content of the adenomas are not in accordance with pharmacological and genetic data showing that treatment with the endoglin neutralizing antibody TRC105 reduced αSMA-positive tumor content in an *in vivo* breast cancer model [38]. Additionally, endoglin heterozygous mice displayed reduced αSMA-positive content in prostate cancer [39]. A major difference between

the studies mentioned earlier and our current research is that heterozygote endoglin deletion and TRC015 treatment affect all cells expressing endoglin, which might explain the differential effects observed compared to fibroblast-specific endoglin deletion.

In summary, these data show that fibroblast specific loss of endoglin increases intestinal adenoma formation in a model for colitis associated cancer. These lesions show increased stromal accumulation and altered immune cell infiltration, which might be involved in the increased carcinogenesis. Further studies should reveal the exact role of the altered immune cell infiltration.

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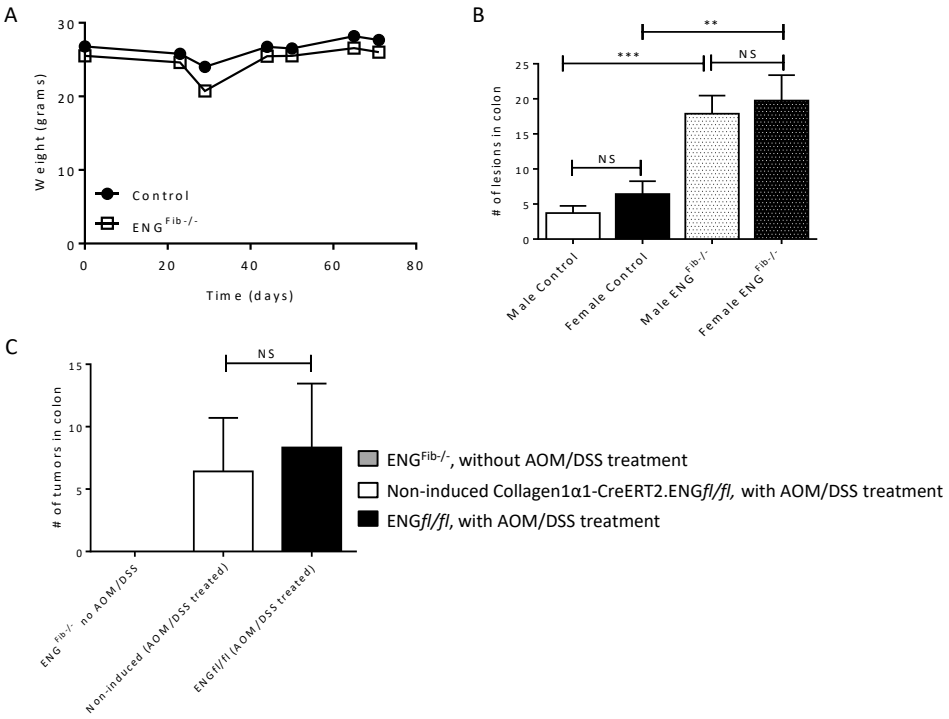
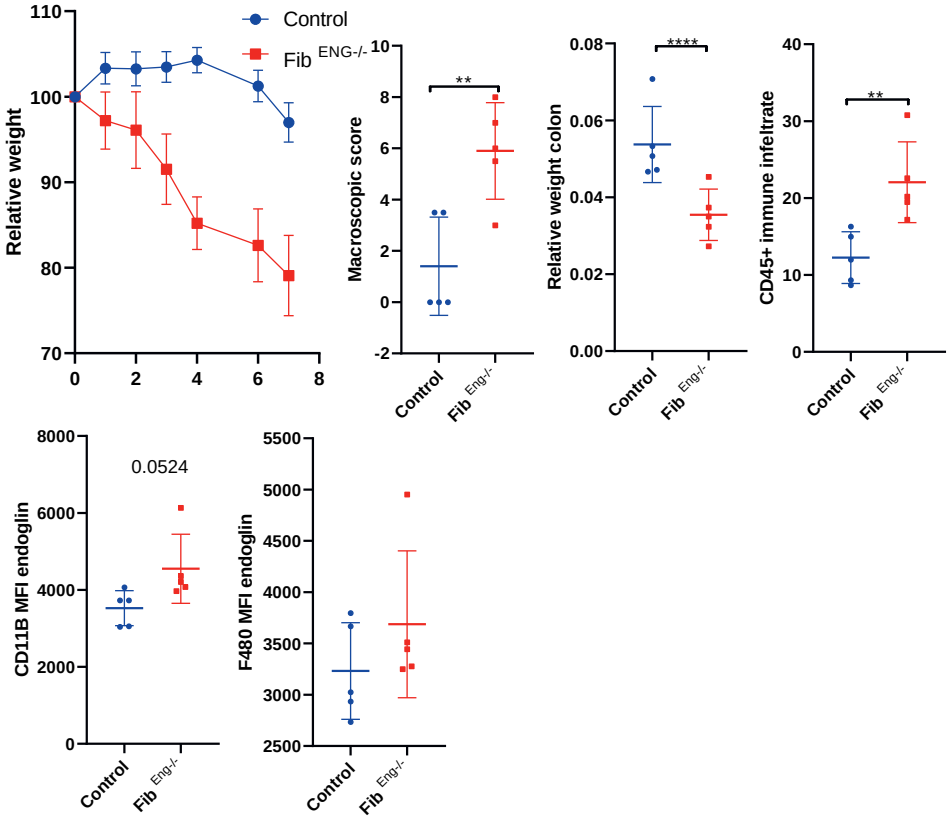
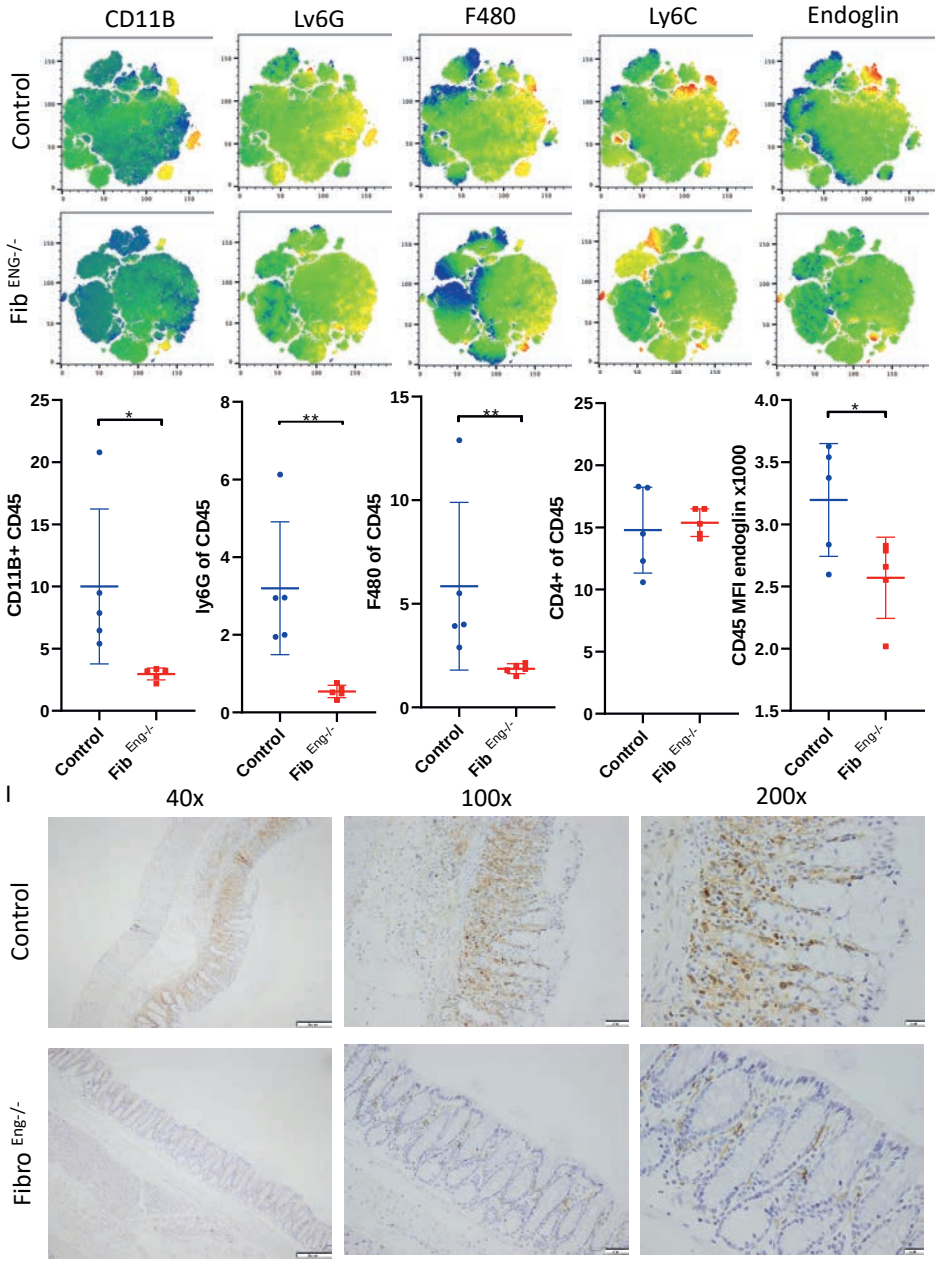


Supplementary figure 1 A. Mouse weights dropped during DSS supplementation, but recovered during the two weeks on normal drinking water. **B.** The number of lesions observed in the AOM/DSS model is independent of animal sex, in both the control and ENGFib-/- group. **C.** Fibroblast-specific endoglin deletion without AOM/DSS treatment did not induce neoplastic growth over the course of 13 weeks (n=8). ENGfl/fl mice, which received tamoxifen, showed similar number of lesions as non-induced Collagen1α1-CreERT2. ENGfl/fl mice after AOM/DSS (n=9).



Supplementary figure 2. collagen1α2-CreERT2.ENGfl/fl mice



Supplementary figure 3. collagen1α2-CreERT2.ENGfl/fl mice

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Targeting endoglin expressing cells in the tumor microenvironment does not inhibit tumor growth in a pancreatic cancer mouse model

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ABSTRACT

Background

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer and is known to have low immunogenicity and an immunosuppressive microenvironment. It is also characterized by high accumulation of dense stroma, composed of mostly cancer-associated fibroblasts (CAFs). Multiple subsets of CAFs are described, with one of them expressing the Transforming Growth Factor (TGF)-β co-receptor endoglin. In previous work, we and others, have shown that endoglin-expressing CAFs stimulate tumor progression and metastasis. Therefore, in this study, we set out to investigate the role of endoglin-expressing CAFs in pancreatic cancer progression.

Methods

First, we investigated the expression of endoglin on CAFs in both human tissues as well as a mouse model for PDAC. Since CAF-specific endoglin expression was high, we targeted endoglin by using the endoglin neutralizing antibody TRC105 in the murine KPC model for PDAC.

Results

Although some signs of immune activation were observed, TRC105 did not affect tumor growth. Since 90% of the CD8+ T-cells expressed the immune checkpoint PD-1, we investigated the combination with a PD1 checkpoint inhibitor, which did not enhance therapeutic responses. Finally, genetic deletion of endoglin from collagen1a1 expressing cells also did not affect the growth of the mouse KPC tumors.

Conclusions

Our results show that although endoglin is highly expressed on PDAC-CAFs and signaling is efficiently inhibited by TRC105, this does not result in decreased tumor growth in the KPC model for pancreatic cancer.

Keywords

1. endoglin 2. PDAC 3. cancer-associated fibroblasts 4. TRC105 5. stroma 6. KPC

BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal forms of cancer with a 5-year survival rate of only 7%. The most common mutations are activating mutations in KRAS (95%), loss of P53 (75%) and/or Smad4 (55%). This type of cancer is known to have low immunogenicity and to display an immunosuppressive microenvironment. This has resulted in the fact that immunotherapy through checkpoint inhibition has shown limited clinical success. The immune system has a crucial role in cancer progression and PDAC is capable of using various mechanisms for immune evasion, such as recruitment of regulatory immune cells, the secretion of immunosuppressive chemokines and different, as well as the expression of cell-surface proteins, such as programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte associated protein 4 (CTLA4) and colony-stimulating factor (CSF)-1.[1] Next to the immune component of the tumor microenvironment (TME), PDAC is typically characterized by high accumulation of non-tumor cells together called the tumor stroma, which has been correlated to the poor survival of PDAC patients,[2] but also of various other solid tumors like breast-,[3] and colorectal cancer.[4] Cancer-associated fibroblasts (CAFs) are the most abundant cell type in the tumor stroma and exhibit diverse functions, including extracellular matrix deposition and remodeling.[5] CAFs can influence tumor progression and metastasis, for example via interactions with cancer cells and infiltrating immune cells.[6] Therefore, CAFs have been proposed as a potential target for therapeutic interventions in PDAC.[7]

More recent work has, however, revealed that multiple subsets of CAFs exist in PDAC,[8, 9] which can stimulate or inhibit tumor progression. This was further emphasized by showing that the depletion of all α-SMA expressing cells in a murine model for PDAC can increase tumor aggressiveness.[10]

Our recent work in colorectal cancer revealed a subset of α-SMA positive CAFs that express the Transforming Growth Factor (TGF)-β co-receptor endoglin. The abundance of endoglin-expressing CAFs was related to invasive behavior and increased risk of metastasis in colorectal cancer and in a murine model for prostate cancer.[11]·[12] Endoglin is a transmembrane co-receptor for TGF-β ligands (mainly bone morphogenetic protein (BMP)-9) and originally described for its crucial role in angiogenesis. Later work revealed a significant role of endoglin beyond the endothelial cell.[13] TRC105 (Carotuximab, Tracon Pharmaceuticals, Inc) is a human endoglin neutralizing antibody, blocking endoglin-BMP9 interactions. We and others have shown in multiple pre-clinical models that TRC105 inhibits angiogenesis,[14, 15] tumor growth,[16] and metastasis,[11, 17] and induces antibody-dependent cellular cytotoxicity (ADCC) in mice.[18]

Based on our previous findings that TRC105, next to targeting endothelial cells, also targets endoglin-expressing CAFs and regulatory T-cells (Tregs), we explored if endoglin could serve as a potential target to improve PDAC outcomes.

In this study, we investigated the expression of endoglin on CAFs in samples from patients with PDAC. Next, we evaluated the therapeutic and immune-modulating effects of the endoglin neutralizing antibody TRC105 in the murine derived $Kras^{G12D/+}$ LSL-$Trp53^{R172H/+}$ Pdx-1-Cre (KPC) syngeneic transplantation model for pancreatic cancer.[19] Our results show that although endoglin is highly expressed on PDAC-CAFs and signaling is efficiently inhibited by TRC105, this does not result in decreased tumor growth in the KPC model.

METHODS

Cell Culture

The mouse PDAC cell line KPC-3 ($Kras^{G12D/+}$ LSL-$Trp53^{R172H/+}$ Pdx-1-Cre),[19] (kindly supplied by the department of Immunology, LUMC) with a targeted insertion of codon-optimized Luc-2 (pGL4.10 [luc2], (Promega Leiden, the Netherlands), mouse MC38 cells (kindly supplied by the department of Immunology, LUMC) and primary fibroblasts were all cultured in DMEM/F12 glutamax medium (Invitrogen, Landsmeer, the Netherlands), with 10% fetal bovine serum (FBS) (Gibco, Bleiswijk, the Netherlands), 0.01 M HEPES, 0.1 µg/ml Gentamycin, 40U/ml Penicillin and 40 µg/ml Streptomycin (all Invitrogen Landsmeer, the Netherlands) at 37°C and 5% CO2. Mouse endothelial 2H11 cells (kindly supplied by Dr. Sanchez-Duffhues, department of Cell and Chemical Biology) and human PDAC cell lines MIA PaCa-2 and PANC-1 (both cell lines obtained from ATCC, Manassas, VA, USA) were cultured in DMEM medium with 10% FBS, Penicillin and 40 µg/ml Streptomycin at 37°C and 5% CO2. The human PDAC cell line BxPC-3 (ATCC) in RPMI-1640 (Invitrogen, Landsmeer, the Netherlands), with 10% FBS, Penicillin and 40 µg/ml Streptomycin at 37°C and 5% CO2. Human endothelial (ECRF) cells (kindly supplied by Dr. Fontein, AMC Amsterdam) were cultured as described before.[20] Both primary human and mouse-derived fibroblasts were isolated by mechanically dissociating the tumor and culturing the tumor pieces using the culture medium described above. Fibroblasts grew from the tissue fragments and were used between passage 4 and 10. Fibroblasts were characterized by qPCR for expression of α-SMA and vimentin and the absence of CD31, CD45 and cytokeratin. Primary cells were characterized as indicated above. All cells were tested monthly and directly before *in vivo* use for mycoplasma contamination by PCR. The MC38 cell line was authenticated by STR profiling.

Western blot

Fibroblasts were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8.0), 2 mM EDTA, 1 mM NaVO4, 10 mM NaF and 1 mM sodium orthovandate (BDH Laboratory, Poole Dorset, UK)). Protein content was determined by DC protein assay according to the manufacturer's protocol (BioRad

Hercules, USA). Western blot analysis was performed as described before.[14] Membranes were incubated overnight with primary antibodies against endoglin (R&D systems, Abington, UK), phosphorylated Smad1/5/8 (both Cell Signaling Technologies, Leiden the Netherlands). Blots were stripped and reprobed with an antibody against actin (Millipore, Amsterdam, the Netherlands) antibody as a loading control.

Animal experiments

All performed animal procedures were approved by the Central Authority for Scientific Procedures on Animals (CCD). For all experiments, female C57/Bl6 jico mice (Jackson) were used, which were allowed to acclimatize for 7 days before the start of the experiment. Both genders were used. Thirty minutes before the surgery, the mice received a subcutaneous injection of 0.1 mg/kg buprenorphine (Indivior North Chesterfield, USA). Mice were sedated using isoflurane (Pharmachemie B.V. Haarlem, the Netherlands) and an incision of 1 cm was made in the skin and peritoneal wall separately, after which the pancreas was elevated. 10.000 KPC-3-Luc2 cells in 50 ul PBS were injected in the tail of the pancreas. Afterwards, the pancreas was carefully placed back, and the peritoneal wall and skin were closed separately. Mice were randomized to treatment groups and treatment was given intraperitoneally (i.p.) twice a week with either 15 mg/kg bodyweight TRC105 (TRACON Pharmaceuticals, San Diego, USA) or human IgG control (BioXcell, West Lebanon, USA). For the combination therapy mice were injected with anti-endoglin as described above and twice a week with either anti-PD-1 (clone J43, 10 mg/kg bodyweight, i.p. injection) or hamster IgG (both BioXcell, West Lebanon, USA). Mice were monitored twice a week using bioluminescent imaging. Mice were sacrificed 28 days after tumor cell transplantation. The tumor was taken out and measured using a caliper. Researchers were blinded to treatment groups when analyzing tumors. The tumor was divided for histology, flow cytometry analysis and snap-frozen for RNA and protein isolation.

Inducible fibroblast-specific endoglin knock-out

To obtain tamoxifen inducible, fibroblast-specific endoglin knock-out mice, mice, in which exon 5-6 of the endoglin gene are flanked by LoxP sides,[21] were crossbred with mice expressing cre-recombinase under control of the Collagen1a1 promotor (Tg(Col1a1-cre/ERT2)1Crm (Jackson Laboratory, Bar Harbor, USA) to obtain a Col1a1$^{eng-/-}$. Genotyping (Supplementary Figure 1) was performed for the presence of endoglin floxed sides as described,[21] and for CRE using the protocol 19078 (Jackson laboratories). To induce recombination, mice received 50 µl of a 100 mg/ml tamoxifen (Sigma-Aldrich, Zwijndrecht, the Netherlands) solution in sunflower oil through oral gavage on three sequential days. Control mice received sunflower oil. One day after the last Tamoxifen dose KPC tumor cells were injected orthotopically as described above.

Immunohistochemistry

Tissue was fixed, dehydrated, and processed to paraffin as described previously.[18] Five µm section were immunohistochemically stained using primary antibodies; goat anti-human endoglin (BAF 1097, R&D systems, Abington, UK) and goat anti-mouse endoglin (BAF 1320, R&D systems, Abington, UK), mouse anti-α-SMA (clone: 1A4/ ASM-1, Progen, Heidelberg, Germany) mouse anti-pan-cytokeratin (clone: PKC-26, Sigma-Aldrich, Zwijndrecht, the Netherlands) and rabbit anti-vimentin (clone: D21H3, Cell Signaling Technologies, Leiden, the Netherlands). In short, sections were deparaffined and endogenous peroxidase was blocked using 0.3% H_2O_2 in methanol, rehydrated followed by the antigen retrieval by boiling sections in 0.1M sodium citrate (pH 6.0) buffer. Next, the sections were washed (1%BSA/PBS) and stained with primary antibodies overnight. The next day the slides were washed and incubated with biotinylated secondary antibodies (DAKO, Carpinteria, USA), washed and incubated with vectastain complex (Vectorlabs, Peterborough, UK). The color was developed using 3,3'Diaminobenzidine (Dako, Carpinteria, USA). Nuclear staining was performed using Hematoxylin (EMD Millipore Corporation, Amsterdam, the Netherlands). Slides were dehydrated and mounted using entellan (Merck, Darmstadt, Germany). Representative pictures were taken with an Olympus BX51TF microscope (Olympus Life Science Solutions, Zoeterwoude, the Netherlands). Image quantification was performed using ImageJ software (NIH, Bethesda, MD, USA),[11] and researchers analyzing the tissues were blinded to treatment groups. To quantify the number of blood vessels 10x magnification of the endoglin staining was used and the number of vessels in 3-5 fields of view (FOV) were counted. For other stainings, the relative stained area was calculated in 3-5 fields per tumor. For human pancreatic tumors, CAF-specific endoglin expression was scored on a scale of 1 to 4 (1: 0-10%; 2: 10%–25%; 3: 25%–50%, and 4: >50% endoglin positive CAFs vs total amount of CAFs) in a blinded manner by two independent observers (LH and MP).

Immunofluorescent staining was performed as described before.[11] In short, sections were deparaffined and and antigen retrieval was performed as described above. Slides were incubated with the primary antibodies followed by incubation with goat anti mouse alexa-488 (Thermo Fisher Scientific, Landsmeer, the Netherlands) and streptavidin PE (BioLegend San Diego, USA) for α-SMA and endoglin stainings respectively for 30 minutes. Next slides were, washed and mounted with prolong gold (Thermo Fisher Scientific, Landsmeer, the Netherlands). Pictures were taken using a Confocal microscope, LICA SP8 Lightning was used and pictures were processed using LICA LAS-X software.

Flow cytometry

Tumors were disrupted with scissors and incubated in 375 µg/ml Liberase TL solution (Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in DMEM/F12 containing

10% FCS for 30 minutes at 37°C. To obtain single cells the suspension was filtered through Falcon™ Cell Strainers with 70 µm pore size (Thermo Fisher Scientific, Landsmeer, the Netherlands) and washed in FACS buffer (0.5% bovine serum albumin (BSA), 0.02% NaN_3 in PBS). Cells were stained with antibodies described in supplementary table 1 for 45 minutes at room temperature, washed 2x with FACS buffer, and measured on the BD LSRII (BD bioscience, Vianen, the Netherlands) as described before.[18] Flow cytometry data analysis was performed using Flowjo 10 software (BD bioscience, Vianen, the Netherlands).

RT-qPCR

Samples were homogenized using a TissueLyser according to manufacturers' protocol (Qiagen, Hilden, Germany). RNA was isolated from the tissue lysate using Nucleospin RNA kit (Bioké, Leiden, the Netherlands), according to manufacturers' instructions. RNA concentration was determined using the nanodrop 3300 (Thermo Scientific, Breda, the Netherlands). Next, complementary DNA synthesis was performed using 1 µg RNA using the RevertAid First Strand cDNA synthesis kit, according to manufacturers' instructions (Thermo Scientific Breda, the Netherlands). Quantitative PCR analyses were performed as described before,[18] using primers as described in supplementary table 2 (Invitrogen Landsmeer, the Netherlands). All values were normalized for GAPDH expression.

ELISA

Part of the tumor (10-20 mg) was lysed with RIPA buffer as described above, supplemented with a proteinase inhibitor cocktail (Roche, Indianapolis, USA). Subsequently, the tissues were disrupted and homogenized with the TissueLyser and centrifuged for 10 minutes at 11.000 rcf. Protein concentrations were determined with the DC Protein Assay. To investigate tissue TGF-β1 and TRC105 concentrations, enzyme-linked immunosorbent assays (ELISA) were performed. The TGF-β1 ELISA was performed as described before.[22] For the TRC105 ELISA Maxisorp flat bottom 96 well plates (NuncTM, Thermo Scientific, Breda, the Netherlands) were coated with 0.1ug/well recombinant human endoglin (R&D systems, Abington, UK) in 0.2M carbonate-bicarbonate pH 9.4 overnight at 4°C. Next, (and after each step) the plate was washed with PBS/0.05% Tween20 (PBST, Merck, Darmstadt, Germany). Subsequently, plates were blocked with assay diluent (1% BSA in PBST) for 1 hour and incubated with the samples for 2 hours and washed. Thereafter, 0.01667ug/ml goat anti-human IgG conjugated HRP antibody (Bethyl Laboratories Montgomery, USA) was added for 60 minutes. After washing, the plate was incubated with substrate TMB (Thermo Fisher Scientific, Landsmeer, the Netherlands) buffer for 12 minutes in dark. 2N H_2SO_4 was added to stop the reaction and the absorbance was read at 450 nm using the cytation-5 plate Reader (Biotek, Winooski, USA).

Statistical analysis

Data indicate mean ± standard deviation (SD). Differences between groups were calculated using Students' t-test, Mann-Whitney analysis, or ANOVA where appropriate using GraphPad Prism 8 software. P values ≤0.05 were considered statistically significant.

RESULTS

Endoglin is highly expressed on CAFs in human and mouse pancreatic tumors

To investigate endoglin expression on CAFs, 25, non-pretreated human pancreatic tumors and normal human pancreatic tissue were immunohistochemically stained for endoglin, α-SMA (a marker for activated fibroblasts), cytokeratin (a marker for epithelial cells) and vimentin (a stromal marker). High accumulation of α-SMA-expressing cells with an elongated phenotype was observed in pancreatic tumors (Figure 1A). Endoglin expression was seen on both α-SMA positive cells as vimentin positive cells and was absent in cytokeratin-expressing cells, suggesting a substantial endoglin positive subset of CAFs in pancreatic tumors (Figure 1A), next to the highly positive endothelial cells. In normal pancreatic tissue mostly endothelial endoglin expression with limited positive fibroblasts were observed (Figure 1A, lower panel). No difference in distribution of CAF-specific endoglin expression was observed when tumor borders were compared to tumor cores (Supplementary Figure 2A and 2B). Colocalisation of endoglin and α-SMA was confirmed by immunofluorescent double staining (Figure 1B). Next CAF-specific endoglin expression was scored on a scale of 1 to 4 in a cohort of PDAC patients. The majority of the tumors analyzed (20/25 tumors were evaluable) were scored 3 or 4 indicating high CAF-specific endoglin expression (Supplementary Figure 3).

Next, we investigated the presence of endoglin-expressing CAFs in a murine model for pancreatic cancer and in normal murine pancreas tissue. KPC-3 cells were injected orthotopically, once tumors were 5x5x5 mm mice were sacrificed and tumors were stained for cellular markers. KPC tumors were characterized by significant stromal accumulation (Figure 2A), as described before.[19] Next to expression of endoglin on endothelial cells, endoglin staining was present on elongated, spindle like cells colocalized with the α-SMA and vimentin staining. In normal murine pancreas tissue mostly endothelial endoglin expression was observed (Figure 2A, lower panel). Colocalisation of endoglin and α-SMA was confirmed by immunofluorescent double staining (Figure 2B). Isotype controls shows no aspecific staining of the antibodies on human and mouse tissues (Supplementary Figure 2C and 2D). These data indicate endoglin expression on CAFs in human and mouse PDAC tissues, rendering mouse models a suitable tool to study the role of endoglin-expressing CAFs in pancreatic cancer.

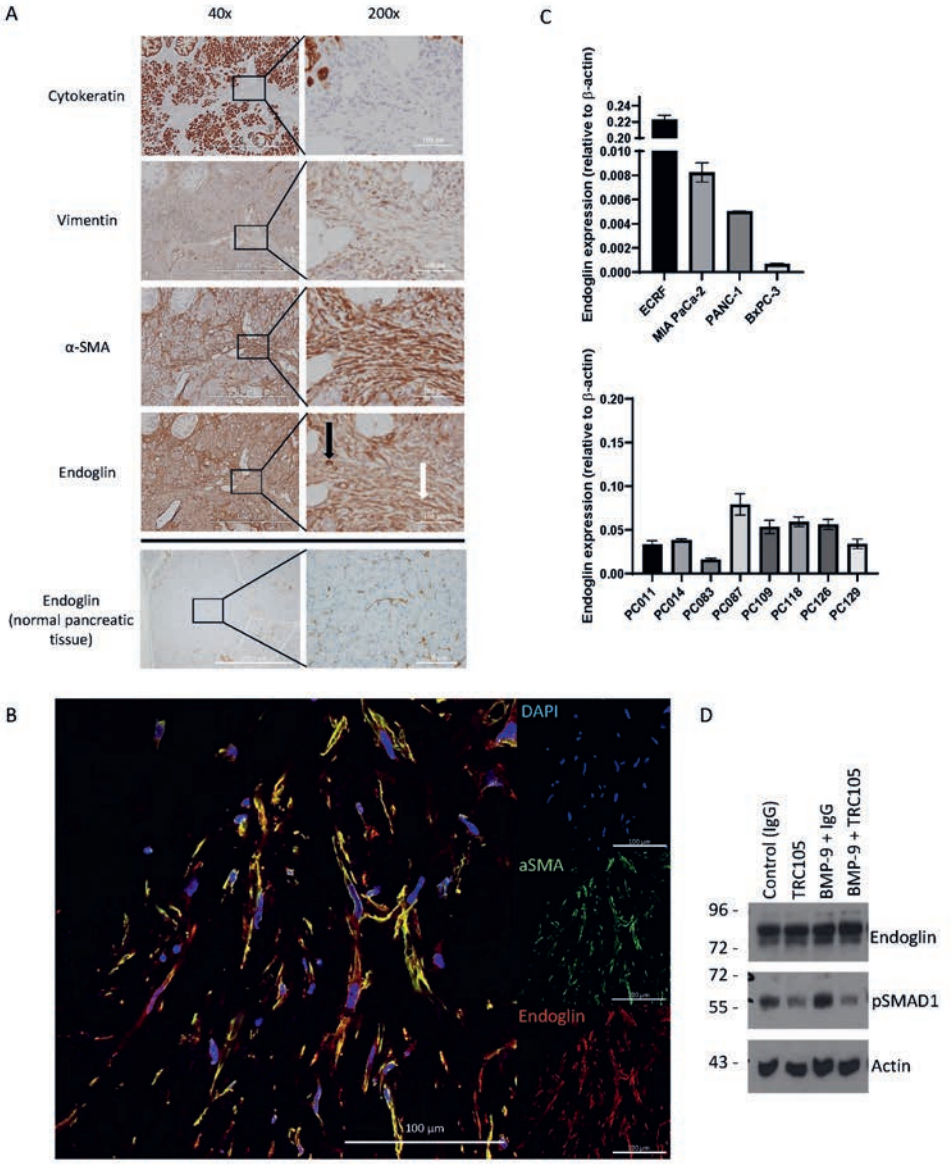

Figure 1. Endoglin is highly expressed on CAFs in human pancreatic tumors. (**A**) Representative images of human pancreatic cancer (representative from n = 25 PDAC patients) and normal pancreas stained for α-SMA, endoglin, cytokeratin, and vimentin. Endothelial cells (black arrow) and endoglin expressing CAFs (white arrow). (**B**) Immunofluorescent double staining for α-SMA and endoglin in human PDAC tumors. (**C**) Endoglin mRNA expression by human cells; ECRF endothelial cells, MIA PaCa-2, PANC-1 and BxPC-3 PDAC cells and 8 patient derived primary pancreatic CAFs. (**D**) Endoglin protein expression on human pancreatic fibroblasts. Basal and BMP9-induced downstream signaling (pSMAD1) was inhibited with TRC105 (full-length blot shown in Supplementary figure 4A–C).

To further investigate endoglin expression on CAFs, human and mouse primary pancreatic tumors were cultured to isolate CAFs. Fibroblasts were confirmed to be positive for vimentin and α-SMA and negative for CD31 (endothelial marker), CD45 (leukocyte marker) and EpCAM (epithelial marker) by qPCR. CAFs cultured from both mouse and human primary tumors showed high endoglin mRNA expression (Figure 1C and 2C).

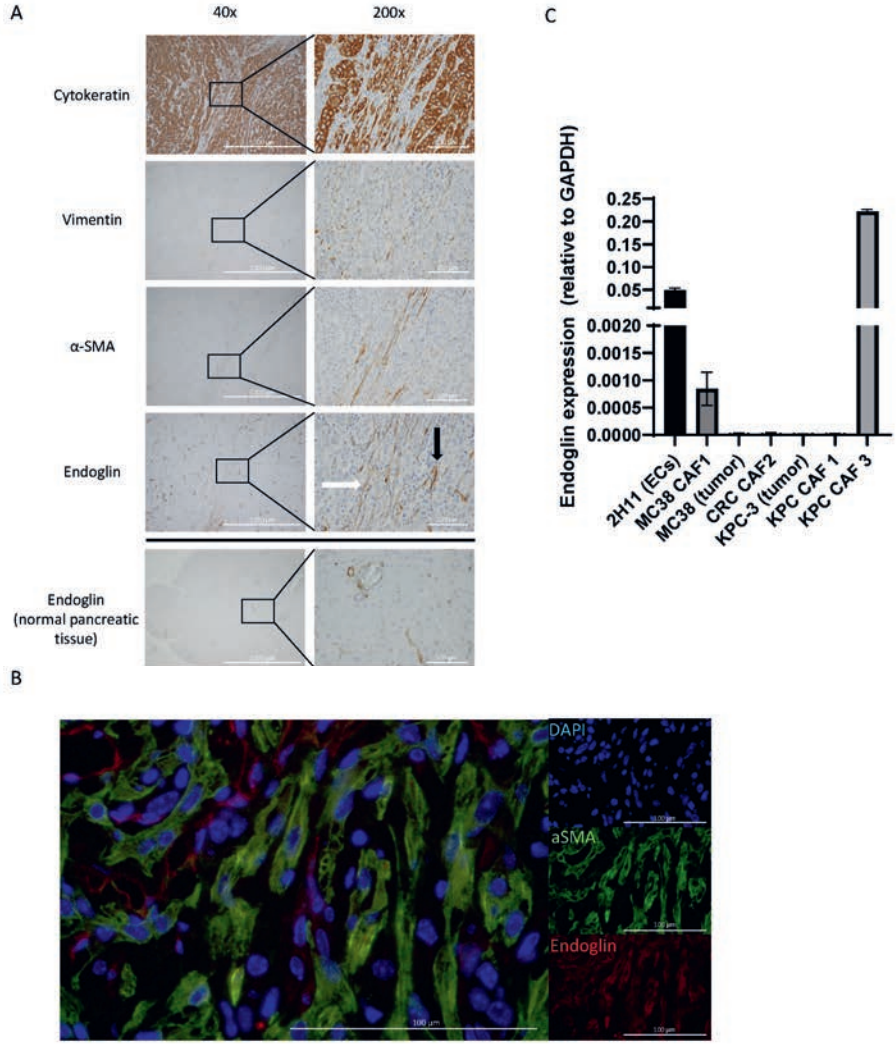

Figure 2. Endoglin is highly expressed on CAFs in mouse pancreatic tumors. (**A**) Representative images of mouse pancreatic tumors (KPC) (representative from n = 5) and normal pancreas stained for α-SMA, endoglin, cytokeratin, and vimentin. Endothelial cells (black arrow) and endoglin expressing CAFs (white arrow). (**B**) Immunofluorescent double staining for α-SMA and endoglin in mouse KPC tumors. (**C**) Endoglin mRNA expression by mouse cells; 2H11 endothelial cells, MC38 colorectal cancer, and KPC-3 pancreatic cancer cells, CAFs isolated from colorectal and pancreatic tumors.

Furthermore, endoglin expression was not detected on mouse KPC as well as MC38 colorectal tumor cells. Low but detectable endoglin expression was detected on MIA PaCa-2 and PANC-1 (both harboring a KRAS mutation) pancreatic cancer cells while low endoglin expression was detected on BxPC-3 cells (wildtype KRAS). Next to the epithelial tumor cells, analysis of eight patient derived CAFs showed detectable expression of endoglin *in vitro*. Absolute mRNA levels of endoglin varied among the isolated CAF subsets. These data show that endoglin is expressed on CAFs in human and mouse pancreatic tumors and *in vitro* on pancreatic cancer derived CAFs, while being absent on epithelial tumor cells.

TRC105 inhibits BMP-9 induced signaling in vitro

Since endoglin can bind BMP-9 and induce downstream signaling, we investigated if the endoglin neutralizing antibody TRC105 was able to inhibit endoglin signaling in pancreatic fibroblasts. High basal phosphorylation of SMAD1, a downstream target of endoglin signaling, was observed, which could be partially inhibited by TRC105 (Figure 1D, Supplementary Figure 4A-C). Stimulation with BMP9 strongly increased SMAD1 phosphorylation, which could be inhibited by TRC105, signifying that indeed TRC105 can bind endoglin on fibroblasts and inhibit BMP-9-induced endoglin-mediated downstream signaling *in vitro*.

TRC105 does not affect tumor growth in a murine model for pancreatic cancer

To investigate the therapeutic potential of anti-endoglin therapy in PDAC we injected murine KPC cells orthotopically in mice. After 14 days mice were treated with TRC105 as described and sacrificed 28 days post tumor implantation. The data revealed that there were no significant differences in either tumor volume (Figure 3A) or tumor weight (Figure 3B) upon TRC105 treatment. Since TRC105 acts, next to inhibiting ligand binding, via immune dependent mechanisms, we assessed immune cell infiltration by flow cytometric analysis on these tumors. Tumors were characterized by low CD45+ immune cell infiltration (4% of the live cell population in IgG control mice), which was hardly affected by TRC105 treatment (5%) (Figure 3C). Although the total percentage of CD45+ infiltrating immune cells did not change we observed a significant increase in the percentage CD8+ cytotoxic T-cells in tumors from TRC105 treated mice (Figure 3D), which, are instrumental in the therapeutic effects of TRC105.[16, 18]

Since endoglin is highly expressed on endothelial cells and previous research showed decreased blood vessel formation upon TRC105 treatment,[16, 17] we investigated the number of tumor blood vessels. No differences were observed in the number of endoglin-expressing blood vessels upon TRC105 therapy (Figure 3E and 3F). Next, the total stromal content of the tumors was analyzed using vimentin staining. These results revealed that total vimentin levels were slightly increased in the KPC tumors treated

with TRC105, although not significantly (p=0.087, Figure 3E and 3G). This could imply that fibroblast proliferation or immune infiltration is increased by TRC105. However, probably due to the high biological variation, this did not reach statistically significance. Finally, we determined the number of α-SMA expressing CAFs in the tumors, which did not differ between control and TRC105 treated mice (Figure 3E and 3H).

Pancreatic tumors are known for their high intratumoral pressure and low penetrance of therapeutic compounds.[23] To investigate therapeutic TRC105 levels are reached in the tumor, we determined intratumoral TRC105 concentrations by ELISA. High intratumoral accumulation of TRC105 (Figure 3I) was observed, indicating that therapeutic levels of TRC105 are present in the tumor. Taken together, these data show that TRC105 penetrates mouse PDAC tumors, increases the percentage of CD8+ cytotoxic T-cells, but does not inhibit tumor growth or affect CAF density.

Combining TRC105 with anti-PD1 does not increase therapeutic responses

Previously we have shown that the therapeutic effects of TRC105 are dependent on infiltrating immune cells.[18] Given the low percentage of CD45+ cells in the KPC tumors, this might hamper therapeutic responses. Therefore, we generated luciferase expressing KPC-3 tumor cells (KPC-luc2), enabling bioluminescent visualization and increasing immunogenicity of the tumor. In a pilot study, KPC-luc2 tumors showed reduced tumor growth, accompanied by an increased number of immune cells, of which a fraction expressed the T-cell activation markers LAG3, TIM3, and PD-1, and altered expression of cytokines compared to non-luciferase expressing KPC tumors (Supplementary Figure 5A-E).

Since 80-90% of the T-cells expressed PD-1 (Supplementary Figure 5D) and we have previously shown that combined TRC105/PD1 therapy shows increased therapeutic efficiency,[13] we investigated the combination in this model. KPC-luc2 cells were injected orthotopically and 14 days after tumor inoculation mice were randomized based on bioluminescent signal from the tumor cells (Supplementary Figure 5F), after which therapy was started. Tumor growth was followed by bioluminescent imaging. This was shown not to be representative due to de novo pigment formation on the shaved mouse skin, blocking bioluminescent signal (Supplementary Figure 5G). Therefore, the bioluminescent signal did not correlate to the tumor volume in this experiment (Supplementary Figure 5H). At the end of the experiment, mice were sacrificed and tumor volume was determined by caliper. No significant differences were detected between the treatment groups (Figure 4A). Although no differences were detected in tumor volume, there was a clear accumulation of TRC105 in the tumor as measured by ELISA (Figure 4B), while no correlation was observed between the TRC105 levels and the tumor volume (Supplementary Figure 5I).

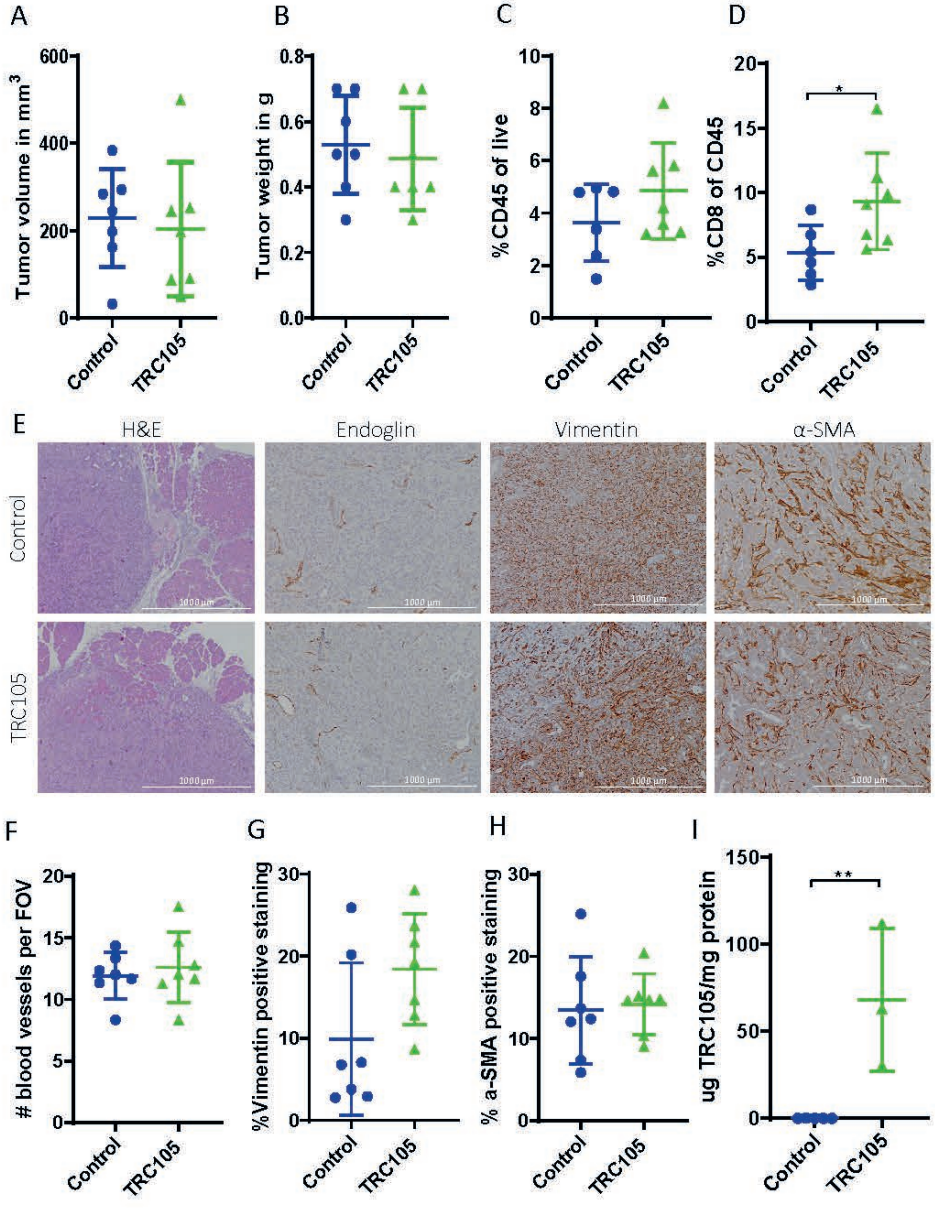

Figure 3. TRC105 does not affect tumor growth in a murine KPC-3 model for pancreatic cancer. (**A**) Tumor volume in mm_3 and (**B**) tumor weight upon 13 days of therapy (28 days after tumor inoculation, n = 7 animals per group). (**C**) Percentage of intratumoral CD45+ cells (gated from live gate) by using flow cytometry. (**D**) Percentage of CD8+ T-cells (from CD45 gate, n = 6-7 mice per group. (**E**) Representative histological images and quantifications of endoglin (**F**), vimentin (**G**) and α-SMA (**H**) (n = 7 animals per group). (**I**) Intratumoral TRC105 levels in tumor lysates determined by ELISA (n = 5 control, n = 3 TRC105). All graphs represent mean ± SD. Student's *T*-test was performed to calculate differences indicated in the graphs *p = <0.05 **p = <0.01.

Next, the tumor immune infiltrate was examined by flow cytometry. Although most cell populations did not differ between groups (Figure 4D-I), a slightly decreased percentage of CD4+ CD25+ cells (Treg-like cells) was detected in TRC105 treated groups (Figure 4F), albeit not statistically significant. Since Tregs are one of the major producers of TGF-β in pancreatic tumors,[24] we investigated tumor TGF-β1 levels by ELISA. Surprisingly, increased tumor TGF-β1 levels were detected in the TRC105 and TRC105/PD1 treated mice (Figure 4C).

Early treatment with TRC105 after tumor inoculation changes the tumor microenvironment without affecting tumor growth

Since the growth speed of KPC tumors is very high, this might limit the opportunities for therapeutic interventions. To investigate if an earlier start of treatment could enhance therapeutic benefits, TRC105, PD1, or combination therapy was initiated 1 day after tumor transplantation and continued twice per week for 28 days. At the end of the experiment, no significant differences in tumor volume were observed between all groups (Figure 5A). Although tumor volumes did not differ, some changes were observed in the immune composition of the tumors. No differences were detected in the total immune infiltrate (Figure 5B), but a non-significantly increased (p=0.08) percentage of CD3+ T-cells was observed in the TRC105 group (Figure 5C), composed of both the CD4+ and CD8+ population (p=0.19 and p=0.06 respectively, Figure 5D and 5E). A trend towards similar changes was also visible in the TRC105/PD1 combination therapy group. Although the number of total CD4+ T-cells was increased, the percentage of CD25+ CD4+ Tregs cells was slightly decreased upon TRC105 monotherapy (p=0.087, Figure 5F). Due to the altered presence of immune cells, we analyzed cytokine levels by qPCR. mRNA expression analysis revealed altered cytokine expression (IL-1β, IL-2, IL-6, IL-10, TNFα, INFγ, Granzyme B) in tumor homogenates (Figure 5G) upon combination therapy. Interestingly, especially increased granzyme B mRNA expression was observed upon TRC105/PD1 therapy, as we previously have seen in colorectal cancer models.[18] Finally, we investigated the stroma composition by immunohistochemistry. No differences were observed in the abundance of α-SMA, endoglin, vimentin and cytokeratin expressing cells (Figure 5H). These data indicate that treatment with TRC105 or a combination with PD-1 elicits increased cytokine expression and immune cell infiltration, but this is not sufficient to induce therapeutic responses.

Col1a1-specific endoglin deletion alters immune cell composition without affecting tumor growth

To further investigate the effects of endoglin expression on fibroblasts in pancreatic tumors we generated an inducible, Collagen1a1 driven endoglin knock-out mouse ($Col1a1^{Eng-/-}$). CRE-mediated recombination was induced by three consecutive days

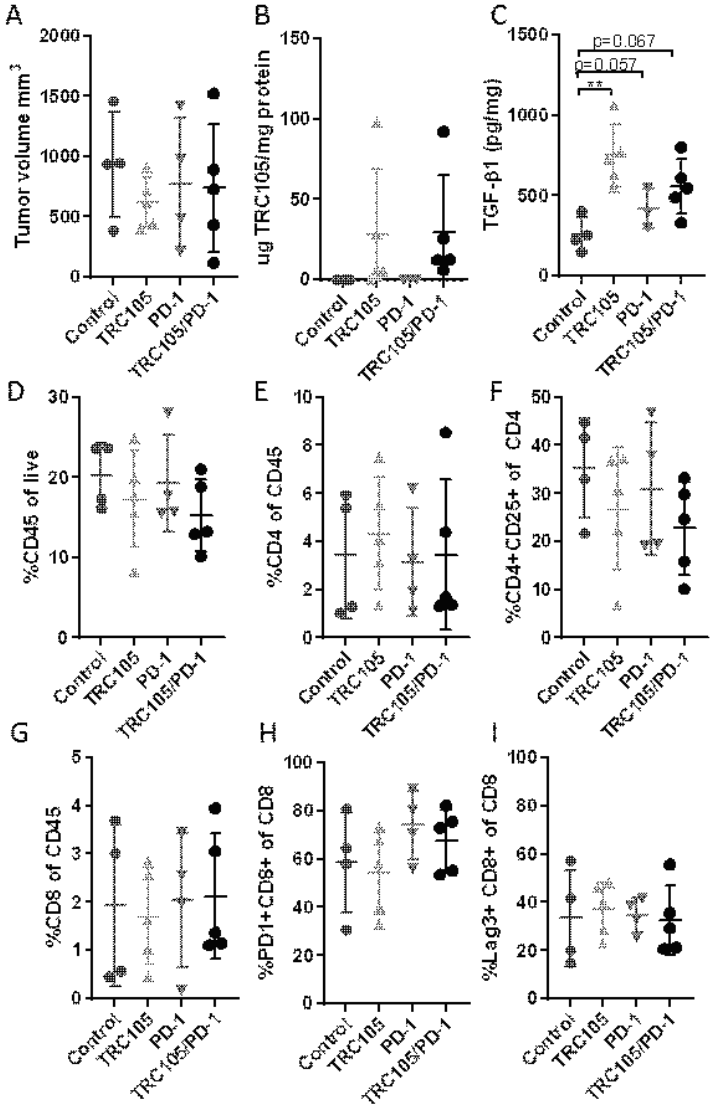

Figure 4. Combining TRC105 with anti-PD1 does not increase therapeutic responses in KPC-3luc2 tumors. (**A**) Tumor volume in mm_3 upon 13 days of therapy and 28 days after tumor inoculation (n = 4-5 mice per group). (**B**) Intratumoral levels of TRC105 and (**C**) TGF-β1 determined by ELISA (n = 3-5 per group due to limited amount of sample). (**D**) Total percentage of infiltrating CD45+ immune cells, (**E**) percentage of CD4+ T-cells out of CD45+ gate and (**F**) CD4+ CD25+ Treg-like cells out of the CD4+ gate. (**G**) Percentage of CD8+ T-cells and expression of PD-1 (**H**) and LAG3 (**I**) on CD8+ cells (n = 4-5 mice per group). All graphs represent mean ± SD. One-way ANOVA was used to calculate differences.

of tamoxifen administration, after which KPC-luc2 tumor cells were injected orthotopically. The Col1a1 driven endoglin deletion did not affect endothelial endoglin expression (Supplementary Figure 6). After 28 days, mice were sacrificed

and tumor volumes were measured. No differences in tumor volume were observed between wildtype and Col1a1$^{Eng-/-}$ mice (Figure 6A). Additionally, the abundance of α-SMA and endoglin expressing cells did not differ between the groups (Figure 6B). Next, we analyzed the effects of Col1a1-specific endoglin deletion on recruitment of tumor infiltrating immune cells by flow cytometry. The presence of CD45+ immune cells was similar between the controls and the Col1a1$^{eng-/-}$ mice (Figure 6C). Although no significant differences were found in the percentage of the total number of CD3+ T-cells (Figure 6D), the percentage of CD8+ T-cells decreased, with a concomitant increase in the percentage of CD4+ T-cells (Figure 6E and 6F). The activation markers LAG-3 and PD-1 on the CD8+ T-cells did not differ between the two groups (Figure 6G and 6H). Finally, mRNA expression analysis for a range of cytokines revealed no differences between Col1a1-specific endoglin knock-out mice and controls (Figure 6I). These data show that Col1a1 driven deletion of endoglin increases the percentage of CD4+ T-cells, without affecting KPC-luc2 tumor growth *in vivo*.

DISCUSSION

In this study, we evaluated endoglin as a potential target for the treatment of pancreatic cancer. Although endoglin was highly expressed on both human- and mouse CAFs in pancreatic tumors, no changes in tumor volume were observed when targeting endoglin by TRC105 or genetically deleting endoglin from Col1a1 expressing cells, although some changes in the immune infiltrate were observed.

Although previous data in colorectal cancer mouse models showed that endoglin in combination with PD-1 was very effective in reducing tumor growth,[18] this could not be achieved in the KPC model for pancreatic cancer. Pancreatic cancer is characterized by high stromal accumulation, which is thought to limit the success rates of many current treatment options which are effective in other solid cancers. This might be due to forming a physical stromal barrier limiting the number of immune cells or drugs that can enter the tumor.[25] Therefore, targeting CAFs might result in a degradation of this physical barrier, thereby increasing therapeutic efficacy. In this study, we showed that although a dense stroma was present, TRC105 accumulated within the tumor and induced changes in immune cell composition- and activation up on TRC105 and combination therapies. Surprisingly, this did not lead to therapeutic effects. An explanation might lie in the presence of different CAF subsets.

It has become clear that CAFs are a very diverse population of cells with multiple CAF subtypes and functions.[26]·[27] Attempts to classify CAFs have led to proposed subsets of inflammatory CAFs (iCAFs) and myofibroblastic CAFs (myCAFs) in PDAC.[28] iCAFs are characterized by low expression of α-SMA and high expression of IL-6, whereas myCAFs are characterized by high expression of α-SMA and low

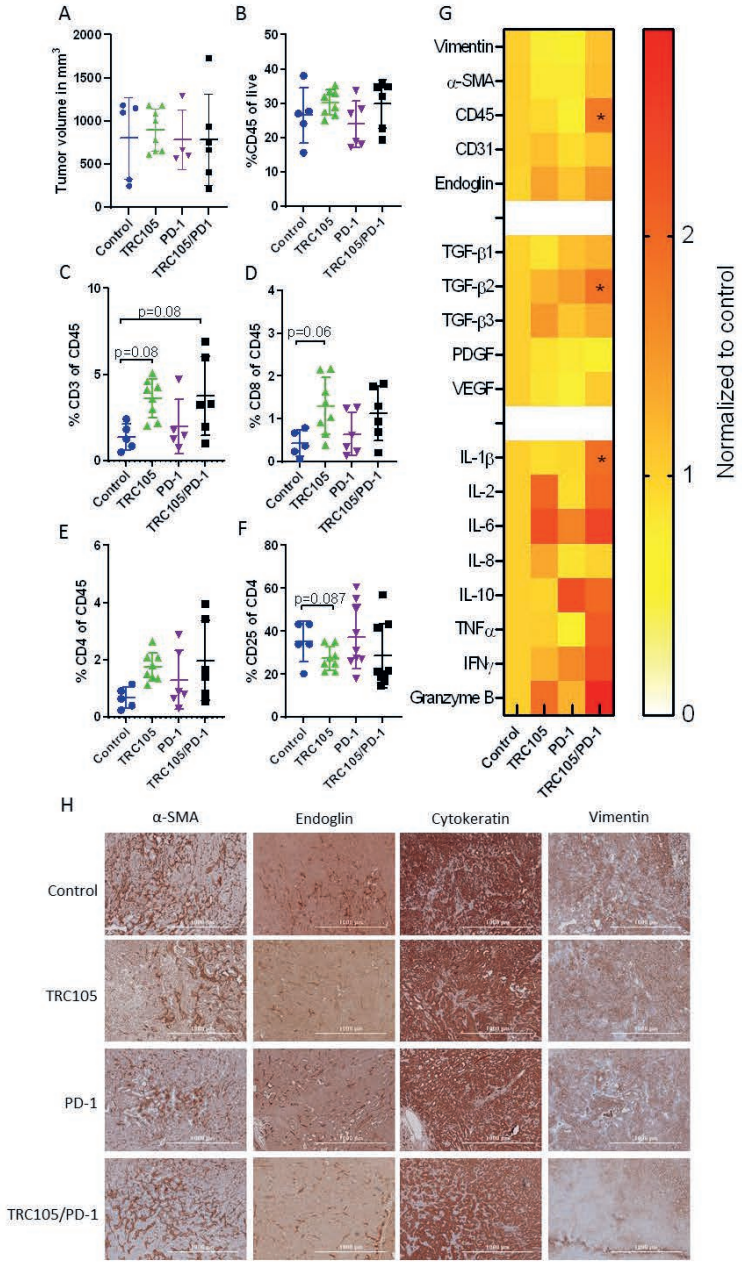

Figure 5. Early treatment with TRC105 does not affect tumor growth but changes the tumor microenvironment. (**A**) Tumor volume in mm_3 upon 27 days of therapy and 28 days after tumor inoculation (n = 5-8 mice per group). (**B**) Percentage of infiltrating immune cells (CD45+). (**C**) CD3+, (**D**) CD8+ and (**E**) CD4+ cells out of CD45 gate. (**F**) Intratumoral CD4+ CD25+ Treg-like cells out of CD4 gate (n = 5-8 mice per group). (**G**) Heatmap summarizing qPCR data normalized to the control group of different cytokines, growth factors and stromal markers (n = 5-8 mice per group). (**H**) Representative histological pictures of α-SMA, endoglin, cytokeratin and vimentin staining (n = 5-8 mice per group). All graphs represent mean ± SD. One-way ANOVA was used to calculate statistical differences. *p = <0.05.

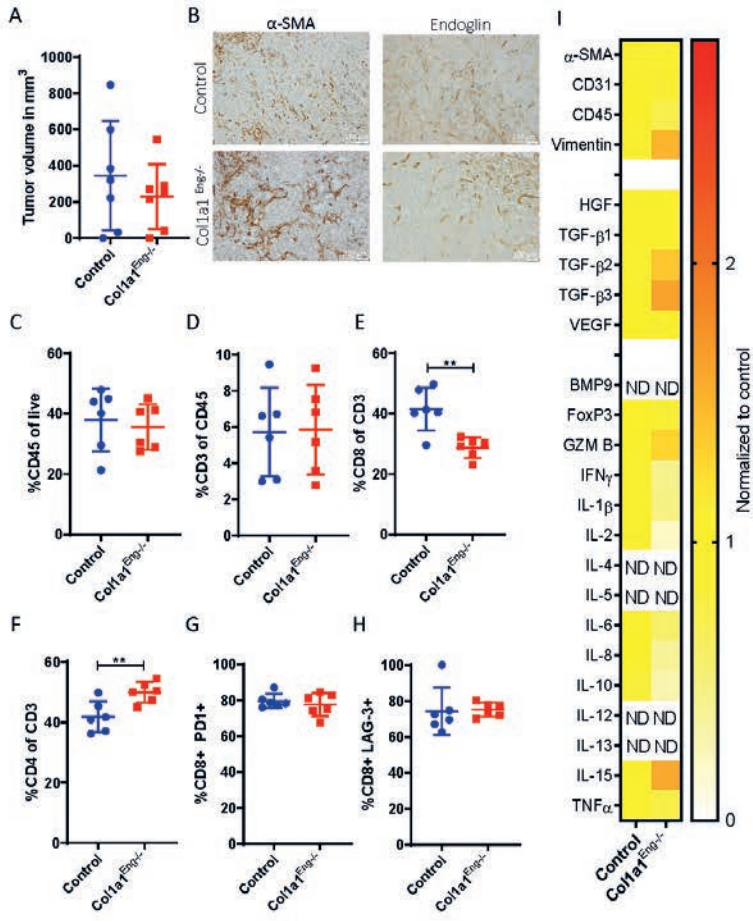

Figure 6. Col1a1 specific endoglin knock-out does not affect tumor growth but alters immune cell composition. (**A**) Tumor volume in mm_3 after 28 days of tumor inoculation (n = 7 mice per group). (**B**) Representative pictures of histological samples stained with α-SMA and endoglin (n = 6 mice per group). (**C**) CD45+ immune infiltrate and (**D**) CD3 + T-cells (from CD45+ gate). (**E**) CD8+ and (**F**) CD4+ cells from (from CD3+ gate). (**G**) Percentage CD8+ PD1+ cells (from CD8+ gate). (**H**) Percentage of CD8+ LAG-3+ cells (from CD8+ gate) (n = 6 mice per group). (**I**) Heatmap summarizing qPCR data normalized to the control group of different cytokines growth factors and stromal markers (n = 6 mice per group) ND in the graph indicates not-detectable. All graphs represent mean ± SD. Student's *T*-test was performed to calculate significances indicated in the graphs **p = <0.01.

expression of IL-6. ICAFs have been shown to promote PDAC progression, whereas myCAFs restrict tumor progression.[29] In our experiments, decreased α-SMA and increased IL-6 mRNA levels in the mice treated with TRC105 were detected (Figure 5G and Supplementary Figure 7). Interestingly, increased IL-6 protein levels were also seen in the serum of patients treated with TRC105,[30] which might suggest an increase of iCAFs upon TRC105 therapy. Although IL-6 is not only produced by CAFs, IL-6 can promote tumor growth, angiogenesis,[31] and invasion.[32-34] Interestingly,

PD-L1 blocking in combination with anti-IL-6 therapy reduced tumor progression in murine pancreatic cancer.[35] Since increased IL-6 has been observed in both mice and humans treated with TRC105, a combination of TRC105 with anti-IL-6 might thus be relevant to study.

Next to the suggested myCAFs and iCAF subsets, multiple other subsets of CAFs were described, including the endoglin-expressing CAF subset in prostate- and colorectal cancer.[11, 12] In breast cancer, we could show decreased α-SMA-expressing CAFs upon endoglin targeting with TRC105 *in vivo*.[14] Endoglin has also been described to play a role in in liver fibrosis and cardiac fibrosis.[36, 37] Surprisingly, targeting endoglin with TRC105 or by means of Col1a1 driven deletion of endoglin did not affect the number α-SMA expressing cells, indicating alternative mechanisms in PDAC.

CAFs in PDAC and KPC tumors have been reported to reduce the migration of cytotoxic (CD8+) T-cells.[38] Interestingly Col1a1 driven endoglin deletion promoted the infiltration of CD4+ T-cells in the tumor and decreased CD8+ T-cells. In contrast, increased CD8+ T-cells were found upon TRC105 therapy, suggesting that TRC105 enables the migration and activation of CD8+ T-cells. This might be due to altering the tumor microenvironment by the targeting of endoglin positive blood or lymphatic vessels or endoglin expressing Tregs.[18, 39] This was not observed in the Col1a1 driven endoglin knockout mice, confirming that TRC105 was instrumental in increasing the intratumoral CD8+ T-cell count. Although TRC105 was not able to reduce the α-SMA expressing cells in the KPC tumors, other therapies like Focal Adhesion Kinase (FAK) targeting reduced fibrosis in KPC tumors making them susceptible to anti-PD-1 therapy,[40] stressing the importance of combining therapies targeting multiple components of the tumor microenvironment.

Next to targeting CAFs, our recent work showed specific targeting of endoglin expressing Tregs by TRC105,[18] a phenomenon which was also observed in patients treated with TRC105.[41] Interestingly, in this study, we also observed a trend towards a decreased percentage of CD4+CD25+ Tregs upon TRC105 treatment ($p=0.087$), which, however, did not affect tumor growth (Figure 5A). In pancreatic cancer, it was recently described that a complete depletion of FoxP3-expressing Tregs increases carcinogenesis by reducing intratumoral TGF-β1 levels.[24] However, our data show an increase in intratumoral TGF-β1 levels, which might be due to many cells in the tumor microenvironment producing TGF-β1. Although the role of Tregs in PDAC is yet unclear, recent work showed that disrupting the homing of Tregs via CCR5 or targeting Tregs using anti-OX40 resulted in sustained anti-tumor responses in PDAC.[42, 43] Interestingly, both CCR5 and OX40 are described to promote fibrosis.[44, 45] Moreover, targeting OX40 blocks tissue fibrosis, which is induced by activated fibroblasts.[45, 46] These findings highlight the importance and successes of targeting CAF subsets within the pancreatic tumors, opening new opportunities for PDAC.

CONCLUSIONS

In conclusion, while high endoglin expression was observed on CAFs in pancreatic cancer, targeting endoglin by TRC105 as monotherapy, in combination with PD1 checkpoint inhibitors, or by genetic deletion of endoglin from Col1a1 expressing cells did not inhibit tumor growth in the KPC model for pancreatic cancer. Interesting changes in immune cell infiltration might open up opportunities to explore the role of endoglin further. Additional studies will be required to investigate the delicate balances and effects of changes in the tumor microenvironment driving pancreatic tumor progression.

List of abbreviations

Pancreatic ductal adenocarcinoma (PDAC); Cancer-associated fibroblasts (CAFs); Transforming growth factor (TGF); Bone morphogenetic protein (BMP); Antibody-dependent cellular cytotoxicity (ADCC); Regulatory T-cells (Tregs); $Kras^{G12D/+}$ LSL-$Trp53^{R172H/+}$ Pdx-1-Cre (KPC); Fetal bovine serum (FBS); Fields of view (FOV); Bovine serum albumin (BSA); Enzyme-linked immunosorbent assays (ELISA); Standard deviation (SD); Luciferase expressing KPC-3 tumor cells (KPC-luc2 tumor cells); Collagen1a1 driven endoglin knock-out mouse ($Col1a1^{Eng-/-}$ mouse); Inflammatory CAFs (iCAFs); Myofibroblastic CAFs (myCAFs); Focal Adhesion Kinase (FAK)

Declarations

Ethics approval and consent to participate

All experiments using human material were performed according to the guidelines of the Medical Ethical Committee of the LUMC, and conducted in accordance to the Declaration of Helsinki and the Code of Conduct for responsible use of Human Tissue and Medical Research, as drawn up by the Federation of Dutch Medical Societies in 2011. This code permits the further use of coded residual (historical) tissue and data from the diagnostic process for scientific purposes. Permission is granted by implementing an opt-out procedure for the patients, written informed consent is in that case not needed. All experimental protocols were approved by the Medical Ethical Committee of Leiden-Den Haag-Delft (METC-LDD). The use of the aforementioned primary cells was approved by the Medical Ethical Committee of Leiden-Den Haag-Delft (METC-LDD). Human cadaveric donor pancreata were procured through a multiorgan donor program. Pancreatic tissue was used in our study if the pancreas

could not be used for clinical pancreas or islet transplantation, according to national laws, and if research consent was present. All animal experiments were executed in accordance with responsible science with animals (2021) and reviewed by the animal welfare body Leiden. The license was issued by the Central Authority for Scientific Procedures on Animals (CCD) based on a positive advice by the Animal Ethics Committee Leiden (AVD116002017858 and AVD11600201571). All animals were housed and cared for in accordance with the Experiments on Animals Act (Wod, 2014). All experiments were performed and are reported according to the ARRIVE guidelines and the code of practice cancer research.

Consent for publication

Not applicable

Availability of data and materials

All data and used materials are available upon request from the corresponding author.

Competing interests

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Authors' contributions

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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Targeting endoglin expressing regulatory T cells in the tumor microenvironment enhances the effect of PD1 checkpoint inhibitor immunotherapy

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ABSTRACT

Endoglin is a coreceptor for Transforming Growth factor (TGF)-β ligands that is highly expressed on proliferating endothelial cells and other cells in the tumor microenvironment (TME). Clinical studies have noted increased programmed cell death (PD)1 expression on cytotoxic T-cells in the peripheral blood of cancer patients treated with TRC105, an endoglin targeting antibody. In the current study we investigated the combination of endoglin antibodies (TRC105 and M1043) with an anti-PD1 antibody in four preclinical mouse models representing different stages of cancer development. In all models, the combination of endoglin antibody and PD1 inhibition produced durable tumor responses, leading to complete regressions in 30-40% of the mice. These effects were dependent on the presence of Fc-y receptors, indicating the involvement of antibody-dependent cytotoxic responses and the presence of CD8+ cytotoxic T cells. Interestingly, treatment with the endoglin antibody TRC105 significantly decreased the number of intratumoral regulatory T cells (Tregs). Endoglin expressing Tregs were also detected in human colorectal cancer specimens. Taken together these data provide a rationale for combining TRC105 and anti-PD1 therapy and provide additional evidence of endoglin's immunomodulatory role.

Keywords
Endoglin, TRC105, TGFbeta, tumor microenvironment, PD1, Tregs

INTRODUCTION

The tumor microenvironment (TME) is composed mainly of cancer-associated fibroblasts (CAFs), infiltrating immune cells and blood vessels, which are partly enveloped by pericytes. Endothelial cells in the newly formed blood vessels in the TME highly express the transforming growth factor (TGF)-β co-receptor endoglin, which has been correlated with poor prognosis and metastatic disease in many solid tumors [1, 2]. Upon stimulation with ligands, including bone morphogenetic protein (BMP) and TGF-β, endoglin promotes endothelial cell proliferation and migration, via signaling through phosphorylation of the SMAD-1 signaling molecule. In previous work, we and others demonstrated that endoglin is also expressed on CAFs, in particular at the invasive margin of colorectal tumors [3] and on mononuclear cells including lymphocytes and activated macrophages [4, 5].

TRC105 (Carotuximab, Tracon Pharmaceuticals, Inc.) is a humanized IgG1 endoglin neutralizing antibody, which has been studied in multiple clinical trials in oncology and age-related macular degeneration (AMD). In pre-clinical cancer models, we and others have shown that treatment with TRC105 inhibits angiogenesis, tumor growth and metastases [6-10]. Notably, TRC105 treatment might engage antibody dependent cellular cytotoxicity (ADCC) [11] and is more potent in immunocompetent mice compared to immunodeficient mice, indicating the active involvement of the immune system [7]. TRC105 binds to human endoglin with high avidity and inhibits BMP9 binding, but binds much less avidly to mouse endoglin, with consequently less inhibition of murine BMP9 binding. Therefore, a mouse-specific endoglin targeting rat IgG1 antibody, M1043, has been developed for pre-clinical studies. This antibody efficiently inhibits BMP9 induced endoglin signaling in mice [12].

Program cell death receptor 1 (PD1/CD279) is an immune checkpoint molecule which plays an important role in preventing self-reactive T-cell responses [13]. The PD1 ligands PD-L1 and PD-L2 are found on tumor cells, fibroblasts, and myeloid cells [14]. Inhibition of PD1 re-activates the tumor immune responses and PD1 antibodies have been approved for the treatment of melanoma [15], non-small cell lung cancer [16], renal cell carcinoma [17]. Additionally, anti-PD1 therapy has been approved for cancer patients with high microsatellite instability (MSI-H) representing the first tissue agnostic companion diagnostic approved by the FDA. In clinical trials, treatment with TRC105 increased PD1 expression on CD8+ T-cells in the blood of cancer patients [18]. Since the effects of TRC105 have been described to be immune-dependent and PD1 expression is increased upon TRC105 treatment, we hypothesized that a combination of TRC105 with anti-PD1 antibody therapy might enhance therapeutic responses.

In this study, we evaluated the effects of endoglin targeting antibodies in combination with a PD1 antibody in mouse models representing different cancer stages. We

compared the efficiency and mechanism of antibodies binding to human and mouse endoglin (TCR105 and M1043, respectively), and investigated the effects of combination treatment. Our data show enhanced therapeutic effects when combining endoglin antibodies with PD1 inhibitors, resulting in prolonged anti-tumor responses which are dependent on ADCC and CD8+ cytotoxic T-cells. Moreover, we present evidence that targeting endoglin-expressing regulatory T-cells in the TME reactivates the immunosuppressed tumor microenvironment.

MATERIALS AND METHODS

Cell culture

The C57BL/6 murine colon adenocarcinoma cell line MC38 and a BALB/c CT26, which stably expresses a codon optimized luciferase construct [3], were routinely cultured in DMEM/ F12 Glutamax with 10mM HEPES, 50µg/ml gentamicin, 100IU/ml Penicillin and 100µg/ml Streptomycin (Invitrogen, Landsmeer, NL), supplemented with 10% fetal calf serum (FCS) (Gibco, ThermoFisher Scientific, Waltham, MA, USA). Cells were tested directly before *in-vivo* use for mycoplasma contamination by PCR analysis.

Mice

In this study, 8 to 10 weeks old male C57BL/6 or BALB/c mice (Jackson Laboratories, Bar Harbor, Maine, USA) were used. Mice were maintained at the central animal facility at the Leiden University Medical Centre under standard conditions. All mice were treated twice a week with intraperitoneal (IP) injections of 10mg/kg bodyweight M1043 or 15mg/kg bodyweight TRC105 (both kindly supplied by TRACON Pharmaceuticals, San Diego, CA, USA) or a human IgG control (inVivoMAB, BioXcell, West Lebanon, NH, USA). For the combination studies, mice were additionally treated twice a week with either an anti-PD1 (clone J43, 10mg/kg body weight, IP injection) or a hamster IgG control (both inVivoMAB, BioXcell, West Lebanon, NH, USA). To investigate the FcReceptor interactions we investigated the therapeutic efficacy of TRC105 and TRC105 in combination with PD1 in an FcRI/II/III/IV KO mice [19] as described above. CD8 and CD4 dependent effects were studied using 200ug CD8 depleting antibody and 50ug for CD4 depletion (anti-CD8 Clone 2.42 and anti-CD4 clone GK1.5 in house production followed by potein G column purification) given one day prior to the therapeutic antibodies CD8 and CD4 depletion was checked using flow cytometry of the blood samples the day of treatment. The depleting antibody was given once a week during the entire experiment.

In order to investigate the tumor growth and survival, mice were subcutaneously injected with 250.000 MC38 or CT26 tumor cells. When palpable tumors were present treatment was started as described above and tumor volume was assessed by

caliper measurement. Mice were sacrificed when tumors reached 1500 mm^3. To investigate changes in the tumor immune infiltrate a short-term experiment was performed. Mice were subcutaneously injected with 250.000 tumor cells and when tumors were 5x5x5 mm therapy was started. Ten days after start treatment, when tumor sizes were still comparable, mice were sacrificed and after cardiac injection with 2 mM EDTA (Merck, Darmstadt, Germany) to eliminate the blood from the vessels, the tumors were collected and processed for histology, flow cytometry, RNA and protein isolation. For the M1043 studies in subcutaneous MC38 mice a total of forty female 6 to 8 week-old C57BL/6J mice were inoculated subcutaneously with $2.5x10^5$ CEA2 expressing MC38 colon carcinoma cells [20]. Once tumors reached 100 mm^3, mice were randomized into four treatment groups: 1) Isotype control (rat IgG2a, clone 2A3, Catalog # BE0089, BioXcell, West Lebanon, NH, USA); 2) 5 mg/kg M1043 (provided by Tracon Pharma, San Diego, CA, USA); 3) Anti-mouse PD1 antibody (RMP1-14, catalog # BE0146, BioXcell) at fixed dose of 150 g per mouse; and 4) Combination of M1043 and anti-PD1 antibodies at the aforementioned doses. Drugs were injected intraperitoneally every two days and tumor sizes monitored. When tumors reached 2000mm^3, mice were sacrificed according to institutional animal use guidelines (Duke University).

For imaging, mice were injected intravenously (iv) with 0.1mg TRC105 labeled with a near-infrared fluorescent dye 800CW according to the manufacturer (LI-COR Biosciences, Lincoln, Ne, USA). Mice were imaged using the Pearl Impulse Small Animal Imager (LI-COR Biosciences, Lincoln, Ne, USA) 24- and 48-hours post-injection, after which mice were sacrificed. Finally, tumors were also imaged *ex-vivo*.

For the orthotopic implantation, subcutaneous tumors were grown and upon reaching 0.5 cm^3 mice were sacrificed, and the donor tumor were divided in 1-2 mm^3 pieces. These pieces were transplanted to the caecum wall of a recipient mice [21] In short, mice were sedated using isoflurane followed by a small incision in the center of the abdomen. The caecum was isolated and a small piece of tumor (1x1x1mm) was attached to the caecal wall, followed by the closure of the peritoneal wall and skin. Bioluminescent imaging was performed as described before. In short mice received an IP injection with 100mg/kg luciferin (D-luciferin sodium salt, Synchem, Altenburg, Germany) and were subsequently imaged on the IVIS lumina-II (Perkin Elmer, Waltham, USA) signal. The signal was quantified using the living image software. Eight days post-transplantation mice were randomized based on equal bioluminescent signal after which treatment was started. Thirty-six days after transplantation mice were, imaged, sacrificed and the tumor volume was measured using a caliper.

To investigate early stages of cancer development, the Azoxymethane (AOM) Dextran Sodium Sulphate Sodium (DSS) colitis-associated cancer model was used. Wildtype C57/Bl6 jico mice received one IP injection with 10mg/kg azoxymethane (Sigma, Zwijndrecht, the Netherlands), which induces aberrant DNA methylations in the

colon and liver [22]. In order to accelerate tumor formation, mice were subsequently exposed to three, 7-day cycles of 1,5% DSS (MP Biomedicals, Santa Ana, CA, USA) supplied in the drinking water with 2week intervals. Mouse weights were monitored every other day. In previous studies we observed small colonic lesions 48 days after AOM injections. Therefore, therapy was started at this time point. At the end of the experiment (84 days) mice were sacrificed by cervical dislocation and colons and livers were obtained. The number of colonic lesions was counted and one lesion per mouse was used for flow cytometric analysis. The remaining material was fixed in 4% buffered formaldehyde (Added Pharma, Oss, The Netherlands) and embedded in paraffin (Leica Biosystems, Richmond, IL, USA).

Flow cytometry

Tumors were mechanically disrupted and incubated for 15 minutes at 37°C in DMEM containing 1mg/ml liberase TL (Roche, Woerden, The Netherlands). Single-cell suspensions were prepared by mincing the tumors through a 70-µm cell strainer (BD Bioscience, Breda, the Netherlands). For cell surface staining, cells were resuspended in FACS buffer (PBS + 0.5% bovine serum albumin (BSA Sigma, Zwijndrecht, The Netherlands) + 0.05% sodium azide (pharmacy, Leiden University Medical Center). Cells were stained with Life death UV marker, specific antibodies indicated in supplementary table 1 (mouse) and -2 (human) or MC38 specific tetramers (kindly provided by Kees Franken department of Immunohematology and Blood Transfusion, LUMC) for 1 hour. After incubation cells were washed 3 times with FACS buffer and analyzed on an LSRII (BD Bioscience, Breda, the Netherlands). For the FoxP3 staining, the eBioscience Foxp3/Transcription Factor staining buffer set was used according to the one-step protocol for intranuclear proteins. Data analyses were performed using Flowjo 10.0.6 (Flowjo, data analysis software, Ashland, OR, USA).

Histology

Four µm sequential sections were deparaffinized and stained with hematoxylin and Eosin (HE) or processed for immunohistochemistry as described before [10]. In short sections were deparaffinized, blocked in 0.3% hydrogen peroxidase (H_2O_2, Merck, Darmstadt, Germany) in methanol for 20 minutes. Next slides were rehydrated, and antigen retrieval was performed by boiling in 0.01M sodium citrate pH6.0 for 10 minutes. Slides were washed and incubated with primary antibodies against CD31 (1:1600, Santa Cruz, Dallas, TA, USA), α- smooth muscle actin (SMA) (1:1600, Progen, Heidelberg, Germany), endoglin (1:100, R&D systems Minneapolis, MN, USA) Foxp3 (1:25, Thermo fisher, Bleiswijk, the Netherlands) diluted in 1% PBS/BSA) overnight at room temperature in a humidified box. The next day slides were incubated with appropriate biotinylated secondary antibodies (DAKO, Glostrup, Denmark) or anti-

Goat-alexa488 (Abcam) and anti-Rat-alexa568 (Invitrogen) for immunofluorescent staining slides were mounted with prolong gold anti fade (Thermo fisher) including DAPI. For IHC slides were incubated for 30 minutes at room temperature using Vectastain complex (Vector Labs, Burlingame, CA, USA). Slides and color developed with the DAB+ reagent (DAKO, Glostrup, Denmark) for 10 minutes. Nuclei were counterstained with Mayers Haematoxylin (Merck, Darmstadt, Germany) and slides were rinsed in tap water , dehydrated and mounted using Entellan (Merck, Darmstadt, Germany). Finally, pictures were taken with an Olympus BX51 light microscope equipped with an Olympus DP25 camera using the program CellSense and analyzed using ImageJ. For confocal microscopy LICA SP8 Lightning was used and pictures were processed using LICA LAS-X software.

Statistical analysis

Statistical analysis was performed using GraphPad/Prism software, version 7.0 (Graphpad Prism Software, Inc. La Jolla, CA, USA) All data are presented as mean +/- standard deviation unless otherwise indicated. Differences in survival were assessed by the Log-rank/Mantel-Cox test. For all others when normally distributed, Students t-test, and ANOVA or Kruskal-Wallis (non parametric) were used to test for significant differences, as indicated in the figure legends. P-values <0.05 were considered statistically significant.

Ethical approvals

All experiments using human material were performed according to the code of conduct for responsible use of human tissue and medical research as drawn up by the federation of the Dutch medical societies in 2011, guidelines of medical ethical committee of the LUMC and conducted in accordance to the declaration of Helsinki.

All animal experiments were approved by the national Dutch animal ethics committee under project license number AVD116002017858 and AVD11600201571 and accordance with rules and regulation of the animal welfare body of the LUMC. Animal experiments conducted at Duke University were performed in the Duke Preclinical Translational Research Unit in accordance with the Institutional Animal Care and Use Committee (IACUC) of Duke University and Duke University Medical Center.

RESULTS

Combined endoglin/PD1 targeting reduces tumor burden in a chemically induced colorectal cancer model

To investigate if endoglin targeting can decrease tumor burden in early stage colorectal tumor development and if therapeutic effects can be enhanced together with checkpoint inhibition, we employed an azoxymethane (AOM) dextran sulfate sodium (DSS) colitis-associated cancer model. These mice show high-grade adenomas with dysplasia, but without invasion through the basement membrane (Supplementary figure 1A/B/C). Mice were either treated with endoglin antibody (TRC105 or M1043) and/or PD1 antibody or appropriate IgG controls starting at day 48 after AOM injection. At the end of the experiment (day 84) mice were sacrificed and the number of colonic lesions was counted. Treatment with TRC105 significantly reduced the number of lesions compared to control, which was further reduced by combination with a PD1 antibody (Figure 1A/B). The size of the remaining lesions was also significantly reduced by the TRC105/PD1 combination compared to IgG controls (Figure 1C). Since TRC105 binds to mouse endoglin with low affinity, we also used M1043, a specific mouse endoglin neutralizing antibody. In contrast to TRC105, M1043 monotherapy did not reduce the number of lesions in this experiment, although the combination with PD1 was highly effective in reducing the lesion count and size (Figure 1 D/E/F). Taken together these data show that targeting endoglin by using TRC105 can reduce tumor burden in an early stage tumor model and that these effects can be enhanced by combining endoglin and PD1 targeting antibodies.

TRC105/anti-PD1 therapy inhibits orthotopic MC38 tumor growth

To investigate the effects of combination therapy in a more advanced cancer model, we used a MC38 syngeneic orthotopic transplantation model. In short, part of a subcutaneously grown MC38 tumor, expressing codon-optimized luciferase, was transplanted onto the caecal wall of recipient mice (Supplementary figure 2A). Bioluminescent imaging was performed at day 8 after tumor transplantation, after which mice were allocated into treatment groups based on equal bioluminescent signal (Supplementary figure 2B). Tumor specific T-cell effects were assessed at day 17 (9 days after start treatment) by flow cytometry for the MC38 specific neo-epitopes ADPGK and DPAGT-1 tetramers [23], (gating strategy in Supplementary figure 2C). CD8+ DPAGT-1 positive cells increased slightly in the TRC105, PD1, and combination-treated mice compared to controls. M1043 alone did not induce tumor specific T-cells (Supplementary Fig 2D). A similar trend was present in ADPGK recognizing CD8 T-cells, but did not reach statistical significance (Supplementary figure 2E). At the end of the experiment (36 days post tumor transplantation) mice were sacrificed

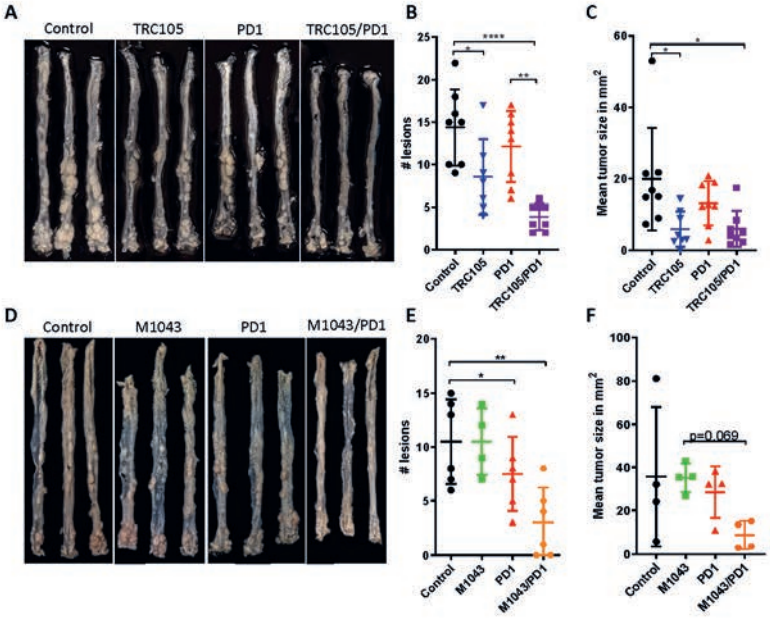

Figure 1. Combined endoglin/PD1 targeting reduces tumor burden in a chemically induced colon cancer model. Azoxymethane (AOM)/dextran sodium sulphate (DSS) model to study early colon tumor development. From day 48 on mice were treated twice weekly with anti-endoglin antibodies or control IgG and twice a week with anti PD-1 or IgG control until day 84 when mice were sacrificed. **A.** Images obtained from the mouse colon at the end of the experiment (84 days) showing multiple lesions in the distal colon. B. Quantification of the number of colonic lesions upon treatment with control IgG, anti-PD1, anti-endoglin (TRC105), and the TRC105/PD1 combination. Combination treated mice show significantly reduced tumor formation compared to monotherapy and IgG controls (one way ANOVA). C. Tumor volume measurements showing significantly smaller tumors in the combination treated mice (Kruskal-Willis test for multiple comparison). D. Images obtained from the distal mouse colon at the end of the experiment showing multiple lesions. E. Quantification of the number of lesions showing a significant decrease in the combination group (M1043/PD-1) compared to the IgG controls (one way ANOVA). F. Tumor volume measurement of the colonic lesions (Kruskal-Willis test for multiple comparison). Data shown are representative from two or more independent experiments with 6-8 mice per group. * P≤0.05 ** P<0.01 **** P<0.0001.

and the tumor volume was determined by caliper measurements. In contrast to M1043, TRC105 and PD1 antibody monotherapy and the combination of M1043 and PD1 antibody treatment resulted in reduced tumor volume compared to control IgG. Strikingly, the combination of TRC105 and PD1 antibody resulted in a more profound reduction of the tumor volume by bioluminescent imaging and caliper measurements (Figure 2A/B). Remaining tumors were processed for histological analysis and stained for H&E, the pan-endothelial marker CD31 (Figure 2A, middle panel) and endoglin (Figure 2A, right panel), using an antibody recognizing a non-overlapping endoglin epitope. No significant differences were detected in the number of CD31 or endoglin expressing blood vessels (Figure 2C/D) in the remaining tumors. Of note, vessel density could not be assessed in mice with complete tumor regressions and differences in tumor size were not considered in the analysis.

Combined, these data indicate that TRC105/PD1 antibody therapy induced marked anti-tumor responses in an orthotopic MC38 colon cancer model and TRC105 seemed more effective in tumor inhibition compared to M1043 therapy.

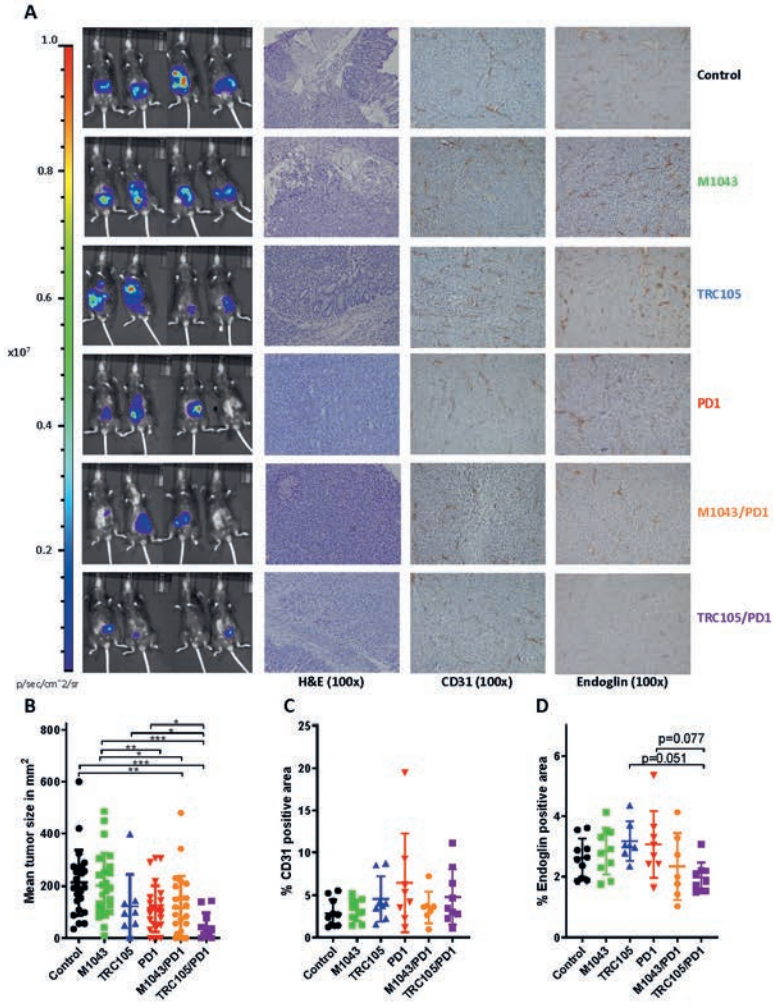

Figure 2. Endoglin/PD-1 therapy inhibits orthotopic tumor growth. Tumors were orthotopically transplanted in recipient mice and after engraftment and randomization, mice were treated twice a week with anti-endogin or control IgG and twice a week with anti PD-1 or IgG control. **A**. Bioluminescent imaging (left panel) at the end of the experiment showing anti-tumor responses. The right panel shows (immuno) histochemical analysis for H&E, CD31 and endoglin. **B.** Quantification of the tumor volume at the end of the experiment showing significantly smaller tumors upon combination therapy, especially in the TRC105/PD-1 group. Data are from 2 independent experiments with 8-20 mice per group (one way ANOVA) **C.** Quantification of tumor CD31 staining showing no significant difference in the number of CD31 positive cells (Kruskal-Willis test for multiple comparison) **D.** Quantification of intratumoral endoglin staining showing no significant differences between treatment groups. Quantifications are from at least two independent experiments (one way ANOVA). * P≤0.05 ** P<0.01 *** P<0.001.

TCR105/PD1 therapy efficiently reduces tumor growth and induces memory anti-tumor responses

In order to assess the immune responses in more detail, we evaluated the therapeutic effectivity of the mouse endoglin antibody M1043 with PD1 antibody in a subcutaneous MC38 model. A significant delay in tumor outgrowth was observed in the combination therapy treated mice and resulting complete tumor regression in 20% of the mice in the combination therapy group (Figure 3A and Supplementary figure 3A). Given the fact that TRC105 binds murine endoglin with lower avidity, we confirmed that TRC105 can still bind to mouse endoglin and accumulates in subcutaneous mouse tumors. Therefore we labelled TRC105 with the near-infrared dye CW800. Upon intravenous injection in mice bearing subcutaneous MC38 tumors, tumor accumulation of TRC105 was observed 24h and 48h post injection as well as *ex vivo* (Figure 3B).

The subcutaneous MC38 model was used to directly compare the efficiency of M1043 and TRC105 versus an isotype control. These data demonstrate significantly more therapeutic efficacy of TRC105 compared to M1043 (Supplementary figure 3B). Therefore, we focused further studies on TRC105/PD1 only. The therapeutic benefit of TRC105/PD1 antibody therapy was assessed in MC38 and CT26 subcutaneous tumor models in C57BL/6 and Balb/c mice, respectively. TRC105 or PD1 antibody monotherapy delayed tumor growth and prolonged survival, but effects were more pronounced in animals who received TRC105/PD1 combination therapy in both the MC38 (Figure 3C) and CT26 model (Figure 3D). Complete tumor responses were observed in 30-40% of the mice in both models. To investigate memory anti-tumor responses, the surviving, tumor-free mice were injected again with $2.5*10^5$ MC38 or $2.5*10^5$ CT26 cells 60 days after the initial tumor cell injection. Importantly, no tumor outgrowth was observed after re-challenge with tumor cells, implying that a memory anti-tumor response was induced. Our data show that combined TRC105/PD1 antibody therapy delays tumor growth compared to either antibody alone, induces complete and sustained regression in both the MC38 and CT26 subcutaneous tumor models, and prevents tumor growth after re-challenge with tumor cells.

TRC105/PD1 antibody therapeutic effects are ADCC dependent

We next investigated the underlying mechanism for the activity of the TRC105/PD1 antibody combination. TRC105 can mediate ADCC, which requires binding to Fc-receptors. We therefore investigated whether the TRC105 and TRC105/PD1 antibody effects were dependent on Fc-receptor binding in $FcγR^{KO}$ mice. MC38 bearing C57Bl6 and $FcγR^{KO}$ mice injected with human IgG showed similar tumor outgrowth and survival (supplementary figure 3C). Notably, the activity of TRC105 and TRC105/PD1 combination therapy was completely abolished in $FcγR^{KO}$ mice (Figure 3E/F) indicating, that the therapeutic effects of TRC105 and the TRC105/PD1 combination are dependent on Fcγ receptor binding *in vivo*.

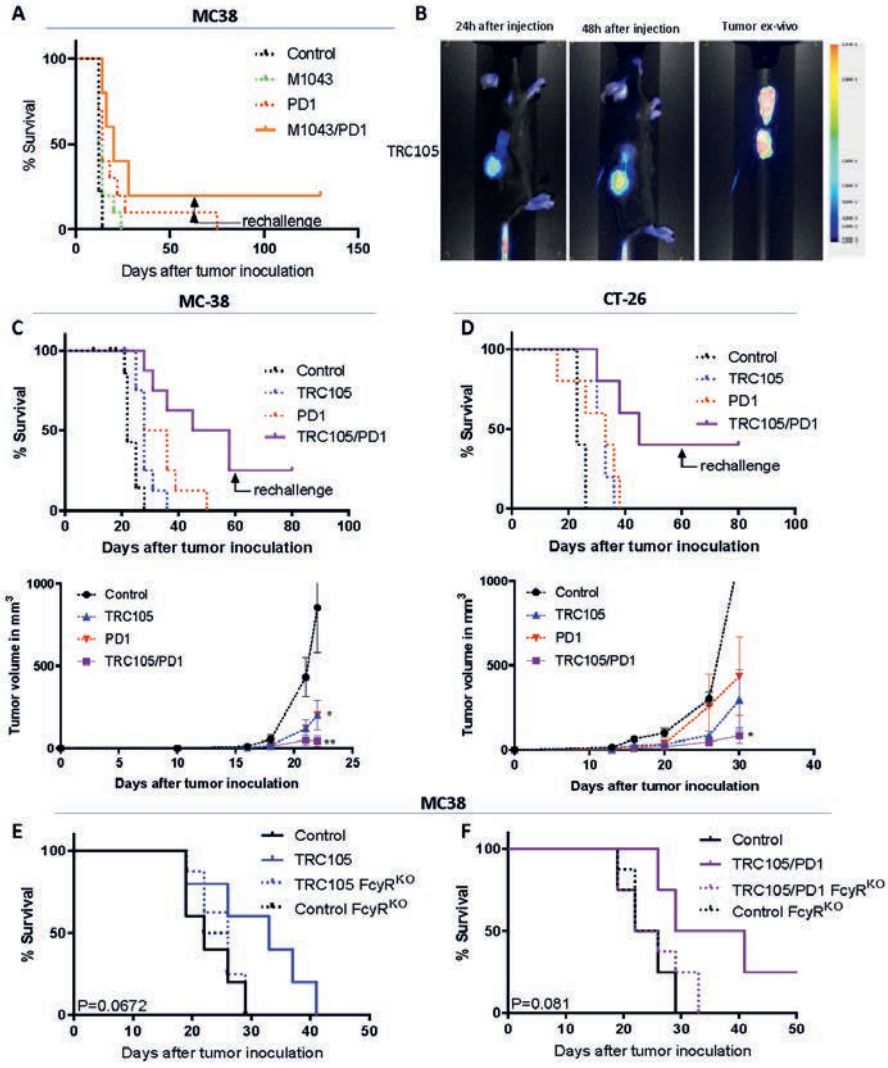

Figure 3. TRC105/PD-1 therapy efficiently reduces tumor growth, induces memory T cell responses and is dependent on FcyR expression. When tumors were palpable mice were treated twice a week with anti-endoglin or control IgG and twice a week with anti PD-1 or IgG control **A.** A combination of M1043 and PD1 results in significantly improved mouse survival and induced memory T cell responses, since rechallenge with tumor cells does not result in tumor outgrowth. **B.** Intravenously administered, CW800 labeled TRC105 shows high tumor accumulation in subcutaneous MC38 tumors (representative image from 2 mice). **C.** Combination treatment with TRC105/PD1 significantly increases survival of mice bearing MC38 (n=7-8 mice per group) or (**D**) CT26 subcutaneous tumors (n=5 mice per group). **E.** Therapeutic effects of TRC105 or combination therapy (**F**) are diminished in a FcyRI,II,III,IV KO mouse, indicating the involvement of ADCC (n=5-8 mice per group). Statistical analysis includes Log-rank/Mantel-Cox test for survival analyses, one way ANOVA for multiple comparison on day 22 for C and day 30 for D. * P≤0.05 ** P<0.01.

TRC105/PD1 therapy requires T cell infiltration and activity

Next, we assessed changes in immune cell infiltrate upon combination treatment. Subcutaneous MC38 tumors of similar size (Supplementary figure 4A) nine days after start of treatment were evaluated to exclude potential effects of tumor volume on the composition of the immune infiltrate. Immunohistochemistry showed decreased numbers of Ki67+ proliferating cells, accompanied by increased numbers of apoptotic, cleaved caspase-3 positive cells (Supplementary figure 4B), indicating anti-tumor responses. The number of endoglin-positive blood vessels was unaffected at this time point (Supplementary figure 4C). Treatment with TRC105 or the combination of TRC105/PD1 antibody increased the number of intratumoral CD8+ T cells (Figure 4A). Moreover, we observed a significant increase in the number of activated, granzyme B+/CD8+ T cells upon TRC105 or PD1 or TRC105/PD1 antibody combination therapy in the circulation (Figure 4B). Activation of T cells was further confirmed by mRNA expression analysis of the tumor tissue, showing that granzyme B mRNA expression was particularly increased following TRC105/PD1 antibody combination treatment (Figure 4C and 4D). Increased numbers of tumor infiltrating CD8+ T cells was confirmed by immunohistochemical analysis (Figure 4E). Protein levels of VEGF, INF-y and TGFβ-1 within the tumor lysates did not differ although levels varied considerably between and within groups (supplementary figure 4E). Taken together these data indicate that an increased number of CD8+ T cells were recruited and activated upon TRC105/PD1 combination therapy.

To further investigate if CD8+ T cells are instrumental for the therapeutic responses, MC38 cells were subcutaneously injected in mice and a CD8+ depleting antibody was given before the initiation of TRC105 or TRC105/PD1 antibody treatment. This resulted in a significantly reduced number of circulating CD8+ T cells (supplementary figure 4F) in these groups. In T cell depleted mice, therapeutic responses induced by TRC105 or TRC105/PD1 antibody combination were abolished compared to control IgG treated, mice (Figure 4 F/G). These data suggest that the therapeutic effect of combination therapy is dependent on the activity of CD8+ T cells and involves their recruitment and activation.

Targeting of FOXP3 immune subsets by endoglin targeted therapy

On the ninth day, subcutaneous MC38 tumor experiments anti-endoglin therapy did not cause significant differences in the number of peripheral blood $Treg^{CD4+CD25+Foxp3+}$cells pre- and post- treatment or between the treatment groups (Figure 5A). However, we observed a significantly decreased percentage of regulatory T cells in the tumor in the TRC105 and TRC105/PD1 antibody treated mice, compared to the control IgG and PD1 antibody monotherapy treated mice (Figure 5B). Moreover, the CD8+/Treg ratio significantly increased to a more beneficial ratio to reach anti-tumor effects in the combination therapy treated mice (Figure 5C). A

striking increase in endoglin expression on tumor localized Tregs was present, compared to circulating Tregs in these mice (Figure 5D). Endoglin expression was present only on a subpopulation of Tregs within the tumor and not on conventional CD4+ T cells (Figure 5E). Treatment with TRC105 significantly reduced the number of intratumoral CD25+/Foxp3+ cells (Figure 5F).

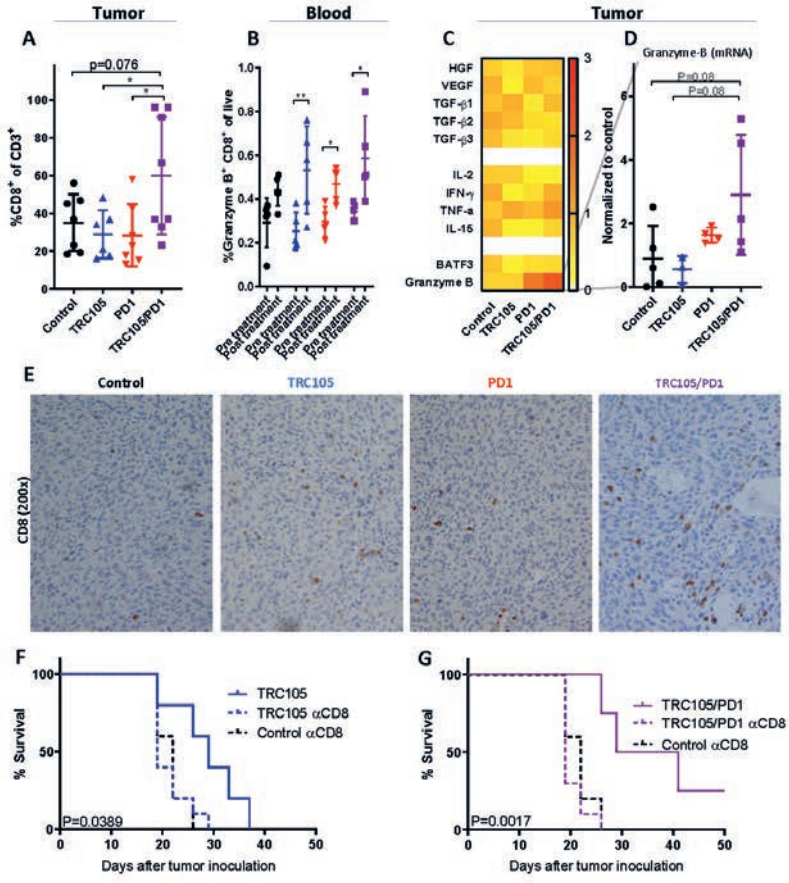

Figure 4. TRC105/PD-1 therapy requires CD8+ T-cell infiltration. When tumors were palpable mice were treated twice a week with anti-endoglin or control IgG and twice a week with anti PD-1 or IgG control. After 9 days of treatment mice were sacrificed and the immune infiltrate was examined **A.** Upon treatment with TRC105/PD1 combination therapy the percentage of intratumoral CD8+ T cells is increased (mean of 2 independent experiments, n=4 mice per group per experiment, one way ANOVA). **B.** Increased number of circulating granzyme B+ CD8+ T cells post-treatment (n=3-5 mice per group, paired t-test). **C.** Heatmap showing mRNA expression of growth factors and genes involved in immune regulation, normalized to control IgG treated mice (n=3-5 mice per group). Increased granzyme B expression within the tumor in combination therapy treated mice is observed (**D**) **E.** Immunostaining revealing increased CD8+ T cells throughout the tumors upon treatment with TRC105/PD-1 (one way ANOVA). The therapeutic effects of TRC105 monotherapy (**F**) and combination therapy (**G**) were completely depending on CD8+ T cells (n=5 (control)-10 (CD8 depleted) mice per group, Log-rank/Mantel-Cox test). * P≤0.05 ** P<0.01.

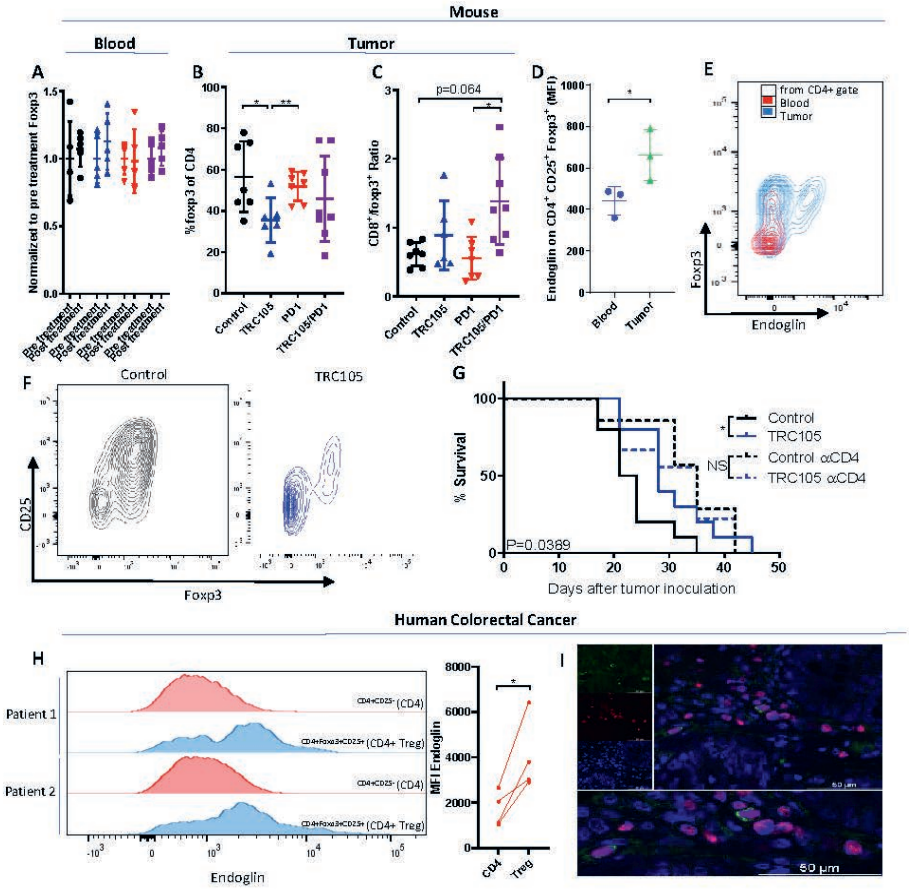

Figure 5. Endoglin expressing FoxP3 cells are detected intratumorally and are targeted by anti-endoglin therapy. A. The number of FoxP3 cells in the circulation of tumor bearing mice did not change pre- and post- treatment (n=5 mice per group, paired t-test). **B.** A significant decrease in intratumoral Tregs (FoxP3+CD25+CD4+) was observed (one-way ANOVA), resulting in increased CD8/FoxP3 tumor ratios (**C**, mean of 2 independent experiments, n=4 mice per group per experiment, Kruskal-Wallis test for multiple comparison). **D/E**. FACS analysis revealed higher endoglin expression on intratumoral FoxP3 cells, compared circulating FoxP3 cells from the same mouse (mean fluorescent intensity, n=3 mice per group, t-test). **F.** Flow cytometry plot of TRC105 treated MC38 tumors, showing a decrease in CD25+/Foxp3+ cells upon TRC105 treatment. **G.** TRC105 significantly enhances survival of mice bearing MC38 tumors (p=0.039), which is lost upon depletion of CD4+ cells (p=0.039 vs p=0.37 respectively, n=7-10 mice per group, Log-rank/Mantel-Cox test). **H.** In human colorectal cancer samples endoglin expression on intratumoral Tregs was observed by flow cytometry analysis (representative image from n=4 patients, paired t-test). **I.** Immunohistochemical analysis shows colocalization of endoglin (green) with FoxP3 (red) in a subset of FoxP3 cells in human CRC tissues (representative image from n=4 patients). * P≤0.05 ** P<0.01.

To further investigate the targeting of Foxp3+ Tregs we depleted CD4+ cells, compromising the Foxp3+ population. MC38 cells were subcutaneously injected in mice and a CD4+ depleting antibody was given before the initiation of TRC105 treatment. This resulted in a loss of circulating CD4+ T cells (supplementary figure

5C) in the depleted groups. Mice not showing CD4+cell depletion (indicated in red) were excluded from the experiment. In control mice TRC105 effectively delayed tumor growth (p=0.039) as shown before. However, the therapeutic effects of TRC105 are lost once the CD4+ T cells were depleted (Figure 5G, p=0.37). These data suggest that therapeutic TRC105 effects are CD4 cell dependent and most probably involves targeting endoglin expressing Foxp3+/CD4+ cells.

Finally to illustrate the potential translational relevance of our findings, we investigated whether endoglin is also expressed on Tregs in a limited number of human colorectal cancer tissues by using flow cytometry. High endoglin expression was seen on a subset of $Tregs^{CD4+CD25+Foxp3+}$ compared to the $CD4+^{CD25-Foxp3-}$ population within the tumor (Figure 5H). To confirm our flow cytometry findings, we performed immunofluorescent double staining for endoglin and Foxp3 on colorectal cancer tissue sections (Figure 5I). These data revealed high endoglin expression of a subset of intratumoral Tregs. Taken together, our data confirm that a subset of Foxp3 cells co-express endoglin in mouse colorectal tumors and can also be detected in human CRC.

DISCUSSION

In this study, we demonstrated that combined therapy with anti-endoglin and anti-PD1 antibodies significantly increases the therapeutic efficacy in several pre-clinical cancer models, including subcutaneous, orthotopic, and chemically induced colorectal cancer models. The endoglin antibody TRC105 acted principally via immune dependent mechanisms, where both CD8. the Fc receptors played instrumental roles in the therapeutic response. In addition we identified endoglin expressing Tregs in mouse and human colorectal cancer tissue, which also seem to be targeted by TRC105, as the effects are lost when CD4+ cells are depleted.

TRC105 was initially described in the late 1990s [8, 9], and subsequently has been studied in Phase 2 and Phase 3 clinical trials. Since high endoglin expression has been reported on angiogenic endothelial cells and endothelial endoglin expression has been linked to disease progression and prognosis [2], targeting endoglin seems a logical therapeutic approach in solid tumors. Indeed, we and others have shown that anti-endoglin therapy inhibits tumor growth and metastasis formation in various cancer models [6, 8-10, 24]. Furthermore, targeting the VEGF pathway activates alternative pathways, of which endoglin has been frequently reported [10, 25-27].

It was surprising that M1043 was less effective in reducing tumor growth in the presented tumor models. M1043 is a mouse-specific endoglin targeting antibody, which efficiently inhibits downstream BMP9 induced signaling [12] and supplementary figure 5), while TRC105 less effectively inhibits mouse BMP9 binding to endoglin. Rat IgG1 is, however not capable of inducing ADCC, compared to other isotypes,

making it an interesting approach to change the M1043 isotype to Mouse IgG2a, thereby creating an antibody which blocks ligand binding but also induces ADCC.
Fc-mediated effects have been clearly shown for human IgG subtypes and the human IgG1 antibody TRC105, which is able to bind mouse Fc-receptors [28]. Moreover FcR mediated ADCC appeared crucial for the mechanism of action of endoglin antibody in our tumor models. In humans, the TRC105 IgG isotype may bind with higher affinity to Fc Receptors compared to mouse Fc receptors, even further enhancing ADCC-responses. Previous studies have shown that the therapeutic PD1 antibody effects are independent on Fc receptor binding [29]. These data also indicate that evoking an immune response might be a more important mechanism of action for endoglin antibodies than inhibiting BMP9 binding.
In addition to endothelial cells, endoglin expression has been reported on (cancer-associated) fibroblasts in prostate cancer [30] and colorectal cancer [3]. Endoglin expression seems to be important for fibroblast survival *in-vitro* and stimulates metastatic dissemination [3]. Furthermore endoglin expression has also been described on macrophages, where it seems to be important for differentiation and phagocytosis [31]. In the current study we show that endoglin is expressed by regulatory T-cells, posing them as a novel target for endoglin targeted therapy. Although endoglin expression on CD4+ cells has also been observed by others [32-34], endoglin expression on Treg cells has only been described before in an *in-vitro* setting in which Tregs were cultured with adipose derived mesenchymal stromal cells [34]. Strikingly, we observed that endoglin expression on Tregs is significantly increased in tumor tissue compared to the peripheral circulation. Furthermore, we show that endoglin targeted therapy decreased the number of Tregs in tumors, thereby potentially contributing to the tumor responses. Depletion of Tregs within the tumor using antibody therapy has also been shown by others, for example using anti-CD25 [35] and anti-CTLA-4 antibodies [36] in mice. Since endoglin therapy targets multiple subsets of cells within the tumor microenvironment (i.e., proliferating endothelial cells, fibroblasts and Tregs), TRC105 may more efficiently inhibit tumor growth and metastasis compared to other anti-angiogenic therapies. The fact that endoglin expressing Tregs could be detected in colorectal cancer tissue might explain the data from a previous clinical trial showing a significantly decreased number of circulating Tregs upon TRC105 therapy [18]. Although we could not show a clear endoglin positive population in the blood of mice, we were able to detect endoglin expressing Tregs in the blood of healthy volunteers (data not shown).
In conclusion, in this study we show that combining endoglin with PD1 targeted therapy in four preclinical cancer models strongly increases therapeutic effects. We propose a model in which TRC105 binds to endoglin expressing endothelial cells, fibroblasts, and endoglin expressing Tregs within the tumor, evoking a FcR dependent ADCC response. Consequently, the increased recruitment and activity of CD8+ cells

evokes sustained tumor regression and produce a memory T cell response. Results from a phase 1b dose-escalation study of carotuximab (TRC105) in combination with nivolumab (anti-PD1) in patients with metastatic Non-Small Cell Lung Cancer (NCT03181308) should reveal if this combination strategy is effective in human patients.

Conflict of interest

This study was supported by a sponsored research agreement from TRACON Pharmaceuticals. C.P. Theuer has ownership interest (including patents) in TRACON Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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Author contributions

MS performed experiments, designed and analyzed experiments and wrote the manuscript. MK, RA, BK, MT, YL, MP, LH, CS and MB performed and analyzed the experiments. AN, CT, CS, and JH revised the manuscript and provided critical feedback on experiments. LH and MF designed experiments, supervised the project and revised the manuscript.

All supplementary figures and tables are available online

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Tumor-draining lymph nodes are pivotal in PD-1/PD-L1 checkpoint therapy

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ABSTRACT

PD-1/PD-L1 checkpoint therapy for cancer is commonly considered to act by reactivating T cells in the tumor microenvironment. Here, we present data from 2 mouse tumor models demonstrating an essential involvement of tumor-draining lymph nodes in PD-1 and PD-L1 therapeutic efficacy. Immune activation induced by checkpoint treatment was predominantly observed in the tumordraining, but not nondraining, lymph nodes and was reflected in local accumulation of CD8+ T cells. Surgical resection of these lymph nodes, but not contralateral lymph nodes, abolished therapy induced tumor regressions and was associated with decreased immune infiltrate in the tumor microenvironment. Moreover, inhibitor FTY720, which locks lymphocytes in lymph organs, also abrogated checkpoint therapy, suggesting that the tumor-draining lymph nodes function as sites of T cell invigoration required for checkpoint blockade therapy. Now that PD-1/PD-L1 checkpoint treatment is applied in earlier clinical stages of cancer, our preclinical data advocate for enrolling patients with their tumor-draining lymph nodes still in place, to optimally engage the antitumor immune response and thereby enhance clinical benefit.

INTRODUCTION

Blocking antibodies against PD-1 and PD-L1, named checkpoint molecules, shows exceptional clinical responses in cancer patients and has already become a standard-of-care treatment in a still-increasing number of cancer types [1]. Generally, PD-1/PD-L1 checkpoint blockers are thought to invigorate T cells within the tumor microenvironment (TME), where PD-L1 is expressed on tumor cells and infiltrating myeloid cells. Indeed, therapeutic responses generally correlate with high T cell infiltrate, PD-L1 expression, and tumor mutational load [2]. More recent studies have pointed to systemic factors, such as frequencies of myeloid cells, lymphocytes, and eosinophils in peripheral blood [3–5], and we and others have shown that PD-L1 expression on tumor cells is not a prerequisite for successful checkpoint therapy [6–9]. However, the exact mode of action in vivo is still poorly understood. Thus far, studies on the role of tumor-draining lymph nodes (TDLNs) for checkpoint therapy are limited. TDLNs are the first sites of metastasis and are therefore often resected when invaded by tumor cells. Lymph nodes (LNs) play important roles in the generation and regulation of immune responses to pathogens and autoantigens [10]. TDLNs have been described to contain both tumor effector as well as suppressor immune components [11], and it has been shown that tumor antigens drain primarily to TDLNs, more specifically via transportation by CD103+ DCs, which leads to priming of T cells [12–14]. We here investigate the role of TDLNs in checkpoint therapy of solid tumors in preclinical mouse models, by analyzing effects of checkpoint therapy on TDLN immune content and surgically resecting TDLNs before checkpoint therapy.

RESULTS

We set out to evaluate the role of TDLNs in PD1/PD-L1 therapy by analyzing immune cell composition of TDLNs and nondraining lymph nodes (NDLNs). MC38 colon carcinoma cells were inoculated subcutaneously in the flank of syngeneic mice, and TDLNs (inguinal and axillary LNs) were isolated for in vitro analyses, after verification of drainage with fluorescent imaging (data not shown). LNs at the opposite flank of the mice served as internal, nondraining controls. We observed that CD11b+ myeloid cells in the TDLNs of untreated tumor-bearing mice expressed higher PD-L1 levels as compared with levels in NDLNs, suggesting that active immune suppression is ongoing (Figure 1A). A strong increase in cellularity was observed in TDLNs 3 days after the start of PD-1–blocking treatment (Figure 1B). This swelling did not cause gross irregularities in LN architecture (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/ jci.insight.124507DS1). PD-1 treatment resulted in an increase of the total number of CD8+ T cells in TDLNs.

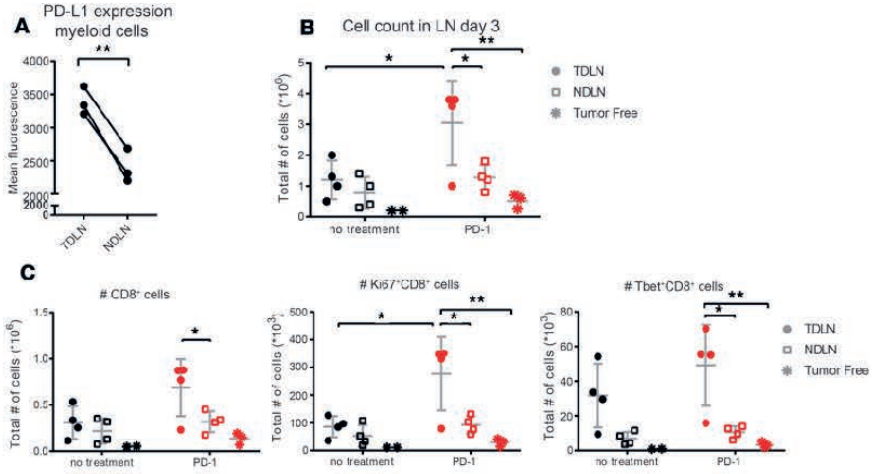

Figure 1. Immune activation takes place in tumor-draining, but not in nondraining, lymph nodes. (**A**) TDLNs (inguinal and axillary) and NDLNs (opposite flank) were isolated when subcutaneous MC38 tumors reached an average size of 100 mm^3 and were analyzed with flow cytometry. Mean fluorescence expression of PD-L1 on myeloid cells ($CD19^-CD11b^+$) is shown. Statistical difference was calculated with a paired 2-tailed t test. (**B** and **C**) Three days after PD-1 Ab treatment, lymph nodes were analyzed for (**B**) immune cell counts and numbers of (**C**, left) $CD8^+$ T cells, (**C**, middle) proliferating $CD8^+$ T cells, and (**C**, right) T-bet+ $CD8^+$ T cells. Statistical differences were analyzed with 2-way ANOVA. All data represent mean ± SEM from 1 experiment (n = 4 per group) out of 3 independent experiments with similar outcome (*P < 0.05, **P < 0.01).

Activated CD8+ T cells were more abundant in TDLNs compared with NDLNs as indicated by the proliferation marker Ki67 and transcription factor T-bet (Figure 1C). In contrast, hardly any T cell activation was found in NDLNs or LNs from tumor-free, PD-1–treated mice, indicating that vigorous T cell immune activation after PD-1–blocking Ab treatment within TDLNs depends on the presence of tumors. This suggested that PD-1 immunotherapy could activate T cells in the TDLNs, which may contribute to the antitumor response. By surgically resecting inguinal and axillary TDLNs just before the start of PD-1 treatment, we evaluated the actual contribution of TDLNs in therapeutic efficacy. In the absence of TDLNs, the treatment efficacy was strongly diminished (Figure 2, A and B), compared with mock surgery controls. We previously demonstrated that local administration, close to the tumor site, of immunomodulatory Abs, such as agonistic anti-CD40 and blocking anti–CTLA-4, decreased toxicity but sustained the treatment effect (15, 16). PD-1–blocking Ab was also operational in this setting (Figure 2C), suggesting that neighboring communication between tumor and TDLN is critical. We then reasoned that draining of tumor-derived antigens to the TDLNs might be involved in this system and resected the TDLNs even before tumor inoculation. In this setting, PD-1 efficacy was completely abrogated, whereas resection after tumor settlement, as applied thus far, still showed residual tumor control (Figure 2C). To control for a nonspecific effect

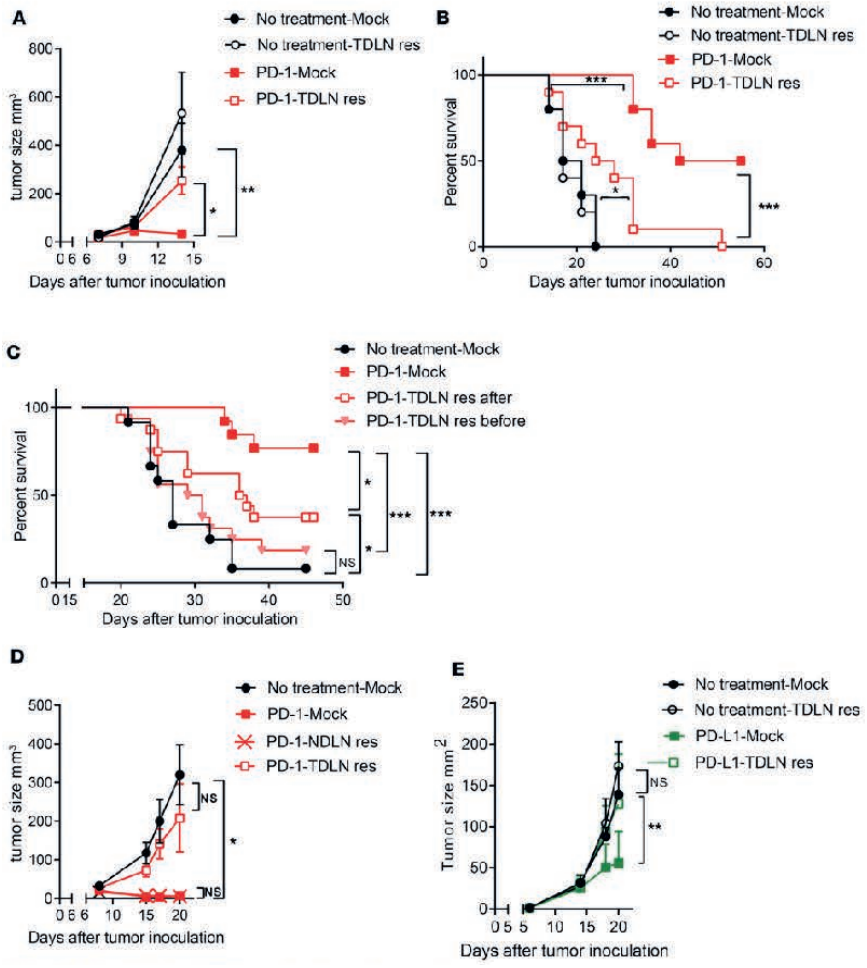

Figure 2. Essential role of TDLNs in checkpoint therapy. Mice bearing subcutaneous tumors in the right flank were treated with checkpoint blocker therapy immediately following lymph node resection surgery or mock surgery. (A–D) C57BL/6 mice bearing MC38 tumors were treated with PD-1–blocking Ab immediately following lymph node resection surgery. (**A**) Average outgrowth of mice treated with PD-1 Ab systemically (2 × 100 πg i.p.). Average ± SEM are depicted. Statistical analysis of average tumor outgrowth difference on day 14 was calculated by 1-way ANOVA, 10 mice per group. Res: resection. (**B**) Survival of mice treated with PD-1 Ab systemically (2 × 100 πg i.p.). Statistical difference was analyzed with log-rank test, 10 mice per group. (**C**) Survival of mice treated with PD-1 Ab systemically (2 × 100 πg i.p.). TDLNs were resected 7 days after ("res after") or 1 day before ("res before") tumor inoculation. Pooled data of 2 independent experiments, with 16 mice per group. Statistical difference was analyzed with log-rank test. (**D**) Average tumor outgrowth of mice treated with local injection of low-dose PD-1 Ab (1 × 50 πg s.c.), with TDLNs or NDLNs resected before PD-1 treatment, 8 mice per group. Average ± SEM are depicted. Statistical difference in average tumor size on day 20 was calculated by 1-way ANOVA. (**E**) BALB/c mice bearing subcutaneous CT26 tumors in the right flank were treated with PD-L1 Ab systemically. Average tumor outgrowth ± SEM are depicted. One representative experiment is shown from 2 performed, with 8 mice per group. Statistical analysis of average tumor size on day 20 was calculated with 1-way ANOVA (*P < 0.05, **P < 0.01, ***P < 0.005, NS: nonsignificant).

of disrupting the lymphoid system, we resected the NDLN at the opposite flank, which did not influence the therapeutic efficacy (Figure 2D and Supplemental Figure 2). Finally, we applied this treatment setting with PD-L1–blocking Ab in another colon tumor model on a distinct genetic background, CT26 in BALB/c mice. In this independent model, PD-L1 blockade displayed an identical dependency on the presence of the TDLNs (Figure 2E). These results suggested that T cell trafficking was required for therapeutic efficacy; we therefore treated tumor-bearing mice with PD-1 therapy in the presence of the S1P receptor inhibitor FTY720, which locks T cells in lymphoid organs. Efficacy of FTY720 administration was shown by decrease in T cell content of peripheral blood (Figure 3A). Importantly, FTY720 mitigated the therapeutic efficacy of PD-1 treatment (Figure 3B), suggesting that therapeutic efficacy is not solely based on reactivation of T cells within the TME but that influx of T cells from elsewhere dominantly contributes to this therapy. Next we analyzed the TME of tumor-bearing mice treated with PD-1–blocking Ab with or without TDLN resection. We found a strong decrease of CD45+ immune infiltrate in the TME of TDLN-resected mice, pointing at a role for TDLNs in trafficking of immune cells to

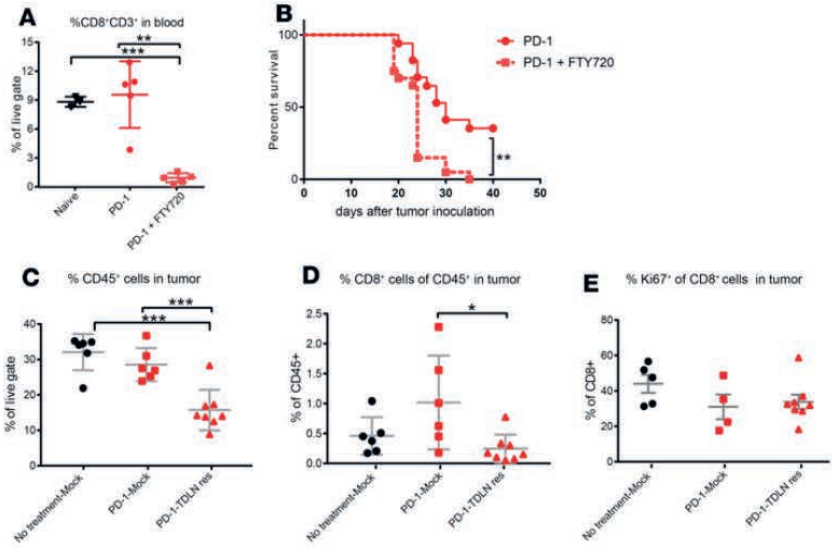

Figure 3. Increase of intratumoral CD8⁺ T cell numbers after PD-1 treatment is abrogated in the absence of TDLNs. (**A**) $CD3^+CD8^+$ content in peripheral blood of mice treated with FTY720. Mean ± SEM are depicted. Statistical differences were calculated with 1-way ANOVA. (**B**) Survival of mice treated with PD-1–blocking Ab with or without FTY720. Pooled data of 2 comparable experiments with 16 mice per group are shown. Statistical analysis was done by log-rank test. (**C–E**) Analysis of subcutaneous MC38 tumors at day 13 after PD-1 treatment, with or without TDLN resection immediately before PD-1 treatment. (**C**) Percentage of $CD45^+$ cells out of live gate. (**D**) Percentage of $CD8^+$ cells out of $CD45^+$ gate. (**E**) Percentage of $Ki67^+$ cells out of $CD8^+$ cells. Statistical analysis was performed with 1-way ANOVA, 5–8 mice per group (*P < 0.05, **P < 0.01, ***P < 0.005); 1 representative experiment is shown out of 2 performed.

the tumor (Figure 3C). Additionally, significantly more CD8+ T cells were found in the TME of TDLN-proficient versus TDLN-deficient mice after PD-1 treatment. Proliferation marker Ki67 did not differ between CD8+ T cells of these groups, suggesting that influx of cells, rather than enhanced proliferation of T cells within the TME, caused this difference. Together, our data indicated that PD-1 blockade reinvigorates CD8+ T cells in the TDLNs, resulting in an influx of these effector cells to the TME.

DISCUSSION

In conclusion, we show that TDLNs are key regulators in the antitumor immune response and control the magnitude of therapeutic efficacy of PD-1– and PD-L1–blocking Ab treatment in mouse models. This is in agreement with a previous study analyzing the role of VEGF-C. Increased VEGF-C in the tumor caused enhanced lymph drainage, which was associated with increased antitumor immune response and stronger effect of immunotherapy [17]. These data strongly support our findings, suggesting a potential reservoir of activating tumor-specific immune cells within TDLNs. Harnessing this empowering effect of TDLNs might benefit the clinical outcome of checkpoint blockade therapy and might ideally be tested in early-stage cancer patients where the tumor and TDLNs are still in place. Applying therapy before resection surgery, often termed neoadjuvant treatment, is already performed for chemotherapy and radiotherapy to reduce tumor size before surgery and to pre-evaluate therapy response. For immunotherapy, this strategy is only starting to be explored [18–21]. The potential of TDLNs is demonstrated in the clinical study of Koster et al in which stage I and II melanoma patients were treated with immune-stimulating TLR9 agonist CpG in the scar of tumor resection, before TDLN resection. Patients displayed stronger tumor-specific immune responses and longer recurrence-free survival than placebo-injected patients [22]. Recently, in a preclinical mouse study, treatments with PD-1 combined with other agents in an adjuvant (the tumor and TDLN are resected) or neoadjuvant setting (the tumor and TDLN are in place) were compared and showed improved outcomes for neoadjuvant treatment [18]. The enhanced tumor-specific T cell response and decrease of metastatic recurrence was attributed to the activation of immune cells within the TME. In light of our findings, it is conceivable that the TDLNs, which were also surgically resected in the adjuvant group, were responsible for this, but this was not taken into account. Sentinel LN resection is still common clinical practice in many forms of cancer to determine disease staging and consecutive therapy options, and when metastases are detectable in the sentinel LN, resection of several, if not all, draining LNs is often performed. There is ongoing debate on the value of this strategy, with regard to

predictive value, metastasis risk, and consequences for treatment options [23]. However, the immunological implications of LN resection are not evaluated here. We now show that ignoring a potential positive role of TDLNs in immunotherapy for cancer should be studied with care in clinical settings.

METHODS

Mice and cell lines

C57BL/6 mice and BALB/c mice were purchased from Charles River and housed under specified pathogen-free conditions in animal facilities of Leiden University Medical Center. MC38 and CT26 cells were cultured in IMDM (Lonza) containing 8% FCS (Greiner), 100 IU/ml penicillin/streptomycin (Gibco), 2 μM glutamin (Gibco), and 25 μM 2-mercaptoethanol. Cell lines were mycoplasma tested and MAP tested before the start of experiments.

Tumor inoculation

Tumors were inoculated by subcutaneous injection in the right flank of 250,000 MC38 cells or 100,000 CT26 cells in 100 μl PBS. Tumor outgrowth was measured in 3 dimensions, until mice had to be sacrificed due to tumor burden, according to local ethical guidelines.

Treatments

Tumor-bearing mice were treated on days 7 and 10 after tumor inoculation by intraperitoneal injection of 200 μg PD-1–blocking Ab (clone RMP1-14 from Bio X Cell or BioLegend) or PD-L1– blocking Ab (clone 10F.9G2, Bio X Cell), or peritumoral subcutaneous injection of 50 μg PD-1–blocking Ab (clone RMP1-14, Bio X Cell). Tumor size was checked 2 or 3 times a week and measured in 3 dimen-sions. FTY720 treatment was given intraperitoneally, 25 μg in saline, on days 6, 8, 10, 13, 16, and 20. Retention of lymphocytes in lymphoid organs was confirmed on day 16 (before subsequent injection) in peripheral blood by flow cytometry (Supplemental Figure 2B).

Surgical resection

Mice received buprenorphine (Temgesic) painkiller preoperatively (3 μg per mouse, subcutaneously), after which they were anesthetized with isoflurane inhalation and the right flank was subsequently shaved. Small incisions were made in the groin area (for inguinal) and armpit (for axillary). Inguinal or axillary LNs were located using blunt forceps and resected using sharp forceps. Control animals were mock resected; similar incisions were made but LNs were left in place. Incisions were sutured using Novosyn Quick suture 6/0 (B. Braun), and mice were placed back in their cage.

Flow cytometry

Cell surface staining was performed using the following Abs: CD8α (clone 53-6.7), CD3ε (clone 145-2c11), CD11b (clone M1/70), CD45.2 (clone 104), and PD-L1 (clone MIH5). Dead cells were excluded based on 7-aminoactinomycin D (Invitrogen). After surface staining, cells were fixed with Foxp3 staining kit (eBioscience), and cells were stained with Ki67 (clone B56) and T-bet (clone eBio4B10). Examples of gating strategies are depicted in Supplemental Figure 3. Samples were analyzed with LSR II cytometer (BD Biosciences) using FACSDiva software (BD Biosciences) and FlowJo software (Tree Star Inc). For flow cytometry analyses of TME, mice were perfused with PBS/EDTA (2 μM) to exclude blood content. Tumors were isolated, minced with scalpels, and incubated with 2.5 mg/ml Liberase TL (Roche) for 20 minutes at 37°C, and single-cell suspensions were made using 70-μm cell strainers (BD Biosciences).

Histology

MC38-bearing mice were left untreated or received 200 μg of PD-1 Ab intraperitoneally on day 7 after tumor inoculation. Inguinal TDLNs and NDLNs were isolated 3 days later and fixed in formalin dehydrated in series of increasing amounts of ethanol. Tissue was embedded in paraffin, sequentially sectioned, and stained with an H&E. Photos were taken using an Olympus DX51 light microscope and Olympus cellSens software. From each organ, the largest section was chosen.

Data availability

All data are available from the corresponding authors upon reasonable request.

Statistics. GraphPad Prism 7 software was used for all statistical analyses. The means of groups were compared using t test or ANOVA (depending on how many groups, and survival differences in Kaplan-Meier curves were analyzed by log-rank test; all tests were 2-tailed. Differences were considered statistically significant at P value less than 0.05 (*$P < 0.05$, **$P < 0.01$, ***$P < 0.005$). For flow cytometry analysis of TME, Grubbs' test for outliers was performed on tumor size within their groups, and significant outliers were removed from all analyses.

Study approval. All animal experimentations were approved by and conducted according to guidelines of the Netherlands Association of Animal Experimentation Committees (Central Animal Testing Commission, the Hague, the Netherlands).

Author contributions

MFF, MS, PK, and MGMC performed experiments and acquired and analyzed data. MFF, MS, LJACH, MK, TVH, and FO interpreted data. MFF, TVH, and FO conceived and

designed the study. MFF, MS, TVH, and FO wrote the manuscript. All authors contributed to reviewing and revising the manuscript.

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8

General discussion

SUMMARY OF MAIN OBSERVATIONS

Although the role of endothelial endoglin expression has been extensively been studied and related to tumor progression, the role of endoglin expression on non-endothelial cells has only recently drawn significant attention. The role of endoglin beyond the endothelium is the central theme of the studies described in this thesis. The current scientific view of endoglin beyond the endothelium is summarized in **Chapter 2.** Reviewing recent literature on the role of non-endothelial endoglin expression revealed that this is still unclear and that many contradictory results have been published. However, endoglin seems to be expressed particularly, but not exclusively, on cells sensitive to Transforming Growth Factor β (TGF-β), like fibroblasts, monocytes, regulatory T cells, and some tumor cells. **Chapter 3** shows that endoglin is expressed on Cancer Associated Fibroblasts (CAFs) at the invasive front of colorectal cancer (CRC) tissues, where it regulates invasion and stimulates tumor metastasis. Surprisingly, we found an opposite role for endoglin expression on fibroblasts in the early stages of CRC carcinogenesis as shown in **Chapter 4**. Fibroblast specific deletion of endoglin led to an increased number of colonic adenomas. Interestingly, this was accompanied by altered myeloid responses within the intestine. To further investigate the endoglin expressing CAFs in a more advanced tumor model, a pancreatic cancer mouse model was used in **Chapter 5.** These data showed that targeting of endoglin expressing cells in the tumor microenvironment does not inhibit tumor growth. To further explore the crosstalk between endoglin and immunomodulatory molecules, we investigated a novel therapeutic strategy by targeting endoglin with the endoglin neutralizing TRC105 combined with an antibody against the immune checkpoint inhibitor programmed cell death (PD)-1 in **Chapter 6**. In addition to increased therapeutic efficiency, these data also revealed an endoglin expressing subset of Tregs in the tumor microenvironment, which can be targeted by the endoglin neutralizing antibody TRC105. Targeting endoglin-expressing Tregs enhanced the effect of PD1 checkpoint inhibitor immunotherapy. Finally, in **Chapter 7**, we investigated the role of the tumor-draining lymph nodes during PD-1/PD-L1 checkpoint inhibitor therapy. These data revealed that tumor draining lymph nodes play a pivotal role during immunotherapy, strengthening the current view on the application of neoadjuvant immunotherapy in cancer treatment.

Endoglin

Endoglin was initially identified in 1985, expressed on a pre-B leukemia cell line [1]. Most later studies, however, were almost exclusively focused on the role of endoglin in angiogenesis. Endoglin is highly expressed by activated endothelial cells and plays a crucial role in (developmental) angiogenesis [2]. Loss of endoglin in mice results in an embryonic lethal phenotype around embryonic day 10.5, due to impaired

vascular and cardiac development [3, 4]. Endoglin is a homodimeric transmembrane receptor composed of disulfide bond-linked subunits of 95 kDa [5]. Endoglin has a short cytoplasmic domain, which exposes its co-receptor function [6]. Therefore, it requires additional receptors to induce signaling. Activation of the activin receptor-like kinase (ALK)1 and ALK5 pathways leads to the downstream phosphorylation of the smad1/5/8 pathway, resulting in the transcription of distinctive target genes. Interestingly endothelial endoglin expression can be regulated by TGF-β, bone morphogenetic protein (BMP)-9 [7], and hypoxia [8]. As discussed in **Chapter 2**, more recent scientific work has shown endoglin expression on a variety of cells. Although studies of endoglin on cells beyond the endothelium have increasingly been published, contradictory results have been reported, which might partially be explained by the experimental setup and culture conditions. For example, in **Chapter 3**, we show that endoglin knockout in cultured fibroblasts results in a senescent-like phenotype implying that fibroblasts need endoglin to survive in cell culture. Furthermore, the vast majority of cultured fibroblasts express endoglin, whereas in normal tissue, endoglin expression is low to non-detectable. This suggests that endoglin is hard to study in cultured cells.

Endoglin, a negative regulator of the TGF-β pathway?

The main cell types that express endoglin are endothelial cells, pro-B-Cells, a subset of monocytes, regulatory T-cells (Tregs), keratinocytes, fibroblasts including CAFs, mesenchymal stromal cells (MSCs), and some epithelial cells. Many, if not all, of these cell types, are dependent on TGF-β for their differentiation or cell maintenance. As indicated above, endoglin expression in endothelial cells can be regulated by its ligands and hypoxia, whereas for other cells this has not yet been established. In endothelial cells endoglin has been reported to act as a negative regulator of the TGF-β/ALK5 pathway. Stimulation of the endoglin dependent/ALK1 signaling pathway, indirectly inhibits the TGF-β ALK5 signaling pathway, thereby stimulating endothelial cell proliferation [9]. Next to the endoglin expressing CAFs, in **Chapter 6** we show an endoglin expressing subset of Tregs. Interestingly, in contrast to the suppressive signal mediated by the TGF-β in T-cells, cross-linking of endoglin substantially enhanced T-cell proliferation, indicating that endoglin by itself mediates signal transduction via activation of ERK 1/2 leading to T-cell proliferation [10].

In CAFs, we have shown that inhibiting endoglin signaling prevents the invasive behavior of CAFs. Furthermore, targeting endoglin signaling resulted in decreased experimental liver metastasis in a mouse model, suggesting that endoglin contributes to the invasive behavior of CAFs. In humans, endoglin expressing CAFs were found to correlate with decreased metastasis-free survival in stage II CRC. Moreover, high levels of TGF-β in patients with colorectal cancer is associated with disease progression [11, 12]. Since targeting of these endoglin expressing CAFs resulted in decreased

metastasis formation, it seems likely that endoglin does not have a negative feedback function in CAFs as shown in other cells. Another hypothesis is that since TGF-β and hypoxia regulate endoglin, the observed decrease in metastasis-free survival is dependent on TGF-β and endoglin as a bystander effect. The exact role of endoglin on CAFs is still unknown and needs to be further investigated in the future.

MSCs as CAF precursors

CAFs are a key component of the tumor microenvironment (TME) with distinctive functions, including matrix deposition and remodeling, signaling interactions with cancer cells, and crosstalk with infiltrating leukocytes [13]. Some studies describe that CAFs can be derived from Mesenchymal Stem Cells (MSCs) [14, 15], which might explain the expression of endoglin on a subset of fibroblasts since endoglin is one of the criteria for defining MSCs [16]. MSC were first identified in the bone marrow and can differentiate into mesenchymal tissue such as bone, adipose tissue and cartilage. [17] More recent research has shown a possible role during inflammation, immune response, wound healing and cancer progression [17]. In our patient samples and mouse models, we observed a subset of, potentially MSC-derived, endoglin expressing CAFs. Previous work has been shown that a significant proportion of CAFs can be derived from the bone marrow [18]. Interestingly, these bone marrow derived fibroblasts expressed higher levels of TGF-β 1 [18]. Moreover, the activation of fibroblasts by TGF-β [19] family ligands promotes the activity of the smad transcription factors, which drives the expression of alpha Smooth Muscle Actine (αSMA), an activation marker of fibroblasts [20]. in **Chapter 3**, we describe that these αSMA positive endoglin positive CAFs are responsible for the metastasis of CRC tumor cells to the liver of the mice. Targeting these CAFs using TRC105 resulted in decreased formation of experimental metastasis in mice. This same reduction in metastatic formation was observed in breast cancer once TRC105 was administrated [21]. Assessing the effects of endoglin in tumor progression using TRC105 will not discriminate between endoglin targeting of CAFS, Endothelial cells or other cells in the TME. Therefore, we explored the effects of endoglin in a fibroblast specific endoglin knockout mouse in **Chapter 4**. Interestingly we found increased tumorigenesis when we genetically deleted endoglin from the fibroblasts. In early stage lesions at the end of the experiment, we observed an increase in the macrophages and neutrophils. This indicates that there is a role for endoglin expressing fibroblasts and immune cell recruitment in the bowel. However, our data showed that depleting neutrophils had no effect on the tumorigenesis and therefore that neutrophils seem not to be responsible for the increased tumorigenesis.
Interestingly, in the first Dextran Sulfate Sodium (DSS) cycle, we found decreased myeloid cells (CD11B+) both in the blood and the intestines. Especially the Ly6C population was significantly reduced upon fibroblast specific endoglin deletion. This

indicates a potential protective role for Ly6C+ population. Others have shown that once the Ly6C high population was introduced into the blood, it restored the DSS induced damage [18], indicating that these Ly6C cells are partially responsible for the intestinal integrity during DSS induces colitis. Further research is necessary to investigate the role of myeloid cells upon fibroblast specific endoglin deletion and increased lesion formation up on Azoxymethane (AOM) DSS induced tumorigenesis. Endoglin on fibroblasts might have a dual role like TGF-β, which acts in a tumor preventative manner during early tumorigenesis and a pro-tumorigenic manner in the late stages of tumor development and metastasis. However, the dual role of TGF-β for fibroblasts is yet to be determined.

Targeting CAFs

CAFs are one of the most abundant cell types in the TME and are thought to have a prominent role in cancer pathogenesis. Mechanistically, CAFs secrete cytokines, chemokines and growth factors and are responsible for Extracellular Matrix (ECM) remodeling enabling cancer cells to invade through the TME [22]. Therefore, CAFs have been an obvious target in solid tumors and extensively studied. As described above, αSMA is a marker for a CAF subset called myofibroblast like CAFs (myCAF). Depletion of all αSMA positive myofibroblasts in a genetic Pancreatic ductal adenocarcinoma (PDAC) mouse model resulted, increased aggressiveness of the tumors, an influx of Tregs, increased epithelial–mesenchymal transition (EMT) marker expression and increased stemness of the pancreatic cancer cells. This resulted in enhanced tumor progression and subsequently reduced mouse survival [23]. Both in CRC and PDAC, high endoglin expressing CAFs were observed, targeting them in CRC reduced metastatic spread. However, in PDAC this did not seem to be the case. In vitro, CAFs isolated from both human and mouse pancreatic tumors showed high endoglin expression both *in-vitro* as *in-vivo*. Once targeted with TRC105, we could not detect any significant differences in αSMA expressing cells. Furthermore, no differences were found in tumor growth and immune influx of mouse bearing pancreatic tumors. Changes in immune cells were observed in CRC upon targeting with TRC105 (**Chapter 6)**. This striking difference between these 2 tumor types, both displaying high endoglin expressing cells might be explained the tumor mutational burden which is higher in the MC38 (CRC) tumor cells compared to the pancreatic tumor cells (KPC-3). This mutational burden leads to more immunogenic antigens that can in turn lead to immune influx and responsiveness to immunotherapy. Therefore, since TRC105 did not improved survival in mice, this needs to be further investigated. As shown in the **Chapter 6**, especially the Fc receptors play an important role in the efficacy of TRC105 in CRC, which was not determined in the pancreatic models. Next to endoglin targeting by TRC105, we used a fibroblast specific endoglin knockout mouse to investigate fibroblast specific endoglin deletion.

This fibroblast-specific endoglin deletion did not affect tumor volume and the cytokine profile in the tumor, suggesting that endoglin expressing CAFs do not contribute to the development and progression of pancreatic cancer. Interestingly others have shown that the depletion of a subset of CAFs expressing Fibroblast Activated Protein (FAP) effectively inhibits pancreatic tumor growth [24]. The depletion of the FAP-expressing cells also increased the anti-tumor effects of α-CTLA-4 and α-PD-L1, indicating that FAP positive cells can cause immune suppression. This indicates that targeting CAF subsets can considerably enhance anti-tumor responses. It remains to be elucidated why targeting of the abundantly present endoglin expressing subsets does not affect tumor growth in PDAC. In addition to targeting CAFs other possible interventions are altering CAF activation or function, CAF normalization, and ECM normalization [25]. The diverse function of CAFs and the interconvertibility of subtypes presents a challenge for CAF targeting agents. Many of the CAF targeting therapies are now undergoing clinical testing in phase I, II and III trials. However, none of the CAF targeted therapies has yet been approved for clinical use [26, 27].

Immune modulation

Immune modulation in cancer refers to a range of therapies aimed to eradicate cancer by using the immune system. There are multiple types of immune modulation, of which the immune checkpoint inhibitors are the most successful and widely accepted in the clinic. they belong to one of the most promising cancer therapies of the 21st century. Reactivating the immune system is an essential tool to target tumors that are not responding to conventional treatment. Currently, a hand full of antibodies have been approved for clinical use. Some of them target the PD-1/PD-L1 interactions and are currently approved to treat melanoma and lung cancer patients. **Chapter 7** describes the essential contribution of the tumor-draining lymph nodes (TDLNs) to therapy efficiency of immunotherapy in mouse models. Once the TDLNs are removed, mice fail to respond to therapy. Interestingly, others have shown that these TDLNs were responsible for enhanced anti-tumor T cell immunity by seeding the tumor with progenitor T cells resulting in improved tumor control [28]. In **Chapter 6** we have shown that TRC105, combined with anti-PD-1, increases the number and activation of T-cells, significantly enhancing the survival of mice induced with colorectal cancer. Interestingly, we observed a significant decrease in the percentage of Tregs accompanied by an influx of CD8+ T-cells. When we investigated endoglin expression on Tregs, we found a subset of endoglin expressing Tregs, signifying that TRC105 can directly bind Tregs and could induce antibody-dependent cellular cytotoxicity (ADCC) in mouse models for CRC. Decreased Tregs were also observed in the blood of patients treated with TRC105 [29].

Taken together, this suggests that TRC105 is not only an anti-angiogenic antibody but possibly also acts as an immunoregulatory antibody by targeting the Tregs within the TME of CRC tumors. With increasing knowledge of endoglin expression beyond the endothelium, it might be that endoglin targeting directly targets other cell types. In cancer, TRC105 has been clinically evaluated. Although encouraging results have been published [30], a recent phase III trial in angiosarcomas (TAPPAS trial) showed no differences in the progression-free survival between the standard of care Votrient and a combination of Votrient and TRC105 in advanced angiosarcoma. Further research is needed to gain more knowledge of responders and non-responders to therapy. Liu and Paauwe et al. [30] give an exciting overview of pre-clinical and clinical targeting of endoglin.
Remarkably, some tumor cells might be directly targeted with TRC105 since many reports have shown endoglin expressing tumor cells. Although endoglin's role on tumor cells is under debate and might be tumor type specific, targeting with TRC105 might induce ADCC in tumor cells. Supporting data has been found in urothelial sarcoma patients treated with TRC105 in which a decrease in circulating tumor cells (CTCs) was observed [29]. Several reports describe the loss of epithelial endoglin to be pro tumorigenic [31, 32]. In contrast, other reports have described a pro-tumorigenic role for endoglin expression on epithelial cancer cells [33][34][35]. As demonstrated, the role of endoglin is not fully understood and needs to be further investigated.

In conclusion, the role of endoglin on CAFs might depend on the stage of the tumor, acting in an anti-tumor manner in the developmental stage of cancer and pro-tumor manner during the metastatic process. Targeting endoglin has shown promising results hampering angiogenesis, metastatic spread, and acting as an immunoregulatory antibody in pre-clinical models. Although TRC105 failed to prove efficacious in a phase III study, the novel approach of targeting endoglin has the potential to become a valuable cancer treatment strategy by targeting multiple cell types that contribute to the TME.

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Appendix

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Nederlandse samenvatting

In Nederland krijgen ca 12.000 mensen de diagnose darmkanker per jaar. Samen met de kankercellen vormen vele andere “normale” cellen en moleculen de tumor. Al deze cellen en moleculen bij elkaar wordt het tumor micromilieu genoemd. In dit proefschrift worden diverse onderzoeken naar de rol van het tumor micromilieu in zowel darmkanker als alvleesklierkanker behandeld, met daarbij speciale aandacht voor een receptor genaamd Endoglin. Dit proefschrift heeft tot doel de rol van Endoglin als een mogelijk doelwit op verschillende celtypes binnen de Tumor Micromilieu (TME) van solide tumoren te ontrafelen.

Na een algemene introductie in **hoofdstuk 1** wordt in **hoofdstuk 2** een overzicht gegeven van de huidige literatuur over Endoglin en haar rol op cellen in het tumor micromilieu. Endoglin wordt in de literatuur niet alleen beschreven op nieuwgevormde bloedvaten in de tumor, maar ook op steuncellen genaamd fibroblasten, de kankercellen zelf en bepaalde immuuncellen genaamd macrofagen. De rol van Endoglin op deze cellen is controversieel en er zijn veel tegenstrijdige bevindingen over de uiteindelijke rol van Endoglin. Echter hebben de cellen die Endoglin tot expressie brengen allemaal iets gemeen, namelijk dat ze voorkomen in een omgeving met veel TGF-β. TGF-β is een groeifactor die een remmende werking heeft op tumorcellen in de vroege fase van kanker. Echter naarmate de kanker vordert heeft TGF-β een stimulerend effect op tumorgroei. TGF-β zorgt voor een tumor micromilieu dat ervoor zorgt dat de kankercellen overleven door het aantrekken van fibroblasten en aan het immuunsysteem ontsnappen. Endoglin maakt onderdeel uit van de TGF-β signaleringsroute.

In **hoofdstuk 3** is onderzocht wat de rol van Endoglin is op fibroblasten in darmkanker. In dit hoofdstuk beschrijven we dat fibroblasten die Endoglin tot expressie brengen zorgen voor een verhoogde kans op uitzaaiing van de darmkankercellen naar de lever. We hebben dit onderzocht op verschillende manieren. In kweekbuisjes hebben we laten zien dat tumorcellen sneller naar fibroblasten migreren met Endoglin dan naar fibroblasten zonder Endoglin. Deze fibroblasten werden vervolgens samen met kankercellen geïnjecteerd in zebravis embryo’s, wat resulteerde in een verhoogde migratie van tumorcellen richting de lever van de vis. Vervolgens hebben we in muizen getest of het toedienen van een medicijn tegen Endoglin de vorming van uitzaaiingen van darmkanker in de lever kon voorkomen. Deze verschillende dierproeven suggereren dat Endoglin een belangrijke rol speelt op fibroblasten tijdens de uitzaaien van darmkanker naar de lever. De volgende stap zou zijn om dit te verifiëren in mensen.

Omdat fibroblast specifieke Endoglin expressie uit ons onderzoek in hoofdstuk 3 bleek een belangrijke rol te spelen bij de uitzaaiing van kanker hebben we dit verder onderzocht in **hoofdstuk 4.** Endoglin werd selectief op fibroblasten genetisch uitgeschakeld (knockout) waardoor Endoglin niet meer tot expressie gebracht kan worden op deze cellen. Door gebruik te maken van een vroeg stadium darmkankermodel uitgaande van een darmontsteking (colitis) werden tot onze verrassing meer tumoren waargenomen in de muizen zonder Endoglin. In deze tumoren werd een verhoogd aantal neutrofielen (ontstekingscellen) gevonden. Om te onderzoeken of deze neutrofielen betrokken zijn bij de formatie van meer tumoren in ons muismodel hebben we de cellen gedepleteerd (verwijderd). Echter bleek dat neutrofielen niet verantwoordelijk waren voor de verhoogde tumor vorming. Uit vervolgexperimenten bleek dat tijdens de eerste episode van darmontsteking een verminderde hoeveelheid "myeloïde" afweercellen gevonden in zowel de darmen als in het bloed van de muis. Dit zou erop kunnen wijzen dat er een vertraagde immuunrespons plaats vindt in de muizen zonder endoglin. Samenvattend laten de resultaten in dit hoofdstuk laten zien dat Endoglin op fibroblasten een belangrijke rol speelt bij de formatie van ontsteking-gemedieerde tumoren, mogelijk door een vertraagde immuunrespons.

De rol van Endoglin werd verder onderzocht in een model voor alvleeskliertumoren in **hoofdstuk 5.** Ondanks dat Endoglin hoog tot expressie komt in deze tumoren bleek dat in dit model geen therapeutisch effect te zien was van het medicijn gericht tegen Endoglin (TRC105), zoals we met meer conventionele therapieën hebben gezien. De rol van Endoglin in dit model is dus mogelijk beperkter, moeilijker te bestuderen of er zijn additionele mechanismen die een rol spelen, waardoor de therapie niet goed werkt.

Hoewel we in het eerste gedeelte van dit proefschrift voornamelijk hebben gekeken naar de rol van Endoglin op fibroblasten hebben we ons in de volgende hoofdstukken voornamelijk gefocust op de interactie met het afweersysteem. In **hoofdstuk 6** hebben we de combinatie TRC105 en immunotherapie (in de vorm van anti-PD-1 antilichamen) onderzocht in verschillende darmkanker muismodellen. We vonden we dat de combinatie van Endoglin medicatie en immunotherapie significant beter werkte dan beide monotherapieën. In de tumor van de muizen behandeld met TRC105 vonden we een afname in het aantal afweer onderdrukkende regulatoire T-cellen. Door deze regulatoire T-cellen dus uit de tumor te verwijderen werd de tumor beter aangevallen door het immuunsysteem, zeker in combinatie met anti-PD-1 welke het immuunsysteem verder motiveert om de tumorcellen aan te vallen. De antitumor effecten van het Endoglin medicijn bleken voornamelijk veroorzaakt te worden door antilichaam gemediteerde cellulaire doding (ADCC). Dit gebeurt in

combinatie met de aanwezige CD8+ Cytotoxische T-afweercellen, welke verantwoordelijk zijn voor het doden van de tumorcellen. De combinatie van TRC105 en anti-PD-1 is dus superieur aan de monotherapieën door de aanwezigheid van CD8+ T-cellen en ADCC in muizen met darmkanker. Deze CD8+ T-cellen worden geactiveerd in de lokale lymfeklieren om de tumor te herkennen.

In **hoofdstuk 7** hebben we naar de rol van deze lokale lymfeklieren gekeken tijdens immunotherapie. Lymfeklieren spelen een belangrijke rol bij het activeren van T-cellen. De rol van lokale lymfeklieren was echter nooit onderzocht. Muizen werden ingespoten met darmkankercellen, de lokale lymfeklieren werden vervolgens verwijderd en immunotherapie werd gestart. Bij muizen waar de lymfeklieren werden verwijderd groeide de tumor significant sneller dan bij muizen die de lokale lymfeklieren nog hadden. Deze bevindingen bevestigen dus hoe belangrijk de lokale lymfeklieren zijn bij de behandeling van kanker met immunotherapie, met mogelijk ook groet implicatie voor patiënten die chirurgie ondergaan.

Tenslotte wordt in **hoofdstuk 8** een algemene discussie gegeven, waarbij boven beschreven hoofdstukken geïntegreerd besproken worden. De conclusie is dat de rol van Endoglin op fibroblasten mogelijk een verschillende rol speelt tijdens de verschillende tumorstadia. Het aanvallen van Endoglin met het medicijn TRC105 heeft bemoedigende resultaten laten zien bij zowel uitzaaiing van de tumor, en als een mogelijk immunoregulatoire therapie. Echter is er meer onderzoek nodig en voornamelijk naar de rol van Endoglin in mensen en het behandelen met het Endoglin medicijn TRC105.

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Curriculum vitae

Mark Johannes Adrianus Schoonderwoerd was born on May 18th, 1990, in Utrecht, The Netherlands. After graduating in 2007 from the Rientjes MAVO in Maarssen, he started his MBO study which he finished in 2011. Next he started Clinical Laboratory science/ Medical technology at the university of applied sciences (Hogeschool) Leiden, which he completed in 2014. Mark started his master's animal biology and disease models at the Leiden university which he completed in 2016. Directly thereafter Mark started his PhD studies in the group of Dr. Luuk Hawinkels and co-supervised by Dr. Marieke Fransen and Prof. Dr. James Hardwick at the Leiden University Medical Center. During his PhD studies, Mark received the "Best abstract award" at the Dutch Experimental Gastroenterology and Hepatology (DEGH) meeting in 2017. During his PhD studies he co-founded the startup company Levels Diagnostics, aimed to limit the unnecessary use of antibiotics. Currently Levels is having 4 employees and several externally funded projects investigating the use of biomarkers in multiple disease types. In October 2020 Mark started as medical science liaison at Bristol Myers Squibb responsible for melanoma, renal cell cancer and bladder cancer in the northern part of the Netherlands. In this role he is the medical expert in the listed tumor types supporting multiple clinical studies and novel treatment strategies for cancer patients.

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