Blocking angiopoietin-2 promotes vascular damage and growth inhibition in mouse tumors treated with small doses of radiation

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Running title: Ang2 blocking improves radiotherapy efficacy

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Abstract

Abnormal vasculature in tumors leads to poor tissue perfusion and cytostatic drug delivery. Although drugs inducing vascular normalization, e.g., angiopoietin-2 (Ang2)-blocking antibodies, have shown promising results in preclinical tumor models, clinical studies have so far shown only little efficacy. Since Ang2 is known to play a protective role in stressed endothelial cells, we tested here if Ang2 blocking could enhance radiation-induced tumor vascular damage. Tumor-bearing mice were treated with anti-Ang2 antibodies every three or four days starting three days before 3x2 Gy or 4x0.5 Gy whole-body or tumor-focused radiation. Combination treatment with anti-Ang2 and radiation improved tumor growth inhibition and extended the survival of mice with melanoma or colorectal tumors. Single-cell RNA sequencing revealed that Ang2 blocking rescued radiation-induced decreases in T cells and cells of the monocyte/macrophage lineage. In addition, anti-Ang2 enhanced radiation-induced apoptosis in cultured endothelial cells. In vivo, combination treatment decreased tumor vasculature and increased tumor necrosis in comparison with tumors treated with monotherapies. These results suggest that a combination of Ang2 blocking antibodies with radiation increases tumor growth inhibition and extends the survival of tumor-bearing mice.

Significance: Findings offer a preclinical rationale for further testing of the use of radiation in combination with Ang2 blocking antibodies to improve the overall outcome of cancer treatment.

Introduction

Almost half of all cancer patients receive radiation therapy as a curative or palliative treatment. Although radiation is commonly used in the treatment of many types of tumors, for example breast, lung, brain, prostate and rectal cancers (1), several tumor types show resistance to radiation therapy, compromising treatment efficacy. In addition, radiation sensitivity of the surrounding healthy tissues often limits the use of radiation therapy.

Radiation damages not only tumor cells but also cells forming the tumor microenvironment, including immune and endothelial cells. Previous studies have shown that the radiation-induced vascular damage occurs mostly in immature tumor vessels (2). Low doses of radiation have been shown to stimulate vessel formation (2), whereas high doses of microbeam radiation have been shown to damage preferentially tumor vessels, preserving the normal vasculature (3). The radiation-induced vessel damage increases hypoxia, activating hypoxia-inducible factor 1 (HIF1). This increases the expression of vascular endothelial growth factor (VEGF), which promotes the growth of abnormal vessels in tumors (4, 5).

Tumor vessels are malformed and structurally defective, which leads to their dysfunction, plasma leakage into the tumor stroma, poor tissue perfusion and compromised tissue oxygenation (6-8). The efficacy of radiation depends on a number of factors, of which oxygen concentration in the target tissue is important, since radiation produces highly reactive oxygen radicals that cause DNA damage and cell death (9). Hypoxia in tumor tissue counteracts radiation therapy, and the increased interstitial fluid pressure resulting from leaky tumor vessels has been reported to reduce the delivery of cytostatic drugs to the tumors (10, 11). Angiogenesis inhibitors, including inhibitors of VEGF and VEGF receptors, and vascular disrupting agents, such as combretastatin, have been tested as modifiers of the tumor vasculature in association with radiation therapy (12). Anti-VEGF agents can improve tumor response to radiation, presumably by normalizing the tumor vasculature, and thereby reducing vascular leak, tumor hypoxia, and radiation resistance (2, 12).

Besides VEGF and its receptors, the endothelial angiopoietin (Ang) growth factors and their Tie receptors regulate physiological and pathological angiogenesis and vascular remodeling (13). The constitutively expressed ligand Ang1 acts as a stabilizer of blood vessels (14) and has been shown to protect endothelial cells from radiation-induced apoptosis *in vitro* (15). In contrast, Ang2 is a dual, inducible and context-dependent autocrine modulator, which is involved in vessel destabilization (13). However, previous findings have indicated that Ang2 protects stressed endothelial cells from apoptosis in

several tumor models by activating Tie2, thereby limiting the anti-vascular effects of VEGF inhibition (16, 17).

Tissue hypoxia and proinflammatory signals are known to induce Ang2 expression in endothelial cells (13, 18). Ang2 levels are increased in many types of human tumors, for example in colorectal cancer (13). In some cases, such as in melanoma, non-small-cell lung cancer and neuroblastoma, induction of Ang2 expression has been shown to correlate with disease progression (19-21). *In vivo*, a single 10 gray (Gy) dose of radiation increased Ang2 mRNA and protein in brain tissue, while decreasing VEGF, Tie2 and Ang1 levels (22). Monoclonal antibodies that neutralize Ang2 and VEGF tend to normalize tumor blood vessels and inhibit tumor growth (23). In addition to Ang2 blocking, Tie1 deletion has also been shown to decrease tumor growth (24). However, therapeutic efficacy of Ang2 blocking antibodies in clinical use has been so far limited (13, 25, 26).

In this study, we report the discovery that Ang2 blocking in combination with small doses of radiation leads to increased tumor vascular damage and to decreased tumor growth.

Materials and Methods

Mice and tumor models. 18–20-week-old male C57BL/6JRj mice from Janvier and the tumor cell lines B16-F0 (a generous gift from Dr. Sirpa Jalkanen in 2012) and MC38-GFP (a generous gift from Dr. Jeffrey Schlom in 2013) were used for the mouse allograft experiments. 20-week-old male and female NOD *scid* gamma mice (NSG; NOD.Cg-Prkdc^{scid} Il2rg$^{tm1/wjl}$/SzJ, 005557) from the Jackson Laboratory were injected with human LS174T cells (a generous gift from Dr. Ragnhild A. Lothe and Dr. Olli Kallioniemi in 2015) in the tumor xenograft experiments. Due to the radiation sensitivity of the NSG mice, they were euthanized five days after the last dose of radiation. All experiments were approved by the National Animal Experiment Board in Finland (ESAVI/6306/04.10.07/2016 and ESAVI/7945/04.10.07/2017).

LS174T cells passage 6-10 were cultured in DMEM-F12 (BE04-687F/U1, Lonza), containing penicillin/streptomycin and 10 % fetal bovine serum (S181B-500, Biowest), and MC38-GFP and B16-F0 cells, both in passages 6-10, in DMEM (BE12-707F, Lonza) containing 2-mM L-glutamine (25-005-Cl, Corning), penicillin/streptomycin and 10% fetal bovine serum. Cell lines were not authenticated or tested for *Mycoplasma*. For *in vivo* tumor experiments, 1 x 10^6 tumor cells (passage 6-10) were injected subcutaneously. Tumor growth was monitored by manual measurements with a caliber in mice under inhalation anesthesia (isoflurane). Tumor volume was calculated as length x width x thickness in mm^3. Tumor growth time (TGT) represents the time in days starting from the first day of treatment, until the tumor reached the total volume of 2500 mm^3 (B16-F0) or 2000 mm^3 (MC38). Tumor growth delay (TGD) was calculated as: $TGT_{treatment} - TGT_{control\ no\ radiation}$.

Antibody injections and radiation. When the tumors formed, their volumes were measured and mice were randomized into the different treatment groups according to their tumor size. As previously reported (27, 28), intraperitoneal injections of 10 mg/kg of Ang2 blocking antibody (MEDI3617 or 3.19.3) or isotype control antibody were started three days before the first radiation dose. The radiation source was the gamma irradiator OB29/4 (STS, Braunschweig, Germany, isotype Cs137) at the dose of 1.4 Gy/min.

Histology and immunohistochemistry. Hematoxylin and eosin (H&E) staining was used to analyze necrotic areas in tumor sections. Immunohistochemical stainings were done using antibodies for endomucin (sc-65495, 1:500, Santa Cruz), CD31 (553370, 1:250, BD Pharmigen), smooth muscle actin (α-SMA, C6198, 1:500, Sigma-Aldrich), Erg (ab133264, 1:250, abcam), laminin (RB-082-AO, 1:500, Thermo Scientific), Glut-1 (07-1401, 1:500, Merck) and Caix (ab15086/ab108351, 1:500, abcam), followed by Alexa Fluor-conjugated secondary antibodies (Molecular Probes). Deparaffinization employed the xylene substitute (Tissue-Tek, Tissue-Clear, 1466, SAKURA) for 3x5 min plus rehydration in an alcohol series (2 x 100%, 2 x 96%, 1 x 70% and 1 x 50% for 3 min each). After heat-

induced epitope retrieval, the sections were blocked for endogenous peroxidase activity using H_2O_2 and for nonspecific binding using TNB (NEL700001KT, PerkinElmer). Primary antibodies were incubated in TNB overnight at +4°C. After TNT washes, the sections were incubated in the appropriate species-specific ImmPRESS kit (MP-7401, MP-7402, MP-7405, VECTOR laboratories) secondary antibodies for 30 min, washed with TNT and PBS, treated with AEC for 10 min, hematoxylin stained and mounted with Aquatex (1.08562.0050, Millipore). Images were scanned using 3DHISTECH Pannoramic 250 FLASH II digital slide scanner, and unprocessed digital images were analyzed using Pannoramic 250 Scanner Software. The images were modified to optimize visualization using Fiji software.

Analysis of Caspase-3/7–positive cells and cell cycle phase. Human umbilical vein endothelial cells (HUVEC) passage 6-10 were cultured on 6-well plates coated with gelatin. 24 h after subculture, the growth medium was replaced with medium supplemented with antibodies (MEDI3617 or isotype control antibody, 2 μg/ml) plus IncuCyte Caspase-3/7 reagent (4440, 4704, 1:1000, Essen BioScience) for 15 min, followed by radiation with 4 Gy x 1. Images were taken 24 h and 48 h later with Thermo Fisher EVOS FL inverted epifluorescence microscope. The original images were processed and analyzed using Fiji software. For cell cycle analysis, HUVECs were cultured for 24 h in endothelial growth medium supplemented with either anti-Ang2 (MEDI3617) or control antibody, and then radiated with a 4 Gy single radiation dose. On the following day, cells were detached with a brief trypsin treatment (Trypsin-EDTA, 25200056, Thermo Fisher Scientific) and fixed with cold 70% ethanol. After at least four-hour incubation in -20^{O}C, cells were washed with HBSS (14175-053, Gibco) + 2% FBS once, treated with 0.1 mg RNase A at +37^{O}C for 30 min and then stained with 20 μg of propidium iodide for 30 min in RT. Cells were analyzed with BD Accuri^{TM} C6 Flow Cytometer, and the cell cycle phases were determined with FlowJo.

Single-cell RNA sequencing and data analysis. B16-F0 tumor cells were injected into C57BL/6Jrj mice, and six days later, the mice were randomized into the treatment groups.

Anti-Ang2 was dosed every 3 days starting from day 6, and 2 Gy daily doses of tumor focused-radiation were given on days 9–11. Five days after the last radiation dose, the mice were euthanized and tumors were harvested for single-cell RNA sequencing (scRNA seq). Each sample was pooled from 2-6 tumors. Tumors were dissociated in HBSS (14175-053, Gibco) supplemented with 1 mg/mL collagenase type 1 (LS004196, Worthington), 1 mg/mL collagenase H (11074032001, Roche), 4 mg/mL dispase II (04942078001, Sigma) and 1000 U/mL benzonase (sc-202391, ChemCruz) for 30 min at +37°C, followed by 15 min incubation with Trypsin-EDTA (25200056, Thermo Fisher Scientific) at +37°C and red blood cell lysis buffer (ACK Lysing Buffer, A1049201, Gibco) for 10 minutes in RT. Cells in 0.04% BSA-HBSS were analyzed using the Chromium Single Cell 3′RNA-sequencing system (10x Genomics, Pleasanton, CA, USA) with the Reagent Kit v3 according to the manufacturer's instructions. Multiplex libraries were sequenced on the Illumina NovaSeq 6000 system. The Cell Ranger v 2.1.1 mkfastq and count pipelines (10x Genomics, Pleasanton, CA, USA) were used to demultiplex and convert Chromium single-cell 3' RNA-sequencing barcodes and to read data to FASTQ files and generate aligned reads and gene–cell matrices. Reads were aligned to the mouse reference genome mm10. Seurat R package 3.1.1 was used for quality control, filtering, and analysis of the data. Cells were filtered based on UMI counts and the percentage of mitochondrial genes. Cells with more than 10–15 % of mitochondrial genes were filtered out. The expression matrix was further filtered by removing genes with expression in less than three cells and cells with less than 200 expressed genes. The final dataset was down-sampled to include 2,000 cells per sample. To be able to compare the samples to each other, we performed a principal component analysis (PCA) to identify shared correlation structures and aligned the dimensions using dynamic time warping. After this, we performed clustering using UMAP and set the resolution at 0.5.

RNA extraction and qPCR analysis. RNA of dissociated melanoma tumors was extracted with NucleoSpin RNA II kit (Macherey-Nagel #740955) according to the manufacturer's instructions. Cells for RNA extraction were harvested from the samples used for scRNA seq. cDNA was synthesized with cDNA Synthesis Kit (Thermo Fischer Scientific #4368814) according to the manufacturer's instructions. Gene expression analysis was

performed by quantitative PCR using following primers: Cd4_fw: 5' TAGCAACTCTAAGGTCTCTAAC, Cd4_rec: 5'GATAGCTGTGCTCTGAAAA, Cd8_fw: 5'CCTTCAGAAAGTGAACTCTAC, Cd8_rev: 5'CCAGATGTAAATATCACGGC. Mouse *Gapdh* was used as a housekeeping gene.

Statistical Analyses. For each *in vivo* analysis, data from all mice in a treatment group was pooled, analyzed using the Mann–Whitney test and presented as mean +/- standard error of mean (SEM). *In vitro* experiments were repeated two to four times, data was pooled from all experiments, analyzed using the Mann–Whitney test and presented as mean +/- standard error of mean (SEM). GraphPad PRISM 7 was used for the statistical analyses. Statistical significance, marked by p-value * < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001, is indicated in the figure legends.

Results

Ang2 is critical for the survival of endothelial cells after radiation. Since Ang2 has been shown to have a protective role in stressed endothelial cells (16), we speculated that Ang2 could also be critical for the survival of endothelial cells after radiation. To test this, we exposed cultured HUVECs to 2 Gy dose of radiation on two consecutive days and analyzed ANG2 RNA 24 h after radiation. Although radiation increased ANG2 RNA only slightly, radiation in combination with Ang2 blocking increased ANG2 RNA very significantly, reflecting stress in the endothelial cells induced by this combination treatment (**Fig. 1A**). To study the possible effect of Ang2 on endothelial cell survival after radiation, we next supplemented the endothelial growth medium with Ang2 blocking (MEDI3617) or isotype control antibody, plus the IncuCyte Caspase-3/7 reagent, radiated the cultures with single dose of 4 Gy radiation, and 24 and 48 h thereafter, determined the percentage of caspase-3/7–positive apoptotic cells. We found that the combination caused significantly more apoptosis than either treatment alone (**Fig. 1B**). However, γH2AX staining for detection of DNA damage did not indicate differences between radiation and anti-Ang2 plus radiation treated HUVECs, indicating that Ang2 blocking does not sensitize endothelial cells to radiation-induced DNA damage (**Fig. 1D, Supplementary Fig. S1A**). To analyze how the combination treatment affects endothelial cell proliferation, HUVECs

were treated for 24 h with the antibodies and subjected to a 4 Gy radiation dose, followed by staining for the Ki67 on the next day. The results showed that the anti-Ang2 plus radiation treated cultures had more cells in the G_0-phase (Ki67 negative) than cultures treated with either radiation or antibodies alone (**Fig. 1C, D**). This result was further supported by flow cytometry analysis of propidium iodide (PI) stained HUVECs, which showed that there were more endothelial cells in the G_0/G_1 cell cycle phase in the combination treated cultures than in the other cultures (**Fig. 1E, Supplementary Fig. S1B-E**). These results indicated that anti-Ang2 increases radiation-induced endothelial cell cycle arrest and cell death.

Low doses of radiation in combination with Ang2 blocking inhibit melanoma tumor growth. To test if Ang2 blocking plus radiation-induced endothelial cell death could lead to tumor growth inhibition, we tested the effect of combination treatment to subcutaneous B16-F0 melanoma allografts in C57BL/6JRj mice. Anti-Ang2 (MEDI3617) injections were started five days after the tumor cell implantation and continued than every 3 days, and whole-body radiation was given on days 8-10, when the tumors had grown to an average size of 140 mm^3. Three daily doses of 2 Gy whole-body radiation induced only a trend of tumor growth inhibition (**Fig. 2A**). This effect was of similar magnitude as the effect of anti-Ang2 antibodies (**Fig. 2A**). Although the monotherapies did not show significant tumor growth inhibition, the tumor-bearing mice subjected to a combination treatment with anti-Ang2 plus radiation showed a significant improvement of tumor growth inhibition (**Fig. 2A**).

Anti-Ang2 treatment combined with radiation extends the survival of melanoma tumor-bearing mice. To test the long-term effects of the combined anti-Ang2 plus radiation treatment, the B16-F0 allografts in the 4 treatment groups were allowed to grow until they reached a total tumor volume of 2500 mm^3, when the mice were euthanized. However, since the tumors in the combination treatment group did not seem to progress to meet the euthanization criteria, antibody treatment was discontinued on day 44, when all mice in the other treatment groups had already been euthanized. The termination of Ang2

antibody treatment accelerated tumor growth in the combination treatment group, and by day 63, all tumor volumes in the combination treatment group had reached a volume of 2500 mm^3 (**Fig. 2B, D**). The tumor growth delay (TGD) in the anti-Ang2 monotherapy group was on average 5 days, in the radiation monotherapy group 13 days, and in the combination treatment group 34 days (**Fig. 2C**).

We then repeated the experiment by starting the anti-Ang2 (MEDI3617) antibody treatment on day 3 after tumor implantation, when the tumor volume was about 15 mm^3 on average, and whole-body radiation was given on days 6-8. In this experiment, the antibodies were injected every fourth day. Although both monotherapies resulted in a significant tumor growth inhibition, the combination treatment again significantly improved both tumor growth inhibition and host survival (**Fig. 2E-H**). Notably, anti-Ang2 treatment 1) given only three days before radiation and on first radiation day, 2) starting on the first radiation day, or 3) starting on following day of last radiation dose resulted in shorter survival than our standard combination treatment (**Supplementary Fig. S2A-D**). We conclude that for optimal results, anti-Ang2 treatment should be started before radiation and continued thereafter.

Additive effect of anti-Ang2 and radiation in colorectal allografts. In order to study if the effect of the combination treatment could be reproduced in another tumor type, we next analyzed growth of subcutaneous MC38 colorectal carcinoma (CRC) allografts subjected to the treatments. To independently confirm our findings, we further used another monoclonal Ang2 blocking antibody (3.19.3) (28). The Ang2 blocking antibodies were injected every third day starting on day eight, when the tumor volume was 25 mm^3 on average, and 2 Gy whole-body radiation doses were given on days 11-13. Radiation monotherapy strongly suppressed MC38 allograft growth and increased the survival of the tumor-bearing mice, whereas anti-Ang2 monotherapy resulted only in a trend of slower tumor growth (**Fig. 3A-D**). Yet, the combination treatment with anti-Ang2 plus radiation significantly increased host survival when compared to the monotherapies (**Fig. 3B, C**). The tumor growth delay in the anti-Ang2 monotherapy group was on average 5 days, in

the radiation monotherapy group 22 days, and in the combination treatment group 32 days (**Fig. 3C**).

Effect of the combination treatment in severely immunodeficient mice. Recent research has indicated that the results of chemotherapy often depend on the adaptive immune response, whereas in the case of radiotherapy its role is less clear (29, 30). In order to investigate if the inhibitory effect of Ang2 blocking in combination with radiation works in severely immunodeficient mice, we injected LS174T cells subcutaneously and allowed the tumors to develop to an average volume of 135 mm^3 (day 16), after which the mice were injected with anti-Ang2 (MEDI3617) or control antibody every third day. Due to the high sensitivity of the NSG mice to radiation, only a 0.5 Gy radiation dose was administered daily over 4 consecutive days starting on day 19. We found that radiation and anti-Ang2 monotherapies decreased tumor growth: the combination treatment significantly increased tumor growth inhibition in the first experiment, but in a repeated experiment only a trend of additional inhibition was found (**Supplementary Fig. S3A-F**). This suggested that adaptive immunity may improve the outcome of the combination treatment.

Anti-Ang2 improves tumor growth inhibition in response to focused radiation. We next tested if the results obtained with whole-body radiation plus anti-Ang2 could be reproduced with tumor-focused radiation (TF-IR). B16-F0 allografts (approximately 80 mm^3) in otherwise lead-shielded mice were radiated with a 2 Gy daily dose on days 12-14. Anti-Ang2 (MEDI3617) injections were started three days before the first radiation dose, and were continued every three days. The results showed that tumor growth delay in anti-Ang2 and radiation monotherapy groups was one and two days respectively, whereas the delay was 13 days in the combination treatment group, indicating that TF-IR increases tumor growth inhibition by anti-Ang2 highly significantly (**Supplementary Fig. S4A-D**). Similar results were obtained in a repeated experiment: anti-Ang2 monotherapy, radiation monotherapy and the combination treatment induced tumor growth delays were 6, 3 and 18 days, respectively (**Supplementary Fig. S4E-H**). Thus, the mice treated with the

combination therapy lived on average 6-8 times longer than mice treated with either monotherapy, even when tumor TF-IR was used.

Ang2 blocking does not sensitize mice to radiation-induced adverse effects. To analyze possible adverse effects of the combination treatment, the wellbeing of the mice was regularly monitored during the experiments. Although one of the most sensitive tissues to radiation-induced damage is the intestine, none of the mice developed diarrhea in any of the experiments. Furthermore, the reduction in body weight in the mice treated with the combination treatment did not significantly differ from that in mice treated with radiation monotherapy 7 or 10 days after the last dose of radiation (**Supplementary Figure 5A-F**). In the whole-body radiation experiments, 7% (3/44) of the radiation monotherapy treated mice and 2% (1/54) of the combination treated mice had to be euthanized based on decreased body weight (> 20%), whereas none of the mice which received tumor-focused radiation met the euthanization criteria. These results indicated that Ang2 blocking did not sensitize the mice to major radiation-induced adverse effects.

Radiation increases vascular pruning induced by anti-Ang2. To see if the combination treatment had affected the tumor vasculature as expected based on the *in vitro* experiments, we studied the tumor blood vessels by immunostaining endothelial cells (endomucin plus CD31), pericytes (NG2) and smooth muscle cells (SMC). Consistent with previous findings (27, 31), the pericyte and SMC coating of tumor vessels was increased by anti-Ang2 in the LS174T and B16-F0 tumors when analyzed five days after the last radiation dose (**Supplementary Fig. S6A-D**). The vascular analysis further indicated that both anti-Ang2 and radiation monotherapy decreased vascular density in the LS174T and B16-F0 tumors, and that the effect of the combination treatment was significantly stronger than the effect of the monotherapies five days after the last dose of radiation (**Fig. 4A-C**). Staining of the endothelial Erg protein and basement membrane laminin confirmed that the combination treatment led to increased loss of vascular endothelium from the tumors (**Supplementary Fig. S6E-J**). A similar effect of the combination treatment was observed in the B16-F0 and MC38 tumors harvested at the experimental endpoint (**Fig. 4D-F**).

Anti-Ang2 treatment rescues radiation-induced loss of inflammatory cells. In order to analyze the tumor microenvironment in mice treated with TF-IR plus anti-Ang2, B16-F0 tumor cells were injected into C57BL/6Jrj mice, and six days later, the mice were randomized to the treatment groups. Anti-Ang2 was dosed every 3 days starting from day 6, and 2 Gy daily doses of tumor-focused radiation were given on days 9-11. Five days after the last radiation dose, the mice were euthanized and tumors were harvested for single-cell RNA sequencing (scRNA seq) (**Fig. 5A**). ScRNA seq analysis of 2000 cells per treatment group revealed less Cd4+ and Cd8+ T cells and cells of the monocyte/macrophage lineage in the TF-IR group than in the non-radiated groups, but not in the combination treatment group (**Fig. 5B, C, Supplementary Fig. S7D**). QPCR from total RNA was consistent with the rescue of the radiation-induced decrease of the Cd4 and Cd8 T cells in the combination treatment group (**Supplementary Fig. S7A, B**). These results indicated that Ang2 blocking protects T cells and monocytes/macrophages from radiation-induced damage. ScRNA seq analysis also revealed that endothelial Ang2 expression was higher in all the treatment groups than in the control group, with highest levels in the combination treated group (**Supplementary Fig. S7C)**.

Increased necrosis in the combination treated tumors. To analyze if anti-Ang2 treatment led to increased tumor tissue hypoxia before radiation, we injected pimonidazole intraperitoneally to the tumor-bearing mice, and stained pimonidazole-thiol adducts of hypoxic cells in the tumor sections. As additional markers of hypoxia, we stained for the hypoxia-inducible proteins carbonic anhydrase IX (Caix) and glucose transporter 1 (Glut1). In tumors isolated before radiation, there was no significant difference in the pimonidazole-thiol adducts, Caix or Glut1 expression between control and anti-Ang2 antibody–treated B16-F0 allografts in either of two different experiments, indicating that the blocking of Ang2 did not increase tumor hypoxia before radiation (**Supplementary Fig. S8A, D**). ScRNA seq analysis of the B16-F10 tumors five days after the radiation showed that the hypoxia markers Caix and Glut1 were expressed in a greater fraction of tumor cells in the TF-IR monotherapy group than in the other treatment groups. This indicated again that Ang2 blocking rescued radiation-induced hypoxia in the melanoma cells (**Supplementary Fig. S8B, C**). This may be due to the decrease of oxygen consumption after cell death in

the combination treatment group, since H&E stainings showed either a trend or significantly more necrosis in the combination treatment group than in the other treatment groups five days after radiation and at mouse termination timepoints (**Fig. 6A-F**).

Discussion

Based on our results, Ang2 seems to have a protective function against radiation-induced endothelial cell damage, and when Ang2 is blocked, radiation leads to enhanced vascular pruning, thus resulting in increased tumor growth inhibition. This effect of the combination treatment was evident in all three tumor models used, and it was associated with increased host survival in both melanoma and CRC models. Importantly, increased survival was also observed in the combination-treated group when tumor-focused radiation was used.

Although we did not detect significantly increased hypoxia before radiation or five days after the last radiation dose in the tumor cells, the anti-Ang2 plus radiation-treated tumors were more necrotic than tumors in the other treatment groups five days after the radiation and at mouse termination. It is possible that our analysis at the selected timepoints does not allow for detection of transient hypoxia upon decrease of tumor vasculature, but in such cases, the hypoxia may be rapidly compensated for by the simultaneous increase in tumor cell death that decreased tumor oxygen consumption.

In our experiments, the anti-Ang2 plus radiation-induced decrease of the tumor vasculature was evident both five days after the last dose of radiation and at the endpoint of the survival experiments. We found that Ang2 activity was critical for endothelial cell survival also in culture, since the anti-Ang2–treated endothelial cells showed more apoptosis and less proliferating cells after radiation than the cultures treated with radiation only. Previous studies have indicated that Ang2 can act as an autocrine endothelial survival factor in stressed conditions (16), an activity that the Ang2 antibodies likely neutralized in the tumor-bearing mice and in cultured endothelial cells.

To improve the overall outcome of cancer therapy, radiation sensitizers have been tested that not only increase the local tumor cell death induced by radiation, but also induce tumor cell death in distant metastases (32). Most of the radiation sensitizers used so far have been chemotherapeutic agents, which reduce proliferating cells in both normal and tumor tissues by inducing DNA damage, inhibiting DNA repair, promoting cell cycle arrest, or apoptosis and re-oxygenation (33). Furthermore, preclinical studies in which radiation has been combined to immune checkpoint blockade have shown promising abscopal effects (34, 35). Several preclinical studies have indicated that VEGF blocking agents combined with radiation can provide additive inhibition of growth in human and murine tumor models (36, 37). Such a concept has been advanced to clinical trials, but it has not yet led to clinical applications (2), although the anti-VEGF antibody plus radiation treatment was well tolerated in both preclinical and in clinical studies (2, 38). In our experiments, blocking Ang2 in combination with small doses of radiation increased the survival of the tumor-bearing mice. Since the blocking of Ang2 has been shown to decrease tumor growth and metastasis in mouse tumor models (31), combining anti-Ang2 treatment with radiation could inhibit tumor growth not only in primary tumors but also in metastases.

Besides anti-VEGF treatments, also drugs targeting the Tie2 signaling pathway have also been tested to improve the effect of radiation therapy (39). Goel *et al*. showed that pre-treatment with vascular endothelial protein tyrosine phosphatase (VE-PTP) inhibitor, which increases the activation of Tie2, decreased breast carcinoma tumor growth and increased tumor doubling time by 2.5 days after a single radiation dose of 20 Gy (39). In our experiments, anti-Ang2 blocking antibodies in combination with radiation delayed tumor growth in a CRC model by 10 days and in a melanoma model on average by 16 days, when compared to radiation monotherapy-induced delay in tumor growth. This indicates, that even very small doses of radiation can reduce tumor growth when combined with the anti-Ang2 blocking treatment. This could be beneficial in the treatment of cancer patients since the side effects of radiation on healthy tissue in the radiation field often limits the radiation dose. Anti-Ang2 could perhaps allow for the use of lower radiation doses, with less side effects and increased tumor growth inhibition.

Currently, there is strong interest in new drug combinations that lead to "synthetic lethality" of tumor cells (40), and this especially concerns pathways that interact with the anti-tumor immune responses. Ang2 serum concentrations have been shown to predict poor survival of patients receiving CTLA4 or PD1 immune checkpoint blocking antibodies, both of which increase Ang2 levels in serum (41). In their paper, Schmittnaegel et al. showed that dual Ang2 and VEGF inhibition in combination with the anti-PD-1 immune checkpoint inhibitor results in improved tumor growth control (42). The authors concluded that immune cells are essential in determining the outcome of anti-angiogenic treatments. In our experiments, both the Ang2 blocking antibody and TF-IR increased endothelial Ang2 expression *in vivo*, with an additive effect in the combination treatment group. In addition, at the same time, the blocking antibody inhibited the TF-IR-induced decrease in the tumor infiltrating T-cells, especially Cd8 T cells, and monocytes/macrophages, supporting the findings of Schmittnaegel et al. (42).

Reasons for the increased recruitment of immune cells to the tumors likely include immune-attracting signals induced by the increased tissue damage in the combination-treated tumors and subsequent vascular normalization five days after the radiation in the anti-Ang2 monotherapy and anti-Ang2 plus IR treatment groups. In addition, the tumor-focused radiation decreases radiation-induced damage to the bone marrow compared to the whole-body radiation, which further enables the recruitment of immune cells to the tumors. The combination treatment also showed some signs of efficacy in NSG mice, which represent the most immune-compromised xenograft model available. Of note, one of the mutations in the NSG mice inhibits the non-homologous end joining (NHEJ) DNA repair mechanism, and this sensitizes them to radiation-induced damage. Our results indicated that the blocking of Ang2 may also increase the efficacy of radiation therapy in these conditions, making it possible that it could work even in the absence of adaptive immunity.

Based on our results, the anti-Ang2 plus radiation treatment should be further tested in transgenic and PDX tumor models, and if successful, in a clinical trial. Furthermore, anti-PD-1/PD-L1 could be tried for the improvement of the efficacy of the treatment with Ang2 blocking antibodies plus radiation, especially since the combination treatment increased

tumor infiltration by the cytotoxic Cd8+ T cells and since previous studies have showed synergistic effects when antiangiogenic treatment has been combined with immune checkpoint therapies (43).

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Figure legends

Figure 1. Ang2 is critical for endothelial cell survival after radiation-induced damage. A and B, HUVECs were plated and allowed to grow for 24 h, after which growth medium was replaced and anti-Ang2 (MEDI3617) or isotype control antibody (2 μg/ml) was added. 15 minutes later, the cells were either sham-radiated or radiated with either 2 Gy x 1 (**A**)

or 4 Gy x 1 (**B**). On the following day, the cells were again either sham-radiated or radiated with 2 Gy x 1 (**A**). ANG2 RNA was measured in three replicate experiments 24 h after the last radiation dose. **B,** The percentage of caspase-3/7–positive HUVECs was analyzed 24 and 48 h after 4 Gy radiation. **C**–**E**, HUVECs were plated, allowed to grow for 24 h in growth medium supplemented with either anti-Ang2 (MEDI3617) or isotype control antibody (2 μg/ml), and then radiated with 4 Gy x 1. On the following day cells were stained for either Ki67 or propidium iodide (PI). The percentage of Ki67-negative cells was counted from four replicate experiments (**C, D**), and the cell cycle phase was analyzed from PI staining and flow cytometry (**E**). Mean + SEM for each treatment group and for each cell cycle phase: control no IR: G_0/G_1 39 + 4, S 44 + 6, G_2/M 18 + 2, anti-Ang2 no IR G_0/G_1 44 + 3, S 38 + 1, G_2/M 18 + 2, ctrl + IR G_0/G_1 59 + 11, S 18 + 5, G_2/M 23 + 15, anti-Ang2 + IR G_0/G_1 67 + 12 , S 14 + 1, G_2/M 20 + 11 (**E**). * p-value < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001. Scale bar 50 μm.

Figure 2. Increased melanoma growth inhibition and extended survival in mice treated with a combination of Ang2 blocking antibodies and a small dose of radiation. B16-F0 melanoma cells were injected subcutaneously into C57BL/6Jrj mice. Anti-Ang2 (MEDI3617) or control antibody (red arrows) was injected either every third day starting from day 5 after the implantation of tumor cells, until the indicated timepoint (green arrow, day 41), (**A**–**D**) or every fourth day starting from day 3 after the implantation of tumor cells (**E**–**H**). The mice received a total of 6 Gy whole-body radiation (IR, black arrows) in three equal fractions on days 8, 9 and 10 (**A**–**D**) or on days 6, 7 and 8 (**E**–**H**). Mice were euthanized when the total tumor volume reached 2500 mm^3. **C and G,** Tumor growth delay (TGD, compared to control no IR treatment group) was calculated for each treatment group. Number of mice per group (**A**–**D**): ctrl no IR: 14, anti-Ang2 no IR: 12, ctrl + IR: 6, anti-Ang2 + IR: 11, (**D**–**H**): ctrl no IR: 12, anti-Ang2 no IR: 10, ctrl + IR: 7, anti-Ang2 + IR: 13. * p-value < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001.

Figure 3. CRC tumor growth inhibition and extended survival in mice treated with anti-Ang2 plus radiation. MC38 CRC cells were injected subcutaneously into

C57BL/6Jrj mice. Anti-Ang2 (clone 3.19.3) or control antibody (red arrows) was given every third day starting on day 8 after the implantation of the tumor cells, until the end of the experiment. The mice received a total of 6 Gy whole-body radiation in three equal fractions on days 11, 12 and 13 (black arrows). The mice were euthanized when the total tumor volume reached 2000 mm^3. **A,** Tumor growth was measured every three days starting on day 8. The figure shows the tumor growth curves in each treatment group, until the first mouse was euthanized from non-radiated (black and red) and radiated (blue and green) treatment groups. **B,** Survival of the mice. **C,** Tumor growth delay compared to ctrl no IR group. In **D**, are shown all individual tumor growth curves. Number of mice per group: ctrl no IR: 9, anti-Ang2 no IR: 14, ctrl + IR: 10, anti-Ang2 + IR: 9. * p-value < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001.

Figure 4. Quantification of tumor vessels in mice treated with anti-Ang2 plus radiation. A and **B**, Five days after the last fraction of radiation, mice were euthanized. Tumor sections were stained for endomucin and CD31, and the endothelial area was quantified. **C,** Representative images from LS174T tumor sections stained for endomucin plus CD31. Arrowheads point to the few remaining capillaries in the combination-treated tumor sections. Scale bar 0.4 mm. **D, E** and **F**, Quantification of endomucin plus CD31 staining area in B16-F0 and MC38 tumors after mouse euthanization. * p-value < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001.

Figure 5. Ang2 blocking inhibits radiation-induced decrease of T cells and cells of the monocyte/macrophage lineage. A, B and **C,** B16-F0 cells were injected subcutaneously into C57BL/6Jrj mice. After randomizing of the mice into different treatment groups on day six, anti-Ang2 injections were started and continued every three days. Daily radiation doses of 2 Gy were given on days 9–11. The mice were euthanized and tumors were harvested for scRNA seq analysis on day 16. **B**, Cell populations based on single-cell RNA clusters. **C,** Percentages of Cd8+ and Cd4+ T cells, fibroblasts (FB), tumor cells (TC) and monocytes/macrophages (Mo/Mø) in the treatment groups. Number of tumors per treatment group: ctrl no IR: 6, anti-Ang2 no IR: 6, ctrl + TF-IR: 2, anti-Ang2 + TF-IR: 6.

Figure 6. Anti-Ang2 treatment combined with radiation increases tumor necrosis. A and **B,** Five days after the last fraction of whole-body radiation, the mice bearing the indicated tumors were euthanized and tumor sections were stained with H&E. Quantification of tumor necrosis in LS174T (**A**) and B16-F0 tumors (**B). C, D, E** and **F,** B16-F0 and MC38 allografts excised at experiment endpoint were stained with H&E. Quantification of tumor necrosis in B16-F0 allografts after whole-body radiation (**C, F**) or after tumor-focused radiation (**D**) and in MC38 allografts treated with whole-body radiation (**E**). **F,** Representative images of B16-F0 tumor sections stained with H&E. Yellow dots encircle the main necrotic areas. Scale bar 1 mm. * p-value < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001.