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Blocking Angiopoietin-2 Promotes Vascular Damage and Growth Inhibition in Mouse Tumors Treated with Small Doses of Radiation

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4 **Blocking angiopoietin-2 promotes vascular damage and growth**
5 **inhibition in mouse tumors treated with small doses of radiation**
6

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18 Running title: Ang2 blocking improves radiotherapy efficacy
19

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23

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25
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27
28

29 **Abstract**

30

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

47

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

51

52 **Introduction**

53

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

59

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

69

70 Tumor vessels are malformed and structurally defective, which leads to their dysfunction,
71 plasma leakage into the tumor stroma, poor tissue perfusion and compromised tissue
72 oxygenation (6-8). The efficacy of radiation depends on a number of factors, of which
73 oxygen concentration in the target tissue is important, since radiation produces highly
74 reactive oxygen radicals that cause DNA damage and cell death (9). Hypoxia in tumor
75 tissue counteracts radiation therapy, and the increased interstitial fluid pressure resulting
76 from leaky tumor vessels has been reported to reduce the delivery of cytostatic drugs to the
77 tumors (10, 11). Angiogenesis inhibitors, including inhibitors of VEGF and VEGF
78 receptors, and vascular disrupting agents, such as combretastatin, have been tested as
79 modifiers of the tumor vasculature in association with radiation therapy (12). Anti-VEGF
80 agents can improve tumor response to radiation, presumably by normalizing the tumor
81 vasculature, and thereby reducing vascular leak, tumor hypoxia, and radiation resistance
82 (2, 12).

83

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

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

93

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

104

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

107

108 **Materials and Methods**

109

110 **Mice and tumor models.** 18–20-week-old male C57BL/6JRj mice from Janvier and the
111 tumor cell lines B16-F0 (a generous gift from Dr. Sirpa Jalkanen in 2012) and MC38-GFP
112 (a generous gift from Dr. Jeffrey Schlom in 2013) were used for the mouse allograft
113 experiments. 20-week-old male and female NOD *scid* gamma mice (NSG; NOD.Cg-
114 Prkdc^{scid} Il2rg^{tm1/wjl}/SzJ, 005557) from the Jackson Laboratory were injected with human
115 LS174T cells (a generous gift from Dr. Ragnhild A. Lothe and Dr. Olli Kallioniemi in
116 2015) in the tumor xenograft experiments. Due to the radiation sensitivity of the NSG mice,
117 they were euthanized five days after the last dose of radiation. All experiments were
118 approved by the National Animal Experiment Board in Finland
119 (ESAVI/6306/04.10.07/2016 and ESAVI/7945/04.10.07/2017).

120

121 LS174T cells passage 6-10 were cultured in DMEM-F12 (BE04-687F/U1, Lonza),
122 containing penicillin/streptomycin and 10 % fetal bovine serum (S181B-500, Biowest),
123 and MC38-GFP and B16-F0 cells, both in passages 6-10, in DMEM (BE12-707F, Lonza)
124 containing 2-mM L-glutamine (25-005-Cl, Corning), penicillin/streptomycin and 10%
125 fetal bovine serum. Cell lines were not authenticated or tested for *Mycoplasma*. For *in vivo*
126 tumor experiments, 1×10^6 tumor cells (passage 6-10) were injected subcutaneously.
127 Tumor growth was monitored by manual measurements with a caliber in mice under
128 inhalation anesthesia (isoflurane). Tumor volume was calculated as length x width x
129 thickness in mm^3 . Tumor growth time (TGT) represents the time in days starting from the
130 first day of treatment, until the tumor reached the total volume of 2500 mm^3 (B16-F0) or
131 2000 mm^3 (MC38). Tumor growth delay (TGD) was calculated as: $\text{TGT}_{\text{treatment}} -$
132 $\text{TGT}_{\text{control no radiation}}$.

133

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

140

141 **Histology and immunohistochemistry.** Hematoxylin and eosin (H&E) staining was used
142 to analyze necrotic areas in tumor sections. Immunohistochemical stainings were done
143 using antibodies for endomucin (sc-65495, 1:500, Santa Cruz), CD31 (553370, 1:250, BD
144 Pharmingen), smooth muscle actin (α -SMA, C6198, 1:500, Sigma-Aldrich), Erg (ab133264,
145 1:250, abcam), laminin (RB-082-AO, 1:500, Thermo Scientific), Glut-1 (07-1401, 1:500,
146 Merck) and Caix (ab15086/ab108351, 1:500, abcam), followed by Alexa Fluor-conjugated
147 secondary antibodies (Molecular Probes). Deparaffinization employed the xylene
148 substitute (Tissue-Tek, Tissue-Clear, 1466, SAKURA) for 3x5 min plus rehydration in an
149 alcohol series (2 x 100%, 2 x 96%, 1 x 70% and 1 x 50% for 3 min each). After heat-

150 induced epitope retrieval, the sections were blocked for endogenous peroxidase activity
151 using H₂O₂ and for nonspecific binding using TNB (NEL700001KT, PerkinElmer).
152 Primary antibodies were incubated in TNB overnight at +4°C. After TNT washes, the
153 sections were incubated in the appropriate species-specific ImmPRESS kit (MP-7401, MP-
154 7402, MP-7405, VECTOR laboratories) secondary antibodies for 30 min, washed with
155 TNT and PBS, treated with AEC for 10 min, hematoxylin stained and mounted with
156 Aquatex (1.08562.0050, Millipore). Images were scanned using
157 3DHISTECH Panoramic 250 FLASH II digital slide scanner, and unprocessed digital
158 images were analyzed using Panoramic 250 Scanner Software. The images were modified
159 to optimize visualization using Fiji software.

160

161 **Analysis of Caspase-3/7–positive cells and cell cycle phase.** Human umbilical vein
162 endothelial cells (HUVEC) passage 6-10 were cultured on 6-well plates coated with
163 gelatin. 24 h after subculture, the growth medium was replaced with medium supplemented
164 with antibodies (MEDI3617 or isotype control antibody, 2 µg/ml) plus IncuCyte Caspase-
165 3/7 reagent (4440, 4704, 1:1000, Essen BioScience) for 15 min, followed by radiation with
166 4 Gy x 1. Images were taken 24 h and 48 h later with Thermo Fisher EVOS FL inverted
167 epifluorescence microscope. The original images were processed and analyzed using Fiji
168 software. For cell cycle analysis, HUVECs were cultured for 24 h in endothelial growth
169 medium supplemented with either anti-Ang2 (MEDI3617) or control antibody, and then
170 radiated with a 4 Gy single radiation dose. On the following day, cells were detached with
171 a brief trypsin treatment (Trypsin-EDTA, 25200056, Thermo Fisher Scientific) and fixed
172 with cold 70% ethanol. After at least four-hour incubation in -20°C, cells were washed
173 with HBSS (14175-053, Gibco) + 2% FBS once, treated with 0.1 mg RNase A at +37°C
174 for 30 min and then stained with 20 µg of propidium iodide for 30 min in RT. Cells were
175 analyzed with BD AccuriTM C6 Flow Cytometer, and the cell cycle phases were
176 determined with FlowJo.

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

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

204 **RNA extraction and qPCR analysis.** RNA of dissociated melanoma tumors was extracted
205 with NucleoSpin RNA II kit (Macherey-Nagel #740955) according to the manufacturer's
206 instructions. Cells for RNA extraction were harvested from the samples used for scRNA
207 seq. cDNA was synthesized with cDNA Synthesis Kit (Thermo Fischer Scientific
208 #4368814) according to the manufacturer's instructions. Gene expression analysis was

209 performed by quantitative PCR using following primers: Cd4_fw: 5'
210 TAGCAACTCTAAGGTCTCTAAC, Cd4_rec: 5'GATAGCTGTGCTCTGAAAA,
211 Cd8_fw: 5'CCTTCAGAAAGTGAAGTCTAC, Cd8_rev:
212 5'CCAGATGTAAATATCACGGC. Mouse *Gapdh* was used as a housekeeping gene.

213 **Statistical Analyses.** For each *in vivo* analysis, data from all mice in a treatment group was
214 pooled, analyzed using the Mann–Whitney test and presented as mean +/- standard error
215 of mean (SEM). *In vitro* experiments were repeated two to four times, data was pooled
216 from all experiments, analyzed using the Mann–Whitney test and presented as mean +/-
217 standard error of mean (SEM). GraphPad PRISM 7 was used for the statistical analyses.
218 Statistical significance, marked by p-value * < 0.05, ** < 0.005, *** < 0.0005, **** <
219 0.0001, is indicated in the figure legends.

220

221 **Results**

222

223 **Ang2 is critical for the survival of endothelial cells after radiation.** Since Ang2 has
224 been shown to have a protective role in stressed endothelial cells (16), we speculated that
225 Ang2 could also be critical for the survival of endothelial cells after radiation. To test this,
226 we exposed cultured HUVECs to 2 Gy dose of radiation on two consecutive days and
227 analyzed ANG2 RNA 24 h after radiation. Although radiation increased ANG2 RNA only
228 slightly, radiation in combination with Ang2 blocking increased ANG2 RNA very
229 significantly, reflecting stress in the endothelial cells induced by this combination treatment
230 (**Fig. 1A**). To study the possible effect of Ang2 on endothelial cell survival after radiation,
231 we next supplemented the endothelial growth medium with Ang2 blocking (MEDI3617)
232 or isotype control antibody, plus the IncuCyte Caspase-3/7 reagent, radiated the cultures
233 with single dose of 4 Gy radiation, and 24 and 48 h thereafter, determined the percentage
234 of caspase-3/7–positive apoptotic cells. We found that the combination caused significantly
235 more apoptosis than either treatment alone (**Fig. 1B**). However, γ H2AX staining for
236 detection of DNA damage did not indicate differences between radiation and anti-Ang2
237 plus radiation treated HUVECs, indicating that Ang2 blocking does not sensitize
238 endothelial cells to radiation-induced DNA damage (**Fig. 1D, Supplementary Fig. S1A**).
239 To analyze how the combination treatment affects endothelial cell proliferation, HUVECs

240 were treated for 24 h with the antibodies and subjected to a 4 Gy radiation dose, followed
241 by staining for the Ki67 on the next day. The results showed that the anti-Ang2 plus
242 radiation treated cultures had more cells in the G₀-phase (Ki67 negative) than cultures
243 treated with either radiation or antibodies alone (**Fig. 1C, D**). This result was further
244 supported by flow cytometry analysis of propidium iodide (PI) stained HUVECs, which
245 showed that there were more endothelial cells in the G₀/G₁ cell cycle phase in the
246 combination treated cultures than in the other cultures (**Fig. 1E, Supplementary Fig. S1B-**
247 **E**). These results indicated that anti-Ang2 increases radiation-induced endothelial cell
248 cycle arrest and cell death.

249

250 **Low doses of radiation in combination with Ang2 blocking inhibit melanoma tumor**
251 **growth.** To test if Ang2 blocking plus radiation-induced endothelial cell death could lead
252 to tumor growth inhibition, we tested the effect of combination treatment to subcutaneous
253 B16-F0 melanoma allografts in C57BL/6JRj mice. Anti-Ang2 (MEDI3617) injections
254 were started five days after the tumor cell implantation and continued than every 3 days,
255 and whole-body radiation was given on days 8-10, when the tumors had grown to an
256 average size of 140 mm³. Three daily doses of 2 Gy whole-body radiation induced only a
257 trend of tumor growth inhibition (**Fig. 2A**). This effect was of similar magnitude as the
258 effect of anti-Ang2 antibodies (**Fig. 2A**). Although the monotherapies did not show
259 significant tumor growth inhibition, the tumor-bearing mice subjected to a combination
260 treatment with anti-Ang2 plus radiation showed a significant improvement of tumor growth
261 inhibition (**Fig. 2A**).

262

263 **Anti-Ang2 treatment combined with radiation extends the survival of melanoma**
264 **tumor-bearing mice.** To test the long-term effects of the combined anti-Ang2 plus
265 radiation treatment, the B16-F0 allografts in the 4 treatment groups were allowed to grow
266 until they reached a total tumor volume of 2500 mm³, when the mice were euthanized.
267 However, since the tumors in the combination treatment group did not seem to progress to
268 meet the euthanization criteria, antibody treatment was discontinued on day 44, when all
269 mice in the other treatment groups had already been euthanized. The termination of Ang2

270 antibody treatment accelerated tumor growth in the combination treatment group, and by
271 day 63, all tumor volumes in the combination treatment group had reached a volume of
272 2500 mm³ (**Fig. 2B, D**). The tumor growth delay (TGD) in the anti-Ang2 monotherapy
273 group was on average 5 days, in the radiation monotherapy group 13 days, and in the
274 combination treatment group 34 days (**Fig. 2C**).

275

276 We then repeated the experiment by starting the anti-Ang2 (MEDI3617) antibody
277 treatment on day 3 after tumor implantation, when the tumor volume was about 15 mm³
278 on average, and whole-body radiation was given on days 6-8. In this experiment, the
279 antibodies were injected every fourth day. Although both monotherapies resulted in a
280 significant tumor growth inhibition, the combination treatment again significantly
281 improved both tumor growth inhibition and host survival (**Fig. 2E-H**). Notably, anti-Ang2
282 treatment 1) given only three days before radiation and on first radiation day, 2) starting on
283 the first radiation day, or 3) starting on following day of last radiation dose resulted in
284 shorter survival than our standard combination treatment (**Supplementary Fig. S2A-D**).
285 We conclude that for optimal results, anti-Ang2 treatment should be started before
286 radiation and continued thereafter.

287

288 **Additive effect of anti-Ang2 and radiation in colorectal allografts.** In order to study if
289 the effect of the combination treatment could be reproduced in another tumor type, we next
290 analyzed growth of subcutaneous MC38 colorectal carcinoma (CRC) allografts subjected
291 to the treatments. To independently confirm our findings, we further used another
292 monoclonal Ang2 blocking antibody (3.19.3) (28). The Ang2 blocking antibodies were
293 injected every third day starting on day eight, when the tumor volume was 25 mm³ on
294 average, and 2 Gy whole-body radiation doses were given on days 11-13. Radiation
295 monotherapy strongly suppressed MC38 allograft growth and increased the survival of the
296 tumor-bearing mice, whereas anti-Ang2 monotherapy resulted only in a trend of slower
297 tumor growth (**Fig. 3A-D**). Yet, the combination treatment with anti-Ang2 plus radiation
298 significantly increased host survival when compared to the monotherapies (**Fig. 3B, C**).
299 The tumor growth delay in the anti-Ang2 monotherapy group was on average 5 days, in

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

302

303 **Effect of the combination treatment in severely immunodeficient mice.** Recent research
304 has indicated that the results of chemotherapy often depend on the adaptive immune
305 response, whereas in the case of radiotherapy its role is less clear (29, 30). In order to
306 investigate if the inhibitory effect of Ang2 blocking in combination with radiation works
307 in severely immunodeficient mice, we injected LS174T cells subcutaneously and allowed
308 the tumors to develop to an average volume of 135 mm³ (day 16), after which the mice
309 were injected with anti-Ang2 (MEDI3617) or control antibody every third day. Due to the
310 high sensitivity of the NSG mice to radiation, only a 0.5 Gy radiation dose was
311 administered daily over 4 consecutive days starting on day 19. We found that radiation and
312 anti-Ang2 monotherapies decreased tumor growth: the combination treatment significantly
313 increased tumor growth inhibition in the first experiment, but in a repeated experiment only
314 a trend of additional inhibition was found (**Supplementary Fig. S3A-F**). This suggested
315 that adaptive immunity may improve the outcome of the combination treatment.

316

317 **Anti-Ang2 improves tumor growth inhibition in response to focused radiation.** We
318 next tested if the results obtained with whole-body radiation plus anti-Ang2 could be
319 reproduced with tumor-focused radiation (TF-IR). B16-F0 allografts (approximately 80
320 mm³) in otherwise lead-shielded mice were radiated with a 2 Gy daily dose on days 12-14.
321 Anti-Ang2 (MEDI3617) injections were started three days before the first radiation dose,
322 and were continued every three days. The results showed that tumor growth delay in anti-
323 Ang2 and radiation monotherapy groups was one and two days respectively, whereas the
324 delay was 13 days in the combination treatment group, indicating that TF-IR increases
325 tumor growth inhibition by anti-Ang2 highly significantly (**Supplementary Fig. S4A-D**).
326 Similar results were obtained in a repeated experiment: anti-Ang2 monotherapy, radiation
327 monotherapy and the combination treatment induced tumor growth delays were 6, 3 and
328 18 days, respectively (**Supplementary Fig. S4E-H**). Thus, the mice treated with the

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

331

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

344

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

359

360 **Anti-Ang2 treatment rescues radiation-induced loss of inflammatory cells.** In order to
361 analyze the tumor microenvironment in mice treated with TF-IR plus anti-Ang2, B16-F0
362 tumor cells were injected into C57BL/6Jrj mice, and six days later, the mice were
363 randomized to the treatment groups. Anti-Ang2 was dosed every 3 days starting from day
364 6, and 2 Gy daily doses of tumor-focused radiation were given on days 9-11. Five days
365 after the last radiation dose, the mice were euthanized and tumors were harvested for
366 single-cell RNA sequencing (scRNA seq) (**Fig. 5A**). ScRNA seq analysis of 2000 cells per
367 treatment group revealed less Cd4+ and Cd8+ T cells and cells of the
368 monocyte/macrophage lineage in the TF-IR group than in the non-irradiated groups, but not
369 in the combination treatment group (**Fig. 5B, C, Supplementary Fig. S7D**). QPCR from
370 total RNA was consistent with the rescue of the radiation-induced decrease of the Cd4 and
371 Cd8 T cells in the combination treatment group (**Supplementary Fig. S7A, B**). These
372 results indicated that Ang2 blocking protects T cells and monocytes/macrophages from
373 radiation-induced damage. ScRNA seq analysis also revealed that endothelial Ang2
374 expression was higher in all the treatment groups than in the control group, with highest
375 levels in the combination treated group (**Supplementary Fig. S7C**).

376

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

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

394

395 **Discussion**

396

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

403

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

411

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

420

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

437

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

451

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

465

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

478

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

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

486

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488

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510

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633
634 **Figure legends**

635
636 **Figure 1. Ang2 is critical for endothelial cell survival after radiation-induced damage.**
637 **A and B**, HUVECs were plated and allowed to grow for 24 h, after which growth medium
638 was replaced and anti-Ang2 (MEDI3617) or isotype control antibody (2 µg/ml) was added.
639 15 minutes later, the cells were either sham-radiated or radiated with either 2 Gy x 1 (**A**)

640 or 4 Gy x 1 (**B**). On the following day, the cells were again either sham-radiated or radiated
641 with 2 Gy x 1 (**A**). ANG2 RNA was measured in three replicate experiments 24 h after the
642 last radiation dose. **B**, The percentage of caspase-3/7-positive HUVECs was analyzed 24
643 and 48 h after 4 Gy radiation. **C–E**, HUVECs were plated, allowed to grow for 24 h in
644 growth medium supplemented with either anti-Ang2 (MEDI3617) or isotype control
645 antibody (2 µg/ml), and then radiated with 4 Gy x 1. On the following day cells were stained
646 for either Ki67 or propidium iodide (PI). The percentage of Ki67-negative cells was
647 counted from four replicate experiments (**C, D**), and the cell cycle phase was analyzed from
648 PI staining and flow cytometry (**E**). Mean + SEM for each treatment group and for each
649 cell cycle phase: control no IR: G₀/G₁ 39 + 4, S 44 + 6, G₂/M 18 + 2, anti-Ang2 no IR
650 G₀/G₁ 44 + 3, S 38 + 1, G₂/M 18 + 2, ctrl + IR G₀/G₁ 59 + 11, S 18 + 5, G₂/M 23 + 15,
651 anti-Ang2 + IR G₀/G₁ 67 + 12, S 14 + 1, G₂/M 20 + 11 (**E**). * p-value < 0.05, ** < 0.005,
652 *** < 0.0005, **** < 0.0001. Scale bar 50 µm.

653

654 **Figure 2. Increased melanoma growth inhibition and extended survival in mice**
655 **treated with a combination of Ang2 blocking antibodies and a small dose of radiation.**

656 B16-F0 melanoma cells were injected subcutaneously into C57BL/6Jrj mice. Anti-Ang2
657 (MEDI3617) or control antibody (red arrows) was injected either every third day starting
658 from day 5 after the implantation of tumor cells, until the indicated timepoint (green arrow,
659 day 41), (**A–D**) or every fourth day starting from day 3 after the implantation of tumor cells
660 (**E–H**). The mice received a total of 6 Gy whole-body radiation (IR, black arrows) in three
661 equal fractions on days 8, 9 and 10 (**A–D**) or on days 6, 7 and 8 (**E–H**). Mice were
662 euthanized when the total tumor volume reached 2500 mm³. **C and G**, Tumor growth delay
663 (TGD, compared to control no IR treatment group) was calculated for each treatment
664 group. Number of mice per group (**A–D**): ctrl no IR: 14, anti-Ang2 no IR: 12, ctrl + IR: 6,
665 anti-Ang2 + IR: 11, (**D–H**): ctrl no IR: 12, anti-Ang2 no IR: 10, ctrl + IR: 7, anti-Ang2 +
666 IR: 13. * p-value < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001.

667

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

670 C57BL/6Jrj mice. Anti-Ang2 (clone 3.19.3) or control antibody (red arrows) was given
671 every third day starting on day 8 after the implantation of the tumor cells, until the end of
672 the experiment. The mice received a total of 6 Gy whole-body radiation in three equal
673 fractions on days 11, 12 and 13 (black arrows). The mice were euthanized when the total
674 tumor volume reached 2000 mm³. **A**, Tumor growth was measured every three days
675 starting on day 8. The figure shows the tumor growth curves in each treatment group, until
676 the first mouse was euthanized from non-radiated (black and red) and radiated (blue and
677 green) treatment groups. **B**, Survival of the mice. **C**, Tumor growth delay compared to ctrl
678 no IR group. In **D**, are shown all individual tumor growth curves. Number of mice per
679 group: ctrl no IR: 9, anti-Ang2 no IR: 14, ctrl + IR: 10, anti-Ang2 + IR: 9. * p-value < 0.05,
680 ** < 0.005, *** < 0.0005, **** < 0.0001.

681

682 **Figure 4. Quantification of tumor vessels in mice treated with anti-Ang2 plus**
683 **radiation.** **A** and **B**, Five days after the last fraction of radiation, mice were euthanized.
684 Tumor sections were stained for endomucin and CD31, and the endothelial area was
685 quantified. **C**, Representative images from LS174T tumor sections stained for endomucin
686 plus CD31. Arrowheads point to the few remaining capillaries in the combination-treated
687 tumor sections. Scale bar 0.4 mm. **D**, **E** and **F**, Quantification of endomucin plus CD31
688 staining area in B16-F0 and MC38 tumors after mouse euthanization. * p-value < 0.05, **
689 < 0.005, *** < 0.0005, **** < 0.0001.

690

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

700

701 **Figure 6. Anti-Ang2 treatment combined with radiation increases tumor necrosis. A**
702 **and B,** Five days after the last fraction of whole-body radiation, the mice bearing the
703 indicated tumors were euthanized and tumor sections were stained with H&E.
704 Quantification of tumor necrosis in LS174T (**A**) and B16-F0 tumors (**B**). **C, D, E** and **F,**
705 B16-F0 and MC38 allografts excised at experiment endpoint were stained with H&E.
706 Quantification of tumor necrosis in B16-F0 allografts after whole-body radiation (**C, F**) or
707 after tumor-focused radiation (**D**) and in MC38 allografts treated with whole-body
708 radiation (**E**). **F,** Representative images of B16-F0 tumor sections stained with H&E.
709 Yellow dots encircle the main necrotic areas. Scale bar 1 mm. * p-value < 0.05, ** < 0.005,
710 *** < 0.0005, **** < 0.0001.