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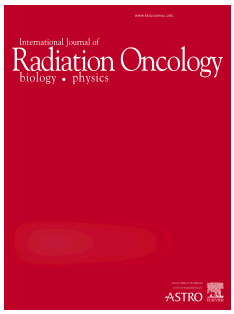

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PII: S0360-3016(20)31185-8

DOI: https://doi.org/10.1016/j.ijrobp.2020.05.028

Reference: ROB 26362

To appear in: *International Journal of Radiation Oncology • Biology • Physics*

Received Date: 13 March 2020

Revised Date: 7 May 2020

Accepted Date: 18 May 2020

Please cite this article as: Brown JM, Radiation Damage to Tumor Vasculature Initiates a Program that Promotes Tumor Recurrences, *International Journal of Radiation Oncology • Biology • Physics* (2020), doi: https://doi.org/10.1016/j.ijrobp.2020.05.028.

Radiation Damage to Tumor Vasculature Initiates a Program that Promotes Tumor Recurrences

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Running title: Damaged tumor vasculature promotes recurrences

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Conflict of interest: none

Funding: None for this review

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Abstract

This review, mostly of preclinical data, summarizes the evidence that radiation at doses relevant to radiotherapy initiates a pathway that promotes the reconstitution of the tumor vasculature leading to tumor recurrence. The pathway is not specific to tumors; it promotes repair of damaged and ischemic normal tissues by attracting proangiogenic cells from the bone marrow. For radiation of tumors the pathway comprises: 1) Radiation causes loss of endothelial cells and reduced tumor blood perfusion leading to increased tumor hypoxia and increased levels of hypoxia inducible factor-1 (HIF-1). Alternatively increased HIF-1 levels may arise by reactive oxygen species (ROS) production caused by tumor reoxygenation, 2) Increased HIF-1 levels lead to increased levels in the tumor of the chemokine stromal cell-derived factor-1 (SDF-1, CXCL12), which captures monocytes/macrophages expressing the CXCR4 receptor of CXCL12, 3) The increased levels of tumor associated macrophages (TAMs) become highly proangiogenic (M2 polarized) and restore the tumor vasculature thereby promoting tumor recurrence.

The relevance of this pathway for radiotherapy is that it can be blocked in a number of different ways including by inhibitors of monocytes/macrophages, of HIF-1, of CXCL12, of CXCR4, and of CSF-1R, the latter of which is responsible for the M2 polarization of the TAMs. All of these inhibitors produce a robust enhancement of the radiation response of a wide variety of preclinical tumor

models. Further, the same inhibitors actually provide protection against radiation damage of several normal tissues. Some of these pathway inhibitors are available clinically and a first-in-human trial of the CXCR4 inhibitor, plerixafor, with radiotherapy of glioblastoma has yielded promising results including an impressive increase in local tumor control. Further clinical trials are warranted.

Introduction

The possibility that the radiation response of tumors could be enhanced by damage to the tumor vasculature has long been recognized (1). In particular several investigators have proposed that the high doses per fraction (> 8 -10 Gy) delivered in stereotactic body radiotherapy (SBRT) could cause damage to the tumor vasculature that amplifies tumor cell kill (2-4). The evidence for this amplified tumor cell kill from vascular damage by high doses is by two possible mechanisms, (a) induction of vascular shutdown and ischemia leading to further tumor cell death in the 2-4 days following irradiation (3, 5), and (b) ischemia/reperfusion inhibiting homologous recombination repair of the radiation-induced DNA damage in the tumor cells thereby increasing tumor cell kill (2). Whether either of these mechanisms is responsible for the success of SBRT is controversial (6).

However, separate from this controversy, accumulating evidence suggests another consequence of radiation damage to tumor blood vessels: namely that vascular damage initiates a sequence of events that attracts proangiogenic cells, principally from the bone marrow, which promote recovery of the vasculature and tumor recurrence. This is the subject of this review. This sequence of events is

not a tumor-specific phenomenon; the seminal study of Asahara and colleagues showed that proangiogenic cells originating in the bone marrow are attracted to ischemic sites and promote recovery of the damaged or occluded vasculature (7). Subsequently many preclinical and clinical studies have shown that bone marrow derived cells (BMDCs) can speed the recovery of ischemia-damaged tissue (8). The purpose of this review is to examine the evidence that the pathway leading to recruitment of BMDCs initiated by damaged tumor vasculature not only contributes to tumor recurrence after radiotherapy but that it can be interrupted to improve tumor response. The following sections outline the evidence for each of the steps in this pathway.

Radiation damages tumor vasculature and reduces blood perfusion.

Large single doses of irradiation (> 8 -10 Gy) damage the tumor vascular and reduce blood perfusion. This can occur rapidly (within 24 hours) (2, 4, 9) possibly due to endothelial cell apoptosis or endothelial-induced vasoconstriction, but more commonly in a delayed manner over 1-3 weeks after irradiation (10-15), as assessed by blood perfusion or loss of endothelial cells stained with the endothelial cell marker CD31. Because lower doses of irradiation do not produce significant damage to the vasculature or reduced vessel perfusion it is commonly assumed that this is a phenomenon only occurring at large doses (2, 5). However this ignores the cumulative effect of multiple daily doses: indeed Zywietz and colleagues showed that prolonged daily doses of 3 Gy to a rat rhabdomyosarcoma produced destruction of the vessel walls by 3 weeks, and one week after the end of the radiation treatment, at which time the tumor had

accumulated 75 Gy, the tumor capillaries showed complete necrosis (16). One caveat in extrapolating the timing of the vasculature effects from experimental animals to man is the much slower growth and lower levels of proliferation of the endothelial cells in human tumors (17). However, though the vasculature changes may be delayed in human tumors there is no reason to believe that similar effects do not occur.

The damaged vasculature and reduced perfusion increase tumor hypoxia and HIF-1

Reduced perfusion through tumor vessels would be expected to increase tumor hypoxia and several investigators have reported such increases in tumor hypoxia following both single dose and fractionated irradiation of experimental tumors (2, 9, 12-15, 18, 19). The situation is less clear with conventional fractionation of human tumors. Using oxygen needle electrode measurements Brizel and colleagues reported no changes in oxygenation of head and neck cancers after 10 – 15 Gy compared to baseline results (20). Cooper and colleagues also reported no changes in the oxygenation of cervix cancers after 40-45 Gy delivered in 20 fractions over 4 weeks (21). On the other hand Stadler and colleagues found reduced tumor oxygenation in head and neck cancers after both 3 weeks of chemoradiotherapy as well as at the end of the 70 Gy treatment regime (22). Note that these clinical measurements were taken during or soon after conventional fractionation and blood flow changes take time to develop, so it may be that changes would occur later than was measured. To date there are

no data on tumor oxygenation after high dose SBRT which would be expected to produce more rapid changes.

Several of the preclinical studies that have reported increased tumor hypoxia after radiation also showed increased levels of the hypoxia inducible factor-1 (HIF-1) (11-13). HIF-1 is a transcription factor that regulates many genes involved in the homeostatic responses of cells and tissues to reduced oxygen levels. It is composed on two subunits HIF-1α and HIF-1β. HIF-1β, also known as the ary hydrocarbon nuclear translocase (ARNT) protein is ubiquitously expressed, whereas HIF-1α □□ rapidly degraded by oxygen. At low levels of oxygen□ HIF-1α □□comes stabilized, levels increase and it binds to its partner HIF-1β □□□ translocates to the nucleus where it transcribes the genes involved in the cell's response to hypoxia. Thus increased levels of hypoxia caused by irradiation lead to increased levels of HIF-1 and its downstream targets.

However, it appears that radiation of tumors can increase HIF-1 separately from radiation-induced hypoxia. Moeller and colleagues (23) using a window chamber model in mice showed that HIF-1 was induced rapidly (within 24 hours) by irradiation (5 Gy daily x 2) but for the most part HIF-1 was not associated with tumor hypoxia. Rather, the induced HIF-1 was associated with tumor regions expected to be reoxygenated after irradiation and could be abrogated using a small molecule mimetic of superoxide dismutase (SOD) to inhibit reactive oxygen species (ROS). Further the authors demonstrated that preventing the induction of HIF-1 by scavenging ROS produced a significant enhancement of vascular damage. This implies that HIF-1 or its downstream

transcripts either provides protection against radiation-induced vascular damage or promotes its recovery. Consistent with this they showed that treating mice with the HIF-1 inhibitor YC-1 following irradiation of flank tumors produced a major sensitization of the tumors (Fig 1).

Increased HIF-1 levels generates CXCL12 which promotes uptake of BMDCs into tumors

A common feature of irradiated experimental tumors, first noted by Stephens and colleagues (24), and subsequently demonstrated by a variety of investigators (12, 14, 15, 25-29), is the increased levels of bone marrow derived cells (BMDCs) following irradiation. This has also been reported for human glioblastoma (12) and oral squamous cell carcinoma (14). These BMDCs are primarily monocytes/macrophages and are positive for the markers CD11b and F4/80. Typically the increase in these CD11b+ cells begins 1-2 weeks following irradiation concurrently with increased CXCL12 levels in the tumors (12) and can last for several weeks (Fig 2).

Increased uptake of BMDCs after vascular damage is not a tumor specific phenomenon: it has been reported following irradiation of brain (30), bone marrow (31), and lung, both in mice (32) and humans (33). The general phenomenon of the homing of BMDCs to injured tissue was first reported by Asahara and colleagues who showed that putative endothelial cell progenitors from the bone marrow are attracted to ischemic sites in normal tissues undergoing active angiogenesis (7). It was then established that stromal cell-derived factor-1 (SDF-1 or CXCL12) was the important mediator of the

recruitment of stem and progenitor cells to injured tissue by showing that expression of CXCL12 in injured tissue correlated with stem cell recruitment and tissue repair (34, 35). Ceradini and colleagues then showed that CXCL12 gene expression was regulated by HIF-1 and that increased CXCL12 expression increased the homing to and capture of CXCR4 positive progenitor cells in ischemic tissue (36). The idea that stem and progenitor cells from the bone marrow could enhance the recovery of ischemic tissues has led to many studies, on the use of these cells in the treatment of critical limb ischemia and cardiovascular diseases (37, 38).

However, in addition to the possibility that BMDCs can help to reverse vasculature damage or insufficiency in normal tissues, the process also has relevance to tumors. It means that in addition to angiogenesis, by which tumor blood vessels are formed from the sprouting of local vessels, tumors can also develop or repair blood vessels from circulating cells, a process known as “vasculogenesis”. Under normal conditions this is a secondary pathway for the formation of tumor blood vessels and can be regarded as a “backup” pathway if angiogenesis is inhibited. In a model system to investigate the importance of vasculogenesis to tumor growth if angiogenesis is blocked by irradiation Ahn and Brown (25) showed that tumors could only grow in a locally pre-irradiated site if the mice had functional bone marrow by which to permit vasculogenesis. This implies that vasculogenesis is the primary way for a tumor to grow after irradiation, and suggests that the influx of BMDCs into irradiated tumors is the means by which this is accomplished. This then suggests an important concept

for radiotherapy, namely that after radiation, the increased hypoxia in the tumors makes the tumors similar to ischemic tissues, and therefore incorporate circulating proangiogenic cells that help to rescue the radiation-damaged tumor vasculature and promote tumor recurrence. Kioi and colleagues (12) provided direct evidence for this: They showed that 15 Gy to subcutaneous xenografts temporarily reduced tumor blood flow which returned to normal by 3 weeks after irradiation. However, the restoration of normal blood flow was prevented using plerixafor, which abrogates the interaction of CXCL12 with its CXCR4 receptor on monocytes and endothelial progenitor cells. A similar lack of return to normal tumor blood flow after irradiation was reported in a clinical trial of glioblastoma using the same CXCR4 antagonist (39). Already mentioned is that Moeller and colleagues demonstrated that preventing the induction of HIF-1 by scavenging ROS produced a significant enhancement of tumor vascular damage (23).

There is also clinical evidence that expression levels of CXCL12 correlate with tumor hypoxia and prognosis (40, 41). The further question of whether pre-treatment levels of CXCL12 correlate with outcome from radiotherapy cannot be determined as HIF-1 levels (which drive CXCL12 levels) correlate with radiotherapy response due to the intrinsic radioresistance of hypoxic cells.

In addition to the induction of CXCL12 in tumors after irradiation by increased levels of hypoxia and HIF-1, studies with cells *in vitro* have shown that CXCL12 can also be induced by radiation in both a HIF-1-dependent and independent mechanism (42), and may be related to the presence of micronuclei in irradiated cells that have undergone post-irradiation mitosis (43). One possible

mechanism for the radiation induction is through histone modification of the CXCL12 promoter region (44). However, the data showing the profound effect of HIF-1 inhibition either chemically (12, 23) or genetically (12, 45) on tumor response to irradiation argues that the primary mechanism for the induction of CXCL12 is by increased HIF-1 levels post-irradiation.

The increased levels of BMDCs in tumor following irradiation are macrophages polarized to the M2 phenotype

As noted above the majority of the BMDCs that infiltrate tumors after irradiation are CD11b and F4/80 positive and are identified as macrophages termed tumor associated macrophages (TAMs). TAMs derive from circulating monocytes and exhibit a wide spectrum of phenotypes in tumors loosely categorized as along an M1 to M2 spectrum from proinflammatory (M1) to anti-inflammatory and proangiogenic (M2). There is considerable evidence that in tumors recurring after irradiation the spectrum is heavily biased towards the proangiogenic M2 phenotype (12, 14, 46), and the same is true for relapsing tumors after chemotherapy (47). Consistent with the hypothesis that it is the M2 macrophages that are responsible for the reconstitution of the tumor vasculature following irradiation Okubo and colleagues showed that tumor cells implanted into pre-irradiated sites, which prevents local angiogenesis, grew when implanted with M2 macrophages but did not when implanted with M1 macrophages (14).

In addition to TAMs radiation also increases the uptake of myeloid-derived suppressor cells (MDSCs) in the irradiated tumors (29, 48) recruited by tumor secreted chemokines including CXCL12 (49). MDSCs comprise a mixed

population of polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs), which act to inhibit the activity of CD8 T-cells. In tumors M-MDSCs predominate and differentiate into TAMs that are highly proangiogenic (49, 50). In highly immunogenic tumors there is evidence that following irradiation the initial rise in MDSCs and TAMs is overwhelmed by the subsequent rise in cytotoxic T-cells (48). This raises the question, still to be answered, of whether the proangiogenic program following vascular injury by radiation is dampened in tumors that are immunogenic and have a robust influx of cytotoxic T-cells following irradiation. As a corollary to this is the possibility that blocking the CXCL12/CXCR4/7 pathway, which prevents the influx of immunosuppressive myeloid cells including MDSCs and TAMs into the irradiated tumor, will enhance tumor immunity. This has been reported with an autochthonous model of pancreatic ductal adenocarcinoma in mice (51). Enhanced anti-tumor immunity could also make an abscopal effect more likely and would be an additional advantage of blocking the pathway.

Direct evidence that radiation initiates a program that promotes tumor recurrence.

The evidence outlined above suggests the following sequence of events: 1) Radiation causes vascular damage and diminished tumor blood perfusion leading to increased tumor hypoxia and increased HIF-1 levels. Alternatively increased HIF-1 levels may arise by ROS production caused by reoxygenation, 2) Increased HIF-1 levels lead to increased levels in the tumor of the chemokine CXCL12, which captures monocytes/macrophages expressing the CXCR4

receptor of CXCL12, 3) The increased levels of macrophages in the tumors become M2 polarized that are highly proangiogenic and restore the tumor vasculature thereby promoting tumor recurrence. Damage to tumor vasculature also occurs with other agents including vascular disruptive agents (VDA's) such as combretastatin (52), some chemotherapy drugs (47, 53) and sustained or aggressive antiangiogenic therapy (54). The sequence of events is shown diagrammatically in Fig 3.

There is considerable direct evidence for this pathway following tumor irradiation as follows:

a) Inhibitors of HIF-1 interrupt the pathway.

Kioi and colleagues (12) treated mice bearing the intracranially implanted U251 xenograft with the HIF-1 inhibitor NSC-134754 for 21 days started immediately following 15 Gy tumor irradiation and demonstrated a complete absence of the irradiation-induced influx of TAMs into the tumors. They further showed that the HIF-1 inhibitor, while not affecting the growth of non-irradiated tumors, prevented the recurrence of the tumors after irradiation (Fig 4). To demonstrate this was not an off target effect of the drug they showed that knockdown of HIF-1 in the tumor cells also inhibited recurrence of the tumors after irradiation. The YC-1 HIF-1 inhibitor also has a marked radiosensitizing effect on the radiation response of an experimental tumor (23) (Fig 1). This is also the case for the HIF-1 inhibitor PX-478 (55). Williams and colleagues also showed the impact of a deficiency of HIF-1 by demonstration that tumors grown from hepatoma cells lacking HIF-1β (and therefore HIF-1 activity) were more

sensitive to radiation than wild-type tumors (45).

b) Inhibition of CXCL12 and CXCR4 interrupts the pathway thereby radiosensitizing tumors

A number of investigators have shown that chemical or genetic means to disrupt the interaction of CXCL12 with its receptor CXCR4 following irradiation enhance the radiation response of a variety of experimental tumors (Reviewed in (56)). The fact that the increased radiation response occurs by blocking the CXCL12/CXCR4 pathway after irradiation suggests that changes in intrinsic radiation sensitivity or in tumor oxygenation are unlikely to be mechanisms for the increased tumor radiation response. One conclusion that emerges from all the data is that blocking the CXCL12/CXCR4 pathway following irradiation produces an increase in tumor radiation response irrespective of tumor type,. This suggests that neither the tumor type nor the tumor's genetic makeup are critical factors for radiation enhancement, which is consistent with the hypothesis that the protection afforded by CXCL12 induction is a universal response of tissues and tumors to injury.

Of the studies discussed above two are highlighted as the most clinically relevant. In the first study Milosevic and colleagues (29, 57) treated two different orthotopically-implanted patient derived cervix tumors in mice with 15 daily fractions of 2 Gy delivered locally to the tumors with weekly cisplatin with or without the CXCR4 antagonist plerixafor infused either concurrently or following the radiation and cisplatin treatment. A major increase in tumor response to radiation and cisplatin was seen in the animals treated with plerixafor with the

biggest response seen in the group treated with plerixafor subsequent to radiation (Fig 5) consistent with the model that BMDCs infiltrate tumors following irradiation.

In the second example Liu and colleagues (58) irradiated chemically-induced brain tumors in rats with or without the specific CXCL12 inhibitor NOX-A12 (olaptesed pegol) following irradiation and found that CXCL12 inhibition produced both a large prolongation of median survival time and a major shrinkage to undetectable sizes of the brain tumors (Fig 6). Similar efficacy in enhancing the radiation response of the chemically induced brain tumors in rats was shown by Walters and colleagues (59) using inhibitors of CXCR7, which is necessary for the interaction of CXCL12 with CXCR4.

Consistent with the need to block the CXCL12/CXCR4/7 pathway or deplete macrophages after irradiation, the one negative result that showed no increased tumor radiosensitivity after macrophage deletion used macrophage depletion by injection of clodronate liposomes 1 day prior to irradiation and did not continue the depletion after irradiation (60).

c) Blocking the switch of TAMs from an M1 to an M2 phenotype also radiosensitizes tumors

As shown in Fig 3 a key component of the pathway is the conversion of the tumor infiltrating monocytes into highly proangiogenic TAMs and TEMs. As noted earlier the ratio of M1 to M2 TAMs is heavily biased towards M2 TAMs in tumors recurring after radiation. This is often seen in the large proportion of TIE2-expressing macrophages (TEMs), which are highly proangiogenic and at the

extreme M2 end of the M1/M2 spectrum (61). The M2 polarization is driven by the radiation-induced expression of colony stimulating factor-1 (CSF-1) and IL-34, the ligands for the receptor CSF-1R on macrophages (62-64) and can be prevented by CSF-1R antagonists. Thus a prediction of the model in Fig 3 is that CSF-1R antagonists would interrupt the radiation protective pathway and radiosensitize tumors. This has been demonstrated by Xu and colleagues (65) who showed that the CSF-1R inhibitor PLX3397 given to mice bearing the BM-1 prostate tumor both during and following 5 daily doses of 3 Gy produced a major enhancement of the radiation response. Similar results were obtained using PLX3397 by Stafford and colleagues (64) with a patient derived xenograft of glioblastoma irradiated with a single dose of 12 Gy.

d) Neutralization or elimination of monocytes/macrophages enhances tumor response to irradiation

Tumor associated macrophages (TAMs) polarized to the M2 phenotype are hypothesized to be responsible for the reconstitution of the radiation damaged vasculature and early tumor recurrence (Fig 3). This hypothesis therefore predicts that elimination or neutralization of monocytes/macrophages in the tumor-bearing host would inhibit tumor recurrence after irradiation. This has been demonstrated. Ahn and colleagues showed that CD11b neutralizing antibodies both eliminated the influx of myeloid cells in FaDu human carcinoma tumors recurring after 20 Gy irradiation and enhanced the response of the tumors to irradiation (66). Consistent with this result Kioi and colleagues showed that macrophage depletion with carrageenan in mice bearing U251 intracranially

implanted tumors also prevented the recurrence of the irradiated tumors (12). Macrophage depletion also enhances the response of a variety of experimental tumors to several chemotherapeutic agents (Reviewed in (67-69)).

e) Tumor irradiation without vascular depletion does not induce hypoxia or CXCL12 or promote the influx of BMDCs into the tumors

In an innovative study Kane and colleagues (18) compared the effects of standard radiotherapy (SRT) (20 Gy in 10 daily fractions over 12 days) with hyper fractionated, or pulsed radiotherapy (PRT), on the response of subcutaneous Lewis lung carcinoma allografts and on the microenvironment of the tumors. The PRT consisted of giving each of the 2 Gy daily fractions as ten 0.2 Gy doses each separated by 3 minutes. The total dose and overall treatment time of the two modalities was identical. The authors found that SRT depleted the tumor vasculature leading to tumor hypoxia but PRT did not. Further they showed that SRT induced high levels of CXCL12 in the tumors but the levels following PRT were unchanged compared to non-irradiated tumors. In addition the post-irradiation levels of BMDCs in the tumors was higher in the SRT treated tumors than in the PRT treated tumors. Finally, despite their much greater fractionation the tumor response of the PRT treated tumors was significantly greater than that of the SRT treated tumors (Fig 7). These data support the overall model that a damaged tumor vasculature initiates the program of increased hypoxia, increased HIF-1, increased CXCL12 and tumor influx of BMDCs that is protective against the response of tumors to irradiation.

Implications for radiation therapy

Of the several ways of interrupting the pathway initiated by depletion of tumor vasculature outlined above probably the most suitable for clinical use would be ones that block the CXCL12/CXCR4/7 pathway or the conversion of the tumor infiltrating monocytes into M2 macrophages. Blocking the pathway further upstream (for example by inhibiting HIF-1) or by depleting monocytes/macrophages would likely affect too many other processes. Discussed below are clinical trials using the available antagonists of the CXCL12/CXCR4 interaction or of CSF-1R, which is responsible for the transformation of macrophages from M1 to M2 polarization.

Plerixafor is a small molecule CXCR4 antagonist that has been used clinically as an acute dose to mobilize hematopoietic stem cells from the bone marrow for bone marrow transplantation. However, it has recently been tested in a phase I/II 4-week continuous infusion with standard chemoradiotherapy for previously untreated glioblastoma (39). The authors found that the treatment was well tolerated and showed promising results: In addition to increasing the expected median survival time of 15 months to 22 months they showed that there was a diminished blood supply in the high dose irradiated area in the plerixafor treated patients and a very high rate of local control. These results are consistent with the preclinical studies of this drug with radiation and were sufficiently promising to warrant a further clinical trial (NCT03746080).

Another inhibitor of the CXCL12/CXCR4/7 pathway is olaptesed pegol (olaptesed, NOX-A12), which is a PEGylated L-oligoribonucleotide that binds and neutralizes CXCL12 and is currently in a phase I/II trial with newly diagnosed

glioblastoma of unmethylated MGMT promoter status in three clinics in Germany (NCT04121455).

A barrier to widespread use of these currently used inhibitors of the CXCl12/CXCR4/7 pathway with radiotherapy is the fact that both plerixafor and olaptesed pegol require weeks of continuous infusion following irradiation. This adds considerable inconvenience and complexity to the treatment. Fortunately, several oral antagonists of CXCR4 and CXCR7 are currently in development and should speed the testing of this strategy in radiotherapy.

PLX3397 is a small molecule inhibitor of CSF-1R and in preclinical models has been shown to prevent the conversion of M1 to M2 TAMs and to improve the response to radiation (64). It is also in a phase Ib/II clinical trial added to standard therapy of newly diagnosed glioblastoma (NCT01790503).

A relevant question regarding the possibility of blocking the CXCL12/CXCR4/7 pathway for radiation therapy is whether the effect might be more marked with stereotactic body radiotherapy (SBRT). This seems likely as vascular damage would be expected to be greater than for conventional radiotherapy because (a) the individual doses are higher in SBRT, (b) the biologically effective doses (BEDs) are higher, and (c) the overall time is shorter in SBRT giving less time for tissue repair during the treatment. To date there are no data on any of the components of the pathway following SBRT.

The fact that tumor radiation in the absence of damage to the tumor vasculature produces enhanced radiation response (see Figure 7) raises the possibility that the ultra high dose rates (> 60Gy/s) delivered in FLASH

radiotherapy, because they preferentially protect normal tissues compared to conventional dose rates (70, 71), would spare the tumor vasculature and so enhance the tumor response. In fact though multiple normal tissues are spared by FLASH compared to conventional irradiation there is no evidence of a change in tumor response (70). It would thus appear that there is little or no sparing of the damage to the tumor vasculature. However, this could be fruitful avenue for a more careful study.

Since the dose delivered to the tumor in conventional radiation therapy is largely determined by the maximum dose that can be delivered safely to the immediately surrounding normal tissue it is important to know whether the inhibitors of the pathway outlined in Fig 3 also enhance the radiation response of these critical normal tissues. In fact there is considerable evidence that not only do these inhibitors not enhance the radiation response they actually provide considerable radiation protection (reviewed in (56)). In brief, radioprotection has been shown for normal mouse skin (66, 72), for the GI tract and rectum of mice (57), for the brain (73), and for mouse lung (32).

What could be the scientific basis for the fact that inhibition of the tumor radioprotective pathway should both potentiate tumor response and protect normal tissues? Inflammation contributes to the radiation injury of normal tissues (74), and M1 macrophages, which are highly pro-inflammatory, migrate to irradiated tissues thereby enhancing inflammation and radiation response (30). Thus a strategy to prevent macrophage trafficking to irradiated tissues should lessen radiation injury. On the other hand, TAMs, particularly those in tumors

recovering from radiation, are largely alternatively activated or M2 macrophages, which are highly proangiogenic. Thus excluding these macrophages would reduce the ability of the irradiated tumor to restore a functioning vasculature, and therefore prevent recurrence. Kioi and colleagues confirmed this by demonstrating that plerixafor infusion, which blocked the influx of TAMs following radiation, prevented the restoration of blood flow in irradiated mouse tumors (12).

Summary

Tumor Irradiation with doses comparable to those used in radiotherapy depletes the tumor vasculature over a period of days to weeks depending on the radiation dose and the growth rate of the tumors and vasculature. This reduces perfusion of the blood vessels and increases tumor hypoxia. As a consequence levels of the transcription factor hypoxia inducible factor-1 (HIF-1) rise and a number of genes regulated by HIF-1 are turned on. HIF-1 can also be activated by reactive oxygen species (ROS) as a result of reoxygenation of previously hypoxia tumor regions. The increased HIF-1 levels respond to the increased tumor hypoxia by activating normal pathways that attempt to promote blood vessel growth in the damaged tissue to counteract the induced hypoxia. One of these pathways attracts bone marrow derived cells (BMDCs), principally monocytes/macrophages into damaged sites by increasing levels of stromal cell-derived factor-1 (SDF-1 or CXCL12), a downstream target of HIF-1. For the tumor blood vessels depleted by irradiation these attracted monocytes become highly proangiogenic M2 macrophages and accelerate the restoration of the tumor blood flow thereby promoting tumor recurrence.

This review summarizes the evidence for this pathway and highlights the many studies in which inhibitors of the pathway, including agents that are clinically available, prevent the radiation-induced accumulation in tumors of M2 tumor associated macrophages (TAMs) and enhance the radiation response of the tumors. Evidence is also presented that not only does interruption of this pathway increase tumor response to irradiation but it also protects normal tissue, giving it a strong possibility of improving the therapeutic ratio of radiation therapy. Early clinical trials are underway

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Figure Captions

Figure 1: inhibition of HIF-1 enhances the tumor response to irradiation. HCT116 human colon carcinoma tumor was grown subcutaneously in nude mice and irradiated with 2 doses of 5 Gy separated by 24 hr. starting on day 12. The HIF-1 inhibitor was intraperitoneally injected daily (5 mg/kg) starting on day 15 (arrow) and stopped after 2 weeks (arrowhead). From (23) with permission.

Figure 2: Irradiation causes loss of endothelial cells, increased hypoxia and influx of CD11b+ myeloid cells in an oral squamous cell carcinoma xenograft model. (a) Growth curve of OSC-19 subcutaneous tumors treated with 12 Gy local irradiation on day 10. (b) Loss of CD31+ endothelial cells in the tumor vascular as a function of time after irradiation. (c) Increased tumor hypoxia as determined by density of the hypoxia marker pimonidazole as a function of time after irradiation. (d) Influx of CD11b+ myeloid cells in the tumors as a function of time after irradiation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control. Modified from (14).

Figure 3: The proposed pathway from tumor irradiation to recurrence. 1) Radiation (and VDAs and antiangiogenic drugs) damages tumor vasculature, reduces tumor blood perfusion, and increases tumor hypoxia and HIF-1 levels. Alternatively increased HIF-1 levels arise by ROS production caused by tumor reoxygenation, 2) Increased HIF-1 levels lead to increased tumor levels of CXCL12, which capture CXCR4 expressing monocytes arising from the bone

marrow 3) The monocytes in the tumors become M2 polarized macrophages (TAMs and TEMs), which are highly proangiogenic and help restore the tumor vasculature thereby promoting tumor recurrence. Modified from De Palma and Lewis (67)

Figure 4: The HIF-1 inhibitor NSC-134754 prevents the radiation-induced influx of bone marrow derived CD11b+ monocytes into intracranially implanted U251 tumors and blocks tumor recurrence. (a) Dose dependent increase of BMDCs in the irradiated tumors. Tumor bearing nude mice with green fluorescent protein (GFP) bone marrow received whole brain irradiation at 0, 8, or 15 Gy on day 22. Scale bar: 50 μm. (b) Immunohistochemistry of tumor sections stained for all leukocytes (CD45) and for myeloid cells (CD11b) showing a major increase in myeloid cells after irradiation. (c) Quantification of CD11b+ and F4/80+ cell influx in tumors of b. Error bars indicate SEM. **P < 0.01, ***P < 0.001 versus control. (d) The HIF-1 inhibitor injected daily (5 mg/kg/day) started immediately after irradiation of the intracranial U251 glioblastoma prevents recurrence of the tumor. Error bars indicate SD. *P < 0.05. Modified from (12)

Figure 5: The CXCR4 antagonist plerixafor enhances the chemo-radiation response of two orthotopic patient-derived cervix cancer models and is most effective when given after radiation. (a) The study schema in which groups were treated with radiation (RT) alone (30 Gy over 3 weeks) focused on the cervix implanted tumors in immune deficient mice, or radiation with cisplatin

(4 mg/kg i.p one day per week during the radiation treatment) (RTCT) either alone or combined with the CXCR4 antagonist plerixafor (continuous infusion at 5 mg/kg/day) given either concurrently with or following irradiation. (b) and (c) Individual growth curves of the two different patient derived orthotopic cervix tumors treated according to the color scheme shown in a. Tumor size was measured weekly by CT and time was measured from the first treatment. (b) includes a comparison of concurrent with adjuvant plerixafor. Plerixafor given for 3 weeks after RTCT gave significantly more tumor growth delay compared to RTCT and concurrent plerixafor for 3 weeks ($p = 0.018$). From (29) with permission.

Fig 6: Addition of the CXCL!2 antagonist NOX-A12 following irradiation of ENU-induced brain tumors produces complete responses by MRI. Rats born following *In utero* ENU-treatment were imaged by MR starting on day 130 of age, and repeated every 2 weeks until death. Rats were distributed into the various treatment groups so as to have approximately equal total tumor volumes in each group at the start of treatment. NOX-A12 was injected subcutaneously at 10 mg/kg every 2 days for 10 weeks starting after the 20 Gy whole brain irradiation. Temozolomide (TMZ) (5 mg/kg i.p.) was given 5 days/week for 3 weeks. From (58) with permission.

Fig 7: Hyper-fractionated irradiation (PRT) produces greater tumor response to irradiation compared to standard fractionation (SRT) with no vascular damage, increased hypoxia, CXCL12 (SDF-1) or tumor uptake of

BMDCs. Standard radiotherapy (SRT) was 20 Gy in 2 Gy fractions given 5 days/week for 2 weeks. With hyper-fractionated or pulsed radiotherapy (PRT) each of the 2 Gy fractions was given as 10 doses of 0.2 Gy each separated by 3 minutes. (a) The PRT treated tumors have a significantly greater tumor response than the SRT treated tumors during the second week of radiation. (b) Vessel density assessed by CD31 staining was significantly reduced in the SRT but not in the PRT-treated tumors. (c) Tumor hypoxia assessed by pimonidazole staining was increased in the SRT but not in the PRT-treated tumors. (d) Tumor levels of SDF-1 are significantly higher in the SRT treated tumors compared to controls and to the PRT treated tumors. (e) Levels of BMDCs assessed by flow cytometer measurements of tumor infiltrating CD45 cells are significantly higher in the SRT than the PRT- treated tumors. Modified from (18).

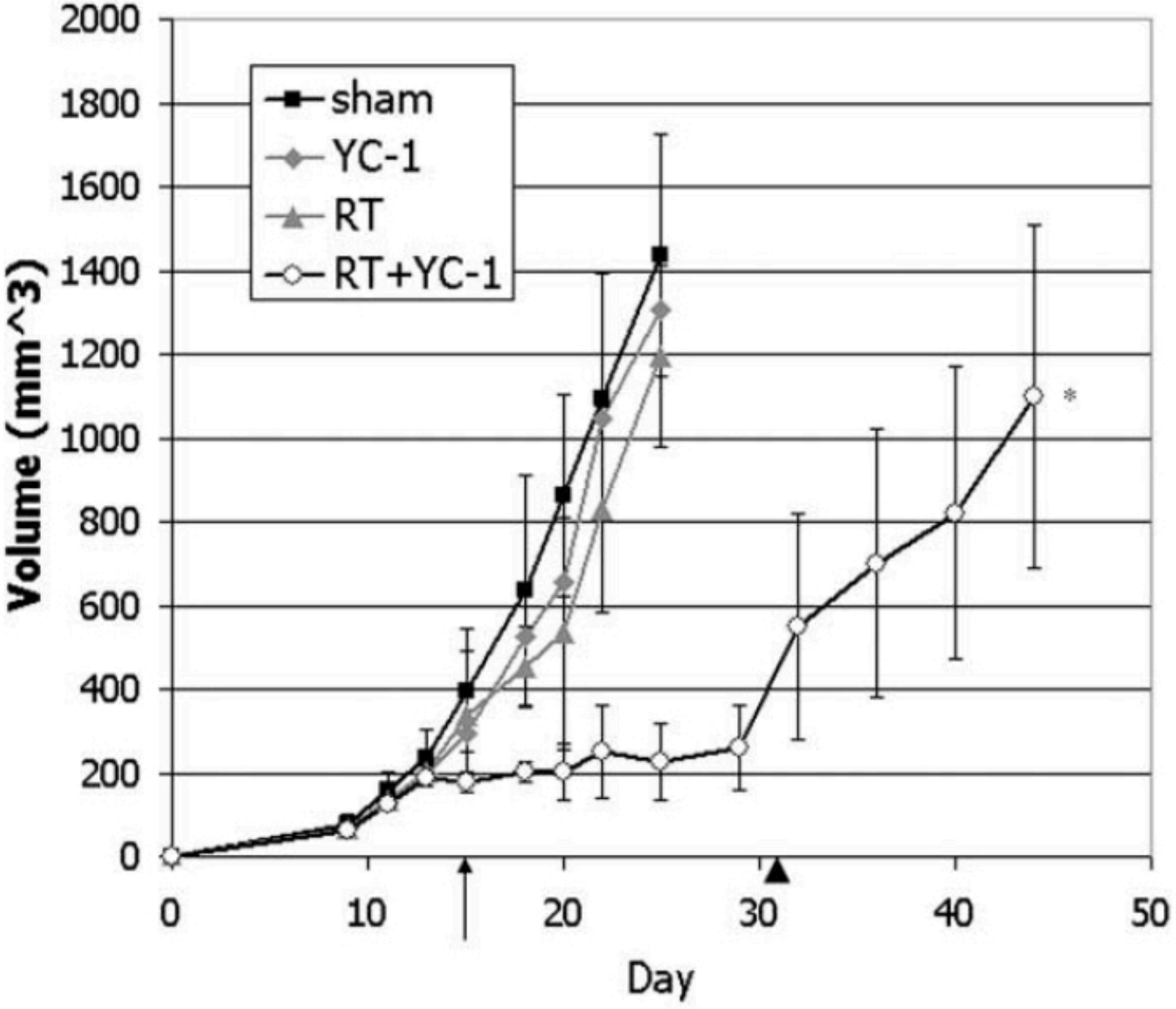

2000
1800
1600
1400
1200
1000
800
600
400
200
0
Volume (mm^3)
sham
YC-1
RT
RT+YC-1
*
0
10
20
30
40
50
Day

Fig 1

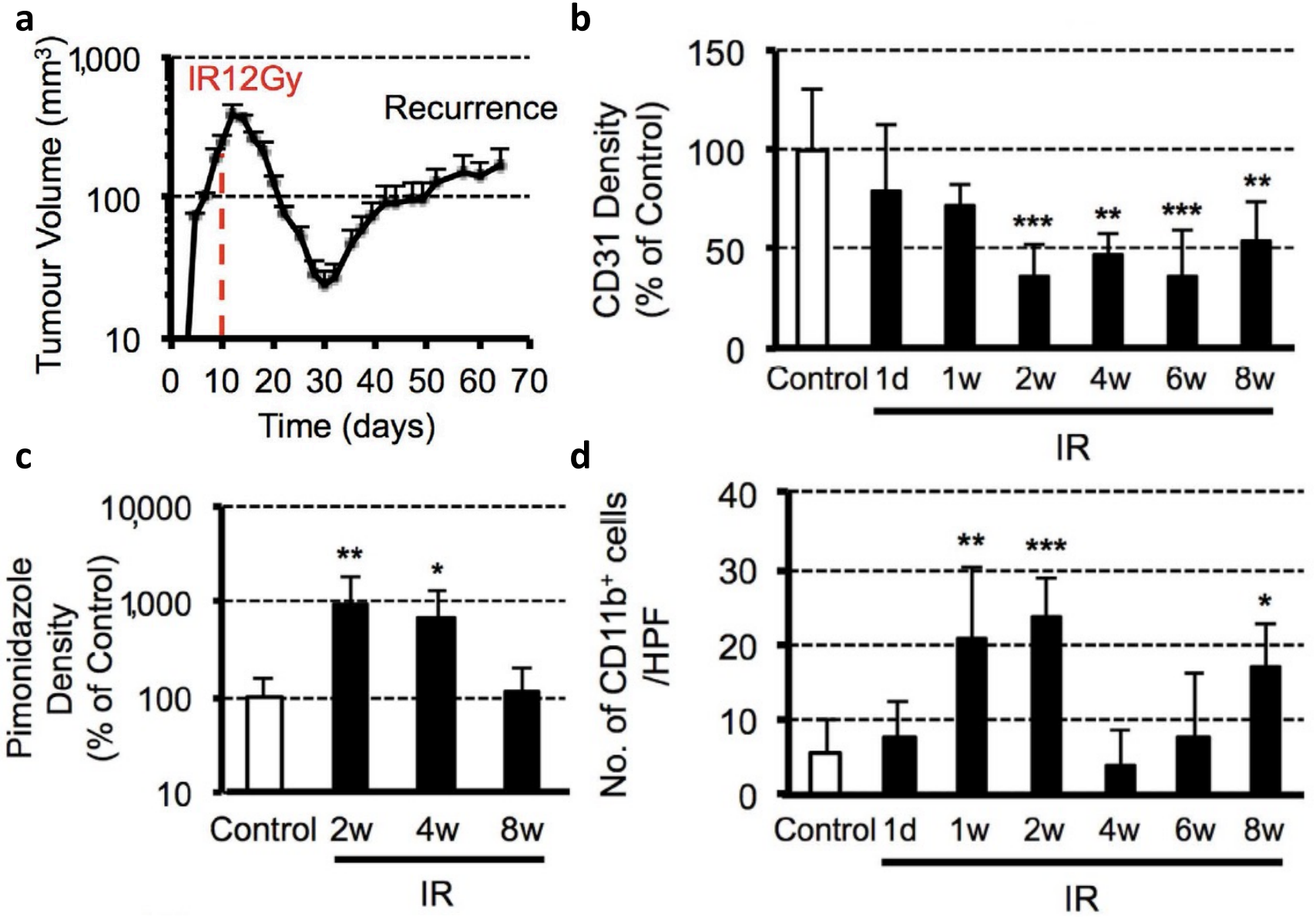

a
Tumour Volume (mm3)
1,000
100
10
IR12Gy
Recurrence
0 10 20 30 40 50 60 70
Time (days)
b
CD31 Density (% of Control)
150
100
50
0
***
**
***
**
Control 1d 1w 2w 4w 6w 8w
IR
c
Pimonidazole Density (% of Control)
10,000
1,000
100
10
**
*
Control 2w 4w 8w
IR
d
No. of CD11b+ cells /HPF
40
30
20
10
0
**
***
*
Control 1d 1w 2w 4w 6w 8w
IR

Fig 2

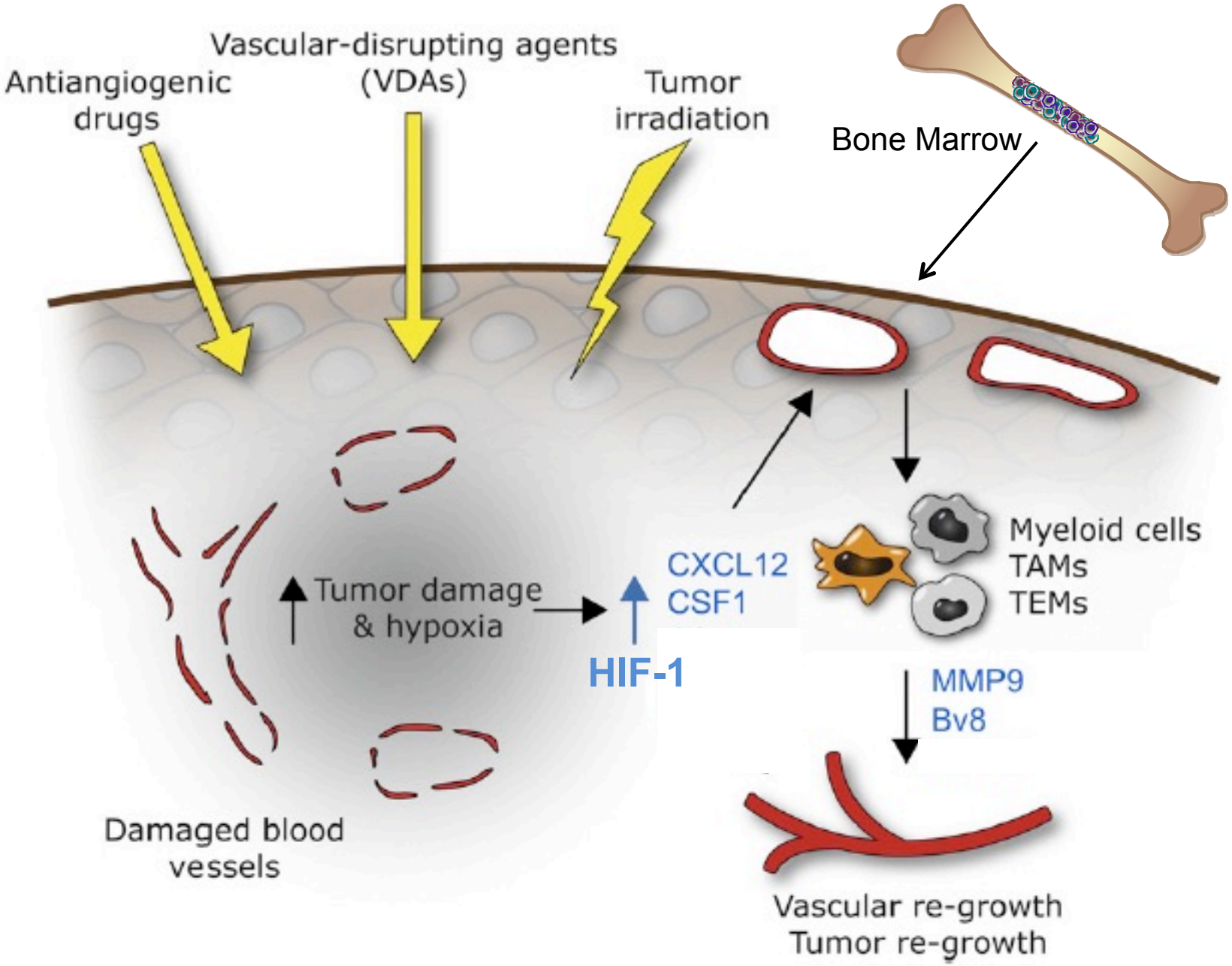

Antiangiogenic drugs
Vascular-disrupting agents (VDAs)
Tumor irradiation
Bone Marrow
Tumor damage & hypoxia
HIF-1
CXCL12
CSF1
Myeloid cells
TAMs
TEMs
MMP9
Bv8
Damaged blood vessels
Vascular re-growth
Tumor re-growth

Fig 3

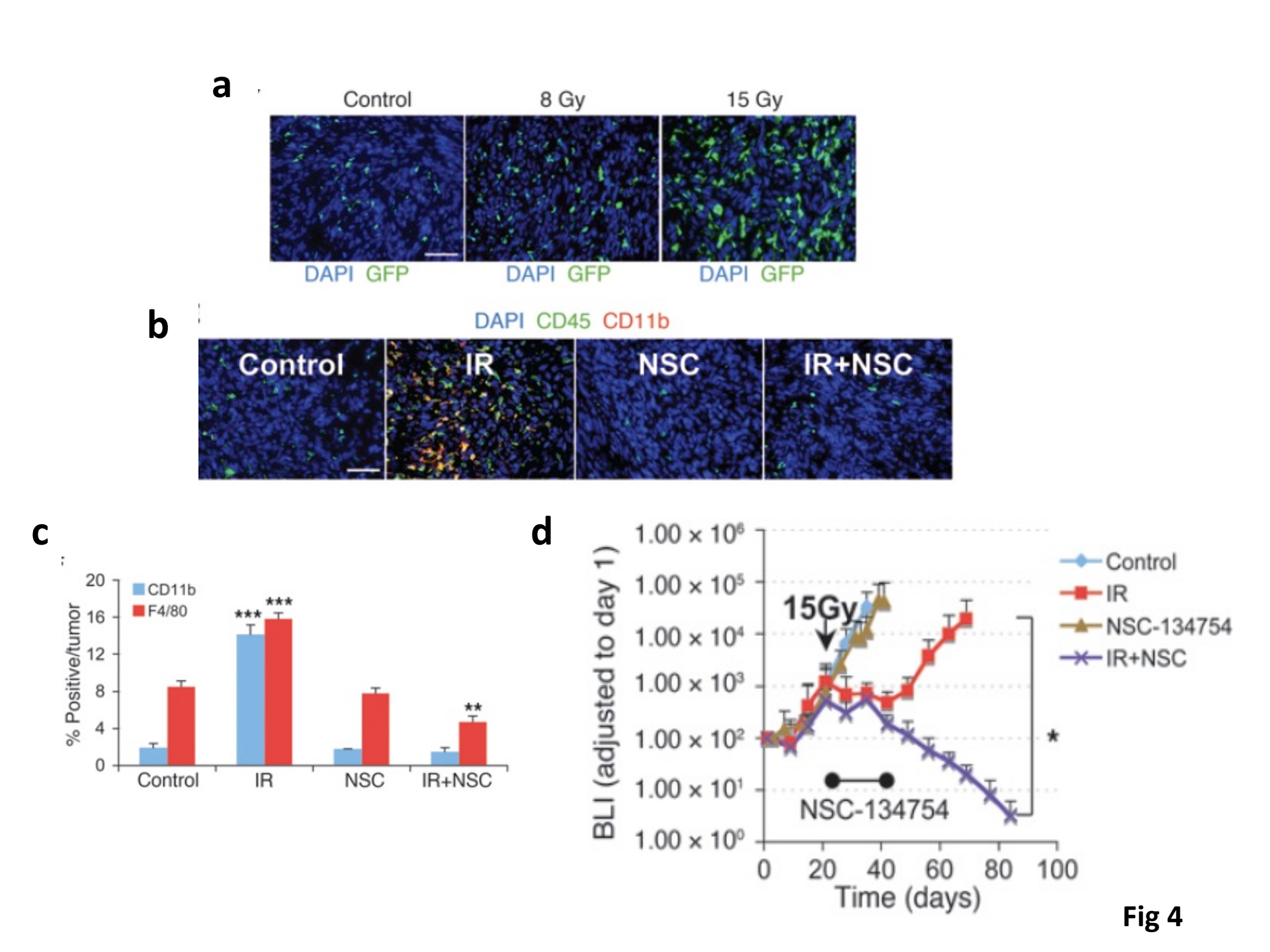

a
Control
8 Gy
15 Gy
DAPI GFP
DAPI GFP
DAPI GFP
b
DAPI CD45 CD11b
Control
IR
NSC
IR+NSC
c
% Positive/tumor
CD11b
F4/80
***
***
**
Control
IR
NSC
IR+NSC
d
BLI (adjusted to day 1)
15Gy
NSC-134754
*
Time (days)
Control
IR
NSC-134754
IR+NSC

Fig 4

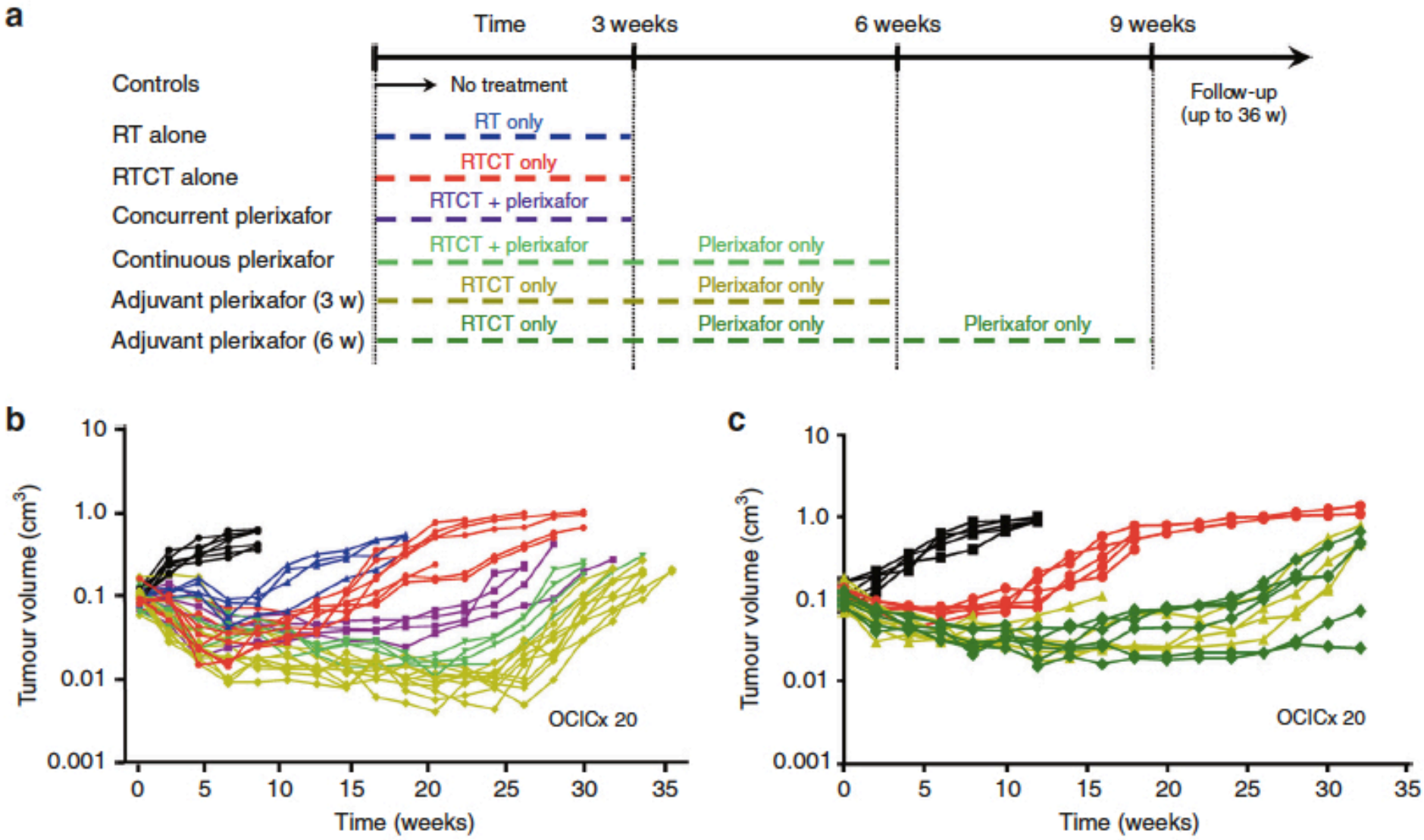

a
Time
3 weeks
6 weeks
9 weeks
Controls
No treatment
Follow-up
(up to 36 w)
RT alone
RT only
RTCT alone
RTCT only
Concurrent plerixafor
RTCT + plerixafor
Continuous plerixafor
RTCT + plerixafor
Plerixafor only
Adjuvant plerixafor (3 w)
RTCT only
Plerixafor only
Adjuvant plerixafor (6 w)
RTCT only
Plerixafor only
Plerixafor only
b
Tumour volume (cm3)
10
1.0
0.1
0.01
0.001
0
5
10
15
20
25
30
35
Time (weeks)
OCICx 20
c
Tumour volume (cm3)
10
1.0
0.1
0.01
0.001
0
5
10
15
20
25
30
35
Time (weeks)
OCICx 20

Fig 5

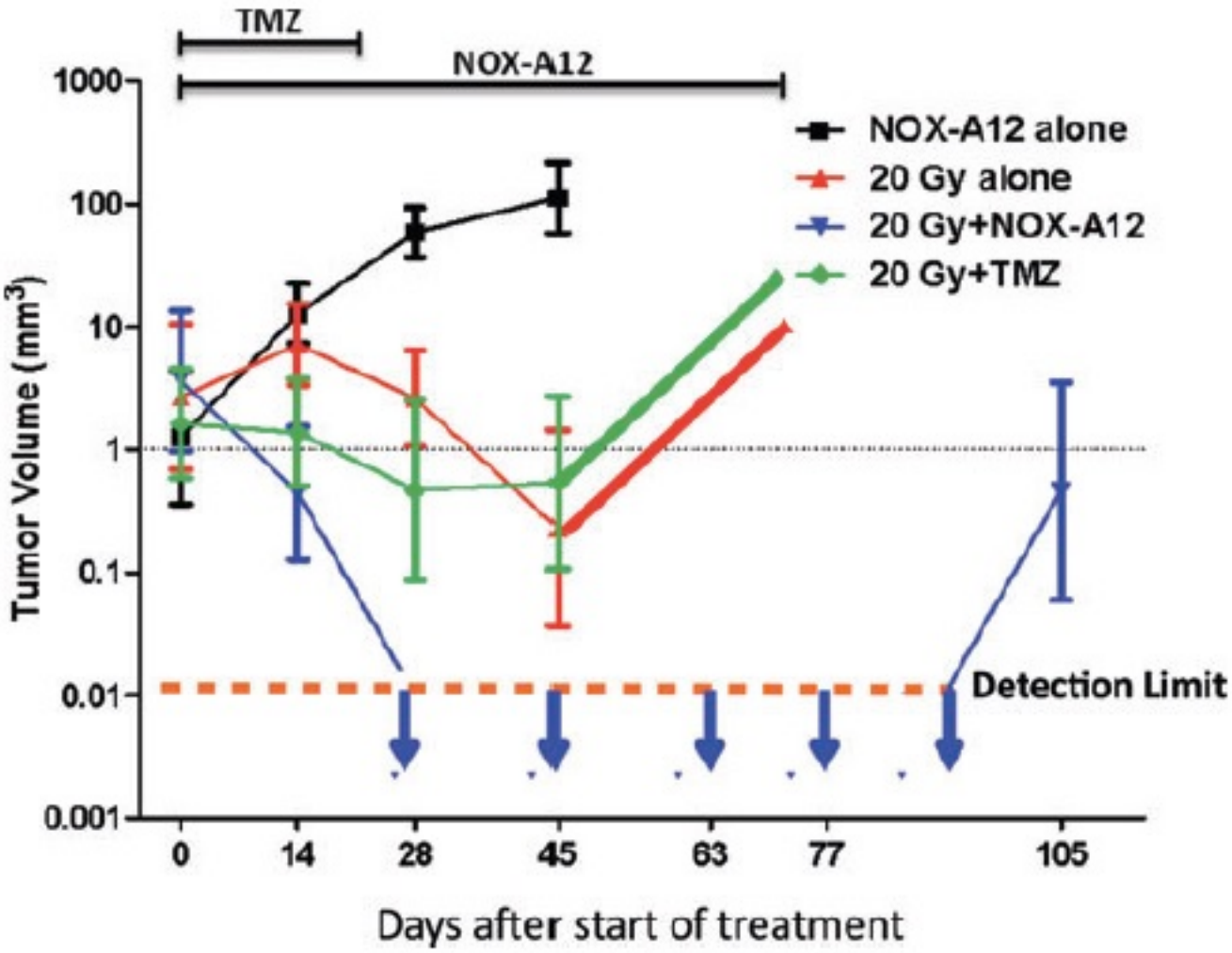
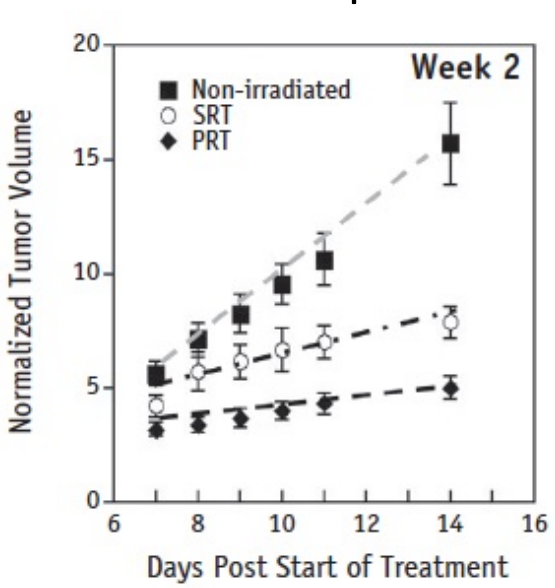
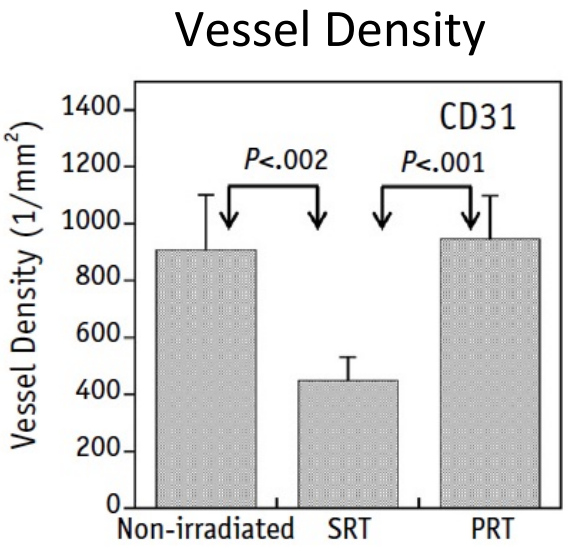

TMZ
NOX-A12
NOX-A12 alone
20 Gy alone
20 Gy+NOX-A12
20 Gy+TMZ
Tumor Volume (mm3)
1000
100
10
1
0.1
0.01
0.001
Detection Limit
0
14
28
45
63
77
105
Days after start of treatment

Fig 6

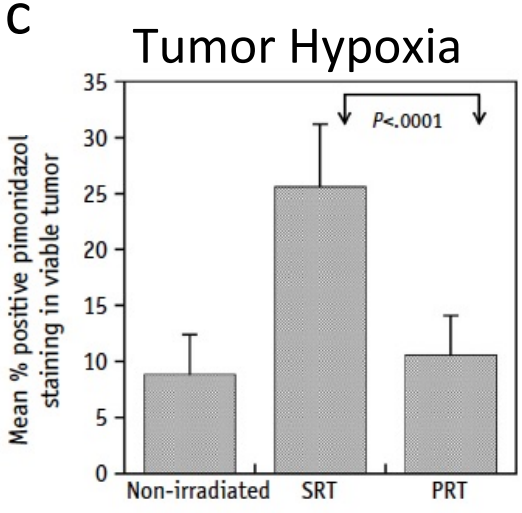
a


Tumor Response
Week 2
Non-irradiated
SRT
PRT
Normalized Tumor Volume
Days Post Start of Treatment
0
5
10
15
20
6
8
10
12
14
16

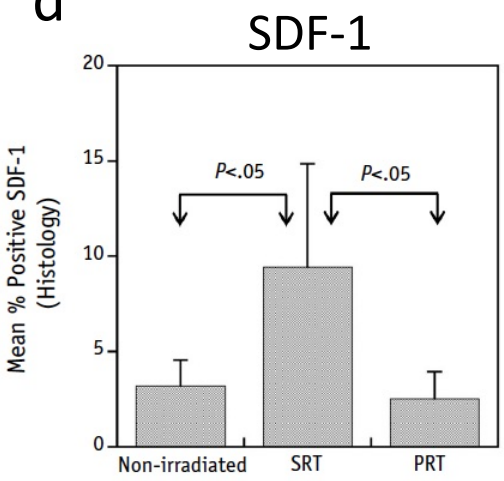
b


Vessel Density
CD31
P<.002
P<.001
Vessel Density (1/mm²)
0
200
400
600
800
1000
1200
1400
Non-irradiated
SRT
PRT

c


Tumor Hypoxia
P<.0001
Mean % positive pimonidazol staining in viable tumor
0
5
10
15
20
25
30
35
Non-irradiated
SRT
PRT

d


SDF-1
P<.05
P<.05
Mean % Positive SDF-1 (Histology)
0
5
10
15
20
Non-irradiated
SRT
PRT

e

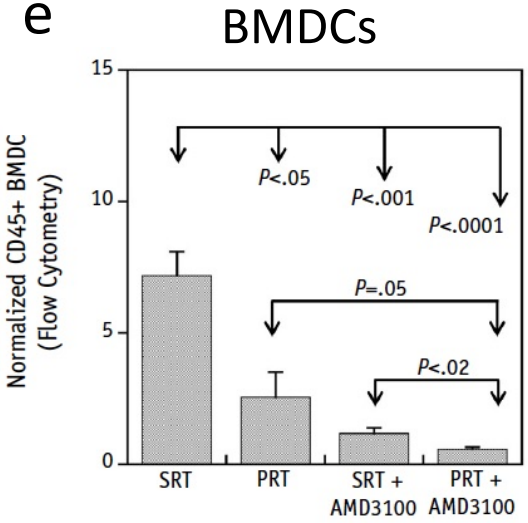

BMDCs
P<.05
P<.001
P<.0001
P=.05
P<.02
Normalized CD45+ BMDC (Flow Cytometry)
0
5
10
15
SRT
PRT
SRT + AMD3100
PRT + AMD3100

Fig 7